Cryopreservation and intracytoplasmic sperm injection with bovine epididymal spermatozoa

Carlos Andres Guerrero
Louisiana State University and Agricultural and Mechanical College, cguerr1@lsu.edu

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations

Part of the Animal Sciences Commons

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_dissertations/1835

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Doctoral Dissertations by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
CRYOPRESERVATION AND INTRACYTOPLASMIC SPERM INJECTION WITH BOVINE EPIDIDYMAL SPERMATOZOA

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Interdepartmental Program of Animal and Dairy Sciences

by

Carlos A. Guerrero
B.S., Louisiana State University, 2002
August 2006
ACKNOWLEDGMENTS

I would like to start by thanking my major professor and advisor, Dr. Robert A. Godke, for accepting me in his Reproductive Physiology Program. I want to thank him for the encouragement, patience, guidance, friendship and financial support he has given me. Most importantly, I want to thank him for the magnificent scientific training he gave me over the past 4 years. He made me the hard working person I am today. I really enjoyed working with him, and thanks again for allowing me to learn such specialized skills in the field of reproduction.

The completion of this dissertation has definitely been a challenge and could not have been accomplished without the assistance and support from many individuals. I want to thank the members of my committee (Dr. Stanley Leibo, Dr. Jill Jenkins, Dr. John Lynn and Dr. Kenneth Bondioli) for constantly providing advice and suggestions for my research.

Special thanks to Dr. Kenneth Bondioli for sharing with me his extensive experience in the area of reproductive physiology. He constantly helped me overcome problems that I had in my work. Thank you for helping me and other graduate students at the laboratory with many protocols and procedures. I feel honored to have worked with a scientist of your prestige. Also, thanks to Dr. Lynn for helping me out with his microscopy skills. He was always available and willing to help me with my research. Special thanks to Dr. Jill Jenkins for teaching me flow cytometry. Thanks for always being worried and interested about my research. You were always there for me as a professor and as a friend.

I would like to thank the Director of the Embryo Biotechnology Laboratory, Mr. Richard Denniston, for keeping running smoothly and up to date this state-of-the-art research facility. He always gave me his support and friendship. The laboratory will miss you when you retire.

Thanks to all the fellow graduate students and student workers at the Laboratory, who offered there help in all projects undertaken. Special thanks to Angelica Giraldo, for helping me with research projects and revising my abstracts. Thanks for your friendship! Also, thanks to Dr. Marina Sansinena, for teaching the art of micromanipulation and laboratory procedures. Thanks to Jesse for being there for me all the time. Keep working hard and you will reach your goals. Thanks to Casey Ballard and Jason Smith, for helping me with the embryo transfer of my ICSI embryos.

I would like to thank the Theriogenology group (Dr. Bruce Eilts, Dr. Dale Paccamonti and Dr. Sara Lyle) from the LSU School of Veterinary Medicine for their participation at our weekly research meetings. Thanks to Bryan for providing me with biological tissues at the local abattoir,
to Jerry for providing fresh chicken eggs for my cryopreservation projects and to Dr. David Donze for allowing me to use his tissue sonicator.

Last but not least, I want to thank my Mother, Maria Islena Espinosa, and Father, Carlos Leonardo Guerrero, for their infinite emotional and financial support. Without your unconditional support, the pursuit of this degree would have been impossible. I love you very much and hope that some day I can repay you for everything you have done for me. Also, my Brother, Jorge Enrique Guerrero, for being such an excellent friend and brother. I am proud of you for everything you have accomplished.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ........................................................................................................ ii

LIST OF TABLES ................................................................................................................ vi

LIST OF FIGURES .............................................................................................................. vii

ABBREVIATIONS USED IN THE DISSERTATION ............................................................. xii

ABSTRACT ......................................................................................................................... xiii

CHAPTER I  INTRODUCTION ............................................................................................. 1

CHAPTER II  LITERATURE REVIEW ................................................................................... 3
  Functions of the Epididymis .............................................................................................. 3
    Maturation ....................................................................................................................... 3
    Transport ....................................................................................................................... 3
    Maintenance and Storage ............................................................................................ 4
    Protection ..................................................................................................................... 4
  Collection of Epididymal Sperm .................................................................................... 5
    Incisions ....................................................................................................................... 5
    Mincing ......................................................................................................................... 5
    Flushing ....................................................................................................................... 5
    PESA .......................................................................................................................... 6
    MESA .......................................................................................................................... 6
  The Use of Epididymal Sperm for Assisted Reproductive Techniques ......................... 6
    Artificial Insemination (AI) ........................................................................................ 6
    In Vitro Fertilization (IVF) ........................................................................................ 9
    Intracytoplasmic Sperm Injection (ICSI) .................................................................... 12
  Intracytoplasmic Sperm Injection in Cattle .................................................................. 16
  Multicolor Flow Cytometry for Sperm Quality Evaluation ............................................ 21
    Viability ....................................................................................................................... 22
    Capacitation ............................................................................................................... 24
    Acrosome Integrity ..................................................................................................... 25
    Mitochondrial Function .............................................................................................. 26
    DNA Integrity ............................................................................................................. 28

CHAPTER III EFFECT OF STORAGE AND CRYOPROTECTANT ADDITION AND REMOVAL AT 4°C ON MOTILITY AND PLASMA MEMBRANE INTEGRITY OF BOVINE CAUDAL EPIDIDYMAL SPERM ........................................ 30
  Introduction .................................................................................................................... 30
  Materials and Methods ................................................................................................. 32
    Experimental Design .................................................................................................. 32
    Experimental Procedure ........................................................................................... 33
  Statistical Analysis ....................................................................................................... 39
  Results ............................................................................................................................. 39
  Discussion ....................................................................................................................... 46
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Mean overall motility values for individual males during a 5-day exposure to different concentrations of CPAs at 4°C.</td>
</tr>
<tr>
<td>3.2</td>
<td>Mean progressive motility values for individual males during a 5-day exposure to different concentrations of CPAs at 4°C.</td>
</tr>
<tr>
<td>3.3</td>
<td>Mean plasma membrane integrity values for individual males during a 5-day exposure to different concentrations of CPAs at 4°C.</td>
</tr>
<tr>
<td>4.1</td>
<td>Testicular and epididymal values from bovine testes collected postmortem.</td>
</tr>
<tr>
<td>5.1</td>
<td>Testicular and epididymal measurements from bovine testes collected postmortem.</td>
</tr>
<tr>
<td>5.2</td>
<td>Morphology values (mean%±SEM) of bovine epididymal sperm prior to and post-cryopreservation.</td>
</tr>
<tr>
<td>6.1</td>
<td>Percent plasma membrane integrity (mean±SEM) of post-thaw bovine epididymal sperm before and after dilution from the freezing extender.</td>
</tr>
<tr>
<td>6.2</td>
<td>Percent acrosome integrity (mean±SEM) of post-thaw bovine epididymal sperm before and after dilution from the freezing extender.</td>
</tr>
<tr>
<td>6.3</td>
<td>Percent mitochondrial activity (mean±SEM) of post-thaw bovine epididymal sperm before and after dilution from the freezing extender.</td>
</tr>
<tr>
<td>6.4</td>
<td>Percent plasma membrane integrity (mean±SEM) of post-thaw bovine epididymal sperm during a 4-hour incubation period at 37°C.</td>
</tr>
<tr>
<td>6.5</td>
<td>Percent acrosome integrity (mean±SEM) of post-thaw bovine epididymal sperm during a 4-hour incubation period at 37°C.</td>
</tr>
<tr>
<td>6.6</td>
<td>Percent mitochondrial activity (mean±SEM) of post-thaw bovine epididymal sperm during a 4-hour incubation period at 37°C.</td>
</tr>
<tr>
<td>6.7</td>
<td>Percent progressive motility (mean±SEM) of post-thaw bovine epididymal sperm during a 4-hour incubation period at 37°C.</td>
</tr>
<tr>
<td>7.1</td>
<td>Effect of activation treatments on pronuclear formation of epididymal sperm-injected bovine oocytes.</td>
</tr>
<tr>
<td>7.2</td>
<td>Effect of activation treatments on the embryonic development of epididymal sperm-injected bovine oocytes.</td>
</tr>
<tr>
<td>7.3</td>
<td>Total cell number (mean±SEM) of day-8 bovine blastocysts derived from ICSI using different activation treatments.</td>
</tr>
</tbody>
</table>
3.1 The process of scrotum dissection. A. Pair of bovine testes inside the scrotum. B. Dissection of the scrotum to retrieve the testes ................. 34

3.2 The process of cauda epididymides dissection. A. Pair of bovine testes outside the scrotum and tunica vaginalis. B. Three pairs of testes showing the dissected cauda epididymides. Each cauda was dissected from the testis by cutting the vas deferens and corpus epididymis .................... 36

3.3 The process of epididymal sperm retrieval from a dissected cauda epididymis. The cauda was placed on a 100 mm plastic dish for 10 minutes to allow sperm to swim out into the collection medium .................... 37

3.4 Effect of cryoprotectant toxicity during 5 days of storage at 4°C on overall motility of bovine epididymal sperm. GLY = glycerol and EG = ethylene glycol ................................................................. 41

3.5 Effect of cryoprotectant toxicity during 5 days of storage at 4°C on progressive motility of bovine epididymal sperm. GLY = glycerol and EG = ethylene glycol ................................................................. 42

3.6 Effect of cryoprotectant toxicity during 5 days of storage at 4°C on plasma membrane integrity of bovine epididymal sperm. GLY = glycerol and EG = ethylene glycol ................................................................. 45

4.1 Representative plasma membrane and acrosome integrity triple stain assay. Sperm that exhibit green fluorescence (SYBR 14) are considered membrane intact (viable), those that exhibit red fluorescence (PI) were considered to have damaged membranes (nonviable) and those exhibiting yellow fluorescence (PE-PNA) were considered acrosome reacted ......................................................... 58

4.2 Flow cytometric out-gating of egg yolk particles based on scatter properties after staining frozen-thawed epididymal sperm with SYBR 14, PI and PE-PNA. Sperm were selected (R1) based on size, gating out small particles ......................................................................................... 59

4.3 PI (red) and SYBR 14 (green) fluorescence. Remaining egg yolk particles having sperm-like scatter properties were gated out based on DNA fluorescence. Events that had either positive PI and/or SYBR 14 (R1) fluorescence were selected as DNA positive events. Particles exhibiting no DNA fluorescence (lower left corner) were considered to be nonsperm events ......................................................................................... 59

4.4 SYBR 14 (viable) and PI (nonviable) flow cytometric dot plot illustrating frozen-thawed epididymal sperm after gating out all nonsperm events. Acquisitions of 10,000 events were made from positive green and red fluorescence ......................................................................................... 60

4.5 Flow cytometric graph illustrating PE-PNA (orange) and PI (red) fluorescence. Events exhibiting high orange fluorescence were recorded as sperm with
reacted acrosomes. Quadrants were set to identify viable acrosome intact sperm (LL), viable acrosome reacted sperm (LR), nonviable acrosome intact sperm (UL) and nonviable acrosome reacted sperm (UR).………………............. 60

4.6 Examples of two pairs of bull testes with abnormal morphology...............................63

4.7 Percent progressive motility (mean±SEM) of prefreeze and post-thaw bovine epididymal sperm cryopreserved with glycerol and ethylene glycol.............................64

4.8 Prefreeze and post-thaw percent progressive motility values (mean±SEM) of bovine epididymal sperm from individual bulls cryopreserved with glycerol or ethylene glycol.................................................................................65

4.9 Percent membrane integrity (mean±SEM) of prefreeze and post-thaw bovine epididymal sperm cryopreserved with glycerol and ethylene glycol........................67

4.10 Prefreeze and post-thaw percent membrane integrity (mean±SEM) of bovine epididymal sperm from individual bulls cryopreserved with glycerol and ethylene glycol.................................................................................68

4.11 Post-thaw percent reacted acrosomes (mean±SEM) derived from the viable and nonviable populations of bovine epididymal sperm cryopreserved with glycerol and ethylene glycol.................................................................69

4.12 Percent acrosome integrity values (mean±SEM) of prefreeze and post-thaw bovine epididymal sperm from individual bulls cryopreserved with glycerol and ethylene glycol.................................................................................71

5.1 Flow cytometric out-gating of egg yolk particles based on scatter properties after staining frozen-thawed bovine epididymal sperm with SYBR 14 and MitoTracker Red. Sperm were selected (R1) based on size, gating out small particles……………….......................................... 85

5.2 MitoTracker Red (red) versus SYBR 14 (green) fluorescence. Remaining egg yolk particles having sperm-like scatter properties were gated out based on DNA fluorescence. Events that had SYBR 14 (R1) positive fluorescence were gated out and used for analysis................................................................................. 85

5.3 Flow cytometric dot plot illustrating MitoTracker Red (red) and SYBR 14 (green) fluorescence from sperm of a bull exhibiting high mitochondrial activity. Graph displays an epididymal sperm sample (10,000 sperm) with 69% of its population having active mitochondria. ................................................................. 87

5.4 Flow cytometric dot plot illustrating MitoTracker Red (red) and SYBR 14 (green) fluorescence from sperm of a bull exhibiting low mitochondrial activity. Graph displays an epididymal sperm sample (10,000 sperm) with only 17% of its population having active mitochondria................................................................. 87

5.5 Flow cytometric dot plot illustrating green versus red fluorescence of acridine orange in the SCSA®. Graph displays bovine sperm with no DNA damage as green and sperm with denatured DNA (R1) fluorescing red..................................................... 89
5.6 Flow cytometric histogram illustrating red fluorescence of acridine orange in the SCSA®. Sperm in the M1 region are exhibiting DNA damage (% COMP)............................................................................................................89

5.7 Post-thaw percent overall motility (mean±SEM) of bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation……………………………………………………………..93

5.8 Post-thaw percent progressive motility (mean±SEM) of bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation……………………………………………………………..94

5.9 Post-thaw percent membrane integrity (mean±SEM) of bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation……………………………………………………………..96

5.10 Post-thaw percent reacted acrosomes (mean±SEM) derived from the viable and nonviable populations of bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation........97

5.11 Percent acrosome integrity (mean±SEM) of post-thaw bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation……………………………………………………………..98

5.12 Percent mitochondrial activity (mean±SEM) of post-thaw bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation……………………………………………………………..100

5.13 Percent DNA damage (mean±SEM) of post-thaw bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation……………………………………………………………..101

6.1 Membrane integrity values (mean±SEM) of post-thaw bovine epididymal sperm before and after one step dilution for removal of glycerol. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation…………………………………………………..118

6.2 Membrane integrity values (mean±SEM) of post-thaw bovine epididymal sperm before and after one step dilution for removal of glycerol. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation…………………………………………………..119

6.3 Percent acrosome integrity (mean±SEM) of post-thaw bovine epididymal sperm before and after one step dilution for removal of glycerol. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation…………………………………………………..121

6.4 Percent acrosome integrity (mean±SEM) of post-thaw bovine epididymal sperm before and after one step dilution for removal of glycerol. The bars represent sperm from individual males harvested with seminal plasma
6.5 Percent mitochondrial activity (mean±SEM) of post-thaw bovine epididymal sperm before and after one step dilution for removal of glycerol. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation.

6.6 Percent mitochondrial activity (mean±SEM) of post-thaw bovine epididymal sperm before and after one step dilution for removal of glycerol. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation.

6.7 Membrane integrity values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation.

6.8 Membrane integrity values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation.

6.9 Acrosome integrity values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation.

6.10 Acrosome integrity values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation.

6.11 Mitochondrial activity values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation.

6.12 Mitochondrial activity values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation.

6.13 Progressive motility values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation.

6.14 Progressive motility values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation.
7.1 Bovine cumulus oocyte complexes (COCs) received from a commercial supplier at 21 hours of maturation. A. COC at 20X magnification. B. COCs at 10X magnification..........................146

7.2 Immobilization of a motile bovine epididymal sperm with the microinjection pipette prior to sperm microinjection. Arrow for the tail of the sperm.............150

7.3 The piezo ICSI procedure (40X magnification). A. An oocyte with the polar body in the 12 o’clock position held by the negative pressure of the holding pipette before sperm injection. B. The zona pellucida drilled by applying piezo pulses. A small cylinder of the zona pellucida can be observed outside the oocyte. The sperm is moved to the tip of the pipette before penetrating the oolema. C. The pipette is mechanically moved forward to stretch the oolema. A single piezo pulse is applied to break the oolema. D. The sperm is pushed forward by applying positive pressure to the injector. E. Excess medium is removed and the injection pipette is moved out from the oocyte. F. A small amount of ooplasm at the place of injection is removed to allow sealing of the oolema...............................................151

7.4 Cytolyzed bovine oocytes degenerated 4 to 5 hours after sperm injection. A. Arrows indicates cytolyzed oocytes (20X magnification). B. Cytolyzed oocyte (40X magnification).................................................................154

7.5 Oocyte stained with aceto-orcein 18 hours after the ICSI procedure. Male and female pronuclei are indicated by arrows.........................................................155

7.6 An example of a day-3 bovine embryo (8- to 16-cells) derived from epididymal sperm-injected oocytes..............................................................159

7.7 Examples of a day-8 blastocysts derived from epididymal sperm-injected oocytes. Blastocysts are hatching through the opening made by the microinjection pipette.................................................................160

7.8 An example of a day-8 blastocyst stained with Hoechst 33342. A. Day-8 blastocyst at 20X magnification. B. An overlay of the stained cells of the same day-8 blastocyst in panel A.................................................................163

7.9 Ultrasound images of a 50-day frozen-thawed epididymal sperm ICSI-derived fetus..........................................................164
ABBREVIATIONS USED IN THE DISSERTATION

AI – artificial insemination
ART – assisted reproductive techniques
ATP – adenosine triphosphate
BO – Brackett Oliphant medium
BSA – bovine serum albumin
BSP – bovine seminal plasma proteins
CASA – computer assisted sperm analysis system
CAM – calcein acetoxy methyl ester
CFDA – 6-carboxylfluorescein diacetate
CKIA – computerized karyometric image analysis
CMFDA – 6-carboxymethylfluorescein diacetate
COC – cumulus oocyte complex
EYT-GC – egg yolk Tris-glucose citric acid monohydrate extender
FBS – fetal bovine serum
FDA – fluorescein diacetate
FITC – fluorescein isothiocyanate
GFP – green fluorescent protein
HCG – human chorionic gonadotropin
ICI – intra cervical insemination
ICSI – intracytoplasmic sperm injection
IUAI – intrauterine artificial insemination
IUI – intrauterine insemination
IVF – in vitro fertilization
IVM – in vitro maturation
LN₂ – liquid nitrogen
MPF – maturation promoting factor
MHV – mouse hepatitis virus
PE – phycoerythrin
POEC – porcine oviductal epithelial cell monolayer
PI – propidium iodide
PNA – Arachis hypogaea agglutinin
PSA – Pisum sativum agglutinin
PVP – polyvinylpyrrolidone
R123 – rhodamine 123
TCM – tissue culture medium
TLH – tyrodes lactate HEPES medium
ABSTRACT

Recently, interest in the preservation of epididymal sperm as a potential source of valuable genes for genome resource banks has escalated. The development of a successful protocol to recover and cryopreserve sperm harvested from the epididymides would salvage germplasm from genetically valuable males that are injured and can no longer mate or have unexpectedly died and can be used as a model for the preservation of male gametes from endangered species. In a series of experiments, epididymal sperm was successfully harvested, cryopreserved and used for intracytoplasmic sperm injection. In Experiment I, ethylene glycol was found to cause significantly (P<0.05) less osmotic damage to bovine sperm during a one step addition and/or removal at 4°C as compared with glycerol in all concentrations evaluated. Furthermore, prolonged exposure (5 days at 4°C) of ethylene glycol was found to be less toxic than glycerol to sperm. In Experiment II, it was demonstrated that glycerol was more effective than ethylene glycol in providing protection against freezing injury during the cryopreservation process in the concentrations evaluated. In Experiment III, it was demonstrated that epididymal sperm retrieval using seminal plasma is beneficial to enhance sperm overall and progressive motility characteristics and to protect it from morphological abnormalities derived from the freezing process. In Experiment IV, a one step dilution process for removal of glycerol from cryopreserved epididymal sperm was found to significantly affect plasma membrane integrity and mitochondrial function of sperm previously exposed to seminal plasma. However, seminal plasma exposure did not have any significant detrimental effect on acrosome integrity. Furthermore, it was demonstrated that the longevity and survivability in vitro during a 4-hour incubation period at 37°C of post-thaw epididymal sperm exposed to seminal plasma prior to cryopreservation was not compromised when compared with the control extended sperm. In Experiment V, we have demonstrated that fertilization, blastocyst and fetal development could be achieved with cryopreserved bovine epididymal sperm by intracytoplasmic sperm injection (ICSI). To our knowledge, this is the first report in the United States and second in the world to use bovine epididymal sperm for ICSI. We achieved far markedly improved blastocyst rates over those results recently reported in the first study originating in Japan.
CHAPTER I

INTRODUCTION

The unexpected loss of breeding animals of high genetic value or zoological interest for any reason, such as loss of libido, reproductive tract injury, or death, can be costly in terms of the potential loss of genetically valuable germplasm. This loss can be reduced by harvesting spermatozoa (more commonly referred to as sperm) from the epididymis and using assisted reproductive techniques (ART) to continue propagating these valuable genetics over an extended period of time.

Assisted reproductive techniques, such as artificial insemination (AI), in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have been used over the years in an effort to bypass critical fertilization steps, bypass breeding problems, increase animal production efficiencies, overcome infertility and to propagate valuable genetics. Moreover, the use of ART procedures represent a plausible strategy to circumvent sexual incompatibility, eliminate the risk associated with animal transport and provide a method of using cryopreserved germplasm to infuse genes from wild animals into captive breeding populations (Stachecki et al., 1994). The objective of this research project was to develop a successful protocol for the cryopreservation of bovine epididymal sperm. Furthermore, we wanted to use these valuable samples to produce embryos and calves by developing an efficient ICSI protocol for cattle.

Reports on the use, cryopreservation, fertility and characteristics of bovine epididymal sperm are limited. Even with all the research over the years to improve the survivability of bovine sperm after cryopreservation, there is still a decline of ~50% in sperm post-thaw viability due primarily to temperature and osmotic effects (Thomas et al., 1998). Furthermore, due to intermale variation sperm from some bulls still can not be cryopreserved with present bovine protocols.

ICSI can be defined as the process of mechanically inserting a whole sperm or an isolated sperm head into the ooplasm of an oocyte. This becomes a powerful technique when only a few sperm are available for insemination. Since ICSI requires far fewer sperm to fertilize the same number of oocytes than does IVF, it is potentially valuable for using semen samples more effectively (Horiuchi et al., 2002). The use of postmortem epididymal sperm with ICSI will allow a more effective use of valuable genetic material. This technique has become important in the area of human infertility.
and in studies of the molecular mechanisms of fertilization. ICSI has become one of the
most successful techniques in humans for achieving fertilization and subsequent embryo
development in cases of male sterility (Palermo et al., 1992). In contrast, the use of ICSI
in farm animals to date is far from satisfactory. The embryo developmental rates of
sperm-injected oocytes are still markedly lower than after conventional IVF. The
blastocyst rates reported for ICSI using bovine sperm range from 1% to 20% (Goto et
al., 1990; Hamano et al., 1999; Horiuchi et al., 2002).

We must take into consideration that epididymal and ejaculated sperm are two
biochemically and functionally different cell types. Ejaculated sperm differ from
epididymal sperm in respiration rate (Hammerstedt et al., 1993), number of heparin-
binding sites (Nass et al., 1990), ability to capacitate (Miller et al., 1990), morphology
(Hewitt et al., 2001), types of proteins bound to the plasma membrane (Lee et al., 1985)
and they display different motion characteristics (Gooaverts et al., 2006). Thus, ART
protocols used for ejaculated sperm will likely need to be modified for epididymal sperm.

Once we develop a successful protocol to preserve bovine epididymal sperm
from postmortem animals and develop an efficient ICSI procedure to use these valuable
sperm more effectively, research can be conducted to modify these protocols to best fit
other species, including farm animal and endangered species.
CHAPTER II
LITERATURE REVIEW

FUNCTIONS OF THE EPIDIDYMIS

The epididymis is generally divided into three functional regions; caput (head), corpus (body) and cauda (tail), where sperm undergo physiological and functional maturation (Bedford, 1975). Sperm present in the testis or in the caput epididymis are inmotile and immature, while those that reach the cauda are generally more motile and mature and are thought to be capable of fertilization (Toshimori, 2003).

Maturation

Sperm maturation refers to the change in functional capacity that occurs in sperm during their transit through the epididymis (Cooper, 1999). Modification of sperm to achieve maturation requires enzymes and transfer proteins that are secreted from the epididymal epithelium into the luminal fluid (Amann et al., 1993). Changes in sperm associated with maturation fall into the following four areas, the relative importance of which likely depends on the species (Amann et al., 1993).

- Modification of the DNA-protein complex of the nucleus to minimize the probability of environmental damage or premature degradation.
- Modification of the plasma membrane, mitochondria, and fibrous and microtubular components of the middle and principal pieces to enable energy transduction for coordinated contractions of tail and motility.
- Development of surface characteristics enabling prolonged survival within the female reproductive tract.
- Stabilization of the plasma and acrosome membranes and development of multiple binding proteins to bind with ovum receptors.

Transport

Sperm transport is a consequence of frequent pendular contractions of smooth muscle fibers in the wall of the duct within the head and body of the epididymis and low frequency segmental contractions of periducal smooth muscle in the tail of the epididymis (Jaakola and Talo, 1983; Turner, 1991). This spontaneous muscular activity is augmented by adrenergic, cholinergic and purinergic nerve fibers along with blood-borne angiotensins, vasopressin and oxytocin (Cooper, 1999). Although some sperm are
transported distally more rapidly than others, a typical bovine sperm is transported through the caput and corpus of the epididymides in 2 to 4 days (Amann, 1972). Then, sperm are transported through the cauda of the epididymides in 6 to 14 days or longer, with this interval being influenced by frequency of ejaculation (Amann et al., 1993).

**Maintenance and Storage**

Storage and maintenance of fertile sperm is the primary role of the tail of the epididymis. The number of sperm stored varies according to the reproductive pattern, social and mating behavior of a given species (Amann, 1981). In all species known, sperm are stored in a quiescent state before ejaculation. This gamete reserve permits ejaculation of larger numbers of sperm than the daily testicular production (Copper, 1999). Once the sperm reserve is full, existing mature sperm are expelled through the ductus deferens and the urethra either into the urine or via spontaneous ejaculation. The two primary mechanisms for sperm storage (Copper, 1999) are:

1. **Enforcing Metabolic Quiescence**
   - The lowering of luminal sodium ion concentration (prevents proton efflux and a rise in pH that triggers motility).
   - High concentration of sperm in billions/ml.
   - Secretion of viscous mucoprotein that restrict sperm movement.
   - Steady production of acids keeping intracellular sperm pH low.

2. **Prevention of Premature Sperm Activation**
   - Protection of sperm membranes by the secretion of decapacitation factor(s).
   - Sperm aggregation, including the pairing of sperm in New World marsupials and rouleaux formation of New World eutherians (e.g., flying squirrel, guinea pig and Naked Tail armadillo). The pairing and rouleaux formation involve stacking of acrosome membranes for protecting this liable structure during transit (Bedford, 1979).

**Protection**

It is the responsibility of the epididymal epithelium to maintain stored sperm in a viable state. This responsibility includes the rapid elimination of harmful metabolic by-products, toxic substances, prevention of oxidative stress and protection from the immune system (Hinton et al., 1995).
A series of enzymes produced by the epididymal epithelium, such as glutathione-s-transferase, superoxide dismutase, glutathione peroxidase and catalase are important for the elimination of reactive oxygen species and for the removal of toxicants (Cooper, 1999; Hinton et al., 1995). Protease inhibitors, such as cystatin-related epididymal-specific protein prevents damage by lytic enzymes leaking from degenerating sperm (Hinton et al., 1996). The blood-epididymis barrier is very important for the protection of sperm against the immune system (Pollanean and Cooper, 1994). Sperm acquire many different surface antigens during their development and immune system exposure of these surface antigens often mounts an immune response (Pollanean and Cooper, 1994).

**COLLECTION OF EPIDIDYMAL SPERM**

There are five basic procedures for the collection of cauda epididymal sperm. Three are used for harvesting sperm from deceased animals and two developed specifically for live animals, and are often applied to humans.

**Incisions**

This method of collection is one of the fastest and easiest to perform. The cauda is excised from the testis as aseptically as possible. Several longitudinal incisions are made on the distal end of the cauda to expose the sperm to the outer environment. Sperm are rinsed from the cauda with 3 ml of isotonic sperm wash media into a 100 mm petri-dish. The cauda is placed on the dish for 5 minutes to allow the sperm to swim out into the medium. This process has a good sperm recovery rate, however, there is chance of contamination from blood during the process.

**Mincing**

The cauda epididymis is placed on a 100 mm petri-dish and minced for 10 minutes. Then 3 ml of sperm wash medium is added to the dish. The sample is filtered and centrifuged twice. This method provides a high sperm recovery rate, however, there is a high chance of contamination from blood and tissue. A longer process is required to filter and remove pieces of tissue and debris from the sperm sample. Viability of sperm can be affected due to centrifugation and exposure to blood components.

**Flushing**

The cauda is excised from the testis. A blunt needle (size depends on species) is inserted into the vas deferens. A syringe is attached with 3 ml of sperm wash medium
and pressure is applied by depressing the plunger. Sperm is collected in a 15 ml test tube. This method provides the highest recovery rate and the least contamination.

**PESA**

Percutaneous epididymal sperm aspiration (PESA) (Craft et al., 1995a) is a relatively simple procedure involving removal of sperm directly from the epididymis by percutaneous needle aspiration. The advantages to this technique are that it can be performed without surgical scrotal exploration, it can be repeated easily, it is low cost and it does not require an operating microscope or the expertise in microsurgery (Patrizio, 2000).

**MESA**

Microsurgical epididymal sperm aspiration (MESA) (Patrizio et al., 1988) is the microsurgical incision in a single epididymal tubule, very carefully prepared by microsurgical exploration under an operating microscope. The aspiration is performed using a special microsurgical capillary system. This procedure causes minimal trauma to the epididymal tubular system (Schwarzer et al., 2003b). This approach provides a higher recovery rate than PESA, however, it is more invasive and expensive (Patrizio, 2000).

**THE USE OF EPIDIDYMAL SPERM FOR ASSISTED REPRODUCTIVE TECHNIQUES**

**Artificial Insemination (AI)**

Barker and Gandier (1957) reported the birth of an equine foal using epididymal sperm by AI. Epididymal sperm were frozen in milk diluent with 10% glycerol and stored at -79°C for 30 days. This was the first recorded birth resulting from the use of frozen-thawed stallion epididymal sperm and AI. Decades later, Morris et al. (2002) reported 9 pregnancies from 20 mares using fresh epididymal sperm deposited directly into the utero-tubal papilla, and they reported 9 pregnancies in 45 mares using the same procedure with frozen-thawed equine epididymal sperm.

Barker (1954) reported the first pregnancy using frozen-thawed bull epididymal sperm for AI. However, birth of the calf was not reported. Sperm were added to milk diluent with 10% glycerol. The sample was placed in a refrigerator at 5°C for an equilibration period of 18 hours and further cooled to -79°C in 1 ml ampoules. Amann and Griel (1973) compared the fertility of bovine caudal epididymal sperm with ejaculated sperm by AI. Insemination with 200 x 10^6 sperm into the uterine body of 106
cows resulted in 84% and 94% fertilized ova for caudal epididymal sperm and ejaculated sperm, respectively. More recently, it was reported that bovine epididymal sperm stored at 5°C were viable for at least 60 hours when used for AI (Foote, 2000).

A 41-day pregnancy following AI with cryopreserved epididymal sperm harvested from a gaur (*Bos gaurus*) bull that had been euthanized was reported (Hopkins et al., 1988). Sperm were extracted 27 hours postmortem, diluted in an egg yolk-based extender supplemented with 7% glycerol, placed at 12.5 cm above liquid nitrogen vapor for 10 minutes and then plunged in liquid nitrogen for storage. Bartels et al. (2001) maintained eland epididymal sperm at 4°C for 5 hours postmortem before cryopreservation. A live birth was then achieved following AI. More recently, Soler et al. (2003a) reported an average fertility rate of 56% in Red deer using frozen-thawed epididymal sperm subjected to different thawing procedures before AI.

In the rabbit, no difference was reported in the fertility after AI of sperm extracted from the caudae epididymides when compared with its ejaculated counterpart after they were incubated for 20 hours or for a longer interval in the female (Dott et al., 1966). However, Cummins and Orgebin-Crist (1971) found that rabbit sperm from the caudae epididymides fertilized 52% of oocytes when artificially inseminated at the same time as human chorionic gonadotropin injection or 9 to 13 hours before ovulation in the recipients, while ejaculated sperm fertilized 84% of oocytes under same experimental conditions. Furthermore, Overstreet and Bedford (1976) reported 32 and 28 pregnancies in does artificially inseminated with caudal epididymal and ejaculated sperm, respectively, demonstrating that both were equally competent to support embryonic and fetal development. Earlier, Adams (1975) produced offspring from 2 wild rabbits (*Oryctolagus cuniculus*), which did not breed under laboratory conditions, using AI with caudal epididymal sperm. These does were inseminated directly into the uterus performing a laparotomy, however 17 other does inseminated into the vagina using epididymal sperm produced no offspring.

Blandau and Rumery (1964) compared fertilization rates in the rat after AI using caput and caudal epididymal sperm. Fertilization rates were 8% and 93% for the caput and the cauda, respectively. Orihuela et al. (1999) studied sperm migration into and through the oviduct following AI with epididymal sperm at different stages of the follicle cycle in the rat. Nakatsukasa et al. (2001) reported birth of 41 normal offspring born from
9 female rats that were artificially inseminated with frozen-thawed epididymal sperm. Sperm was frozen in a medium composed of 23% egg yolk, 8% lactose monohydrate, 10% Tris and 0.7% Equex Stem. This was the first successful production of rat offspring using epididymal sperm cryopreserved at -196°C. Recently, live young were produced by AI with cryopreserved epididymal sperm extracted from closed colonies (Jcl:SD and Jcl:Wistar), inbred (BN/Crj, F3441 DuCrj, LEW/Crj, Long-Evans and WKY/NCrj), mutant (Zitter [WTC.ZI-zi] and Tremor [TRM]), transgenic (human A-transferase [A] and green fluorescent protein [GFP]) strains of rats (Nakatsukasa et al., 2003).

Blash et al. (2000) reported the birth of one healthy kid from 20 inseminated does by AI with frozen-thawed goat epididymal sperm. Epididymal sperm was obtained at necropsy from goats and equilibrated with 20% egg yolk, 7% glycerol, 2.42% Tris, 1.38% citric acid, 5.5 mg tylosin and 1% fructose. Samples were cooled to 5°C for 4 hours, placed into a -80°C for 15 minutes and then plunged into liquid nitrogen.

In swine, live piglets were reported using AI and epididymal sperm harvested from different regions of the epididymis (Holtz and Smidt, 1976). In this study, fertilization rates improved significantly from the caput to the cauda regions.

Marks et al. (1994) reported the birth of a canine puppy after AI using frozen-thawed epididymal sperm extracted postmortem to preserve the breeding line of a 9-year old male boxer. Recently, Hori et al. (2004) compared the fertilizing ability of frozen-thawed caudal epididymal and ejaculated sperm in beagle dogs. The post-thaw motility was slightly lower for epididymal sperm, but the difference was not significant. However, the pregnancy rate (16.7% and 90%) after AI was much lower for epididymal than for ejaculated sperm, respectively.

Timing of AI in marsupials is critical because fertilization must occur before mucin coats the oocyte during passage through the oviduct (Rodger and Bedford, 1982a). Fertilization after intrauterine artificial insemination (IUAI) has been achieved using epididymal sperm in the Brushtail possum (Trichosurus vulpecula) but no births were reported (Molinia et al., 1998). Recently, the birth of two Tammar wallabies (Macropus eugenii) were reported using IUAI and epididymal sperm (Paris et al., 2004). These young represent the first macropodids born by AI and first marsupials conceived from using epididymal sperm.
Morrel et al. (1997) used cooled epididymal sperm for AI in Marmoset monkeys (*Callithrix jacchus*). Epididymal sperm was deposited in the cervix of 18 Marmoset monkeys near the time of expected ovulation using 1, 2 or 3 inseminations. Six of 18 females conceived, resulting in the first reported births following AI in this species.

In humans, extraction of epididymal sperm and intrauterine insemination (IUI) has been used to treat male infertility with obstructive azoospermia. In one report, from 66 IUI cycles, 3 pregnancies resulted giving birth to 3 healthy infants (Qiu et al., 2003). Pregnancies have also been achieved in humans using intracervical inseminations (ICI) using epididymal sperm (Cooper, 1990).

**In Vitro Fertilization (IVF)**

Goto et al. (1989) used frozen-thawed bovine epididymal sperm to examine variation among bulls in fertilization and embryo development rates in vitro. Bovine epididymides were transported from a local abattoir within 2 hours at 25°C to the laboratory. Sperm samples were frozen in egg yolk tris citrate extender supplemented with 6.5% glycerol. The fertilization rates ranged from 55.2 to 64.3% and the day 7 to day 8 blastocyst rates ranged from 9% to 12% among bulls, respectively. Subsequently, Graff et al. (1996) reported 2 pregnancies obtained from IVF with noncapacitated epididymal bovine sperm. Recently, Herbert and co-workers (2005) (unpublished data) produced 5 live calves from IVF with bovine epididymal sperm harvested from epididymides stored at 4°C for 24 hours.

Herrick et al. (2004) used springbok (*Antidorcas marsupialis*) frozen-thawed epididymal sperm for IVF to test its fertilizing ability. Epididymal sperm were extracted following managed culls or hunts within 4 hours of death. From 132 in vitro matured oocytes, only 4 had zona penetration. Winger et al. (1997) reported fertilization of blesbok (*Damaliscus dorcas phillipsi*) oocytes using frozen-thawed epididymal sperm. In African Buffalo (*Syncerus caffer*), epididymal sperm was harvested from testes stored at 4°C for up to 24 hours, and used for IVF. Cleavage and morula rates were 50% and 65%, respectively (Shaw et al., 1995). Unfortunately, no live births have been reported from these wild species.

Boar epididymal sperm show a higher resistance to cold shock than ejaculated sperm, suggesting that they may be more suitable for cryopreservation (Losley and Bogart, 1944). Jiang et al. (1991) reported the birth of live piglets after the transfer of 2-
to 4-cell embryos produced by in vitro matured porcine follicular oocytes and IVF using frozen-thawed epididymal sperm. Rath and Niemann (1997) compared the use of porcine frozen-thawed epididymal and ejaculated sperm for IVF obtained from identical boars. Epididymal sperm were harvested immediately postmortem and cryopreserved in an egg yolk lactose-based extender supplemented with 2% glycerol. The fertilization (59.7% compared with 16%) and cleavage rates (21% compared to 5.3%) were higher for frozen-thawed epididymal sperm than for frozen-thawed ejaculated sperm, respectively. Subsequently, Kikuchi et al. (1998) studied the influence of prolonged storage at 4°C of boar epididymides on IVF. They reported lower oocyte penetration rates for sperm collected on days 1, 2 and 3 from stored epididymides (12, 13 and 2%, respectively) than control sperm (40%) collected from nonrefrigerated epididymides.

Recently, Romar et al. (2003) evaluated the effects of adding porcine oviduct epithelial cell (POEC) monolayer during IVF. Frozen-thawed epididymal sperm were used to avoid replicate variability. They reported an increase in oocyte penetrability in the presence of POEC. However, a lower blastocyst rate was found with POEC incubation during IVF than without POEC monolayer (21% and 14%, respectively).

Ogawa et al. (1972) reported the successful IVF of rabbit ova by epididymal sperm in a synthetic medium without the need of follicular fluid, which in the past, was normally required to induce capacitation in ejaculated sperm. They obtained a 59% fertilization and a 23% blastocyst rate. Later, it was found that capacitation could be induced in rabbit epididymal sperm by washing them twice (15 minute interval) in isotonic or hypertonic defined medium (Brackett et al., 1978; Hosoi et al., 1981).

Niwa et al. (1985) studied the penetration rates of cat epididymal sperm for IVF. They reported that 90% to 100% of the ova were penetrated between 0.5 and 5 hours post-IVF. Female and male pronuclei were observed as early as 4 hours post-insemination. Freistedt et al. (2001) used cat epididymal sperm and IVF to test the effect of season and ovarian status on the efficiency to produce embryos. They reported that although embryos could be produced year round, the efficiency was significantly affected by season. Blastocyst rates produced from January to September and from October to December were reported to be 28% and 18%, respectively.

In the field of transgenic production, the ability to maintain the genetic contribution of the male after death is remarkably important. The first report of IVF using
goat epididymal sperm was in 1988 (Song and Iritani, 1988). They examined the effects of length of pre-incubation and concentration during pre-incubation of fresh epididymal sperm on IVF rates. The highest fertilization rates (57%) were obtained when sperm had been pre-incubated at a concentration of \(50 \times 10^7\) sperm/ml for 6 hours in a modified Krebs Ringer bicarbonate solution. Blash et al. (2000) compared frozen-thawed epididymal and ejaculated sperm for IVF. They reported 40% and 38% cleavage rates, with 6% and 4% developing into blastocysts using frozen-thawed caprine epididymal and ejaculated sperm, respectively.

Today, most of the basic research in mammalian genetics is undertaken in mice and rats. The production of mice with transgenes and mutant genes is common, and an abundance of valuable genomes are available for analysis (Szczygiel et al., 2002). Due to the difficulty in collecting ejaculated sperm from mice and rats, most of the research has been done using epididymal sperm. Toyoda and Chang (1974) used fresh rat epididymal sperm for IVF. A total of 203 cleaved embryos were transferred to 14 pseudopregnant rats. A total of 43 live young were born.

Birth of live mouse pups have been reported using fresh (e.g., Miyamoto and Chang, 1972; Songsasen et al., 1997) and frozen-thawed epididymal sperm (e.g., Songsasen et al., 1997; Sztein et al., 2000; Kawase et al., 2002). Lacham-Kaplan and Trounson (1994) showed that the fertilizing ability of mouse epididymal sperm decreases significantly from the caput to the cauda. Fertilization rates for caput, corpus and caudal epididymal sperm were 10, 30 and 90%, respectively. Bongso and Trounson (1996) co-cultured mouse epididymal sperm from the caput, corpus and cauda with epithelial cells of their own region or more distal regions. They found that the presence of more distal epithelial cells of the mouse epididymis increases the ability of caput and corpus, but not cauda sperm, to fertilize zona-free oocytes. It was reported that epididymal sperm collected from mouse cadavers kept at room temperature up to 24 hours can fertilize oocytes in vitro (Christian et al., 1993; Songsasen et al., 1998). Sperm collected 15 and 24 hours after death resulted in fertilization rates of 25% and 20%, respectively. The birth of 3 live female pups was reported after transfer of 11 blastocysts fertilized with epididymal sperm harvested from cadavers after 24 hours of death. Subsequently, Kishikawa et al. (1999) harvested epididymal sperm from mouse cadavers stored up to 15 days at 4°C. Sperm motility decreased from 88% (day 0) down to 66% (day 5), 32%
(day 10) and 8% (day 15). Day-5 epididymal sperm had a fertilization rate of 26%. However, the sperm extracted from day 10 and day 15 had limited ability to fertilize oocytes (2.8% and 2.7%, respectively). Tada et al. (1994) used epididymal sperm to fertilize mouse vitrified oocytes. A 54% fetal development resulted after embryo transfer. Sato and Ishikawa (2004) stored epididymal sperm at room temperature (22°C) for 5 days in M2 medium and then used it for IVF. They reported a 7% fertilization rate with 12.5% of the transferred embryos resulting in live mouse pups. Scavizzi and Raspa (2004) used epididymal sperm extracted from infected mice with mouse hepatitis virus (MHV) to investigate its transmission by IVF. They reported that transmission was not found when sperm from infected testes were used for IVF. These results suggested that sperm do not transmit infection from actively infected animals and that IVF could be considered a virus cleansing procedure.

In humans, epididymal sperm and IVF are used to treat patients who suffer from azoospermia due to congenital absence of the vas deferens or secondary extended obstruction of the spermic ducts (Bladou et al., 1991). Also, it is important to freeze epididymal sperm to avoid additional epididymal surgery in the male. Pregnancies have been obtained by IVF using epididymal sperm (e.g., Temple-Smith et al., 1985; Silber et al., 1987, 1988; Silber, 1988; Bladou et al., 1991; Oates et al, 2004 and others). Bladou et al. (1991) reported 5 pregnancies (35%) with embryos produced by IVF using caput epididymal sperm. They found that caput epididymal sperm used for IVF gave a higher rate of embryo degeneration (>50%) after embryo transfer than caudal epididymal sperm. Patrizio et al. (1995) reported the birth of a healthy girl using frozen-thawed epididymal sperm after conventional IVF. Belker et al. (2001) reported a live birth from embryos produced by IVF using epididymal sperm retrieved from a moribund man. Tzeng et al. (1996) reported the birth of a healthy girl by MESA and IVF.

**Intracytoplasmic Sperm Injection (ICSI)**

ICSI has become an important technique in the areas of infertility and for the study of the molecular mechanisms of fertilization. This approach has become one of the most successful techniques in humans for achieving fertilization and subsequent embryo development in cases of male sterility (Palermo et al., 1992). ICSI has helped the understanding of the early events of fertilization such as capacitation, the acrosome...
reaction, the mechanisms of sperm oocyte interaction, sperm-induced oocyte activation, pronucleus formation and the control of the first cell cycle (Keskintepe et al., 1997).

Caudal epididymal sperm for ICSI has been used extensively in the mouse (e.g., Markert, 1983; Iritani et al., 1988; Ahmadi et al., 1995; Kimura and Yanagimachi, 1995; Ron-El et al., 1995; Suzuki and Yanagimachi, 1997; Moreira et al., 2003; Nagai et al., 2003; Baart et al., 2004). Kishikawa et al. (1999) produced 6 mouse pups after ICSI with immotile epididymal sperm harvested from cadavers stored at 4°C for 20 days. Forty two embryos were transferred and 6 implanted. When valuable male animals die unexpectedly and facilities for sperm cryopreservation are not immediately accessible, temporal storage of cadavers in a regular refrigerator followed by ICSI may help to preserve the genome of individuals. Suzuki and Yanagimachi (1997) compared the use of conventional ICSI with a novel piezo electrically driven pipette using mouse epididymal sperm. They reported that although the efficiency of the piezo ICSI is much more efficient, the inefficiency of the conventional ICSI could be improved by performing the operation in a medium with high serum concentration. Kawase et al. (2005) studied the effect of storing freeze-dried mouse epididymal sperm at either 4°C or -80°C on embryo development after ICSI. They reported blastocyst rates of 21% and 62% for freeze-dried epididymal sperm stored for 3 months, and 13% and 59% for sperm stored for 6 months at 4°C and -80°C, respectively. Yanagimachi et al. (2004) reported the birth of fertile mouse offspring after injecting epididymal sperm from an infertile male into oocytes of normal females.

Re-establishment of mouse strains used for mutagenesis and transgenesis has been hindered by difficulties in freezing sperm. The use of epididymal sperm and ICSI enables the production of embryos for the restoration of mouse lines using low quality sperm. Lacham-Kaplan et al. (2003) produced live offspring from inbred and hybrid mouse strains using ICSI with epididymal sperm frozen without cryoprotectant. Li et al. (2003) reported the birth of 26 live mutant mouse pups after ICSI using epididymal sperm. They rescued 4 mutant lines that had become infertile and unresponsive to conventional IVF by using ICSI. Moreira et al. (2004) reported the efficient generation of transgenic mice by ICSI using epididymal sperm. They produced offspring carrying the YAC transgene.
Hirabayashi et al. (2002) reported the birth of 18 transgenic rat pups carrying the green fluorescent protein gene (GFP) from 168 transferred ICSI embryos using epididymal sperm. Transgenic male rats carrying the GFP gene were used as the sperm donors. Said et al. (2003) studied embryo development of rat oocytes after ICSI with epididymal sperm heads. They reported 18% blastocyst rate and 6 pups were born after embryo transfer.

Goto et al. (1990) used immobilized, killed epididymal sperm to fertilize IVM bovine oocytes by ICSI. From 507 oocytes injected, 1.8% reached the blastocyst stage. One healthy calf was born after embryo transfer. This is the first and only report of birth of a calf using epididymal sperm and ICSI.

Ng et al. (2002) used Cynomolgus monkey (*Macaca fascicularis*) epididymal sperm to produce embryos by ICSI. They reported 61% and 27% fertilization and pregnancy rates, respectively. Four monkeys became pregnant resulting in the birth of two healthy female offspring.

Richings et al. (2004) reported the use of ICSI with Tammar wallaby (*Macropus eugenii*) epididymal sperm. Oocytes were assessed for the presence of pronuclei and polar body extrusion and in vitro development was monitored for up to 4 days, resulting in 3 of 4 follicular oocytes and 4 of 8 tubal oocytes cleaving. Embryo development halted at the 4 to 8 cell stages. To date, this is the only known report of ICSI in marsupials.

Pushett et al. (2000) compared the developmental competence of cat embryos developed by IVF and ICSI using frozen-thawed epididymal sperm. They reported higher fertilization and blastocyst rates using ICSI from cryopreserved epididymal sperm than by IVF. Bogliolo et al. (2001) studied the developmental competence of in vitro matured (IVM) cat oocytes after ICSI with frozen-thawed epididymal sperm. A 7% blastocyst rate was reported 7 days after sperm-injection.

Probst and Rath (2003) reported the birth of 13 male piglets from sex-sorted frozen-thawed epididymal sperm and ICSI. Flow cytometrically sorted Y-chromosome bearing sperm were injected into in vivo matured oocytes, activated with 1.2 pl of a 30 mM CaCl$_2$ solution. Approximately 85 fertilized oocytes were transferred surgically into 4 recipient females.

Zheng et al. (2004) used fresh epididymal sperm collected from a fertile male rabbit to compare different sperm injection methods into oocytes. They reported that the
The highest blastocyst rate was achieved when the needle tip was pushed across half the diameter of the oocyte, and when oolema breakage was achieved by repeated aspiration and expulsion. Deng and Yang (2001) reported the birth of 7 live young from the transfer of 113 ICSI embryos to 6 rabbit recipients. The fertilization and blastocyst rates obtained were 78% and 39%, respectively.

Epididymal sperm for ICSI has been extensively used in humans for the last decade to treat male caused infertility (e.g., Tournaye et al., 1994; Devroey et al., 1995; Patrizio et al., 1995; Oates et al., 1996; Cha et al., 1997; Holden et al., 1997; Friedler et al., 1998; Garrels et al., 1998; Nudell et al., 1998; Pasqualotto et al., 2002; DeVos et al., 2003; Horak et al., 2003; Rhouma et al., 2003; Lin et al., 2004; Nicopoulos et al., 2004 and others). The ICSI procedure has revolutionized the treatment of male infertility because ICSI is effective not only in cases of severe oligozoospermia but also in cases of azoospermia, providing higher fertilization, pregnancy and implantation rates than that of standard IVF (Schwarzer et al., 2003a). Live births have been reported in humans by ICSI using frozen-thawed epididymal sperm recovered by PESA (Jin et al., 2004) and MESA (Drouineaud et al., 2003). Nagy et al. (1995) compared the use of human ejaculated, fresh and frozen-thawed epididymal and testicular sperm for ICSI. Fertilization rates were reported to be 56% for fresh, 56% for frozen-thawed epididymal and 48% for testicular sperm were used for the injection but these rates were significantly lower than 70% for ejaculated sperm.

Schwarzer et al. (2003a) reported results from 1,079 ICSI cycles with aspirated epididymal and testicular sperm. Epididymal sperm were used in 172 cycles and testicular sperm or spermatids in 907 cycles. Retrieved sperm were used after cryopreservation or immediately after aspiration. No significant difference was found between epididymal (frozen-thawed or fresh) and testicular sperm. A 28% birth rate resulted from these treatments. Ludwig and Katalinic (2003) analyzed 2,809 pregnancies with 3,199 fetuses/children reported in Germany from ICSI using either testicular, epididymal or ejaculated sperm. No statistical difference resulted from the origin of sperm. Bonduelle et al. (2002) reported the birth of 2,840 ICSI children using either ejaculated, epididymal or testicular sperm from 1983 to 1999 in Belgium. A year earlier, Abdul-Jalil et al. (2001) had reported the first ongoing clinical twin pregnancy resulting from ICSI using sperm retrieved by PESA into IVM oocytes. In recent years the recovery
of immature oocytes from unstimulated ovaries followed by IVM was found to be an attractive alternative to conventional IVF in the treatment of female infertility.

Burrello et al. (2003) evaluated the impact of sperm aneuploidy on ICSI outcome. Patients were divided into two groups based on sperm aneuploidy (<25% or >75% aneuploidy). Significantly higher clinical pregnancy (75 versus 34%; P<0.001) and implantation (34 versus 13%; P<0.001) rates were reported for the low and high aneuploidy frequency groups, respectively. Recently, Ramos et al. (2004) reported the use of computerized karyometric image analysis (CKIA) to select normal epididymal sperm for ICSI from males with obstructive azoospermia. CKIA evaluates DNA stainability, chromatin texture (nuclear condensation) and morphology. A two-fold increase in selecting normal sperm for ICSI is achieved by this system.

**INTRACYTOPLASMIC SPERM INJECTION IN CATTLE**

The first report of ICSI in cattle (Younis et al., 1989) was performed 13 years after the development of the basic ICSI technique by Uehara and Yahagimachi (1976). In this report, sperm decondensation and cleavage development of sperm-injected oocytes were evaluated using in vitro capacitated sperm and IVM oocytes. They reported a 2% cleavage rate (2/101) when oocytes were not exogenously activated. In addition, they stated that only 21% (21/101) of injected oocytes contained a sperm inside the ooplasm at the time of evaluation. The addition of 1 µM calcium ionophore (A23187) for 5 minutes to artificially activate the oocytes increased sperm decondensation 3 hours after sperm-injection from 0 to 37% (11/30) and cleavage rates 48 hours post-injection from 2 to 36% (5/14). A year later, Goto et al. (1990) reported the production of the first calf by ICSI using immobilized, killed cryopreserved bovine epididymal sperm. Oocytes were chemically activated post-injection with 50 µM calcium ionophore (A23187). This was the first report to evaluate embryonic development of ICSI embryo up to the blastocyst stage. Of 507 sperm-injected oocytes, only 9 (1.8%) reached the blastocyst stage.

The high inefficiency of the ICSI procedure in cattle led researchers to evaluate different chemical activation treatments and sperm pretreatments in order to increase pronuclear formation and blastocyst rates. Li et al. (1999) investigated the effect of activating sperm-injected oocytes with ethanol. In this study, sperm were decapitated by sonication. They reported a 15.1% and 26.4% sperm decondensation and pronuclei
formation at 18 to 20 hours post-injection, respectively. The use of sperm heads for ICSI will enable the use of flow cytometrically sorted sexed semen for gender preselection. Hamano et al. (1999) reported the birth of 8 male and 2 female calves using flow cytometrically sex sorted sperm heads. Oocytes were activated for 5 minutes in 7% ethanol once before and twice after sperm injection (at 30 and 60 minutes). Cleavage and blastocyst rates were 46.6% and 6.9%, respectively. They obtained a 20.8% pregnancy rate (10/48) from the blastocysts nonsurgically transferred into recipient females.

Sperm pretreatments were evaluated by Wei and Fukui (1999) in pursue for an answer for the low fertilization rates of ICSI oocytes in cattle. They investigated the effect of sperm type (dead, immotile or motile), sperm mechanical pretreatment (tail cutting or scoring) and sperm chemical pretreatment (heparin, heparin and caffeine, calcium ionophore (A23198) or dithiothreitol (DTT) on fertilization rates 20 hours after sperm injection. No significant difference was found when dead (21.8%), immotile (20.8%) or motile sperm (11.3%) without mechanical pretreatment were injected into oocytes. However, the rate of pronuclear formation with motile sperm tended to be lower than those with dead or immotile sperm. When motile sperm was used, tail scoring (36.4%) yielded higher pronuclear formation than the control at 11.3%. However, no significant difference was found between tail scoring (36.4%) and tail cutting (22.8%). All chemical pretreatment agents used significantly increased the formation of the male pronucleus 20 hours post-injection. Pretreatment with calcium ionophore (A23187) (58.9%) or DTT (61.3%) resulted in higher fertilization rates than those pretreated with heparin alone (32.8%) or the control (23.2%). Fertilization rates were also higher with heparin and caffeine treatment (46.4%) than in the control. In other studies DTT has also been demonstrated to enhance bovine male pronuclear formation and blastocyst rates (Rho et al., 1998a; Wei and Fukui, 2002; Galli et al., 2003; Ock et al., 2003).

The development of the piezo ICSI technique in the mouse by Kimura and Yanagimachi (1995) led to a dramatic increase in fertilization and blastocyst rates when compared with those of the conventional technique. With this novel technique, the zona pellucida and oolema are penetrated by rapid vibrations produced at the pipette tip caused by longitudinal wavelength produced by a piezo actuator. One of the main advantages of the piezo technique is that it causes less deformation to the oocyte during
injection. The oolema is easily broken without suction of the ooplasm and produces less ooplasm leakage increasing oocyte survival post sperm injection. Katayose et al. (1999) were the first to apply this technique in cattle and reported a dramatic increase in fertilization rates with piezo ICSI (55%) when compared with the conventional technique (0.05%) when no exogenous chemical activation was used. The addition of 50 µM calcium ionophore (A23187) to activate the oocytes increased fertilization rates for the conventional technique to 15%, while no beneficial effect was found in the piezo ICSI group (58%).

In contrast, a year later Suttner et al. (2000) reported in cattle no significant difference between the piezo and conventional ICSI techniques as assessed by pronuclear formation, cleavage and blastocyst rates. They evaluated different chemical agents to activate sperm injected oocytes. Ionomycin followed by 6-dimethylaminopurine (DMAP) produced the highest quantity of blastocysts when compared with calcium ionophore (A23187) and ionomycin followed by cycloheximide. This is in agreement with others where the highest quantities of blastocysts were produced by using ionomycin followed by DMAP (Ock et al., 2003). However, the use of ionomycin followed by DMAP was found to produce up to 67% chromosomal abnormalities as assessed by polyploidy and mixploidy (Ock et al., 2003). This high percentage of abnormal embryos was slightly reduced by allowing a 3 hour delay in DMAP treatment (46% mixoploid and polyploid). The occurrence of chromosomal abnormalities may be due to inhibition of extrusion of the second polar body, with some nuclei presumably reentering S-phase of the cell cycle without having passed through metaphase.

Horiuchi et al. (2002) produced 5 healthy calves from the transfer of 10 blastocysts activated with 7% ethanol 4 hours post-injection. They reported that ~60% of the sperm-injected oocytes were activated by the spermatozoon as assessed by second polar body extrusion 4 hours post-piezo ICSI. It was concluded that treating activated oocytes 4 hours post-injection with 7% ethanol for 5 minutes, increased subsequent embryo development. By treating activated oocytes with or without ethanol, they obtained 20% and 11.9% blastocyst rates, respectively. Moreover, they demonstrated that using killed sperm readily affects embryo development to the blastocyst stage when compared with motile sperm immobilized prior to injection. Only 0.8% of activated oocytes reached the blastocyst stage when killed sperm were used.
Fujinami et al. (2004) studied the effects of artificial activation with ethanol on kinetics of maturation promoting factor (MPF) activity and development of bovine oocytes following ICSI. They reported that MPF activity decreases immediately after sperm injection but elevates temporarily at 6 hours post-injection. This temporary elevation of MPF was inhibited when sperm-injected oocytes were treated with ethanol 4 hours post-injection. They obtained 14 and 4% blastocyst rates when sperm-injected oocytes were treated with or without ethanol 4 hours after sperm injection, respectively.

Wei and Fukui (2002), using the piezo ICSI technique, were the first to report the birth of 3 calves from ICSI derived embryos without any exogenous oocyte activation. They felt that the poor outcome in bovine ICSI could be improved by making technical improvements. The oocyte ooplasm was made clearer by centrifugation, the sperm tail was cut leaving the midpiece intact and the amount of polyvinylpyrrolidone (PVP) was reduced from 10% to 4% to ensure proper delivery of sperm into the oocytes. With these adjustments, they reported 78.2, 71.8 and 22.7% fertilization, cleavage and blastocyst rates, respectively.

Subsequently, Galli et al. (2003) also concluded that exogenous oocyte activation did not improve the development of piezo ICSI embryos. They obtained 15, 6 and 17% blastocyst rates when sperm-injected oocytes were not treated or were treated with ionomycin or ionomycin followed by cycloheximide, respectively. One healthy calf was produced by the nonsurgical transfer of 11 nontreated piezo ICSI blastocysts into 6 recipient females.

Rho et al. (2004) reported the use of frozen-thawed bovine oocytes for ICSI. Oocytes were cryopreserved by vitrification with copper electron microscope grid. They reported that blastocyst rates of 9.8% and 16.3% were significantly different when using frozen-thawed and fresh oocytes, respectively. They detected a significant difference (P<0.05) between the treatment groups. Total cell counts on day 8 post-injection were 99±19 and 124±21 for frozen-thawed and fresh oocytes, respectively. It was concluded that frozen-thawed bovine oocytes were suitable for ICSI to produce transferable embryos.

Freeze drying sperm has been attempted as an alternative method to preserve sperm in humans, (Katayose et al., 1992), hamsters (Hoshi et al., 1994), rabbits (Hoshi et al., 1994), mice (Wakayama and Yanagimachi, 1998), cattle (Keskintepe et al., 2002),
swine (Kwon et al., 2004) and rats (Hirabayashi et al., 2005). Live offspring have been reported in mice (Wakayama and Yanagimachi, 1998; Kusakabe et al., 2001; Kaneko et al., 2003; Ward et al., 2003), rabbits (Liu et al., 2004) and rats (Hirabayashi et al., 2005) after sperm injection of oocytes with freeze dried sperm.

Freeze drying is a procedure designed to achieve preservation by restricting the active water in a biological system. In this process frozen material is dried through the sublimation of ice (Keskintepe et al., 2002). In contrast, maintenance of cryopreserved sperm is higher in cost due to the need of a constant supply of liquid nitrogen (Kawase et al., 2005). Moreover, in some geographical areas, LN₂ is very costly or in some cases, not available to store sperm. Freeze drying allows the storage of sperm at room temperature or in a household refrigerator and sperm can be reconstituted by the addition of water when needed for ICSI (Wakayama and Yanagimachi, 1998). In cattle, Keskintepe et al. (2002) reported cleavage and blastocyst rates for oocytes injected with lyophilized sperm and activated with ionomycin followed by DMAP of 63.3% and 29.6%, respectively. No significant difference was detected with lyophilized sperm when compared with frozen-thawed sperm (72.4% and 34.2% cleavage and blastocyst rates, respectively).

Recently, heat drying was tried as an alternate way to preserve sperm (Lee and Niwa, 2006). The male gamete has been shown previously to have thermostability (Yanagida et al., 1991). Hoshi et al. (1992) reported that injecting rabbit sperm heated to 60°C for 30 minutes into oocytes resulted in the development of embryos up to the 8 cell stage. In addition, live offspring were produced in mice by injecting sperm that had been heated at 56°C for 30 minutes (Cozzi et al., 2001). The ability of sperm to withstand heating, led investigators to evaluate heat drying as a method to preserve sperm. It is a much simpler and less expensive way to preserve sperm than freeze drying and conventional cryopreservation (Lee and Niwa, 2006). In cattle, Lee and Niwa (2006) reported the development of ICSI blastocysts from sperm that had been heat dried in a conventional oven at 50°C for 8 hours and stored at 4°C for up to 1 year. However, a significant decline in blastocysts was found when heat-dried sperm was stored for longer than 1 month before ICSI when compared with that of the control sperm.

The highest pregnancy and birth rates to date were reported for piezo ICSI bovine embryos treated with 7% ethanol 4 hours after sperm injection (Oikawa et al.,
This study compared activation protocols for blastocyst development. They reported 8.0, 29.4 and 40.1% blastocyst rates for sperm-injected oocytes nontreated, treated with 7% ethanol 4 hours post-injection or with ionomycin followed by DMAP, respectively. Although the percentage of blastocysts was higher for ionomycin followed by DMAP group. The subsequent pregnancy and birth rates were significantly higher for the ethanol-treated group. One (12.5%) of the 11 recipients that received blastocysts produced following the ionomycin-DMAP activation became pregnant. However, blastocysts from the ethanol-treated group resulted in 10 of 17 (58.8%) pregnant recipient females. The birth rate per transferred embryo was 47.4% and 9.2% for the ethanol and ionomycin-DMAP-treated piezo ICSI oocytes, respectively. More recently, Horiuchi (2006) reported that 24 calves were produced by the nonsurgical transfer of 61 ICSI-derived blastocysts activated with ethanol 4 hours after sperm injection.

With the exception of the birth of a few calves from sperm-injected oocytes without the need of an exogenous activation stimulus (Wei and Fukui, 2002; Galli et al., 2003), ICSI techniques have failed to produce expected rates of embryonic and fetal development (Malcuit et al., 2006). Recently, Malcuit et al. (2006) attributed the ‘ICSI problem’ in bovine to a failure of the injected spermatozoon to initiate and maintain prolonged [Ca\(^{2+}\)] oscillations required for subsequent embryonic development. They noted that less than 10% of sperm-injected oocytes displayed any [Ca\(^{2+}\)] responses. It is thought that some of the signaling mechanisms that lead to the activation of the phosphoinositide pathway and generation of oscillations during natural fertilization are not replicated by the ICSI procedure. The possibilities are that the release of the sperm factor or the function and activation of the sperm factor after release may be compromised after ICSI (Malcuit et al., 2006). It was recently demonstrated that injected bovine sperm does not undergo comparable dissolution of the sperm perinuclear theca, as occurs during IVF (Sutovsky et al., 1997; Sutovsky et al., 2003).

**MULTICOLOR FLOW CYTOMETRY FOR SPERM QUALITY EVALUATION**

Most laboratory assays used to assess the fertilizing capacity of a semen sample before its use, have relied in the evaluation of motility, morphology and sperm concentration. Unfortunately, these routinely used laboratory assays have not been very successful in predicting the fertilizing capacity of sperm in a given sample (Gillian et al., 2005). Correlations between sperm motility and fertility and/or sperm morphology and
fertility have been reported to range from 0.15 to 0.83 and 0.06 to 0.86, respectively (Graham et al., 1980; Kjaestad et al., 1993; Stalhammar et al., 1994; Janaskuskas et al., 2003). Sperm require a number of events to occur to achieve fertilization, such as, ATP production for sperm movement, achieve hyperactivation, ability to capacitate, undergo the acrosome reaction, a functional plasma membrane, have the ability to recognize and bind the zona pellucida and possess a normal DNA complement (Gillian et al., 2005).

Multicolor flow cytometry is a powerful tool for evaluating the quality of sperm in a given sample. This technology works by forcing individual sperm cells into a confined stream of fluid that passes through the beam of a laser that will cause any fluorescent stain associated with the sperm cell to fluoresce (Graham, 2001). Photomultiplier tubes permit the determination of specific wavelengths emitted by individual compartments in each sperm cell that have been previously stained with a fluorescent dye (Graham and Moce, 2005). This permits the observation of physical characteristics including cell size, shape and internal complexity (Gillian et al., 2005). The advantage of flow cytometry is that thousands of cells can be evaluated in less than a minute, the amount of fluorescent stain associated with cells is measured in an unbiased manner, it offers a high level of accuracy and experimental repeatability, and can evaluate multiple sperm characteristics at the same time (Graham, 2001; Gillian et al., 2005). Moreover, fixed or live sperm cells can be used for evaluation and a large variety of fluorochromes are currently available to measure most sperm characteristics (Graham and Moce, 2005). Using flow cytometric assays, cell viability, capacitation status, acrosome integrity, mitochondrial function and DNA integrity can be evaluated.

Viability

Cell viability is referred in most cases to whether or not the plasma membrane of the spermatozoon is intact. One of the consequences of membrane disruption is the loss of important intracellular components, such as metabolic enzymes and ATP (Graham and Moce, 2005). In addition, plasma membrane integrity status is of extreme importance due to its role in interacting with the female genital tract, the outer vestments of the cumulus oocyte complex and fusion with the ooplasm (Rodriguez-Martinez, 2003). Many different fluorochromes have been used to assess the integrity of the sperm plasma membrane. This can be achieved by using fluorochromes that select viable or nonviable sperm. Viable sperm can be measured using membrane permeable stains,
which are converted into a nonpermeant fluorescent compound by esterases in the cytoplasm of ‘live’ cells. The most used enzyme based compounds are fluorescein diacetate (FDA) (Resli et al., 1983), 6-carboxylfluorescein diacetate (CFDA) (Garner et al., 1986), 6-carboxylmethylfluorescein diacetate (CMFDA) (Thomas and Garner, 1994) and calcein acetoxy methyl ester (CAM) (Kato et al., 2002). However, most recent assessments of bovine sperm viability have used the membrane permeable DNA fluorochrome, SYBR 14 (Garner et al., 1994). Viability assessments using nucleic acid stains are considered less variable than enzyme-based stains. Enzyme-based stains suffer from time dependency problems because they are based on enzyme substrate conversion to a fluorescent product (Garner et al., 1995). Also, sperm DNA is considered to be a more appropriate cellular target due to its stainability and staining uniformity (Gillian et al., 2005).

The percentage of nonviable cells in a sperm sample can be measured using membrane impermeable nucleic acid stains, which identify nonviable sperm by entering cells with ruptured membranes. The most commonly used are Hoechst 33258 (Pintado et al., 2000), ethidium homodimer-1 (Althouse and Hopkins, 1995), YO-Pro (Kavak et al., 2003) and propidium iodide (PI) (Matyus et al., 1984). Each fluorochrome has different excitation and emission ranges making it suitable for multicolor assays. Nowadays, viability using a flow cytometer is routinely assessed by dual staining sperm with the combination of a membrane permeable and membrane impermeable fluorochrome. The two most common combinations are CFDA with PI or SYBR 14 and PI. CFDA is converted to a green fluorescent molecule by esterases in the cytoplasm of viable sperm, while PI is a red fluorescent molecule that labels the nucleus of sperm with damaged membranes. SYBR 14 is also a green fluorescent molecule that labels the nucleus of membrane intact sperm. By staining with any of these two combinations, two populations of sperm can be identified. Events exhibiting brighter green fluorescence in the FL1 channel are considered ‘live’ sperm, while events exhibiting high red fluorescence in the FL3 channel are considered ‘dead’ sperm.

Before 1986, the supravital stains used to assess viability, such as eosin Y alone or in combination with nigrosin or elanin blue, were not appropriate for analyzing cryopreserved sperm because glycerol interfered with the stains (Mixner and Saroff, 1954). Sperm from bulls, boars, dogs, horses, mice and humans were evaluated to
examine the use of CFDA in combination with PI using flow cytometry (Garner et al., 1986). They demonstrated that motile sperm retained the CFDA fluorochrome throughout the cytoplasm of the cell and did not stain with PI. However, some immotile sperm retained the CFDA fluorochrome in the cytoplasm. The authors claim that this small population of sperm was composed of moribund sperm. Moreover, the percentage of sperm that were positive for CFDA increased after a swim-up procedure. They found a high correlation in the percentage of CFDA-positive sperm and progressive motility. In addition, they reported that the major advantage of using this dual staining protocol was that cryopreserved sperm containing glycerol could be analyzed.

Garner et al. (1994) were the first to use SYBR 14 in combination with PI to evaluate viability in sperm. They validated the assay by evaluating mixtures of nonviable and viable sperm. Viable bovine sperm were prepared by filtration through glass wool and Sephadex to remove nonviable sperm. A sample from the viable population was killed by repeated freezing and thawing without cryoprotectants. A mixture of 100:0, 75:25, 50:50, 25:75 and 0:100 of the filtered:killed samples were used to validate the viability assay. The percentages of viable sperm for the ratios evaluated, as determined by the green fluorescence of the SYBR 14 emitted in the FL1 channel, were: 85.1, 68.8, 39.8, 20.7, and 1.4%, respectively. Subsequently, Garner et al. (1995) evaluated the viability assay in the mouse, boar, human, ram and rabbit. They reported that this flow cytometric assay detected subtle differences between males in all the species evaluated. To date, this staining combination has been shown to be a reliable way for determining the proportions of viable and nonviable sperm in a given semen sample (Donoghue et al., 1995; Maxwell et al., 1996; Garner et al., 1997; Maxwell and Johnson, 1997; Thomas et al., 1998; Merkies et al., 2000; Garner et al., 2001; Love et al., 2003; Guerrero et al., 2006; Saragusty et al., 2006). Viability stains can be used in combination with other stains for evaluating multiple quality traits at the same time by using a flow cytometer capable of detecting 3 or more colors.

**Capacitation**

For a sperm to undergo the acrosome reaction and achieve fertilization, the sperm must first become capacitated. Capacitation is the process in which changes occur within the sperm, such as cholesterol efflux from the plasma membrane (Langlais and Roberts, 1985), increase in intracellular [Ca^{2+}] (Parrish et al., 1999), bicarbonate,
protein phosphorylation and a decrease in intracellular pH. Cholesterol efflux and the increase in disorder in lipid packaging leads to an increase in plasma membrane fluidity (Gadella et al., 1999), which can be measured with the hydrophobic fluorochrome Merocyanine 540 (M540). These changes lead to destabilization of the membrane, which eventually leads to the acrosome reaction required to penetrate the outer vestments of the oocyte. M540 is believed to detect a decreased packing order of phospholipids in the outer leaflet of the plasma membrane lipid bilayer (Williamson et al., 1983). Kavak et al. (2003) reported that the quantity of capacitated sperm increased significantly after the incubation of bull sperm in a bicarbonate containing medium. It is thought that membrane fluidity changes precede the increase in intracellular [Ca\(^{2+}\)], making M540 a better method for evaluating the early events of capacitation (Rathi et al., 2001).

**Acrosome Integrity**

An intact acrosome is required for a sperm to penetrate the cumulus oophorus cells and to attach and penetrate the zona pellucida of the oocyte at the time of fertilization (Franklin et al., 1970). The acrosome reaction involves vesiculation of part of the outer acrosome membrane and the overlaying plasma membrane (Bedford, 1968). After vesiculation and release of the hydrolytic enzymes stored in the acrosome, some sugar moieties, such as α-mannose and β-galactose remain exposed to the external environment. There are a large number of plant lectins currently available that can be used to detect reacted acrosomes by attaching to exposed sugar moieties in the reacted acrosome compartment. A strong correlation between fertility and the percentage of bovine sperm exhibiting intact acrosomes has been previously reported (Berndtson et al., 1981).

Flow cytometry can be used to detect the percentage of sperm exhibiting a reacted acrosome by using plant lectins labeled by a fluorescent probe (Gillian et al., 2005). The most common plant lectins used to evaluate acrosome integrity are *Pisum sativum* agglutinin (PSA) (Graham et al., 1990) and *Arachis hypogaea* agglutinin (PNA) (Vasquez et al., 1990). PSA binds to α-mannose and β-galactose moieties in the acrosome compartment, while PNA binds more specifically to β-galactose moieties. Since these lectins are not membrane permeable, only acrosome reacted or damaged sperm will fluoresce (Farlin et al., 1992). The use of PSA to assess acrosome integrity has been widely used (e.g., Cross and Meizel, 1989; Graham et al., 1990; Kinger et al.,
1995; Margalit et al., 1997; Pena et al., 1999; Pena et al., 2001), however, PNA is preferred because it exhibits less nonspecific binding to other areas of the sperm and has less affinity to egg yolk particles when working with cryopreserved sperm (Dalimata and Graham, 1997; Thomas et al., 1997).

Plant lectins can be conjugated to many different commercially available fluorescent probes in order to make them suitable for multicolor flow cytometric assays (Graham, 2001). PI in combination with fluorescein isothiocyanate (FITC) PSA (Sukardi et al., 1997) or FITC-PNA (Rathi et al., 2001) has been widely used to discriminate between nonviable acrosome reacted and viable acrosome reacted sperm. Recently, Nagy et al. (2003) developed a triple fluorochrome staining technique that involves the well validated SYBR 14 and PI, with PNA conjugated to phycoerythrin (PE). PE fluorescent moiety was used instead of FITC, because its fluorescence emission could be measured independently from that of SYBR 14 or PI. This novel technique was shown to assess viable acrosome intact, viable acrosome reacted, nonviable acrosome intact and nonviable acrosome reacted sperm in a single assay.

Acrosome integrity can also be measured using the acidophilic probe LysoTracker Green DND-26 (LysoTracker). This probe works by labeling acidic organelles, such as the intact acrosome. Thomas et al. (1997) found a high correlation between LysoTracker and the lectin PNA for determining acrosome integrity of bovine sperm. In addition, they reported a high correlation between the percentages of sperm stained positive for LysoTracker and sperm displaying normal acrosome ridges.

Mitochondrial Function

Motility is currently the most commonly used to evaluate sperm quality in commercial bull stations. At best it is an indicator or an indirect measure of metabolic activity and cell viability (Hallap et al., 2005). Mitochondria are the major organelles for the production of adenosine triphosphate (ATP) required for sperm motility (Bartoov et al., 1980). ATP production is very important for maintaining flagellar movement for a long period of time. This is required for sperm to reach the site of fertilization (Windsor, 1997). Changes in mitochondrial membrane potential has been proposed to be a good indicator of sperm functional impairment (Pena et al., 2003). Pyruvate and lactate are produced from glycolysis in the flagella and diffused to the midpiece where mitochondria metabolize them to produce ATP via respiration (Mukai and Okuno, 2004). Flagellar
movement is the product of dynein ATPase activity that is localized along the entire flagellum and depends on the supply of ATP (Mukai and Okuno, 2004).

There are ~100 mitochondria in the midpiece of each mammalian sperm, and they can be visualized with various fluorochromes (Hallap et al., 2005). The evaluation of mitochondrial function in a sperm sample should give us a more objective assessment of the energetic status of the cells (Martinez-Pastor et al., 2004). Furthermore, mitochondrial activity has been related to cell motility (Auger et al., 1989; Garner et al., 1997; Thomas et al., 1998; Garner and Thomas, 1999; Garner et al., 2001; Martinez-Pastor et al., 2004; Nunez et al., 2004; Hallap et al., 2005; Januskauskas et al., 2005), viability (Garner et al., 1997; Januskauskas et al., 2005) and fertility (Kasai et al., 2002). A number of commercially available fluorochromes have been used to assess mitochondrial status of sperm. Rhodamine 123 (R123) is a cationic compound that excites at 488 nm and emits at 515-575 nm (Evenson et al., 1982). R123 is transported into actively respiring mitochondria and their accumulation in the mitochondria causes them to fluoresce green (Graham, 2001). The mitochondrial inhibitors, monensin and rotenone, were shown to decrease the uptake of R123 by sperm (Graham et al., 1990). In humans, Auger et al. (1989) found a strong correlation between sperm motility and R123 uptake during a 24-hour incubation period. Also, Graham et al. (1990) reported that R123 was accumulated and retained most intensively in fully active bovine sperm mitochondria. Mitochondria from nonviable sperm did not uptake R123. They demonstrated that sperm treated with sodium fluoride (to inhibit glycolysis) displayed positive R123 staining as a positive control. R123 is considered not to be very reliable due to its low sensitivity, variability within samples and background staining (Garner et al., 1997). In addition, its fluorescence is lost when mitochondria experience losses of membrane potential, thus limiting its use for fixation. R123 also has been reported to have a higher nonspecific affinity to other parts of the sperm than other mitochondrial fluorochromes (Garner et al., 1997).

MitoTracker Probes offers a wide range of mitochondrion selective probes, such as MitoTracker Orange CMTMROS, MitoTracker Red CMXRos and MitoTracker Deep Red 633. These probes differ in spectral characteristics making them suitable for multicolor flow cytometric analyses. These fluorochromes work by accumulating in active mitochondria and are well retained during fixation. They contain a mildly thiol-reactive
chloromethyl moiety that is responsible for keeping the fluorochrome associated with the mitochondria after fixation. Hallap et al. (2005) used MitoTracker Deep Red 633 in combination with SYBR 14 to evaluate mitochondrial activity in bovine sperm. They reported a high correlation with motility both after cryopreservation and the swim-up procedure. Furthermore, no interference with glycerol or egg yolk from the freezing extender was observed. Although functioning mitochondria are stained with these fluorochromes, these stains do not permit one to differentiate among mitochondria that exhibit different respiratory rates (Graham, 2001).

The lipophilic cationic fluorescent carbocyanine dye, JC-1, has been used to evaluate mitochondrial membrane potential in sperm in the human (Kasai et al., 2002), bull (Garner et al., 1997; Garner and Thomas, 1999), stallion (Grevance et al., 2000), rat (Grevance et al., 2001), boar (Huo et al., 2002), Iberian Red deer (Martinez-Pastor et al., 2002) and ram (Martinez-Pastor et al., 2004). This fluorochrome permits the distinction of mitochondria exhibiting low or high membrane potential (Thomas et al., 1998). This fluorochrome can reversibly change its emission from green to red with increasing mitochondrial membrane potential (Garner and Thomas, 1999). In mitochondria with low membrane potential, JC-1 forms monomers which emit in the green wavelength when excited at 488 nm. In mitochondria exhibiting high membrane potential, JC-1 forms aggregates emitting in the orange-red wavelength when excited at 488 nm (Pena et al., 2003). In humans, in vitro fertilization rates were reported to be higher for sperm exhibiting high mitochondrial membrane potential (Kasai et al., 2002). Furthermore, a relationship was found between high membrane potential and fertility in humans (Troiano et al., 1998). However, JC-1 appears to be not suitable for multi-parameter assays due to its emission in both the green and red wavelengths. Moreover, Garner et al. (1999) reported that it does not work effectively on frozen-thawed bovine sperm, probably due to interactions with egg yolk particles from the freezing extender.

DNA Integrity

The nucleus of normal sperm has a highly condensed chromatin structure (Yanagimachi and Noda, 1970). The major function of the male sperm cell is to transfer genetic information to the future zygote. In mammals, the sperm nucleus is transcriptionally inactive (Imschenetzky et al., 2003) and its chromatin is compacted at a degree of condensation 6-fold higher compared with a somatic cell nucleus (Ward and
Coffey, 1991). This is achieved by the replacement of somatic histones by a series of basic proteins and finally by protamines (Ward and Coffey, 1991). This structure of protamine complexed DNA is likely important for protection of the genetic information (Ward and Coffey, 1991). Various studies have demonstrated that chromatin structure is related to the fertilizing ability of sperm, serves as a biomarker for exposure to toxic chemicals and to detect disturbances during spermatogenesis (Evenson, 1986; Ballachey et al., 1987; Dobrinski et al., 1994; Evenson and Jost, 1994). DNA fragmentation can be caused during spermatogenesis by apoptosis caused by activation of endonucleases or during ejaculation by reactive oxygen species (Evenson, 1999).

The DNA integrity of sperm can be determined by the sperm chromatin structure assay (SCSA®) (Evenson et al., 1980). The SCSA® is a flow cytometric assay that utilizes the metachromatic properties of the dye acridine orange (AO), staining single stranded DNA red and double stranded DNA green (Evenson et al., 1980). It is based on the principle that sperm with an abnormal chromatin structure have a greater susceptibility to physical induction of partial DNA denaturation in situ (Darzynkiewicz et al., 1975). The extent of DNA denaturation following acid treatment is measured by the ratio of red to total fluorescence providing an index of normality to abnormality. The percentage of sperm exhibiting denatured DNA has been related to high, moderate or very low fertility rates when a semen sample has DNA damage between 0 to 15%, 16 to 29% and >30%, respectively (Evenson and Jost, 2000). The SCSA® has been used in the bull (Evenson et al., 1980; Ballachey et al., 1987; Bocheneck et al., 2001; Januskauskas et al., 2003), rat (Evenson et al., 1983), mouse (Evenson et al., 1993; Sailer et al., 1997), boar (Evenson et al., 1994; Boe-Hansen et al., 2005), Rhesus monkey (Sivashanmugam and Rajalakshmi, 1997), stallion (Love and Kenney, 1998; Love, 2005; Dias et al., 2006), human (Spano et al., 1999; Larson et al., 2000; Saleh et al., 2002; Boe-Hansen et al., 2006; Evenson, 2006), rabbit (Gogol et al., 2002), cat (Penfold et al., 2003), ram (Peris et al., 2004) and dog (Nunez-Martinez et al., 2005), and has shown a good correlation with fertility in mice (Evenson et al., 1980), bulls (Evenson et al., 1980; Ballachey et al., 1988; Karabinus et al., 1990; Sailer et al., 1996), boars (Evenson et al., 1994), stallions (Love and Kenney, 1998) and humans (Evenson et al., 1999; Spano et al., 2000; Evenson and Jost, 2000; Virro et al., 2004).
CHAPTER III

EFFECT OF STORAGE AND CRYOPROTECTANT ADDITION AND REMOVAL AT 4°C ON MOTILITY AND PLASMA MEMBRANE INTEGRITY OF BOVINE CAUDAL EPIDIDYMAL SPERM

INTRODUCTION

Cryopreservation of sperm harvested from the epididymides would salvage germplasm from genetically valuable males that are injured and can no longer mate or have unexpectedly died. Over the past few decades, most research on sperm cryopreservation has focused on comparative studies, such as changing cryoprotectant (CPA) concentration, additives, cooling and warming rates and extender composition (Holt, 2000). Although these studies have helped to improve sperm quality in a fixed set of circumstances, they fail to identify important problems in sperm cryobiology, and are of limited use in performing major advances in this area (Holt, 2000). In an effort to develop a cryopreservation protocol for epididymal sperm, there needs to be a better understanding of the underlying fundamental cryobiological properties of epididymal sperm. Studies of membrane permeabilities, osmotic tolerance limits and on theoretical approaches to predict minimally damaging cooling protocols have been successful in allowing post-thaw sperm recovery (Gao et al., 1995; Guthrie et al., 2002).

Since the first use of glycerol for sperm cryopreservation by Polge et al. (1949), the development of a more complete understanding of the mechanisms by which CPAs protect cells during cooling and warming has been emphasized (Gilmore et al., 1997). It is well known that the addition of CPAs is essential for sperm optimal survival following the freezing process. However, CPAs can cause loss in sperm viability due to osmotic damage or from true chemical toxicity. The addition of CPAs before freezing and their removal after warming creates an anisotonic environment, resulting in potentially damaging osmotically driven cell volume changes (Guthrie et al., 2002). During the addition of permeating CPAs, sperm are exposed to a hypertonic environment, resulting in cell shrinkage. During CPA removal, sperm are exposed to a hypotonic environment, resulting in cell swelling. Such changes in solution osmolality can cause loss in the functional integrity of sperm (Guthrie et al., 2002; Ball and Vo, 2001). It has become evident that sperm from males of different species possess different sensitivities to
osmotic damage. Thus, it is of critical importance that the osmotic behavior of bovine epididymal sperm be characterized to develop a successful cryopreservation protocol.

Tolerance to osmotic stress during the addition and removal of CPAs is highly predictive of sperm survival after cryopreservation (Holt, 2000). This suggests that sperm survival can be optimized by minimizing osmotic shock. There is limited information regarding osmotic properties and cryopreservation of bovine caudal epididymal sperm. Epididymal sperm and its ejaculated counterpart likely react differently to cryoprotective agents due to differences in morphology and membrane properties. Cytoplasmic droplets found on epididymal sperm are thought to cause an increase in plasma membrane damage and cause bent tails during cryopreservation. Theoretical predictions indicated that the optimal CPA would be one that could permeate the cell in the shortest time causing the least amount of volume excursion during the addition and removal of the CPA (Gilmore et al., 1995; Gao et al., 1995; Gilmore et al., 1997). Thus, this problem might be overcome by using CPAs with higher membrane permeabilities.

Considerable research efforts in rams (Curry and Watson, 1994), humans (Gilmore et al., 1995; Gilmore et al., 1997), bulls (Liu and Foote, 1998; Guthrie et al., 2002), boars (Gilmore et al., 1998), mice (Phelps et al., 1999) and stallions (Ball and Vo, 2001) have focused the attention to the osmotic stress attributed to the relative permeabilities of different CPAs and water. However, no information is yet available regarding addition and removal of CPAs in bovine epididymal sperm.

The use of ethylene glycol to cryopreserve different cell types has widely increased after its first use with human erythrocytes by Lovelock (1954). Ethylene glycol was found to have the highest membrane permeability of the most commonly used CPAs (Gilmore et al., 1997; Gilmore et al., 1998). In swine, the rapid movement of ethylene glycol through the plasma membrane was shown to cause less volume excursions than glycerol (Gilmore et al., 1998). In the present study, we tested the effects of addition and removal of two different concentrations of ethylene glycol and the commonly used CPA, glycerol to select the cryoprotectant that offers the least osmotic damage to epididymal sperm for future cryopreservation studies.

Another problem associated with permeating CPAs is the toxicity that they may incur in sperm during the equilibration process prior to cryopreservation. Due to the
different protocols used in research laboratories, the length of sperm exposure to CPAs varies widely. Sperm are usually in contact with permeating cryoprotectants for hours before freezing. Prolonged exposure to cryoprotectants will likely shorten the life span of sperm due to chemical toxicity. Field conditions often preclude the immediate availability of liquid nitrogen, necessitating the development of alternative short term storage methods. To our knowledge, no study has investigated the quality of bovine epididymal sperm during cool storage (4°C) for an extended length in a common Tris based extender. In addition, the toxicity of prolonged exposure of different concentrations of glycerol and ethylene glycol to bovine epididymal sperm during cool storage has not been evaluated to date.

The objectives of this study were: (1) to assess the effect of addition and removal of different concentrations of permeating cryoprotectants to epididymal sperm at 4°C (2) to test the toxicity caused by permeating cryoprotectants during prolonged exposure to epididymal sperm during cool storage and (3) to investigate viability parameters of epididymal sperm stored at 4°C for 5 days.

MATERIALS AND METHODS

Experimental Design

Experiment 3.1

This experiment was conducted to determine the effect of the addition and the removal of different concentrations of permeating CPAs on subsequent sperm quality. Testes pairs were obtained from 10 mature bulls of various breed types at a local abattoir and transported to the laboratory within 4 to 6 hours postmortem. Epididymal sperm were harvested by multiple incisions from the caudae epididymides of each pair of testes, then pooled and cooled in egg yolk Tris-glucose citric acid monohydrate extender (EYT-GC) to 4°C at ~0.1°C/minute.

Pooled sperm from each bull were allocated to each of five treatment groups: Control (no CPA), 7% glycerol (GLY), 14% GLY, 7% ethylene glycol (EG) and 14% EG. Replicate samples (n=2) from each bull were diluted 1:1 in EYT-GC extender containing twice the final desired concentration of CPA. After 10 minutes, each sample was diluted directly into EYT-GC at 4°C. Motility was assessed using a computer assisted semen analysis system and sperm plasma membrane integrity was evaluated using fluorescent microscopy following staining with SYBR 14 and propidium iodide.
Experiment 3.2

This experiment was conducted to determine the toxic effect of permeating CPAs during 5 days of storage at 4°C on sperm quality. Replicate samples (n=2) from Experiment 3.1 were diluted 1:1 in EYT-GC extender containing twice the final desired concentration of CPA and stored at 4°C for 5 days. A 0.5 ml sample from each treatment group was taken on days 0, 1, 3 and 5 and diluted directly into Brackett-Oliphant medium to assess sperm quality. Motility was assessed using a computer assisted semen analysis system and plasma membrane integrity was determined using fluorescent microscopy.

Experimental Procedure

Testes Collection

Bovine testes were collected as pairs from mature mix breed bulls (n=10) at an abattoir in Robert, Louisiana. Testes were transported to the Louisiana State University Veterinary School individually packed in Ziploc® plastic bags (Johnson & Son, Inc., Racine, WI) in a Styrofoam box at a temperature that ranged between 25°C to 28°C within 4 to 6 hours postmortem. Immediately upon arrival to the laboratory, each pair of testes was dissected away from its scrotum and tunica vaginalis (Figure 3.1). Testes were positioned with the vas deferens facing ventrally and the corpus facing up to distinguish the side of origin of each testis. Bulls with anatomically abnormal testes were not used in this study.

Medium Preparation and Use

Egg yolk Tris-glucose citric acid monohydrate extender (EYT-GC) (250 mM Tris (T-6066, Sigma-Aldrich Inc., St. Louis, MO), 69 mM glucose (6152, Sigma-Aldrich Inc., St. Louis, MO), 81 mM citric acid monohydrate (C-1909, Sigma-Aldrich, St. Louis, MO) and 20% (v/v) dried egg yolk (E-0625, Sigma-Aldrich, St. Louis, MO) was prepared with the addition of 50 µg/ml of gentamicin (15750-060, Gibco, Grand Island, NY). Extender was centrifuged at 20,000 x g in an ultra-fast centrifuge to remove large egg yolk components. The supernatant was placed in a 50 ml plastic tube (BCT-P50RS, Biologix, Shawnee Mission, KS) and warmed up to 37°C in a water bath 1 hour prior to epididymal sperm retrieval.
Figure 3.1. The process of scrotum dissection. A. Pair of bovine testes inside the scrotum. B. Dissection of the scrotum to retrieve the testes.
Brackett-Oliphant medium (BO) was prepared as previously described (Brackett and Oliphant, 1975). Briefly, BO-A stock solution contains 150.2 mM NaCl (S-5886, Sigma-Aldrich, St. Louis, MO), 3.0 mM CaCl₂·2H₂O (C-7902, Sigma-Aldrich, St. Louis, MO), 1.0 mM NaH₂PO₄·H₂O (S-9638, Sigma-Aldrich, St. Louis, MO), 0.6857 mM MgCl₂·6H₂O (M-2393, Sigma-Aldrich, St. Louis, MO) and 0.1 ml of 0.5% phenol red (P-0290, Sigma-Aldrich, St. Louis, MO) in a final volume of 500 ml of Milli-Q water. BO-B stock solution contains 154.0 mM NaHCO₃ (S-8875, Sigma-Aldrich, St. Louis, MO) and 0.04 ml of 0.5% phenol red in a final volume of 200 ml of Milli-Q water.

On the day of use, BO-AB medium was prepared by mixing 38 ml of BO-A stock and 12 ml of BO-B stock with the addition of 1.2 mM pyruvic acid (P-4562, Sigma-Aldrich, St. Louis, MO), 3 mg/ml of bovine serum albumin (Fraction V, A-4503, Sigma-Aldrich, St. Louis, MO) and 50 µ/ml of gentamicin. Medium was warmed in an incubator at 37°C 1 hour prior to epididymal sperm collection.

Epididymal Sperm Retrieval

Each cauda was dissected from the testis by cutting the vas deferens and corpus epididymis (Figure 3.2). Upon dissection, each cauda was rinsed with 0.9% saline to remove any remaining materials before epididymal sperm extraction. Epididymal sperm were harvested by making 5 to 6 incisions using a surgical blade (MDS-15110, Medline Industries Inc., Mundelein, IL) in the cauda epididymides. The incisions were rinsed with 4 ml of BO medium into a 60 mm Falcon® plastic petri dish (3037, Becton Dickinson Labware, Franklin Lakes, NJ). The caudae were placed on the 60 mm plastic dish for 10 minutes to allow sperm to swim out into the medium (Figure 3.3). The sperm solution was transferred into a 15 ml plastic tube (2071-024, VWR International Inc., Sugar Land, TX). Specimens were centrifuged twice at 500 x g for 5 minutes to wash the sperm. The pellet was resuspended in 8 ml of EYT-GC extender and placed for 15 minutes in a 37°C water bath for sperm quality analyses.

Epididymal Sperm Analyses

Sperm analyses included plasma membrane integrity, overall motility and progressive motility. A 100-µl sample from the sperm solution was placed in a 1.7 ml plastic micro-centrifuge tube (2070-355, VWR International Inc., Sugar Land, TX) and diluted 1:10 sperm solution:EYT-GC for epididymal sperm quality analyses.
Figure 3.2. The process of cauda epididymides dissection. A. Pair of bovine testes outside the scrotum and tunica vaginalis. B. Three pairs of testes showing the dissected cauda epididymides. Each cauda was dissected from the testis by cutting the vas deferens and corpus epididymis.
Figure 3.3. The process of epididymal sperm retrieval from a dissected cauda epididymis. The cauda was placed on a 100 mm plastic dish for 10 minutes to allow sperm to swim out into the collection medium.
Plasma membrane integrity was assessed using the LIVE/DEAD® Sperm Viability Kit (L-7011, Molecular Probes Inc., Eugene, OR). The kit consists of two nucleic acid stains. SYBR 14 (1 mM solution in DMSO, Component A of the LIVE/DEAD® Sperm Viability Kit) is a permeable nucleic acid stain, which labels membrane intact cells green. PI (2.4 mM solution in H₂O, Component B of the LIVE/DEAD® Sperm Viability Kit) is a nonpermeable nucleic acid stain, which labels membrane ruptured sperm red. For analysis, 250 µl of the diluted sperm sample were placed in a 0.65 ml plastic micro-centrifuge tube (20170-293, VWR International Inc., Sugar Land, TX). Staining was conducted by the addition of 400 nM SYBR 14 and 24 µM PI to the sperm solution. The stained samples (n=10) were incubated for 10 minutes in the dark at 37°C and mixed gently before analyses.

For plasma membrane integrity, samples (n=10) were analyzed by counting 300 sperm with an inverted microscope (Nikon Diaphot, Tokyo, Japan) equipped with epi-fluorescent illumination, a fluorescein isothiocyanate (FITC) filter set and a heated stage.

Sperm progressive motility was assessed by computer assisted sperm analysis (CASA, Minitube of America, Verona, WI) equipped with a heated stage.

Epididymal Sperm Cooling

Sperm concentration was adjusted to 120 x 10⁶ cells/ml using a hemacytometer by the addition of more extender. The 15 ml plastic conical tube containing the epididymal sperm sample was placed in a 250 ml Pyrex® glass beaker (13912-207, VWR International Inc., Sugar Land, TX) containing water. The beaker was placed in a refrigerator for cooling to 4°C for 4 hours.

The cooled sperm sample was thoroughly mixed using a plastic pipette and placed equally (3 ml) into 5 previously cooled plastic tubes. Specimens were allocated into 5 treatment groups: Control (no CPA), 7% GLY, 14% GLY, 7% EG and 14% EG. Replicate samples (n=2) of cooled epididymal sperm were diluted 1:1 in EYT-GC extender containing twice the final desired concentration of CPA. After being exposed for 10 minutes, a 0.5 ml sample from each treatment group was removed and diluted directly into EYT-GC at 4°C. Duplicate samples from each treatment group were stored at 4°C for 5 days. A 0.5 ml sample from each treatment group was removed on days 0, 1, 3 and 5 and then diluted directly into BO medium to assess CPA toxicity.
Subsequently, plasma membrane integrity and motility were analyzed as described above to evaluate the effect of addition and/or removal and toxicity of the different concentrations of CPAs.

**Statistical Analysis**

Variances in membrane integrity and overall and progressive motility were statistically analyzed by ANOVA and the differences between bulls were calculated by a Tukey Multiple Comparisons test. The sperm parameters are expressed as mean±SEM per treatment group. A P<0.05 value was considered significant in this study. Data were analyzed using SigmaStat Statistical Software Version 2.0.

**RESULTS**

Experiment 3.1

**Overall and Progressive Motility**

The mean initial overall motility and progressive motility values before epididymal sperm cooling were 75.1±1.3% and 58.9±2.4%, respectively. After cooling to 4°C, these mean values declined to 72.9±1.6% and 53.5±3.2%, respectively. No significant detrimental effect (P>0.05) was detected during the cooling process of epididymal sperm as assessed by both motility parameters. After CPA addition and removal, the mean overall motility values for each treatment group were: 49±3.8, 23±4.5, 64±2.8, 48 ±4.4% for 7% GLY, 14% GLY, 7% EG and 14% EG, respectively. The mean progressive motility values for each treatment group were: 30±3.8, 10±3.9, 46±3.9 and 31±4.0% for 7% GLY, 14% GLY, 7% EG and 14% EG, respectively. No significant decline in sperm overall and progressive motility was detected with the addition and removal of 7% EG. However, a significant decline (P<0.05) in both motility parameters was noted in the 14% EG group, 7% GLY group and 14% GLY group.

**Membrane Integrity**

No significant damage to the plasma membrane of sperm was detected during the cooling process. Plasma membrane integrity values of epididymal sperm before and after cooling to 4°C were 83.2±1.1% and 78.1±2.5%, respectively. After CPA addition and removal, the mean plasma membrane integrity values for each treatment group were: 54±3.0, 29±3.0, 70±2.5 and 48±3.7% for 7% GLY, 14% GLY, 7% EG and 14% EG treatment groups, respectively. No significant difference was detected in the 7% EG group when compared with the control group. However, the plasma membrane was
damaged significantly in the 14% EG and 7% and 14% GLY groups when compared with the control group.

Due to intermale variations, membrane integrity values ranged from 60 to 84%, 33 to 74%, 35 to 74% and 18 to 46% for 7% EG, 14% EG, 7% GLY and 14% GLY treatment groups, respectively. There was a high variability in percentage of sperm with intact membranes between males exposed to the same treatment groups. Some bulls were apparently more sensitive to osmotic damage caused by the high concentration of CPAs than others.

**Experiment 3.2**

**Overall and Progressive Motility**

The mean overall and progressive motility values during the 5 days of cool storage are shown in Figure 3.4 and Figure 3.5, respectively. No significant decline in overall and progressive motility was detected during the first 24 hours of storage for the control and 7% EG-treated group. However, a significant decline (P<0.05) in overall and progressive motility was found after CPA addition on day 0 for the 14% EG-treated group and the 7% GLY- and 14% GLY-treated groups. The control group had a decline in overall motility of 47.4% during the 5 days of storage, while the 7% EG, 14% EG, 7% GLY and 14% GLY treatment groups had a decline of 42.3, 62.1, 63.9 and 74.8%, respectively.

After 5 days of storage, progressive motility declined to 7.8, 12.0, 2.5, 2.2 and 0% in the control, 7% EG, 14% EG, 7% GLY and 14% GLY treatment groups, respectively. Progressive motility was more severely affected than overall motility with less than 20% progressively motile sperm in all CPA-containing treatment groups on day 3 of cool storage. The highest loss in progressive motility was observed in the 14% GLY-treated group, which had a decline in progressive motility to less than 10% after the addition of the CPA on day 0 and further declined to less than 3% after 24 hours of cool storage.

Mean overall motility and progressive motility values for individual bulls during the 5 day cool storage period are shown in Table 3.1 and Table 3.2, respectively. A significant difference (P<0.05) was detected among males in both motility parameters during the 5 day cool storage period. Epididymal sperm from 40% of the males were
Figure 3.4. Effect of cryoprotectant toxicity during 5 days of storage at 4°C on overall motility of bovine epididymal sperm. GLY = glycerol and EG = ethylene glycol.
Figure 3.5. Effect of cryoprotectant toxicity during 5 days of storage at 4°C on progressive motility of bovine epididymal sperm. GLY = glycerol and EG = ethylene glycol.
Table 3.1. Mean overall motility values for individual males during a 5-day exposure to different concentrations of CPAs at 4°C

<table>
<thead>
<tr>
<th>Bull No.</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONT</td>
<td>GLY 7%</td>
<td>GLY 14%</td>
<td>EG 7%</td>
</tr>
<tr>
<td>1</td>
<td>73.0</td>
<td>35.0</td>
<td>20.0</td>
<td>50.0</td>
</tr>
<tr>
<td>2</td>
<td>66.0</td>
<td>49.0</td>
<td>38.0</td>
<td>57.0</td>
</tr>
<tr>
<td>3</td>
<td>66.0</td>
<td>30.0</td>
<td>10.0</td>
<td>63.0</td>
</tr>
<tr>
<td>4</td>
<td>69.0</td>
<td>45.0</td>
<td>9.0</td>
<td>50.0</td>
</tr>
<tr>
<td>5</td>
<td>75.0</td>
<td>64.0</td>
<td>46.0</td>
<td>73.0</td>
</tr>
<tr>
<td>6</td>
<td>80.0</td>
<td>55.0</td>
<td>20.0</td>
<td>77.0</td>
</tr>
<tr>
<td>7</td>
<td>70.0</td>
<td>60.0</td>
<td>15.0</td>
<td>67.0</td>
</tr>
<tr>
<td>8</td>
<td>75.0</td>
<td>38.0</td>
<td>10.0</td>
<td>65.0</td>
</tr>
<tr>
<td>9</td>
<td>80.0</td>
<td>65.0</td>
<td>44.0</td>
<td>70.0</td>
</tr>
<tr>
<td>10</td>
<td>75.0</td>
<td>50.0</td>
<td>15.0</td>
<td>65.0</td>
</tr>
</tbody>
</table>

CPAs = cryoprotectants; Trt = treatment; CONT = control; GLY = glycerol; EG = ethylene glycol.
Table 3.2. Mean progressive motility values for individual males during a 5-day exposure to different concentrations of CPAs at 4°C

<table>
<thead>
<tr>
<th>Bull No.</th>
<th>Trt</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cont</td>
<td>GLY 7%</td>
<td>GLY 14%</td>
<td>EG 7%</td>
<td>EG 14%</td>
</tr>
<tr>
<td>1</td>
<td>40.0</td>
<td>18.0</td>
<td>10.0</td>
<td>27.0</td>
<td>36.0</td>
</tr>
<tr>
<td>2</td>
<td>45.0</td>
<td>34.0</td>
<td>20.0</td>
<td>39.0</td>
<td>35.0</td>
</tr>
<tr>
<td>3</td>
<td>40.0</td>
<td>19.0</td>
<td>3.0</td>
<td>39.0</td>
<td>20.0</td>
</tr>
<tr>
<td>4</td>
<td>45.0</td>
<td>20.0</td>
<td>0.0</td>
<td>26.0</td>
<td>5.0</td>
</tr>
<tr>
<td>5</td>
<td>60.0</td>
<td>45.0</td>
<td>33.0</td>
<td>50.0</td>
<td>45.0</td>
</tr>
<tr>
<td>6</td>
<td>63.0</td>
<td>33.0</td>
<td>5.0</td>
<td>60.0</td>
<td>30.0</td>
</tr>
<tr>
<td>7</td>
<td>55.0</td>
<td>38.0</td>
<td>0.0</td>
<td>55.0</td>
<td>40.0</td>
</tr>
<tr>
<td>8</td>
<td>60.0</td>
<td>13.0</td>
<td>0.0</td>
<td>54.0</td>
<td>30.0</td>
</tr>
<tr>
<td>9</td>
<td>69.0</td>
<td>50.0</td>
<td>28.0</td>
<td>60.0</td>
<td>46.0</td>
</tr>
<tr>
<td>10</td>
<td>58.0</td>
<td>30.0</td>
<td>0.0</td>
<td>50.0</td>
<td>19.0</td>
</tr>
</tbody>
</table>

CPAs = cryoprotecants; Trt = treatment; Cont = control; GLY = glycerol; EG = ethylene glycol.
Figure 3.6. Effect of cryoprotectant toxicity during 5 days of storage at 4°C on plasma membrane integrity of bovine epididymal sperm. GLY = glycerol and EG = ethylene glycol.
more vulnerable to the CPA than other males, as noted in a rapid depression of motility parameters.

Membrane Integrity

The mean values for sperm exhibiting intact membranes during the 5 days of cool storage are shown in Figure 3.6. No significant damage to the sperm plasma membrane was detected during the first 24 hours of storage for the control and 7% EG-treated group when compared with the 7% GLY, 14% GLY and 14% EG treatment groups. However, a significant increase (P<0.05) in the percentage of sperm with ruptured membranes was found immediately after the addition of CPA and further increased after 24 hours of storage for the 7% GLY, 14% GLY and 14% EG treatment groups. The highest reduction in membrane integrity was detected during the first 24 hours of storage for all 4 CPA treatment groups. The control group had a decline in membrane integrity of 31.5% during the 5 days of storage, while the 7% EG, 14% EG, 7% GLY and 14% GLY treatment groups had a decline of 37.9, 63.1, 54.3 and 72.5%, respectively.

The highest loss in membrane integrity was found in the 14% GLY-treated group. In the 14% GLY treatment group, membrane integrity of epididymal sperm declined from 83.2% to 29.0% on day 0 and further declined to 10.9% on day 5 of cool storage.

Mean plasma membrane integrity values for individual bulls during the 5 day cool storage period are shown in Table 3.3. A significant difference (P<0.05) was found among 40% of the males used in this study. There was a high variability in the percentage of sperm with intact membranes between males subjected to the same treatment group for all treatments.

**DISCUSSION**

Glycerol is currently used as the cryoprotectant of choice to cryopreserve bull sperm. The use of alternative permeating cryoprotectants with higher membrane permeabilities might help reduce the osmotic damage caused to sperm during their addition prior to freezing and removal after warming.

We found that the addition and removal of glycerol and ethylene glycol at 4°C resulted in a concentration dependent loss of motility and membrane integrity. Ethylene glycol produced the least harmful effects when compared with glycerol in the concentrations evaluated in this study. These results are in agreement with those
Table 3.3. Mean plasma membrane integrity values for individual males during a 5-day exposure to different concentrations of CPAs at 4°C

<table>
<thead>
<tr>
<th>Bull No.</th>
<th>Trt</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONT</td>
<td>GLY 7%</td>
<td>GLY 14%</td>
<td>EG 7%</td>
<td>EG 14%</td>
</tr>
<tr>
<td>1</td>
<td>79.0</td>
<td>58.0</td>
<td>36.0</td>
<td>70.0</td>
<td>68.0</td>
</tr>
<tr>
<td>2</td>
<td>77.0</td>
<td>58.0</td>
<td>42.0</td>
<td>65.0</td>
<td>56.0</td>
</tr>
<tr>
<td>3</td>
<td>64.0</td>
<td>54.0</td>
<td>23.0</td>
<td>65.0</td>
<td>46.0</td>
</tr>
<tr>
<td>4</td>
<td>79.0</td>
<td>51.0</td>
<td>26.0</td>
<td>64.0</td>
<td>33.0</td>
</tr>
<tr>
<td>5</td>
<td>80.0</td>
<td>56.0</td>
<td>30.0</td>
<td>78.0</td>
<td>41.0</td>
</tr>
<tr>
<td>6</td>
<td>80.0</td>
<td>50.0</td>
<td>23.0</td>
<td>79.0</td>
<td>43.0</td>
</tr>
<tr>
<td>7</td>
<td>72.0</td>
<td>49.0</td>
<td>18.0</td>
<td>65.0</td>
<td>39.0</td>
</tr>
<tr>
<td>8</td>
<td>70.0</td>
<td>35.0</td>
<td>19.0</td>
<td>60.0</td>
<td>40.0</td>
</tr>
<tr>
<td>9</td>
<td>90.0</td>
<td>74.0</td>
<td>46.0</td>
<td>84.0</td>
<td>53.0</td>
</tr>
<tr>
<td>10</td>
<td>90.0</td>
<td>50.0</td>
<td>27.0</td>
<td>74.0</td>
<td>54.0</td>
</tr>
</tbody>
</table>

CPAs = cryoprotectants; Trt = treatment; CONT = control; GLY = glycerol; EG = ethylene glycol.
reported for ejaculated bull and human sperm by Guthrie et al. (2002) and Gilmore et al. (1997), respectively. Both groups found that the removal of ethylene glycol produced the least harmful effects to sperm motility compared with that of dimethyl sulfoxide and glycerol.

In our study, maximum survival as assessed by measurements of motility and membrane integrity was achieved with spermatozoa exposed to 7% ethylene glycol. Almost identical intermediate levels of survival were observed with sperm exposed to 7% glycerol or 14% ethylene glycol. The lowest survival was found for sperm exposed to the 14% glycerol treatment.

There was a high variability in percentage of sperm with intact membranes between males exposed to the same treatment groups. Some bulls were apparently more sensitive to osmotic damage caused by the high concentration of CPAs than others.

Ethylene glycol has been reported to permeate the plasma membrane of human sperm four times as fast as glycerol (Gilmore et al., 1997). When a permeable cryoprotectant is added to sperm, a hypertonic environment is created. The cell shrinks as water leaves the cytoplasm into the extracellular environment and then swells as the cryoprotectant enters the cell and water re-enters to maintain osmotic equilibrium. During removal, the cell swells due to an influx of water and then returns to isosmotic volume after the cryoprotectant and water leaves the cell.

In this study, the rapid movement of ethylene glycol through the plasma membrane might have helped reduce shrinking and swelling of sperm reducing plasma membrane damage and depression of motility to the sperm. The osmotic damage caused by high concentration of cryoprotectants or cryoprotectants with low membrane permeabilities can be reduced by step wise addition and dilution (Katkov et al., 1998).

Guthrie et al. (2002) reported that motility of ejaculated bull sperm was very sensitive to osmotic changes. For bull sperm to maintain 90% motility of their isosmotic value, sperm must be maintained within 92 to 103% of its original isosmotic volume. Conversely, human sperm has a higher osmotic tolerance and can maintain motility within 75 to 110% of its original isosmotic volume (Gao et al., 1995), while motility in the boar is depressed outside 97 to 102% of its isosmotic volume (Gilmore et al., 1998). Swelling of sperm was reported to be more detrimental to survival than shrinking.
(Pommer et al., 2002). Therefore, most of the damage to sperm is likely during the removal rather than during the addition of cryoprotectants. Due to the osmotic tolerance differences between species and cell types, the osmotic tolerance limits of epididymal sperm must be determined in an effort to reduce osmotic damage.

The protective effects of permeating cryoprotectants are so impressive that little attention has been focused on the toxic effects of these chemical agents. Some cell types are more vulnerable to damage caused by permeating cryoprotectants than others. Hammerstedt and Graham (1992) reported that glycerol can affect physical features in the cytoplasm, permeability and stability of the membrane bilayer, metabolism and noncovalent attachment of proteins to the sperm surface. However, the biochemical mechanisms of this type of toxicity are unknown.

To our knowledge, this is the first report to study the toxic effect of prolonged incubation of epididymal sperm with different concentrations of permeable cryoprotective agents. Ethylene glycol at low concentrations produced no evident toxic effect during the 5 days of cool storage, as assessed by sperm viability and both motility parameters. However, when the CPA concentration was increased overall and progressive motility was reduced and the percentage of membrane ruptured sperm was elevated. In the present study, 7% and 14% glycerol was found to be more toxic than 7% and 14% ethylene glycol concentrations. At the low concentration of glycerol (7%), overall and progressive motility and membrane integrity were significantly reduced during storage. At the high concentration of glycerol (14%), progressive motility was impaired after 24 hours of storage and membrane integrity was reduced to less than 20%. The instant decline of motility and membrane integrity on day 0 was likely caused by osmotic damage rather than true chemical toxicity. However, possible chemical toxicity by the cryoprotectants can not be ruled out.

Toxicity of permeating cryoprotectants has been reported in mouse (Sztein et al., 2001) and rooster sperm (Hammerstedt and Graham, 1992). Mouse sperm do not tolerate the intracellular presence of permeating CPAs as well as sperm from other species (Sztein et al., 2001). Nonpermeable CPAs, such as raffinose and trehalose are well tolerated by mouse sperm (Storey et al., 1998). It is thought that permeable cryoprotective agents affect mouse sperm due to true chemical toxicity rather than to osmotic damage. Furthermore, glycerol has also been reported to increase membrane
disruptions in rooster sperm resulting in a reduction in fertility (Hammerstedt and Graham, 1992).

In this study, motility and membrane integrity were the end points to assess toxicity. The presence of permeable cryoprotectants might also affect other sperm components lowering the functional integrity of the sperm, which were not assessed by the assays used in this study. Poor fertility was reported in mice when sperm were frozen using glycerol despite having good post-thaw sperm motility (Sztein et al., 1992). A more rigorous study to try to understand the biochemical mechanism of toxicity caused by permeating CPAs is needed in the future.

These results indicate that the use of ethylene glycol as a CPA may reduce toxicity and osmotic damage to bovine epididymal sperm over that of glycerol. Future research must evaluate its efficacy as a cryoprotectant for bovine epididymal sperm.
CHAPTER IV

THE USE OF ETHYLENE GLYCOL AND GLYCEROL FOR THE CRYOPRESERVATION OF BOVINE CAUDAL EPIDIDYMAL SPERM

INTRODUCTION

The development of a protocol to cryopreserve sperm harvested from the epididymides will enable the propagation of gametes from valuable animals that may die unexpectedly. Recently, interest for the preservation of epididymal sperm as a potential source of valuable genes for genome resource banks has escalated. In cattle, most research has concentrated on improving freezing protocols for ejaculated bull sperm. Reports on the use, characteristics, cryopreservation and fertility of bovine epididymal sperm are limited. Even with all the work to improve the survivability of bovine sperm after cryopreservation, there still remains ~50% decrease in sperm viability due to temperature and osmotic effects (Thomas et al., 1998). Moreover, it is well known that sperm from some bulls still can not be successfully cryopreserved with present day protocols due to individual bull to bull variation. It has recently been emphasized that there is marked diversity in the cryobiological response of sperm among different mammalian species and among different individuals within a species (Agca and Crister, 2002).

Epididymal sperm has been cryopreserved in various species, including swine (Rath and Niemann, 1997), dogs (Stilley et al., 1999), goats (Blash et al., 2000), mice (Koshimoto et al., 2000), humans (Patrizio, 2000) and horses (Tiplady et al., 2002) with variable results.

Research on the cryopreservation of bovine epididymal sperm is very limited. Barker (1954) reported the first pregnancy using frozen-thawed bull epididymal sperm by artificial insemination. Later, cryopreserved epididymal sperm was used for in vitro fertilization (Goto et al., 1989) and intracytoplasmic sperm injection (Goto et al., 1990). However, no data are available on the survivability and quality of epididymal sperm in regards to post-thaw membrane integrity and acrosome integrity.

Most sperm are lethally damaged during the cooling and warming process of cryopreservation in the absence of cryoprotective additives (CPAs) (Ashwood-Smith and Farrant, 1980). The most widely used permeating CPAs since the 1950s have been glycerol, dimethyl sulfoxide, ethylene glycol and propylene glycol. Permeating CPAs
function through colligative properties, which help maintain solute concentrations inside and outside the cells at nondamaging levels (Gilmore et al., 1997). However, CPAs produce a hyperosmolar environment to sperm cells inducing potentially damaging volume changes. Sperm survival can be optimized by avoiding or minimizing osmotic damage (Holt, 2000). Although functional differences exist between epididymal and ejaculated sperm, it is proposed that epididymal sperm still require the presence of similar components during the freezing process.

From early studies it is well known that the cryoprotective ability of any CPA varies widely across different cell and tissue types (Karow, 1969). Therefore, an optimal CPA for cryopreservation of bovine epididymal sperm needs to be determined.

Glycerol is routinely used at concentrations ranging form 5 to 8% to cryopreserve bovine ejaculated sperm. Recently, the high membrane permeability of ethylene glycol was shown to reduce the damage caused to the plasma membrane of bovine caudal epididymal sperm during its addition and/or removal when compared with that of glycerol (Guerrero et al., 2006). Gilmore et al. (1997) indicated that the optimal CPA would be one that could permeate the cell in the shortest time causing the least amount of volume excursions during its addition and removal. Ethylene glycol was chosen as a candidate for the cryopreservation of bovine epididymal sperm in our study because of its high membrane permeability.

Sperm are intricate cells that require a number of criteria to be met to achieve fertilization. It is generally agreed that an intact acrosome and plasma membrane are required for the sperm to transverse through the cumulus cells, zona pellucida and finally fuse with the oolema of the oocyte.

The discovery of a variety of fluorochromes has made possible the analysis of sperm at a functional, biochemical and ultrastructural level. Most fluorochromes have been used by staining and examining cells with fluorescent microscopy. However, microscopic analysis only measures a small number of sperm (200-300) within a population, is time consuming and is considered subjective (Gillian et al., 2005). By adapting these fluorochromes for flow cytometry, various sperm attributes can be measured faster and on a larger scale (10,000-50,000).

The aim of all semen analysis procedures is to predict the potential for fertility in a fast, objective and accurate way. The use of flow cytometry to assess sperm quality
has greatly improved the objectivity, accuracy and reproducibility of the quality evaluation compared with traditional microscopy methods (Waterhouse et al., 2004).

Garner et al. (1994) developed a simple dual nucleic acid stain combination to assess viability of frozen-thawed sperm in the presence of egg yolk particles. The two stains, SYBR 14 and propidium iodide (PI) have the same cellular target making it less variable than enzyme-based stains. Sperm DNA are believed to be a more appropriate cellular target due to their stainability and staining uniformity (Garner et al., 1996). These probes stain viable membrane intact sperm green, while nonviable membrane damaged sperm stain red. After cell death, the PI rapidly overwhelms the fluorescence exhibited by the SYBR 14 (Thomas et al., 1998).

Acrosome integrity has been measured using plant lectins conjugated to a fluorescent probe. These lectins bind to sugar moieties exposed in reacted acrosome membranes. *Arachis hypogaea* agglutinin (PNA) and *Pisum sativum* agglutinin (PSA) are the most used plant lectins. However, PNA is believed to display less nonspecific binding to other areas of the sperm and also to egg yolk particles, leading researchers to favor PNA over PSA (Graham, 2001; Nagy et al., 2003).

Recently, Nagy et al. (2003) developed a triple fluorochrome staining technique which involves the well validated SYBR 14 and PI, with PNA conjugated to phycoerythrin (PE). The PE fluorescent moiety was used instead of fluorescein isothiocyanate (FITC), because its fluorescence emission could be measured independently from that of SYBR 14 or PI. This novel technique was shown to assess viable acrosome intact, viable acrosome reacted, nonviable acrosome intact and nonviable acrosome reacted sperm in a single assay.

In the present study, this novel triple fluorochrome technique was duplicated for the first time to determine the effect of ethylene glycol and glycerol on the cryopreservation of bovine caudal epididymal sperm.

**MATERIALS AND METHODS**

**Experimental Design**

**Experiment 4.1**

Paired testes were obtained from mature mixed breeds of beef and dairy bulls (n=10) at a local abattoir and transported to the laboratory within 4 to 6 hours postmortem. Epididymal sperm were harvested by multiple incisions from the caudae
epididymides of testes from each bull, pooled and then placed in egg yolk Tris-glucose citric acid monohydrate extender (EYT-GC). Specimens were allocated into two treatment groups: 7% ethylene glycol or 7% glycerol. Samples were diluted slowly over a period of 30 min 1:1 in EYT-GC medium containing twice the final desired concentration of cryoprotectant (CPA) and subsequently frozen over liquid nitrogen vapors. Plasma membrane and acrosome integrity were evaluated by the same technician using multicolor flow cytometry. Progressive motility was assessed subjectively with a light microscope. The experiment was repeated three times with each replicate on a different day.

Experimental Procedure

Testes Collection

Bovine testes were collected as pairs from mature mixed breed bulls (n=10) at an abattoir in Robert, Louisiana. Testes were individually packed in Ziploc® plastic bags (Johnson & Son Inc., Racine, WI) and transported to the Louisiana State University Embryo Biotechnology Laboratory, St. Gabriel, Louisiana, in a Styrofoam box (at a temperature that ranged between 25°C to 28°C) within 4 to 6 hours postmortem. Immediately upon arrival to the laboratory, each pair of testes was dissected away from its scrotum and tunica vaginalis. Testes were positioned on the table with the vas deferens facing ventrally and the corpus facing up to distinguish the side of origin of each testis. The weight of the testis was measured with a Pelouze® balance scale (Model SP5) before carefully dissecting the cauda epididymis.

Medium Preparation

Egg yolk Tris-glucose citric acid monohydrate extender (EYT-GC) (250 mM Tris (T-6066, Sigma-Aldrich Inc., St. Louis, MO), 69 mM glucose (6152, Sigma-Aldrich Inc., St. Louis, MO), 81 mM citric acid monohydrate (C-1909, Sigma-Aldrich, St. Louis, MO) and 20% (v/v) egg yolk) was prepared with the addition of 50 µg/ml of gentamicin (15750-060, Gibco, Grand Island, NY) and filtered through a 0.45 µm Nalgene® syringe filter (190-2545, Nalge Company, Rochester, NY) to remove egg yolk materials. Egg yolks were obtained from fresh chicken eggs at the Louisiana State University Poultry research Farm. The extender was placed in a 37°C water bath for at least 3 hours prior to epididymal sperm retrieval.
Epididymal Sperm Retrieval

Upon dissection, each cauda was weighed and rinsed with 0.9% saline before epididymal sperm extraction. Epididymal sperm were harvested by making 5 to 6 incisions using a surgical blade (size 10, MDS-15110, Medline Industries Inc., Mundelein, IL) in the cauda epididymis. The incisions were rinsed with 4 ml of EYT-GC into a 60 mm Falcon® plastic petri dish (3037, Becton Dickinson Labware, Franklin Lakes, NJ). The sperm solution was transferred into a 15 ml plastic tube (20171-024, VWR International Inc., Sugar Land, TX) and placed for 15 minutes in a 37°C water bath for sperm quality analyses.

Epididymal Sperm Analyses

Sperm analyses included plasma membrane integrity, acrosome integrity and progressive motility. A 100-µl sample from the sperm solution was placed in a 1.7 ml plastic micro-centrifuge tube (20170-355, VWR International Inc., Sugar Land, TX) and diluted 1:10 sperm solution:EYT-GC for the epididymal sperm quality analyses.

Plasma membrane integrity was assessed using the LIVE/DEAD® Sperm Viability Kit (L-7011, Molecular Probes Inc., Eugene, OR). The kit consists of two nucleic acid stains. SYBR 14 (1 mM solution in DMSO, Component A of the LIVE/DEAD® Sperm Viability Kit) is a permeable nucleic acid stain, which labels membrane intact cells green. PI (2.4 mM solution in H₂O, Component B of the LIVE/DEAD® Sperm Viability Kit) is a nonpermeable nucleic acid stain, which labels membrane ruptured sperm red. For analysis, 250 µl of the diluted sperm sample were placed in a 0.65 ml plastic micro-centrifuge tube (20170-293, VWR International Inc., Sugar Land, TX). Staining was carried out by the addition of 400 nM SYBR 14 and 24 µM PI to the sperm mixture. The stained sample was incubated for 10 minutes in the dark at 37°C and remixed gently before analysis.

Acrosome integrity was assessed using the lectin peanut agglutinin conjugated to FITC (FITC-PNA) (L-7381, Sigma-Aldrich Inc., St. Louis, MO) and the nucleic acid stain PI. PNA labels acrosome reacted sperm green, while PI discriminates between viable and nonviable sperm. For analysis, 250 µl of the diluted sperm sample were placed in a 0.65 ml plastic micro-centrifuge tube. A final concentration of 5 µg/ml of FITC-PNA and
24 µM PI were added. The stained sample was incubated for 15 to 20 minutes in the dark at 37°C and mixed gently before analysis.

For plasma membrane and acrosome integrity, each sample was analyzed by counting 300 sperm cells with an inverted microscope (Nikon Diaphot, Tokyo, Japan) equipped with epi-fluorescence, a FITC filter set and a heated stage.

Sperm progressive motility was assessed subjectively by the same technician at 20X magnification using an inverted Nikon Diaphot microscope equipped with Hoffman optics and a heated stage.

Sperm Cooling, Cryopreservation and Thawing

Sperm concentration was determined using a hemacytometer and adjusted to 100 x 10⁶ cells/ml by the addition of more extender. The 15 ml plastic conical tube containing the epididymal sperm sample was placed in a 250 ml Pyrex® glass beaker (13912-207, VWR International Inc., Sugar Land, TX) containing water. The beaker was placed for 4 hours in a refrigerator for cooling the sperm samples to 4°C.

Specimens from each bull were then allocated into two treatment groups: 7% ethylene glycol (E-9129, Sigma-Aldrich Inc., St. Louis, MO) or 7% glycerol (G-5516, Sigma-Aldrich Inc. St. Louis, MO). Samples were diluted slowly over a period of 30 minutes 1:1 in EYT-GC extender containing twice the final desired concentration of CPA and subsequently loaded into previously cooled 0.5 ml straws (005569, Cassou straw, IMV Technologies, Minneapolis, MN). Straws were placed in a custom built rack 2 cm over liquid nitrogen (LN₂) vapors for 10 minutes before being plunged in LN₂. Between 15 to 20 of these 0.5 ml straws were frozen per bull.

Sperm samples were stored for 6 months prior to thawing. On the day of analysis, 50 ml of Dulbecco’s phosphate-buffered saline (D-PBS) (14287-080, Gibco, Grand Island, NY) were placed in a 50 ml plastic tube (BCT-P50RS, Biologix, Shawnee Mision, KS) and warmed in a 37°C incubator 1 hour prior to sperm dilution. Three straws per animal were thawed in a water bath at 37°C for 40 seconds. The contents of each straw (25 x 10⁶ sperm) were placed in a 15 ml plastic tube and diluted 1:10 sperm sample:D-PBS before motility and multicolor flow cytometric analyses.

Multicolor Flow Cytometry

Assessment of plasma membrane and acrosome integrity were carried out using a novel triple stain protocol in the presence of egg yolk components (Nagy et al., 2003).
with minor modifications. The staining protocol consisted of SYBR 14, PI and the lectin peanut agglutinin conjugated to PE (R-PE-PNA) (p-44, Biomaeda Corporation, Foster City, CA). A final concentration of 100 nM SYBR 14 solution, 4 µg/ml of R-PE-PNA (1 mg/ml of stock solution in a buffer composed of 50 mM sodium phosphate and 0.05% sodium azide, pH 7.0, and also containing 0.1 mM [Ca\textsuperscript{2+}] and [Mn\textsuperscript{2+}] ions) and 12 µM PI solution were added to 250 µl of diluted sperm sample. The samples were thoroughly mixed and incubated at 37°C in the dark for 30 to 40 minutes and then remixed before flow cytometric analysis.

Measurements were completed on a FACSCalibur flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA). Forward and side scatter values were recorded on a linear scale, while fluorescent values were recorded on a logarithmic scale. The three fluorochromes were excited at 488 nm with a 20 mW argon laser. Plasma membrane ruptured cells were PI positive, and their red fluorescent signal was detected on the FL3-channel using a 610 nm long pass filter. Membrane intact cells were SYBR 14-positive, and its green fluorescent signal was detected on the FL1-channel using a 530/30 nm band pass filter (Figure 4.1).

Acrosome reacted cells were PE-PNA positive, and its orange fluorescent signal was detected at 585/42 nm on the FL2-channel (Figure 4.1). Three color compensations were set according to the FACS\textsuperscript{®} training manual (Becton Dickinson Biosciences, San Jose, CA). Nonsperm events (egg yolk components) were gated from the analyses as judged on scatter properties when detected in the forward and side scatter detectors (Figure 4.2). Moreover, events with similar scatter characteristics to sperm but with low PI and SYBR 14 fluorescence were also gated out (Figure 4.3). Sperm acquisitions were made using CellQuest software (Becton Dickinson, San Jose, CA). The flow cytometer was kept at a low flow of less than 300 sperm/second. The recording of scatter and fluorescent properties of all events stopped when 10,000 gated events were recorded.

Density and dot plots drawn for data analysis were generated by WinMDI 2.8 (free software by J. Trotter, available for downloading at http://www.facs.scripps.edu/software.html). On SYBR 14 (FL1) and PI (FL3) dot plots, regions were drawn to determine the percentage of viable and nonviable sperm (Figure 4.4). On PE-PNA (FL2) and PI (FL3) dot plots, quadrants were set to measure the percentage of sperm displaying intact plasma membranes and acrosomes (LL), intact plasma membranes
Figure 4.1. Representative plasma membrane and acrosome integrity triple stain assay. Sperm that exhibit green fluorescence (SYBR 14) are considered membrane intact (viable), those that exhibit red fluorescence (PI) were considered to have damaged membranes (nonviable) and those exhibiting yellow fluorescence (PE-PNA) were considered acrosome reacted.
Figure 4.2. Flow cytometric out-gating of egg yolk particles based on scatter properties after staining frozen-thawed epididymal sperm with SYBR 14, PI and PE-PNA. Sperm were selected (R1) based on size, gating out small particles.

Figure 4.3. PI (red) and SYBR 14 (green) fluorescence. Remaining egg yolk particles having sperm-like scatter properties were gated out based on DNA fluorescence. Events that had either positive PI and/or SYBR 14 (R1) fluorescence were selected as DNA positive events. Particles exhibiting no DNA fluorescence (lower left corner) were considered to be nonsperm events.
Figure 4.4. SYBR 14 (viable) and PI (nonviable) flow cytometric dot plot illustrating frozen-thawed epididymal sperm after gating out all nonsperm events. Acquisitions of 10,000 events were made from positive green and red fluorescence.

Figure 4.5. Flow cytometric graph illustrating PE-PNA (orange) and PI (red) fluorescence. Events exhibiting high orange fluorescence were recorded as sperm with reacted acrosomes. Quadrants were set to identify viable acrosome intact sperm (LL), viable acrosome reacted sperm (LR), nonviable acrosome intact sperm (UL) and nonviable acrosome reacted sperm (UR).
and reacted acrosomes (LR), ruptured plasma membranes and intact acrosomes (UL) and ruptured plasma membranes and reacted acrosomes (UR) (Figure 4.5).

Statistical Analysis

Variances in testicular parameters, membrane integrity, acrosome integrity and progressive motility were statistically analyzed by ANOVA and the differences between bulls were evaluated by a Tukey Multiple Comparisons test. In addition, a t-test was used to verify differences in sperm parameters before and after cryopreservation. The sperm parameters are expressed as mean±SEM per treatment group. A P<0.05 was considered significant in this study. Data were analyzed using SigmaStat Statistical Software Version 2.0.

RESULTS

Experiment 4.1

Testicular and Epididymal Weights

Testicular and caudae epididymal weights for individual bulls are shown in Table 4.1. Both weights from the right testis of bull number 10 were not included in the mean values due to their abnormal weights (low). Testes and caudae epididymides weights ranged from 241 to 490 g and 5 to 16 g for the bulls used in this study, respectively. There was an intermale variability in both testicular parameters analyzed. No significant difference (P>0.05) was detected between bulls for both testicular parameters.

A total of 32 pairs of testes were obtained from the abattoir. Only 10 bulls of the 32 obtained showed anatomically normal testis morphology, sperm production and progressive motility greater than 60%. From the 22 bulls not used, 5 had no evidence of sperm production, 7 had low sperm concentration and 10 had impaired motility and high amount of morphological abnormalities. Of those 22 animals, 10 displayed abnormal testicular morphology (Figure 4.6).

Progressive Motility

The mean initial progressive motility value after epididymal sperm retrieval was 66±2.1% for all the 10 bulls used in this study. Cryopreservation using both CPAs had a significant detrimental effect (P<0.05) on sperm forward motility. The mean post-thaw progressive motility values for samples frozen in 7% GLY and 7% EG were 31±3.3 and 5±0.8%, respectively (Figure 4.7). Progressive motility post-thaw for sperm frozen in 7%
### Table 4.1. Testicular and epididymal values from bovine testes collected postmortem

<table>
<thead>
<tr>
<th>Bull No.</th>
<th>Testicle weight (g)</th>
<th>Cauda weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>1</td>
<td>338</td>
<td>325</td>
</tr>
<tr>
<td>2</td>
<td>450</td>
<td>490</td>
</tr>
<tr>
<td>3</td>
<td>322</td>
<td>290</td>
</tr>
<tr>
<td>4</td>
<td>349</td>
<td>370</td>
</tr>
<tr>
<td>5</td>
<td>250</td>
<td>241</td>
</tr>
<tr>
<td>6</td>
<td>254</td>
<td>252</td>
</tr>
<tr>
<td>7</td>
<td>242</td>
<td>254</td>
</tr>
<tr>
<td>8</td>
<td>301</td>
<td>300</td>
</tr>
<tr>
<td>9</td>
<td>308</td>
<td>269</td>
</tr>
<tr>
<td>10*</td>
<td>92</td>
<td>470</td>
</tr>
</tbody>
</table>

Mean (±SEM) 312.6±21.5 326.1±28.4 8.7±1.0 9.3±0.9

*Right testis not used in the study due to low weight and no sperm production. Means values were not significantly different (P>0.05).
Figure 4.6. Examples of two pairs of bull testes with abnormal morphology.
Figure 4.7. Percent progressive motility (mean±SEM) of prefreeze and post-thaw bovine epididymal sperm cryopreserved with glycerol and ethylene glycol. a,b,cMean values with different letters are significantly different (P<0.05).
Figure 4.8. Prefreeze and post-thaw percent progressive motility values (mean±SEM) of bovine epididymal sperm from individual bulls cryopreserved with glycerol or ethylene glycol. a, b, c Means with different letters within bull are significantly different (P<0.05).
GLY achieved a higher survivability than sperm frozen in 7% EG as assessed by post-thaw progressive motility.

The mean progressive motility values for individual bulls prior to and post-cryopreservation are shown in Figure 4.8. Sperm frozen in 7% GLY had a higher progressive motility than sperm frozen in 7% EG after cryopreservation. However, sperm from bull number 3 had little or no survivability post-thaw when compared with sperm from the other bulls used using both CPAs in the study. Progressive motility declined from 60 to 3.3% and from 60 to 0% when frozen in 7% GLY and 7% EG, respectively. Post-thaw motility of sperm frozen in 7% GLY ranged from 25 to 40%, while sperm frozen in 7% EG only ranged from 0 to 10%.

Membrane Integrity

The mean initial plasma membrane integrity value after epididymal sperm retrieval was 83±1.5% for all the bulls used in this study. The mean percentage of sperm with intact membranes after thawing was significantly (P<0.05) higher in samples frozen in 7% GLY when compared with 7% EG (Figure 4.9). Moreover, there was a significant decline in percent intact plasma membranes after cryopreservation using both CPAs.

The mean intact membrane values for individual bulls prior to and post cryopreservation are shown in Figure 4.10. Sperm from bull number 4, 9 and 10 frozen in 7% GLY had the highest number of viable sperm after thawing. These were significantly different (P<0.05) in the incidence of live sperm after thawing from the remaining bulls. Moreover, sperm from bull number 3 frozen in 7% GLY had the lowest membrane integrity values of the 7% GLY-treated group. Conversely, only sperm from bull number 1, 4 and 10 frozen in 7% EG had intact membrane values between 10 and 20%. These males had significantly higher sperm with intact membranes than those of the remaining bulls in the 7% EG-treated group.

Acrosome Integrity

The overall mean prefreeze acrosome integrity value was 93.6±0.3% for all the bulls used in this study. Overall, sperm frozen in 7% GLY and 7% EG treatment groups had 81.2±2.0% and 73.3±3.4% intact acrosomes, respectively. No significant difference was detected in acrosome integrity between the 7% GLY and 7% EG treatment groups. However, there was a significant decline (P<0.05) in post-thaw acrosome integrity in the
Figure 4.9. Percent membrane integrity (mean±SEM) of prefreeze and post-thaw bovine epididymal sperm cryopreserved with glycerol and ethylene glycol. a,b,c Means with different letters are significantly different (P<0.05).
Figure 4.10. Prefreeze and post-thaw percent membrane integrity (mean±SEM) of bovine epididymal sperm from individual bulls cryopreserved with glycerol and ethylene glycol. a,b,c Mean values with different letters are significantly different (P<0.05).
Figure 4.11. Post-thaw percent reacted acrosomes (mean±SEM) derived from the viable and nonviable populations of bovine epididymal sperm cryopreserved with glycerol and ethylene glycol. a,b Mean values with different letters are significantly different (P<0.001).
The overall mean number of live sperm with intact acrosomes was higher in the 7% GLY-treated group than in the 7% EG-treated group. There were 2.1±0.3% and 0.3±0.1% viable sperm with damaged acrosomes post-cryopreservation in the 7% GLY and 7% EG treatment groups, respectively (Figure 4.11). In contrast, the number of nonviable sperm with damaged acrosome membranes was lower in the 7% GLY-treated group (16.5±1.5%) when compared with the 7% EG-treated group (26.0±2.1%). These means were not statistically different (P>0.05). However, a difference (P<0.001) was detected between the quantity of damaged acrosomes derived from membrane ruptured than membrane intact sperm in both the 7% EG and 7% GLY treatment groups.

The mean acrosome integrity values for individual bulls before and after cryopreservation are shown in Figure 4.12. Bull number 4, 5 and 10 in both treatment groups did not have a significant decline in percent intact acrosomes after cryopreservation as was the case for bull number 1, 2, 3, 6, 7, 8 and 9. Overall, there was a high variability in acrosome integrity between bulls subjected to the same treatments.

**DISCUSSION**

Reports comparing the quality of the sperm acrosome and the plasma membrane of bovine caudal epididymal sperm cryopreserved from postmortem bulls are limited. In this experiment, sperm progressive motility, acrosome and viability parameters of epididymal sperm were evaluated before and after cryopreservation. Previous reports in cats (Goodrowe and Hay, 1993), pigs (Kikuchi et al., 1998), mice (Songsasen et al., 1998), cattle (Foote, 2000), horses (James et al., 2002), dogs (Yu and Leibo, 2002) and sheep (Kaabi et al., 2003) and in exotic animals such as gaur (Hopkins et al., 1988), rhinoceros (Lubbe et al., 2000), zebra (Lubbe et al., 2000), eland (Bissett and Bernard, 2005), Flying fox (De Jong et al., 2005), Common wombat (MacCallum and Johnston, 2005), Roe deer (Martinez-Pastor et al., 2005) and Red deer (Soler et al., 2005) have focused on collection and/or cryopreservation of epididymal sperm from cooled testes stored up to 7 days. In these studies most of the procedures use one epididymis to evaluate sperm quality (control) and the sperm from the other testis is stored or frozen, which increases the chance for error. Recently, Gooaverts et al. (2006) have indicated
Figure 4.12. Percent acrosome integrity values (mean±SEM) of prefreeze and post-thaw bovine epididymal sperm from individual bulls cryopreserved with glycerol and ethylene glycol. a,b,c Mean values with different letters within bulls are significantly different (P<0.05).
that sperm from both bovine caudae epididymides within the same male were not comparable in most quality parameters.

In our experiment, it was decided to harvest sperm in the shortest time possible (4 to 6 hours) without lowering the temperature of the testes to 4°C. Various reports have shown a lowered fertilizing ability in vitro of sperm that has was kept in testes stored at low temperatures (Hay and Goodrowe, 1993; Kikuchi et al., 1998). Our aim was to successfully develop a protocol to cryopreserve bovine epididymal sperm. Therefore, we wanted to obtain sperm that were not exposed to the stress imposed by low-temperature storage and possible degeneration caused by postmortem tissue necrosis.

In the present study, progressive motility, acrosome integrity and plasma membrane integrity of frozen-thawed bovine epididymal sperm cryopreserved with two different CPAs were evaluated. Our results show that 66%, 94% and 83% of sperm harvested from postmortem bulls have progressive motility, intact acrosome membranes and intact plasma membranes, respectively. A detrimental effect of cryopreservation in the survivability of epididymal sperm post-thaw was clearly evident from the use of both glycerol and ethylene glycol as CPAs. Furthermore, sperm frozen in glycerol showed a higher survivability post-thaw compared with that of ethylene glycol. These results differ from those of various reports from other species using glycerol and ethylene glycol to cryopreserve ejaculated sperm (Mahadevan and Trounson, 1983; Alvarenga et al., 2000; Li et al., 2005; Rota et al., 2005; Sanitani et al., 2005). For example, Mahadevan and Trounson (1983) reported a similar cryoprotective effect between 7.5% glycerol and 7.5% ethylene glycol for cryopreservation of human ejaculated sperm.

In contrast, Glander and Colditz (1981) reported a higher survivability of frozen-thawed human ejaculated sperm as assessed by motility using glycerol compared with that of ethylene glycol at three different concentrations (3.5%, 7% and 12%). Rota et al. (2005) found a higher post-thaw survivability in canine ejaculated sperm cryopreserved with ethylene glycol than with glycerol. Santiani et al. (2005) reported no difference between glycerol and ethylene glycol for the cryopreservation of llama ejaculated sperm. However, they reported plasma membrane integrity values as high as 20% post-thaw for ethylene glycol. There range in values were similar to the results obtained in our study.
In sperm from Cynomolgus monkeys, ethylene glycol was reported to provide a similar cryoprotective effect as glycerol (Li et al., 2005). Also, Alvarenga et al. (2000) and Squires et al. (2004) found similar cryoprotective effects between glycerol and ethylene glycol in post-thaw equine sperm. In contrast, Mantovani et al. (2002) noted a significantly higher progressive motility in equine ejaculated sperm frozen with glycerol than with ethylene glycol. They reported a detrimental effect of ethylene glycol on progressive motility and membrane integrity at concentrations higher than 3%. In our study, a 7% CPA concentration was used on the epididymal sperm, which could have contributed to the decline in progressive motility, acrosome integrity and membrane integrity post-thaw. It should be noted that bovine ejaculated sperm is routinely frozen in CPA concentrations ranging from 5 to 8%, while in equine CPA concentrations used are generally lower.

In previous studies we have verified that ethylene glycol at a 7% concentration was not toxic to bovine epididymal sperm (Guerrero et al., 2006). This demonstrates that ethylene glycol does not visibly affect bovine epididymal sperm during its addition sequence prior to cryopreservation. It may be that ethylene glycol at lower concentration (7%) does not provide protection against freezing injury.

In such species as mice, rats, pigs and horses ejaculated sperm are more sensitive to CPAs than in other species (Sztein et al., 2001). In mice, higher post-thaw survival of epididymal sperm has been obtained without the use of permeating CPAs (Sztein et al., 2001). In contrast, bovine sperm require a higher concentration of permeating CPA to successfully survive the freezing process. Therefore, the 7% concentration of ethylene glycol used in our study may have been too low to provide enough protection for bovine epididymal sperm. Furthermore, the same freezing rate was used for both CPAs. It is possible that there is an interaction between CPA and freezing rate. Ethylene glycol migh have a different optimum freezing rate than glycerol.

The success obtained with glycerol as a CPA to cryopreserve epididymal sperm was shown to vary between bulls. Thus far, the understanding of the factors associated with intermale variation in most species is very sparse.

We have demonstrated that the routine protocol to cryopreserve bovine ejaculated sperm has to be modified to increase the survivability of post-thaw epididymal sperm. It must be taken into account that ejaculated and epididymal sperm are
morphologically and functionally different (Hewitt et al., 2001). Hammerstedt (1993) has shown that bull and ram epididymal sperm consume less ATP when compared with that of ejaculated sperm. Recently, Goovaerts et al. (2006) demonstrated that bovine epididymal sperm display a lower motility than ejaculated sperm from the same bull when assessed by Computer Assisted Sperm Analysis System (CASA). Membrane properties between epididymal and ejaculated sperm also differ, which may affect sperm survival after cooling and freezing (Rath and Niemann, 1997). Moreover, the distal cytoplasmic droplet found in epididymal sperm is thought to make them more vulnerable to tail damage post-thaw (personal observation, unpublished). This morphological difference may influence their susceptibility to osmotic damage during the cryopreservation process. It has been shown that the fertilizing ability of canine epididymal sperm is compromised after cryopreservation due to an increase in the percent of sperm with abnormal morphology (Hori et al., 2004). However, no studies have been reported to date to assess a direct comparison between the freezability of epididymal and ejaculated sperm obtained from the same male.

We have successfully adapted the flow cytometric triple fluorochrome technique for use with bovine epididymal sperm. This system has provided us with data showing acrosome integrity from membrane damaged (nonviable) and membrane intact (viable) sperm. Most dual stain techniques adapted to the flow cytometer to assess acrosome integrity are compromised due to misreading sperm with egg yolk particulates. By using the triple stain technique, egg yolk particles can successfully be removed from sperm events by making gates based on DNA positive events. In the present study, acrosome integrity readings were based only on events showing positive DNA staining.

Most sperm with reacted acrosomes were found to come from the nonviable population than from the viable population in both CPA treatments (P<0.001). This indicates that the plasma membrane is more sensitive to damage by the freezing process than the acrosome compartments in epididymal sperm. Furthermore, the plasma membrane was shown to be compromised prior to the acrosome. Most live sperm were found to have an intact acrosome post-thaw. This is important for a sperm to be able to penetrate the layer of cumulus cells and the zona pellucida surrounding an oocyte during the fertilization process.
Factors that could have affected the success obtained with glycerol as a CPA were that the testes were obtained from abattoir derived bulls. Bulls sent to be slaughtered are sent there for numerous reasons, including low fertility. We confirmed this by only using 10 bulls of 32 males that showed epididymal sperm motility parameters higher than 60%.

It can be concluded that ethylene glycol at low concentrations does not provide adequate protection against freezing injury during cryopreservation of bovine caudal epididymal sperm. Moreover, the routine protocol used to cryopreserve bovine ejaculated sperm must be improved to successfully cryopreserve sperm harvested from postmortem animals. More research is needed to establish what concentration of CPA is the optimal for the cryopreservation of bovine epididymal sperm.
CHAPTER V
THE EFFECT OF ADDITION OF BOVINE SEMINAL PLASMA TO BOVINE CAUDAL EPIIDIDYMAL SPERM PRIOR TO CRYOPRESERVATION

INTRODUCTION

Few studies on the cryopreservation of bovine epididymal sperm have been reported. Recovery of epididymal sperm from postmortem animals will enable the propagation of valuable gametes from genetically superior animals that may die or have been injured. Development of a successful protocol to retrieve and cryopreserve bovine epididymal sperm can be used as a model for the preservation of male gametes from endangered bovid species.

In a recent study, Gooaverts et al. (2006) reported lower sperm motility characteristics of bovine epididymal sperm when compared with ejaculated sperm. The main difference between these two cell types is the presence or absence of accessory sex gland fluids. Seminal plasma is a complex mixture of fluids from the testis, epididymis and accessory sex glands and is used as a vehicle for sperm into the female reproductive tract. Furthermore, it provides sperm with nutrients, a variety of biochemical components for the regulation of sperm function post-ejaculation (Strzezek et al., 1992) and factors that alter the sperm surface (Polakoski et al., 1982) and modulate the fertilizing ability of sperm (Amann and Griel, 1974).


Studies examining the post-thaw influence of seminal plasma on the survivability of sperm have been contradictory. One report stated that removal of seminal plasma was necessary for sperm cryosurvival (Amann and Pickett, 1987). In contrast, other reports indicate that seminal plasma is beneficial for sperm survivability post-thaw (Aurich et al., 1996, Katila et al., 2002). Graham (1994) reported that the addition of seminal plasma to washed bovine ejaculated or epididymal sperm had no effect on...
motility post-thaw. However, post-thaw motility was higher for epididymal sperm when treated with seminal plasma than for ejaculated sperm derived from the same male.

Removal of cytoplasmic droplets before cryopreservation may help reduce the percentage of broken midpieces and circular swimming patterns normally found in post-thaw epididymal sperm. It is probable that this morphological difference between epididymal and ejaculated sperm renders epididymal sperm more susceptible to osmotic damage during the cryopreservation process. It has been shown that the fertilizing ability of canine epididymal sperm is compromised after cryopreservation due to an increase in the percentage of sperm exhibiting abnormal morphology (Hori et al., 2004).

Selivanova et al. (1937) proposed that seminal plasma and its rate of dilution were the primary cause of cytoplasmic droplet detachment. Moreover, Bialy and Smith (1958) showed that seminal vesicle fluid reduces the quantity of distal droplets from bovine caudal epididymal sperm. Subsequently, a phospholipid binding protein was identified in the bovine ampulla and seminal vesicles, but not in epididymal fluid, which was capable of liberating droplets from mature and immature sperm from various species (Matousek and Kysilka, 1980). It is known that fewer sperm have droplets in the ejaculates of boars, rams, bulls and bucks compared with epididymal sperm suggesting that the droplets are removed near or prior to the time of ejaculation (Cooper, 2005).

Recently, Hori et al. (2005) reported a significant decrease in cytoplasmic droplets in canine epididymal sperm recovered with prostatic fluid. They reported that epididymal sperm motility, membrane integrity and pregnancy rates were higher after thawing when sperm was recovered with prostatic fluid.

Extended exposure of bovine epididymal sperm to seminal plasma have been reported to have detrimental effects on sperm viability (Way et al., 2000). In contrast, the effects of epididymal sperm retrieval with seminal plasma followed by a short incubation and its complete removal by density gradient centrifugation before cryopreservation has not been carefully evaluated.

Flow cytometry is a valuable tool to evaluate frozen-thawed sperm since it is an objective technique. This methodology allows accurate detection of small differences between samples, with high repeatability and statistical reliability (Pena, 2000). Studies on the use of flow cytometry with bovine epididymal sperm are uncommon. We have previously adapted the triple fluorochrome staining technique (SYBR 14/PI/PE-PNA)
developed by Nagy et al. (2003) for the evaluation of frozen-thawed epididymal sperm. This technique allows the accurate detection of sperm membrane integrity and acrosome integrity in the presence of egg yolk particulates.

Sperm motility depends greatly on the energetic status of the cell. Since the mitochondria of the sperm midpiece generate energy to support movements, changes in mitochondrial membrane potential have been suggested as a good indicator of functional sperm impairment (Pena et al., 2003). Furthermore, mitochondrial function has been related to fertility in some species (human: Kasai et al., 2002; stallion: Kirk et al., 2005).

Often variation in fertility found between individual males can not be adequately explained by conventional methods of sperm quality evaluation. This led to speculations that nonmorphological defects in sperm may be implicated in reduced fertility (Bedford et al., 1973). Normal sperm development leads to a chromatin structure in which DNA is fully resistant to denaturation (Gillian et al., 2005). Defects in the structure of chromatin can severely reduce fertility, impair embryo development, increase spontaneous abortions as well as birth defects (Evenson et al., 1980; Evenson and Jost, 2000; Cordelli et al., 2005).

The DNA of sperm with an abnormal chromatin structure is susceptible to denaturation and the extent of this abnormality can be detected using the metachromatic properties of acridine orange in the sperm chromatin structure assay (SCSA®) (Evenson et al., 1980). This well validated method has had good correlation with male fertility in a number of species, including mice (Evenson et al., 1980), cattle (Ballachey et al., 1987; Karabinus et al., 1990; Januskauska et al., 2001; Januskauskas et al., 2003), pigs (Evenson et al., 1994), horses (Love and Kenney, 1998) and humans (Hashimoto et al., 2003; Boe-Hansen et al., 2006; Chohan et al., 2006; Erenpreiss et al., 2006).

Testing sperm for their susceptibility to DNA denaturation would increase our ability to eliminate males with potentially low fertility. No information has been found on chromatin stability from sperm of abattoir-derived bulls.

The objective of this study was to assess the effect of absence or presence of seminal plasma during bovine caudal epididymal sperm retrieval as regard sperm morphology, progressive motility, viability, mitochondrial function and chromatin stability post cryopreservation. Furthermore, we will optimize the use of the SCSA® and of
MitoTracker Red CMXRos in combination with SYBR 14 for the assessment of chromatin stability and mitochondrial function of bovine epididymal sperm derived from postmortem abattoir bulls.

**MATERIALS AND METHODS**

**Experimental Design**

Experiment 5.1

Paired testes were obtained from mature bulls (n=10) at a local abattoir and transported to the laboratory within 3 to 5 hours postmortem. Caudal epididymal sperm were retrieved by multiple incisions from the caudae epididymides from each pair of testes, sperm from each bull was pooled and placed in either egg yolk Tris-glucose citric acid monohydrate extender (EYT-GC) (Treatment A) or bovine seminal plasma (Treatment B). Epididymal sperm was incubated for 30 minutes before removal of seminal plasma by centrifugation. Sperm from each treatment were then cryopreserved in EYT-GC extender supplemented with 7% glycerol. Prefreeze membrane integrity and acrosome integrity was evaluated by fluorescence microscopy. Post-thaw membrane integrity, acrosome integrity, mitochondrial function and DNA integrity were evaluated using multicolor flowcytometry. Overall and progressive motility were assessed subjectively under a light microscope. Three replicate samples were evaluated per animal each on different days.

**Experimental Procedure**

**Testes Collection**

Testes were collected as pairs from mature mixed breed bulls at an abattoir in Robert, Louisiana. The testes were then transported within 3 to 5 hours to the Louisiana State University Embryo Biotechnology Laboratory individually packed in Ziploc® plastic bags (Johnson & Son Inc., Racine, WI) in a Styrofoam box with the temperature ranging from 28°C to 29°C. After arrival to the laboratory, each pair of testes was dissected away from its scrotum and tunica vaginalis. Testes were positioned on the table with the vas deferens facing down and the corpus facing up to distinguish the side of origin of each testis. The weight of the testis was measured with a Pelouze® balance scale (Model SP5) before carefully dissecting free the cauda epididymis.
Medium Preparation

Egg yolk Tris-glucose citric acid monohydrate extender (Liu et al., 1998) (EYT-GC) (250 mM Tris (T-6066, Sigma-Aldrich Inc., St. Louis, MO), 69 mM glucose (6152, Sigma-Aldrich Inc., St. Louis, MO), 81 mM citric acid monohydrate (C-1909, Sigma-Aldrich, St. Louis, MO) and 20% (v/v) egg yolk) was prepared with the addition of 50 µg/ml of gentamicin (15750-060, Gibco, Grand Island, NY) and filtered through a 0.45 µm Nalgene® syringe filter (190-2545, Nalge Company, Rochester, NY) to remove large egg yolk materials. Egg yolks were obtained from fresh eggs at the Louisiana State University Poultry Research Farm. The extender was placed in a 37°C water bath for 2 to 3 hours prior to harvesting the epididymal sperm.

Seminal Plasma Collection and Processing

Ejaculates for seminal plasma extraction were collected from mature beef bulls (n=6) at a commercial bull stud (Genex Corporation, Baton Rouge, Louisiana). Ejaculates were collected with an artificial vagina (AV) and placed in a 37°C water bath for motility analysis. Ejaculates were transported to the Embryo Biotechnology Laboratory at 20° to 25°C for seminal plasma collection and storage.

Collections with more than 50% progressively motile sperm were pooled and used as part of this experiment. Samples were centrifuged at 2,000 x g in a general purpose centrifuge (Model 5682 GP8R, Thermoforma, Marietta, OH) for 20 minutes. The supernatant was placed into a new 15 ml plastic tube (20171-024, VWR International Inc., Sugar Land, TX) and the sperm pellet was discarded. The centrifugation procedure was repeated twice. The supernatant of each sample was filtered through a 0.8 µm Acrodisc® syringe filter (PN 4618, Pall Corporation, East Hills, NY) for the complete removal of any remaining sperm. Filtered seminal plasma from the six bulls was thoroughly mixed together (pooled) and stored in 2 ml aliquots in a -20°C freezer. On the day of use, 3 vials of the seminal plasma were thawed in a 37°C water bath ~30 minutes prior to epididymal sperm extraction.

Epididymal Sperm Retrieval

After dissection, each cauda epididymis was weighed with a balance scale and washed from remaining materials with 0.9% saline before epididymal sperm retrieval. Epididymal sperm were harvested by making 5 incisions using a surgical blade (size 10; MDS-15110, Medline Industries Inc., Mundelein, IL) in the cauda epididymis. Half of the
sperm was scooped out from the incisions with a new surgical blade and placed in a 60 mm Falcon® plastic petri dish (3037, Becton Dickinson Labware, Franklin Lakes, NJ) containing 2 ml of EYT-GC extender (Treatment A). The other half was placed (with a new blade) in another 60 mm plastic petri-dish containing 2 ml of seminal plasma (Treatment B). Each sperm treatment sample was transferred into separate 4 ml plastic tubes and placed in a 37°C water bath for 30 minutes.

Assessment of Morphology

After the incubation period and after cryopreservation, a small droplet of each sperm sample was mixed in ~20 µl of eosin-nigrosin morphology stain (Lane Manufacturing Inc, Denver, CO) previously prepared on a precleaned microscope slide (3051, Gold Seal® Products, Portsmouth, NH). Using a new slide, a smear was prepared by spreading the sperm-stain mixture over the surface of the slide. Smears were allowed to air dry for ~24 hours before evaluation. Smears were evaluated under light microscopy using a 1,000X oil immersion objective (Nikon, Tokyo, Japan). A minimum of 200 sperm per treatment sample were evaluated to determine the percentages of lose heads, abnormal heads, broken necks, bent tails, presence of distal and/or proximal droplets. In this experiment, the presence of distal droplets was not recorded as sperm abnormality at the time of evaluation.

Seminal Plasma Removal

Samples were placed over a two column sperm separation medium (99264, ISolate®, Irvine Scientific, Santa Ana, CA) (1 ml lower and 1 ml upper layer) in a 15 ml plastic tube and centrifuged for 20 minutes at 300 x g to remove seminal plasma. After centrifugation, the ISolate® upper layer with the seminal plasma or extender was removed. The lower layer containing the sperm suspension (1 ml) was resuspended in 8 ml of Tyrodes Lactate HEPES (TL-H) (04-616F, Cambrex Bio Science Walkersville Inc., Walkersville, MD) to wash sperm and remove silica particles. The sperm mixture was then centrifuged at 350 x g for 5 minutes and resuspended in EYT-GC extender.

Epididymal Sperm Analyses Prior to Cryopreservation

Sperm samples were analyzed for plasma membrane integrity, acrosome integrity, overall motility and progressive motility. A 100-µl sample from the sperm mixture was placed in a 1.7 ml plastic micro-centrifuge tube (20170-355, VWR
International Inc, Sugar Land, TX) and diluted 1:10 sperm solution: EYT-GC for epididymal sperm quality analysis.

Plasma Membrane Integrity

Plasma membrane integrity was assessed using the LIVE/DEAD® Sperm Viability Kit (L-7011, Molecular Probes Inc., Eugene, OR)). The kit consists of two nucleic acid stains. SYBR 14 (1 mM solution in DMSO, Component A of the LIVE/DEAD® Sperm Viability Kit) is a permeable nucleic acid stain that labels membrane intact sperm green. PI (2.4 mM solution in H₂O, Component B of the LIVE/DEAD® Sperm Viability Kit) is a nonpermeable nucleic acid stain which labels membrane ruptured sperm red. For analysis, 250 µl of the diluted sperm sample from each treatment group per bull were placed in a 0.65 ml plastic micro-centrifuge tube (20170-293, VWR International Inc., Sugar Land, TX). Staining was completed by the addition of 100 nM SYBR 14 and 24 µM PI to the sperm solution. The stained sample was incubated for 10 minutes in the dark at 37°C and mixed gently before analysis.

Acrosome Integrity

Acrosome integrity was assessed using the lectin peanut agglutinin conjugated to FITC (FITC-PNA) (L-7381, Sigma-Aldrich Inc., St. Louis, MO) and the nucleic acid stain PI. PNA labels acrosome reacted sperm green, while PI discriminates between viable and nonviable sperm. For analysis, 250 µl of the diluted sperm sample from each treatment group per bull were placed in a 0.65 ml plastic micro-centrifuge tube. The final concentration was 5 µg/ml of FITC-PNA and 24 µM PI. The stained sample was incubated for 15 to 20 minutes in the dark at 37°C and mixed before analysis.

For plasma membrane and acrosome integrity, each sample (n=10 bulls per treatment) was analyzed by counting 300 sperm cells with an inverted microscope (Nikon Diaphot, Tokyo Japan) equipped with epi-fluorescent illumination, a FITC filter set and a heated stage.

Progressive motility

Sperm progressive motility was assessed subjectively before cooling on each treatment for each of the 10 bulls by the same technician at 20X magnification using an inverted microscope (Nikon Diaphot, Tokyo, Japan) equipped with Hoffman optics and a heated stage.
Sperm Cooling, Cryopreservation and Thawing

Sperm concentration was adjusted to 70 x 10^6 cells/ml using a hemacytometer by the addition of more EYT-GC extender. The 15 ml plastic tubes containing the epididymal sperm samples were placed in a 250 ml Pyrex® glass beaker (13912-207, VWR International Inc., Sugar Land, TX) containing water at room temperature. The beaker was placed for 4 hours in a refrigerator for cooling the sperm samples to 4°C.

Samples were diluted slowly over a period of 30 minutes 1:1 in EYT-GC medium containing 14% glycerol and subsequently loaded into previously cooled 0.5 ml straws (005569, Cassou straw, IMV Technologies, Minneapolis, MN). Straws were placed in a custom built rack 2 cm over liquid nitrogen (LN2) vapors for 10 minutes before being plunged in LN2. A total of 10 to 15 straws were frozen per bull.

Sperm samples were stored for 2 to 3 months prior to thawing. On day of analysis, 50 ml of TL-H were placed in a 50 ml plastic tube (BCT-P50RS, Biologix, Shawnee Mision, KS) and warmed in a 37°C incubator 1 hours prior to sperm dilution. Straws were thawed in a water bath at 37°C for 40 seconds. The contents of each straw were placed in a 15 ml plastic tube and diluted 1:5 sperm sample:TL-H before assessing sperm morphology, sperm motility and multicolor flow cytometric analyses.

Multicolor Flow Cytometry

Flow cytometric measurements were carried out on a FACSCalibur flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA). Forward and side scatter values were recorded on a linear scale, while fluorescent values were recorded on a logarithmic scale for all assays, unless otherwise stated. All fluorochromes were excited at 488 nm with a 20 mW argon laser. Density and dot plots drawn for data analysis were generated by WinMDI 2.8 (software by J. Trotter, available for downloading at http://www.facs.scripps.edu/software.html).

Assessment of Plasma Membrane and Acrosome Integrity Post-Cryopreservation

Assessment of plasma membrane and acrosome integrity were completed using a triple stain protocol in the presence of egg yolk particles (Nagy et al., 2003) with minor modifications. The staining protocol consisted of SYBR 14, PI and the lectin peanut agglutinin conjugated to PE (R-PE-PNA) (p-44, Biomeda Corporation, Foster City, CA). At a final concentration of 100 nM SYBR 14 solution, 4 µg/ml of R-PE-PNA (1 mg/ml of stock solution in a buffer composed of 50 mM sodium phosphate and 0.05% sodium
azide, [pH 7.0], and also containing 0.1 mM [Ca$^{2+}$] and [Mn$^{2+}$] ions) and 12 µM PI solution were added to 250 µl of diluted sperm sample. Samples were thoroughly mixed and incubated at 37°C in the dark for 30 to 40 minutes and then gently remixed before flow cytometric analysis.

Plasma membrane ruptured sperm were PI positive, and their red fluorescent signal was detected on the FL3-channel using a 610 nm long pass filter. Membrane intact sperm were SYBR 14 positive, and their green fluorescent signal was detected on the FL1-channel using a 530/30 nm band pass filter. Acrosome reacted sperm were PE-PNA positive, and its orange fluorescent signal was detected at 585/42 nm on the FL2-channel. Three color compensations were set according to the FACS® training manual (Becton Dickinson Biosciences, San Jose, CA). Nonsperm events (egg yolk particles) were gated out of the analyses, as judged on scatter properties detected in the forward and side scatter detectors. Moreover, events with similar scatter characteristics to sperm but with low PI and SYBR 14 fluorescence were also gated out in the procedure. Sperm acquisitions were made using CellQuest software (Becton Dickinson, San Jose, CA, USA). The flow cytometer was kept at a low flow of less than 300 sperm/second. The recording of scatter and fluorescent properties of all events stopped when 10,000 gated events were recorded.

On SYBR 14 (FL1) and PI (FL3) dot plots, regions were drawn to determine the percentage of viable and non viable sperm cells. On PE-PNA (FL2) and PI (FL3) dot plots, quadrants were set to measure the percentage of sperm showing plasma membrane intact and acrosome intact sperm (LL), plasma membrane intact and acrosome reacted sperm (LR), plasma membrane ruptured and acrosome intact sperm (UL) and plasma membrane ruptured and acrosome reacted sperm (UR).

**Assessment of Mitochondrial Function Post-Cryopreservation**

Assessment of mitochondrial function was carried out using a novel dual stain protocol in the presence of egg yolk particles developed in our laboratory. The staining protocol consisted of SYBR 14 and MitoTracker Red CMXRos (MITO) (Molecular Probes Inc., Eugene, OR, USA). A 1mM MITO stock solution was prepared in DMSO. Then a 20 µM working solution was prepared in TL-H before sample preparation. For staining, 100 nM SYBR 14 and 100 nM MITO were added to 250 µl of diluted sperm.
Figure 5.1. Flow cytometric out-gating of egg yolk particles based on scatter properties after staining frozen-thawed bovine epididymal sperm with SYBR 14 and MitoTracker Red. Sperm were selected (R1) based on size, gating out small particles.

Figure 5.2. MitoTracker Red (red) versus SYBR 14 (green) fluorescence. Remaining egg yolk particles having sperm-like scatter properties were gated out based on DNA fluorescence. Events that had SYBR 14 (R1) positive fluorescence were gated out and used for analysis.
Samples were thoroughly mixed and incubated at 37°C in the dark for 30 minutes and then remixed before flow cytometric analysis.

Detector FL-1 was used to detect SYBR 14 fluorescence (green). MITO fluorescence was detected on detector FL-3. Data acquisition and compensations were set, as described previously. The egg yolk particles were gated out based on scatter properties (Figure 5.1) and on particles showing high green fluorescence (Figure 5.2). Acquisitions were stopped after recording 10,000 SYBR 14 positive events.

On SYBR 14 (FL-1) and MITO (FL-3) dot plots, regions were drawn to determine the percentage of sperm with active mitochondria and sperm with no mitochondrial function. Sperm positive events showing a high red fluorescence were determined as viable sperm with active mitochondrial membrane potential (Figure 5.3). Sperm positive events showing a low red fluorescence were assessed as sperm with mitochondria that had lost their transmembrane potential (Figure 5.4).

Assessment of DNA Integrity Post-Cryopreservation

DNA integrity was assessed using acridine orange (AO) (15855-0, Sigma-Aldrich, St. Louis, MO) in the sperm chromatin structure assay (SCSA), as described by Evenson (1980), with minor modifications. The utility of AO is based on its metachromatic emission of fluorescence upon excitation. AO intercalated into double stranded (dsDNA) nucleic acids fluoresces green. AO bound to single stranded (ssDNA) nucleic acids emits a red fluorescence (Ballachey et al., 1987). When used with a flow cytometer, the green and red fluorescence can be separated to indicate the amount of ssDNA in the sperm sample. Evaluation of abnormal chromatin structure was assessed as the increased susceptibility of sperm to acid-induced denaturation in situ. Quantification was completed by measuring the percent of cells that shift from green to red fluorescence. Cells outside the green population are determined as cells outside the main population (COMP). COMP can be analyzed either by a flow cytometric dot plot or histogram. The dot plot was used for analysis in this study.

Diluted sperm samples were further diluted 1:5 with TNE buffer (0.01 M tris (hydroxymethyl) aminomethane (Tris) (15453-3, Sigma-Aldrich, St. Louis, MO), 0.15 M NaCl (S5886, Sigma-Aldrich, St. Louis, MO) and 1 mM disodium ethylenediaminetetra acetate [EDTA] [E-5134, Sigma-Aldrich, St. Louis, MO], pH 7.4) to a total of 0.2 ml. Samples were maintained on ice at all times. Sperm were subjected to partial DNA
Figure 5.3. Flow cytometric dot plot illustrating MitoTracker Red (red) and SYBR 14 (green) fluorescence from sperm of a bull exhibiting high mitochondrial activity. Graph displays an epididymal sperm sample (10,000 sperm) with 69% of its population having active mitochondria.

Figure 5.4. Flow cytometric dot plot illustrating MitoTracker Red (red) and SYBR 14 (green) fluorescence from sperm of a bull exhibiting low mitochondrial activity. Graph displays an epididymal sperm sample (10,000 sperm) with only 17% of its population having active mitochondria.
denaturation in situ by mixing the 0.2 ml of diluted sperm sample with 0.4 ml of a low pH detergent solution (0.15 M NaCl, 0.1% Triton X-100 [T-9284, Sigma-Aldrich, St. Louis, MO] and 0.08 N HCl [SA48-1, Fisher Scientific, Fair Lawn, NJ], pH 1.4). Exactly 30 seconds later, 1.2 ml of AO staining solution (0.2 M NaH2PO4 [S-5011, Sigma-Aldrich, St. Louis, MO], 1 mM disodium EDTA, 0.15 M NaCl, 0.1 M citric acid monohydrate [C-1909, Sigma-Aldrich, St. Louis, MO], 6 µg/ml of AO, pH 6.0) were added to the sperm sample. The stained samples were analyzed 3 minutes later by flow cytometry.

Fluorescent values were recorded on a linear scale. Detector FL-1 was used to detect the green fluorescence emitted by dsDNA. Detector FL-3 was used to detect the red fluorescence emitted by ssDNA. The standard bull (CSS 7H5188, Genex Cooperative Inc., Shawano, WI) used for in vitro fertilization at the Embryo Biotechnology Laboratory was used to set up the machine and as a control. Data acquisition and compensations were set, as described previously. Most egg yolk particles were gated out by drawing a region on particles showing a low green and red fluorescence. Acquisitions were stopped after recording 10,000 gated events.

On the red (FL-3) and green (FL-1) dot plot (Figure 5.5) and the (FL-3) histogram (Figure 5.6) regions were drawn to determine the percentage of COMP. Sperm events showing red fluorescence (% COMP) were determined as sperm with damaged DNA.

Statistical Analysis

Variances in testicular parameters, morphology, membrane integrity, acrosome integrity, mitochondrial function, % COMP and overall and progressive motility were statistically analyzed by ANOVA and the differences between bulls were calculated by a Tukey Multiple Comparisons test. Additionally, a t-test was used to verify differences within each sperm parameter before and after cryopreservation. The sperm parameters are expressed as mean±SEM per treatment group. A P<0.05 was considered significant in this study. Data were analyzed using SigmaStat Statistical Software Version 2.0.

RESULTS

Experiment 5.1

Testicular and Epididymal Weights

Testicular and caudae epididymal weights for individual bulls are shown in Table 5.1. Weights from the right testis of bull number 5 were not included in the mean values due to the abnormally low weight. Testes weights ranged from 152 to 455 g and caudae
Figure 5.5. Flow cytometric dot plot illustrating green versus red fluorescence of acridine orange in the SCSA®. Graph displays bovine sperm with no DNA damage as green and sperm with denatured DNA (R1) fluorescing red.

Figure 5.6. Flow cytometric histogram illustrating red fluorescence of acridine orange in the SCSA®. Bovine sperm in the M1 region are exhibiting DNA damage (% COMP).
### Table 5.1. Testicular and epididymal measurements from bovine testes collected postmortem

<table>
<thead>
<tr>
<th>Bull No.</th>
<th>Testis weight (g)</th>
<th>Cauda weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>1</td>
<td>340</td>
<td>326</td>
</tr>
<tr>
<td>2</td>
<td>249</td>
<td>253</td>
</tr>
<tr>
<td>3</td>
<td>251</td>
<td>439</td>
</tr>
<tr>
<td>4</td>
<td>306</td>
<td>367</td>
</tr>
<tr>
<td>5*</td>
<td>90</td>
<td>455</td>
</tr>
<tr>
<td>6</td>
<td>317</td>
<td>364</td>
</tr>
<tr>
<td>7</td>
<td>368</td>
<td>379</td>
</tr>
<tr>
<td>8</td>
<td>218</td>
<td>243</td>
</tr>
<tr>
<td>9</td>
<td>152</td>
<td>154</td>
</tr>
<tr>
<td>10</td>
<td>184</td>
<td>145</td>
</tr>
</tbody>
</table>

Mean±SEM: 265±24.3, 312±34.6, 8.6±1.2, 9.7±1.4

*Weight from the right testis and cauda of bull number 5 were not included in the total mean values due to the abnormally low weight. Means from testes and cauda weights were not significantly different (P>0.05).
epididymides weights ranged from 3 to 18 g. No significant difference (P>0.05) was
detected between bulls in both testicular parameters. There was a high intermale
variability in both testicular weights analyzed.

Morphology

Overall, epididymal sperm harvested with the addition of seminal plasma had
80.6±4.9% of sperm with normal morphology and was significantly higher (P<0.05) than
with without the addition of seminal plasma at 57.5±5.8%. Incubation in seminal plasma
significantly improved sperm quality, reducing the quantity of distal cytoplasmic droplets
and bent tails both prior to and post-cryopreservation (Table 5.2). However, the
incidence of other forms of sperm abnormalities were not increased or decreased by
treating sperm with bovine seminal plasma.

After cryopreservation, sperm previously exposed to seminal plasma had
76.2±3.3% of sperm with normal morphology compared with 39.9±3.7% in the
nonexposed treatment group. Cryopreservation significantly (P<0.05) increased the
percentage of sperm displaying bent tails and increased distal cytoplasmic droplet
detachment in both treatments.

Although the quantity of distal droplets was reduced after incubation in seminal
plasma, the extent of this removal was different among bulls. High distal cytoplasmic
droplet detachment was observed in 70% of the bulls after incubation in seminal plasma
while the remaining 30% showed no significant effect.

Overall and Progressive Motility

The mean initial overall motility and progressive motility values for all the bulls
used in this study after epididymal sperm retrieval were 75.5±1.7% and 68.0±2.1%,
respectively. Cryopreservation had an overall detrimental effect (P<0.05) on post-thaw
sperm motility for sperm harvested with or without seminal plasma treatment groups.
The mean overall post-thaw motility values for samples retrieved with or without seminal
plasma were 47.5±2.1% and 40.5±2.1%, respectively. In addition, the mean progressive
post-thaw motility values for samples retrieved with or without seminal plasma were
39.6±2.4% and 20.3±1.8%, respectively. Epididymal sperm retrieved with seminal
plasma achieved a significantly higher post-thaw overall and progressive motility than
the control (no seminal plasma).
Table 5.2. Morphology values (mean%±SEM) of bovine epididymal sperm prior to and post-cryopreservation

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Normal</th>
<th>Distal droplets</th>
<th>Proximal droplets</th>
<th>Bent tails</th>
<th>Abnormal heads</th>
<th>Detached heads</th>
<th>Broken necks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Pre</td>
<td>57.5±5.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>65.8±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.1±3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.9±5.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.2±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.2±2.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>39.9±3.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52.7±3.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47.3±4.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.2±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.1±1.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Seminal plasma</td>
<td>Pre</td>
<td>80.6±4.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>45.9±1.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.8±3.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.7±0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.5±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.4±1.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Post</td>
<td>76.2±3.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36.9±2.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.0±1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.9±3.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.7±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.3±1.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup>Means with different superscripts within columns are significantly different (P<0.05).
Figure 5.7. Post-thaw percent overall motility (mean±SEM) of bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation. a,b,c Means with different letters within bull are significantly different (P<0.05).
Figure 5.8. Post-thaw percent progressive motility (mean±SEM) of bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation. a,b,cMeans with different letters within bull are significantly different (P<0.05).
The mean overall and progressive motility values for individual bulls prior to and post-cryopreservation are shown in Figures 5.7 and 5.8. Epididymal sperm collected from animals harvested with seminal plasma had a higher (P<0.05) overall and progressive motility than controls after thawing. However, bull number 6 and 7 showed no difference (P>0.05) in overall motility between treatments. Furthermore, bull number 9 had a higher post-thaw overall motility in the control treatment than in sperm retrieved with seminal plasma.

Membrane Integrity

The mean initial plasma membrane integrity value for the bulls used in this study before cryopreservation was 81.2±2.1%. The mean percentages of post-thaw sperm with intact membranes were 52.1±2.4% and 53.0±2.0% when retrieved with or without seminal plasma, respectively. No significant difference was detected between treatments. In contrast, there was a significant decline (P<0.05) in the percentage of sperm with intact plasma membranes after cryopreservation in both sperm treatment groups.

The mean intact membrane values for individual bulls prior to and post-cryopreservation are shown in Figure 5.9. Bull number 7 had the highest number of viable sperm after thawing in both treatments, while bull number 9 had the lowest membrane integrity values prior to and post-cryopreservation of the bull group.

Acrosome Integrity

Overall, the mean prefreeze acrosome integrity value was 92.0±0.5%. Epididymal sperm harvested with seminal plasma contained 75.0±3.1% intact acrosomes, compared with 80.6±2.2% in the control group. No statistical difference was detected between treatment groups. However, there was a significant decline (P<0.05) in acrosome integrity after the cryopreservation process in sperm retrieved with or without seminal plasma.

The mean numerical value of viable sperm with intact acrosomes was higher (52.7±2.0%) when epididymal sperm were not exposed to seminal plasma but not different than that for sperm exposed to seminal plasma (51.5±2.4%). Sperm exposed to seminal plasma prior to cryopreservation had a mean of 0.6±0.1% viable sperm with damaged acrosomes after thawing, while the control sperm had 0.3±0.1%. Furthermore, the numerical value of post-thaw nonviable sperm with reacted acrosomes was higher.
Figure 5.9. Post-thaw percent membrane integrity (mean±SEM) of bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation. a,b,c Means with different letters within bull are significantly different (P<0.05).
Figure 5.10. Post-thaw percent reacted acrosomes (mean±SEM) derived from the viable and nonviable populations of bovine epididymal sperm harvested with extender (no seminal plasma) or seminal plasma prior to cryopreservation. a,b Means with different letters within treatment are significantly different (P<0.05).
Figure 5.11. Percent acrosome integrity (mean±SEM) of post-thaw bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation. a,b,c Different letters within bulls indicate significantly different (P<0.05).
(23.7±3.0%) in sperm exposed to seminal plasma but not different than that for sperm not exposed to seminal plasma (18.1±1.8%) prior to cryopreservation (Figure 5.10). In contrast, a higher quantity (P<0.001) of sperm with damaged acrosomes was found from the nonviable than from the viable sperm population in sperm retrieved with or without seminal plasma.

The mean acrosome integrity values for individual bulls before and after cryopreservation are shown in Figure 5.11. Bull number 8, 9 and 10 had a significantly higher (P<0.05) acrosome integrity when sperm were not exposed to seminal plasma before freezing. However, bull number 1 had a higher acrosome integrity when exposed to seminal plasma prior to cryopreservation. There was an intermale variation noted in acrosome integrity post-thaw with males being exposed to the same treatments.

Mitochondrial Function

The overall mean percentages of post-thaw sperm with functioning mitochondria were 49.3±2.0% and 47.9±1.7% when retrieved with or without seminal plasma prior to cryopreservation, respectively. No significant (P>0.05) difference was detected between treatments.

The overall mean values for sperm exhibiting functioning mitochondria for individual bulls post-cryopreservation are shown in Figure 5.12. Bull number 1, 4 and 7 had a significantly higher percentage of sperm with active mitochondria after thawing when sperm were exposed to seminal plasma prior to the freezing process compared with the control extended sperm. However, bull number 9 had lower mitochondrial function post-thaw when sperm were exposed to seminal plasma prior to cryopreservation compared with the not exposed to seminal plasma treatment group.

DNA Integrity

The overall mean values of post-thaw sperm exhibiting chromatin damage were 1.6±0.3% and 2.5±0.5% when harvested with or without seminal plasma prior to cryopreservation, respectively. The range of chromatin damage when sperm were retrieved with or without seminal plasma were 0.9 to 3.5% and 0.8 to 7.8%, respectively.

The mean values of post-thaw sperm exhibiting chromatin damage for individual bulls are shown in Figure 5.13. Bull number 1 had significantly (P<0.01) less DNA damage when sperm were briefly exposed to seminal plasma prior to the
Figure 5.12. Percent mitochondrial activity (mean±SEM) of post-thaw bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation. a,b Means with different letters within bull are significantly different (P<0.05).
Figure 5.13. Percent DNA damage (mean±SEM) of post-thaw bovine epididymal sperm harvested with either extender (no seminal plasma) or seminal plasma prior to cryopreservation. a,b Means with different letters within males are significantly different (P<0.05).
cryopreservation process compared to sperm not exposed to seminal plasma. There was a variation noted between males exposed to the same treatment.

**DISCUSSION**

The number of studies conducted on the cryopreservation of bovine epididymal sperm is very limited and, to the best of our knowledge, none has reported the effect of epididymal sperm retrieval with seminal plasma followed by a short incubation and its complete removal before cryopreservation.

In the work described herein we have demonstrated that the use of seminal plasma to harvest bovine epididymal sperm is beneficial to enhance sperm quality post-cryopreservation. Seminal plasma significantly improved overall and progressive motility (P<0.05) characteristics when compared with the control (no seminal plasma) and increased the quantity of sperm exhibiting normal morphology post-thaw. However, seminal plasma exposure prior to cryopreservation had neither beneficial nor detrimental effects on viability, mitochondrial function and DNA integrity after thawing.

Ejaculated sperm have been reported to differ from epididymal sperm in respiration (Hammerstedt et al., 1993), heparin-binding sites (Nass et al., 1990), ability to capacitate (Miller et al., 1990), morphology (Hewitt et al., 2001) and proteins bound to the plasma membrane (Lee et al., 1985). Recently, bovine ejaculated sperm have been shown to display different motion characteristics than bovine epididymal sperm when assessed by computer-assisted sperm analysis (Gooaverts et al., 2006). Epididymal sperm exhibits lower straight line and average path velocity and higher amplitude of the head, which could hinder the penetration of the zona pellucida during fertilization (Suarez et al., 1991).

In stallions, epididymal sperm have been shown to display progressive motility similar to ejaculated sperm. However, the fertility of stallion epididymal sperm was much lower than ejaculated sperm after cryopreservation (Tiplady et al., 2002). Also, canine epididymal sperm have been reported to exhibit lower motility and pregnancy rates than ejaculated sperm post-cryopreservation (Hori et al., 2004). Recently, the addition of prostatic fluid to canine epididymal sperm prior to freezing enhanced motility, membrane integrity and pregnancy rates after cryopreservation (Hori et al., 2005).

Various groups have reported seminal plasma factors that support or enhance sperm motility and fertility (Baas et al., 1983; Okamura and Sugita, 1983; Killian et al.,
1993; Bellin et al., 1998; Love, 2005). However, seminal plasma is also known to contain factors that inhibit motility (Iwamoto and Gagnon, 1988), inhibit capacitation (Chang, 1957) and negatively affect viability of sperm during extended incubation (Way et al., 2000). These contradictory reports may be due to seminal plasma having different roles in different species. Some species might only use it as a vehicle of transportation into the female reproductive tract, while others might need it as a final maturation step prior to fertilization.

In the present study, we found no detrimental effect in post-thaw viability when epididymal sperm were harvested with seminal plasma. Most studies in the bull regarding effects of seminal plasma on sperm have been made during long term incubation and have not studied its possible cryoprotective effect. We avoided long term incubation to minimize its possible detrimental effects. No difference in viability was noted for epididymal sperm harvested and incubated for 30 minutes in seminal plasma before cryopreservation compared with that of the control extender before cryopreservation. In contrast, Way et al. (2000) found a 20% decrease in sperm viability as soon as seminal plasma was added to bull epididymal sperm.

In the present study, seminal plasma enhanced both post-thaw overall and progressive motility characteristics of epididymal sperm. This is in agreement with a recent study reported were motility was greatly improved post-thaw when canine epididymal sperm was harvested with prostatic fluid (Hori et al., 2005). This might have been due to beneficial factors in seminal plasma as well as the reduction in cytoplasmic droplets prior to cryopreservation. In the present study, the motion characteristics of sperm incubated with seminal plasma both pre- and post-thaw was not assessed. However, faster and straighter progressive motility was observed after sperm incubation with seminal plasma.

Henault et al. (1995) demonstrated that incubation of epididymal sperm with seminal plasma increased their ability to penetrate bovine oocytes in vitro. A possible explanation may be that seminal plasma factors might have enhanced straight line motility and reduced head amplitude increasing cumulus cells and zona pellucida penetration rates. Also, seminal plasma might have caused modifications in the plasma membrane that enhanced zona pellucida recognition and binding. This further verifies
that bovine seminal plasma might have uses other than transportation of sperm into the female reproductive tract that are still unknown.

The overall mean number of epididymal sperm possessing droplets after extraction was 65.8%. This value is in close agreement with 62.9% having droplets reported by Branton and Salisbury (1947) but is lower than the 84% with droplets reported later by Bialy and Smith (1957). This difference could be accounted to different extenders used, dilution rates and possibly male to male variation. Furthermore, our results are in agreement with previous studies (Selivanova et al., 1937; Bialy and Smith, 1958), that reported a decrease in cytoplasmic droplets after incubation of bovine epididymal sperm in seminal plasma. Our results showed a significant decrease (P<0.05) in cytoplasmic droplets when epididymal sperm was harvested and incubated in seminal plasma at 37°C for 30 minutes. The extent of this reduction in cytoplasmic droplets was found in some bulls more than others. Part of the difference could be accounted for by the inherent bull differences (Bialy and Smith, 1957).

In the present study, cryopreservation caused a significant elevation in the percentage of epididymal sperm with abnormal morphology only when sperm had not been exposed to seminal plasma prior to the freezing process. This could be due to the reduction of cytoplasmic droplets when sperm was incubated in seminal plasma prior to cryopreservation. However, 1 of 10 bulls showed no significant reduction in cytoplasmic droplets after incubation with seminal plasma and had no elevation of post-thaw morphological abnormalities. Thus, we propose that unknown factors in seminal plasma are perhaps protecting sperm from osmotic or mechanical damage during cryopreservation.

Bent tails were reported to increase in rooster sperm during the removal of glycerol post-thaw (Westfall and Howarth, 1977). From previous observations at the laboratory (unpublished observations), cytoplasmic droplets are thought to increase the sensitivity of sperm to osmotic changes and to mechanical stress rendering them more vulnerable to morphological damage at the tail and midpiece after thawing. These observations were confirmed in this study, when the amount of bent tails increased significantly after thawing in the treatment not exposed to seminal plasma. These results are in agreement with those in a recent study using canine epididymal sperm, where an
elevation in morphological sperm abnormalities was noted post-cryopreservation (Hori et al., 2004).

The lower progressive motility we observed in epididymal sperm not treated with seminal plasma might have been due to the increase in bent tails post-thaw. However, post-thaw epididymal sperm previously exposed to seminal plasma were observed to possess faster velocity and straighter motility when compared with progressively motile sperm from the control treatment. An increase in bent tails and circular swimming patterns post-thaw have been observed in cattle and White Tail deer epididymal sperm (personal observations, unpublished) cryopreserved with a routine freezing protocol used for ejaculated sperm. This may be due to unknown biochemical components in seminal plasma that permanently modify sperm enhancing motility characteristics.

A sperm motility factor found in bovine seminal plasma has been reported to restore motility in nonmotile bovine sperm (Bass et al., 1983). In elephants, equine seminal plasma was shown to initiate motility while almost no motility (<1%) was initiated by the addition of extender (Schmitt et al., 2005). In humans, zinc and fructose levels in seminal plasma were reported to be correlated with motility (Fuse et al., 1999; Patel et al., 1988). Further investigations should include fractionation of bovine seminal plasma to identify potential chemical components that enhance progressive motility.

Some of the published protocols developed to evaluate sperm function with a flow cytometer appear to be compromised for an accurate analysis of frozen-thawed sperm. This is likely due to interactions of the fluorochromes with egg yolk particles of the freezing extender leading to misinterpretation of the results. The development of the SYBR 14/PI/PE-PNA triple stain protocol by Nagy et al. (2003) allows the easy removal of egg yolk particulates found in the freezing extender by gating using scatter properties and by particles exhibiting DNA staining.

Although various fluorochromes are being used to assess mitochondrial function in sperm, there are several problems connected with them for the evaluation of frozen-thawed sperm. Some of the most commonly used fluorochromes involve Rhodamine 123 (R123), MitoTracker Green, JC-1 and recently MitoTracker Deep Red 633. R123 is considered not reliable due to its low sensitivity, variability within samples and background staining (Garner et al., 1997). In addition, its fluorescence is lost when mitochondria experiences losses of membrane potential, thus limiting its use for fixation.
R123 also has been reported to have a higher nonspecific affinity to other parts of the sperm than JC-1 and MitoTracker Green (Garner et al., 1997).

MitoTracker Green labels mitochondria regardless of membrane potential. Its main use is to measure size and structure of mitochondria (Molecular Probes Handbook). Therefore, it is not a reliable fluorochrome to evaluate mitochondrial activity in sperm.

The usefulness of JC-1 is that this carbocyanine dye is capable of discriminating between mitochondria exhibiting high membrane potential from those possessing relatively low membrane potentials. However, by previous observations in our laboratory and by those of Garner et al. (1999), it does not work properly on frozen-thawed sperm due to interactions with egg yolk particles. MitoTracker Deep Red was shown to be reliable to evaluate mitochondrial function in frozen-thawed sperm (Hallap et al., 2005). However, a conventional flow cytometer equipped with a 488 nm argon laser can not be used to excite this fluorochrome due to its high excitation wavelength.

We report for the first time the use of MitoTracker Red CMXRos in combination with SYBR 14 to assess mitochondrial function in frozen-thawed epididymal sperm. This combination allows us to gate nonsperm events depending on scatter properties and DNA stainability.

Mitochondrial function in the present study was correlated to viability and overall sperm motility, however, mitochondrial function was not correlated to progressive motility. Previous studies have reported a high correlation between bovine ejaculated sperm mitochondrial activity and motility (Januskauskas et al., 2005). However, our results show that an increase in morphological abnormalities, such as bent tails, can hinder sperm from moving in a progressive manner even though they exhibit functionally active mitochondria.

Here we report the use of the well validated SCSA® for the evaluation of bovine epididymal sperm. To our knowledge, this is the first report to assess DNA damage from abattoir-derived bulls. Although we discarded those bulls with low motility (<50%) and high levels of abnormalities, this assay may also help discard bulls with spermatogenic disturbances that can not be uncovered by conventional methods. Also, no detectable detrimental effect on DNA was found in post-thaw bovine epididymal sperm previously exposed to seminal plasma when compared with the control sperm (no seminal plasma).
We found that some bulls possess chromatin that is more sensitive to denaturation than other bulls. It was noted that one bull possessed a significantly lower (P<0.01) percent of denatured DNA when it was harvested with seminal plasma. During the 30-minute incubation period, seminal plasma might have provided the necessary antioxidants to help protect DNA from free radicals (Love, 2005). Furthermore, the sperm nucleus absorbs zinc from seminal plasma during ejaculation (Bjorndahl et al., 1986). Zinc was shown to enhance chromatin stability (Kvist et al., 1987). Sperm DNA from that one bull might have been more susceptible to DNA damage when compared with the other males used in this study. Furthermore, one can not preclude that unknown factors in the seminal plasma might help protect or stabilize sperm nuclear chromatin.

The effect of seminal plasma from highly fertile bulls compared with low fertility bulls should be carefully studied, since incubation of sperm from a low fertility bull with seminal plasma from a highly fertile bull has been shown to increase the oocyte penetration rates in vitro (Heneault et al., 1995). Killian et al. (1993) have shown that two proteins (26 and 55 kDa) in seminal plasma predominate in higher fertility bulls, while Bellin et al. (1994) found two proteins (16 kDa each) that predominate in lower fertility bulls. The use of seminal plasma from highly fertile bulls might help enhance even more the protective effect during cryopreservation. It should not be overlooked that seminal plasma might help improve survivability of cryopreserved sperm from ‘lower freezer’ bulls.

We propose that sperm retrieval in the presence of seminal plasma is beneficial to enhance bovine epididymal sperm motility and to protect it from morphological abnormalities derived during the freeze-thaw process. Further research is needed to evaluate the performance and longevity of epididymal sperm exposed to seminal plasma during incubation post-thaw to address any possible long term effects.
CHAPTER VI

EFFECT OF DILUTION AND INCUBATION OF POST-THAW BOVINE EPIDIDYMAL SPERM HARVESTED WITH SEMINAL PLASMA PRIOR TO CRYOPRESERVATION

INTRODUCTION

Cryopreservation of bovine epididymal sperm is still very inefficient. Recently, the quality of post-thaw bovine epididymal sperm has been enhanced by a short exposure to seminal plasma prior to cryopreservation (unpublished, personal observations). Seminal plasma produces physiological changes to sperm preparing it for capacitation (Miller et al., 1990) and fertilization (Bellin et al., 1998). Osmotic injury can occur when cryopreserved epididymal sperm are diluted directly into an isotonic medium after thawing. When sperm treated with glycerol are abruptly placed in an isotonic environment, the intracellular environment is hyperosmotic relative to the extracellular environment. The membrane permeability of glycerol is lower than that of water. Therefore, there is a net influx of water resulting in an increase in cell volume (Ball and Vo, 2001). This can cause cell lysis and damage to the plasma membrane due to the fast water movement across the membrane (Curry and Watson, 1994). Osmotic tolerance of sperm to hyperosmotic conditions differs between species. Post-hyperosmotic stress is characterized by an irreversible loss of motility, membrane integrity and mitochondrial function (Ball and Vo, 2001).

A one step addition and removal of glycerol to cooled bovine epididymal sperm have been shown to be detrimental (Guerrero et al., 2006). Step-wise addition of glycerol to ejaculated sperm prior to cryopreservation is done routinely to prevent osmotic damage. However, glycerol is routinely removed from post-thaw ejaculated sperm in a one step dilution process. Swelling of sperm was reported to be more detrimental than shrinking, which indicates the importance to further evaluate the removal than the addition of cryoprotectants (Pommer et al., 2002). Although multiple studies have demonstrated the theoretical benefits of a stepwise dilution to remove permeating cryoprotectants from post-thaw sperm (e.g., Gilmore et al., 1995; Gao et al., 1995; Gilmore et al., 1998; Liu and Foote, 1998; Phelps et al., 1999; Ball and Vo, 2001; Agca et al., 2005), studies examining this effect experimentally are rather uncommon (Gilmore et al., 1997; Wessel and Ball, 2004). No studies have been found on the sensitivity of post-thaw bovine epididymal sperm to a one step removal of glycerol.
Moreover, epididymal sperm previously exposed to seminal plasma prior to cryopreservation may be more sensitive to osmotic changes due to physiological modifications caused to the plasma membrane by biochemical components found in seminal plasma (Harrison et al., 1992).

In the stallion, stepwise removal of glycerol from fresh ejaculated sperm prior to freezing prevented the detrimental effects on membrane integrity and motility caused by a rapid, one step removal (Wessel and Ball, 2004). However, they found no beneficial effect from stepwise dilution for removing glycerol from cryopreserved sperm. In humans, slow dilution for removal of glycerol from cryopreserved sperm resulted in a higher recovery of motile cells than when glycerol was removed abruptly (Gilmore et al., 1997). In the domestic cat, multi-step dilution with isotonic medium for the removal of glycerol after cryopreservation of sperm minimized loss of sperm motility and membrane disruption (Pukazhenthi et al., 2002). Sperm from various species are more susceptible to osmotic damage caused by the hyperosmolar environment caused by glycerol, therefore, stepwise dilution might help reduce its detrimental effects. In mice, no more than 0.3 M glycerol is used in freezing extenders because of its toxicity and osmotic damage caused at the 1.0 M concentration usually used in other species (Katkov et al., 1998). However, stepwise dilution of glycerol minimized osmotic shock and allowed a high percentage of mouse sperm to survive up to 0.8 M glycerol concentration (Katkov et al., 1998).

In most mammals, fertilization requires that a sufficient number of sperm survive in the female tract until ovulation. Cryopreservation exposes sperm to different stress conditions resulting in a reduction in the proportion of sperm surviving for fertilization, as well as altering functional capacity of those sperm that do survive (Watson, 2000). Even the best cryopreservation protocols allow lethal and sublethal cryoinjury affecting the efficiency of sperm to achieve fertilization (Holt, 2000). Assessing longevity of cryopreserved sperm is of primary importance to know if the sperm would be able to last long enough to achieve fertilization. Individual bulls that produce semen capable of maintaining a competent population of sperm over time are generally the ones that achieve acceptable fertilization rates, even with a lower number of sperm in the inseminate (Den Daas, 1997). No studies have assessed the survivability and longevity of bovine epididymal sperm subjected to 4 hours of stress in vitro. Moreover, it is not
known if epididymal sperm previously exposed to seminal plasma prior to cryopreservation will have a longer or shorter life span.

The use of multicolor flow cytometry to assess membrane integrity, acrosome integrity and mitochondrial function will allow the detection of subtle differences between treatments. The overall objectives of this two part study were: (1) to assess the effects of one step removal of glycerol from cryopreserved bovine epididymal sperm previously exposed to seminal plasma and (2) to assess epididymal sperm longevity during a 4-hour incubation period at 37°C.

**MATERIALS AND METHODS**

**Experimental Design**

**Experiment 6.1**

This experiment was conducted to evaluate the effect of a one step dilution for removal of glycerol from epididymal sperm that had been previously harvested without seminal plasma (Treatment A) or with seminal plasma (Treatment B). Paired testes were obtained from mature bulls (n=10) at a local abattoir and transported to the laboratory within 3 to 5 hours postmortem. Caudal epididymal sperm from each pair of testes were harvested from the caudae epididymides, pooled and then split evenly into two treatments. Each of the two treatment samples was incubated for 30 minutes in either egg yolk tris-glucose citric acid monohydrate extender (EYT-GC) (Treatment A) or bovine seminal plasma (Treatment B). The seminal plasma in Treatment B was then removed by centrifugation. Treatments A and B were cryopreserved in EYT-GC extender supplemented with 7% glycerol. Three treatment replicates per bull were analyzed before and after a one step dilution (1:5) for removal of glycerol. Plasma membrane, acrosome integrity and mitochondrial function were evaluated by the same technician using multicolor flow cytometry.

**Experiment 6.2**

This experiment was designed to evaluate the survivability of post-thaw epididymal sperm when exposed to a 4-hour in vitro stress test. Duplicate diluted post-thaw epididymal sperm samples of both Treatments A and B from Experiment 6.1 were incubated at 37°C for 4-hours. Three treatment replicates per bull were evaluated at both 0 hours and 4 hours post-incubation. Progressive motility was assessed subjectively with a light microscope. Three replicates were repeated on different days. Plasma
membrane, acrosome integrity and mitochondrial function were assessed by the same technician using multicolor flowcytometry.

**Experimental Procedure**

**Testes Collection**

Bovine testes were collected as pairs from 10 mature mixed breed bulls at an abattoir in Robert, Louisiana. Testes were transported to the Louisiana State University Embryo Biotechnology Laboratory individually packed in Ziploc® plastic bags (Johnson & Son Inc., Racine, WI) in a Styrofoam box (temperature ranging from 28° to 29°C) within 3 to 5 hours postmortem. Upon arrival to the laboratory, testes were dissected away from its scrotum and tunica vaginalis. Then, testes were positioned on a table with the vas deferens facing ventrally and the corpus epididymis facing dorsally to distinguish the side of origin of each testis.

**Medium Preparation**

Egg yolk Tris-glucose citric acid monohydrate extender (EYT-GC) (Liu et al., 1998) (250 mM Tris (T-6066, Sigma-Aldrich Inc., St. Louis, MO), 69 mM glucose (6152, Sigma-Aldrich Inc., St. Louis, MO), 81 mM citric acid monohydrate (C-1909, Sigma-Aldrich, St. Louis, MO) and 20% (v/v) egg yolk) was prepared with the addition of 50 µg/ml of gentamicin (15750-060, Gibco, Grand Island, NY). The extender was filtered through a 0.45 µm Nalgene® syringe filter (190-2545, Nalge Company, Rochester, NY) to remove large egg yolk particulates. Egg yolks from fresh chicken eggs were obtained at the Louisiana State University Poultry Research Farm. The extender was placed in a 37°C water bath 2 to 3 hours prior to epididymal sperm retrieval.

**Seminal Plasma Collection and Processing**

Ejaculates for seminal plasma extraction were collected from mature beef bulls (n=6) at a commercial bull stud (Genex Corporation, Baton Rouge, LA). Ejaculates were collected with an artificial vagina and placed in a 37°C water bath for progressive motility analysis. Samples with more than 50% progressively motile sperm were used for the experiment. Ejaculates were transported to the Embryo Biotechnology Laboratory at 20° to 25°C to harvest the seminal plasma.

Semen samples were centrifuged at 2,000 x g in a general purpose centrifuge (Model 5682 GP8R, Thermoforma, Marietta, OH) for 20 minutes. The supernatant was placed into a new 15 ml plastic tube (20171-024, VWR International Inc., Sugar Land,
and the sperm pellet was discarded. This centrifugation procedure was repeated twice on the seminal plasma. The supernatant of each sample was filtered through a 0.8 µm Acrodisc® syringe filter (PN 4618, Pall Corporation, East Hills, NY) for the complete removal of any remaining sperm. Filtered seminal plasma from the 6 bulls was thoroughly mixed together and stored in 2 ml aliquots in a -20°C freezer. On the day of use, 3 vials of seminal plasma were thawed in a 37°C water bath 30 minutes prior to harvesting the epididymal sperm.

Epididymal Sperm Retrieval

After dissection, caudae epididymides were rinsed with 0.9% saline to remove any remaining materials before epididymal sperm retrieval. Epididymal sperm were harvested by making 5 incisions using a surgical blade (size 10, MDS-15110, Medline Industries Inc., Mundelein, IL) in each cauda epididymis. Half of the sperm was scooped out from the incisions with a new surgical blade to a 60 mm Falcon® plastic petri dish (3037, Becton Dickinson Labware, Franklin Lakes, NJ) containing 2 ml of EYT-GC extender (Treatment A). The remaining half of the sperm sample was placed with a new blade in another 60 mm plastic dish containing 2 ml of seminal plasma (Treatment B). Each sperm mixture was then transferred into separate 4 ml plastic tubes and placed in a 37°C water bath for 30 minutes.

Seminal Plasma Removal

Samples were placed over a two-column sperm separation medium (99264, ISolate®, Irvine Scientific, Santa Ana, CA) (1 ml lower layer and 1 ml upper layer) in a 15 ml plastic tube and centrifuged for 20 minutes at 300 x g to remove seminal plasma. After centrifugation, the ISolate® upper layer with the seminal plasma or egg yolk was removed. The lower layer containing the sperm suspension (1 ml) was resuspended in 8 ml of Tyrodes-Lactate HEPES (TL-H) (04-616F, Cambrex Bio Science Inc., Walkersville, MD) to wash sperm and remove silica particles. Sperm suspension was then centrifuged at 350 x g for 5 minutes and resuspended in EYT-GC extender for both Treatments A and B.

Sperm Cooling, Cryopreservation and Thawing

Sperm concentration was adjusted to 70 x 10⁶ cells/ml using a hemacytometer by the addition of more EYT-GC extender. The 15 ml plastic tubes containing the epididymal sperm samples were placed in a 250 ml Pyrex® glass beaker (13912-207,
VWR International Inc., Sugar Land, TX) containing water at room temperature. The beaker was placed in a refrigerator for 4 hours to cool the sperm samples to 4°C.

Samples were diluted slowly over a period of 30 minutes 1:1 in EYT-GC medium containing 14% glycerol and subsequently loaded into previously cooled 0.5 ml straws (005569, Cassou straw, IMV Technologies, Minneapolis, MN). The straws were placed in a custom built rack 2 cm over liquid nitrogen (LN₂) vapors for 10 minutes before being plunged in LN₂. A total of 10 to 15 straws were frozen per bull (35 x 10⁶ cells/ml).

Sperm samples were stored for 2 to 3 months prior to thawing. On the day of sperm analysis, 50 ml of TL-H supplemented with 3 mg/ml of bovine serum albumin (Fraction V, A-7906, Sigma-Aldrich, St. Louis, MO) were placed in a 50 ml plastic tube (BCT-P50RS, Biologix, Shawnee Mission, KS) and warmed in a 37°C incubator 1 hour prior to sperm dilution. Straws were thawed in a water bath at 37°C for 40 seconds and the contents of each straw were placed in a 15 ml plastic tube. Two 50 µl droplets of undiluted sperm were placed in each of two 5.5 ml plastic tubes. One tube was left undiluted while the other was diluted 1:5 sperm sample:TL-H before multicolor flow cytometric analyses. Duplicate diluted samples of each treatment were placed in an incubator at 37°C for 4 hours to expose thawed sperm to an in vitro stress test. After the incubation period, sperm motility, membrane integrity, acrosome integrity and mitochondrial function were assessed.

Multicolor Flow Cytometry

A FACSCalibur flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA) was used for sperm measurements. Forward and side scatter values were recorded on a linear scale, while fluorescent values were recorded on a logarithmic scale for all assays unless otherwise stated. All fluorochromes were excited at 488 nm with a 20 mW argon laser. Density and dot plots drawn for data analysis were generated by WinMDI 2.8 (software by J. Trotter, available for downloading at http://www.facs.scripps.edu/software.html).

Assessment of Plasma Membrane and Acrosome Integrity

Assessment of plasma membrane and acrosome integrity were completed using a triple stain protocol in the presence of egg yolk particulates (Nagy et al., 2003) with minor modifications. The staining protocol consisted of SYBR 14, PI and the lectin peanut agglutinin conjugated to PE (R-PE-PNA) (p-44, Biomedia Corp., Foster City, CA).
A final concentration of 100 nM SYBR 14, 4 μg/ml of R-PE-PNA (1 mg/ml of stock solution in a buffer composed of 50 mM sodium phosphate, and 0.05% sodium azide, [pH 7.0] and also containing 0.1 mM [Ca²⁺] and [Mn²⁺] ions) and 12 μM PI solution were added to 250 μl of diluted sperm suspension. Sperm samples were thoroughly mixed and incubated at 37°C in the dark for 30 to 40 minutes and then remixed gently by pipetting before flow cytometric analysis.

Plasma membrane ruptured cells were PI positive, and their red fluorescent signal was detected on the FL3-channel using a 610 nm long pass filter. Membrane intact sperm were SYBR 14 positive, and its green fluorescent signal was detected on the FL1-channel using a 530/30 nm band pass filter. Acrosome reacted sperm were PE-PNA positive, and its orange fluorescent signal was detected at 585/42 nm on the FL2-channel. Three color compensations were set according to the FACS® training manual (Becton Dickinson Biosciences, San Jose, CA). Nonsperm events (egg yolk particulates) were gated out of analyses based on scatter properties detected in the forward and side scatter detectors. Furthermore, events with similar scatter characteristics to sperm but with low PI and SYBR 14 fluorescence were also gated out. Sperm acquisitions were made using CellQuest software (Becton Dickinson, San Jose, CA). The flow cytometer was kept at a low flow of less than 300 sperm/second. The recording of scatter and fluorescent properties of all events stopped when 10,000 gated events were recorded.

On SYBR 14 (FL1) and PI (FL3) dot plots, regions were drawn to determine the percentage of viable and non viable sperm. On PE-PNA (FL2) and PI (FL3) dot plots, quadrants were set to measure the percentage of sperm showing plasma membrane intact and acrosome intact sperm (LL), plasma membrane intact and acrosome reacted sperm (LR), plasma membrane ruptured and acrosome intact sperm (UL), and plasma membrane ruptured and acrosome reacted sperm (UR).

Assessment of Mitochondrial Function

Assessment of sperm mitochondrial function was conducted using a novel dual stain protocol in the presence of egg yolk particles developed in our laboratory. The staining protocol consisted of SYBR 14 and MitoTracker Red CMXRos (MITO) (Molecular Probes Inc., Eugene, OR). A 1 mM MITO stock solution was prepared in DMSO. Then a 20 μM working solution was prepared in TL-H before sample preparation. For staining, 100 nM SYBR 14 and 100 nM MITO were added to 250 μl of
diluted sperm. Samples were thoroughly mixed and incubated at 37°C in the dark for 30 minutes and then remixed gently before flow cytometric analysis.

Detector FL-1 was used to detect SYBR 14 fluorescence (green). MITO fluorescence was detected on detector FL-3. Data acquisition and compensations were set as described previously. The egg yolk particles were gated out based on scatter properties and on particles showing high green fluorescence. Acquisitions were stopped after recording 10,000 SYBR 14 positive events.

On SYBR 14 (FL-1) and MITO (FL-3) dot plots, regions were drawn to determine the percentage of sperm with active mitochondria and sperm with no mitochondrial function. Sperm positive events showing a high red fluorescence were determined as viable sperm with active mitochondrial membrane potential. Sperm positive events showing a low red fluorescence were assessed as sperm with mitochondria that had lost their transmembrane potential.

**Statistical Analysis**

Variances in progressive motility, membrane integrity, acrosome integrity and mitochondrial function were statistically analyzed by ANOVA and the differences between bulls were calculated by a Tukey Multiple Comparisons test. Additionally, a t-test was used to verify differences in sperm parameters before and after cryopreservation. The sperm parameters are expressed as mean±SEM per treatment group. A P<0.05 value was considered statistically significant in this study. All data were analyzed using SigmaStat Statistical Software Version 2.0.

**RESULTS**

**Experiments 6.1**

**Membrane Integrity**

The mean overall percentage of sperm with intact membranes after thawing was significantly (P<0.05) higher in undiluted samples when compared to the diluted samples in the seminal plasma-exposed treatment (Treatment B) (Table 6.1). However, no difference was detected before or after dilution for removal of glycerol in the control extender group (Treatment A). Furthermore, the mean percent intact plasma membranes in undiluted and diluted samples between both treatments (Treatments A and B) were not significantly different.
Table 6.1. Percent plasma membrane integrity (mean±SEM) of post-thaw bovine epididymal sperm before and after dilution from the freezing extender

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of bulls</th>
<th>Plasma membrane integrity (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Undiluted</td>
<td>Diluted</td>
<td></td>
</tr>
<tr>
<td>Extender</td>
<td>10</td>
<td>57.9±2.0\textsuperscript{a}</td>
<td>53.1±2.0\textsuperscript{a}</td>
<td></td>
</tr>
<tr>
<td>Seminal Plasma</td>
<td>10</td>
<td>62.2±2.1\textsuperscript{a}</td>
<td>51.5±2.2\textsuperscript{b}</td>
<td></td>
</tr>
</tbody>
</table>

Extender = Treatment A; Seminal Plasma = Treatment B. \textsuperscript{a,b}Means with different superscripts within rows are significantly different (P<0.05).
The mean intact membrane values for undiluted and diluted sperm samples from individual bulls harvested without seminal plasma (Treatment A) or with seminal plasma (Treatment B) or are shown in Figures 6.1 and 6.2, respectively. In both Treatments A and B, bull number 2 appears to be the most affected by the one step dilution process, while bull number 8 and 10 were the least affected by the one step dilution process.

**Acrosome Integrity**

The overall mean acrosome integrity values for undiluted and diluted post-thaw sperm harvested with seminal plasma (Treatment B) or without seminal plasma (Treatment A) prior to cryopreservation are shown in Table 6.2. The mean number of viable sperm with intact acrosomes was not significantly different (P>0.05) before or after dilution between both Treatments A and B. There were 0.2±0.3% and 0.6±0.1% viable post-thaw sperm with damaged acrosomes before and after dilution, respectively, for sperm harvested with seminal plasma (Treatment B). These values accounted for 0.1±1% and 0.3±1% for undiluted and diluted sperm post-cryopreservation in the control group (Treatment A). The numerical value of nonviable sperm with damaged acrosomal membranes was lower before and after dilution in the control group (Treatment A) than in the seminal plasma group (Treatment B). These values accounted for 21.4±1.5% before dilution and 24.9±1% after dilution for sperm exposed to seminal plasma (Treatment B), as compared with 14.9±1% and 19.2±1% before and after dilution, respectively, for sperm not exposed to seminal plasma (Treatment A). However, this difference was not statistically significant (P>0.05). Furthermore, a difference (P<0.001) was detected between the quantity of damaged acrosomes derived from nonviable than from the viable sperm population in both Treatments A and B.

The mean acrosome integrity values for individual bulls before and after dilution for sperm harvested without seminal plasma (Treatment A) or with seminal plasma (Treatment B) are shown in Figures 6.3 and 6.4, respectively. Bull number 6 in the seminal plasma group (Treatment B) and bull number 1 in the control group (Treatment A) were the most affected by the dilution process. There was an intermale variation in acrosome integrity between bulls subjected to the same treatments.

**Mitochondrial Activity**

The overall mean percentage of sperm with functioning mitochondria post-thaw was significantly (P<0.05) higher in undiluted samples than in diluted samples in the
Figure 6.1. Membrane integrity values (mean±SEM) of post-thaw bovine epididymal sperm before and after one step dilution for removal of glycerol. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation. a,b Means with different superscripts within males are significantly different (P<0.05).
Figure 6.2. Membrane integrity values (mean±SEM) of post-thaw bovine epididymal sperm before and after one step dilution for removal of glycerol. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation. a,b Means with different superscripts within males are significantly different (P<0.05).
Table 6.2. Percent acrosome integrity (mean±SEM) of post-thaw bovine epididymal sperm before and after dilution from the freezing extender

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of bulls</th>
<th>Acrosome integrity (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Extender</td>
<td>10</td>
<td>Undiluted</td>
<td>85.1±1.5</td>
<td>80.6±2.2</td>
</tr>
<tr>
<td>Seminal Plasma</td>
<td>10</td>
<td>Diluted</td>
<td>78.6±3.2</td>
<td>75.0±3.1</td>
</tr>
</tbody>
</table>

Extender = Treatment A; Seminal Plasma = Treatment B. Means were not significantly different (P>0.05).
Figure 6.3. Percent acrosome integrity (mean±SEM) of post-thaw bovine epididymal sperm before and after one step dilution for removal of glycerol. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation. a,bMeans with different superscripts within males are significantly different (P<0.05).
Figure 6.4. Percent acrosome integrity (mean±SEM) of post-thaw bovine epididymal sperm before and after one step dilution for removal of glycerol. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation. a,b Means with different superscripts within males are significantly different (P<0.05).
seminal plasma treatment group (Treatment B). However, no difference was detected before or after dilution for removal of glycerol in the control treatment group (Treatment A) (Table 6.3). In addition, there was no significant difference in the percentage of sperm exhibiting active mitochondria in undiluted and diluted samples between both Treatments A and B.

The mean values of sperm exhibiting functioning mitochondria for individual bulls before and after dilution for sperm harvested without seminal plasma (Treatment A) or with seminal plasma (Treatment B) are shown in Figures 6.5 and 6.6, respectively. Bull number 1, 3, 4 and 7 had significantly higher post-thaw sperm displaying active mitochondria in the undiluted sample when sperm were exposed to seminal plasma (Treatment B) prior to the freezing process. However, bull number 9 had lower mitochondrial function in the undiluted than in the diluted post-thaw sperm sample when sperm were exposed to seminal plasma (Treatment B) prior to cryopreservation.

**Experiment 6.2**

Membrane Integrity

Incubation significantly (P<0.05) affected the post-thaw integrity of the plasma membrane when epididymal sperm was harvested with seminal plasma (Treatment B) or without seminal plasma (Treatment A) prior to cryopreservation (Table 6.4). However, no difference in the percentage of sperm with intact membranes was detected between Treatments A and B after 4 hours of incubation.

The mean membrane integrity values for sperm before and after being exposed to the 4-hour in vitro stress test for epididymal sperm retrieved without seminal plasma (Treatment A) or with seminal plasma (Treatment B) prior to cryopreservation are shown in Figures 6.7 and 6.8, respectively. These values indicate that some bulls were more sensitive to the 4-hour incubation period than the remaining bulls. Furthermore, cryopreserved sperm from some males apparently deteriorate faster than others when exposed to an in vitro stress test.

Acrosome Integrity

Acrosome integrity was significantly (P<0.05) affected by the 4-hour incubation period in both Treatments A and B (Table 6.5). No difference was detected in acrosome integrity at 0 hours post-cryopreservation between both Treatments A and B. However,
Table 6.3. Percent mitochondrial activity (mean±SEM) of post-thaw bovine epididymal sperm before and after dilution from the freezing extender

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of bulls</th>
<th>Mitochondrial activity (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Undiluted</td>
<td>Diluted</td>
<td></td>
</tr>
<tr>
<td>Extender</td>
<td>10</td>
<td>54.1±1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.1±1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Seminal Plasma</td>
<td>10</td>
<td>61.2±2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.3±2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Extender = Treatment A; Seminal Plasma = Treatment B. 
<sup>a,b</sup>Means with different superscripts within rows and columns are significantly different (P<0.05).
Figure 6.5. Percent mitochondrial activity (mean±SEM) of post-thaw bovine epididymal sperm before and after one step dilution for removal of glycerol. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation. a,bMeans with different superscripts within males are significantly different (P<0.05).
Figure 6.6. Percent mitochondrial activity (mean±SEM) of post-thaw bovine epididymal sperm before and after one step dilution for removal of glycerol. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation. a,b Means with different superscripts within males are significantly different (P<0.05).
Table 6.4. Percent plasma membrane integrity (mean±SEM) of post-thaw bovine epididymal sperm during a 4-hour incubation period at 37°C

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of bulls</th>
<th>Plasma membrane integrity (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 hours</td>
<td>4 hours</td>
<td></td>
</tr>
<tr>
<td>Extender</td>
<td>10</td>
<td>52.7±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.1±1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Seminal Plasma</td>
<td>10</td>
<td>51.5±2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.3±1.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Extender = Treatment A; Seminal Plasma = Treatment B.
<sup>a,b</sup>Means with different superscripts within rows are significantly different (P<0.05).

Table 6.5. Percent acrosome integrity (mean±SEM) of post-thaw bovine epididymal sperm during a 4-hour incubation period at 37°C

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of bulls</th>
<th>Acrosome integrity (%)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 hours</td>
<td>4 hours</td>
<td></td>
</tr>
<tr>
<td>Extender</td>
<td>10</td>
<td>80.6±2.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.1±2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Seminal Plasma</td>
<td>10</td>
<td>75.3±3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49.0±1.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Extender = Treatment A; Seminal Plasma = Treatment B.
<sup>a,b,c</sup>Means with different superscripts within rows and columns are significantly different (P<0.05).
Figure 6.7. Membrane integrity values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation. a,b Means with different superscripts within males are significantly different (P<0.05).
Figure 6.8. Membrane integrity values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation. a,bMeans with different superscripts within males are significantly different (P<0.05).
there was significantly lower acrosome integrity for sperm exposed to seminal plasma (Treatment B) when compared with the control group (Treatment A) after 4 hours of incubation.

   The overall mean acrosome integrity values for sperm at 0 and at 4 hours post-incubation for sperm retrieved without seminal plasma (Treatment A) or with seminal plasma (Treatment B) are shown in Figures 6.9 and 6.10, respectively. Cryopreserved sperm from some males suffer acrosome damage faster than others when exposed to a 4-hour in vitro stress test.

Mitochondrial Activity

   Exposing sperm to stress during incubation significantly (P<0.05) affected the percentage of sperm exhibiting active mitochondria in both Treatments A and B (Table 6.6). However, no difference in the percentage of sperm with functioning mitochondria was detected between both Treatments A and B at both 0 and 4 hours of incubation. The mean values for sperm displaying functioning mitochondria for individual males at 0 and 4 hours post-incubation for sperm retrieved without seminal plasma (Treatment A) or with seminal plasma (Treatment B) are shown in Figures 6.11 and 6.12, respectively. Cryopreserved sperm from some males displayed a faster reduction in the percentage of functioning mitochondria than others when exposed to a 4-hour in vitro stress test in both Treatments A and B.

Progressive Motility

   Sperm exposed to seminal plasma (Treatment B) displayed significantly higher (P<0.05) post-thaw progressive motility than the nonexposed control group (Treatment A), both before and after the incubation period (Table 6.7). The 4-hour incubation significantly reduced progressive motility in both Treatments A and B. The mean values for sperm displaying progressive motility for individual males at both 0 and 4 hours post-incubation for sperm retrieved without seminal plasma (Treatment A) or with seminal plasma (Treatment B) are shown in Figures 6.13 and 6.14, respectively. Some bulls were more affected by incubation than others as observed in a decline in progressive motility overtime. Sperm exposed to seminal plasma (Treatment B) maintained a higher progressive motility overtime when compared with the control group (Treatment A).
Figure 6.9. Acrosome integrity values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation. a,bMeans with different superscripts within males are significantly different (P<0.05).
Figure 6.10. Acrosome integrity values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation. a,bMeans with different superscripts within males are significantly different (P<0.05).
Table 6.6. Percent mitochondrial activity (mean±SEM) of post-thaw bovine epididymal sperm during a 4-hour incubation period at 37°C

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>No. of bulls</th>
<th>Mitochondrial activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 hours</td>
</tr>
<tr>
<td>Extender</td>
<td>10</td>
<td>47.1±1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Seminal Plasma</td>
<td>10</td>
<td>49.3±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Extender = Treatment A; Seminal Plasma = Treatment B.
<sup>a,b</sup>Means with different superscripts within rows and columns are significantly different (P<0.05).

Table 6.7. Percent progressive motility (mean±SEM) of post-thaw epididymal sperm during a 4-hour incubation period at 37°C

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>No. of bulls</th>
<th>Progressive motility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 hours</td>
</tr>
<tr>
<td>Extender</td>
<td>10</td>
<td>20.3±1.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Seminal Plasma</td>
<td>10</td>
<td>40.3±2.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Extender = Treatment A; Seminal Plasma = Treatment B.
<sup>a,b,c,d</sup>Means with different superscripts within rows and columns are significantly different (P<0.05).
Figure 6.11. Mitochondrial activity values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation. *a,b* Means with different superscripts within males are significantly different (P<0.05).
Figure 6.12. Mitochondrial activity values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation. a,b Means with different superscripts within males are significantly different (P<0.05).
Figure 6.13. Progressive motility values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with the extender (Treatment A) prior to cryopreservation. a,b Means with different superscripts within males are significantly different (P<0.05).
Figure 6.14. Progressive motility values (mean±SEM) of post-thaw bovine epididymal sperm before and after a 4-hour incubation period at 37°C. The bars represent sperm from individual males harvested with seminal plasma (Treatment B) prior to cryopreservation. a,b Means with different superscripts within males are significantly different (P<0.05).
DISCUSSION

In the present study the one step dilution process for removal of glycerol from cryopreserved epididymal sperm significantly affected plasma membrane integrity and mitochondrial function of sperm previously exposed to seminal plasma. However, exposure to seminal plasma did not produce any significant detrimental effect in acrosome integrity when compared with the control treatment.

Seminal plasma has been shown to cause cholesterol efflux from the plasma membrane to prepare sperm for capacitation, acrosome reaction and fertilization (Osheroff et al., 1999; Iborra et al., 2000). The major protein fraction of bovine seminal plasma is represented by a family of closely related proteins designated BSP proteins (Manjunath and Sairam, 1987). These proteins represent ~65% of the total proteins in seminal plasma (Bergeron et al., 2004). Also, these proteins have been shown to interact with choline phospholipids in the plasma membrane and to potentiate sperm capacitation induced by heparin (Therien et al., 1995) by stimulating cholesterol and phospholipids efflux from the plasma membrane (Bergeron et al., 2004). We propose that BSP proteins likely make the sperm more sensitive to osmotic changes during the removal of glycerol when compared to sperm not exposed to seminal plasma during the harvesting process.

During cryopreservation, sperm experience membrane perturbations and membrane lipid and protein reorganization (Steponkus et al., 1983). High membrane cholesterol levels inhibit crystallization of membrane hydrocarbons chains at low temperatures (Rottem et al., 1973). This enhances membrane fluidity at low temperatures and helps prevent damage to membranes as they undergo the phase transition (Purdy and Graham, 2004). Treating sperm with cholesterol before cryopreservation has resulted in the recovery of a higher proportion of membrane intact and motile sperm in the bull (Purdy and Graham, 2004; Moce and Graham, 2006) and the stallion (Moore et al., 2005). The short exposure of epididymal sperm to seminal plasma prior to cryopreservation might have enhanced plasma membrane modifications that rendered sperm more sensitive and vulnerable to dilution post-thaw. In the stallion, rapid removal of glycerol was reported to cause a relative hyposmotic shock upon dilution into isotonic medium resulting in loss of sperm motility, viability and mitochondrial function (Ball and Vo, 2001). The rapid movement of water and glycerol
across the plasma membrane appears to account, in part, to the decline in membrane integrity and mitochondrial function (Ball and Vo, 2001). Stepwise dilution with isotonic media or multistep dilution with media of fixed molarities might help reduce the osmotic damage caused by one step removal of glycerol.

In our second experiment, the long term effects of seminal plasma on post-thaw epididymal sperm were assessed after a 4-hour incubation period at 37°C. Incubation significantly decreased progressive motility, membrane integrity, acrosome integrity and mitochondrial function of post-thaw epididymal sperm harvested with or without seminal plasma prior to cryopreservation. Membrane integrity and mitochondrial function did not differ between the two treatments after 4 hours of incubation. However, acrosome integrity was significantly lower at 4 hours of incubation when sperm were retrieved with seminal plasma than without seminal plasma. In contrast, motility was higher at 4 hours post-incubation for post-thaw epididymal sperm harvested with seminal plasma than without seminal plasma.

In this study, we have demonstrated that the longevity of bovine epididymal sperm harvested with seminal plasma prior to cryopreservation is not compromised when compared with the extended sperm (control). To our knowledge this is the first study to experimentally report that post-thaw bovine epididymal sperm are able to survive a 4-hour stress test. In the stallion, no detrimental effect was noted on motility at 90 minutes post-thaw when 5% seminal plasma was added to the freezing extender (Moore et al., 2005). However, it has been shown that increasing the content of equine seminal plasma to 20% resulted in a significant decline in motility (Moore et al., 2005). In our study, seminal plasma was completely removed 30 minutes after epididymal sperm retrieval. This short incubation period could have helped reduce any long term detrimental effects of seminal plasma.

Seminal plasma provides sperm with nutrients, a variety of biochemical components for the regulation of sperm function (Strzezek et al., 1992) and factors that alter the sperm surface (Polakoski et al., 1982). In addition, it has biochemical components that permanently stimulate sperm enhancing motility characteristics. A sperm motility factor found in bovine seminal plasma has been reported to restore motility in nonmotile sperm (Bass et al., 1983). In our study, this sperm motility factor might have increased sperm motility when compared with the treatment retrieved with
the extender. Seminal plasma has been shown to prepare sperm for capacitation and the acrosome reaction. Thus, bovine epididymal sperm in our study might have been capacitated faster than those in the control treatment during incubation. A higher percentage of acrosome reacted sperm was found after 4 hours of incubation in the seminal plasma exposed treatment. This might have been due to membrane modifications caused by biochemical components in seminal plasma. Viable epididymal sperm exhibiting damaged acrosomes after 4 hours were higher in the sperm seminal plasma treatment than in the control treatment. However, these treatments were not significantly different. It has been suggested that the reduced longevity and fertilizing ability of cryopreserved sperm is due to accelerated capacitation (Perez et al., 1996).

In future experiments, membrane fluidity and calcium levels should be investigated to assess the early stages of capacitation and determine if seminal plasma is producing any significant effects. Further studies are needed to assess the effect of harvesting epididymal sperm with seminal plasma from highly fertile bulls and with seminal plasma collected by either artificial vagina or electroejaculation. Collection techniques have been reported to affect the biochemical composition of bovine seminal plasma (Bennett and Dott, 1967; Seidel and Foote, 1969). Furthermore, seminal plasma might help improve survivability of cryopreserved sperm from 'lower freezer' bulls.
CHAPTER VII
THE USE OF CRYOPRESERVED BOVINE CAUDAL EPIDIDYMAL SPERM FOR INTRACYTOPLASMIC SPERM INJECTION (ICSI)

INTRODUCTION

ICSI is a procedure of mechanically inserting a whole sperm or an isolated sperm head into the ooplasm of an oocyte. Since ICSI requires far fewer sperm to fertilize the same number of oocytes than in vitro fertilization (IVF), it is potentially valuable especially when a limited number are available (Keefer et al., 1990). The use of postmortem epididymal sperm for ICSI will allow a more effective use of valuable gametes if a breeding male unexpectedly dies.

In recent years, ICSI has been extensively used to treat male factor infertility in humans, with thousands of babies born to date. Its importance in human infertility relies on its ability to use semen samples with low sperm counts, low motile sperm and for sperm with increased percentages of morphological abnormalities. In addition, sperm can be used from patients with previous vasectomies by using testicular or epididymal sperm. The ICSI procedure has revolutionized the treatment of male infertility providing higher fertilization, pregnancy and implantation rates than standard IVF (Schwarzer et al., 2003). ICSI bypasses the normal process of fertilization, where motile sperm are needed to reach the oocyte, penetrate the cumulus cells, bind and penetrate the zona pellucida and subsequently fuse with the oolema. This process requires the sperm to be progressively motile, to be able to capacitate, undergo an acrosome reaction and transform the plasma membrane for fertilization to be completed. Since ICSI bypasses the initial steps of fertilization, nonfunctional or even ‘dead’ sperm with a normal DNA complement can be used to fertilize an ovum and produce healthy offspring (Goto et al., 1990).

Since the first report of ICSI in hamsters (Uehara and Yanagimachi, 1976), this technique has been used in various mammalian species. Live offspring have been obtained using the ICSI procedure in the rabbit (Hosoi et al., 1988), cow (Goto et al., 1990), human (Palermo et al., 1992), mouse (Kimura and Yanagimachi, 1995), sheep (Catt et al., 1996), horse (Cochran et al., 1998), cat (Gomez et al., 2000), pig (Martin, 2000), rat (Hirabayashi et al., 2002), Cynomolgus monkey (Ng et al., 2002), goat (Wang et al., 2003) and hamster (Horiuchi, 2006).
The efficiency of ICSI in cattle has been very limited because of the necessity for additional oocyte activation before or after the sperm injection procedure (Suttner et al., 2000). In contrast to mouse and human oocytes, bovine oocytes generally do not activate by mechanical stimulus of the injection pipette (Goto et al., 1990). In mice, ~70% of sperm-injected oocytes reach the blastocyst stage (Kimura and Yanagimachi, 1995), while in cattle only between 1 to 20% develop into blastocysts (Goto et al., 1990; Horiuchi et al., 2002). In contrast to humans, bovine sperm apparently need to be pretreated by chemicals, immobilized before ICSI by freeze-thawing without cryoprotectants or scoring the tail before injection to increase sperm head DNA decondensation and pronuclear formation (Wei and Fukui, 1999). A detailed explanation for these differences among various species has not been reported.

In mammals, the fertilizing sperm produces a series of long-lasting [Ca\(^{2+}\)] oscillations, which initiate shortly after sperm-oocyte fusion and can last up to 24 hours. This continuous rise in [Ca\(^{2+}\)] is required for normal oocyte activation and subsequent embryo development. It is believed that a ‘sperm factor’ is delivered to the ooplasm by sperm at the time of fusion, which activates the phosphoinositide pathway (Swann, 1990). Recently, it was demonstrated that sperm injection into bovine oocytes produces abnormal [Ca\(^{2+}\)] responses and oocyte activation (Malcuit et al., 2006). Less than 10% of the sperm-injected oocytes display any [Ca\(^{2+}\)] responses. It is thought that the function or activation of the sperm factor may be compromised after ICSI, thus leading to incomplete [Ca\(^{2+}\)] oscillations (Malcuit et al., 2006). This explains in part the need for additional exogenous chemical activation of bovine sperm-injected oocytes.

Bovine oocytes for ICSI have been exogenously activated using electric stimulation (Hwang et al., 2000), ethanol (Horiuchi et al., 2002; Fujinami et al., 2004; Oikawa et al., 2005), calcium ionophore (A23187) (Goto et al., 1990; Keefer et al., 1990) ionomycin (Chung et al., 2000; Keskintepe et al., 2002), ionomycin followed by DMAP (Rho et al., 1998; Chung et al., 2000; Keskintepe et al., 2002; Ock et al., 2003; Li et al., 2004; Oikawa et al., 2005) and ionomycin followed by cyclohexamide (Galli et al., 2003; Rho et al., 2004). However, developmental rates of activated oocytes are still markedly lower than after conventional IVF. Although there has been low ICSI efficiency in cattle, there have been <35 calves reported after transfer of ICSI-derived blastocysts into
recipient females (Goto et al., 1990; Hamano et al., 1999; Horiuchi et al., 2002; Wei and Fukui, 2002; Galli et al., 2003; Oikawa et al., 2005; Horiuchi, 2006).

Epididymal sperm have been used for ICSI in humans (Craft et al., 1995), mice (Kimura and Yanagimachi, 1995), pigs (Kolbe and Holtz, 1999), cats (Pushett et al., 2000), Cynomolgus monkeys (Ng et al., 2002), rats (Said et al., 2003), the Tammar wallaby (Richings et al., 2004) and rabbits (Zheng et al., 2004). There has been only one report of the use of bovine epididymal sperm for ICSI (Goto et al., 1990). In the latter report, only 1.8% of 507 sperm-injected oocytes developed into blastocysts. One healthy calf was born after transfer of 7 blastocysts into recipient females.

To our knowledge, pronuclear formation of epididymal sperm-injected oocytes has not been evaluated in cattle. The low blastocyst development reported for epididymal sperm-injected oocytes might have been due to low sperm head decondensation of epididymal sperm. It is important to assess sperm head decondensation and pronuclear formation of epididymal sperm before focusing on blastocyst development. Also, due to chemical activation of sperm-injected oocytes in present procedures, it can not be ruled out that some of the 7-day embryos produced to date were of parthenogenetic origin.

Ejaculated and epididymal sperm may act differently when mechanically injected into oocytes due to their biochemical and physiological differences. Ejaculated sperm differ from epididymal sperm in respiration (Hammerstedt et al., 1993), motion characteristics (Gooaverts et al., 2006), heparin-binding sites (Nass et al., 1990), ability to capacitate (Miller et al., 1990), morphology (Hewitt et al., 2001) and proteins bound to the plasma membrane (Lee et al., 1985). In humans, epididymal sperm are readily capable of fertilizing oocytes by IVF, but the pregnancy rates are found to be significantly lower than for ejaculated sperm (Hirsh et al., 1994).

The objectives of this study were: (1) to assess pronuclear formation of bovine epididymal sperm-injected oocytes activated using two different protocols, (2) to assess embryo development up to the blastocyst stage of epididymal sperm-injected oocytes using two different chemical activation protocols and (3) to produce pregnancies from the transfer of blastocysts derived from bovine epididymal sperm-injected oocytes.
MATERIALS AND METHODS

Experimental Design

Experiment 7.1

This experiment was conducted to determine pronuclear formation in bovine sperm-injected oocytes subjected to two different activation protocols using both cryopreserved epididymal sperm and ejaculated sperm. Oocytes were obtained from a commercial source and upon arrival oocytes exhibiting the presence of the first polar body were selected and randomly allotted to one of six treatment groups. Treatments A and B both consisted of the injection of a sperm head derived from cryopreserved ejaculated sperm directly into the ooplasm, and served as controls for this study. In Treatment A (n=4 replicates), sperm-injected oocytes (n=93) were chemically activated in 7% ethanol in Tissue Culture Medium 199 (TCM) for 5 minutes. In Treatment B (n=3 replicates), sperm-injected oocytes (n=54) were chemically activated by exposure to 5 μM ionomycin in CR1aa medium for 5 minutes followed by incubation in 10 μg/ml cycloheximide in CR1aa medium for 5 hours.

In Treatments C and D, a sperm head derived from cryopreserved epididymal sperm was directly injected into the ooplasm of a matured bovine oocyte. The sperm-injected oocytes (n=108) in Treatment C (n=4 replicates) were chemically activated 4 hours post-injection in 7% ethanol in TCM for 5 minutes. In Treatment D (n=3 replicates), the sperm-injected oocytes (n=65) were chemically activated by exposure to 5 μM ionomycin in CR1aa medium for 5 minutes followed by incubation in 10 μg/ml cycloheximide in CR1aa medium for 5 hours.

Treatments E and F both consisted of sham-injected oocytes and served as parthenogenetic controls for this study, where a similar volume of 10% polyvinylpyrrolidone (PVP) in human tubal fluid medium was injected directly into the ooplasm. Sham-injected oocytes (n=63) in Treatment E (n=4 replicates) were chemically activated 4 hours post-injection in 7% ethanol in TCM for 5 minutes. In Treatment F (n=4 replicates), the sham-injected oocytes (n=59) were chemically activated by exposure to 5 μM ionomycin in CR1aa medium for 5 minutes followed by incubation in 10 μg/ml cycloheximide in CR1aa medium for 5 hours.

The sperm-injected oocytes and sham-injected controls were then cultured in CR1aa culture medium. Pronuclear formation was assessed 18 to 20 hours post-sperm
injection by fixing sperm-injected oocytes in acetic alcohol overnight. Fixed oocytes were stained with 1% aceto-orcein, and the presence of male and female pronuclei were examined with a bright field inverted microscope.

Experiment 7.2

This experiment was conducted to assess embryo development up to the blastocyst stage of sperm-injected oocytes activated with two different activation protocols using epididymal sperm. Oocytes were also obtained from a commercial source and upon arrival oocytes exhibiting the presence of the first polar body were selected and randomly allocated to one of four treatment groups. Treatments A and B consisted of injection of a single immobilized epididymal sperm into the ooplasm of a matured oocyte. Treatments C and D (both controls) consisted of sham-injected oocytes, where a similar volume of 10% polyvinylpyrrolidone (PVP) in human tubal fluid medium was injected directly into the ooplasm.

Sperm-injected oocytes (n=118) in Treatment A (n=4 replicates) were chemically activated 4 hours after sperm injection in 7% ethanol in TCM for 5 minutes. In Treatment B (n=3 replicates), sperm-injected oocytes (n=75) were activated by exposure to 5 µM ionomycin in CR1aa medium for 5 minutes followed by 10 µg/ml cycloheximide in CR1aa medium for 5 hours.

Sham-injected oocytes (n=45) in Treatment C (n=4 replicates) were chemically activated 4 hours post-injection in 7% ethanol in TCM for 5 minutes. In Treatment D (n=3 replicates), the sham-injected oocytes (n=35) were chemically activated by exposure to 5 µM ionomycin in CR1aa medium for 5 minutes followed by incubation in 10 µg/ml cycloheximide in CR1aa medium for 5 hours.

Sperm-injected and sham-injected oocytes from all four treatment groups were cultured in CR1aa medium from day 0 to day 3 post-injection and then in CR1aa medium supplemented with 5% fetal bovine serum (FBS) from day 3 to day 8 of culture. Cleavage and blastocyst formation rates were assessed on day 3 and day 8 of culture, respectively.

Experimental Procedure

Oocyte Preparation

Bovine oocytes were obtained from a commercial supplier (BoMed Inc., Madison, WI and Concho Valley Genetics, San Angelo, TX) in groups of 50 and matured in a
Figure 7.1. Bovine cumulus oocyte complexes (COCs) received from a commercial supplier at 21 hours of maturation. A. COC at 20X magnification. B. COCs at 10X magnification.
portable incubator at 39°C during overnight travel (Figure 7.1). Upon arrival to the Embryo Biotechnology Laboratory, shipping vials were removed from the portable incubator and stored in a 5% CO₂ incubator in humidified air at 39°C until use. After 21 hours of in vitro maturation, cumulus cells were removed by vortexing the cumulus oocyte complexes for 4 minutes in Tyrodes lactate HEPES medium (TLH) (04-616F, Cambrex Bio Science Walkersville Inc., Walkersville, MD) supplemented with 1 mg/ml of hyaluronidase (H-3506, Sigma-Aldrich Inc., St. Louis, MO). After vortexing, oocytes were washed through two Falcon® 35 x 10 mm plastic petri dishes (353001, Becton Dickinson, Franklin Lakes, NJ) of mTLH containing 10% (v/v) FBS (SH30070.02, Hyclone, Logan, UT) and 50 µg/ml of gentamicin (15750-060, Gibco, Grand Island, NY) to stop the enzymatic action of the hyaluronidase.

Mature oocytes with an extruded first polar body were selected with a dissecting microscope and placed in 500 µl of mTLH in a 1.7 ml plastic micro-centrifuge tube (20170-355, VWR International Inc., Sugar Land, TX). Oocytes were centrifuged at 6,000 x g for 5 minutes in a micro-centrifuge (AccuSpin, Fisher Scientific) to clear the ooplasm to facilitate the microinjection procedure. Centrifuged oocytes were placed in TCM 199 (11150-067, Gibco, Grand Island, NY) containing Earle’s salts, L-glutamine, 2,200 mg/l of sodium bicarbonate, 5% FBS and 50 µg/ml of gentamicin until the time of use.

Sperm Preparation

Both frozen-thawed ejaculated and epididymal bovine sperm were used for Experiment 7.1. Ejaculated sperm were obtained from a Holstein bull (CSS 7H5188, Genex Cooperative Inc., Shawano, WI) with proven in vitro fertilization ability. Epididymal sperm were harvested from an abattoir-derived mature beef bull. No historical information was available.

Straws (0.5 ml) of both ejaculated sperm and epididymal sperm were thawed in a 37°C water bath for 40 seconds. Thawed sperm were carefully layered over 2 ml of both lower and upper layer of ISolate® sperm separation medium (99264, Irvine Scientific, Santa Ana, CA) in a 15 ml plastic centrifuge tube (Corning, New York, NY). The tube was then centrifuged at 300 x g for 20 minutes to obtain the motile fraction of sperm and to remove egg yolk components to prevent sperm from sticking into the microinjection pipette. The supernatant was discarded and the pellet and 500 µl of the lower layer were
resuspended in 8 ml of mTLH medium supplemented with 3 mg/ml of bovine serum albumin (Fraction V, A-7511, Sigma-Aldrich Inc., St. Louis, MO), 1 mg/ml of caffeine sodium (C-4144, Sigma-Aldrich Inc., St. Louis, MO) and 50 µg/ml of gentamicin and centrifuged twice at 350 x g for 5 minutes. After centrifugation, the supernatant was removed and the remaining pellet was resuspended in 1 ml of mTLH and placed in a water bath at 37°C until time of use.

Sperm Head Preparation

For sperm head preparation, the 1 ml of sperm-TLH medium was placed on ice and transported to Louisiana State University, Life Sciences Building for tail removal. A 500 µl sample of the specimen was placed in a 1.7 ml micro-centrifuge tube and sonicated at 20% power in a tissue sonicator for 1 second. The sample was kept on ice to prevent the temperature from rising during sonication. With this procedure, 70 to 80% of the sperm had their tails removed. Sperm heads were washed twice by centrifugation at 350 x g for 5 minutes in mTLH and stored on ice until use.

Microinjection Apparatus

Holding pipettes were purchased from Cook (V-HPIP-1015, Cook, Australia). They had a 1 mm taper with a 35° angle and a polished end with outer and inner diameters of 120 µm and 20 µm, respectively. The holding pipette was connected to a mineral oil-filled plastic tubing of a Narishige microinjector unit. The injection pipettes were purchased from Humagen (PIEZO-8-25, Charlottesville, VA). They consisted of a 6 mm taper with a 25° angle and a blunt end with an inner diameter of 8 to 9 µm. A small volume of mercury (~1 µl) was placed in the proximal end of the injection pipette. Then, with an air-filled syringe the mercury was pushed forward around 0.5 cm to leave an air column between the mercury and the proximal end of the pipette which was connected to an oil-filled Eppendorf microinjector (Cell Tram, Eppendorf).

Sperm Injection Procedure

Sperm injection was performed in the lid of a Falcon® 60 mm plastic petri dish (353002, Becton Dickinson, Franklin Lakes, NJ) on a heated stage at 37°C with 400X magnification using an inverted Nikon microscope equipped with Hoffman optics and Narishige micromanipulators. Each of four microdrops were aligned in the center of the lid and covered with mineral oil (330779, Sigma-Aldrich Inc., St. Louis, MO). The first two droplets (3 µl) contained a 1:1 mixture of 10% (v/v) polyvinylpyrrolidone (PVP).
(reconstituted in isotonic HEPES buffered human tubal fluid medium) (99311, Irvine Scientific, Santa Ana, CA) and mTLH to give a final PVP concentration of 5% (v/v).

A small volume of the sperm mixture was transferred into the two droplets by using a pulled glass pipette. The third droplet was a 50 µl oblong droplet of TLH supplemented with 10% FBS for oocyte micromanipulation. The last droplet was 30 µl in volume and contained 10% (v/v) PVP for pipette washing after each injection procedure. No more than 10 oocytes were placed in the TLH-FBS droplet at a time.

Immediately before sperm injection, a motile sperm was randomly selected from one of the PVP-TLH droplets and immobilized by scoring its tail with the tip of the injection pipette against the bottom of the dish (Figure 7.2). The sperm was aspirated tail first into the injection pipette by applying gentle suction. When a sperm head was used instead of whole sperm, 3 sperm heads were aspirated into the injection pipette at the same time to decrease manipulation time of the oocytes.

In this study, ICSI was performed with a piezo unit (Piezo-Drill, Burleigh, Germany) (Figure 7.3), as previously described (Kimura and Yanagimachi, 1995). Briefly, a MII oocyte was picked up by the holding pipette by applying gentle suction and the oocyte was rotated with the injection pipette until the polar body was located at either the 6 o’clock or the 12 o’clock position. The injection pipette was then placed in close contact with the oocyte at the 3 o’clock position. The zona pellucida was drilled by applying several piezo pulses while a light negative pressure was applied to it. When the injection pipette had passed the zona pellucida, a cylindrical piece of the zona inside the injection pipette was expelled into the medium outside the oocyte. After the injection pipette was reinserted through the hole in the zona, the sperm or sperm head was pushed slowly until it was positioned very close to the tip of the injection pipette before penetrating the ooplasm. The pipette was advanced mechanically into the ooplasm until it reached the center of the oocyte. A single piezo pulse was applied to break the oolema. Then, the injection pipette was moved forward slightly and the sperm was expelled slowly in the ooplasm with a minimum volume of medium. The pipette was removed slowly while applying light negative pressure. The injection procedure lasted 3 to 5 minutes per oocyte. Sham injections were performed in the same manner, except that no sperm was injected. After 8 to 10 oocytes were injected, they were washed twice in mTCM and placed into their respective activation treatment groups.
Figure 7.2. Immobilization of a motile bovine epididymal sperm with the microinjection pipette prior to sperm microinjection. Arrow for the tail of the sperm.
Figure 7.3. The piezo ICSI procedure (40X magnification). A. An oocyte with the polar body in the 12 o’clock position held by the negative pressure of the holding pipette before sperm injection. B. The zona pellucida drilled by applying piezo pulses. A small cylinder of the zona pellucida can be observed outside the oocyte. The sperm is moved to the tip of the pipette before penetrating the oolema. C. The pipette is mechanically moved forward to stretch the oolema. A single piezo pulse is applied to break the oolema. D. The sperm is pushed forward by applying positive pressure to the injector. E. Excess medium is removed and the injection pipette is moved out from the oocyte. F. A small amount of ooplasm at the place of injection is removed to allow sealing of the oolema.
Oocyte Activation Treatments

Sperm-injected and sham-injected oocytes were subjected to two different activation treatments. After injection procedure, oocytes in Treatments A, C and E were cultured for 4 hours in TCM supplemented with 5% FBS and 50 µg/ml of gentamicin. Oocytes were then treated for 5 minutes in 7% (v/v) ethanol (060602, AAPER Alcohol and Chemical, Shelbyville, KY) in TCM 199 containing 1 mg/ml PVP-40 (P-0930, average molecular weight 40,000, Sigma-Aldrich Inc., St. Louis, MO). The remaining sperm-injected oocytes in Treatments B, D and F were exposed to 5 µM ionomycin (I-9657, Sigma-Aldrich Inc., St. Louis, MO) in TCM 199 for 5 minutes followed by exposure to 10 µg/ml of cycloheximide (C-7698, Sigma-Aldrich Inc., St. Louis, MO) in CR1aa medium supplemented with 3 mg/ml bovine serum albumin Fraction V (A-7511, Sigma-Aldrich Inc., St. Louis, MO).

Embryo Culture

CR1aa medium was prepared as described previously (Rosenkrans and First, 1994). The CR1aa stock solution was prepared by adding 114.7 mM NaCl (S-5886, Sigma-Aldrich Inc., St. Louis, MO), 1.6 mM KCl (P-5405, Sigma-Aldrich Inc., St. Louis, MO), 0.39 mM pyruvic acid (P-4562, Sigma-Aldrich Inc., St. Louis, MO), 26.2 mM NaHCO₃ (S-8875, Sigma-Aldrich Inc., St. Louis, MO), 2.5 mM L (+) lactic acid (L-4388, Sigma-Aldrich Inc., St. Louis, MO), 0.5195 mM glycine (G-8790, Sigma-Aldrich Inc., St. Louis, MO), 0.5051 mM L-alanine (A-7469, Sigma-Aldrich Inc., St. Louis, MO) and 0.2 ml of 0.5% phenol red per 100 ml of Milli-Q water. The stock solutions were kept unfiltered at 4°C for up to 2 months.

CR1aa culture medium for day 0 to day 3 of in vitro culture was prepared by adding 1% (v/v) of Modified Eagle Medium (MEM) amino acid solution (10X, 11140050, Gibco Laboratories, Grand Island, NY), 2% (v/v) of Basal Medium Eagle (BME) amino acid solution (50X, B-6766, Sigma-Aldrich Inc., St. Louis, MO), 1.1 mM L-glutamine (G-5763, Sigma-Aldrich Inc., St. Louis, MO), 3 mg/ml of BSA and 50 µg/ml of gentamicin to the CR1aa stock solution. CR1aa culture medium from day 3 to day 8 was the same as from day 0 to day 3 of culture but with the addition of 5% FBS. The medium was prepared the night before use and placed in a 5% CO₂ incubator at 39°C to allow proper temperature and CO₂ equilibration.
Sperm-injected oocytes were cultured in groups of 10 in 50 µl drops of CR1aa medium at 39°C in an atmosphere of 5% CO₂, 7% O₂ and 88% N₂. Cleavage and blastocyst rates were assessed on day 3 and day 8 of in vitro culture, respectively.

Assessment of Fertilization

Pronuclear formation was assessed 18 to 20 hours after ICSI. Oocytes were mounted in groups of five between a precleaned Gold Seal® microscope slide (3051, Gold Seal® Products, Portsmouth, NH) and a coverslip (size 18 x 18, 12-540A, Fisher Scientific, Pittsburgh, PA) and fixed with methanol:acetic acid (3:1, v/v) for at least 24 hours. Fixed oocytes were stained with 1% aceto-orcein (O-7380, Sigma-Aldrich Inc., St. Louis, MO) and unstained with acetic acid:glycerol:water (1:1:2 v/v) for easier evaluation of the sperm. An oocyte with two polar bodies and two pronuclei were considered as fertilized. In this study, an oocyte with two polar bodies, one pronucleus and a sperm head were considered to be activated. Also, an oocyte displaying an enlarged sperm head was considered as decondensing sperm head.

Blastocyst Staining

Day-8 blastocysts that were not transferred into recipient females were stained in 1 mg/ml of Hoechst 33342 (B-2261, Sigma-Aldrich Inc., St. Louis, MO) in TLH medium for 15 minutes. After staining, blastocysts were washed twice in separate 75 µl droplets of TLH to remove excess stain. Embryos were placed between a slide and coverslip for cell counts. Total number of cells was counted using an inverted microscope (Nikon Diaphot, Tokyo, Japan) equipped with an ultraviolet light and a Hoechst filter.

Statistical Analysis

Embryo developmental stages were analyzed across treatments by the Chi-square test and Fisher’s Exact Probability test. Total cell counts for blastocysts are expressed as mean value±SEM for each treatment group. Means were considered significantly different at P<0.05 in this study. All data were analyzed using SigmaStat Statistical Software Version 2.0.

RESULTS

Experiment 7.1

Pronuclei formation of sperm-injected oocytes was evaluated following aceto-orcein staining. At 18 to 20 hours post-sperm injection, oocytes that had degenerated
Figure 7.4. Cytolyzed bovine oocytes degenerated 4 to 5 hours after sperm injection. A. Arrows indicates cytolyzed oocytes (20X magnification). B. Cytolyzed oocyte (40X magnification).
Figure 7.5. Oocyte stained with aceto-orcein 18 hours after the ICSI procedure. Male and female pronuclei are indicated by arrows.
Table 7.1. Effect of activation treatments on pronuclear formation of epididymal sperm-injected bovine oocytes

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Sperm source</th>
<th>No. injected</th>
<th>No. survived</th>
<th>DSH</th>
<th>1 PN</th>
<th>2 PN</th>
<th>Total activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>Epi</td>
<td>108</td>
<td>97(90)a</td>
<td>8(8)</td>
<td>40(41)a</td>
<td>30(31)a</td>
<td>70(72)a</td>
</tr>
<tr>
<td></td>
<td>Ejac</td>
<td>93</td>
<td>86(93)a</td>
<td>5(6)</td>
<td>24(28)b</td>
<td>37(43)b</td>
<td>61(71)a</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>63</td>
<td>60(95)a</td>
<td>-</td>
<td>27(45)a</td>
<td>1(2)c</td>
<td>28(47)b</td>
</tr>
<tr>
<td>Iono+Cyclo</td>
<td>Epi</td>
<td>65</td>
<td>46(71)b</td>
<td>3(6)</td>
<td>15(33)b</td>
<td>14(30)a</td>
<td>29(63)a</td>
</tr>
<tr>
<td></td>
<td>Ejac</td>
<td>54</td>
<td>35(65)b</td>
<td>2(6)</td>
<td>11(31)b</td>
<td>13(37)b</td>
<td>24(69)a</td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>59</td>
<td>43(73)b</td>
<td>-</td>
<td>21(48)b</td>
<td>2(4)c</td>
<td>23(53)b</td>
</tr>
</tbody>
</table>

Iono+Cyclo = ionomycin treatment followed by cycloheximide, Epi = epididymal sperm, Ejac = ejaculated sperm, DSH = decondensed sperm head, 1 PN = one pronucleus, 2 PN = two pronuclei.

Three to four replicates per treatment group. a,b,c Values with different superscripts within columns are significantly different (P<0.05).
(Figure 7.4) were removed from the study. Only morphologically normal oocytes were fixed for cytological assessments. The proportion of oocytes exhibiting male and female pronuclei and two polar bodies (successfully fertilized) (Figure 7.5) was significantly higher (P<0.05) when ejaculated sperm were used instead of epididymal sperm (43% and 31%, respectively) (Table 7.1). However, significant difference was only found when ethanol was used to exogenously activate the oocytes in place of ionomycin followed by cycloheximide. Furthermore, no statistical difference was found in the percentage of successfully fertilized oocytes when ionomycin followed by cycloheximide was used as the activation protocol when both ejaculated and epididymal sperm were used.

The proportion of oocytes with one pronucleus was significantly higher (P<0.05) when ethanol was used as activation protocol in the epididymal sperm-injected treatment group compared with ionomycin followed by cycloheximide treatment. However, no significant difference in the proportion of oocytes exhibiting one pronucleus was detected when ejaculated sperm or sham injection were used. In more than 90% of sperm-injected oocytes exhibiting one pronucleus a condensed sperm head was observed. In sham-injected oocytes only 48% and 54% of oocytes formed a single pronucleus, however, 2% and 5% of the oocytes formed two pronuclei when activated with ethanol and ionomycin followed by cycloheximide, respectively. A significant difference (P<0.05) was noted between the percentage of oocytes forming two pronuclei between the sham-injected groups (Treatments E and F) and sperm-injected groups (Treatments A, B, C and D) regardless of activation protocol used.

The proportion of cytolyzed oocytes was significantly greater (P<0.05) when ionomycin followed by cycloheximide (Treatments B, D and F) was used compared with ethanol activation (Treatments A, C and E). The percentage of cytolyzed oocytes was not found to increase due to the source of sperm.

Experiment 7.2

Results of embryonic development from epididymal sperm-injected oocytes are shown in Table 7.2. The percentage of sperm-injected oocytes reaching the blastocyst stage (Figure 7.7) was significantly greater (P<0.05) when ethanol (14%) was used compared with ionomycin followed by cycloheximide (4%) or the sham-injected control (0%) groups. No difference in cleavage rates were found between the two different
Table 7.2. Effect of activation treatments on the embryonic development of epididymal sperm-injected bovine oocytes

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Sperm injected</th>
<th>No. injected</th>
<th>No. survived</th>
<th>2-4 cell</th>
<th>4-8 cell</th>
<th>8-16 cell</th>
<th>Total cleaved</th>
<th>Total BLST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>+</td>
<td>118</td>
<td>108(92)a</td>
<td>19(18)a</td>
<td>33(31)</td>
<td>26(24)a</td>
<td>78(72)a</td>
<td>15(14)a</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>45</td>
<td>40(89)a</td>
<td>13(33)b</td>
<td>9(23)</td>
<td>0</td>
<td>22(55)b</td>
<td>0</td>
</tr>
<tr>
<td>Iono + Cyclo</td>
<td>+</td>
<td>75</td>
<td>55(73)b</td>
<td>26(47)b</td>
<td>11(20)</td>
<td>4(7)b</td>
<td>41(74)a</td>
<td>2(4)b</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>35</td>
<td>26(74)b</td>
<td>6(38)b</td>
<td>3(19)</td>
<td>0</td>
<td>9(56)b</td>
<td>0</td>
</tr>
</tbody>
</table>

Iono + Cyclo = ionomycin followed by cycloheximide; BLST = blastocysts.
Three to four replicates per treatment group. a,b,cValues with different superscripts within columns are significantly different (P<0.05).
Figure 7.6. An example of a day-3 bovine embryo (8- to 16-cells) derived from epididymal sperm-injected oocytes.
Figure 7.7. Examples of day-8 blastocysts derived from epididymal sperm-injected oocytes. Blastocysts are hatching through the opening made by the microinjection pipette.
activation treatment groups. However, a significantly higher percentage of oocytes cleaved in the sperm-injected oocytes when compared with the sham-injected control, regardless of activation treatment. A significantly higher percentage of epididymal sperm-injected oocytes reached the 8- to 16-cell stages (Figure 7.6) when activated with ethanol (28%) compared with ionomycin followed by cycloheximide (7%) at 72 hours of embryo culture. No oocyte in the sham-injected control group developed further than the 4- to 8-cell stages in culture.

Furthermore, a significantly higher (P<0.05) proportion of ICSI oocytes were observed at the 2- to 4-cell stages at 72 hours of culture when ionomycin followed by cycloheximide treatment was used compared with ethanol treatment.

The mean total cell numbers of the stained blastocysts (Figure 7.8) that were not transferred into recipient females are shown in Table 7.3. No difference was found between treatments likely due to the low number of blastocyst in each group.

**Experiment 7.3**

Nine day-8 blastocysts were nonsurgically transferred into recipient beef females on day 7 of the estrous cycle. Of the 9 blastocysts, 2 were excellent Grade 1 embryos transferred to 2 recipients and 7 Grade 1 to Grade 2 embryos were transferred to 3 recipients. One ongoing 50-day pregnancy (with a heart beat) resulted from the transfer of one of the 2 Grade 1 blastocysts transferred (Figure 7.9).

**DISCUSSION**

We have demonstrated that normal fertilization and blastocyst development could be achieved with cryopreserved bovine epididymal sperm by piezo ICSI. Although this is not the first report of ICSI in bovine using epididymal sperm, the blastocyst success rate in this study is considerably higher at 14% than that reported previously by Goto et al. (1990) at 1.8%. It should be noted that Goto et al. (1990) used epididymal sperm that had been ‘killed’ by repeated freeze-thawing without cryoprotectants. Recently, Horiuchi et al. (2002) reported that embryonic development was drastically reduced when ‘killed’ sperm by multiple freeze-thawing was used compared with ‘live’ sperm that had been immobilized prior to the microinjection procedure. Also, our higher success could be, in part, for the use of a piezo drill to penetrate the oolema to assure the injection of sperm into the ooplasm.
Table 7.3. Total cell number (mean±SEM) of day-8 bovine blastocysts derived from ICSI using different activation treatments

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of embryos</th>
<th>Total no. of cells/embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>5</td>
<td>96±4.2</td>
</tr>
<tr>
<td>Ionomycin + cyclohexamide</td>
<td>2</td>
<td>80±4.5</td>
</tr>
</tbody>
</table>
Figure 7.8. An example of a day-8 blastocyst stained with Hoechst 33342. A. Day-8 blastocyst at 20X magnification. B. An overlay of the stained cells of the same day-8 blastocyst in panel A.
Figure 7.9. Ultrasound images of a 50-day frozen-thawed epididymal sperm ICSI-derived fetus.
Reports to date indicate that pronuclear formation and blastocyst rates following ICSI with bovine ejaculated sperm range from 20 to 60% and 7 to 20%, respectively (Keefer et al., 1990; Wei and Fukui, 1999; Hamano et al., 1999; Keskintepe et al., 2002; Horiuchi et al., 2002; Galli et al., 2003; Ock et al., 2003; Rho et al., 2004; Li et al., 2004). The marked difference in pronuclear formation and blastocyst rates between laboratories may be because of different protocols used for pretreating sperm prior to microinjection, including if whole sperm or sperm heads and if ‘dead’ or ‘live’ sperm are used, scoring or cutting the tail before sperm injection and treating sperm with different chemicals to accomplish disruption of plasma membrane and/or reduction of disulfide bonds. Also, embryo manipulation, the use of conventional or piezo ICSI, expertise of the technician and embryo culture play an important role in the success of the oocyte microinjection procedure.

This is the first report that evaluated pronuclear formation of bovine ICSI oocytes using epididymal sperm. A significantly higher fertilization rate was achieved, however, when ejaculated rather than epididymal sperm was used in our procedure. This difference in fertilization rates between ejaculated and epididymal sperm was only noted depending on the activation protocol employed. No difference in fertilization rates was found between activation treatments when the same sperm source (either ejaculated or epididymal) was used. Furthermore, activation with ethanol produced higher fertilization rates for ejaculated sperm than for epididymal sperm compared with ionomycin followed by cycloheximide.

The lower pronuclear formation for epididymal sperm in this study may rely on the difference in maturation status between ejaculated sperm and epididymal sperm. Disruption of the plasma membrane to achieve nuclear decondensation and the release of the sperm factor may be somehow compromised for epididymal sperm. The plasma membrane is modified and prepared for fertilization by biochemical components in seminal plasma at the time of ejaculation in the bull. Seminal plasma has been shown to cause cholesterol efflux from the plasma membrane to prepare sperm for capacitation, acrosome reaction and fertilization (Osheroff et al., 1999; Iborra et al., 2000). Furthermore, it can not be ruled out that unknown factors in seminal plasma play an important role in ‘modifying’ sperm for the fertilization process.
Heneault et al. (1995) demonstrated that incubation of sperm from a low fertility bull with seminal plasma from a highly fertile bull increased the oocyte fertilization rates in vitro. Two proteins in seminal plasma of 26 and 55 kDa each have been shown to predominate in higher fertility bulls (Killian et al., 1993). Thus, the role of seminal plasma in preparing sperm for fertilization at the time of ejaculation can not be overlooked.

In addition, the differences in fertilization rates for IVF and ICSI due to a bull effect using ejaculated sperm have to be considered. In a study using different bulls for conventional IVF, fertilization rates have been reported to vary from 35 to 96% (Niwa and Ohgoda, 1988). Recently, Wei and Fukui (1999) indicated that the ability of microinjected sperm to decondense and form male pronuclei varies greatly between bulls. In our study, epididymal sperm were harvested from testes obtained from abattoir derived bulls with no previous reproductive history. This effect has to be considered when comparing and analyzing the results reported herein.

Sperm pretreatment might help enhance sperm head decondensation and pronuclear development of epididymal sperm-injected oocytes. The use of chemicals, such as calcium ionophore, Triton-X 100 and dithiothreitol, can be used to increase the rate of acrosome reaction, plasma membrane permeabilisation and sperm head decondensation (Keefer et al., 1990; Malcuit et al., 2006; Wei and Fukui, 1999).

We found a significantly higher number of oocytes to degenerate 4 to 5 hours after ICSI when they were activated with ionomycin followed by cycloheximide than with the ethanol treatment. One difference between the two activation protocols was that the oocytes in the ethanol group were kept for 4 hours in a medium containing 5% fetal bovine serum before activation. Proteins, growth factors and unknown biochemical components in serum might help with the ‘wound healing’ of the oolema post-ICSI (Suzuki et al., 1997). Suzuki et al. (1997) reported that a medium with high concentration of serum was beneficial for the sealing process of the oolema after sperm injection in the mouse. We are in agreement that serum-rich medium likely enhances the survival of the oocyte after the injection procedure. This, in part, further demonstrates that the bovine oocyte is very sensitive to the piercing of the microinjection pipette. We can not rule out that the effect of the ICSI procedure is one of the reasons for the less than optimal success in fertilization and embryo development rates of this technique in cattle.
The present study indicates that activation of bovine oocytes following ICSI by ethanol was better in terms of blastocyst production than activation by ionomycin followed by cycloheximide. The blastocyst rate of 14% obtained in our study using epididymal sperm was in the range of 7 to 20% blastocysts reported over the years for ejaculated sperm by other investigators (Hamano et al., 1999; Horiuchi et al., 2002; Galli et al., 2003; Rho et al., 2004). We agree with previous investigations where ethanol was reported to be the best activation treatment for bovine ICSI with ejaculated sperm when applied 4 hours after sperm injection (Horiuchi et al., 2002; Fujinami et al., 2004; Oikawa et al., 2005).

Recently, Horiuchi (2006) reported that more than 24 calves have been produced by ethanol-activated ICSI oocytes using ejaculated sperm. In contrast, no calves have been reported to date in ionomycin followed by cycloheximide-activated ICSI oocytes. It has been reported that ~60% of the oocytes post-ICSI were activated by the sperm when assessed by the extrusion of the second polar body 4 hours after sperm injection (Horiuchi et al., 2002; Galli et al., 2003). This shows, in part, that a protein synthesis inhibitor, such as cycloheximide, is not required during the first 4 to 5 hours after ICSI for proper oocyte activation. Cycloheximide might rather affect embryo development or enhance parthenogenesis of the oocytes not activated by the initial signal of the sperm.

The level of maturation promoting factor (MPF) activity was reported to decrease upon sperm injection and temporarily increase after 4 to 6 hours post-ICSI (Fujinami et al., 2004). Activation of bovine oocytes with ethanol 4 hours after sperm injection has been shown to maintain the low level of MPF activity until 20 hours for the next cell cycle, whereas, this elevation in MPF was not found in bovine oocytes without activation (Fujinami et al., 2004). This rise in MPF may promote cleavage and subsequent embryo development (Horiuchi, 2006). In addition, microtubules around the male and female pronuclei were reported to be more defined and better developed when ethanol was used to activate bovine oocytes 4 hours after ICSI compared with that of no activation (43% vs. 73%) (Horiuchi, 2006). However, the exact mechanism by which ethanol provides better subsequent embryo development remains largely unknown.

We can conclude that postmortem epididymal sperm can be collected from genetically valuable males and used for the production of blastocysts using ICSI. At the time of this writing 9 ICSI-derived bovine blastocysts were nonsurgically transferred into
5 recipient females resulting in a 50 day ongoing pregnancy. Further investigations should evaluate the fertilizing ability of epididymal sperm harvested from testes that have been stored for different time periods after death.
CHAPTER VIII

SUMMARY AND CONCLUSIONS

The primary objectives of these series of experiments were to develop a successful protocol for cryopreservation and intracytoplasmic sperm injection (ICSI) using bovine caudal epididymal sperm. Information on the cryopreservation and use for ICSI of epididymal sperm is limited. The development of a protocol to recover and cryopreserve sperm from postmortem animals will allow the propagation of valuable gametes for future generations increasing the ‘life span’ of an individual male. Developing a successful ICSI protocol will allow the use of these rare and valuable cryopreserved sperm samples for the production of future offspring.

In the first experiment, the effect of a one step addition and/or removal of different concentrations of permeable cryoprotectants (CPAs) and their toxic effects during cool storage were assessed. Results showed that ethylene glycol caused less osmotic damage to sperm during addition and/or removal at 4°C when compared with glycerol in all concentrations evaluated. Maximum survival was achieved with sperm exposed to 7% ethylene glycol. Furthermore, extended exposure (5 days at 4°C) of ethylene glycol was found to be less toxic than glycerol to epididymal sperm. We conclude that ethylene glycol, used as a cryoprotectant, may help minimize toxicity and osmotic damage to bovine epididymal sperm.

In the second experiment, the cryoprotective effect of glycerol and ethylene glycol was evaluated. Results indicated that glycerol was far more effective in providing protection against freezing injury during the cryopreservation process than ethylene glycol.

In the third experiment, the effect of exposing epididymal sperm to seminal plasma prior to cryopreservation was evaluated. It was demonstrated that epididymal sperm retrieval using seminal plasma is clearly beneficial to enhance sperm overall and progressive motility characteristics and to protect it from morphological abnormalities derived from the freeze-thaw process.

In the fourth experiment, the effects of a one step removal of glycerol from cryopreserved bovine epididymal sperm previously exposed to seminal plasma and its longevity during a 4-hour incubation period at 37°C were assessed. The results indicated that a one step dilution process for removal of glycerol from cryopreserved epididymal
sperm affected the integrity of the sperm plasma membrane and the function of the mitochondria of sperm previously exposed to seminal plasma. However, dilution did not have any significant detrimental effect on the integrity of the acrosome. Furthermore, we demonstrated that the longevity in vitro of post-thaw epididymal sperm exposed to seminal plasma prior to cryopreservation showed no evidence of being compromised when compared with epididymal sperm that was not exposed to seminal plasma.

In the final series of trials, an effort was made to evaluate fertilization and blastocyst development rates from bovine epididymal sperm-injected oocytes. The results demonstrated that acceptable fertilization and blastocyst development rates could be achieved with cryopreserved bovine epididymal sperm by intracytoplasmic sperm injection. Secondly, good quality ICSI-derived blastocysts were nonsurgically transferred to evaluate if the protocols developed in our study would result in confirmed pregnancies. One ongoing pregnancy at 50 days resulted from a day-8 Grade 1 blastocyst.

We can conclude that postmortem epididymal sperm can be collected from genetically valuable males and used for the production of blastocysts using ICSI. This is the first report to evaluate epididymal sperm quality using multicolor flow cytometry. In addition, we have developed a successful protocol to cryopreserve bovine caudal epididymal sperm. Furthermore, we have developed a successful ICSI protocol and technique for generating embryos from sperm harvested from postmortem animals. The protocols developed and designed herein can be adapted to best fit other species of interests, including humans and endangered species.
LITERATURE CITED


spermatozoa on the developmental rate of embryos transferred at the 2-cell stage. Biol. Reprod. 66:381-385.


and testicular sperm obtained from patients with obstructive and nonobstructive azoospermia. J. Urol. 167:1753-1756.


Phelps, M. J., J. Liu, J. D. Benson, C. E. Willoughby, J. A. Gilmore, and J. K. Critser. 1999. Effects of Percoll separation, cryoprotective agents, and temperature on


Saragusty, J., H. Gacitua, R. King, and A. Arav. 2006. Post-mortem semen cryopreservation and characterization in two different endangered gazelle species (Gazella gazella and Gazella dorcas) and one subspecies (Gazella gazelle acaiae). Theriogenology (In press).


VITA

Carlos Andres Guerrero was born on June 23, 1980, in Cali, Valle, Colombia, to Maria Islena Espinosa and Carlos Leonardo Guerrero. Carlos has an older brother, Jorge Enrique Guerrero, who resides in Boston, Massachusetts, and works as a physician. Carlos grew up in Cali, Colombia, where he attended the British school, Colegio Colombo Britanico. Following graduation in 1998, Carlos came to the United States to pursue a career in the area of animal science. Carlos enrolled at Louisiana State University, in Baton Rouge, Louisiana, in the fall of 1998. During his junior year, Carlos met Dr. Robert A. Godke, Boyd Professor of Reproductive Physiology, who involved him in various reproductive physiology projects at the Embryo Biotechnology Laboratory, St. Gabriel, Louisiana. After receiving his bachelor degree in animal science in May 2002, Carlos started to pursue his doctoral degree under the guidance of Dr. Robert A. Godke. He is a candidate for the Doctor of Philosophy degree in reproductive physiology in the Department of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana.