1991

Effects of Chronic Administration of Phenylbutazone on Reproduction in the Mare.

Mareth Ellsworth
Louisiana State University and Agricultural & Mechanical College

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Effects of chronic administration of phenylbutazone on reproduction in the mare

Ellsworth, Mareth, Ph.D.
The Louisiana State University and Agricultural and Mechanical Col., 1991
EFFECTS OF CHRONIC ADMINISTRATION OF PHENYLButAZONE 
ON REPRODUCTION IN THE MARE

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 in Veterinary Medical Sciences 
(Physiology Option)

by 

Mareth Ellsworth 
B.A., University of California, 1970 
May, 1991
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Phenylbutazone is a non-steroidal anti-inflammatory drug that acts by inhibiting prostaglandin synthesis. Prostaglandins are associated with crucial aspects of reproduction. In 5 experiments, mares were used to test the effects of phenylbutazone on reproduction. In all experiments, treatment was 2 g phenylbutazone or 10 ml physiological saline intravenously (control).

In Experiment I, 9 mares were phenylbutazone-treated for 180 consecutive days and teased. Ovaries were palpated and cyclicity was verified by circulating progesterone concentrations. Thirty-six cycles were evaluated. Mares were then divided into treatment and control groups. Estrous mares were artificially inseminated. Non-surgical embryo collection was performed 7 to 8 days post-ovulation. Three fertilized ova were collected from control mares and 1 from a treated mare. Results suggested that phenylbutazone had no effect on estrual behavior, cyclicity or ovulation.

In Experiment II, 8 mares were phenylbutazone-treated daily for 33 days. Blood samples and corresponding uterine secretions were obtained daily. Only purulent samples of uterine secretion were positive for phenylbutazone.

In Experiment III, 7 mares were placed on a switchback experimental design, teased daily and cyclicity was verified by circulating progesterone concentrations. On day 4 post-ovulation, mares were subjected to uterine biopsy and subsequent estrous behavior was determined by teasing. Days from biopsy to induced
estrus for controls and treatment were significantly different (P<0.025). Results of Experiments II and III suggested that phenylbutazone accumulated in inflamed uterine tissue and inhibited prostaglandin synthesis.

In Experiments IV & V, 14 mares were teased daily. Estrous mares were mated. Treatment started on the first day of estrus and continued daily until embryo collection on day 10 post-ovation. Embryos (blastocysts) were photographed to evaluate morphological development, incubated in Dulbecco's phosphate-buffered saline and then homogenized to determine total protein concentrations. Culture medium was assayed for prostaglandin synthesis. There was no significant difference between embryos recovered from treated or control mares for total prostaglandin synthesis. There was, however, a reduced concentration of PGF$_2$α, PGE$_2$ and PGI$_2$ in embryos from treated mares compared with controls. Embryos from treated mares were significantly smaller (P<0.05), but contained more total protein than control embryos. Treatment did not affect ovulation, fertilization or embryo viability.
INTRODUCTION

Phenylbutazone is a non-steroidal anti-inflammatory drug widely used in equine medicine. It is used to restore performance in competitive horses, to relieve musculoskeletal diseases and to comfort chronically sore horses, such as brood mares. Its therapeutic effectiveness and toxic parameters have been clearly defined (Tobin, 1979a). Non-steroidal anti-inflammatory drugs (1) inhibit enzymes that act in the synthesis of prostaglandins and therefore reduce tissue prostaglandins; (2) normally accumulate in stomach, kidney, small intestines and inflamed tissues; (3) reduce pain, fever and swelling in inflamed tissues (Tobin, 1979a).

Phenylbutazone has been shown to inhibit both the cyclooxygenase and hydroperoxidase enzyme systems that are necessary for the synthesis of prostaglandins from arachidonic acid. Prostaglandins actively participate in many crucial reproductive processes. When the non-toxic effective dose of phenylbutazone (2 g) is injected intravenously it takes ≈30 minutes to transverse the blood-brain barrier, distribute throughout the horse and to begin blockage of formation of the prostaglandins (Tobin, 1979a). In previous studies, it was assumed that phenylbutazone was potentially capable of entering uterine tissue interrupting prostaglandin synthesis and altering normal cyclic function of the corpus luteum. In previous investigations in this laboratory, researchers administered 2 g phenylbutazone intravenously to mares for 8 to 21 days, beginning on day 14 and day 1 of the estrous cycle, respectively. Results showed no significant differences in plasma progesterone profiles and
interestrous intervals between phenylbutazone-treated and physiological saline-treated mares.

The viable embryo exerts biochemical signals to the maternal unit that permit development of a histotrophic uterine environment through the continued secretion of progesterone from the corpus luteum. Conceptus secretory proteins play a role in directing the embryo to synthesize major arachidonic acid metabolites associated with bovine conceptus viability, development and implantation (Thatcher et al., 1989). There are reports that verify production of prostaglandins by the harvested embryo in many farm and laboratory animals; however, there are no reports available for similar stage embryos for the mare.

Researchers have determined that prostaglandin antagonists administered to various species inhibited ovulation, luteolysis, ovum transport, blastocyst hatching, embryo implantation, embryo expansion, live birth rate and sharply diminished endometrial prostaglandin concentration and release (see Table 6). However, there was no available information on the viability of the equine embryo in the mare being administered a non-steroidal anti-inflammatory drug.

The experiments undertaken were designed to substantiate previous observations and to determine if chronic administration of phenylbutazone significantly alters fertility in mares.
LITERATURE REVIEW

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

Chemical Properties

Non-steroidal anti-inflammatory drugs (NSAID) are all aromatic-acidic compounds, sometimes referred to as "aspirin-like" drugs. These agents can be subdivided by general chemical structure into several categories: salicylates or derivatives of pyrazolon, fenamic acid, indene or arylalkanoic acids. These agents have, to some degree, analgesic, antipyretic and anti-inflammatory activities. They have been employed in the treatment of arthritis, myositis, tendonitis and other soft-tissue and osseous inflammatory conditions (Short and Beadle, 1978).

All NSAID are carboxylic acids with a pKα of ≈4.5, which gives a very low water solubility (Snow, 1983). They are usually formulated as sodium salts, especially for parenteral administration. Sodium salt formation also increases the speed of absorption following oral administration. Their acidic nature favors accumulation in inflamed tissues (Snow, 1981), which also tend to be acidic (Tobin, 1981b). The acidic nature of these drugs causes them to be between 95 and 99% bound to plasma proteins (Tobin, 1981b). Meclofenamic acid and naproxen are over 99% bound to plasma proteins (Snow, 1981). Although the binding is readily reversible, this property limits NSAID distribution from plasma to other body fluids and tissues and has important implications in drug detection and therapy (Higgins and Lees, 1988). This is important because the non-protein bound or
free drug is associated with biological activity. Prior exposure or concurrent administration of other drugs that are highly protein bound can, because of competition for binding sites, lead to higher free levels of either drug (Snow, 1981). Since these drugs are plasma protein bound they do not pass into saliva. Therefore, saliva testing is not acceptable for detection of NSAID (Tobin, 1981b).

The first NSAID to be synthesized was acetylsalicylic acid (aspirin) at the end of the 19th Century and it is still the most frequently used NSAID in humans. However, it is not popular for use in the horse because of its short duration of action. In 1949, phenylbutazone (the second oldest NSAID) was introduced as a therapeutic agent for man and has since become the dominant NSAID used in the horse (Snow, 1981). Pharmaceutical companies have synthesized a large number of NSAID that are available to both human and equine medicine. A partial list of these agents is presented in Table 1. Only 4 are presently employed for extensive use in the horse (1) the fenamic acid derivative, meclofenamic acid (Arquel®; Snow, 1981); (2) the phenylacetic acid derivative, naproxen (Equiproxen®; Snow, 1981); (3) the clonixin acid derivative, flunixin meglumine (Banamine®; Ciofalo, 1977); (4) the pyrazolone derivative, phenylbutazone (Butazolidin®; Burns, 1953). The chemical structures of these 4 popular equine NSAID are presented in Figure 1. Of these, phenylbutazone remains the most popular and widely used in equine medicine (Tobin, 1979a).
Table 1. Some non-steroidal anti-inflammatory drugs which all appear to share the same basic mechanism of action as phenylbutazone and same general pattern of toxicity

<table>
<thead>
<tr>
<th>Generic</th>
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<tr>
<td>Salicylates</td>
<td>Aspirin</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>Butazolidin</td>
</tr>
<tr>
<td>Oxyphenbutazone</td>
<td>Tandearil</td>
</tr>
<tr>
<td>Naproxen</td>
<td>Equiproxen</td>
</tr>
<tr>
<td>Meclofenamic Acid</td>
<td>Arquel</td>
</tr>
<tr>
<td>Flunixin Meglumine</td>
<td>Banamine</td>
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<tr>
<td>Quinine</td>
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<tr>
<td>Indomethacin</td>
<td>Indocin</td>
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<tr>
<td>Tolmetin</td>
<td>Tolectin</td>
</tr>
<tr>
<td>Niflumic Acid</td>
<td>Nifluril</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>Orudis</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>Motrin</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>Advil</td>
</tr>
<tr>
<td>Mefenamic Acid</td>
<td>Ponstel</td>
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Adapted from Tobin (1981b)
Figure 1. Chemical structure of non-steroidal anti-inflammatory drugs commonly used in equine medicine

Adapted from Booth (1982)
Mechanism of Action

The search for a possible mechanism of action of NSAID was started by Piper and Vane (Vane, 1971). They discovered that lungs could release a previously undetected substance that they called "rabbit aorta contracting substance". When isolated perfused lungs of sensitized guinea-pigs were challenged, rabbit aorta contracting substance, histamine, slow reacting substance in anaphylaxis, prostaglandin E\textsubscript{2} and F\textsubscript{2α} were released. The release of rabbit aorta contracting substance, which could also be provoked by bradykinin and slow reacting substance in anaphylaxis, was antagonized by aspirin-like drugs, as was the evoked broncho-constriction. Results of their experiments suggested that rabbit aorta contracting substance was a prostaglandin or had a structure intermediate between the prostaglandin precursor arachidonic acid and PGE\textsubscript{2} or PGF\textsubscript{2α} (Vane, 1971).

Vane (1971) designed experiments to test the possibility that NSAID inhibit the enzyme(s) which generate prostaglandin. He incubated guinea-pig lung homogenate with arachidonic acid aerobically at 37°C and determined an increase in PGE\textsubscript{2} and PGF\textsubscript{2α} synthesis. To test for inhibition of prostaglandin synthesis, varying amounts of indomethacin, sodium acetylsalicylate or sodium salicylate were added to the incubation flasks. All 3 anti-inflammatory acids inhibited generation of PGF\textsubscript{2α} and PGE\textsubscript{2}-like activity. In all preparations, there was a linear relationship between percentage inhibition and log concentration of indomethacin or aspirin. On a weight basis, indomethacin was 23 times more potent than aspirin as an inhibitor of PGF\textsubscript{2α}, and on a molar basis 47 times more potent. Aspirin was more potent than sodium salicylate as an inhibitor of synthesis of PGF\textsubscript{2α}.
and PGE$_2$-like activity. Results of Vane's experiments determined that the 3 NSAID inhibited synthesis of prostaglandins, but not how the inhibition is brought about.

Since these pioneering reports, the inhibitory action of aspirin-like drugs on prostaglandin production has been amply confirmed and demonstrated in almost all laboratory species and many other biological preparations from tissues throughout the body using a variety of assay techniques (Ferreira and Vane, 1974). Results of experiments conducted by Brune et al. (1976) clearly showed that acidic NSAID (1) are effective in almost every biological system; (2) reach higher concentrations in inflamed tissue than most others throughout the body; (3) are found only in similarly high concentrations in absorptive and excretory organs such as stomach, small intestine and kidney. How NSAID specifically exert their local anti-inflammatory action was, however, undetermined.

Mizuno et al. (1982) proposed the specific mechanism of action of NSAID. The biosynthesis of the prostaglandin family (Figure 2) is initiated by the enzyme reaction of fatty acid cyclooxygenase to convert arachidonic acid to PGG$_2$, followed by the action of prostaglandin hydroperoxidase on PGG$_2$ to form PGH$_2$. The former is a bis-dioxoxygenase reaction, and the latter a peroxidase-like reaction. Mizuno et al. (1982) investigated the effects of various NSAID in general on both cyclooxygenase and hydroperoxidase activity. When tested by the assay of the manganese protoporphyrin-assisted production of PGG$_2$ from arachidonic acid, all the drugs irreversibly inhibited the cyclooxygenase reaction. In contrast, none of the drugs except phenylbuta-zone also inhibited the hydroperoxidase reaction. Aspirin, indometh-
Extracellular Fluid Compartment

<table>
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<th>Acylhydrolases</th>
<th>Triglycerides</th>
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<td>Cell membrane</td>
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<tr>
<td>Proteins</td>
<td>Phospholipids</td>
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Injurious Stimuli (chemical/physical) → Phospholipase A → ARACHIDONIC ACID

Fatty acid cyclooxygenase → PGG₂ → Prostaglandin hydroperoxidase → PGH₂ → PGG₂ → PGE₂, PGI₂

Adapted from Kindahl (1980), Bell et al. (1980) and Chand and Eyre (1977)

Figure 2. Inhibition of prostaglandin biosynthesis by non-steroidal anti-inflammatory drugs (NSAID)
acin and flurbiprofen did protect the hydroperoxidase activity from heat or alkaline pH inactivation. Results raised the possibility that NSAID might also affect the binding of enzyme and heme because heme is a required cofactor of the enzyme in both the cyclooxygenase and hydroperoxidase reactions.

In summary, the mechanism of action for NSAID is the inhibition of cyclooxygenase. Phenylbutazone also inhibits prostaglandin hydroperoxidase (Mizuno et al., 1982). The order of anti-inflammatory potency of commonly used NSAID as determined in laboratory animals is meclofenamic acid > indomethacin > naproxen > phenylbutazone > acetylsalicylic acid (Snow, 1983).

**Pharmacokinetics**

All NSAID appear to be metabolized to inactive metabolites except phenylbutazone. At therapeutic doses about 10% of phenylbutazone is degraded to oxyphenbutazone, that has activity similar to phenylbutazone and is also used therapeutically. The metabolites are then excreted in urine and bile. Only negligible amounts of the drug are secreted in saliva. It is generally considered necessary to determine appropriate dosage and frequency of dosing to maintain a therapeutic circulating plasma level of these drugs during treatment (Snow, 1981). Because of the high plasma protein binding of NSAID and low water solubility, their rate of metabolism is the predominant factor responsible for declining plasma concentrations after administration (Snow, 1983). It is probable that therapeutic concentrations may remain in tissues for a period of time after they are no longer detectable in blood, which may explain a residual effectiveness after
therapy cessation (Snow, 1981).

The plasma half-life following intravenous administration for meclofenamic acid, naproxen and flunixin meglumine in the horse have been reported to be 1.0, 4.7 and 1.6 hours, respectively. For phenylbutazone, the plasma half-life is dose dependent, possibly due to the effect of its active metabolite oxyphenbutazone.

Of the 4 drugs used in the horse, only phenylbutazone and flunixin meglumine are formulated and available for parenteral administration, but all 4 are marketed for oral administration in the form of powders or granules. Phenylbutazone is also prepared in a paste form in a multi-dose applicator (Snow, 1983). Plasma concentrations of NSAID following oral administration can be highly variable (even within one animal) due to breed, age of horse and relationship to feeding time. Giving the drug on an empty stomach results in higher peak plasma concentration and a greater bioavailability than when it is given on a full stomach. Even though these compounds are acidic, most absorption occurs from the duodenum rather than the stomach. Presence of food in the stomach slows gastric emptying and therefore absorption (Snow, 1981). Studies also indicate that under the same conditions, absorption of phenylbutazone and meclofenamic acid may be lower in pony breeds than in Thoroughbreds (Snow, 1983).

In summary of pharmacokinetics, all NSAID are metabolized to inactive metabolites (except phenylbutazone) and are excreted in urine and bile. All preparations are generally administered as a course of treatment lasting 5 to 10 days. Increasing peak plasma concentration is dependent on the rate of drug metabolism, dose rate,
frequency and route of administration, breed and age of the horse and relationship to feed and feeding time. Parenteral administration of flunixin meglumine may be either intravenous or intramuscular. Phenylbutazone and meclofenamic acid must be given only intravenously since intramuscular injection causes severe inflammation.

Toxicity

The NSAID all induce gastrointestinal irritation that often leads to ulceration of mucosal lining of the gastrointestinal tract from the oral cavity to the large intestine. Toxic effects may be due to inhibition of other enzyme systems. Ferreira and Vane (1974) proposed that inhibition of prostaglandin biosynthesis may lead to unwanted side effects in organs that depend upon prostaglandins for normal physiological function. For example, PGE inhibits gastric acid secretion. A locally released prostaglandin may be a mechanism to prevent hyperacidity, which can lead to mucosal damage. Also, the function of locally released prostaglandins in the stomach may be to increase blood flow to the mucosa. Vasoconstriction upon removal of this effect by NSAID may lead to ischaemia, tissue death and bleeding. This proposed cytoprotection property of locally released prostaglandins was demonstrated by Robert et al. (1979). Pretreatment with prostaglandins in rats prevented necrosis of the gastric mucosa when orally given acids, bases and hot water. Therefore, toxic doses of phenylbutazone in horses and ponies may decrease prostaglandin production at the mucosal level and decrease cytoprotection (MacAllister, 1983).

Prolonged bleeding time and inhibition of platelet aggrega-
tion is caused by most NSAID (Tatro et al., 1980). Non-steroidal anti-inflammatory drugs also cause varying degrees of nephrotoxicity with some incidence of papillary necrosis. Some, like phenylbutazone, lead to retention of sodium chloride and water (Ferreira and Vane, 1974). In a retrospective study of 16 horses in which renal papillary necrosis was found at necropsy, it was determined that all had suffered from a variety of diseases in which dehydration and NSAID therapy were prominent features (Gunson, 1983). Papillary necrosis seen in both kidneys of all horses was not associated with clinically apparent renal disease. It was proposed that 2 synergistic etiologic factors contributed to equine renal papillary necrosis. First, blood volume depletion due to dehydration, hemorrhage or water deprivation resulted in dependence on prostaglandins for renal blood flow. Then, antiprostaglandin drugs were able to reduce blood supply to the now prostaglandin dependent vasa recta and cause ischemia of the renal papillae. It was suggested that horses being treated with NSAID must have sufficient water to balance and avoid hypovolemia.

Clinically, the early signs of toxicity are depression, anorexia, weight loss, diarrhea, dehydration and sometimes oral ulceration. The first indication of toxicity on routine blood biochemistry is a progressive decline in total plasma proteins and a rise in plasma urea concentration. With continued treatment, areas of ulceration converge and impaired epithelial integrity leads to absorption of toxins and a terminal shock condition. Marked ulceration of the tongue is often noted (Snow, 1981, 1983).

Toxicity studies of 3 NSAID were conducted by Snow et al. (1983). Phenylbutazone, meclofenamic acid and naproxen were
administered at the recommended dose rate and then at twice the normal dose. Results determined that at the therapeutic dose rate, phenylbutazone may cause a protein-losing gastroenteropathy in ponies; however, the other 2 commonly used NSAID did not exert this effect at either dose regimen.

Potential toxicity of NSAID in mare milk must be considered by the equine veterinary clinician. If a valuable but chronically arthritic brood mare is maintained at low doses of a NSAID, it must be certain that concentrations of the drug are passing into the milk. The NSAID will almost certainly be cleared from the body more slowly in nursing foals than in adult horses and this will increase potential toxicity (Higgins and Lees, 1988).

Clinical Use and Efficacy

All NSAID are proposed to be useful in numerous acute and chronic inflammatory conditions of both soft tissue and skeletal origin. It appears that different NSAID are more effective against some conditions than others. The 4 commonly used NSAID in equine medicine vary in efficacy in specific conditions.

Naproxen (Equiproxen®) is recommended for relief of pain, inflammation and lameness associated with myositis and soft tissue disease (Tobin, 1979b). Kilian et al. (1974) induced myositis "tying up" by injecting lactic acid at 8 sites into the back muscles of horses. These horses were treated with naproxen, which resulted in greatly reduced pain, lameness and tissue swelling. Jones and Hamm (1978) induced equine myositis using this model and proposed that naproxen was superior to phenylbutazone because it gave more rapid
relief of inflammation and associated lameness. Hamm (1978) reported that continuous administration of naproxen in the daily feed during training and racing season of young colts significantly reduced the incidence of musculoskeletal injuries and performance days lost to injuries such as splints, osslets, osteoperiostitis, tendinitis, tenosynovitis and suspensory desmitis.

Flunixin meglumine (Banamine®) appears to be more potent than other equine NSAID and superior for rapid relief of equine colic (Tobin, 1979b). Vernimb and Hennessey (1977) reported 38% of horses treated for colic were improved within 15 minutes. It was reported that animals were eating by this time with normalization of heart and respiratory rates and peristalsis indicated remission of colic and pain. The best results have been obtained in animals affected by flatulent or spastic colic as compared with those with intestinal stasis or circulatory impairment. Houdeshell and Hennessey (1977) demonstrated that parenteral and oral flunixin meglumine is also safe and efficacious for alleviation of pain and inflammation associated with musculoskeletal disorders in the horse. Flunixin meglumine was also reported to be efficacious in treating endotoxic shock in ponies (Turek et al., 1985).

Meclofenamic acid (Arquel®) is an unusual NSAID in that its onset of action is relatively slow, taking from 36 to 96 hours to develop (Tobin, 1979b). It has been demonstrated to be especially effective in the treatment of acute and chronic equine osteoarthritis and certain soft tissue inflammatory conditions affecting the locomo-tor system such as navicular disease, laminitis and bony tissue conditions. The drug accumulates appreciably in synovial fluid,
without altering the physicochemical properties of synovial fluid. The tissue containing the most drug is omental fat with other sites being hair, spleen and articular cartilage. In comparison, meclofenamic acid is superior to phenylbutazone in treating equine osteoarthritis (Riley et al., 1971; Conner et al., 1973).

Phenylbutazone (Butazolidin ®) remains the most popular and widely used NSAID in equine medicine. It is still the standard against which other drugs are compared for clinical effectiveness (Tobin, 1979b). This may be attributed to (1) over 30 years of clinical experience indicating its efficacy in a variety of conditions; (2) considerably lower costs for treatment, especially over extended periods of time; (3) low dose rates; (4) minimal effective blood concentration necessary; (5) establishment of phenylbutazone "Bute" as a household name (Snow, 1981). For these reasons, phenylbutazone was the drug of choice for this study.

Tobin (1979b) summarized that these 4 equine NSAID (1) are safe and effective when given at therapeutic doses; (2) they have a number of broadly similar characteristics in that dosages are approximately equivalent from drug to drug; (3) their time courses of action are broadly similar; (4) their detection in blood and urine is not particularly difficult; but (5) there are subtle differences in clinical effectiveness against certain conditions, that make specific NSAID the drugs of choice for specific conditions. A summary of NSAID properties is presented in Table 2. Pharmacological characteristics of NSAID are summarized in Table 3.
Table 2. Summary of non-steroidal anti-inflammatory drug properties

1. Acidic drugs that accumulate in acidic, inflamed tissues
2. Highly protein bound in plasma
3. Easily detected in urine and plasma; undetectable in saliva
4. Restore normal performance, not a central nervous system stimulant or depressant
5. Safe and effective at therapeutic doses
6. May be used in competitive horses
7. Inhibit enzymes which form prostaglandins and therefore reduce tissue pain and inflammation
8. Reduce fever (antipyretic)
9. Accumulate in stomach, kidney and gastrointestinal tract and tend to produce lesions in these tissues if toxic doses are given
Table 3. Pharmacological characteristics of non-steroidal anti-inflammatory drugs commonly used in equine medicine

<table>
<thead>
<tr>
<th>Drug</th>
<th>Route</th>
<th>Daily dose (mg/kg)</th>
<th>Half-life (hours)</th>
<th>Detection in Urine (hours)</th>
<th>Detection in Plasma (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylbutazone (Butazolidin®)</td>
<td>IV</td>
<td>2.2 to 4.4</td>
<td>3.5</td>
<td>96&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24-48&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>4.4 to 8.8</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flunixin meglumine (Banamine®)</td>
<td>IV,IM</td>
<td>1.1</td>
<td>1.6</td>
<td>48</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naproxen (Equiproxen®)</td>
<td>IV</td>
<td>5.0</td>
<td>4</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>10.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meclofenamic acid (Arquel®)</td>
<td>IV</td>
<td>2.0</td>
<td>6-8</td>
<td>96&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Oral</td>
<td>2.2</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Adapted from Booth (1982)
<sup>b</sup> Gabel et al. (1977)
<sup>c</sup> Maylin (1977)
Use of Non-steroidal Anti-inflammatory Drugs in Competing Horses

Various equestrian federations throughout the world have determined their own regulations governing the use of NSAID in competition horses. The NSAID do not have central nervous system stimulant or depressant actions and therefore are unlikely to affect the optimal performance of a horse. It is accepted that these agents both by their analgesic and anti-inflammatory activities are able to improve performance impaired due to tissue damage (Snow, 1981).

The NSAID are included in a group of drugs referred to as drugs of controlled medication. The goal of a controlled medication program is to smooth out the minor health and musculoskeletal problems that are common to all athletes and to insure that horses are presented at the starting gate fit to race at their best. By allowing specific therapeutic medications to be used, controlled medication programs avoid driving trainers and veterinarians to more extreme, difficult and damaging measures such as freezing, denerving, nerve blocking and intra-articular corticosteroid injections without rest. These are the drugs that many racing commissioners in the 1960s and 1970s chose to allow to be used in performance horses under controlled conditions (Tobin, 1981c).

These drugs are extremely valuable therapeutic agents in performance horses when properly used. Under the proper circumstances, NSAID have the ability to enable a horse to perform more consistently at its innate ability. Therefore, these drugs are of great economic importance in racing. Proponents for the use of NSAID suggested that if their use were restricted, the number of horses that race and compete in shows would be markedly reduced (Tobin,
Therapeutic medication consists of administering an agent to a horse which controls a condition that is interfering with the performance of that horse. The justification for this type of medication is that it allows a horse to run up to its form but not beyond its innate ability. Therefore, a horse with joint or muscle pain may be given a NSAID to control inflammation and pain. The horse trainer's and veterinarian's objective is to keep their horses in the competition. Proper medication can be a critical factor of this objective (Tobin, 1981c).

In January, 1989, the American Horse Show Association (AHSA) Board of Directors unanimously approved a major amendment to the Medications Rule, placing restrictions on the use of NSAID at AHSA recognized competitions. The amendment effective date was December 1, 1989. The purpose of the amendment is to protect the fairness of competition by preventing abuses of drugs and to safeguard the health and well-being of horses and ponies by accommodating the treatment of illness and injury (Lengel, 1989).

This amendment rules (1) when a NSAID is needed, either phenylbutazone or flunixin meglumine should be administered, but not both; (2) when phenylbutazone is administered, not more than 2 g/450 kg body weight/day should be administered, not closer than 12 hours prior to competing and not more than 5 successive days; the dose for a pony is to be reduced accordingly; (3) when flunixin meglumine is administered, not more than 500 mg/450 kg body weight/day should be administered, not closer than 12 hours prior to competing and not more than 5 successive days; the dose for a pony is to be reduced.
accordingly; (4) if both phenylbutazone and flunixin meglumine were administered to a horse or pony during the 7 days prior to competing, then one or both must have been administered no closer than 48 hours prior to competing, in order to achieve an acceptably low trace level (Lengel, 1989).

According to international racing jurisdictions, only the United States and the Rio de Janeiro Jockey Club permit use of NSAID in racing horses. Medication rules regulating use of NSAID vary among states. Each state that permits horse racing determines its own regulations. Some states do not permit any use of NSAID. In many states, there is no permitted medication for 2-year-old horses, but there is restricted use of NSAID in adult horses. Restrictions determine the accepted maximum post-race blood and urine levels of permitted NSAID and the accepted time of administration. Regulations may vary between harness racing and runners (Drug Control Service Policy Review, 1988).

The Louisiana State Racing Commission does not permit any medication for racing 2-year-olds. Of the NSAID, only phenylbutazone and oxyphenbutazone are permitted for use. Post-race urine and blood levels for both drugs are 165 μg/ml and 5 μg/ml, respectively, regardless of time of administration (Louisiana State Racing Commission, 1986).
**PHENYL BUTAZONE IN HORSES**

**Introduction**

Phenylbutazone was first synthesized in 1946 by H. Stengyl in Switzerland and was later marketed as Butazolidin® by J.R. Geigy, A.G., as a therapeutic agent to treat rheumatic diseases (Jeffcott, 1977). Phenylbutazone was widely used in human medicine until it was restricted because it caused deaths from aplastic anemia and agranulocytosis. However, an extensive veterinary market developed to treat equine musculoskeletal disorders, colic and inflammation (Tobin, 1979a). Phenylbutazone has been approved for use in racing horses in most American states. Horses are allowed to be medicated with phenylbutazone while competing in Federation Equestre Internationale shows providing a limit of 4 μg/ml of plasma (Tobin et al., 1986). Phenylbutazone is not a naturally occurring compound (Schubert, 1967) and is classified as a non-steroidal anti-inflammatory drug (Tobin, 1979a).

**Chemistry**

Chemically, phenylbutazone is a pyrazolidine with 2 nitrogen and 3 carbon atoms. It has an acidic nature with a pKa of 4.5, making it soluble in aqueous solutions of pH ≥ 6.0 (therefore also in body fluids). It forms colorless crystals which melt at 104.5 to 106.5°C. It is sparingly soluble in usual organic solvents. Phenylbutazone is rather susceptible to oxidation, the first step being the formation of a hydroxyl group at the 4 position. It has a well defined ultraviolet light spectrum and infrared absorption (Schubert, 1967).
Physically, phenylbutazone is a white or light yellow powder with a bitter taste. It is poorly soluble in water (Barragry, 1973).

**Safety**

Phenylbutazone is a safe drug if administered within therapeutic doses (Tobin et al., 1986). Finocchio et al. (1970) administered the clinically recommended dose of 2 g/450 kg phenylbutazone intravenously to 11 Thoroughbreds and determined that hemoglobin concentration, packed cell volume, serum protein fractionation, serum creatinine concentration and urine pH, osmolarity and creatine concentration were not significantly changed from normal values.

**Recommended Dose and Route of Administration**

The optimal effect can be achieved by 2 g/450 kg (average size horse) intravenously/day or 4 g/450 kg orally/day. Daily dosing is required since the anti-inflammatory effect is not good for more than about 24 hours (Tobin, 1979a). The American Association of Equine Practitioners has recommended that the dose for racing horses be not greater that 2.2 mg/kg intravenously each day, with the last dose occurring not more than 24 hours before post time (Tobin et al., 1986).

First response to the drug can be seen a few hours after administration. Optimum effect is variable but occurs approximately 12 hours after administration. Intravenous injection appears to speed the onset of action on the average by 2 to 4 hours compared with oral use. Onset of action is variable after oral administration depending on whether the horse eats before or after the drug is
given. Optimal clinical effect appears to continue for a variable time, usually for < 48 hours after the last dose, depending on the dose and length of treatment course (Gabel et al., 1977).

Researchers determined that clinical efficacy of doubling the recommended dose of phenylbutazone as a loading dose should be limited to the first 1 or 2 days of treatment only. In this treatment regimen, overall clinical response was good to excellent with no overt signs of phenylbutazone toxicity in any horse (Lees et al., 1983; Taylor et al., 1983a).

Intramuscular injection of phenylbutazone is used in some countries although it is not recommended. Intramuscular injections into the neck region of 2.5 mg/kg phenylbutazone induced heat, tenderness and swelling around the injection site which persisted up to 5 days (Sullivan and Snow, 1982).

For standardization and predictability purposes, 2 g of phenylbutazone intravenously administered daily was chosen for all 5 experiments in this study.

Half-life

The half-life of phenylbutazone is highly variable depending upon dose, dosing regimen, age of recipient and species. In the horse, despite its size, phenylbutazone has approximately the same half-life as in smaller experimental animals.

Usually, the half-life is approximately 3 hours intravenously or 6 hours orally. If the daily dose is increased and the phenylbutazone accumulates in body tissues, the half-life also increases and toxicity can result (Tobin, 1979a). An intravenous dose of 4.4 mg/kg
phenylbutazone results in a plasma half-life of 3 to 4 hours. Doubling the dose almost doubles the half-life (Snow, 1983). Concurrent intravenous administration of phenylbutazone and another related drug, isopyrin (Tomanol) also results in prolongation of both drugs due to competition for the metabolizing enzymes responsible for their degradation (Snow, 1981).

Phenylbutazone exhibits dose-dependent kinetics in the horse. The elimination half-life increases from 3.5 hours at 4.4 mg/kg body weight to 8.6 hours at 10 mg/kg. A possible reason for this could be accumulation of phenylbutazone in the body when high doses are administered. Accumulation of phenylbutazone within the body might partially explain why doses only 1.5 to 2 times the recommended maximum can produce a fatal protein-losing enteropathy in ponies within 10 to 14 days, whereas recommended dose rates seem to be well tolerated clinically by most horses for months or even years (Lees et al., 1985).

Maylin (1974) demonstrated an increase in half-life of phenylbutazone in a daily dosing regimen. He administered 4.4 mg/kg body weight phenylbutazone intravenously to horses daily for 4 days. The half-life of phenylbutazone in plasma was 5.1, 5.3, 5.5 and 6.1 hours following the first, second, third and fourth doses, respectively.

Other reports of the half-life of phenylbutazone being dose dependent include (1) 3.5 hours with 4.4 mg/kg to 6 hours with 17.8 mg/kg (this may reflect saturation of liver enzymes at high dose levels); (2) doubling the dose from 2.2 to 4.4 mg/kg phenylbutazone raised plasma levels by 33%; (3) quadrupling the dose from 2.2 to 8.8 mg/kg phenylbutazone raised the plasma concentration by 300%
Phenylbutazone has demonstrated a wide range in reported half-life among various mammalian species. Phenylbutazone is metabolized in man at a very slow rate with a plasma half-life averaging 3 days. However, in the monkey, dog, rabbit, rat, guinea pig and horse the drug is rapidly metabolized. The half-life of phenylbutazone in the goat and cattle falls in the moderate range (Burns, 1968)(Table 4). In cattle, elimination half-life changes considerably depending on route of administration. Three administration routes were compared using a cross-over design. The half-life of phenylbutazone was 36, 44 and 51 hours for intravenous, oral and intramuscular administration, respectively (Lees et al., 1988a).

In the horse, the half-life of oxyphenbutazone is approximately the same as that of the parent drug, phenylbutazone (Gandal et al., 1969; Barragry, 1973; Lees et al., 1986; Gerken and Sams, 1988). In comparison, the plasma half-life reported for phenylbutazone in the horse has ranged from 3.5 to 10.9 hours, increasing with the administered dose (Soma et al., 1983; Tobin et al., 1986). The plasma half-life of oxyphenbutazone has been reported to be 2.8 to 6.0 hours for doses from 4.4mg/kg to 10mg/kg (Gandal et al., 1969; Lees et al., 1986b; Gerken and Sams, 1988). The plasma half-life of phenylbutazone is increased in horses following administration of oxyphenbutazone. A similar finding has been reported in other species and suggests that oxyphenbutazone contributes to the overall effect of phenylbutazone and inhibits metabolism of phenylbutazone in horses (Booth, 1982; Lees et al., 1986b).

Urinary pH has little effect on the plasma half-life of
phenylbutazone, which is determined mainly by hepatic metabolism and possibly by biliary secretion (Tobin et al., 1986)

Table 4. Species differences in the elimination of phenylbutazone

<table>
<thead>
<tr>
<th>Species</th>
<th>Half-life (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man</td>
<td>$72^a$</td>
</tr>
<tr>
<td>Cattle</td>
<td>$31, 36, 39^b$</td>
</tr>
<tr>
<td>Goat</td>
<td>$15^c$</td>
</tr>
<tr>
<td>Monkey</td>
<td>$8^a$</td>
</tr>
<tr>
<td>Dog</td>
<td>$6^a$</td>
</tr>
<tr>
<td>Horse</td>
<td>$3.5^d, 6^a$</td>
</tr>
<tr>
<td>Rat</td>
<td>$6^a$</td>
</tr>
<tr>
<td>Guinea Pig</td>
<td>$5^a$</td>
</tr>
<tr>
<td>Rabbit</td>
<td>$3^a$</td>
</tr>
<tr>
<td>Pig</td>
<td>$2^e, 6^a$</td>
</tr>
</tbody>
</table>

$^a$ Burns (1968)  
$^b$ De Backer et al. (1980); Lees et al. (1988a); Martin et al. (1984)  
$^c$ Boulos et al. (1972)  
$^d$ Piperno (1968)  
$^e$ Booth (1982)
Absorption

Oral administration of phenylbutazone is often the preferred route because of ease of administration and the irritant action of the drug when injected intravenously and/or perivascularly. A portion of orally administered phenylbutazone is rapidly absorbed from the stomach via diffusion and ion trapping. Since phenylbutazone is a weak acid of high lipid solubility, its absorption would be favored by acid conditions in the stomach and early small intestine of the horse (Traub, 1983; Lees et al., 1986a; Maitho et al., 1986). Gerring et al. (1981) reported detection of phenylbutazone in the plasma of ponies 10 minutes after oral dosing. The most rapid absorption appears to occur from the small intestine, that is likely due to the greater surface area of the intestinal mucosa when compared with the gastric mucosa (Traub, 1983).

Variability has been noted when phenylbutazone is administered orally under similar conditions. Phenylbutazone absorption is subject to marked inter- and intra- animal variations. The rate of absorption may vary both from horse to horse and with the amount and type of food consumed in relation to the time of phenylbutazone administration (Gerring et al., 1981; Lees et al., 1986a).

Rose et al. (1982) investigated the bioavailability of two different oral preparations of phenylbutazone and the effect of the state of stomach contents on the absorption of phenylbutazone. They administered 8.9 mg/kg phenylbutazone in a paste or a traditional powder form either before or after a meal. Ration consisted of a full feed of lucerne chaff, wheaten chaff and bran or partial feed of a small bran mash. Both the paste and powder forms of phenylbutazone
administered before a meal appeared to be almost completely absorbed. Phenylbutazone paste administered after feeding or phenylbutazone powder given in a full feed resulted in lower peak plasma phenylbutazone concentration compared to administration on an empty stomach or relatively empty stomach (small bran mash). They concluded (1) the oral route has a disadvantage of unpredictable absorption even though it is the most convenient; (2) the paste has the advantage to ensure the horse receives the entire dose; (3) the amount of food present in the stomach at the time of phenylbutazone administration has an important effect on absorption; (4) complete absorption of phenylbutazone can be anticipated when given as a powder in a small bran mash or in paste before feeding.

*In vitro* studies have shown that phenylbutazone adsorption onto hay does occur and, therefore, may affect absorption of the drug. Large doses of phenylbutazone generally produce ulcers in the caecum and colon, rather than the stomach. Therefore, delayed absorption might be due to adsorption of phenylbutazone onto roughage in the feed, with subsequent release in the large intestine as a result of fermentative digestive processes (Maitho *et al.*, 1986; Tobin *et al.*, 1986; Lees *et al.*, 1988).

Maitho *et al.* (1986) orally administered a powdered form of phenylbutazone to ponies that had access to hay after dosing. Double peaks in the plasma concentration–time curve were frequently observed. A tentative hypothesis concerning the phenylbutazone absorption was proposed. The administered dose probably dissolved first in the gastrointestinal fluids and a proportion was then available for rapid absorption. This dose portion comprised the
first peak. Then, a variable proportion of the administered dose rapidly became adsorbed onto the hay components and subsequent release did not occur until breakdown of the fibrous, cellulose constituents of the diet that took place by fermentation in the caecum and colon. This phase comprised the second peak.

Three plasma concentration peaks were usually obtained when the experiment was repeated using a phenylbutazone paste formulation. The third peak was proposed to be a feature of the oil:water emulsion of the product. It was proposed that the administered dose became divided into a number of boluses. Dispersal and dissolution of the phenylbutazone would presumably occur at different rates. The erratic absorption from an emulsion would be a consequential concern when phenylbutazone is administered before a competitive equine sport (Lees et al., 1986a).

Sullivan and Snow (1982) studied the absorption of orally administered phenylbutazone (5 mg/kg) in 10 Thoroughbreds, 8 ponies and 4 pony foals. Large variations in area under curve (AUC) and peak plasma concentrations were found both within an animal and within groups of animals. Administration of phenylbutazone (5 mg/kg) following an overnight fast resulted in no difference in AUC (0 to 24 hours) among the 3 groups of animals: mean (± SD) values were 132 ± 68, 107 ± 48 and 98 ± 6, respectively. Feeding before phenylbutazone administration decreased AUC and peak plasma concentration and extended the range of time taken to reach the latter. Repeated, twice daily administration of phenylbutazone (5 mg/kg) resulted in more rapid absorption following the morning than afternoon dose.

Sullivan and Snow (1982) indicated factors that may contribute
to slower absorption of phenylbutazone in horses fed prior to oral administration of phenylbutazone. Slower gastric emptying time is a major factor. Consistency of the roughage (grass versus hay) affects emptying time. Partially digested feed in the stomach interferes with passage of the drug into the small intestine. When horses were fed after oral phenylbutazone administration, most of the drug may have already passed into the small intestine.

When phenylbutazone is injected intravenously, it is rapidly distributed taking approximately 30 minutes to disperse throughout the horse (Tobin, 1979a). Phenylbutazone is capable of transversing the blood-brain barrier (Tobin, 1981a).

Sullivan and Snow (1982) also reported absorption was slower with intramuscular injections of phenylbutazone (2.5 mg/kg) than after oral administration in the fasted animal. They postulated the slower absorption may be caused by the precipitation of the drug into an environment of neutral pH and the presence of only small amounts of fluid restricting redissolution. This slower absorption is in contrast to most drugs which are absorbed more rapidly following intramuscular than oral administration.

After intramuscular administration, peak plasma levels are achieved in 6 to 10 hours. This slower rate is explained by the fact that after intramuscular administration, phenylbutazone is fixed to muscle protein and consequently its absorption is delayed (Barragry, 1973).
Distribution

Tissue concentrations of phenylbutazone had not been investigated until studies conducted by Lees et al. (1987a). Tissue residue and body fluid concentrations of phenylbutazone and oxyphenbutazone were determined 6 hours after intravenous and 6, 12 and 24 hours after oral dosing with 4.4mg phenylbutazone/kg. Tissue cage fluid, peritoneal fluid and synovial fluid concentrations were generally one-third to two-thirds of corresponding concentrations in plasma. Tissue concentrations were even lower, indicating that no tissue cumulation of phenylbutazone or oxyphenbutazone occurred. Concentrations in the adipose tissue could not be determined accurately. The highest phenylbutazone and oxyphenbutazone concentrations were present in kidney tissue and the lowest were detected in muscle and tendon. Intermediate levels were in heart, liver and lung tissue.

These tissue concentration may merely reflect organ blood flow differences rather than tissue differences in binding affinity. There was no tendency for phenylbutazone and oxyphenbutazone to accumulate in high concentrations in any particular tissue. Most tissues were shown to contain undetectable residue levels (< 0.1μg/g) 24 hours after oral dosing with phenylbutazone. Only lung, liver and kidney contained higher concentrations at that time. The relatively good penetration into tissue fluids presumably reflects the ready reversibility of the high degree of plasma protein binding (Lees et al., 1987a).
Plasma Concentration with Intravenous Administration

Maylin (1974) administered 4.4 mg/kg phenylbutazone intravenously to horses daily for 4 days. Plasma concentrations were determined by gas liquid chromatography. Plasma concentrations increased from 18.2 to 31.1 μg/ml 1 hour after the first and fourth doses, respectively. Twenty-four hours after the first and fourth doses, plasma concentrations increased from 0.8 to 2.4 μg/ml, respectively.

Piperno et al. (1968) administered a single dose of 2 g/450 kg phenylbutazone intravenously to 6 horses. Mean plasma concentrations declined from approximately 20 μg/ml a half-hour after administration to about 4 μg/ml 9 hours after administration. They also gave 1 horse 2, 4 and 8 g/450 kg phenylbutazone intravenously in a series. With the 1 hour post-administration value as a reference point, a 2-fold increase in dose (2 to 4 g level) produced a 33% increase in plasma level. A 4-fold increase (2 to 8 g) in dose caused a 300% rise in plasma concentration. Repeated daily doses of 4 g/450 kg phenylbutazone intravenously were given to 5 horses for 3 days. In all horses, slight to moderate elevations in plasma levels occurred. In all experiments by Piperno et al. (1968) peak phenylbutazone plasma concentrations after intravenous administration occurred ≥1 hour post-administration.

Metabolism

The liver is the principle site of phenylbutazone metabolism. Biotransformation of phenylbutazone occurs via hepatic mixed-function oxidase activity. No glucuronide metabolites have been found in the horse. The 4-glucuronide of phenylbutazone has been reported in man
and, therefore, is a potential metabolite in the horse (Tobin et al., 1986).

The 2 major metabolites are hydroxylated derivatives, oxyphenbutazone and \( \gamma \)-hydroxyphenylbutazone. The latter is referred to as the "alcohol metabolite". The 1 minor hydroxylated metabolite is \( \gamma \)-hydroxyoxyphenbutazone. Ring hydroxylation occurs on oxyphenbutazone and side chain hydroxylation occurs on \( \gamma \)-hydroxyphenylbutazone. Side chain and ring hydroxylations occur on \( \gamma \)-hydroxyoxyphenbutazone (Traub, 1983; Tobin et al., 1986; Lees et al., 1987a)(Figure 3). Oxyphenbutazone is an active metabolite which shares many of the pharmacologic actions of phenylbutazone; however, rather high doses (6 to 12 mg/kg; Gerring et al., 1981) are required for effect. Gamma-hydroxyphenylbutazone and \( \gamma \)-hydroxyoxyphenbutazone are presumed to be inactive metabolites. These 3 metabolites are secreted into urine (Traub, 1983; Tobin et al., 1986; Lees et al., 1987).

Because \( \gamma \)-hydroxyphenylbutazone is less tightly bound than either phenylbutazone or oxyphenbutazone it is readily excreted in urine and is the principle metabolite found in urine (14%) after a single intravenous dose of 4.4 mg/kg phenylbutazone. However, if dosing was continued, the proportion of \( \gamma \)-hydroxyphenylbutazone excreted dropped to 6% of the dose. This change in proportion of different metabolites in the urine as dosing proceeds is likely related to the dose-dependent kinetics of phenylbutazone due to inhibition of drug metabolism by one of its metabolites (Maylin, 1977).
Figure 3. Chemical structure of phenylbutazone and its oxidative metabolites; oxyphenbutazone, γ-hydroxyphenylbutazone and γ-hydroxyoxyphenylbutazone

Adapted from Traub et al. (1983) and Tobin et al. (1986)
Oxyphenbutazone has been shown to inhibit the metabolism of phenylbutazone in the rat and horse (Maylin, 1977). It was proposed that a metabolite of phenylbutazone (probably oxyphenbutazone) becomes bound to and inhibits liver microsomal drug metabolism enzymes and alters both the metabolite pattern of phenylbutazone and its plasma half-life (Maylin, 1977). It is also possible that phenylbutazone itself saturates mixed function oxidase to inhibit its own metabolism (Tobin et al., 1986).

Piperno et al. (1968) determined that horses were remarkably uniform in their rate of metabolism of phenylbutazone as indicated by small standard errors (0.2 to 0.6 mg/liter) in plasma concentrations. The greatest variability existed in the first hour after dosing, when decay rate was accelerated. After this time, the decay rate was uniform and logarithmic.

Several studies confirmed that plasma disposition follows a bi-exponential decline (Lees et al., 1983; Lees and Taylor, 1985; Lees et al., 1986; Maitho et al., 1986). By using a sensitive chromatographic method and a frequent blood sampling schedule up to 72 hours, Lees et al. (1987a) investigated but did not observe a third, slower phase of decreasing plasma concentration. Therefore, it seems improbable that phenylbutazone will achieve very high concentrations in tissues since this would be likely to provide a further phase of slower plasma elimination (Lees et al., 1987).

Sex differences did not appear to affect metabolism rate (Piperno et al., 1968), but breed differences appear to affect phenylbutazone metabolism rate. Chay et al. (1984) determined population distributions of phenylbutazone and oxyphenbutazone after oral and
intravenous dosing in Thoroughbred, Standardbred and half-bred horses at pasture. The plasma concentrations of phenylbutazone in half-bred horses were only one-third of those observed in the Thoroughbred and Standardbred horses. Chay et al. (1984) proposed a possible interpretation is that phenylbutazone is metabolized more rapidly in half-bred horses; because phenylbutazone is more toxic in ponies than in Thoroughbred horses (Lees and Mitchell, 1979; Snow et al., 1979, 1980, 1981, 1983; Gerring, et al., 1981; Rose et al., 1982; MacAllister, 1983; Snow and Douglas, 1983), this data may suggest that it is a metabolite of phenylbutazone rather than phenylbutazone itself which is the toxic species.

Feeding times relative to dosing time appear to affect phenylbutazone metabolism. Recovery of phenylbutazone and its metabolites from urine was significantly reduced in the first 24 hours after dosing when horses had free access to hay. This was probably a result of markedly delayed absorption because this did not occur in animals deprived of food for a few hours before and after dosing (Lees et al., 1987a).

Finocchio et al. (1970) suggested various individual factors may affect metabolism of phenylbutazone in the horse. For example, liver microsomal enzyme systems have been shown to be subject to nutritional inadequacy, hepatic glycogen content, stage of pregnancy, stage of enzyme development, circulating hormone concentrations, liver disease, presence of exogenous chemicals and simultaneously administered drugs. Any one or combination of factors may be present in a particular horse such as variations in training, diet and prior or simultaneous drug treatment.
Excretion

Although the pharmacological action of phenylbutazone is over within 24 hours, the time for the horse to eliminate phenylbutazone is much longer (Tobin, 1981b). Phenylbutazone is excreted in the urine in an unchanged form and/or as its 2 major metabolites, oxyphenbutazone and γ-hydroxyphenylbutazone (Maylin, 1974). Renal clearance of phenylbutazone is influenced by the drug being 98% plasma bound and its pKa of 4.5. The acidic nature of phenylbutazone will tend to reduce its reabsorption from the renal tubules, if the tubular luminal fluid is alkaline (Tobin et al., 1986). Only the unbound drug is available for glomerular filtration (Moss, 1973) and the pH of the urine affects excretion rate (Piperno, 1968). Clearance is also affected by the age of the horse (Lees, 1985) and route of administration (Moss, 1972).

Moss (1972) reported that route of administration is an important factor in determining the phenylbutazone excretion pattern. Generally after oral or intravenous administration, plasma levels return to "zero" after about 24 hours, but phenylbutazone persists in the urine for at least 36 hours and its metabolites for more than 48 hours. After intramuscular administration, phenylbutazone appears to persist in urine a much longer and variable period of several days.

Moss (1972) reported that Thoroughbreds during training and certainly after a race produce an acidic urine, but produce a more alkaline urine at rest and light exercise. Moss and Haywood (1973) investigated the significance of urinary pH on phenylbutazone drug clearance time. They administered an oral dose of 5 mg/kg phenylbutazone daily to a pony for 3 days under both alkaline (normal) and
under a condition that induced acidic urine. In alkaline urine, phenylbutazone was detected by a routine screening procedure up to 11 hours after administration and oxyphenbutazone up to 48 hours after dosing. In the acidic urine experiment using the same pony and dose, phenylbutazone was detected up to 48 hours and oxyphenbutazone up to 57 hours. A more exhaustive solvent extraction of the urine detected oxyphenbutazone for 54 and 88 hours in alkaline and acidic urine, respectively. They repeated the experiment dosing 3 ponies with 5 mg/kg phenylbutazone containing 10 microcuries of $^{14}$C. In 1 pony, radioactive material was detected 6 days later in acidic urine. Moss and Haywood (1973) concluded aciduria in the horse prolongs clearance time of phenylbutazone and/or its metabolites.

Houston et al. (1983) determined the effect of urine pH on detection of oxyphenbutazone in racing horses. Urine pH in racing horses may range from pH 4.5 to 10. Distribution tends to be bimodal, with peaks at approximately pH 5 and pH 8. They measured blood and urinary levels of oxyphenbutazone in post-race samples from Thoroughbred horses racing in Kentucky. Data showed that oxyphenbutazone levels in equine blood are not significantly influenced by urine pH, but urine concentrations of oxyphenbutazone are highly dependent on urine pH and may vary up to 500-fold, depending largely on whether the urine is acidic or basic. If the pH of urine was < pH 6, blood and urinary concentrations of oxyphenbutazone were comparable: if the sample pH was > 8, the urinary concentrations of oxyphenbutazone approached 100 µg/ml, while the plasma levels of oxyphenbutazone remained low. Houston et al. (1983) suggested their results represented a classic example of "ion-trapping" phenomenon;
as an acidic drug, the concentrations of oxyphenbutazone are high in basic urines and low in acidic urines.

Houston et al. (1985) conducted a similar experiment to determine the effect of urine pH on detection of phenylbutazone and its metabolites in plasma and urine of Thoroughbred race horses. Analysis of the pH of these post-race urine samples again showed a bimodal frequency distribution. The pH values ranged from 4.9 to 8.7, with peaks at about pH 5.25 and 7.25. This bimodal pattern of urinary pH values was consistent with observations of parallel studies of race horses in England and Japan.

Urinary pH influenced the concentrations of phenylbutazone and its metabolites oxyphenbutazone and γ-hydroxyphenylbutazone found in the urine samples. The concentration of these metabolites found in alkaline urines were 32 to 225 times greater than those found in acidic urines. Again, research by Houston et al. (1985) demonstrated that as acidic drugs, these agents will be largely in their ionized forms at alkaline urinary pH values, and will, therefore, tend to trap in alkaline urine. Plasma concentrations of phenylbutazone and its metabolites, however, were not affected by urinary pH (Houston et al., 1985).

Lees et al. (1985) determined that age affects elimination rate of phenylbutazone in horses. A clinical dose of 4.4 mg/kg phenylbutazone was administered intravenously or orally to 6 ponies. In 3 ponies, 3-years-of age, drug clearance after intravenous administration was almost twice as rapid as in 3 ponies 8 to 10 years-of-age. After oral administration, plasma concentrations were greater in the older ponies, the area under the plasma concentration time curve
being almost twice as high as that of the younger ponies. These researchers stated that this did not result from more efficient absorption but from slower plasma clearance (as demonstrated in the intravenous study). They added that in very young animals (< 8 weeks of age) the plasma clearance of most drugs is slow (as in the older ponies) but is due to immature mechanisms of metabolism in the liver and excretion in the kidney.

Lees et al. (1985) results indicated that only 25% of the administered dose was accounted for by excretion in the urine over 24 hours, the greatest fraction being excreted as oxyphenbutazone and a slightly smaller fraction as γ-hydroxyphenylbutazone. Only 2% of the dose was present in urine as unchanged drug. The remaining 75% remained unaccounted. They speculated that other fates of phenylbutazone may be (1) formation of other unidentified metabolites; (2) excretion via non-renal pathways such as bile; (3) storage of phenylbutazone in tissues with a subsequent slow release which suggests a further elimination phase will occur. Unpublished data of J.B. Taylor and P. Lees (see Tobin et al., 1986) indicated a third phenylbutazone metabolite in horse urine; however, the quantities present were not large and would add only a few percentage points to the 24 hour urinary excretion level (Lees et al., 1985). This third metabolite has been named γ-hydroxyoxyphenylbutazone (Tobin et al., 1986).

Lees et al. (1987a) determined a lack of accumulation of phenylbutazone and oxyphenbutazone in tissues and body fluids and a limited recovery in urine. The fate of at least 50% of the administered intravenous dose of phenylbutazone remained undetermined. Lees et al. (1987a) postulated that up to half of the administered oral and
intravenous doses were probably passed into the gastrointestinal tract as phenylbutazone or metabolites with eventual excretion in feces. This could arise by active secretion in fluids such as bile or by simple passive diffusion across the blood-gastrointestinal tract barrier (Lees et al., 1987a).

Lees et al. (1985) considered that possibly the slower phenylbutazone clearance in 8 to 10-year-old ponies could be related to the 35% greater body weight of the older ponies as compared to the 3-year-old ponies. However, these researchers suggested that this was improbable since data from their laboratory showed that mean AUC values were higher in ponies than in Thoroughbreds weighing approximately twice as much.

Gerring et al. (1981) determined via high performance liquid chromatography the urinary excretion pattern of phenylbutazone and its metabolites after orally dosing horses: doses ranged from 1.1 to 13.2 mg/kg. Analysis of urine specimens demonstrated concentrations of phenylbutazone were similar to or more than those in plasma. However, the concentrations of both metabolites were very much higher in urine than plasma. Gerring et al. (1981) suggested the slow excretion of phenylbutazone may result from tubular reabsorption of the drug, but it is also attributed largely, possibly entirely, to the high degree of plasma protein binding, that limits the amount of phenylbutazone filtered at the glomerulus to a small fraction of the total plasma concentration. Gerring et al. (1981) stated their results made it clear that tubular secretion of phenylbutazone does not occur in the horse (although it has been reported in laboratory animals); however, the hydroxylated phenylbutazone metabolites must
be secreted as suggested by their urine:plasma concentration ratios.

Maylin (1974) administered 4.4 mg/kg phenylbutazone intravenously daily for 4 days. He determined the excretion of phenylbutazone remained relatively constant following 4 consecutive doses. Total percent recovery of phenylbutazone for 24 hours post-administration was 0.9%, 1.7%, 2.1% and 1.6% of the first, second, third and fourth doses, respectively. Concentration of oxyphenbutazone decreased after each successive dose from 14.2%, 10.1%, 8.2% and to 6.4% of the first, second, third and fourth doses, respectively.

Concentration of oxyphenbutazone increased after the second and third doses but decreased after the fourth dose. It accounted for 9.6%, 14.0%, 14.0% and 10.5% of the first, second, third and fourth doses, respectively. The concentration of γ-hydroxyphenylbutazone was greater than oxyphenbutazone for approximately 12 hours after each dose whereas the oxyphenbutazone concentration exceeded that of γ-hydroxyphenbutazone beyond 18 hours. Total urinary excretion of phenylbutazone, oxyphenbutazone and γ-hydroxyphenbutazone accounted for 24.7%, 25.8%, 24.3% and 18.6% of the dose after the first, second, third and fourth doses, respectively. An additional 6.3% of the dose was recovered 24 to 48 hours post-administration.

Toxicity

When phenylbutazone is used in the horse in recommended doses for the recommended period of time, it is a very safe drug. The number of doses administered to horses over approximately 30 years must be infinite but the incidence of reported toxicity is small (Tobin, 1981b). Horses have been kept on the usual maintenance dose
of 2 g/450 kg/day for up to 2 and 3 years with no detectable toxic effects (Gabel, 1977).

Toxicity has been reported most frequently in pony breeds (Lees and Mitchell, 1979; Snow et al., 1979, 1980, 1981, 1983; Gerring et al., 1981; Rose et al., 1982; MacAllister, 1983; Snow and Douglas, 1983). The reason why phenylbutazone produces toxicity at relatively low dose rates in ponies is unknown (Snow and Douglas, 1983). Because ponies were reported to be a phenylbutazone toxicity risk, horse mares were used in all 5 experiments in this study.

The paste preparation is considerably more toxic than the powder preparation. The reason for this is unknown (Snow, 1982). It has been suggested that the bioavailability was greater for the paste and, therefore, would more readily produce toxicity (Snow and Douglas, 1983; Tobin et al., 1986). A lower dose schedule is recommended if the paste preparation is used. Higher dose rates should not be instituted if there is a lack of clinical response (Snow, 1982; Lees and Higgins, 1987).

The principle dangers with phenylbutazone are associated with improper injection. If phenylbutazone is accidentally injected perivascularly it may cause severe inflammation, abscessation and eventual sloughing of the jugular vein (Tobin, 1981b). Rapid intravenous injection of undiluted phenylbutazone (5% solution) will cause phlebitis with fibrosis and sometimes occlusion of the jugular vein. Phenylbutazone should be injected slowly and diluted with saline solution. Since blood levels reached following oral administration are similar to those after intravenous injection, intravenous use of the drug can be justified only in cases when it is important
to attain a more rapid onset of action than via oral administration such as severe trauma, severe pain and selected colic cases (Gabel, 1977) or when standardization and predictability are necessary for drug studies. If phenylbutazone is accidentally injected directly into the carotid artery, the horse immediately becomes excited, prostrated and may die (Tobin, 1981b).

An extensive review of the literature shows there have been few reports of phenylbutazone toxicity in horses. The published reports of phenylbutazone toxicity occurred when researchers elected to give higher than usual doses of phenylbutazone to study its effects (Tobin, 1981b). These reports all conclude that toxic doses of phenylbutazone cause lesions in the entire gastrointestinal tract, liver and kidney. All case reports list the same clinical signs of toxicity: central nervous system depression, anorexia, diarrhea, listlessness and oral ulcerations. The 2 following studies (one involving ponies and one involving horses) are examples of all toxic cases reviewed.

Snow et al. (1980) studied the toxic effects of phenylbutazone by oral administration of 12 mg/kg/day for 8 days to ponies. Blood samples were taken at frequent intervals and necropsies were performed on all ponies. The only consistent clinical biochemical finding was an approximate 25% decrease in total plasma protein. Plasma protein concentration seems to be the best biochemical indicator of phenylbutazone toxicity in the horse (Lees and Higgins, 1986).

Hypoproteinemia was investigated by injecting $^{51}$CrCl$_3$-labeled plasma proteins and calculating the loss. Labeled chromic oxide was given orally and was concentrated in the feces. The results clearly
showed that the phenylbutazone-treated ponies had very high fecal radioactivity compared with the untreated controls indicating a massive loss of plasma protein into the gastrointestinal tract. Necropsies revealed ulceration of the large colon and caecum in varying degrees from few to massive ulcerations. All ponies had tongue ulcerations. Radioactive determinations of gut contents indicated that the site of plasma protein loss was variable throughout the gastrointestinal tract. The plasma protein loss was associated with the gastrointestinal ulceration (Snow et al., 1980).

Gabriel and Martin (1962) chronically administered phenylbutazone to Thoroughbred and Standardbred horses. Necropsies showed ulcerative lesions in the intestines of horses given 8 and 16 g phenylbutazone/day. Ulcerations were also found in the oral mucosa and stomach. Extensive necrotic phlebitis was found in the portal veins and liver degeneration was evident. Autolysis of all tissues submitted at necropsy in a horse given 16 g phenylbutazone/day was so extensive that no definite conclusion could be reached as to the extent of the involvement.

In summary, necropsy reports were extensive, but there was no indication that the phenylbutazone caused any degeneration of the reproductive tract of males or females. It may be assumed that phenylbutazone does not normally accumulate in the reproductive tract.
Phenylbutazone and Inflammation

The acidity of phenylbutazone enables it to accumulate in acidic inflamed tissues (Tobin, 1979a). The primary role of phenylbutazone in treating inflammation in horses is to inhibit a massive prostaglandin synthesis thus reducing the erythema, edema, hyperalgesia and fever of inflammation. Accumulation of phenylbutazone in inflamed tissue is slow. The anti-inflammatory action of phenylbutazone develops slowly because first the preformed tissue prostaglandin concentration must be significantly reduced before phenylbutazone can start blocking synthesis of new prostaglandins (Brune, 1976). After the 30 minutes distribution of an intravenous injection of phenylbutazone in the horse, it then takes ≈3 to 4 hours for the preformed prostaglandin concentration to decline by its own biodegradation followed by the phenylbutazone blockage of newly synthesized prostaglandins. Loss of the signs of inflammation may take up to 12 hours or more. When the plasma level of phenylbutazone declines, the concentration of prostaglandins in inflamed tissues builds back up again and signs of inflammation reappear. Then, it is time for another daily therapeutic dose of phenylbutazone (Tobin, 1979a).

Accumulation of phenylbutazone into cells of inflamed tissues is highly influenced by the variation of pH in both the extracellular and intracellular spaces. Table 5 shows that the normally alkaline pH of the extracellular space (7.4) becomes acidic (6.8) in inflamed tissues and the normally neutral intracellular pH (7.0) becomes slightly alkaline pH (7.2) in cells of inflamed tissue. Therefore, there is a considerable shift of acidic phenylbutazone into a more favorable alkaline pH in cell membranes and intracellular spaces.
Two experimental equine models of acute non-immune inflammation have been developed to enable studies of the biochemical composition and cellular content of exudates. Both are based on the creation of a mild, reproducible and reversible inflammatory reaction that is free from uncontrolled incidental factors and that causes minimal distress to the ponies. The polyester sponge model involves the insertion of small polyester sponge strips soaked in sterile carrageenan solution into subcutaneous neck pouches and their serial removal. The tissue-cage model is based on the initial insertion of a spherical tissue-cage subcutaneously in the neck and the subsequent stimulation with carrageenan of the granulation tissue that lines and permeates the cage. Relationships between prostaglandin formation at the site of inflammation and leucocyte accumulation, enzyme release, total protein content of exudates and the temperature of the lesions have been investigated (Higgins et al., 1987a).

Development of the equine model of acute inflammation facilitated studies of the action of NSAID in horses (Higgins and Lees, 1983; Lees et al., 1986b; Higgins et al., 1987b; Lees et al., 1987b). Higgins and Lees (1983) determined a highly significant (P < 0.001) reduction in prostaglandin E\textsubscript{2} levels in inflammatory exudates from phenylbutazone-treated ponies compared to untreated ponies. Lees and Higgins (1986) determined that phenylbutazone paste significantly reduced skin temperature over the site of the inflammatory lesion for 24 hours and inhibited prostaglandin synthesis for at least 12 hours. There were no significant differences in exudate protein concentration and leukocyte numbers between phenylbutazone-treated and control ponies.
ponies. Phenylbutazone was cleared more slowly from exudate than from plasma. Lees et al. (1986b) utilized this model to determine that phenylbutazone and oxyphenbutazone readily passed into the inflammatory exudate and then were slowly cleared from the exudate.

Table 5. Influence of variation of pH on the relative concentrations of phenylbutazone (PBZ) in neighboring cellular compartments

<table>
<thead>
<tr>
<th>Situation</th>
<th>Extracellular space</th>
<th>Cell membrane</th>
<th>Intracellular space</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>PBZ</td>
<td>PBZ</td>
</tr>
<tr>
<td>Normal</td>
<td>7.4</td>
<td>1.0</td>
<td>5.8</td>
</tr>
<tr>
<td>Inflamed</td>
<td>6.8</td>
<td>1.0</td>
<td>15.8</td>
</tr>
</tbody>
</table>

The values were defined by equilibrium distribution of phenylbutazone between citrate buffers (ionic strength 0.15 M) of different pH values and octanol. The phases were always saturated against each other.

(Adapted from Brune, 1976)
Inflammation Defined

Inflammation has been defined as "the reaction of living tissue to injury, which comprises a series of changes of the terminal vascular bed, of the blood and of the connective tissue that tends to eliminate the injurious agent and to repair the damaged tissue." The reaction which follows interaction between antigen and antibody in an animal which has been in contact with that particular antigen is referred to as "allergic inflammation" (Chand and Eyre, 1977). Inflammation is a fundamental defense mechanism of the body against physical, chemical or infectious injury. Despite its protective nature, the resulting tissue changes may be excessive and damaging (Jones, 1977).

The chemical mediators of inflammation include (1) biogenic amines (histamine, serotonin and catecholamines); (2) vasoactive polypeptides (kinins, cationic proteins, anaphylatoxins, connective tissue activating peptide); (3) lysosomal enzymes; (4) vasoactive lipids (prostaglandins, endoperoxides, thromboxanes, rabbit aorta-contracting substance, slow-reacting substance of anaphylaxis); (5) phospholipid (platelet activating factor); (6) chemotactic substances (eosinophil and neutrophil chemotactic factors of anaphylaxis). The type of principle chemical mediator(s) involved in a particular inflammatory condition depends upon the underlying cause of inflammation and the species (Chand and Eyre, 1977).

Many of these chemical mediators produce, directly or indirectly, inflammation characterized by redness, heat, swelling, pain and
loss of function. Pharmacological agents which inhibit the excessive
defense reactions (e.g. fever, pain and inflammation) have been
called "anti-defense" drugs. The NSAID inhibit inflammation and
fever and are therefore known as "anti-defensive" agents (Chand and
Eyre, 1977).

**Inflammation Process**

The process of inflammation can be initiated in any vascular-
ized part of the body by an injury or insult to living tissue. Then,
the synthesis of eicosanoids is stimulated (Higgins, 1985).

Eicosanoids are derived from naturally occurring eicosapolyen-oic acids, of which arachidonic acid is the most common in domestic
animals. Arachidonic acid is either obtained from the diet or is
synthesized from linoleic acid. It is widely distributed in the body
and is usually stored, covalently bound in its esterified form, in
the phospholipid fraction of cell membranes of most body cells
(Higgins, 1985).

Following an inflammatory stimulus, an acyl hydrolase and
phospholipase A\textsubscript{2} (and possibly others) act to release the arachidonic
acid from its phospholipid pool. This free arachidonate is either
immediately re-esterified or metabolized by 1 of 2 enzyme pathways
(Figures 4 and 5). The cyclooxygenase enzyme system (Figure 4) will
convert the arachidonate to a cyclic endoperoxide PGG\textsubscript{2}, which is
acted upon by another enzyme to yield PGH\textsubscript{2} (a cyclic hydroxyendoper-
oxide) which subsequently gives rise to the primary prostaglandins
(PGD\textsubscript{2}, PGE\textsubscript{2} and PGF\textsubscript{2}\alpha) as well as PGI\textsubscript{2} (prostacyclin) and the non-
prostanoate thromboxanes A\textsubscript{2} (TXA\textsubscript{2}) and TXB\textsubscript{2}. Members of this group
Figure 4. The cyclooxygenase enzyme pathway of arachidonic acid metabolism  

Higgins (1985)
Figure 5. Lipoxygenase enzymes act to convert arachidonic acid to hydroperoxy and hydroxy acids and leukotrienes

Higgins (1985)
of eicosanoids have been implicated in every phase of inflammation (Higgins, 1985).

Prostaglandins induce the signs of inflammation and sensitize tissue including hypothalamus to pain provoking and fever inducing effects of other substances. Sensitivity to the kinins is especially enhanced. They cause exudation of fluid into the inflamed area and intense leukocyte migration due to chemotactic factors (Jones, 1978).

Prostaglandins in Basal Conditions

Prostaglandins are crucial to many body functions and influence the production and release of each other. They modify blood pressure and cardiovascular control. In the lung, prostaglandins influence both circulation and ventilation and may well match the delivery of air and gas to provide gaseous exchange. In the newborn, prostaglandins are instrumental in termination of umbilical blood flow and in diversion of venous blood to the lung. They are involved in many facets of reproduction and regulation of growth hormone. They increase intestinal fluids and gut mobility, often resulting in diarrhea and cramps (Jones, 1978).

Prostaglandins are usually present in the central nervous system and have been implicated in the function of both the central and autonomic systems, especially in mediation or modulation of chemical and other stimuli. Prostaglandins depress behavior and enhance barbiturate action, mediate response to pyrogens, modify food intake, influence cerebral blood flow and modify neurotransmission (Jones, 1977).

None of the synthesized prostaglandins persist long at their
site of formation. They rapidly diffuse to other sites or are converted to various derivatives. Most prostaglandins do not survive one passage through the lungs. Therefore, they do not usually reach the systemic circulation for redistribution (Jones, 1978).

Prostanoids (prostaglandins, prostacyclin and thromboxanes) are not stored in tissues and are very unstable (Higgins, 1985). In aqueous solution at pH 7.5 and 37°C, half-life of the cyclic endoperoxides PGG$_2$ and PGH$_2$ was found to be 4 and 3.5 minutes respectively, before they are converted to primary prostaglandins. The half-life of PGI$_2$ was 4 minutes and that of TXA$_2$ was 32 seconds. These short half-life values require that PGI$_2$ and TXA$_2$ are measured as their degradation products 6-keto-PGF$_{1\alpha}$ and TXB$_2$, respectively. These products have been found to be biologically less active but relatively stable. In vivo, prostaglandins have been shown to be rapidly metabolized to urinary metabolites. More than 95% of infused PGE$_2$ was inactivated after one circulation through the lungs (Higgins, 1985). Only 3% of tritiated PGE$_2$ remained unchanged in the plasma 90 seconds after intravenous administration (Higgins, 1985).
Prostaglandins and Erythema

Prostaglandins $E_1$, $E_2$ and $F_1$ (at higher concentrations) have a direct vasodilating ability that may be responsible for characteristic erythema in inflamed tissue (Short and Beadle, 1978). Erythema results from a local blood pooling due to relaxation of smooth muscles in walls of arterioles and venules (Ferreira and Vane, 1974). This is a long-lasting effect in cutaneous vessels and superficial veins, but lasts only a few minutes in other vascular beds. Prostaglandins may also cause vasodilation by inhibiting the release of adrenergic mediator (Short and Beadle, 1978). They have the ability to counteract the vasoconstriction caused by substances such as norepinephrine and angiotensin (Ferreira and Vane, 1974).

Prostaglandins and Edema

Prostaglandins $E_1$, $E_2$ and $A_2$ can directly cause some degree of localized edema, that is likely caused by increased vascular permeability resulting from contraction of venular endothelial cells. This effect is additive in the presence of other mediators such as histamine and bradykinin. Therefore, prostaglandins have an indirect sensitizing action on the wheal-forming potential of other agents (Short and Beadle, 1978). Experiments have clearly demonstrated that prostaglandin $E$ can sensitize blood vessels (at postcapillary and collecting venules) to the permeability-increasing effects of other edema mediators locally released (Ferreira and Vane, 1974).
Prostaglandins and Pain

Prostaglandins directly induce headache and long-lasting pain when injected in man. They can induce hyperalgesia. Low concentrations of prostaglandins as released in inflammation, alone usually do not invoke pain. However, the addition of histamine or bradykinin causes intense pain. Furthermore, neither histamine nor bradykinin injected alone has been shown to induce pain (Short and Beadle, 1978). It appears prostaglandins must act synergically with histamine and/or bradykinin to cause sensitivity to pain receptors.

Prostaglandins $E_1$ causes long-lasting pain and is 10 times more potent than $PGE_2$. Injections of $PGF_2\alpha$ cause an initial brief pain effect followed by a delayed gradual increase over 4 hours. Subdermal $PGE_1$ infusions mimicking continuous release of mediators at an injury site showed that hyperalgesic effects of prostaglandins were cumulative, since they depended on concentration plus duration of infusions (Ferreira and Vane, 1974).

Prostaglandin $E_1$ must act with histamine to cause pruritus. When infused with bradykinin, there was pain induction rather than itching (Ferreira and Vane, 1974).

Fatty acid hydroperoxides can also induce pain in man. Intensity of pain produced by injections of hydroperoxides of arachidonic, linoleic and linolenic acids was greater than that induced by either the parent fatty acids or $PGE_1$, acetylcholine, bradykinin or histamine. Thus, lipoperoxides formed during prostaglandin biosynthesis may also be important pain-producing substances (Ferreira and Vane, 1974).
PROSTAGLANDINS

1. SENSITIZE PAIN RECEPTORS TO PAIN AGONISTS

**NORMAL**

- **PAIN RECEPTOR**
- **PAIN AGONIST**
- **PROSTAGLANDIN**

**INFLAMED**

- **PAIN RECEPTOR - AGONIST PROSTAGLANDIN COMPLEX**

2. INCREASED PROSTAGLANDIN LEVELS (INJECTION OR WITH INFLAMMATION) CAUSES HYPERALGESIA.

3. BLOCK PROSTAGLANDIN SYNTHESIS: TISSUE RETURNS TO NORMAL (SLOWLY - PROSTAGLANDINS FORMED MUST BREAK DOWN)

Figure 6. Schematic representation of the pain-sensitizing action of prostaglandins. Pain is mediated by certain chemicals which interact with pain receptors. The prostaglandins appear to act by increasing the sensitivity of pain receptors to pain agonists, here represented as a locking of the pain agonists onto the pain receptors. In an inflamed tissue the increased levels of prostaglandin cause the hypersensitivity characteristic of inflamed tissues. Once prostaglandin formation is blocked, the high tissue levels of prostaglandin have to decay over a number of hours before the tissue returns to normal. This is the reason many non-steroidal anti-inflammatory drugs often take several hours to act.

Tobin (1981b)
Prostaglandins and Fever

Prostaglandin $E_1$, $PGF_2\alpha$ and $PGA_1$ are potent pyretic agents. Prostaglandin $E_1$ is the most potent pyrogen known when injected into the cerebral ventricle or the anterior hypothalamus (Short and Beadle, 1978). The hyperthermic effect is dose-dependent, almost immediate and lasts for about 3 hours. Prostaglandin $E_1$ or $E_2$ causes fever acting on the same region on which monoamines and pyrogens act to affect temperature. Fever occurs during induction of human abortion with $PGF_2\alpha$, but it is rather ineffective in cats and rabbits. The pyrogenic action of $PGE_2$ is greater than that of $PGF_2\alpha$ in animals. Generation of a $PGE_2$-like substance in the central nervous system has been measured during fever. Concentrations in the cerebral spinal fluid rise after intravenous pyrogen by 2.5 to 4-fold, sometimes to as much as 35 ng/ml (Ferreira and Vane, 1974). Fever and pain are the first signs of inflammation to be relieved by antiprostaglandin therapy. Edema and erythema are alleviated more slowly (Jones, 1977).

Prostaglandins and Leukotaxis

Prostaglandin $E_1$ has been observed to cause an initial migration of polymorphonuclear (PMN) leukocytes followed by an invasion of mononuclear cells into an area of inflammation. Inhibitors of prostaglandin synthesis have been found to greatly reduce the emigration of monocytes from blood vessels in the inflammatory site in an acute inflammatory reaction (Short and Beadle, 1978). Significant inhibition of polymorphonuclear and mononuclear leukocyte locomotion was produced by indomethacin,
flunixin, phenylbutazone and oxyphenbutazone (Dawson et al., 1987; Sedgwick et al., 1987). Leukotaxis is also demonstrated by other arachidonic acid derivatives, the thromboxanes and hydroxyeicosatetraenoic acid (Short and Beadle, 1978).

Leucocytes enhance eicosanoid-mediated aspects of the inflammatory reaction and its maintenance. During phagocytosis, arachidonic acid metabolites are released. These products mediate inflammatory responses and may be chemotactic for more leucocytes. This cycle of phagocytosis and leucotaxis may continue for as long as debris or the inflammatory stimulus is present (Higgins, 1985).

Prostaglandins and Anti-inflammatory Actions

Prostaglandin F$_2\alpha$ is a potent vasoconstrictor in many vascular beds. It is possible that in some tissues the PGE:PGF ratio determines the degree of inflammation. Some antiprostaglandin drugs alter this ratio in favor of the F series. Prostaglandin E$_1$ and PGE$_2$ have proven to be effective at low concentrations as inhibitors of histamine release in basophils. Lysosomal enzyme release by neutrophils has also been shown to be inhibited at higher levels of PGE$_1$. Prostaglandin E$_1$ and E$_2$ will also inhibit lymphokine secretion by lymphocytes. The development and regression of inflammation probably depend partly upon relative concentrations of pro- and anti-inflammatory prostaglandin activity and possibly on feedback inhibitory mechanisms (Short and Beadle, 1978).
Introduction

Prostaglandin F₂α, PGE₂ and PGI₂ have been documented having an important role in directing estrous cycles, ovulation, fertilization and uterine environment of females. Embryos depend on prostaglandins for hatching, expansion, development and implantation. Researchers have demonstrated these aspects of prostaglandins in female reproduction by administering prostaglandin antagonists and recording various antifertility effects (see Table 6).

Ovulation

Espey (1980) described ovulation as an inflammatory reaction. It was hypothesized that an ovulatory surge of gonadotropin induced an inflammatory condition in mature follicles that brought about the rupture of the ovarian surface. This theory gives prostaglandins the central role as mediators of the inflammatory process. The principle modifications in the ovarian vascular system include an increase in local blood flow (vasodialation) and edema, both effects documented to be caused by PGE. Within minutes of an ovulatory surge of luteinizing hormone there is a significant increase in ovarian circulation associated with the hyperemic condition developed in follicles.

These vascular changes cause the follicle to become edematous to the time of rupture. At the time of ovulation, thecal fibroblasts migrate into the stratum granulosum laying down collagenous support for the mass of developing lutein tissue. Prostaglandins promote fibroblast proliferation. In addition, fibroblasts themselves are a
common source of prostaglandins.

It is well known the ovulatory surge of luteinizing hormone activates the adenylate cyclase system and stimulates cyclic adenosine monophosphate (cAMP) synthesis in follicle cells. Subsequently, cAMP stimulates prostaglandin synthesis by stimulating an acyl hydrolase, which liberates arachidonic acid. Mature follicles begin a cAMP mediated increased secretion of steroids, specifically estrogens that may contribute to the ovulatory process by increasing prostaglandin synthesis (particularly PGF$_2$α) (Espey, 1980).

Prostaglandin F reaches a maximum level in the follicle by ovulation time and then rapidly declines, but PGE continues to be produced for several hours after ovulation (Espey, 1980). Granulosa cells produce significant amounts of prostaglandins, but so do thecal cells that generate PGF. It has been suggested that PGF$_2$α causes rupture of the follicle by increasing ovarian contractility and then vasoconstriction, thus diminishing blood flow to the ovary during the luteal phase of the cycle (Espey, 1980).

There is evidence that mature ovarian follicles contain proteolytic enzymes that have been implicated in inflammatory processes. Fibrinolytic activity increases in the follicle during the hours preceding ovulation. This is associated with production of plasminogen activator in the follicle during ovulation. Cyclic AMP and PGE$_1$ and E$_2$ are also capable of stimulating follicular cells to produce plasminogen activator, which leads to plasmin formation. Plasmin stimulates production of collagenase that is necessary for collagen degradation resulting in the follicle wall becoming flaccid and distensible. The decomposition of follicular connective tissue
accelerates rapidly during the final hour preceding ovulation (Espey, 1980). Prostaglandin F and E appear in the follicular fluid the last few hours before ovulation (Stabenfeldt and Edqvist, 1984). Prostaglandins may contribute to the formation of the collagen decomposition cascade since they are known to stimulate collagenase activity in fibroblasts and related cells (Espey, 1980).

This hypothesis encourages the evaluation of anti-inflammatory agents as potential antifertility agents (Espey, 1980). The Espey hypothesis describing roles of prostaglandins in the mechanism of mammalian ovulation appears to be unchallenged at this time (Lipner, 1988).

**Luteolysis**

Prostaglandin $F_{2\alpha}$ is considered to be the substance that initiates regression of the corpus luteum. Numerous studies have demonstrated that increased $PGF_{2\alpha}$ levels in blood due to either exogenous administration or physiological synthesis and release was concomitant with corpus luteum regression. Prostaglandin $F_{2\alpha}$ is released episodically beginning approximately 14 days after ovulation in large domestic species. Individual surges average 5 to 6 hours in duration with approximately the same interval intervening between surges. Functional luteolysis, involving a decline in progesterone secretion, usually begins 3 to 6 hours after the initiation of $PGF_{2\alpha}$ release. Regression of the corpus luteum is usually complete within 24 to 48 hours in farm animals except in the sow, which has increased amounts of $PGF_{2\alpha}$ several days before the initiation of functional luteolysis (Stabenfeldt and Edqvist, 1984).
Prostaglandin $F_2\alpha$ is synthesized in the uterus and released in a series of pulses, each lasting about an hour with a frequency of about 6 hours (McCracken, 1984). It may pass via general systemic circulation to reach the ovary or be transferred from the utero-ovarian vein to the ovarian artery via local countercurrent type exchange (Stabenfeldt and Edqvist, 1984). The local transfer permits only small amounts of PGF$_2\alpha$ ($\approx 1\%$) to reach the ovary directly (McCracken, 1984). A local route has been found in the ewe and cow and appears to be general circulation in swine and mares. The general route indicates that each uterine horn can influence the life span of the corpus luteum on the contralateral ovary (Stabenfeldt and Edqvist, 1984).

Chronic inflammatory processes in the mare uterus (often resulting in pyometra) often results in destruction of the endometrium and loss of ability to synthesize PGF$_2\alpha$. Depending upon the amount of uterine damage present, luteal function may be prolonged for variable periods of time (days to months) or may be extended indefinitely in cases of severe obliterative endometritis. Most cows and many mares with pyometra have a persistent corpus luteum. The condition does not necessarily lead to a poor fertility prognosis in the cow (Archbald, 1976), but it does in the mare because tissue damage is usually more extensive in the mare compared with the cow. The accumulation of fluid in the uterus in the ewe and cow can also result from a prolonged luteal phase. In general, in large domestic species, abnormalities in estrous cycle lengths are usually related to uterine dysfunction (Stabenfeldt and Edqvist, 1984).

Naturally occurring prolongation of luteal activity in the
nonpregnant mare is one of the most primary causes of infertility. The cause is a failure to release adequate amounts of PGF$_2$α at ≥14 days post-ovulation. Some PGF$_2$α release often occurs with dampening of luteal activity, but not complete luteolysis. Luteal function can persist for months with return to cyclic ovarian activity when the uterus regains the capacity to synthesize and to release PGF$_2$α. In a mare with a pyometra, continual luteal activity has been noted for over a period of 613 days. The usual treatment for such cases is administration of exogenous PGF$_2$α (Stabenfeldt et al., 1980).

Zavy et al. (1978) measured PGF$_2$α concentrations in uterine flushings in cycling mares and demonstrated an abrupt peak on day 14 of the estrous cycle coinciding with the onset of luteal regression. Results showed a 45-fold increase in the amount of PGF$_2$α in uterine flushings from day 4 to day 14 of the estrous cycle. A 3-fold increase was noted from day 12 to day 14 of the cycle.

Prostaglandin is luteolytic in the mare when administered after day 5 of diestrus via either uterine infusion or subcutaneous injection (Douglas and Ginther, 1972; Noden et al., 1974).

Luteotropic Effects of Prostaglandins

Evidence of the luteotropic role of PGI$_2$ during the early development of the bovine corpus luteum was determined by (1) injection of PGI$_2$ directly into the corpus luteum at mid-cycle, which produced a prolonged increase in peripheral plasma progesterone concentrations; (2) PGI$_2$ stimulated progesterone synthesis by luteal cells in vitro; (3) PGI$_2$ synthesis by luteal cells was greatest during the period of early luteal tissue development (days 5 to 10 of
the estrous cycle) and reached a low level by day 15 and remained low during the remainder of the cycle (Hansel and Convey, 1983).

Hansel and Convey (1983) infused either PGE₁ or PGE₂ (both bind to PGI₂ receptors) into either the ipsilateral or contralateral uterine horn of unilaterally ovariectomized ewes and concluded both compounds had antiluteolytic effects. Both PGE₁ and PGE₂ have been shown to inhibit the luteolytic effects of estradiol. Infusions of PGE₂ directly into ovaries transplanted to the neck inhibited the luteolytic effects of concurrently infused PGF₂α (Henderson et al., 1977; Hansel and Convey, 1983). Multiple intra-uterine infusions of PGE₂ also resulted in an increase in peripheral progesterone concentrations and a delay of 2 days in the time at which plasma progesterone declined in the sow although estrous cycle lengths were unaffected (Schneider et al., 1982; Hansel and Convey, 1983).

**Antifertility Effects of Non-steroidal Anti-inflammatory Drugs**

Many studies that implicate prostaglandins in reproductive processes are based on the determination of a response to the administration of prostaglandin synthetase inhibitors and the reversal of that effect by prostaglandin administration. Prostaglandin synthetase inhibitors prolong gestation, inhibit ovulation, reduce gonadotropin releasing hormone release from the hypothalamus and reduce pregnancy and live birth rates (Seeley, 1983). Antifertility effects have been reported when NSAID are administered to various mammalian species (Table 6). Results of these experiments suggest involvement of prostaglandins in these reproductive functions.
Table 6. Antifertility effects of non-steroidal anti-inflammatory drugs (NSAID) administered to various female mammalian species

<table>
<thead>
<tr>
<th>Function of fertility inhibited</th>
<th>NSAID</th>
<th>Animal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ovum transport, implantation</td>
<td>IND</td>
<td>Rabbit</td>
<td>Hodgson, 1976</td>
</tr>
<tr>
<td>2. Blastocyst expansion</td>
<td>IND</td>
<td>Rabbit</td>
<td>Hoffman et al., 1978</td>
</tr>
<tr>
<td>3. Fetal development</td>
<td>IND</td>
<td>Rabbit</td>
<td>Hoffman, 1978</td>
</tr>
<tr>
<td>4. Live birth rate</td>
<td>IND</td>
<td>Deermice</td>
<td>Seeley, 1983</td>
</tr>
<tr>
<td>5. Blastocyst hatching</td>
<td>MF,PA</td>
<td>Mouse</td>
<td>Baskar et al., 1981</td>
</tr>
<tr>
<td>6. Implantation</td>
<td>MF,PA</td>
<td>Mouse</td>
<td>Biggers et al., 1981</td>
</tr>
<tr>
<td>7. Corpus luteum develop</td>
<td>IND</td>
<td>Cow</td>
<td>Hansel &amp; Convey, 1983</td>
</tr>
<tr>
<td>8. Pregnancy</td>
<td>IND</td>
<td>Pig</td>
<td>Kraeling et al., 1985</td>
</tr>
<tr>
<td>10. Luteolysis</td>
<td>IND</td>
<td>Sheep</td>
<td>Lacroix &amp; Kann, 1986</td>
</tr>
<tr>
<td>11. Luteolysis</td>
<td>FM</td>
<td>Pony</td>
<td>Berglund &amp; Sharp, 1983</td>
</tr>
</tbody>
</table>

IND = Indomethacin  
MF = Meclofenamic acid  
PA = Prostynoic acid  
ASA = Acetylsalicylic acid  
FM = Flunixen meglumine  
PG = Prostaglandin
EMBRYO PROSTAGLANDIN SYNTHESIS IN FARM ANIMALS

Introduction

The viable conceptus (embryo and extraembryonic membranes) exerts a biochemical dialogue with the maternal unit to prolong life of the corpus luteum. This dialogue continues the secretion of progesterone essential for a uterine histotroph production. Conceptus secretory proteins direct the synthesis of major prostaglandins that relate to conceptus viability (Thatcher et al., 1989). There are publications on the production of prostaglandins by the harvested embryo in farm animals (Table 7). No reports are presently available in the mare.

Ovine Embryo Prostaglandin Synthesis

Marcus (1981) collected intact embryos from pregnant ewes on day 12 or day 15 of gestation. Embryos were incubated in 1 ml tissue culture medium-199 (TCM-199) containing 10% fetal calf serum and 14C-arachidonic acid for 3 to 10 hours in a shaking water bath at 37°C. The medium was analyzed by thin layer chromatography. Day 12 embryos synthesized approximately equal quantities of PGE₂, PGI₂ and PGF₂α, with PGI₂ being the principle product. It was concluded that maternal recognition of pregnancy in the ewe occurs at least by day 12 post-coitus and involves synthesis of PGI₂ and PGE₂.

Hyland et al. (1982) collected intact embryos from pregnant ewes on days 13, 14 and 15 of gestation. Embryos were homogenized in ice-cold saline or were incubated in Dulbecco’s phosphate-buffered saline with 10% normal sheep serum in Dubnoff shaking incubators at
Table 7. Summary of farm animal embryo prostaglandin (PG) synthesis

<table>
<thead>
<tr>
<th>Reference</th>
<th>Animal</th>
<th>Day</th>
<th>PG assayed for</th>
<th>Major PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marcus et al. (1981)</td>
<td>Ewe</td>
<td>12, 15</td>
<td>E₂, I₂, F₂, E₂, I₂, F₂</td>
<td>PGI₂, E₂</td>
</tr>
<tr>
<td>Hyland et al. (1982)</td>
<td>Ewe</td>
<td>13, 14, 15</td>
<td>E₂, F₂</td>
<td>PGF₂α</td>
</tr>
<tr>
<td>Lacroix &amp; Kahn (1982)</td>
<td>Ewe</td>
<td>14, 23</td>
<td>E₂, F₂, E₂, F₂</td>
<td>PGF₂α, PGE₂</td>
</tr>
<tr>
<td>Lewis &amp; Waterman (1983a)</td>
<td>Sow</td>
<td>16</td>
<td>E₂, F₂</td>
<td>PGE₂</td>
</tr>
<tr>
<td>Shemesh et al. (1979)</td>
<td>Cow</td>
<td>13, 15, 16</td>
<td>E₂, F₂</td>
<td>PGF₂α</td>
</tr>
<tr>
<td>Lewis et al. (1982)</td>
<td>Cow</td>
<td>16, 19</td>
<td>E₂, F₂</td>
<td>PGF₂α</td>
</tr>
<tr>
<td>Lewis &amp; Waterman (1983b)</td>
<td>Cow</td>
<td>19</td>
<td>E₂, F₂</td>
<td>PGF₂α</td>
</tr>
<tr>
<td>Lewis (1984)</td>
<td>Cow</td>
<td>19</td>
<td>E₂, F₂</td>
<td>PGF₂α</td>
</tr>
<tr>
<td>Hwang et al. (1988)</td>
<td>Cow</td>
<td>&gt; 12, 13, &gt; 15</td>
<td>E₂, F₂, I₂, E₂, F₂, I₂, E₂, F₂, I₂</td>
<td>PGE₂*, PGE₂, F₂, PGI₂</td>
</tr>
</tbody>
</table>

*Only PGE₂ was detected*
37°C for 0.5, 4.5 or 8.5 hours. Strips of endometrium were also cultured and prostaglandin E₂ and PGF₂α synthesis was determined by RIA. Prostaglandin F₂α release rates were consistently higher than those of PGE₂ at all incubation stages. Prostaglandin E and PGF₂α output by embryos on a weight basis was up to 200 and 1000 times higher than that from cultured endometrium, respectively. Prostaglandin release declined as the incubation proceeded.

Hyland et al. (1982) stated (1) that the production of higher amounts of PGF than PGE by embryos at a time when luteal maintenance was crucial to pregnancy is difficult to rationalize though similar findings have been recorded; (2) if PGE₂ is a potential anti-luteolysin as suggested in the ewe, its production by embryos may be accompanied by a non-specific increase in PGF secretion; (3) if so, PGE₂ could perhaps counteract the luteolytic effects of PGF₂α at this time.

Lacroix and Kann (1982) incubated ovine day 14 and day 23 intact embryos for 2 hours at 25°C in 1 ml of incubation medium (Krebs-HEPES) under an atmosphere of 95% O₂, 5% CO₂ bubbling in the incubation medium. Prostaglandin F₂α and E₂ synthesis was determined by RIA. Day 14 embryos produced a high prostaglandin concentration of PGF₂α and PGE₂, with PGF₂α being much greater than PGE₂. Day 23 embryos produced much lower concentrations of prostaglandins with PGE₂ being dominant.
Porcine Embryo Prostaglandin Synthesis

Lewis and Waterman (1983a) recovered day 16 blastocysts from pregnant gilts and incubated them in a petri dish containing 15 ml minimum essential medium (MEM) incorporated with radiolabeled arachidonic acid. Incubates were placed into a culture chamber on a rocking platform for 24 hours at 37°C in an atmosphere of 50% N₂:45% O₂:5% CO₂. Aliquots of medium were transferred to storage vials and the tissue was homogenized. Prostaglandin synthesis was determined by high performance liquid chromatography (HPLC). Prostaglandin E₂ was the predominant arachidonic acid product. Blastocysts metabolized 79.8 ± 1.8% of the arachidonic acid added to the MEM. Of the arachidonic acid that was not metabolized by blastocysts during the 24 hour incubation period, 80.2 ± 2.5% was found in the blastocyst homogenates.

Bovine Embryo Prostaglandin Synthesis

Shemesh et al. (1979) collected bovine blastocysts on days 13, 15 and 16 post-mating. Blastocysts from each superovulated cow were placed (1) in 1 ml uterine flushing medium (0.1M-sodium phosphate buffer with 0.1% glucose and bovine serum albumin, 5 mg/ml) and stored at -20°C or (2) in 1 ml TCM-199 supplemented with 5% fetal calf serum and incubated in an incubator at 37°C and continuously flushed with 95% air and 5% CO₂, saturated with water. Prostaglandin F and PGE were determined by RIA. There was a steady rise in PGF and PGE concentrations with increase of embryo age. The prostaglandin concentrations of cultured blastocysts were 4 to 17 times greater than those of uncultured blastocysts. At all 3 gestation ages, PGF
concentration was considerably greater than that of PGE.

Lewis et al. (1982) recovered bovine blastocysts on days 16 and 19 post-mating. Blastocysts were placed in 15 ml MEM and incubated in a culture chamber on a rocking platform for 24 hours at 37°C in an atmosphere of 50% N₂:45% O₂:5% CO₂. Culture medium was assayed for blastocyst prostaglandin synthesis by RIA. Results concurred with those of Shemesh et al. (1979) in that blastocyst production of PGF₂α and PGE₂ increased with day of pregnancy and the quantity of PGF₂α was much greater than PGE₂.

Lewis and Waterman (1983b) recovered day 19 bovine blastocysts and incubated them in 15 ml MEM containing arachidonic acid for 24 hours at 37°C in an atmosphere of 50% N₂:45% O₂:5% CO₂. Metabolism of arachidonic acid was determined by HPLC. Results show a 3-fold increase in PGF₂α synthesis as compared with PGE₂. Lewis (1984) repeated this procedure except blastocysts were incubated in 10 ml MEM and prostaglandins was quantified via RIA. Again there was approximately a 3-fold increase in PGF₂α synthesis as compared with PGE₂.

Thatcher et al. (1984) reported that day 16 bovine conceptuses produced approximately 4 times the amount of PGF₂α as compared with PGE₂. This concurs with other studies indicating bovine conceptuses produce PGF₂α and PGE₂ and that production increased with day of pregnancy. It was proposed that these prostaglandins may be sequestered and compartmentalized within the uterine lumen.

Hwang et al. (1988) determined the profile of arachidonic acid metabolites synthesized by bovine embryos recovered on days 6 through 17. Embryos were incubated in MEM with ³H-arachidonic acid for 15
hours at 37°C in a humidified atmosphere of 5% CO₂ in air. To evaluate the time course of prostaglandin synthesis, day 15 embryos were incubated for 24 hours in 5 ml MEM, but without arachidonic acid. An aliquot of medium was taken at 0, 2, 4, 6, 8, 12, 15 and 24 hours and assayed for PGE₂ and 6-keto-PGF₁α (metabolite of PGI₂) by RIA. Formation of PGE₂ and PGI₂ increased linearly during the 24 hour incubation. Analysis of embryo samples incubated with ³H-arachidonic acid was determined by HPLC. Embryos collected on days 6 through 10 metabolized arachidonic acid primarily to PGE₂. Day 13 embryos metabolized arachidonic about equally to PGE₂ and PGF₂α. The appearance of PGF₂α peaks in other embryo samples occurred between days 12 and 15. The enhanced ability of embryos to synthesize PGF₂α tended to coincide with rapid development of trophoblastic tissue surrounding the embryonic disc. Embryos harvested after day 15 metabolized arachidonic acid to PGI₂ in addition to PGE₂ and PGF₂α. Results indicate there are distinct transitional changes in arachidonic acid metabolism during early stages of development by bovine embryos.

A summary of embryo prostaglandin synthesis studies in ewes, gilts and cows is presented in Table 7. Embryos ranging in age from day 6 to day 23 of gestation were incubated in these studies. Incubation medium was TCM-199, Dulbecco's phosphate-buffered saline or MEM and incubation times ranged from 2 to 24 hours. Prostaglandin synthesis was determined via RIA, thin layer chromatography or HPLC. All embryos were incubated intact, except Hyland et al. (1982) who homogenized one group of embryos. In most studies, researchers also homogenized and cultured endometrial tissue to compare
prostaglandin synthesis. Endometrial prostaglandin synthesis was negligible as compared with that of similar stage embryos.

These findings suggest that endometrial cells harvested with the embryos did not affect prostaglandin concentrations. Hwang et al. (1988) was the only researcher to report washing embryos before incubation. Only Marcus et al. (1981) and Hwang et al. (1988) also assayed for PGI₂ synthesis. There was no absolute pattern of prostaglandin synthesis for various ages of embryos across the species. Embryo prostaglandin synthesis appeared to have transitional changes. The dominant prostaglandin changed as the embryos progressed through the early stages of development.
MARE ESTROUS CYCLE

Introduction

The reproductive cycle of the mare and its related phenomena are unique. Because the mare is susceptible to environmental changes, many so called "normals" that are reported for the mare may actually fluctuate month by month. Such variations deem it necessary to consider every mare as an individual (Belling, 1983).

Breeding Season

Most mares are seasonally polyestrous. Summer is the physiological breeding season when the mare has normal-length cycles, follicular development with ovulations and full uterine growth. Approximately 85 to 95% of all mares exhibit estrus and ovulate regularly in the summer (Ginther, 1979a; Neely, 1983a).

Most mares become anestrus during the cold season because decreased daylight (photoperiod) decreases ovarian activity due to the melatonin effect (Ginther, 1979a). High levels of melatonin secreted by the pineal gland are promoted by darkness and decrease gonadal activity. Only 15 to 25% of all mares are in estrus and ovulate during the months of December, January and February (Ginther, 1979a).

Spring is a transitional period when there is a slow increasing ovarian response to increasing day length. Mares are emerging from winter anestrus. Follicles may or may not ovulate and cycles are often irregular. Uterine epithelium changes from a thin lining of simple cuboidal epithelial cells to a thicker lining due to an increase in height, diameter and number of cells (Ginther, 1979a;
Neely, 1983a; Banks, 1986).

In the Fall there is a slow decrease in ovarian activity due to decreasing day length (Ginther, 1979a). Cycles become irregular, ovarian activity usually terminates in October or November and uterine epithelium regresses and becomes thin again due to a decrease in height, diameter and number of cells (Ginther, 1979a; Banks, 1986).

Some mares cycle all year long, especially those living closer to the equator. A more pronounced period of anestrus is exhibited in mares living at higher latitudes (Ginther, 1979a).

Estrous Cycle

An estrous cycle is defined as the time between the first day of estrus of one cycle and the beginning of estrus of the subsequent cycle whether or not accompanied by ovulation (Ginther, 1979a). The average estrous cycle is 21 days with the normal range reported as 18 to 24 days (Day, 1940). Ranges in estrous cycle have been reported from 12 to approximately 40 days (Belling, 1983).

Estrus is defined as the period when the mare accepts teasing and mounting by the stallion and is normally associated with follicular development and ovulation (Ginther, 1979a; Neely, 1983a). The normal range is 4 to 8 days with ranges from 1 to 40 days reported. The average length of estrus is 5 days (Belling, 1983).

Diestrus is defined as the time between 2 estrous periods when the mare will not accept teasing and mounting by the stallion. It is highly variable due to the life-span of the mare corpus luteum (Ginther, 1979a). The normal range for diestrus is 11 to 17 days.
with a range of 2 to 59 days reported (Belling, 1983).

The normal mare estrous cycle is diagramed in Figure 7. Relative endocrine and PGF$_2$α levels are represented in Figure 8.

**Ovulation**

Ovulation may occur on any day of the cycle, any time of day, may be single or multiple and may vary within a mare from cycle to cycle. Mares may ovulate throughout the year. Ovulation normally occurs 1 to 2 days prior to the end of estrus; however, it may occur on the first or last day of estrus, or the first or second day after the end of estrus (post-estrus ovulation). Ovulation may occur during diestrus in about 5% of cycles without signs of estrus. Most mares ovulate during the night or early morning. Multiple ovulations occur about 20% of the time, may be from the same or both ovaries and may be simultaneous or at intervals (Belling, 1983; Neely, 1983a).

Ovulation occurs only at the ovulation fossa. The ensuing corpus luteum has approximately a 12 day lifetime, with abrupt termination after an increase in PGF$_2$α from the uterus (Ginther, 1979a; Neely, 1983a).
Figure 7. Schematic representation of the mare estrous cycle
FSH = Follicle stimulating hormone  
LH = Luteinizing hormone  
PGF$_2\alpha$ = Prostaglandin $F_2\alpha$  
OV = Ovulation

$P_4$ = Progesterone  
$E_2$ = Estrogen

Figure 8. Endocrine and PGF$_2\alpha$ profile of the mare estrous cycle

Adapted from Stabenfeldt and Hughes (1977) and Ginther (1979b)
Induced Luteolysis in the Mare

Arthur (1970, 1975) and Neely et al. (1975, 1979) reported intrauterine saline infusion in the early diestrous cyclic mare shortened the interestrous interval by inducing premature luteolysis. Plasma progesterone levels decrease within 1 day after infusion and declined to < 1 ng/ml within 4 days. Then, mares returned to estrus. Arthur (1975) infused mares in prolonged diestrus that showed ovulatory estrus within 3 to 9 days. In these experiments, infusion during estrus or late diestrus had no significant effect. It was postulated that the luteolytic effect of early diestrus intrauterine saline was induced by a premature release of uterine PGF$_2$α.

Hurtgen (1975) reported that uterine and/or cervical manipulations caused alterations of the estrous cycle. Several experiments were conducted to evaluate these observations in cycling mares. Hurtgen (1975), Hurtgen and Whitmore (1978) and Hurtgen and Ganjam (1979) tested the effect of specific procedures versus day of estrous cycle it was performed. He grouped estrous cycles as follows (1) control cycles included no cervical or uterine manipulations; (2) cycles included endometrial biopsy and uterine culture (passing a cotton swab through the cervix and rolling it against uterine endometrium) on day 4 post-ovulation; (3) cycles included biopsy and culture on the first and third days after ovulation; (4) cycles included manual dilation of the cervix with the gloved index finger 4 days after ovulation. Serum progesterone concentrations were measured by RIA in all experiments.
In summary, in the first group endometrial biopsy and culture 4 days after estrus resulted in early regression of the corpus luteum that was evidenced by a sharp decline in serum progesterone concentration and a significantly shortened interestrous interval. Estrous cycle length was decreased although the duration of the subsequent estrus was increased. The second and third groups also demonstrated sufficient stimulus to shorten diestrus and lengthen estrus, though the effect was less stimulating than endometrial biopsy when comparing experimental parameters. Double ovulation that required 2 corpora lutea to regress responded in the same manner. Uterine culture on day 4 post-ovulation did not significantly alter estrous cycles. Hurtgen and Whitmore (1979) did induce estrus and ovulation by endometrial biopsy in mares with prolonged diestrus suggesting that regression of persistent luteal tissue was caused by stimulating the release of endogenous PGF$_2$α.

Baker (1981) verified earlier results and also determined that endometrial biopsy (1) during estrus had no effect on the cycle; (2) on day 4 post-ovulation induced premature luteolysis; (3) significantly reduced length of diestrus. Baker (1981) questioned whether luteolysis was induced by the biopsy procedure or by an induced uterine infection because premature luteolysis had been previously induced by bacterial infections (Hughes, 1969), saline infusions (Arthur, 1975; Neely, 1975), cervical manipulation (Hurtgen, 1979a) and exogenous PGF$_2$α (Noden, 1974). Therefore, Baker (1981) cultured endometrial swabs taken before and after the biopsy procedure. No bacterial contamination was apparent or detected by culturing the uterine swabs. These results suggested luteolysis was induced by the
biopsy procedure and was not due to uterine infection.

**Luteolysis in Mares Induced by Endometrial Biopsy**

Hurtgen (1975), Hurtgen and Whitmore (1978), Hurtgen and Ganjam (1979), Hurtgen and Whitmore (1979) and Baker et al. (1981) postulated that endometrial biopsy could potentiate an acute inflammatory response caused by the biopsy and, thus, increase endogenous release of uterine luteolysin PGF$_2 \alpha$ and cause subsequent premature luteal regression. The equine corpus luteum was shown not to be sufficiently mature to respond to the luteolysin until day 4 post-ovulation.

**Technique for Performing Endometrial Biopsy in the Mare**

The mare is restrained in an equine stock as for rectal examination. The gauze-wrapped tail is tied aside and feces are removed manually from the rectum. The perineum and vulvar lips are thoroughly washed 3 times with Betadine surgical scrub, each wash being followed by a thorough rinse with clean, warm water, taking care to wash and rinse the vulvar orifice (Ricketts, 1975; Kenney, 1978).

The tissue specimen is taken with a biopsy punch having alligator-type jaws with a basket 20 x 4 x 3 mm and an overall length of 70 cm (Kenney, 1978). This instrument provides a specimen 2 cm$^2$, which is on average 0.2% of the entire (850 to 1350 cm$^2$) endometrium (Kenney, 1975). A hand is covered first with a plastic sleeve and covered with a sterile disposable glove lubricated with a sterile, surgical jelly (Kenney, 1978). The tip of the sterile biopsy instrument is placed in the palm of the gloved hand.

The vulva lips are spread and the gloved hand cupping the biopsy
punch (jaws closed) is passed through the vagina. The index finger is inserted through the cervix and the instrument passed along the index finger into the uterus. While maintaining the forceps in position, the gloved arm is then withdrawn from the vagina and passed into the rectum where the instrument is palpated and positioned to the desired sites (Ricketts, 1975).

To avoid penetrating the entire uterine wall if a straight bite is taken, the biopsy punch is laid on its side, and the endometrium is pushed into the side of the open jaws and pinched off by closing the jaws. There is usually hemorrhage (1 to 2 ml) at the biopsy site (Kenney, 1978). Endometrial biopsy may be performed any time of the year (Shideler, 1982) and at any stage of the estrous cycle (Kenney, 1978).
HIGH PERFORMANCE LIQUID CHROMATOGRAPHY
AS A METHOD TO ASSAY PHENYL BUTAZONE IN BODY FLUIDS

Introduction

Phenylbutazone was first synthesized in 1946 by H. Stengyl in Switzerland and was later marketed by J. R. Geigy, A.G. (Jeffcott and Coles 1977). This compound was not detected in biological material until 1953 (Burns, 1953). Indiscriminate use of phenylbutazone at race tracks and high performance equestrian events has stimulated interest in developing a fast, reliable, specific method for phenylbutazone detection in various equine biological materials such as plasma, urine and sweat. Since phenylbutazone is almost completely metabolized in the horse, recovery rates are low (Maylin, 1977) and detection can be difficult, inaccurate or even missed. In separate studies, only 3.7% of a 2 g/450 kg intravenous dose was recovered in the first 24 hours (Piperno, 1968).

In another study, only 1 to 2% of the parent drug was recovered in the urine of the horse when dosing 2 g/450 kg intravenously at consecutive 24-hour intervals (Maylin, 1974). The principle metabolites in the horse, oxyphenbutazone and γ-hydroxyphenylbutazone, together accounted for only about 25% of the phenylbutazone administered. The remaining 75% of the parent drug was undetected by gas liquid chromatography (Maylin 1974; Maylin, 1977).
Chromatography History

Burns et al. (1953) pioneered the method for estimating the concentration of phenylbutazone in body fluids. They isolated phenylbutazone from acidified human plasma and urine by extraction into heptane. The drug was then extracted from the heptane solution with aqueous alkali and the phenylbutazone concentration was measured at 265 nm in an ultraviolet spectrophotometer.

Burns et al. (1953) also designed a tissue procedure by homogenizing up to 2 g of tissue in 10 ml of H_2O that followed the same method used for plasma and urine except that it used heptane containing 3% isoamyl alcohol. This is the technique that many equine phenylbutazone researchers have used primarily for 2 to 3 decades (Gabriel, 1962; Piperno, 1968; Gandal, 1969; Finocchio, 1970; Quijano, 1979).

Stevens (1970) developed a faster extraction of phenylbutazone from blood, plasma and liver. He used formic acid to deproteinize the material, hexane instead of heptane for extraction and 263 nm instead of 265 nm to perform the spectrophotometric assay. A 95% recovery yield was reported from plasma and =45% from whole blood or liver. This new method extracted phenylbutazone from water with a 99% yield. It was concluded that the poorer recoveries from biological fluids resulted from plasma protein binding.

Stevens (1970) also collected a blood sample from a human receiving phenylbutazone treatment and divided it into 2 equal portions and estimated the phenylbutazone concentration by both the Burns et al. (1953) method and his own. The same result was obtained by each method, but the Burns et al. (1953) procedure lasted 45 to 50
minutes and the Stevens method could be completed in only 10 to 15 minutes. Equine researchers then started using the Stevens method to estimate phenylbutazone concentration in biological tissues and fluids (Sullivan and Snow, 1982; Rose et al., 1982).

Gas Liquid Chromatography Analysis of Phenylbutazone

Modern advances in chromatography broadened the field to include detection of therapeutic and doping agents. Gas liquid chromatography and high performance liquid chromatography (HPLC) have become popular with equine researchers for phenylbutazone analysis as easier and more specific methods than ultraviolet spectrophotometry.

Bruce et al. (1974) used gas liquid chromatography to measure phenylbutazone concentration in plasma and urine after giving a single, oral dose (8.9 mg/kg) of phenylbutazone to horses either as tablets or granules. Samples were obtained at 0.5 to 72 hours post-administration. The plasma half-life was ≈4 hours. Measurable concentrations of phenylbutazone were present in some horses for 48 hours in plasma and urine. They concluded that gas liquid chromatography analysis of blood and urine for phenylbutazone and oxyphenbutazone is simple, specific and adaptable to analysis of race horses suspected of doping.

Maylin (1974) also used gas liquid chromatography to study the metabolism and pharmacokinetics of phenylbutazone in the horse. Horses were given 4.4 mg/kg phenylbutazone intravenously daily for 4 days. It was reported that (1) phenylbutazone and its metabolites are chemically unstable and decompose readily; (2) urine and blood samples improperly refrigerated or analyzed usually contain several
impurities or decomposition products that must be distinguished from phenylbutazone and metabolites; (3) plasma metabolites could not be assayed accurately at all intervals although they could be detected in most samples; (4) total percent recovery of phenylbutazone in urine for 24 hours post-administration was 0.9%, 1.7% 2.1% and 1.6% of the first, second, third and fourth doses, respectively; (5) plasma concentrations increased from 18.2 μg/ml 1 hour after the first dose to 31.1 μg/ml 1 hour after the fourth dose and increased from 0.8 to 2.4 μg/ml 24 hours after the first and fourth doses, respectively.

High Performance Liquid Chromatography Analysis of Phenylbutazone

Pound et al. (1974) pioneered the analysis of phenylbutazone and its major metabolites in plasma by HPLC. Results of his study demonstrated that HPLC is a sensitive procedure for determination of phenylbutazone concentration in plasma that also offers high specificity, since it differentiates the parent drug from oxyphenbutazone and γ-hydroxyphenylbutazone. Analysis time was less than 7 minutes. No extraneous interfering compounds were extracted from plasma by using n-hexane. Neither of the 2 known metabolites, oxyphenbutazone and γ-hydroxyphenylbutazone, were detected. The output of the ultraviolet detector was linear thus allowing quantitation of phenylbutazone by electronic integration and peak-height measurements. The limit of detection was approximately 0.2 μg/ml of phenylbutazone. Data determined by integration and peak-height measurements showed straight lines over the concentration range of 1.8 to 41.7 μg/ml. Mean recovery values of 99.9 ± 1.7% and 99.3 ± 3.1% were obtained by
integration and peak-height measurement, respectively. It was noted that refrigerated plasma samples containing phenylbutazone were stable for several weeks.

Pound et al. (1974) tested their procedure by doing a comparative analysis of phenylbutazone in plasma using 3 established methods (1) ultraviolet spectrophotometry; (2) gas liquid chromatography; (3) their own HPLC procedure. The overall difference between HPLC and ultraviolet methods was 8.2% and that between gas liquid chromatography and HPLC 8.9%. The ultraviolet method would not distinguish between any side-chain hydroxy- metabolite present and the parent drug. They concluded that their HPLC procedure was a more specific and sensitive method for measurement of phenylbutazone and its major metabolites than classical methods and could form the basis for a method for metabolic studies.

Alvienerie (1980) developed a specific and precise reversed-phase HPLC assay that detected minute quantities of phenylbutazone in horse plasma, urine, saliva and sweat. Recovery from the extraction procedure for both plasma and sweat was 65 ± 2% with a concentration of 100 ng to 10 µg. Concentrations as low as 50 to 100 ng/ml of phenylbutazone in 0.5 to 1.0 ml of body fluids were determined. This reverse-phase HPLC method detected the lowest concentrations of phenylbutazone in horses to date.

Although Pound et al. (1974) did not report detecting oxyphenbutazone or γ-hydroxyphenylbutazone, they developed the method that was the basis for analysis of phenylbutazone and its metabolites utilizing HPLC. Taylor et al. (1981) made modifications and improvements on the Pound method. Their limits of detection were 0.01, 0.05
and 0.10 μg/ml and mean recovery rates in spiked equine plasma were 100.08 ± 1.4%, 90.4 ± 0.9% and 59.74 ± 1.4% for phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone, respectively. Taylor et al. (1981) also reported that phenylbutazone is relatively stable in frozen plasma and urine, but less stable (plasma) and very unstable (urine) when stored at 4°C.

Gerring et al. (1981) tested the Taylor method to determine levels of phenylbutazone and its metabolites in plasma and urine with regard to time and clinical doses administered orally to horses. Gerring et al. (1981) concluded that HPLC is a simple and sensitive method and achieved good recoveries for phenylbutazone and oxyphenbutazone.

During the 1980’s, HPLC methods were developed proving to be superior to previously reported methods. Phenylbutazone is quantitated for forensic purposes by HPLC. The variability of this method within a laboratory is reported to be < 5%, however, between different laboratories it is ± 25% (Tobin et al., 1986).
PREVIOUS EXPERIMENTS LEADING TO FIRST HYPOTHESIS

The basic hypothesis and experiments for studying the effects of chronic administration of phenylbutazone on reproduction in the mare were proposed and undertaken by Dr. Louis F. Archbald and his graduate student Laurence M. Olsen, Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, Louisiana. They conducted 2 preliminary experiments during the 1979 and 1980 breeding seasons.

Prostaglandins, specifically PGF$_2$-$\alpha$, have been shown to be luteolytic in the mare. It has been suggested that the synthesis and release of PGF$_2$-$\alpha$ from the uterus is the primary cause of cyclic luteolysis and consequently controls the estrous cycle of the mare (Stabenfeldt et al., 1980). Archbald (1980) postulated that since phenylbutazone inhibits the cellular synthesis of PGF$_2$-$\alpha$, it could prolong the life span of the corpus luteum and subsequent length of the estrous cycle. Furthermore, it was proposed that prolonged luteal function might alter the uterine environment due to an extended production and circulation of progesterone.

The immunologic competence of the mammalian uterus appears to be steroid hormone-dependent. The progesterone-dominated bovine uterus is more susceptible to bacterial infections than the estrogen-dominated uterus (Black et al., 1953; Rowson et al., 1953). It may be assumed that prolonged luteal function would cause the uterus to be more susceptible to infections. Clinical records indicate that following retirement from the racetrack many mares experience varying degrees of infertility. The possibility exists that previous phenyl-
butazone medication may have been involved in luteal tissue dysfunction, resulting in increased susceptibility of the uterus to infectious organisms. The primary objectives of the first 2 experiments at this station were to evaluate expression of estrual behavior, peripheral plasma progesterone concentration and uterine luminal PGF$_2$α concentrations in mares parenterally treated with phenylbutazone (Archbald, 1980).

Tobin (1979a) reported that in average horses (500 kg body weight) an intravenous dose of 2 to 3 g phenylbutazone daily produces an optimal anti-inflammatory effect. Therefore, in both preliminary experiments and all subsequent experiments, each mare was given intravenously 2 g phenylbutazone which was proposed to be sufficient phenylbutazone to inhibit prostaglandin synthesis (Archbald, 1983).

In the first experiment, 5 mares of mixed breeding between 3 and 4 years of age and 450 to 500 kg body weight were maintained as an experimental group. All mares were teased daily with a stallion to determine onset of estrus (day 0) and rectally palpated daily during estrus to evaluate ovarian follicle development and day of ovulation. Mares were monitored for 2 untreated estrous cycles. Then, each mare served as treatment and control using a switchback experimental design. Between days 13 to 14 of the third estrous cycle each mare was given a daily intravenous dose of either 2 g phenylbutazone as treatment or 10 ml of physiological saline as control until estrus. Blood samples were obtained daily via jugular venipuncture from each mare prior to the daily injection of phenylbutazone or physiological saline. Progesterone concentrations were determined by RIA.
In the second experiment, 8 mares averaging 3 to 4 years-of-age and 450 kg body weight were maintained as an experimental group. All procedures were the same as the first experiment except that treatment began on the first day of estrus and was continued daily until mares exhibited estrus. Also, uterine secretions were collected via cervical approach from each mare on days 0, 4, 8, 12, 14, 16, 18 and 20 of the treatment estrous cycles. Samples were stored at \(-19^\circ C\) until assayed for PGF\(_{2\alpha}\) concentration by RIA.

Results of the first experiment determined that there was no significant difference in estrous cycle lengths within individual mares or across all cycles of all physiological saline-treated mares. Saline-treated estrous cycles were similar in length to pretreatment cycles of each mare. When mean estrous cycle lengths of saline-treated mares were compared with mean estrous cycle lengths of phenylbutazone-treated mares having normal length cycles, no significant differences were noted between the mean cycle lengths of each treatment group. However, signs of estrus were not observed in 2 mares for 34 days during phenylbutazone treatment. Treatment with 5 mg PGF\(_{2\alpha}\) induced overt estrus in these 2 mares on days 2 and 4 post-injection. Estrous cycle lengths of these same 2 mares while being first treated with physiological saline on the switchback design were 24 and 22 days (Archbald, 1980).

Results of the second experiment showed no significant differences in estrous cycle lengths in 75% of the mares treated with physiological saline. The estrous cycle lengths were shorter (cycle was 14 days for each mare) in the 2 remaining mares while treated with physiological saline. However, estrous cycle lengths of normal
duration (cycle was 20 days for each mare) were noted when these 2 mares were treated daily with phenylbutazone in their subsequent cycles (Archbald, 1980).

Prolongation of estrous cycle lengths was recorded for 3 mares following phenylbutazone treatment. However, signs of estrous behavior were observed at the end of the extended cycles in these mares unlike the pattern observed in the 2 mares in the first experiment where estrus was induced with PGF$_2$α following phenylbutazone treatment.

Plasma progesterone profiles determined that 37.5% of the phenylbutazone-treated mares failed to exhibit overt estrus at their predicted time, although progesterone levels had decreased to < 1 ng/ml. The expected time of estrus was based on previous estrous cycle lengths during physiological saline treatment.

In both treatment groups, mean (± SEM) PGF$_2$α concentrations of uterine flushings were between 3.10 ± 0.33 and 3.34 ± 0.40 ng/ml on the first day of estrus, decreased on day 4 and then increased to between 2.71 ± 0.33 and 3.49 ± 0.55 ng/ml on day 14. Values for mares treated with saline then increased on days 16 (3.27 ± 0.22 ng/ml) and 18 (3.47 ± 0.19 ng/ml). These mares exhibited estrus on days 20 to 22 of the saline-treated cycle. In phenylbutazone-treated mares, PGF$_2$α concentrations were significantly lower on days 16 and 18 (< 1 ng/ml) when compared with corresponding days of saline-treated mares. The phenylbutazone-treated cycles from these same 3 mares were 5, 6, and 8 days longer than expected, respectively. Progesterone values of these mares decreased on days 16 and 18 of phenylbutazone-treated cycles, but then increased again before the decline.
Results of the second experiment indicated that there were no significant differences in interestrous intervals between (1) control mares; (2) treatment versus pretreatment cycles of control mares; (3) mares treated with phenylbutazone (mean = 23.1 ± 0.24 days) versus those given saline (20.7 ± 0.28 days). Plasma progesterone profiles of treated mares showed no significant difference compared with controls. Results suggest there was no effect on ovarian cyclicity and plasma progesterone concentration in mares treated with 2 g phenylbutazone intravenously for 21 days. There was no apparent carryover effect of phenylbutazone on subsequent estrous cycle lengths when mares were totally removed from treatment (Archbald, 1983).

Results of these earlier experiments suggested validity in pursuing the effects of parenteral phenylbutazone treatment in mares because some phenylbutazone-treated mares (1) exhibited prolonged cycles; (2) exhibited shortened cycles; (3) did not express estrual behavior even though progesterone concentration was < 1 ng/ml. Because phenylbutazone treatment did significantly decrease uterine PGF$_2$α concentrations on days 16 and 18 of the estrous cycle, it was presumed that phenylbutazone may be able to concentrate in the uterus and alter the estrous cycle of the mare.

Perhaps the phenylbutazone (1) was not potent enough to cause significant luteal tissue dysfunction; (2) did not accumulate in the ovaries or uterus; (3) caused incomplete luteolysis with subsequent recovery of the corpus luteum; (4) did not effectively cause luteal dysfunction when administered for 8 to 21 days.
Therefore, because results of phenylbutazone-treated mares were varied and questions were not answered regarding why the phenylbutazone may not have acted according to the hypothesis, 5 additional experiments were conducted by this research station.
EXPERIMENT I

Estrual Behavior, Ovulation and Fertility of Mares
After Chronic Administration of Phenylbutazone

INTRODUCTION

Results of previous experiments conducted in this laboratory suggested that some of the mares receiving short-term phenylbutazone treatment apparently responded to the treatment by exhibiting prolonged cycles and/or failed to exhibit overt estrus. However, other mares receiving the same treatment did not appear to be affected by the treatment because they exhibited normal cycles and estrual behavior (Archbald, 1980; Archbald, 1983). It was then proposed that possibly long-term phenylbutazone treatment may alter the mare estrous cycle.

OBJECTIVES

The objectives were (1) to test the hypothesis that chronic use of phenylbutazone can cause infertility in mares by inhibiting prostaglandin synthesis; (2) to substantiate previous data collected by this laboratory suggesting that short-term (8 to 21 days) phenylbutazone treatment affected mare fertility; (3) to determine the effects of chronic (182 days) administration of phenylbutazone on estrual behavior, ovulation, luteal function and fertilization in mares; (4) to confirm cyclicity in the mares by using results of progesterone assays used to validate a new commercial, solid-phase, no-extraction $^{125}$I progesterone radioimmunoassay kit (Appendix III).
MATERIALS AND METHODS

Nine mares of mixed breeding, ranging from 3 to 4 years of age and weighing 450 to 500 kg were purchased and maintained under experimental conditions at the Louisiana State University School of Veterinary Medicine. Each mare was given a thorough physical examination before purchasing. Complete reproductive histories of these mares were unknown. All mares tested negative for equine infectious anemia. They were fed 8 liters of mixed grain concentrate/mare/day and free-choice hay and water from December 1, 1980 until August 15, 1981.

On December 1, 1980, each mare was given intravenously 2 g phenylbutazone (Butazolidin®, D-M Pharmaceuticals, Inc., Rockville, MD). This treatment was continued daily until May 31, 1981. Beginning on February 12, 1981, all mares were teased daily by an androgenized gelding, estrous mares were palpated per rectum to evaluate ovarian activity and a heparinized blood sample was collected daily via jugular venipuncture to be used for determination of progesterone concentration via RIA (Appendix III).

Cyclicity (estrus versus diestrus) was confirmed by the progesterone assays outlined in Appendix III. Estrus as detected by teasing was confirmed by correlation with progesterone concentrations < 1 ng/ml.

By June 1, 1981, 8 mares had exhibited at least 2 estrous cycles of normal duration (18 to 24 days). Mares were assigned to one of 2 groups as follows. The first mare to display estrus was a phenylbutazone treatment and the second mare physiological saline. The experimental design for Experiment I is presented in Figure 9.
Mares were alternated via this pattern of grouping until all 9 mares were placed into either the treatment or the control group. The treated group continued to receive 2 g of phenylbutazone daily until the day of embryo collection. The control group received physiological saline treatment.
December 1, 1980, to February 11, 1981, 9 mares were daily administered 2 g phenylbutazone intravenously.

On February 12, 1981, phenylbutazone treatment continued daily for the same 9 mares plus daily teasing, blood collection and ovarian palpation of estrous mares.

June 1, 1981, the same 9 mares were randomly assigned to either continuation of daily phenylbutazone-treatment or physiological saline (PS) control treatment. Teasing and palpation continued and estrous mares mated. Non-surgical embryo collection was performed day 7 or 8 post-ovulation.

Figure 9. Schematic representation of the experimental design for Experiment I
All mares were teased daily for signs of estrual behavior. Any mare of either group that exhibited estrus was artificially inseminated with semen from a fertile stallion. With standard artificial insemination techniques each mare was inseminated on the second day of estrus and on alternate days until no signs of estrus were evident. A standard dose of $500 \times 10^8$ motile spermatozoa from the same stallion was used for all the mares. Within 5 to 10 minutes after insemination, each mare received an intrauterine infusion of 20 ml semen extender prepared as previously described (Pickett and Back, 1973).

The day of ovulation (day 0) was determined by ovarian palpation. Starting 7 to 8 days after ovulation, fertilized ova were collected using an established non-surgical embryo collection technique (Imel et al., 1981). A 30 French Foley catheter with a 30-ml inflatable cuff was manually inserted through the cervix. To ensure a snug fit against the internal os of the cervix, the cuff was inflated with $\approx$30 ml sterile water and pulled caudally. Both uterine horns were filled by gravity flow with 1,000 to 1,500 ml Dulbecco's phosphate-buffered saline solution (Appendix I). The uterus was manipulated per rectum and the medium was collected by gravity flow into an embryo concentrating filter device positioned on top of a sterile 2 liter graduated cylinder. This flushing procedure was performed twice and/or until a fertilized ovum was recovered. If the procedure was successful, the embryo could be detected macroscopically in the filter device. If an embryo was not located macroscopically, the collection medium was searched under low magnification through a dissecting microscope (10x).
Embryos were transferred to a sterile petri dish containing fresh PB-1 holding medium (Appendix II). Subsequently, embryos were stained with fluorescein diacetate using the procedure previously described by Looney et al., 1981.

Percentages were calculated for normal, extended and shortened cycles, embryo recovery rate and embryo flushing medium recovered. Mean (± SEM) cycle length was determined for each treatment mare.

RESULTS

One mare (No. 5) did not exhibit signs of estrus or ovarian activity until June (180 days). The remaining 8 mares exhibited initial signs of estrus between February 12 and February 24, 1981. From February 12 to June 15, 1981, (124 days) 36 cycles were observed (Table 8). Of these, 21 cycles (58.3%) were within the normal range of 18 to 24 days as reported by Belling (1983). Mean cycle length was 23.6 ± 0.98 days. Ten cycles (27.8%) were slightly extended (25 to 28 days) and 3 cycles (8.3%) were very extended (> 30 days). Two cycles (5.6%) were shortened (< 18 days). Thirty-eight ovulations were recorded from 9 mares and of these, 4 mares had double ovulations.

Percentage of Dulbecco's phosphate-buffered saline solution recovered from the uterus during attempts to recover the embryos was 91.7 ± 1.6. An embryo was collected from only 1 of the 5 mares treated with phenylbutazone during the breeding cycle (Table 9). Of the 4 treated mares that an embryo was not collected, 2 had a mucopurulent vaginal discharge when collection was attempted. One control mare also had a mucopurulent vaginal discharge at the time of
attempted collection. An embryo was not recovered from this mare. However, one embryo was recovered from each of the other 3 mares in the control group. In summary, embryos were recovered from 4 of 9 mares (44.4%). Control mares contributed 75% and treated mares 25% of the total embryos recovered.
Table 8. Cyclicity and ovulatory results determined by teasing mares with an androgenized gelding and by palpation of ovaries per rectum of phenylbutazone-treated mares between February 12 and June 15, 1981

<table>
<thead>
<tr>
<th>Mare no.</th>
<th>No. of days/estrous cycle</th>
<th>No. of ovulations</th>
<th>Cycle comment</th>
</tr>
</thead>
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<td>1</td>
<td>18&lt;sup&gt;b&lt;/sup&gt;, 25, 24, 20</td>
<td>3</td>
<td>3 N, 1 SE</td>
</tr>
<tr>
<td>2</td>
<td>26, 17, 19, 18</td>
<td>5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 N, 1 SE, 1 S</td>
</tr>
<tr>
<td>3</td>
<td>25, 22, 24, 21, 20</td>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 N, 1 SE</td>
</tr>
<tr>
<td>4</td>
<td>40*, 21, 23, 18</td>
<td>4</td>
<td>3 N, 1 VE</td>
</tr>
<tr>
<td>5</td>
<td>-- -- 21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>1 N,</td>
</tr>
<tr>
<td></td>
<td>**active ovaries</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>21, 28, 25, 23, 22</td>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 N, 2 SE</td>
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<td>2 N, 2 SE</td>
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<tr>
<td>8</td>
<td>44***, 19, 33, 27</td>
<td>5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 N, 1 SE, 2 VE</td>
</tr>
<tr>
<td>9</td>
<td>28, 19, 15, 26, 19</td>
<td>5</td>
<td>2 N, 2 SE, 1 S</td>
</tr>
<tr>
<td></td>
<td>Total= 36 cycles</td>
<td>38</td>
<td>21 N, 2 S, 10 SE, 3 VE</td>
</tr>
</tbody>
</table>

N = normal cycles (18 to 24 days)
S = short (< 18 days)
SE = slightly extended (25 to 28 days)
VE = very extended (> 30 days)

* 25 days of estrus and 15 days diestrus
** 65 total days of estrus and 29 total days diestrus (no cyclicity)
*** 6 days estrus, 38 days diestrus

<sup>a</sup> double ovulation
<sup>b</sup> follicle regressed

Note: The first cycle recorded is on the left of each row and then sequentially across the row to the right.
Table 9. Embryo collection data for individual mares, intravenous treatment, embryo recovery results and the percent of embryo flushing medium recovered

<table>
<thead>
<tr>
<th>Mare No.</th>
<th>Treatment</th>
<th>Embryo recovered</th>
<th>Clinical evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBZ</td>
<td>No</td>
<td>PM, 94%</td>
</tr>
<tr>
<td>2</td>
<td>PS</td>
<td>No</td>
<td>PM, 95%</td>
</tr>
<tr>
<td>3</td>
<td>PBZ</td>
<td>No</td>
<td>88%</td>
</tr>
<tr>
<td>4</td>
<td>PS</td>
<td>Yes</td>
<td>87%</td>
</tr>
<tr>
<td>5</td>
<td>PS</td>
<td>Yes</td>
<td>93%</td>
</tr>
<tr>
<td>6</td>
<td>PS</td>
<td>Yes</td>
<td>90%</td>
</tr>
<tr>
<td>7</td>
<td>PBZ</td>
<td>Yes</td>
<td>94%</td>
</tr>
<tr>
<td>8</td>
<td>PBZ</td>
<td>No</td>
<td>84%</td>
</tr>
<tr>
<td>9</td>
<td>PBZ</td>
<td>No</td>
<td>PM, 100%</td>
</tr>
</tbody>
</table>

PBZ = daily treatment with 2 g of phenylbutazone
PS = daily treatment with 10 ml physiological saline
PM = pyometra noted at embryo collection
% = percent return of embryo collection medium
DISCUSSION

The decision to administer phenylbutazone to all mares was the result of a limited number of mares and a limited budget. Because results with short-term treatments from previous studies had not been very consistent, it was decided that it might require all of the available mares to find some that might respond to phenylbutazone by demonstrating altered cycles. Results were to be evaluated on the basis of cycles outside of an accepted norm (21 ± 3 days). In retrospect, it is questionable that having control mares would have added much to that information gained.

Mare No. 5 did not exhibit estrual behavior or ovarian activity for several months during the experimental period. It probably was not an effect of treatment with phenylbutazone because when this mare was placed in the experimental herd, it was very thin. However, after being fed grain on a regular basis and since pasture grass was abundant in the spring, it gained weight. This mare exhibited a normal-length cycle at the end of the experimental period and produced a viable control embryo from a typical uterus (Table 9).

During the first part of the experimental period, the intervals between estrus were irregular and erratic. This effect was assumed to reflect the transition period between winter anestrus (when cycles are often longer than later in the year) and the breeding season. Subsequently, the interval between estrus for these 9 mares during the entire period of study was 23.6 ± 0.98 days, which is slightly longer than the reported average of 21 days (Ginther, 1979a; Belling, 1983). However, this cycle mean was within the normal range of 18 to 24 days as reported by Day (1940). Therefore, it was assumed that
administration of phenylbutazone for 180 days (December 1, 1980 to May 31, 1981) did not have a significant effect on the initiation or regularity of estrous cycles.

The mean recovery rate for embryo flushing medium was 92% and was similar to recovery rates reported previously with 93% and 96% by Imel et al. (1981), 97% by Oguri and Tustsumi (1972) and 92% by Douglas (1979). Therefore, the low embryo recovery rate was probably not due to inadequate flushing technique. Medium recovery rates tended to be higher for mares exhibiting pyometra. This was assumed due to other fluid accumulated in the uterus due to inflammation. Active vasodialation with increased permeability of blood vessels resulting in accumulation of a protein-rich fluid and leukocytes making up the inflammatory exudate is a major feature of inflammation (Higgins and Lees, 1984; May et al., 1987).

Embryo recovery rate (44%) was considerably less than those reported in previous reports with 72% and 83% by Imel et al. (1981), 90% by Oguri and Tsutsu (1974) and 65% and 56% by Douglas (1979). The recovery rate was comparable with the 47% recovery rate reported by Oguri and Tsutsu (1972), but greater than the 39% recovery rate reported by Allen and Rowson (1975).

The cause of the mucopurulent vaginal discharge was not ascertained. A review of utero-ovarian palpation records did not give any indication that there was an obvious pyometra during the cycle prior to the cycle that they were mated. Because no abnormalities were observed in the vagina or cervix on vaginoscopic examination, the source of the discharge was likely the uterus. The fact that no embryos were recovered from these mares may have been due to
uterine infection and/or lack of fertilization. However, uterine infection does not necessarily interfere with fertilization in cattle (Archbald, 1976). Forty percent of the treated mares had vaginal discharge. It may or may not have been caused by phenylbutazone because all mares had received phenylbutazone for an extended period of time. Also, semen collection and insemination equipment may have been inadequately sterilized by the methods used (placed in Nolvasan solution and air dried).

The low percentage of embryos recovered could also be stallion related. At the beginning of the experimental breeding season, the stallion was given a complete fertility examination and was considered acceptable. This procedure was not conducted again. Thereafter, each semen collection was simply visualized to evaluate density and measured in a graduated cylinder to estimate semen dose per mare. If only 1 mare was mated per day, artificial insemination was still used to maintain the same protocol for every mare. Possibly, stallion fertility was lowered to less than acceptable for some unknown reason. Others have reported a difference in fertility between stallions (Douglas, 1979).

The low embryo recovery rate (20%) from phenylbutazone-treated mares may also be related to ovum transport. Inhibition of ovum transport in rabbits treated with indomethacin was reported by Hodgson (1976). Possibly the ovum in treated mares did not reach the uterus.

Figure 13 shows an example of an embryo (blastocyst) recovered from one of the mares in this study. The same blastocyst is presented after staining with fluorescein diacetate (Figure 14).
This stain has been used to assess the viability of cell lines cultured in vitro because living mammalian cells take in fluorescein when exposed to fluorescein diacetate (fluorochromasia). Fluorochromasia occurs because fluorescein diacetate, being nonpolar, can readily pass into the cell. There, it is hydrolyzed by esterases to yield fluorescein, which cannot readily cross the cell membrane and, therefore, accumulates intracellularly. The test is a measure of esterase enzyme activity, cell membrane integrity and, indirectly, cell viability (Rotman and Papermaster, 1966).

The fluorescein diacetate stain has been reported not to be detrimental to embryos recovered from other species. Exposure of 2-cell mouse embryos to fluorescein diacetate and ultraviolet light did not alter the rate of implantation or post-implantation development in vivo (Mohr and Trounson, 1980). Also, the fluorescein diacetate viability assay did not inhibit development of hamster and bovine preimplantation embryos (Looney et al., 1981; Hoppe and Bavister, 1983; 1984). In this study, all 4 blastocysts recovered were positive by the fluorescein diacetate test.

The results of this study suggest that chronic treatment with phenylbutazone had little if any effect on estrual behavior, estrous cycle length or ovulation. In addition, it appears that blastocysts from phenylbutazone-treated mares may be viable when recovered from the uterus between 7 and 8 days after ovulation.
Figure 10. Blastocyst recovered from a mare on day 8 post-ovulation (x40). The red stain is derived from the phenol red in PB-1 embryo holding medium.

Figure 11. Blastocyst (Figure 10) stained with fluorescein diacetate (x40). The green stain suggests embryo viability.
EXPERIMENT II

Analysis of Phenylbutazone in Plasma and Uterine Secretions of Mares by High Performance Liquid Chromatography

INTRODUCTION

Since phenylbutazone distributes throughout the horse (Tobin, 1979a) and is capable of traversing the blood-brain barrier (Tobin, 1981a) it was assumed in Experiment I that phenylbutazone was potentially capable of entering uterine tissue following parenteral administration. However, results of Experiment I did not support the hypothesis that long-term phenylbutazone therapy would interfere with prostaglandin synthesis in the uterus and consequently disrupt estrous cycle length in the chronically-treated mare. It was then proposed that possibly phenylbutazone did not have any receptor sites in the mare uterus and did not accumulate there. Consequently, it was decided to evaluate if phenylbutazone was being concentrated in the uterine fluids.

The appearance of a mucopurulent vaginal discharge when embryo collection was attempted in Experiment I prompted the investigation to determine if circulating progesterone concentrations affected the incidence of uterine inflammation. The progesterone-dominated bovine uterus is more susceptible to bacterial infections than the estrogen-dominated uterus (Black et al., 1953; Rowson et al., 1953; Archbald, 1980). Therefore, it was decided to determine if circulating progesterone concentrations affected the incidence of uterine inflammation in the mare.
OBJECTIVES

The objectives were (1) to test the hypothesis that phenylbutazone accumulates in the normal and/or pathological equine uterus by determining concentrations of phenylbutazone in uterine secretions; (2) to determine if progesterone levels affected incidence of uterine inflammation; (3) to determine phenylbutazone concentrations in circulating blood at the time the uterine samples were collected.

MATERIALS AND METHODS

Seven mares of mixed breeding (4 to 6 years of age) averaging 450 kg bodyweight were housed in large pens at the Louisiana State University School of Veterinary Medicine and fed good quality grass hay, trace mineral salt and water ad libitum. Four mares were each given 2 g phenylbutazone (Butazolidin®, 200 mg/ml; D-M Pharmaceuticals, Kansas City, MO.) intravenously (treated group) and 3 mares were given 10 ml sterile physiological saline intravenously daily (control group).

Uterine secretions were obtained 1 day prior to treatment (December 21, 1981) and daily until January 20, 1982. They were obtained by a modified non-surgical collection technique described by Zavy et al., 1978. After perineal washing (Pickett, 1973) a sterile long-sleeved glove was used to insert a 30 French Foley catheter with a 30-ml inflatable cuff through the cervix. To ensure a snug fit against the internal os of the cervix, the cuff was inflated via a sterile syringe with ≥30 ml air and pulled caudally. Sixty ml of sterile physiological saline were infused through the catheter via a sterile syringe into the uterine lumen. The uterus and uterine horns
were manipulated per rectum for 5 minutes after which the flush containing the uterine secretions was slowly massaged out of the uterus, through the catheter and collected by gravity flow into a sterile 60 ml syringe case. Samples were immediately refrigerated standing upright in the syringe case. All of the mares were collected following the same sequence at the same time each day. Collection time was recorded and then each mare was immediately administered intravenously 2 g phenylbutazone or 10 ml physiological saline.

Visual examination of the recovered uterine flushing was then conducted and categorized as follows: (1) clear, appeared to have no uterine particles and looked like the physiological saline infused into the uterus; (2) cloudy, had diffuse white or slightly yellow particles and some sediment; (3) purulent, had a thick white or yellow sediment.

The uterine flushes were allowed to stand overnight in the refrigerator. Every morning the entire volume of each uterine flushing was measured, the supernatant was poured into another sterile 60 ml syringe case for storage at -20°C until analysis and the sediment was discarded. Percent of flushing medium recovered was calculated and recorded.

For the current experiment, sodium heparin and ethylenediaminetetraacetate (EDTA) anticoagulant vacutainers were utilized to harvest jugular blood samples from mares. Ethylenediaminetetraacetate samples were designated to confirm circulating phenylbutazone levels. Sodium heparinized samples were designated to determine circulating progesterone concentrations, but they were also utilized to run duplicate plasma samples because there was a shortage of EDTA.
plasma for the experiment. During the process of assaying plasma samples for phenylbutazone concentrations, it was noted there was a distinct chromatographic peak height difference between EDTA and sodium heparin blood samples even though they were collected one right after the other from the same mare. Therefore, a concurrent experiment evolved to determine if plasma from the 2 anticoagulants gave different phenylbutazone concentration values (Appendix IV).

Heparinized and EDTA blood samples were collected daily from each mare via jugular venipuncture. Progesterone concentrations were analyzed by the solid-phase, no extraction Coat-A-Count assay kit described in Appendix III.

Two preliminary studies were conducted in our laboratory. The first one determined that removing the organic extraction solvent in a stream of air instead of nitrogen did not adversely affect the determination of phenylbutazone concentrations in uterine secretions or plasma (Appendix V). The second one determined that phenylbutazone concentrated in the supernatent rather than the sediment (Appendix VI).

Concentrations of phenylbutazone in uterine samples were determined by HPLC as described for plasma samples by Taylor et al. (1981). The following modifications were made (1) the organic solvent was removed in a stream of air instead of nitrogen; (2) 25 ml aliquots of uterine flushing were used instead of 2 ml; (3) 2 ml 10% (w/w) hydrochloric acid was used instead of 1 ml; (4) residue was dissolved in 50 μl of tetrahydrofuran instead of 150 μl.

A Waters Associates 254 Model-440 liquid chromatograph was used with a prepacked Whatman Partisil PXS 10/25 Column No. 1B separation
column and an HC Pellosil CKS1 guard column.

Statistical analysis was performed using an unpaired Student's t-test (Snedecor and Cochran, 1980) to determine if there was a significant difference in percent of uterine flushing medium recovered from inflamed versus normal uteri in both treated and control groups. A Chi-Square analysis (SAS, 1988) was used to determine if there was a significant difference in incidence of uterine inflammation between phenylbutazone-treated and physiological saline control mares.

RESULTS

A total of 109 samples of uterine secretions were collected from treated mares (n = 4) and were monitored for phenylbutazone. Of these, 53, 28 and 28 secretions were categorized as clear (48.6%), cloudy (25.7%) and purulent (25.7%), respectively. Nine samples were positive for phenylbutazone. Of these, 6 and 3 samples were from purulent and cloudy secretions, respectively. Percentage of secretions positive for phenylbutazone content from phenylbutazone-treated mares was 8.33% (Table 10). Two phenylbutazone positive samples demonstrated a trace estimated at ≤40 and 120 parts per billion, respectively. Concentrations of phenylbutazone in the other 7 uterine secretion samples that tested positive were 3.0, 3.5, 6.0, 12.0, 13.0, 25.0 and 31.5 µg/ml, respectively.

A total of 122 uterine secretion samples from physiological-saline control mares were analyzed for phenylbutazone content and none of these were positive for phenylbutazone. Of the total, 99 (81.1%), 14 (11.5%) and 9 (7.4%) were categorized as clear, cloudy and purulent, respectively (Table 10).
More uterine secretion samples from control mares were analyzed than from treated mares because pretreatment samples were collected from each mare to be used to run standard curves and preliminary HPLC studies (Appendix VIII).

Mares with cloudy and purulent secretions were thought to have uterine inflammation. Inflammation was also evaluated by measuring the volume of secretion recovered. Secretions were grouped as normal uterus (clear) and inflamed (cloudy + purulent). More fluid was recovered from inflamed uteri than the 60 ml physiological saline infused, suggesting fluid accumulation. Means (± SEM) uterine fluid volume recovered from control mares were 45.6 ± 0.2 ml and 129.7 ± 9.8 ml for normal and inflamed uteri, respectively. Mean uterine fluid volume recovered from phenylbutazone-treated mares was 47.5 ± 14.5 ml and 125.4 ± 2.5 ml for normal and inflamed uteri, respectively (Table 11).

Volume of uterine secretions recovered from inflamed uteri was larger (P < 0.01) than from normal uteri in both treatment and control groups (Table 11). Chi-Square analysis determined there was a greater (P < 0.001) incidence of uterine inflammation in mares being treated with phenylbutazone compared with saline-treated control mares.

Results of the progesterone assay determined that 2 of 4 control and 2 of 4 treated mares had active corpora lutea (progesterone values > 1 ng/ml). Two control and 2 treated mares did not have active corpus lutea as determined by progesterone values < 1 ng/ml. Of the 9 uterine secretion samples that were phenylbutazone positive, 7 had a concurrent progesterone value of < 1 ng/ml and 2 had
progesterone values of 17.94 and 18.50 ng/ml, respectively.

Table 10. Results of visual examination of uterine secretions recovered from phenylbutazone-treated and physiological saline (PS) control mares and of high performance liquid chromatography (HPLC) analysis for phenylbutazone (PBZ) content

<table>
<thead>
<tr>
<th>Visual appraisal</th>
<th>Treatment</th>
<th>HPLC analysis (treated mares)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline no.</td>
<td>PBZ no.</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Clear</td>
<td>99</td>
<td>81.1</td>
</tr>
<tr>
<td>Cloudy</td>
<td>14</td>
<td>11.5</td>
</tr>
<tr>
<td>Purulent</td>
<td>9</td>
<td>7.4</td>
</tr>
<tr>
<td>Total</td>
<td>122</td>
<td>109</td>
</tr>
</tbody>
</table>

<sup>a,b</sup>Percents with different superscripts within column are significantly different (P < 0.001)
Table 11. Mean percent and volume of uterine flushing medium recovered from phenylbutazone-treated and physiological saline (PS) control mares

<table>
<thead>
<tr>
<th>Uterine treatment</th>
<th>condition</th>
<th>(n)</th>
<th>Percent (%)</th>
<th>Volume(ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Normal</td>
<td>99</td>
<td>75.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.6</td>
</tr>
<tr>
<td></td>
<td>Inflamed</td>
<td>23</td>
<td>176.8</td>
<td>129.7</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>Normal</td>
<td>53</td>
<td>78.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47.5</td>
</tr>
<tr>
<td></td>
<td>Inflamed</td>
<td>56</td>
<td>180.3</td>
<td>125.4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Flushing significantly different (P \( \leq 0.01 \)) between conditions

<sup>b</sup> 60 ml of physiological saline was infused into the uterus

**DISCUSSION**

Phenylbutazone was detected only in cloudy and purulent uterine secretions from phenylbutazone-treated mares. Results suggested that phenylbutazone accumulated in detectable amounts (HPLC) only in mares with an inflamed uterus. Inflammation was appraised in phenylbutazone positive secretions by presence of a mucopurulent sediment and/or a volume of recovered medium greater (P \( \leq 0.01 \)) than that which was infused into the uterus, which suggested accumulation of other fluids in the uterus as described by Archbald (1976), Higgins and

Results show there was a 2 and 3-fold increase in the number of uterine samples categorized as cloudy and purulent, respectively, for phenylbutazone-treated mares when compared with cloudy and purulent samples from control mares. Results suggest that phenylbutazone treatment enhances \(P \leq 0.001\) the chances of uterine infections in mares. This possibility was also suggested in Experiment I because 40% of the phenylbutazone-treated mares exhibited pyometra at the time of embryo collection when compared to 25% of the control mares.

Results showed a doubling (6 versus 3) of the number uterine fluids samples in which phenylbutazone accumulated in detectible amounts in purulent flushes (indicating heavy inflammation) compared with cloudy flushes (suggesting slight inflammation). An explanation of this finding may be that phenylbutazone adhered to the leucocytes found in the sediment because phenylbutazone is 98% protein bound (Tobin, 1981b).

Phenylbutazone accumulated in detectible amounts in only 9 of the 56 (16.1%) uterine flushings appraised as those from inflamed uteri. This suggests that phenylbutazone does not necessarily accumulate at all times in all mares exhibiting a uterine infection even though it appears that phenylbutazone potentiates a uterine inflammatory condition.

During the course of the experiment, 50% of the treated and 50% of the control mares had active corpora lutea (progesterone > 1 ng/ml). Therefore, it appeared that phenylbutazone had no affect on luteal function.

Progesterone concentrations determined that phenylbutazone
accumulated in inflamed uteri under both high and low progesterone concentrations. Seven of 9 (77.7%) phenylbutazone positive uterine flushings had concurrent progesterone values of < 1 ng/ml while 2 of 9 (22.2%) had high progesterone concentrations. Phenylbutazone accumulation in the inflamed uterus appeared to be associated with low peripheral progesterone concentrations. This finding is unexplained at this time. This phenomenon may or may not have had a physiological basis. It is contrary to that reported in the cow where the progesterone-dominated bovine uterus is more susceptible to uterine infections than the estrogen-dominated uterus (Black et al., 1953; Rowson et al., 1953).

The source of the uterine inflammation was not ascertained. It appeared that it was the result of daily manipulation and intubation of the uterus. All pretreatment samples were clear and the amount of flushing medium recovered was less than that which was infused, which concurred with uterine secretions from normal uteri. This finding confirmed that all uteri were normal before that daily saline infusions started. Therefore, uteri became inflamed due to the daily intubation procedure and was potentiated (P < 0.001) by chronic phenylbutazone treatment.
EXPERIMENT III

Effect of Phenylbutazone on Luteolysis Induced by Uterine Biopsy in the Mare

INTRODUCTION

Results of Experiment I demonstrated that chronic administration of phenylbutazone to mares exhibiting a nonpathological uterus did not affect estrual behavior, ovulation or length of estrous cycle. Results of Experiment II suggested that phenylbutazone accumulated primarily in the inflamed uterus of the mare. It was then proposed that parenterally administered phenylbutazone will accumulate in an inflamed uterus, interrupt prostaglandin synthesis and, therefore, extend the estrous cycle of the mare.

The method chosen to specifically inflame the uterus was endometrial biopsy. Uterine biopsy performed on day 4 post-ovulation shortened the estrous cycle of mares by inducing an inflammation that stimulated an increased synthesis and release of uterine prostaglandins resulting in premature luteolysis (Hurtgen, 1975; Hurtgen and Whitmore, 1978; Hurtgen and Ganjam, 1979; Baker et al., 1981).

OBJECTIVES

The objective of this experiment was to test the hypothesis that parenterally administered phenylbutazone in the mare will accumulate in the uterus that has been inflamed by biopsy and inhibit uterine PGF$_2$α synthesis. Consequently, to evaluate if the phenylbutazone treatment will block the luteolysis induced by the uterine biopsy performed on day 4 post-ovulation and will extend the estrous
MATERIALS AND METHODS

Seven mares of mixed breeding, between 4 and 6 years of age and averaging 450 kg body weight, were purchased and maintained as an experimental group at the Louisiana State University School of Veterinary Medicine. Each mare was given a thorough general physical and breeding soundness examination prior to purchase. Prior to and during the period of study (March through mid-August) mares were fed 8 liters of mixed grain concentrate/mare/day, grass hay and water free choice. All mares were in good body condition at the beginning of treatment and were subsequently maintained in large pasture lots with native grass cover during the study.

Mares were teased every morning with an androgenized gelding to determine the onset of estrus and each estrous mare was rectally palpated to evaluate ovarian structures on a daily basis. Mares were monitored for estrual activity during untreated estrous cycles starting in mid-February. After mares had exhibited 2 normal length estrous cycles (18 to 24 days), they were allotted to treatment on the first day of the third cycle. On the first day of the treatment estrous cycle each mare was administered intravenously 2 g phenylbutazone (Butazolidin, 200 mg/ml; D-M Pharmaceuticals, Kansas City, MO.) in Treatment A. On the first day of the control estrous cycle each mare was administered intravenously 10 ml of sterile physiological saline in Treatment B.

Treatments were continued daily until each mare exhibited an estrous response. Mares were treated with phenylbutazone or physio-
logical saline using a switchback experimental design alternating between Treatment A and Treatment B on successive estrous cycles. The first mare exhibiting a third estrus received phenylbutazone, the second received physiological saline. Remaining mares were alternated into treatments in this manner. Assignments were made until the end of the cyclic season in mid-August. The switchback treatment design was used to aid in reducing individual animal variability within the experiment by allowing each treated mare to also serve as her own control.

The day of ovulation (day 0) was determined by rectal palpation and later verified by peripheral plasma progesterone levels. On day 4 post-ovulation all mares were subjected to uterine biopsy using the instrument and methods previously described (Hurtgen and Whitmore, 1978; Baker et al., 1981). Following uterine biopsy, mares were teased each morning (0800 hours) and then either Treatment A or Treatment B was continued on a daily basis. Estrual behavior was evaluated from the response of each mare to the androgenized gelding and later verified by plasma progesterone concentrations.

Blood samples were collected prior to administration of Treatment A or Treatment B on a daily basis via jugular venipuncture. Circulating progesterone levels on the first day of estrus, on the day of presumed ovulation, on the day of biopsy and on the day of induced estrus verified follicular development, a functional corpus luteum and ensuing luteal regression. Plasma progesterone concentrations were determined by a no-extraction solid-phase RIA (Appendix III). Coefficient of variation for both intra-assay and inter-assay analysis was 5.8% and 7.2%, respectively, for all plasma samples.
evaluated in this study.

Mean plasma progesterone levels for specific days and mean number of days from uterine biopsy to the next estrous period for both treated and control cycles were compared for significance using a Student’s paired t-test (Snedecor and Cochran, 1980).

RESULTS

A total of 19 estrous cycles (10 treated and 9 control cycles) were evaluated (Table 12). Mean number of days (± SEM) from uterine biopsy to the next estrous period was 9.20 ± 0.34 days for treatment cycles and 5.00 ± 0.16 days for control cycles. These mean intervals to estrus were significantly different (P < 0.025) with phenylbutazone treatment cycles longer than control cycles.

Plasma progesterone concentrations verified low concentrations (< 1 ng/ml) at ovulation and at the estrus after uterine biopsy (Figure 12). Ovulation and the presence of functional luteal tissue at the time of uterine biopsy (day 4) were also verified by progesterone levels (> 5 ng/ml).
Table 12. Mean intervals from biopsy to estrus and plasma progesterone (P$_4$) concentrations in mares treated daily with either phenylbutazone (PBZ) or physiological saline (PS)

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment$^a$</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 g PBZ daily</td>
<td>Saline daily</td>
<td></td>
</tr>
<tr>
<td>No. of cycles evaluated$^b$</td>
<td>10</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Mean interval from biopsy to estrus (days)</td>
<td>9.20$^c$</td>
<td>5.00$^d$</td>
<td>±0.34</td>
</tr>
<tr>
<td>Mean plasma P$_4$ concentrations at day 0 (ng/ml)</td>
<td>0.98</td>
<td>0.88</td>
<td>±0.34</td>
</tr>
<tr>
<td>Mean plasma P$_4$ concentrations at day 4 (ng/ml)</td>
<td>15.56</td>
<td>14.21</td>
<td>±1.72</td>
</tr>
<tr>
<td>Mean plasma P$_4$ concentrations at induced estrus (ng/ml)</td>
<td>0.75</td>
<td>0.44</td>
<td>±0.37</td>
</tr>
</tbody>
</table>

$^a$Mean (± SEM)

$^b$A total of 7 cyclic mares allotted to a switchback design

$^c,d$Means with different superscripts in rows were significantly different (P < 0.025)
Figure 12. Plasma progesterone profiles for a typical experimental mare (No. 7) for treatment cycles 2 and 3 using the switchback design. During treatment cycle number 2 this mare was administered 2 g of phenylbutazone (PBZ) daily until it exhibited an estrous response. On the first day of treatment cycle number 3 this mare was administered physiological saline until the subsequent estrus.
DISCUSSION

Results support the hypothesis presented in the objectives of this experiment indicating that treatment with phenylbutazone can delay luteolysis induced by uterine biopsy on day 4 post-ovulation. It seems reasonable to assume that acutely inflamed uterine tissue, resulting from the biopsy, sequestered phenylbutazone in adequate concentrations to inhibit synthesis of uterine prostaglandins. In this regard, it has been previously shown that phenylbutazone does accumulate in inflamed tissues (Brune et al., 1976; Tobin, 1979a). This finding agrees with the results of Experiment II, which showed measurable levels of phenylbutazone in uterine flushings collected from phenylbutazone-treated mares that exhibited an inflamed uterus. Results of Experiment II showed that uterine flushings from phenylbutazone-treated mares containing purulent material also contained measurable levels of phenylbutazone. Uterine flushings from treated and control mares in which there was no evidence of uterine inflammation did not have a detectable level of phenylbutazone.

The occurrence of estrus at 5.0 days following uterine biopsy during the early luteal phase of the equine estrous cycle in the control group (Treatment B) agrees with the findings of others (Hurtgen, 1975; Hurtgen and Whitmore, 1978; Hurtgen and Ganjam, 1979; Baker et al., 1981). Presumably, uterine biopsy caused release of uterine prostaglandin resulting in luteolysis and return to estrus in these mares.

From the results of the present experiment and those previously reported from this laboratory, it is proposed that phenylbutazone will cause inhibition of prostaglandin synthesis with a consequental
blockage of luteolysis only in those mares with an existing acute uterine inflammatory reaction. These previous experiments also demonstrate that the synthesis and cyclic release of uterine prostaglandin from a nonpathological equine uterus appears to be unaffected by parenteral treatment of the mare with phenylbutazone.
EXPERIMENT IV

Effect of Chronic Administration of Phenylbutazone on Fertilization, Embryo Size and Embryo Prostaglandin Synthesis in Mares

INTRODUCTION

Results of Experiment II showed that parenterally administered phenylbutazone accumulated in detectable amounts in mares exhibiting an inflamed uterus. Results from Experiment III suggested that phenylbutazone accumulated in the equine uterus that had been stimulated by biopsy on day 4 post-ovulation and effectively inhibited uterine prostaglandin synthesis that is necessary for luteolysis.

It is proposed that biopsy illicits an inflammatory response. Vasodialation is a major feature of inflammation (Higgins and Lees, 1984; May et al., 1987). Vasodialation is also a prominent response of pregnancy. Ford (1985) reported a 2 to 3-fold increase in uterine blood flow to the gravid uterus of the cow, sow and ewe during the late luteal phase of early pregnancy coinciding with maternal recognition of pregnancy. It was proposed that pregnancy will sequester parenterally administered phenylbutazone to the uterus in adequate concentrations to inhibit uterine and embryo prostaglandin synthesis and consequently inhibit equine embryo development.

In Experiment I, 1 viable embryo was recovered from a phenylbutazone-treated mare. This result demonstrated that fertilization and embryo viability were possible in phenylbutazone-treated mares. Further investigation was deemed necessary to determine if fertility and embryo viability were inhibited in phenylbutazone-treated mares.

An extensive literature search showed no method previously
established for preparing the equine blastocyst for quantification of embryo prostaglandin synthesis.

OBJECTIVES

The objectives of this experiment were to (1) test the hypothesis that increased blood flow to the gravid equine uterus will increase the accumulation of phenylbutazone that will interrupt uterine prostaglandin synthesis and therefore affect early embryonic development; (2) test if phenylbutazone daily administered to pregnant mares will interrupt conceptus prostaglandin synthesis; (3) to substantiate previous data from our laboratory regarding fertility and embryo viability in mares treated with phenylbutazone; (4) to develop an acceptable method to collect and process the equine blastocyst for extraction of embryo synthesized prostaglandins.

MATERIALS AND METHODS

Fourteen mares of mixed breeding (averaging 450 kg body weight) between 4 and 6 years-of-age were used in this experiment. Mares were maintained from May to September on a grass pasture at the Louisiana State University School of Veterinary Medicine with mineralized salt free-choice and supplemented with 8 liters mixed grain/mare/day. Mares were teased daily by a stallion and ovulation was determined using daily rectal palpation once a day at mid-morning. Mares were mated by natural service once or twice a day after detecting a follicle ≥ 30 mm in diameter. Treated mares were administered 2 g phenylbutazone (Butazolidin®; D-M Pharmaceuticals, Kansas City, MO.) intravenously (Treatment A). Control mares were similarly
treated with 10 ml physiological saline (Treatment B). Treatment A or B started on the first day of estrus and continued daily thereafter until the day of embryo collection. Non-surgical embryo collection was performed as described (Imel et al., 1981) starting on days 8, 9 or 10 post-ovulation (day 0 = palpation day of follicle rupture). Dulbecco's phosphate-buffered saline (PBS) with 1% fungizone and antibiotic was used the as flushing medium (Appendix Table I.)

Recovered embryos were immediately placed into a sterile petri dish containing 6 ml flushing medium (Appendix Table II). The embryo was then examined for morphological development under the stereomicroscope. Embryos were then photographed at 6x, 12x, 25x and 50x using a 35 mm Nikon camera attached to a Wild M5D light microscope. Each embryo was then transferred to a capped tube containing 6 ml sterile PB-1 holding medium at room temperature for transport to the laboratory. Embryos were then prepared by one of 3 different methods for prostaglandin synthesis determination by RIA.

The first method was labeled homogenization, incubation and extraction (HIE). The embryo was transferred into a glass homogenizing vial filled with 10 ml PB-1 holding medium and placed on ice. Embryos were homogenized in each method by 10 strokes of a glass teflon homogenizing apparatus attached to a hand drill. One ml aliquots of homogenate were put into plastic tubes and incubated for 0, 5, 10, 20, 30, 40, 50 and 60 minutes in a 37°C shaking water bath. The incubation was stopped by the addition of 20 µl of 5% formic acid. Then, 5 ml of ethyl acetate was added to each tube to extract prostaglandins. The solution was allowed to stand at room tempera-
ture for 30 minutes and the extraction phase was then repeated. The tubes were then evaporated in a stream of N₂, reconstituted in 2 ml phosphate buffered saline-gel and sonicated. Aliquots of 600 µl from each incubation time (set of 3 tubes) were covered and stored at -20°C until assayed by RIA (Hwang, 1985) for PGE₂, PGF₂α and 6-keto-PGF₁α (the PGI₂ metabolite).

The second method was labeled intact embryo incubation and extraction (IIE). One day 8 embryo was put in 10 ml flushing medium in a sterile covered embryo collection dish and incubated in a 37°C incubator for 5, 24, 48 and 72 hours. Another day 8 embryo was incubated in a sterile capped glass vial containing 4 ml PB-1 holding medium in a 37°C water bath for 0, 6, 12 and 18 hours. One ml aliquots of medium were drawn off at the prescribed times, extracted and frozen as previously mentioned.

The third treatment was homogenization (H). The day 10 embryo was homogenized in 4 ml PB-1 holding medium. The homogenate was not extracted but rather frozen in 600 µl aliquots.

Uterine flushing medium recovered along with each embryo was also assayed for prostaglandin concentrations. One ml aliquots from every embryo collection medium were extracted and frozen.

Embryos from control mares were assayed for prostaglandins by RIA as described by Hwang (1985). Results showed a distinct PGE₂ and PGI₂ peak at 5 minutes of incubation for the day 9 HIE embryo. A day 10 HIE embryo peaked at 60 minutes for PGE₂. Consequently, serial incubation time for future HIE embryos was stopped at 30 minutes. The day 8 IIE embryos had a PGE₂ and PGI₂ peak at 24 hours, but the concentration was one-third less than the 5 minute HIE peak. The
homogenized (H) embryo showed a very high PGE$_2$ content compared with HIE and IIE embryos. Results suggested that in order to stay safely within the RIA assay standard curve, day 9 embryos should be collected and processed as HIE for the remainder of Experiment IV.

Three more control and 4 treated embryos were collected on day 9 post-ovulation, processed as HIE, frozen and assayed. Another day 10 control embryo was collected and homogenized (H) but this time in 10 ml PB-1 instead of 4 ml in order to study an appropriate tissue dilution factor. One uterine flushing sample was assayed from all 5 control and 4 treated HIE embryos. All these embryos and uterine flushings were assayed by RIA.

Data was tested for significance using SAS (SAS Institute, 1988) analysis of variance with a split plot design. Data was tested for significance using SAS (SAS Institute, 1988) Student's unpaired t-test.

RESULTS

The 4 embryos from the phenylbutazone-treated mares demonstrated a standard zona pellucida and capsule reaction under microscopic examination (12x). All cellular aspects of the blastocysts appeared normal. However, embryos from the phenylbutazone-treated mares were noticeably smaller and more fragile than embryos from control mares. Embryos from control mares had a thick white capsule, but the embryos from the phenylbutazone-treated mares had a thin translucent capsule. One embryo from a phenylbutazone-treated mare broke apart simply being drawn into the transfer pipette.

Results of the RIA assays for prostaglandins are presented in
Tables 13, 14 and 15. The prostaglandin peak in Table 13 for a day 9 HIE embryo at 5 minutes incubation in the preliminary prostaglandin assay was not demonstrated by any other embryos.

Incubation time was not significant, but there was a highly significant difference of prostaglandin synthesis between embryos from phenylbutazone-treated mares and embryos from saline-treated control mares for PGE$_2$ (P < 0.0001), PGF$_{2\alpha}$ (P < 0.0001) and PGI$_2$ (P < 0.0004). Three of 4 (75%) embryos from phenylbutazone-treated mares synthesized no detectible amounts of PGF$_{2\alpha}$ or PGI$_2$. Prostaglandin E$_2$ appeared to be the dominant prostaglandin synthesized by the day 9 blastocyst from phenylbutazone-treated mares. Data of embryos from control mares shows PGE$_2$ and PGF$_{2\alpha}$ were produced in approximately equal concentrations and PGI$_2$ approximately two-thirds less. A summary of means for the prostaglandin assay is present in Table 17.

Data of embryos from control mares shows another prostaglandin synthesis pattern (Table 14). The day 9 HIE embryo synthesized higher concentrations of PGI$_2$ than PGE$_2$. Day 8 IIIE embryos had synthesis of PGI$_2$ dominating between 0 to 18 hours, but PGE$_2$ was dominant at 24 to 72 hours. The control day 10 H embryo showed another pattern with PGF$_{2\alpha}$ being dominant at near double the concentration of approximately equal concentrations of PGE$_2$ and PGI$_2$.

The 3 prostaglandins assayed were also detected in uterine flushings. There was no significant difference in prostaglandin concentration between uterine flushings recovered from phenylbutazone-treated and saline control mares for all 3 prostaglandins evaluated in this experiment (Table 16).

Results in Table 13 show a peak PGE$_2$ and PGI$_2$ at 24 hours for
day 8 IIE and a much higher prostaglandin synthesis for day 10 HIE than day 9 HIE. A review of the methods and summary of results is presented in Table 14. Homogenization produced a high prostaglandin concentration. It remained undetermined if it was the procedure or the embryo itself that produced the high prostaglandin concentration.
Table 13. Results of preliminary radioimmunoassay for prosta­
glandins E₂ and I₂ synthesized by equine embryos recover­
ed from saline-treated control mares on days 8, 9
or 10 post-ovulation

<table>
<thead>
<tr>
<th>Dayᵃ</th>
<th>Treatmentᵇ</th>
<th>Timeᶜ</th>
<th>PGE₂ ng/ml</th>
<th>PGI₂ ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>HIE</td>
<td>0 minutes</td>
<td>0.62</td>
<td>0.00*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>8.95</td>
<td>18.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0.16</td>
<td>0.13</td>
</tr>
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<td>0.13</td>
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<td>0.11</td>
<td>0.10</td>
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<tr>
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<td>40</td>
<td>0.12</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
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<td>50</td>
<td>0.13</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>0.17</td>
<td>0.44</td>
</tr>
<tr>
<td>10</td>
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<td>-d</td>
</tr>
<tr>
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<td></td>
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<td>10</td>
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<td>10.54</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>IIE</td>
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<tr>
<td></td>
<td></td>
<td>6</td>
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<td></td>
<td></td>
<td>24</td>
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<td>48</td>
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</tr>
<tr>
<td>10</td>
<td>H</td>
<td>-</td>
<td>13.13</td>
<td>-</td>
</tr>
</tbody>
</table>

ᵃNumber of days ovulation to embryo collection
ᵇTreatment to prepare embryo for assay
ˢHIE = Homogenized, Incubated, Extracted
 sIIE = Intact embryo, Incubated, Extracted
 sH = Homogenized
ᶜIncubation time
ᵈAssay not run
*Peak prostaglandin concentration
Table 14. Equine embryo treatment groups and peak prostaglandin E$_2$ concentrations from Table 13

<table>
<thead>
<tr>
<th>Day of cycle</th>
<th>Treatment of embryo</th>
<th>Incubation time of peak PG</th>
<th>PGE$_2$ (ng/ml)</th>
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<tbody>
<tr>
<td>8</td>
<td>IIE$^a$</td>
<td>24 hours</td>
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</tr>
<tr>
<td>9</td>
<td>HIE$^b$</td>
<td>5 minutes</td>
<td>8.95</td>
</tr>
<tr>
<td>10</td>
<td>HIE</td>
<td>60 minutes</td>
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</tr>
<tr>
<td>10</td>
<td>H$^c$</td>
<td>0 minutes</td>
<td>13.13</td>
</tr>
</tbody>
</table>

$^a$IIE = intact embryo incubated and medium extracted  
$^b$HIE = homogenized embryo, medium incubated and then extracted  
$^c$H = homogenized embryo (no incubation or extraction)
Table 15. Prostaglandin synthesized by day 9 post-ovulation embryos recovered from physiological saline (PS) and phenylbutazone treated mares. Embryos were homogenized, incubated and extracted.

<table>
<thead>
<tr>
<th>Mare number</th>
<th>Incubation time (minutes)</th>
<th>PGE₂</th>
<th>PGF₂α</th>
<th>PGI₂</th>
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<tr>
<td><strong>Saline</strong></td>
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<td>28</td>
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<td>0.12</td>
<td>0.14</td>
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<td>0.15</td>
<td>0.12</td>
<td>0.04</td>
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<td>0.16</td>
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</tr>
<tr>
<td>24</td>
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</tr>
<tr>
<td><strong>Phenylbutazone</strong></td>
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<td></td>
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</tr>
<tr>
<td>28</td>
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<tr>
<td></td>
<td>30</td>
<td>0.07</td>
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Table 16. Prostaglandin concentrations for uterine flushings recovered with day 9 equine embryos

<table>
<thead>
<tr>
<th>Mare treatment group</th>
<th>Prostaglandin (ng/ml)</th>
</tr>
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<tr>
<td></td>
<td>PGE₂</td>
</tr>
<tr>
<td>Physiological saline</td>
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<td></td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>0</td>
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</table>
Table 17. Mean prostaglandin concentrations for equine uterine
flushings and day 9 equine embryos from phenylbutazone
(PBZ) treated and physiological saline (PS) control mares

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>n</th>
<th>(\text{PGE}_2) ng/ml</th>
<th>(\text{PGF}_{2\alpha}) ng/ml</th>
<th>(\text{PGI}_2) ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterine flushing(^a)</td>
<td>PS</td>
<td>5</td>
<td>0.066 ± 0.010</td>
<td>0.216 ± 0.121</td>
<td>0.004 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>PBZ</td>
<td>4</td>
<td>0.043 ± 0.025</td>
<td>0.595 ± 0.575</td>
<td>0.113 ± 0.113</td>
</tr>
<tr>
<td>Embryos(^b)</td>
<td>PS</td>
<td>3</td>
<td>0.159 ± 0.007(^c)</td>
<td>0.150 ± 0.007(^c)</td>
<td>0.052 ± 0.008(^c)</td>
</tr>
<tr>
<td></td>
<td>PBZ</td>
<td>4</td>
<td>0.112 ± 0.006</td>
<td>0.012 ± 0.006</td>
<td>0.002 ± 0.007</td>
</tr>
</tbody>
</table>

\(^a\)Mean (± SEM)
\(^b\)Least-Squares Mean (± SEM)
\(^c\)Treatment significantly different (P < 0.001)
DISCUSSION

Results suggest that fertilization was not impaired by parenteral treatment of mares with phenylbutazone. Analysis of embryo photographs suggests that treatment did not affect the zona pellucida reaction and other standard morphological developments. Embryos recovered from phenylbutazone-treated mares appeared viable, but less likely to survive compared with embryos from control mares.

Most of the embryos from phenylbutazone-treated mares were difficult to locate in the flushing medium without a microscope while embryos from control mares were easily visible. Inhibition of embryo expansion appeared to occur in phenylbutazone-treated mares similar to the results reported for the embryos of indomethacin-treated rabbits (Hoffman et al., 1978). Because embryos were not systematically photographed in this experiment to determine diameter, this observation was not statistically evaluated. It was then decided to photograph every embryo in the subsequent experiment to determine its diameter. This apparent size difference also generated interest in determining whether embryo protein content was affected by phenylbutazone treatment of the mare. Thus, future embryos were also to be prepared for the Lowry protein assay.

Results from this experiment suggest that phenylbutazone administered to pregnant mares significantly inhibited embryo synthesis of prostaglandins $F_2\alpha$, $E_2$ and $I_2$. This finding may have been the cause of inhibition of ovum transport, blastocyst hatching, blastocyst expansion, embryo implantation, fetal development and live birth rate reported by other researchers (see Table 6).
The dominant prostaglandin synthesized by equine embryos changed with age of the embryos. This finding concurs with Hwang et al. (1988) who reported transitional changes in prostaglandin synthesis by bovine embryos at different developmental stages.

PGF$_2\alpha$ was the dominant prostaglandin detected in uterine flushings, PGE$_2$ was intermediate and PGI$_2$ was the lowest concentration (Table 16). The dominance of PGF$_2\alpha$ is in agreement with Bergland et al. (1982) and Sharp et al. (1984), who reported a significant increase of uterine luminal PGF$_2\alpha$ synthesis and/or secretion in pregnant mares due to transcervical lavage stimulation in the presence of a prostaglandin antagonist.

Results showed no significant difference in uterine prostaglandin synthesis between phenylbutazone-treated and saline control mares. However, phenylbutazone treatment did significantly affect embryo prostaglandin synthesis. Therefore, the effect of phenylbutazone on diminished size appears to associate with the embryo itself rather than the uterine environment.

The PGE$_2$ and PGI$_2$ peaks at 5 minutes incubation for the HIE embryo was not demonstrated by any other embryos. However, this embryo produced PGE$_2$ concentrations for the other incubation times falling within close range to those assayed at the end of the experiment (Table 15). Therefore, it was concluded that the peak was caused by a laboratory error.

Comparison of the day 10 H embryos in Table 13 and Table 15 homogenized in 4 ml and 10 ml PB-1 holding medium, respectively, suggests that 4 ml is too concentrated and 10 ml is too dilute in order for the embryo prostaglandin concentrations to fall within the
standard curve of the assay. It was concluded that future embryos should be placed in 6 ml PB-1 holding medium for incubation.

Due to overall laboratory time, the HIE method was discarded. Day 10 embryos appear to synthesize the 3 prostaglandins investigated. A decision was made to collect day 10 embryos and incubate them for 24 hours in 6 ml PB-1 holding medium in the subsequent experiment.

Natural service breeding at 6:00 P.M. daily appeared to be more effective than A.M. artificial insemination every other day. When stallion power was available, natural service was conducted twice a day (A.M. and P.M.) for any mare with a ≥ 45 mm follicle.

Damaged embryos were found in the filter apparatus attached on top of the 2 liter collection cylinder after flushing 3 mares on 3 separate attempts. When a mare strongly contracted her abdomen, the flushing medium was expelled so forcefully through the tubing that the embryos broke apart when they hit the filter screen. Therefore, the filter was removed. Outflow tubing was placed directly into the 2 liter cylinder and then embryos gently sank to the bottom of the collection cylinder. The top portion of the medium was then poured off and the lower portion slowly poured through the filter to trap the embryo. Both modifications thereafter enhanced embryo recovery rates and were utilized in the subsequent experiment.
EXPERIMENT V

Effect of Chronic Administration of Phenylbutazone on Fertilization, Embryo Diameter, Embryo Prostaglandin Synthesis and Total Embryo Protein in Mares

INTRODUCTION

Results from Experiment IV substantiated results from Experiment I demonstrating that fertilization and embryo viability were observed in mares being chronically treated with phenylbutazone.

Results of Experiment IV also showed that mare phenylbutazone treatment appeared to inhibit embryo expansion and embryo prostaglandin synthesis. It was proposed to conduct a subsequent experiment utilizing the same protocol as Experiment IV except to also determine embryo diameter and embryo total protein. Methods investigated in Experiment IV that appeared to be the most appropriate were utilized in Experiment V.

OBJECTIVES

The objectives of this experiment were to (1) substantiate data from Experiment IV; (2) determine embryo diameter; (3) quantitate embryo prostaglandin synthesis; (4) quantitate total embryo protein; (5) use the experimental protocol designed from results of Experiment IV.

MATERIALS AND METHODS

Test mares and all experimental procedures were the same as Experiment IV except that all embryo collections were performed on day 10 post-ovulation, a Bivona equine uterine flushing catheter
(Bivona Inc., Gary, IN.) was used to accommodate the larger embryo and modifications were made in embryo processing. Within 15 minutes after embryo collection, the embryo was placed in a sterile petri dish and photographed at 6x, 12x, 25x and 50x magnifications. Embryos were transferred via sterile pipette to a capped sterile glass tube containing 6 ml PB-1 holding medium and incubated in a 37°C water bath for 24 hours. Then, 3 aliquots (1 ml) of medium were transferred to 3 parafilm covered glass vials and stored at -20°C until assayed for prostaglandin content by RIA. The embryo was homogenized in the remaining 3 ml of medium with a glass teflon homogenizing apparatus attached to a Dayton Workshop bench-top drill press (Model 4Z660A) and frozen for Lowry protein determination.

First, 6 embryos from control mares were collected. Then, mares were treated with 2 g phenylbutazone intravenously and 6 embryos were collected from these mares. Because embryos from phenylbutazone-treated mares appeared smaller than those from control mares and it was thought perhaps the diminished size was due to advanced breeding season rather than treatment, additional control embryos were collected, 3 to 4 months after the first embryos were collected from control mares.

Embryo diameters were measured with a Wild stereomicroscope equipped with a 35 mm camera and micrometer to measure across the embryo from zona pellucida border to zona pellucida border with a transparent metric ruler. First, a micrometer was placed under the microscope where the embryos were photographed on top of a 35 mm slide. Slide length and width gradations were counted at 6x and 12x magnifications.
Homogenized embryo samples were thawed at room temperature and assayed according to modified Lowry protein determination (Cooper, 1977). A standard curve was determined using 0 to 250 µg bovine serum albumin (Fraction-V; Sigma Chemical Co.) as standard protein. Samples were diluted 1:40 with 0.1 N NaOH. One-half ml phenol reagent (1N) was added to each tube. Absorbance was read at 540 nm on a Baush and Lomb Spectronic 2000. Data were tested for significance using SAS (SAS Institute, 1988) Student’s unpaired t-test.

RESULTS

Evaluation of embryo photographs showed that parenteral maternal phenylbutazone treatment did not affect fertilization or blastocyst viability. Morphological development appeared typical.

Results of embryo diameters determined at both 6x and 12x magnifications are presented in Table 19. The embryos from phenylbutazone-treated mares were ≈1 mm smaller than embryos from control mares. Mean (± SEM) embryo diameters at 6x and 12x magnifications were 2.54 ± 0.56 mm and 2.42 ± 0.20 mm for embryos from control mares and 1.41 ± 0.18 mm and 1.80 ± 0.27 mm for embryos from phenylbutazone-treated mares, respectively. Embryos from phenylbutazone-treated mares were significantly (P < 0.05) smaller at both 6x and 12x than embryos from controls mares.

Embryo total protein results are presented in Table 19. There was no significant difference in total protein between embryos from phenylbutazone-treated and saline control mares. However, embryos from phenylbutazone-treated mares tended to contain more protein (mean ± SEM) (28.01 ± 0.94 µg) than those from control mares.
Data suggested that as the breeding season advanced, the trend was for protein content to increase whether in embryos from phenylbutazone-treated or saline control mares. Therefore, a linear regression analysis was used to determine if a relationship existed between embryo collection dates throughout the experimental season and protein content. Days of embryo collection were numbered 1 to 117 starting with the first to last embryo collection. There was a highly significant \((P < 0.0007)\) \((r = 0.66)\) correlation between advancement of breeding season and increased embryo total protein content.

Results of prostaglandin embryo synthesis are presented in Table 20. There was no significant difference between embryos from phenylbutazone-treated and saline control mares for prostaglandin synthesis, although PGF\(_2\alpha\) approached significance \((P = 0.06)\). A comparison of mean prostaglandin concentration presented in Table 21 suggests reduced concentrations of all 3 prostaglandins synthesized by embryos from phenylbutazone-treated mares as compared with those from control mares.
Table 18. Diameter at 6x and 12x magnifications and total protein (TP) of embryos recovered on day 10 post-ovulation from physiological saline control and phenylbutazone-treated mares

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Mare#</th>
<th>Treatment</th>
<th>6x(mm)</th>
<th>12x(mm)</th>
<th>TP µg/embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>135</td>
<td>C</td>
<td>2.19</td>
<td>2.74</td>
<td>22.83</td>
</tr>
<tr>
<td>B</td>
<td>139</td>
<td>C</td>
<td>1.17</td>
<td>1.71</td>
<td>23.10</td>
</tr>
<tr>
<td>C</td>
<td>135</td>
<td>C</td>
<td>2.04</td>
<td>2.23</td>
<td>24.23</td>
</tr>
<tr>
<td>D</td>
<td>88</td>
<td>C</td>
<td>2.19</td>
<td>2.57</td>
<td>23.45</td>
</tr>
<tr>
<td>E</td>
<td>2</td>
<td>C</td>
<td>2.34</td>
<td>2.57</td>
<td>24.87</td>
</tr>
<tr>
<td>F</td>
<td>39</td>
<td>C</td>
<td>1.46</td>
<td>1.89</td>
<td>25.37</td>
</tr>
<tr>
<td>M</td>
<td>88</td>
<td>C</td>
<td>2.63</td>
<td>3.26</td>
<td>32.37</td>
</tr>
<tr>
<td>N</td>
<td>88</td>
<td>C</td>
<td>6.28</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G</td>
<td>88</td>
<td>P</td>
<td>1.02</td>
<td>1.20</td>
<td>23.71</td>
</tr>
<tr>
<td>H</td>
<td>46</td>
<td>P</td>
<td>1.46</td>
<td>1.89</td>
<td>30.01</td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>P</td>
<td>1.61</td>
<td>1.97</td>
<td>28.87</td>
</tr>
<tr>
<td>J</td>
<td>139</td>
<td>P</td>
<td>1.17</td>
<td>1.37</td>
<td>28.96</td>
</tr>
<tr>
<td>K</td>
<td>39</td>
<td>P</td>
<td>1.02</td>
<td>1.37</td>
<td>27.12</td>
</tr>
<tr>
<td>L</td>
<td>3</td>
<td>P</td>
<td>2.19</td>
<td>3.00</td>
<td>29.39</td>
</tr>
</tbody>
</table>

C = saline-treated control mares  
P = phenylbutazone-treated mares  
TP = total protein (µg)  
* Embryo too large a diameter to fit in field at 12x and was broken when transferred to the incubating vial in the laboratory.
Table 19. Prostaglandin synthesis of intact incubated equine embryos recovered on day 10 post-ovulation from physiological saline control and phenylbutazone-treated mares

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Mare</th>
<th>Treatment</th>
<th>Prostaglandin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PGE₂</td>
</tr>
<tr>
<td>A</td>
<td>135</td>
<td>C</td>
<td>36.00</td>
</tr>
<tr>
<td>B</td>
<td>139</td>
<td>C</td>
<td>9.24</td>
</tr>
<tr>
<td>C</td>
<td>135</td>
<td>C</td>
<td>17.58</td>
</tr>
<tr>
<td>D</td>
<td>88</td>
<td>C</td>
<td>4.20</td>
</tr>
<tr>
<td>E</td>
<td>2</td>
<td>C</td>
<td>*</td>
</tr>
<tr>
<td>F</td>
<td>39</td>
<td>C</td>
<td>8.18</td>
</tr>
<tr>
<td>G</td>
<td>88</td>
<td>C</td>
<td>1.85</td>
</tr>
<tr>
<td>H</td>
<td>88</td>
<td>P</td>
<td>0.70</td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>P</td>
<td>12.39</td>
</tr>
<tr>
<td>J</td>
<td>139</td>
<td>P</td>
<td>1.83</td>
</tr>
<tr>
<td>K</td>
<td>39</td>
<td>P</td>
<td>3.56</td>
</tr>
<tr>
<td>L</td>
<td>3</td>
<td>P</td>
<td>*</td>
</tr>
</tbody>
</table>

C = saline-treated control mares
P = phenylbutazone-treated mares
* = high concentration and off standard curve

Table 20. Mean concentrations (± SEM) of prostaglandin synthesized by day 10 equine embryos recovered from physiological saline (PS) control or phenylbutazone-treated mares

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>PGE₂</th>
<th>PGF₂α</th>
<th>PGI₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS control</td>
<td>6</td>
<td>12.84 ± 5.12</td>
<td>1.94 ± 0.64</td>
<td>0.78 ± 0.32</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>6</td>
<td>6.23 ± 2.60</td>
<td>0.46 ± 0.15</td>
<td>0.21 ± 0.08</td>
</tr>
</tbody>
</table>
DISCUSSION

Results of Experiment V substantiated results from Experiment IV regarding fertilization, embryo viability, embryo development and embryo prostaglandin synthesis. As in Experiment IV, most of the embryos from phenylbutazone-treated mares were difficult to locate with the naked eye in flushing medium while embryos from control mares were clearly visible.

Embryo photographs showed that fertilization and blastocyst viability were not impaired by mare phenylbutazone treatment. Embryos from phenylbutazone-treated mares appeared to be viable, but less likely to survive compared with embryos from control mares. It appeared that embryo expansion was inhibited. Embryos from phenylbutazone-treated mares were smaller ($P < 0.05$) at both 6x and 12x magnifications than embryos from control mares.

The diminished diameter of the embryos from phenylbutazone-treated mares compared with the diameter of the embryos from control mares coincide with the findings of other researchers (Hoffman, 1978; Hoffman et al., 1978; Lacroix and Kann, 1982). Blastocyst diameters from female rabbits receiving 8 mg indomethacin/kg subcutaneously twice daily on days 4 to 6 of pregnancy were significantly smaller than the diameter of control embryos (Hoffman et al., 1978). Also, when 8 mg indomethacin/kg was administered subcutaneously twice daily to rabbits on days 4 to 7 of gestation nearly complete inhibition of fetal development resulted (Hoffman, 1978). The number of fetuses that survived to day 14 were smaller than controls and their associated decidual and placental tissues weighed less. A lower dose of 3 mg indomethacin/kg administered subcutaneously twice daily on days 4
to 7 of gestation likewise reduced fetal, placental and decidual weights.

Lacroix and Kann (1982) treated ewes on days 7 to 22 of gestation with 300 mg indomethacin resulting in diminished embryo weights on day 23 of treated (67 ± 14 mg) compared with embryo weights of the controls (90.5 ± 28 mg). These results suggest that parenteral maternal treatment with prostaglandin antagonists inhibit embryo development.

Theoretically, embryo diameters at 6x and 12x should be the same size. However, diameters in Table 19 show 12x larger than 6x. Possibly, magnification values differed due to distortion through medium in the petri dish where the embryo was floating when photographed and/or the lens system in the camera and microscope.

Four embryos did not follow the trend of embryos from control mares being ≥1 mm larger than embryos from phenylbutazone-treated mares. Variances from the mean were possibly due to the true age of the embryo. It was possible that some embryos may have been as much as 12 hours older than others due to ovulation palpating error, hour of ovulation, time on day 10 of embryo collection and/or date of embryo collection. For example, if a mare ovulated at 2300 day -1 and was collected day 10 late in the afternoon, the embryo would be at a more advanced growth stage compared with an embryo from a mare that ovulated at 0700 hours on day 0 and was collected at noon day 10.

Witherspoon and Talbot (1970) reported most mares tend to ovulate at night between 2300 and 0700. Neely (1983) reported once the equine embryo enters the uterus, it increases rapidly in size to
By day 8, the vesicle diameter is ≈2 mm (Ginther, 1979). Possibly, the mares producing the smaller control embryos B and F ovulated early in the morning on day 0. They were collected at 1145 and 1245, respectively. Embryos L, M and N were collected in September at 1200, 1230 and 1700 hours, respectively. Embryos M and N from control mares were the last and largest embryos collected and embryo L was the last and largest embryo collected from a phenylbutazone-treated mare. The larger size of these 3 embryos may have been due to an extra half-day growth when compared to those embryos collected early in the day.

Day 10 embryos from phenylbutazone-treated mares were approximately the same size as a normal day 8 embryo. It appeared that as the breeding season advanced, embryos were larger for both treated and untreated mares. However, there was no significant correlation between season and embryo diameter. Perhaps late summer hot ambient temperatures advanced ovulation to early day -1. It is proposed that parenteral maternal phenylbutazone treatment rather than season was the cause of diminished size of embryos from phenylbutazone-treated mares.

Results suggest that maternal phenylbutazone treatment may inhibit embryo prostaglandin synthesis. Results showed reduced concentrations of PGF$_2$α, PGE$_2$ and PGI$_2$ by embryos from phenylbutazone-treated mares compared with those from control mares. The diminished synthesis of prostaglandins in embryos from phenylbutazone-treated mares compared with those from control mares was not significant in Experiment V. However, the difference was highly significant in Experiment IV. The reasons for this discrepancy may
have been due to age of the embryo (day 9 versus day 10) and/or the difference in methods utilized to prepare the embryo for quantification of prostaglandin production.

From these data it appears the prostaglandin synthesized in highest quantity by the day 10 equine embryo is PGE$_2$, with PGF$_2\alpha$ being intermediate and PGI$_2$ the least synthesized. This finding concurs with those from Hwang et al. (1988) who reported bovine embryos < 12 days of age followed the same pattern of prostaglandin synthesis.

In summary, results of this study suggested parenteral maternal phenylbutazone treatment may inhibit embryo expansion and prostaglandin synthesis, but does not affect fertilization, embryo viability and total embryo protein. There is a significant correlation between chronological advancement (June to October) of breeding season and increase in total embryo protein.
SUMMARY OF CONCLUSIONS

In Experiment I, 36 estrous cycles of mares chronically treated with phenylbutazone were evaluated and compared with the reported normal mare estrous cycle range of 18 to 24 days. The majority of cycles (58.3%) were within the average mare estrous cycle range. Cycles that did not fall within the standard range were slightly longer (27.8%) or were very prolonged (8.3%). Three cycles were shorter than usual. The mean (± SEM) estrous cycle length for all 36 estrous cycles evaluated was 23.6 ± 0.98 days. This mean cycle lengths value concurred with previous experiments conducted by Archbald et al. (1983). These researchers used the same mares under the same experimental conditions as Experiment I and reported mean cycle length to be 23.1 ± 0.24 and 20.7 ± 0.28 for phenylbutazone-treated and saline-treated control mares, respectively.

Mean length of the estrous cycles was at the high end of the established mare estrous cycle range. This is likely due to the experimental period including winter months and the early spring transitional phase when cycles are usually longer than normal rather than due to phenylbutazone treatment. However, it should be considered that phenylbutazone may have had an effect on extending some of the estrous cycles. Perhaps phenylbutazone would have inhibited luteolysis in an experimental group housed in the northern latitudes where seasonality has a pronounced effect on mare cyclicity.

Thirty-eight ovulations that included 4 double ovulations were recorded for the same 36 estrous cycles evaluated. Follicular development and ovulation appeared normal. These findings suggest that chronic phenylbutazone treatment of mares did not inhibit
follicular development and ovulation.

After these 36 cycles were evaluated, mares were mated by artificial insemination. A viable embryo was recovered in 75% of the saline-treated control mares. One viable embryo from 5 (20%) phenylbutazone-treated mares was recovered. The pyometra exhibited at embryo collection may have been due to contaminated semen collection and insemination equipment. These same mares were used in Experiment IV but were mated by natural service from a different stallion and did not exhibit any pyometra.

Results from Experiment I could have been substantiated by having control mares for the cyclicity part of the experiment and by more breeding cycles in a switchback design. This was not possible due to lack of both available mares and time to breed and collect embryos before these mares were to be used for another purpose.

Experiment II results determined that phenylbutazone parenterally administered to mares accumulated in detectible levels in uterine flushes recovered from inflamed uteri of mares exhibiting either a high or low concurrent peripheral progesterone concentration. There was a trend for phenylbutazone positive uterine flushes to be from mares with a corresponding low progesterone concentration, as determined in 7 of 9 of the phenylbutazone positive secretions. Treated mares exhibiting a nonpathological uterine environment had no detectible phenylbutazone levels in uterine fluids.

Results suggest that phenylbutazone treatment enhanced (P < 0.001) the incidence of uterine infection. Possibly the pyometra exhibited in Experiment I was potentiated by the phenylbutazone treatment.
Experiment III results suggested that phenylbutazone treatment will cause inhibition of prostaglandin synthesis and blockage of luteolysis only in those mares with an existing uterine inflammatory reaction. The synthesis and cyclic release of uterine prostaglandin from a nonpathological equine uterus appears to be unaffected by parenteral treatment of the mare with phenylbutazone.

Results of Experiment III established a workable model to test the effect of other NSAID or corticosteroids on luteolysis.

Experiment IV results suggested that fertilization was not impaired by parenteral treatment of mares with phenylbutazone. Cellular aspects of the blastocysts recovered from phenylbutazone-treated mares appeared typical; however, the embryos were noticeably smaller and appeared more fragile than those from control mares. Assay results suggested that phenylbutazone significantly inhibited embryo synthesis of prostaglandins.

Results of Experiment IV suggested methods modifications to be used to design Experiment V. The embryo collection filter was removed from the collection system because embryos broke when the conceptus hit the filter screen. Results of the prostaglandin assay led to the decision to collect day 10 embryos and incubate them for 24 hours in 6 ml PB-1 holding medium. It was decided to subsequently photograph future embryos to determine diameter. A homogenization procedure was added to prepare embryos for the Lowry protein assay.

Results of Experiment V showed there was no significant difference between embryos from phenylbutazone-treated and saline control mares for prostaglandin synthesis; however, synthesis of \( \text{PGF}_2 \alpha \) by embryos collected from phenylbutazone-treated mares compared
with embryos from saline control mares approached significance ($P = 0.06$). There was a reduced concentration of all 3 prostaglandins synthesized by embryos from phenylbutazone-treated mares as compared with embryos from saline control mares. Results suggested that maternal phenylbutazone treatment may inhibit embryo prostaglandin synthesis and embryo expansion, but it apparently does not affect fertilization, embryo viability and total protein. There was a significant correlation between an increase in the length of the breeding season as it progressed from June to October and an increase in total amount of protein/embryo. This phenomenon may have been due to loss of embryo protein during the longer storage time of embryos collected early in the season compared with those collected in the fall. However, this may also have had a physiological basis.

Results of all experiments suggest that it would be acceptable to chronically treat a breeding mare exhibiting a nonpathological uterus with phenylbutazone until conception. After fertilization, however, the phenylbutazone may affect embryo development. Further studies are needed to access the affect of chronic treatment of pregnant mares with phenylbutazone on embryo development.
NOTES


Appendix III was published in Theriogenology, December, 1986, Volume 26, pp. 779-793 as part of a validation of this assay for general veterinary use.

Experiment II was presented at the 36th Annual Meeting of Animal Disease Research Workers in the Southern States, March, 1983.

Appendix IV was published in the Journal of Veterinary Pharmacology and Therapeutics 9, 227-229, 1986.

Experiment III was published in Theriogenology, 23, 381-387, 1985.
REFERENCES


# APPENDIX I

## Ingredients of modified Dulbecco's Phosphate-Buffered Saline Solution

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<thead>
<tr>
<th>Ingredient</th>
<th>Amount (g)</th>
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<tr>
<td>CaCl₂·2H₂O</td>
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</tr>
<tr>
<td>MgCl₂·6H₂O</td>
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</tr>
<tr>
<td>NaCl</td>
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</tr>
<tr>
<td>KCl</td>
<td>2.00</td>
</tr>
<tr>
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<td>Glucose</td>
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<tr>
<td>Streptomycin sulfate</td>
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</tr>
<tr>
<td>Na pyruvate</td>
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</tr>
<tr>
<td>Penicillin G, sodium (1,000,000 IU)</td>
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</tr>
<tr>
<td>Fetal calf serum</td>
<td>200 ml</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>q.s. to 10 liters</td>
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Adapted Imel et al. (1981)
### Ingredients of one liter PB-1 embryo holding medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tbody>
<tr>
<td><strong>Stock Solution</strong></td>
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<tr>
<td>Dulbecco's Phosphate Buffered Saline (PBS)</td>
<td>1 liter</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
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</tr>
<tr>
<td>Glucose</td>
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</tr>
<tr>
<td>Sodium pyruvate</td>
<td>0.036 g</td>
</tr>
<tr>
<td>Phenol red</td>
<td>1 ml</td>
</tr>
<tr>
<td><strong>The following ingredients were added to the stock solution just prior to use.</strong></td>
<td></td>
</tr>
<tr>
<td>Fetal Calf Serum (FCS)</td>
<td>10%</td>
</tr>
<tr>
<td>Penicillin (IU/ml)</td>
<td>100</td>
</tr>
<tr>
<td>Streptomycin (µg/ml)</td>
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</tr>
</tbody>
</table>
APPENDIX III

Validation of a new commercial, solid-phase, no-extraction
\(^{125}\)I progesterone radioimmunoassay kit for monitoring
luteal function in the mare

OBJECTIVE

To validate a new commercial, solid-phase, no-extraction \(^{125}\)I
progesterone radioimmunoassay kit for monitoring luteal function in
the mare.

MATERIALS AND METHODS

On December 1, 1980, and daily thereafter, a heparinized blood
sample was collected from each mare in Experiment I via jugular
venipuncture. Samples were immediately placed on ice for storage
until transfer to the laboratory. Samples were then centrifuged.
Plasma was poured into plastic vials and stored at \(-20^\circ\)C until
assayed.

During the sampling interval, mares were monitored via rectal
palpation and teasing to detect ovulation and behavioral estrus.

All samples (n = 547) were assayed by 2 different assay
procedures. First, plasma samples were assayed for progesterone
using a modified procedure of a previously validated RIA method as
described by Abraham et al., 1971. Nano-grade petroleum ether was
used for plasma extraction. Separation of free from bound steroid
was accomplished with dextran-coated charcoal. The specific antiser-
num used was CSU No. 337 (Colorado State University, Fort Collins,
CO). The intra- and inter-assay coefficients of variation were 9 and
10\%, respectively, for a plasma pool averaging 8 ng/ml of progester-
Secondly, a 100-μl volume of plasma was assayed using the commercial kit assay (Coat-A-Count No-Extraction Progesterone Assay, Diagnostic Products Corp., Los Angeles, CA), which had been previously developed for human serum. Seven progesterone standards (0.0, 0.1, 0.5, 2, 10, 20 and 40 ng/ml in human serum) were furnished with the kit assay for construction of the standard curve. In addition, a second set of standards was diluted with stripped equine plasma to determine if plasma components would interfere with the binding of the labeled hormone to the antibody.

A correlation analysis was utilized to evaluate relationships between the 2 assay methods.

RESULTS

An example of plasma progesterone levels from 2 cyclic mares (No. 1 and 3) from Experiment I using both the extraction assay and direct no-extraction assay methods are presented in Figure I. Results from the direct method are regressed against those from the extraction method for the same 2 mares in Figure II. For all 9 mares and all cycles used in the study there was good agreement for progesterone levels up to 2 ng/ml using both assay methods (Figure II). Above that level, the direct method resulted in increasingly higher progesterone values than the extraction method. Correspondingly, the slope of the regression line relating the 2 methods was not the same for all mares (Figure II). One mare in the group (No. 8) had higher progesterone values over the full range of sampling when monitored by the direct method (Figure II). The reason for this could not be
A total of 547 plasma samples from 9 cyclic mares was assayed by both methods. When all data were included, the formula best describing the relationship between the 2 progesterone data sets was exponential with a correlation coefficient of 0.938. The resulting equation was \( y = 1.27(x^{1.076}) \), where \( y \) was the result of the direct method and \( x \) was the result of the extraction method.

When the human serum standards were diluted 1:1 with stripped mare's plasma, the percent bound hormone was reduced to an average of 8.1% below the standards of equal progesterone concentration furnished with the kit (Figure III). When this diluted standard curve was used, the progesterone values for plasma samples were similar to those resulting from the extraction assay. These findings suggest that the 2 assays would have been closer in agreement if the standards were diluted in stripped equine plasma.

Plasma samples with progesterone values ranging between 20 and 28 ng/ml using the direct method were then diluted 1:1 with zero standards furnished with the kit and subsequently reassayed. When the results were corrected for the dilution, values were within 11.3% of the undiluted values, indicating that the assay did provide reproducible progesterone values in this range for mares.
Figure I. Plasma progesterone profile for 2 cyclic mares (No. 1 and 3) assayed by both an extraction, liquid-phase assay and the no-extraction, solid-phase assay. Estrus and ovulation in these mares were determined by teasing and rectal palpation of ovarian structures.
Figure II. Results from a direct assay regressed against results for the same samples assayed by an extraction assay. Data from two mares (No. 1 and 3, see Figure I) were pooled. Values from one mare (No. 8) are plotted separately because the plasma showed exaggerated values across all concentrations compared with other mares.
Figure III. Logit-log plot demonstrating the effect of adding stripped mare plasma at a ratio of 1:1 to the standards on binding of the radioactive progestrone to the antibody.
DISCUSSION

Results of the extraction, liquid-phase progesterone assay validated the use of the new, commercial, no-extraction solid-phase Coat-A-Count progesterone assay kit when exact concentrations are not necessary above 2 ng/ml. The extraction, liquid-phase method presented lower progesterone concentrations when compared with the Coat-A-Count kit.

The direct, solid-phase RIA kit offers a fast, convenient and acceptable method for monitoring luteal function during the estrous cycle of mares. The direct-assay progesterone concentrations may not be in complete agreement with those obtained by the extraction, liquid phase method, especially during diestrus. If absolute values for progesterone are required, an extraction step may be desirable with the direct RIA method. The practice of diluting the kit standards with stripped equine plasma may be an acceptable alternative.

The kit is of potential use to the equine industry for (1) detecting estrus in shy breeders who do not display estrual behavior; (2) to plan ahead for appointment breedings; (3) to confirm diestrus progesterone levels and then plan to feed a commercial progesterone supplement in order to prevent competition mares from coming into estrus during the meet.

The kit appeared to be acceptable for use in future experiments when mare cyclicity was to be evaluated rather than absolute progesterone concentrations.
APPENDIX IV

Effect of sodium heparin and ethylenediaminetetraacetate (EDTA) anticoagulants on phenylbutazone levels in equine plasma detected by high performance liquid chromatography

OBJECTIVE

The objective was to determine whether the anticoagulant used (sodium heparin or EDTA) alters the results from high performance liquid chromatography (HPLC) analysis for phenylbutazone in equine plasma.

MATERIAL AND METHODS

Plasma samples were obtained daily via jugular venipuncture at approximately the same time each morning before feeding. A 15 ml vacutainer (Becton-Dickinson, Rutherford, NJ) containing 286 USP units of sodium heparin was filled and detached and then an EDTA-K₃ (Becton-Dickinson, Rutherford, NJ) tube was placed on the same 20 gauge needle and filled. The vacutainers were immediately placed on ice. After the plasma samples were obtained, each treated mare was given 2 g phenylbutazone intravenously. Within 1 hour the vacutainers were centrifuged in a refrigerated centrifuge for 10 minutes at 2000 rpm. The plasma was immediately poured into sterile vials and stored at -20°C until analysis. Visual inspection revealed that all samples were free of hemolysis.

Concentrations of phenylbutazone in all plasma samples were determined by HPLC as described by Taylor et al., (1981), with minor
modifications. The organic extraction solvent was removed in a stream of air in place of nitrogen (Appendix V). The HPLC solvent (80:20 mixture of n-hexane:tetrahydrofuran containing 0.002% glacial acetic acid) was delivered at a flow rate of 2 ml/minute rather than 100 ml/hour. The same HPLC instrumentation was used as in Experiment II.

Peaks were measured with an ultraviolet absorbance detector at 240 nm and 0.1 absorbance units full scale using a strip chart recorder. The phenylbutazone and hexoestrol (internal standard) peaks were measured to the nearest mm by hand from the strip chart recorder.

Forty-two paired heparin and EDTA plasma samples were selected for analysis. Each sample was processed in duplicate. Pairs were extracted on the same day, dried under an air stream, covered and stored overnight at room temperature. The extracted samples were reconstituted and subjected to chromatography the next day. Extracts of the heparinized sample were always chromatographed first followed by the corresponding EDTA sample.

The phenylbutazone concentration was determined both by using the internal standard method, and by reference to an external standard curve of phenylbutazone alone. A paired Student's t-test (Snedecor and Cochran, 1980) was performed to determine if any difference existed between the mean EDTA and heparin plasma phenylbutazone concentrations by either the internal or external standard methods. Linear regression analysis (Snedecor and Cochran, 1980) contrasting the paired values for the 2 anticoagulants was performed. The regression equation for the internal standard method was:
\[ \text{[phenylbutazone-EDTA]} = 0.862 \text{[phenylbutazone-heparin]} - 0.142 \text{ug/ml.} \]

For the external standard method the equation was:

\[ \text{[phenylbutazone-EDTA]} = 0.819 \text{[phenylbutazone-heparin]} - 0.002 \text{ug/ml.} \]

When the highly significant difference between plasma phenylbutazone levels in samples collected at the same time using different anticoagulants was observed, a subsequent study was conducted. Plasma samples, either EDTA or heparin, collected from untreated mares were separately pooled. Phenylbutazone was added to aliquots of both the plasma pools at 15 known concentrations. The plasma samples were frozen, stored, processed and analyzed as in the first experiment. Peak height ratios and phenylbutazone peak heights of the paired samples at the 15 known concentrations were evaluated using the Student’s paired t-test.

RESULTS

Using either the internal or external standard methods, the heparinized samples gave in higher phenylbutazone values \((P < 0.01)\). Mean plasma phenylbutazone concentrations, calculated with the use of the internal standard, were 5.49 and 4.59 \text{ug/ml} for heparin and EDTA, respectively, and 4.98 and 4.07 \text{ug/ml} by use of the external standard method. For both the internal and external standard methods, a highly significant \((r = 0.96, P < 0.01)\) linear relationship was noted between the phenylbutazone concentrations of paired heparin and EDTA plasma samples.
Results of the subsequent study using pooled mare plasma spiked with phenylbutazone showed no significant differences between the phenylbutazone levels for the heparin or EDTA plasma pools for either the internal or external standard methods (Table I).

Table I. Peak height ratios and peak heights of pooled equine plasma spiked at 15 known phenylbutazone concentrations

<table>
<thead>
<tr>
<th></th>
<th>Peak height ratios</th>
<th>t-test value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Internal standard method</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>0.313 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.452</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.311 ± 0.06</td>
<td>(NS)</td>
</tr>
<tr>
<td><strong>External standard method</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>2.522 ± 0.40</td>
<td>1.007</td>
</tr>
<tr>
<td>EDTA</td>
<td>2.340 ± 0.40</td>
<td>(NS)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean (± SEM)

**DISCUSSION**

The initial study demonstrated an apparent difference in plasma phenylbutazone concentrations dependant on the anticoagulant used during blood collection. Heparinized plasma consistently yielded higher values than EDTA plasma. For several pairs of samples phenylbutazone was detected in the heparin sample, but trace levels or no phenylbutazone was detected in the EDTA sample. However, when pooled plasma was spiked with phenylbutazone to make standard curves, no differences were detected. For both EDTA and heparin plasma, the limit of detection was 0.03 µg/ml of plasma. Therefore, the difference does not appear to be attributable to anticoagulant effects on
the extraction or assay procedure. It has been reported (Taylor et al., 1983) that hemolysis reduces phenylbutazone concentration in plasma. No visible hemolysis was detected in any of the plasma samples used for these studies and does not appear to have been a factor. An area which remains unexplored is the effect of the 2 anticoagulants on erythrocytes which could allow sequestration of phenylbutazone by the erythrocyte. Apparently the erythrocyte and its separation from the phenylbutazone containing plasma is critical to this phenomenon, but no explanation is offered at this time.

The research investigator or regulator of performance horses and the veterinary practitioner should use heparinized plasma for phenylbutazone analyses, or at least specify which anticoagulant was used. Differences observed in absolute levels were significant and the potential failure to detect phenylbutazone in an EDTA plasma sample when it would be detected in heparinized plasma sample must be considered.
APPENDIX V

Preliminary high performance liquid chromatography procedures and modifications used to determine if the published method could be used in Experiment II

Preliminary studies were conducted to determine if the same analysis method described by Taylor et al. (1981) could be successfully used in this experiment to determine the concurrent concentrations of phenylbutazone in peripheral plasma and uterine secretions of mares.

Standard solutions were prepared exactly as the published method and injected several times into the HPLC system. The standard phenylbutazone concentration versus the peak height in centimeters (cm) resulted in a linear relationship as reported. Then, 10 μl of the same standards were used to spike 2 ml aliquots of pooled mare plasma collected in EDTA vacutainers. The exact extraction procedure was carried out, specifically removing the solvent in a stream of nitrogen at room temperature as described. A relatively linear relationship resulted for aliquots with up to 15 μg phenylbutazone/ml plasma. These results indicated that the plasma procedure was successful.

At this time it was decided to see if the same results could be obtained by removing the solvent in a stream of air rather than nitrogen because (1) a limited supply of nitrogen was available at the time; (2) nitrogen was too expensive to buy on a limited budget; (3) 1 extraction alone consumed about one half a nitrogen tank; (4) it would be difficult to keep a large supply of nitrogen on hand considering the number of samples to be analyzed for this experiment.
Duplicate 2 ml aliquots of EDTA plasma were spiked with 10 μl of 4.0 mg/ml, 2.0 mg/ml or 0.4 mg/ml phenylbutazone standards. One set was dried with air and the other with nitrogen. The results indicated that there was little difference in peak height and that the possibility of using air instead of nitrogen should be further explored (Table II).

Table II. First results of plasma phenylbutazone (PBZ) standards dried in either a stream of N₂ or air

<table>
<thead>
<tr>
<th>Plasma Std. Conc. (μgPBZ/ml plasma)</th>
<th>Peak Height (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
</tr>
<tr>
<td>20.0</td>
<td>4.45</td>
</tr>
<tr>
<td>10.0</td>
<td>1.09</td>
</tr>
<tr>
<td>2.0</td>
<td>2.07</td>
</tr>
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</table>

Next, the lowest and highest standard phenylbutazone concentrations were analyzed either dried in air or nitrogen (Table III).
Table III. Second results of plasma phenylbutazone (PBZ) standards dried in either a stream of air or \( \text{N}_2 \)

<table>
<thead>
<tr>
<th>Plasma Standard Concentration (( \mu g \text{PBZ/ml plasma} ))</th>
<th>Peak Height (cm)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
<td>Nitrogen</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>1.60</td>
<td>1.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.65</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.95</td>
<td>1.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.75</td>
<td>1.20</td>
<td></td>
</tr>
<tr>
<td>deleted*</td>
<td>1.10</td>
<td></td>
<td></td>
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Statistics

<table>
<thead>
<tr>
<th></th>
<th>Air</th>
<th>Nitrogen</th>
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<tbody>
<tr>
<td>Mean</td>
<td>1.74</td>
<td>1.22</td>
</tr>
<tr>
<td>SEM</td>
<td>0.15</td>
<td>1.61</td>
</tr>
<tr>
<td>CV</td>
<td>8.91%</td>
<td>13.20%</td>
</tr>
<tr>
<td>t-test</td>
<td>4.8808</td>
<td>(P &lt; 0.005)</td>
</tr>
<tr>
<td>df</td>
<td>7</td>
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*Technical error

<table>
<thead>
<tr>
<th>20.0</th>
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<tbody>
<tr>
<td>4.80</td>
<td>4.70</td>
<td></td>
</tr>
<tr>
<td>4.15</td>
<td>4.50</td>
<td></td>
</tr>
<tr>
<td>5.30</td>
<td>5.10</td>
<td></td>
</tr>
<tr>
<td>5.00</td>
<td>5.35</td>
<td></td>
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<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>4.81</td>
<td>4.91</td>
</tr>
<tr>
<td>SEM</td>
<td>0.49</td>
<td>0.38</td>
</tr>
<tr>
<td>CV</td>
<td>10.10%</td>
<td>7.80%</td>
</tr>
<tr>
<td>t-test</td>
<td>-0.3225 (NS)</td>
<td></td>
</tr>
<tr>
<td>df</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>
These results indicate that at the lower phenylbutazone concentrations there is a significant difference between drying with air and nitrogen, with the mean peak height of the air samples being greater than the nitrogen samples. At the higher phenylbutazone concentrations, no significant difference between the 2 drying processes was demonstrated. Considering the circumstances and these results, it was decided to air dry at room temperature all future samples.

Another standard curve was run with all samples being dried in a stream of air. Two ml aliquots of pooled EDTA mare plasma were spiked with 10 μl of the standard solutions. The standards were made in August, 1982, and this data was collected in December, 1982. It had been reported by previous researchers that the standards could be kept in the refrigerator for at least a month without significant degradation (Burns et al., 1953). Even though peak height versus phenylbutazone concentration resulted in a linear relationship (r = 0.9942) it was decided to avoid contamination and dilution effects (caused by opening and closing the standard vials) by discarding these standards and making new ones. In the future, new standards were to be made and used for only approximately a month.
High performance liquid chromatography preliminary procedure to determine if phenylbutazone concentrates in supernatant or sediment of uterine fluids

Purulent yellow uterine flushing secretions collected from control mares were pooled, measuring 250 ml. The solution was stirred with a magnetic stirrer for 20 minutes. Then, 800 μl of 0.05 mg/ml phenylbutazone standard was added and the mixture was stirred for 30 minutes. Then, it was centrifuged at 2800 rpm for 20 minutes. The supernatant was poured off in 20 ml aliquots into 6 tubes. The sediment pellet was resuspended in the remainder of the supernatant, measuring 125 ml.

The supernatant and resuspended sediment pellet solutions were divided into 25 ml aliquots and were analyzed in duplicate as described in Experiment II. An unpaired Student’s t-test comparing chromatographic phenylbutazone peak heights of the supernatant and the sediment determined that phenylbutazone concentrates in the supernatant ($P < 0.05$) of uterine secretions.
High performance liquid chromatography interassay, intrassay and limit of detection study

Determination of Reproducibility and Standard Curve

An interassay and intrassay study utilizing pooled plasma spiked with the standard phenylbutazone solutions was conducted in triplicate. Discrepancies were minimized in each assay by taking all samples from the same plasma pool, using only the fresh standards, and running the whole set of samples from the same batch of mobile phase. Each time, the samples were extracted one day and allowed to sit covered overnight at room temperature and were assayed the next day. Three sets of triplicate samples were assayed. Two ml of pooled EDTA mare plasma was spiked with 10 μl of standard solutions of 0.00, 0.05, 0.10, 0.20, 0.40, 1.0, 2.0 or 4.0 mg phenylbutazone /ml. Three samples of each concentration were analyzed in each assay.

Determination of Limits and Percent Recovery

The next study was to determine the extraction limit of detection, the HPLC instrument limit of detection and the percent recovery from both EDTA and heparin plasma samples. All samples were again extracted one day, and assayed the next day.

Both EDTA and heparin plasma samples were assayed together under the same experimental conditions to compare results. Two separate plasma assays were conducted. Each plasma sample contained 2 ml aliquots of pooled heparin or pooled EDTA plasma plus a known
amount of a single, 0.1 mg/ml standard solution. Both the EDTA and heparin samples were run in duplicate spiked with 2, 4, 8, 10, 12, 14, 16, 20, 22, 25, 30, 40, 45 or 50 µl aliquots of 0.1 mg/ml standard. Hamilton syringes were used to spike the samples. All plasma residues were reconstituted with 150 µl tetrahydrofuran and 10 µl were injected into the HPLC instrument. An instrument limit of detection was conducted at the same time as the extraction limit of detection in order to determine the percent recovery. All samples in each assay were run with the same batch of mobile phase. Care was taken to maintain the same experimental conditions for all plasma and instrument samples within an assay.
APPENDIX VIII

High performance liquid chromatography preliminary uterine flushing study

The amount of uterine flushing from phenylbutazone-treated mares necessary to elicit a positive response was determined by trial and error. A standard curve and a spiked uterine flushing sample were run with each assay to confirm that the extraction procedure worked. Two, 3, 4 and 5 ml aliquots of uterine flushings were assayed from mares giving a phenylbutazone positive plasma sample. The uterine samples were extracted and analyzed the same as plasma.

All cloudy uterine samples from untreated mares were negative for phenylbutazone. Flushings from 2 treated mares which appeared yellow and contained a sediment of purulent material were assayed in duplicate using 1, 4 and 8 ml aliquots. Plasma samples were phenylbutazone positive, but all uterine flushings were negative. Next, six 25 ml aliquots of flushings classified as clear (2), cloudy (2) or yellow purulent (2) were extracted. The extraction procedure was the same as plasma with the following exceptions (1) 2 ml HCL was used to deproteinize; (2) the residue was reconstituted with 50 µl tetrahydrofuran. All corresponding plasma samples were phenylbutazone positive. The 4 clear or cloudy flushes were negative, but the 2 purulent yellow flushes were phenylbutazone positive, one eliciting a phenylbutazone peak of approximately 40 parts per billion (ppb) and the other 120 ppb. It was then decided that all uterine flushings would be extracted as above in 25 ml aliquots because a positive response was obtained and it was a convenient size considering the
centrifuge and other laboratory equipment available.

The uterine flushing limit of detection and percent recovery study was conducted with pooled uterine flushings from the control mares. Twenty-five ml aliquots of flushings were spiked with 2, 4, 8, 10, 14, 16, 20, 22, 25, 30, 35, 40, 45 or 50 µl of 0.1 mg/ml standard solution. The residue was reconstituted with 50 µl of tetrahydrofuran and 10 µl of each sample were injected into the HPLC machine.

Next, an interassay and intraassay coefficient of variation was conducted on the uterine secretions. Four separate assays were run with 4 samples of 20, 25, 30 or 35 µl of 0.1 mg/ml standard solution in 25 ml aliquots of pooled flushing. Again, the residue was reconstituted with 50 µl tetrahydrofuran and 10 µl of each sample was injected into the HPLC instrument.
VITA

Mareth Ellsworth was born and raised in Dubuque, Iowa, U.S.A. She graduated from The Bishop's School, a private college preparatory boarding school for women located in La Jolla, California.

Ellsworth graduated from the University of California, Berkeley, California. After several years of pursuing her equestrian career, Ellsworth returned to academia and graduated from the Bel-Rae Institute of Animal Technology, Denver, Colorado, as a Colorado state certified animal technician. Ellsworth then enrolled in the preveterinary medicine curriculum for 3 years at Colorado State University, Fort Collins, Colorado. During this time, she participated in the bovine and equine reproduction studies at the Colorado State University Reproduction Station and decided to pursue reproduction with specializing in horses. Ellsworth transferred to the Louisiana State University School of Veterinary Medicine because there was an immediate position available for a graduate student in equine reproduction.

Ellsworth's equestrian career started with horse showing when she was 6 years old. By 10 years old, she was breaking in young horses. Since then, Ellsworth has trained and shown horses in all areas of the United States except the Northeast and in Australia. She has successfully shown horses at the local, regional and national levels. Her expertise has been in hunter pleasure, hunter/jumper, combined training horse trials, dressage, western reining and western pleasure.

During the past 7 years and currently, Ellsworth is the sole owner, manager, trainer and instructor at her own equine stable Red Oak Farm located in Livingston, Louisiana.
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Mareth Ellsworth

Major Field: Veterinary Medical Sciences (Physiology Option)

Title of Dissertation: EFFECTS OF CHRONIC ADMINISTRATION OF PHENYL BUTAZONE ON REPRODUCTION IN THE MARE

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Charles R. Short

Robert A. Drake

O. June Seiter

Mark J. Brey

Zanth C. Kopper

Date of Examination:

11/21/89