Excretory Capability of the Male Reproductive Tract

Cindy Sue Backus

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To the Graduate Council:

I am submitting herewith a dissertation written by Cindy Sue Backus entitled "Excretory Capability of the Male Reproductive Tract." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

Hugo Eiler, Major Professor

We have read this dissertation and recommend its acceptance:

John D. Smalling, J. B. McLaren, Leon N. D. Potgeiter, Fred Hopkins

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)
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[Signatures]

Accepted for the Council:

[Signature]

Vice Provost
and Dean of The Graduate School
EXCRETORY CAPABILITY OF THE MALE REPRODUCTIVE TRACT

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Cindy Sue Backus
May 1989
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ABSTRACT

The objective of this research was to investigate the effect of blood concentrations of different chemical families of substances (markers) when injected intravenously, on the kinetics of their passage into the bovine ejaculate.

Blood and semen were collected simultaneously and were analyzed for a given marker. The selection of markers for this research was based on biomedical significance and degree of chemical complexity. Markers were: Cortisol, progesterone, thyroxine, triiodothyronine, human chorionic gonadotrophin, Calicivirus (feline), penicillin, oxytetracycline, gentamicin, and carnitine.

Steroids, amino acids, glycoprotein, and antibiotics readily passed (within 20 minutes) into the ejaculate from blood. Seminal and blood concentrations of these markers were positively correlated.

The experiment with antibiotics showed that all antibiotics studied gained rapid entry into the ejaculate. It was established by vasectomy that gentamicin exclusively passed through the accessory glands.

Within a given chemical family, markers with a greater affinity for its plasma carrier proteins passed slower than those with smaller affinities. T3 passed faster than T4, and cortisol faster than progesterone.
Within a chemical family the molecular weight of a marker seems to be a secondary factor in determining the rate of transfer into the ejaculate from blood. Cortisol (MW 360) passed faster than progesterone (MW 314).

The relatively complex glycoprotein hormone HCG passed in detectable quantity into the ejaculate from blood. The quantity of HCG in semen was not large enough to cause spermatorrhea when frogs were injected with semen. This frog bioassay for HCG was not sensitive to the small amount of HCG in the semen. This could have been due to the relatively small dose of HCG injected into the bull. Conversely, bioassay experiments showed that all antibiotics used in these experiments were bioactive in semen.

Neither Calicivirus nor carnitine passed into the ejaculate from blood. Due to the complexity of the RNA-containing molecule Calicivirus particle (MW 10 million), it was not expected to pass. However, the fact that carnitine (MW 160) did not pass into semen in spite of a significant increase in blood concentrations was unexpected, since the epididymal source of carnitine is blood. The experiment with carnitine showed that regardless of the high affinity of the epididymis for carnitine, the injection of exogenous carnitine failed to increase (P > 0.05) the carnitine concentrations in the ejaculate. Furthermore, it was found that 79% of seminal carnitine is from epididymal-testicular origin and 21% was derived from accessory gland secretions.
This series of experiments shows that the chemical composition of the ejaculate in the bull is rather fortuitous and depends in part, on the chemical composition of blood around the time of ejaculation. This could have a practical implication when trying to manipulate reproductive efficiency.
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PART I

INTRODUCTION
STATEMENT OF THE PROBLEM

Justification

The male reproductive tract offers no barrier to many chemicals of exogenous origin. A number of such compounds cross into the fluid secreted by the testes and male accessory sex glands and ultimately pass into semen. This peculiar behavior of the male reproductive tract is largely inherent in the nature of the secretory mechanisms which operate in it.

Foreign chemicals are able to penetrate into the ejaculate in several ways. A variety of chemical substances pass into the testicular plasma. The accessibility of testicular plasma to a selective range of compounds has been amply demonstrated thanks to the availability of microsurgical cannulation and catherization techniques which permit the collection of substantial amounts of fluid from different regions of the exocrine system of the testis. Cannulation and micropuncture are used commonly as methods for obtaining epididymal semen and demonstrating the passage of exogenous chemicals into the semen, collected either entire or in separate fractions from distinct regions of the epididymis.

Not only exogenous substances, but a great many natural body constituents are secreted by the accessory glands. The secretion of accessory glands can carry chemicals into the semen.

In order to decide whether a given substance has entered the seminal plasma (together with epididymal, prostatic, vesicular,
bulbourethral, or urethral secretion), it is necessary to analyze each individual secretion separately from each accessory gland, or to collect the semen not as a whole, but in the form of fractions sequentially emitted during the ejaculatory process, and then carry out the analyses on the individual fractions of this "split ejaculate". In farm animals, split ejaculates can be procured easily by means of either an artificial vagina (e.g., in rabbits, stallions, and boars) or an electroejaculator (e.g., in bulls). Vasectomy of course is a useful technique to separate accessory gland secretions from testicular and epididymal secretions.

Certain natural body constituents are formed outside of the epididymis, but enter this organ subsequently from blood, and are accumulated by it. A good example is carnitine, a major natural constituent of epididymal semen. Carnitine is used as an epididymal marker in human clinical andrology. Contrary to carnitine, other body constituents that are excreted into the ejaculate such as glycoproteins, thyroid and steroidal hormones, do not accumulate in any tissue of the male reproductive tract. Finally, a large number of foreign substances such as antibiotics, are readily excreted into the ejaculate. Different kind of viruses have been isolated from semen, but it is not known whether virus particles can pass into the ejaculate directly from blood.

In general, the mechanism of secretion of substances into the ejaculate is ill defined. Many of the above findings are from studies in humans and laboratory animals. Very little of this research has been conducted in the bovine.
Objectives

The objective of this research was to investigate the effect of blood concentrations of different chemical families of substances (markers) on their passage into the bovine ejaculate. The selection of markers for this research was based on biomedical significance and degree of chemical complexity. Markers were:

1) Cortisol and progesterone. These are two small (MW. 360 and 314) steroid molecules with a relatively similar chemical structure, but having different plasma binding affinities. Both are known to pass into the ejaculate.

2) Thyroxine (T4) and triiodothyronine (T3). These are two small endogenous amino acids (MW. 777 and 651) with a relatively similar chemical structure, but they have different binding affinities for blood carrier proteins. T4 and T3 are known to affect sperm metabolism.

3) Human Chorionic Gonadotrophin (HCG). This is a relatively complex and large glycoprotein (MW. 22,000, approximately). It causes spermatorrhea in the frog, testosterone secretion in mammals and ovulatory effect in females. It is cleared by the kidney without loss of bioactivity.

4) Calici virus (feline). This virus represents a large single stranded RNA molecule with a complex protein coat (MW. over 10 million, 40 nanometers in diameter). It is not known to affect the bull reproductive tract.
(5) Penicillin, Oxytetracycline and Gentamicin. These represent three different chemical families of antibiotics. Penicillin consists of a thiazolidine ring and a beta-lactam ring to which is attached a side chain. Oxytetracycline is a polycyclic naphthacenecarboxamide, and gentamicin is an aminoglycoside. The molecular weight of these antibiotics varies between 400 and 800.

(6) Carnitine. Carnitine is a small molecule (MW. 161) related to hydroxybutyric acid that ferries fatty acids into the mitochondria. It is an epididymal marker with regulatory effects on both sperm motility and metabolism.

REVIEW OF LITERATURE

Introduction

The blood-testis barrier, which exists in the male reproductive tract, protects developing spermatozoa from both endogenous and exogenous substances in the blood (Setchell, 1978; Setchell and Waites, 1975). However, several different compounds are found in the ejaculate following their administration to the patient. The anatomical location and mechanism of entry of different compounds into the semen is unknown, but many of their effects are known to be detrimental (Mann and Lutwak-Mann, 1982). The ability to predict the effects of a compound on spermatozoa or fertilization depends on where it is entering the ejaculate and the amount of time it is in
contact with the sperm. Exogenous substances that are found in the ejaculate include chemicals, many antibiotics, drugs, hormones and various microorganisms. This paper will review these compounds and their effects on spermatozoa, the ejaculate and fertility.

Exploration of the excretory pattern of drugs and various chemicals into semen is of interest for many reasons. First, by knowing the relationship between chemical structure of substances and location of their passage into semen, testing procedures can be developed for evaluating the function of the male reproductive tract. Every day, our bodies are becoming exposed to more drugs and chemicals in the air, food and environment. The effects of these chemicals and drugs on reproduction in humans and animals are important. Determining if and how they influence the accessory glands, spermatozoa and spermatogenesis is important in understanding their effects on reproduction. Knowing the mechanism and anatomical location of their passage into the semen will help to better elucidate the influence they will have on reproduction. For example, drugs able to pass the blood-testes barrier will have an effect on spermatogenesis and immature spermatozoa, whereas chemicals passing via the accessory glands will influence only the mature, ejaculated semen and its ability to fertilize the ova.

**Chemicals**

Foreign chemicals are able to penetrate into the testis in several ways. Blood and lymph are the most obvious vehicles. An
alternate route is occasionally provided by the skin (after local application), as shown by the behavior of 'Tris-BP', a major flame-retardant chemical used at one time extensively for impregnating children's clothing (Blum et al., 1978). This mutagen and carcinogen produces testicular atrophy and sterility in rabbits after cutaneous application. More recently, 2,3-dibromopropanol, a metabolite of Tris-BP and a mutagen itself, has been found in urine samples of children who have been wearing Tris-BP treated sleepwear (Blum et al., 1978). Potential adverse reproductive effects of Tris-BP and similar compounds are of special concern to males since the scrotum is known to be more permeable to chemicals than other skin areas (Mann and Lutwak-Mann, 1982). For instance, organophosphates, a group of compounds widely used in agriculture as powerful insecticides, easily penetrates the skin of cattle. When applied to the hairy parts of a bull's skin, they produce only a minor and transient decline in motility of ejaculated spermatozoa, but application to the scrotal skin lowers much more distinctly the quality of ejaculated bovine semen (Mann et al., 1982). Dibromochloropropane (DBCP), a pesticide, has a suppressible effect on human spermatogenesis in factory workers exposed to its production (Potashnik et al., 1987). These men developed oligospermia or azoospermia in response to exposure to DBCP. Researchers have linked an increase in FSH to the severity of damage caused by DBCP, which may have a direct effect on Leydig cells and an effect on seminiferous tubules (Potashnik et al., 1987). These workers also postulated that DBCP may have its effects on germinal epithelium,
impairing the hormonal balance between the Sertoli cells and Leydig cells.

Inhalation of a toxic chemical constitutes yet another route along which that substance or its metabolites might enter the blood and ultimately reach the testis. Inhaled 1,2-dibromo-3-chloropropane, a soil fumigant against nematodes, possesses marked antispermatogenic properties. It caused severe degenerative changes in animal testes and atrophy of germinal epithelium in factory workers who had been chronically exposed to it (Potashnik et al., 1978).

**Antibiotics**

The most studied substances in the literature seem to be the antibiotics. Biswas and workers studied the effect of three different substances, oxytetracycline, acetylarsan (arsenic), and sodium salicylate, on semen characteristics of healthy three-year-old cross-bred rams (Biswas et al., 1980). Treatments were administered for 6-19 days, depending on the substance, and a follow-up on semen characteristics was continued for 60-64 days after treatment.

When oxytetracycline was given at 20 mg/kg (IV) for 10 days, there was a significant increase in sperm with tailless heads together, with decreased sperm motility and sperm viability in the post-treatment period. However, the semen characteristics did return to pretreatment values by day 60 post-treatment.
Acetylarasan given to rams at therapeutic dosage did not adversely affect semen characteristics. However, at three times therapeutic dosage, it produced a significant increase in the incidence of sperm with tailless heads (11.3%) and decrease in motility in days 16 to 52 of the post-treatment period. By day 60, semen characteristics were back to pretreatment values. The incidence of sperm with tailless heads in both instances may be ascribed to the slight increase in body temperature noted following administration of the drug at high dosage (Biswas et al., 1980).

Medication with sodium salicylate in therapeutic and higher dosage did not produce significant changes in different seminal parameters studied (Biswas et al., 1980). One criticism of this study is the small number of animals per group (Biswas et al., 1980). However, it does give the reader an idea of what may occur.

An investigation by Malmborg studied the excretion of ampicillin, erythromycin and doxycycline into human semen after oral administration of each drug (Malmborg et al., 1975). Doxycycline and erythromycin had similar clearance rates from blood and also followed similar excretion patterns into the semen. They were both present in the semen at 2 hours, reached maximal concentrations at 4 to 6 hours and were still present in the semen 24 hours after dosing.

Two hours after oral intake of trimethoprim the concentration of the drug in the seminal plasma was slightly higher than that in blood plasma. This is in agreement with Armstrong and others (1968) who demonstrated that some antibacterials become highly concentrated in the semen when patients had taken repeated doses over 24 hours or
longer. Examples of these include nitrofuratonin, methicillin, and cephalothin.

Armstrong and others (1968) studied the concentration of 16 antibiotics in the serum and semen of patients at 24, 48, 72 hours, 7 days and 14 days from when they began treatment. Drugs were administered daily for 7 days in recommended dosages. Rather than administering a single dose as in Malmborg's study (Malmborg et al., 1975), this work utilized a practical dosing regime normally used in a clinical setting in order to facilitate interpretation of the results by the clinicians.

Armstrong and others (1968) reported results regarding semen/blood ratio. Not only do several of the 16 drugs pass into the semen, but they are present up to 10 times the serum concentration. This observation becomes important when diseased states of the male reproductive tract are considered. In patients with inflammation, such as prostatitis, much more antibiotic passage into the ejaculate occurs (Armstrong et al., 1968). Increased antibiotic passage into the semen in states of inflammation has been documented by Soanes and workers (1963), who gave sulfoxisole to normal patients and patients with prostatitis or benign prostatic hyperplasia. In the normal patients, semen levels of antibiotic were only 40-90% of the blood levels. However, with prostatic inflammation, semen levels were 2.5 times the levels in whole blood (Soanes et al., 1968). Therefore, inflammation permits more antibiotic passage than would occur in normal tissue.
The work by Armstrong and others (1968) is in agreement with Winningham and associates (1968) working with antibacterial drugs in dogs. They found that diffusion of the drugs from the plasma to the prostate fluid was variable. For some antibiotics, the prostatic epithelium serves as a complete barrier to their passage from the plasma to the prostatic fluid, (streptomycin, chloramphenicol) (Winningham et al., 1968). By contrast, other antibiotics are actually concentrated in the prostatic fluid in amounts which exceed the plasma level by several times. Passage of some antibacterials and nonpassage of other antibacterials in this study may be influenced by the lipid solubilities and disassociation constants of each antibacterial agent (Winningham et al., 1968).

Drugs

The passage of drugs other than antibiotics have been investigated in terms of excretion into the seminal fluid. Lutwak-Mann and associates (1967) found thalidomide and its metabolites in rabbit semen after administering C-14 labeled thalidomide to male rabbits. In other studies, thalidomide became bound to rabbit spermatozoa, when added to it in vitro. In addition, progeny of thalidomide pretreated male rabbits exhibited poor neonatal survival, low birth weights and a high incidence of birth defects.

Similarly, male rats that were given methadone produced offspring with low birth weights and diminished neonatal survival (Swanson et al., 1978). Swanson clearly established that methadone
accumulates in the reproductive organs of the rabbit and achieves high concentrations in the semen relative to the levels in the blood. This is in agreement with Gerber and Lynn (1976) who demonstrated that in human subjects, methadone concentrations in semen were 1.8 times the drug levels in the blood.

Another area of interest is the effect of caffeine on spermatozoa and reproduction. Caffeine is present in many foods, drugs and beverages consumed by people and animals. In man, it has been shown to rapidly pass into the ejaculate in concentrations equal to concentrations in the blood (Beach et al., 1982). The in vitro addition of caffeine to semen has been noted to increase the motility, life span and forward progression of especially poor quality spermatozoa. Thus, caffeine may be able to enhance fertility (Hamm et al., 1984; Garbers et al., 1971), not only in freshly ejaculated semen (Harrison, 1978), but also in semen samples stored for AI (Schill et al., 1979; Calamera et al., 1984). Harrison (1978) used caffeine-enhanced semen in order to determine if it was more effective in promoting pregnancy in artificial insemination. This study concluded that although motility was truly stimulated, the increased activity did not result in more pregnancies. In fact, it achieved far less pregnancies than reported by others using AI. The addition of caffeine to spermatozoa was not recommended. In the mouse, caffeine has been found to accelerate the rate at which mouse spermatozoa become capacitated in vitro (Fraser, 1979).

In a reproduction/teratology study by Proctor and Gamble, rats were given solutions of distilled water, brewed regular coffee,
brewed decaffeinated or instant decaffeinated coffee (Nolen, 1982). At concentrations equivalent of 50 cups per day for humans, no deleterious effects were found on general reproduction or on the development of the embryo.

Wichman and associates (Wichman et al., 1983) have found that gossypol, a polycyclic compound isolated from cotton seeds, has a dose dependent inhibitory effect on human sperm motility. This drug also has adverse effects on some of the important metabolic pathways (glycolysis, TCA) used by the spermatozoa. Chronic administration of gossypol to rats was shown to lead to mitochondrial and flagellar damage in testicular and epididymal spermatozoa and to decrease the ATP content. This is what leads to the impaired motility of the spermatozoa. Gossypol is considered by the Chinese to be a safe and reversible male contraceptive if administered in small doses (National Coordinating Group, 1978).

**Hormones**

Several hormones (steroids, proteins, glycoproteins), have been detected in the seminal plasma. Sheth and others (1979) investigated the function of levels of prolactin in semen of monkeys with chlorpromazine (CPZ), a drug known to affect serum prolactin levels (Sheth et al., 1979). When acute treatment with CPZ was carried out, monkeys failed to give a semen sample. This is in agreement with Franks and others (1978) who have found that in clinical situations, high levels of prolactin have been known to cause impotency in men.
Maximum levels of prolactin in serum were found 6 hours after treatment and were back to normal on day 12. These results suggest a considerable lag period between prolactin levels observed for serum and seminal plasma. In other work by the same author (Franks et al., 1978), prolactin was found to stimulate sperm metabolism and fertilizing potential at lower dosages; whereas higher levels of prolactin were inhibitory. This study provides a good illustration of a pharmacological agent that could alter the serum levels of prolactin, which in turn could be reflected in the semen and thereby affect sperm fertilizing potential.

The presence of pituitary gonadotrophins in seminal plasma has also been reported. However, there are conflicting reports as to the relative concentrations of these pituitary hormones in seminal plasma as compared with levels normally found in serum.

DeAloysio and others (1974) reported similar concentrations of LH in serum and semen of humans which is in agreement with Schoenfeld and others (1978) who reported that both LH and FSH concentrations in blood and semen are similar. On the other hand, Sheth and others (1979) reported seminal plasma levels of LH to be significantly higher than those found in serum of humans.

In contrast to this work with humans, Sairam and others (1980) using the bull as a model, have found LH to be highest in the blood and FSH to be 100 times higher in the seminal plasma. In addition to species differences, different assay techniques may also be responsible for this variation. The physiological significance of
finding gonadotrophins in the seminal plasma in not known and their
effect on spermatozoa in these respects remains to be investigated.

According to Mann and Lutwak-Mann (1982) one of the more
significant developments in the biochemistry of semen in the last few
years has been the unequivocal demonstration of several steroid
hormones (testosterone, dihydrotestosterone, androstenedione,
progesterone, estradiol and estrone) in the seminal plasma.
Convincing evidence from studies done by Eiler and others (1977;
1981; 1979; 1987) shows that exogenously administered steroid
hormones reach the bovine ejaculate quickly and in appreciable
quantities. The concentration of steroids in the blood was highest
at 20-minutes and slowly declined until they were back to baseline at
the end of the 3-hour testing period. The clearance pattern of
steroids from the seminal plasma was the same as that for blood with
the highest concentration at 20-minutes and a slow decrease over the
3-hour testing period. Small amino acid hormones, triiodothyronine
(T3) and thyroxine (T4), when administered exogenously, have been
found to pass into the bovine ejaculate, although not as rapidly as
steroid hormones (Eiler et al., 1987). T3 was found to be
transferred faster than T4. Whether or not the high concentration of
T3 and T4 in the ejaculate modifies the characteristics of the
spermatozoa or accessory glands in unknown. However, in vitro
addition of T3 or T4 to spermatozoa does affect fructose metabolism
and fertility rate (Carter, 1932; Schultze et al., 1949).

Many exogenous compounds enter the ejaculate and affect the
spermatozoa directly or indirectly. What are the effects of a
decreased amount of a naturally occurring seminal component of the semen? Carnitine (3-hydroxy-4-trimethylamino-butyric acid), a naturally occurring seminal component, is found in semen of man and several animals, and is mainly epididymal in origin (Tomamichel et al., 1986). Although there is a large concentration of carnitine in the epididymal wall and in the epididymal fluid (Brooks, 1980; Casillas, 1972; Hinton and Hernandez, 1985), carnitine is not synthesized by the epididymus. It is derived from food and from liver biosynthesis (Brooks, 1980). Carnitine is preferentially absorbed by the epididymal cauda from blood, and concentrated in the epididymal fluid to about 2000-times the blood level (Hinton and Setchell, 1980; Hinton and Hernandez, 1985).

The relative uniqueness of carnitine to the epididymus suggests that the study of carnitine in semen, along with citrate and fructose, could be of diagnostic value in epididymal pathology and obstructive azoospermia (Wetterauer, 1980; Casano et al., 1987; Tomamichel et al., 1986; Soufir et al., 1984). In men, the level of testicular or epididymal obstruction correlates well with the carnitine concentration in the semen; the lower the carnitine level, the more distal the obstruction (Tomamichel et al., 1986). Seminal carnitine levels are also reliant on testosterone levels so caution must be used when interpreting low semen carnitine values. Vasectomy, in men, has been shown to cause a decrease, but not total disappearance of carnitine from the ejaculate (Golan et al., 1983; Wetterauer, 1980; Tomamichel et al., 1986). It has been estimated that in men about 58% (Frenkel et al., 1974), 85% (Casano et al.,
1987), and 95% (Wetterauer et al., 1980) of seminal carnitine originated in the epididymis. Studies of vasectomized males and those with obstructive azoospermia at a known level have found carnitine does originate in small quantities from other accessory glands of the male reproductive system. The prostate was found to contribute around 5% and the remaining glands (seminal vesicles, the ampulla and vas deferens) contribute 10% of the free carnitine to semen (Menchini-Fabris et al., 1984).

Carnitine is known to increase pyruvate metabolism which is important in in vitro sperm capacitation (Hutson et al., 1977). Bull spermatozoa have been shown to increase in carnitine concentration as they pass through the epididymal duct (Casillas, 1972; 1973). Therefore, a low carnitine concentration in the ejaculate would indicate an obstruction distal to the epididymus. This is what is seen in vasectomized males. In humans researchers have found seminal free-carnitine concentrations to correlate well with the level of epididymal obstruction (Tomamichel et al., 1986). In a recent study by Casano (1987), lower seminal levels of alpha glucosidase and glycerylphosphorylcholine along with carnitine were correlated with infertile men when compared with controls. However, seminiferous tubule damage also decreases the seminal concentration of these compounds to the range of vasectomized patients; that is, they had a reduction in epididymal function (Casano et al., 1987) along with it.

The kinetics and excretion of carnitine in the reproductive tract of the bull is not reported. It would be reasonable to consider that if carnitine is similarly excreted, it would be useful
determining the level of obstruction in bulls with obstructive azoospermia as it is in men.

**Microorganisms**

Viruses that have been isolated from bovine semen include Bluetongue virus (Howard et al., 1985), Bovine Virus Diarrhea virus, Paravaccinia virus (Johnston et al., 1971), and Infectious Bovine Rhinotracheitis. In humans HTLV-III (a retrovirus) (Ho et al., 1984), cytomegalovirus (Biggar et al., 1983) and Papillomavirus DNA (Ostrow et al., 1986), have all been identified in the semen of men. This could be a mechanism of transmission for these viruses.

Transfer of viral agents via semen, which has not been shown for most viral diseases, has the potential to be an important method of disease perpetuation. For example, ephemeral fever virus may be found in the semen of some bulls; foot and mouth disease virus may be excreted in the semen of some bulls before the onset of clinical disease; and IBR virus may contaminate the semen of bulls as a result of genital infection. In Switzerland (Kupferschmied et al., 1986), a bull stud was identified as the source of spread of IBR through the semen of one of its bulls. The bull in question had tested negative for IBR prior to semen collection for freezing and AI. However, the bull became infected with IBR during the period (several weeks) his semen was being collected and frozen. The nonreturn rate for this bull was 10% lower than other bulls his age. Also a series of cases where, within a single herd, only the animal(s) serviced by this bull
yielded antibodies to IBR. This led to the suspicion that the semen was the source of infection. The semen was tested using a Cornell Semen Test (Schultz et al., 1982), and the bull was found to be positive for IBR. Bluetongue virus has also been isolated from the semen of latently infected bulls (Phillips et al., 1986).

The Cornell Semen Test is able to identify Infectious Bovine Rhinotracheitis virus, Bovine Herpes Mammalitis Virus, Bovine Leukemia Virus, Bluetongue virus and Bovine Virus Diarrhea (Schultz et al., 1982). This test utilizes live animals to test large volumes of pooled semen.

The presence of viruses in the semen demonstrates that this can serve as another mode of transmission of their disease. In many instances, a systemic viremia is followed by a localized infection of the reproductive and accessory organs and would precede viral shedding in the semen. With BTV, the virus is only shed from 7-28 days after infection. During this viremia, the virus can be cultured from blood in high concentrations (Bowen et al., 1985). It is important to know if a virus (a small virus) can pass directly from the blood into the seminal fluid. If it is able to do so, a disease caused by this kind of a virus and having a transient or persistent viremic stage could possibly be transmitted through the semen (AI or naturally).

Hepatitis B Surface Antigen Virus (HBV) has been detected in semen and saliva of infected persons (Scott et al., 1980). Animals inoculated with semen subcutaneously and intravaginally both
developed HBV and antibody to it. Thus, semen and saliva can be infectious and venereal transmission of HBV is possible.

Another example of the transfer of microorganisms into the seminal fluid is the case of *Toxoplasma gondii* in the semen of goats (Dubey et al., 1980). Animals were orally infected with oocysts of *Toxoplasma gondii* and semen was checked for the organism every 2-3 days for 70 days. *Toxoplasma gondii* was first detected in the semen at 7 days after inoculation. Excretion into the semen continued for a period of 52 days for one goat and 16 days for another. *Toxoplasma gondii* has also been found in the semen of rams from 7 to 30 days following subcutaneous inoculation with cysts (Spence et al., 1978).


PART II

PASSAGE OF STEROIDS INTO BOVINE SEMEN: COMPARISON OF ELECTROEJACULATION AND ARTIFICIAL VAGINA COLLECTION METHODS
SUMMARY

Artificial vagina (AV) and electroejaculation (EE) are the two methods used to obtain semen from bulls. The purpose in the present study was to evaluate these two methods of collection when two markers, cortisol and progesterone, were injected IV.

During period one (control measurement), semen was obtained by EE at 0, 20, 60, 120, and 180 minutes. In period two (three days later), bulls were injected with a mixture of cortisol (113 mg) and progesterone (100 mg), and semen samples were obtained by EE. In period three (three weeks later), cortisol and progesterone were injected and semen samples were obtained by AV. Semen was analyzed for progesterone and cortisol using radioimmunoassay procedures.

Seminal plasma concentrations of cortisol and progesterone were maximal at 20 minutes in EE and AV collections. Seminal plasma concentrations of progesterone and cortisol were roughly 50% less in EE than in AV collection. However, the total excretion of progesterone and cortisol per collection was similar in both techniques. Excretion of cortisol was 14 to 33 times greater than that of progesterone.

It was concluded that both cortisol and progesterone pass into the semen in significant concentrations when injected intravenously. Concentrations of markers in the semen when collected by EE were significantly less than those in AV collection. The two methods of semen collection did not differ in total amount of marker present per ejaculate. For this reason, total excretion and not just
concentration of marker in semen should be accounted for when conducting excretion studies.

INTRODUCTION

The physiologic testing of the accessory sex glands in the bull has been based on the analysis of substances synthesized and secreted by the accessory glands, such as fructose and citric acid (Mann and Lutwak-Mann, 1976). Because of the remarkable excretory capability of the ejaculate, there is a potential that the study of the ejaculate excretory profile of selected markers may be helpful in evaluating the physiologic characteristics of the male accessory glands (Mann and Mann-Lutwak, 1982).

It has been shown that the steroidal hormones estrogen, progesterone, and testosterone are excreted into the ejaculate within 20 minutes after they are injected IV into bulls (Eiler and Graves, 1977; Eiler and Graves, 1981). However, the rate of clearance of the different steroids in the ejaculate varied (ie, cortisol gained access into the ejaculate sooner and in larger quantity than did estrogen, progesterone, or testosterone) (Eiler and Graves, 1981; Graves and Eiler, 1977). These observations on steroid excretion offer the possibility of using steroidal hormones as markers for excretion studies.

Artificial vagina (AV) and electroejaculation (EE) are the two procedures used to obtain semen from the bull. The purpose in the present study was to evaluate the effect of these two methods of
collection on the excretion of cortisol and progesterone when they are injected systemically.

MATERIALS AND METHODS

Six 18-month-old virgin Angus bulls were assigned to the present study and were evaluated clinically and by routine semen analysis. All bulls were classified as satisfactory potential breeders as defined by the Society for Theriogenology (Ball et al., 1983).

During period one (control measurement), semen was obtained by EE at 0, 20, 60, 120, and 180 minutes. In period two (three days later), the same bulls were injected with an equimolar mixture of cortisol and progesterone, and then semen samples were obtained by EE at 0, 20, 60, 120, and 180 minutes after injection. Equimolar solutions of cortisol and progesterone were made by dissolving 113 mg of cortisol in 8 ml of ethanol and 100 mg of progesterone (Steraloids Inc, Wilton, NH) in 3 ml of ethanol. The solutions were mixed together, and an equal volume of distilled water (11 ml) was added before the mixture was injected. The cortisol-progesterone solution was injected IV over a one minute period. In period three (three weeks after period two), bulls were injected intravenously with cortisol and progesterone and semen samples were obtained by AV. At the end of the experiment, all semen specimens were centrifuged (3,000 x g for 15 minutes at 5°C), and the sediment was discarded. The sperm-free supernatant was kept frozen (-20°C) until analysis for
cortisol and progesterone. In period one, neither cortisol nor progesterone was detected in the washed sediment.

Hormone assays

Cortisol was measured by radioimmunoassay (Gamma coat, Clinical Assays, Cambridge, Mass.) validated for seminal plasma. In this method, unknown samples and standards were incubated with \(^{125}\text{I}\) cortisol tracer in antibody-coated tubes where the antibody was immobilized on the lower wall of the test tube. After incubation was done for one hour, the contents of the tube were decanted and counted for radioactivity. Protein denaturation or extraction steps were not required. When cortisol concentrations were too high to read from the standard curve, semen samples were diluted with seminal plasma from period one collection, and cortisol concentration from the dilution plasma was subtracted.

The sensitivity of the assay was 7 ng/ml. Intra-assay error, expressed as the coefficient of variation, fluctuated between 1.0% and 8.5%. Interassay error was not determined, because all samples were measured in duplicate in a single assay. When either 5 or 25 ng of cortisol was added to seminal plasma samples (period one), 99% and 103%, respectively, was recovered. Linearity in plasma was tested by assaying 20, 30, and 40 \(\mu\)l of seminal plasma (0 time). Compared with the 20 \(\mu\)l sample, the final concentration of cortisol was 85% in the 30 \(\mu\)l sample and 95% in the 40 \(\mu\)l sample. Consequently, the sample volume was kept constant at 20 \(\mu\)l.
According to the manufacturer, the cortisol antibody used in this assay cross-reacted with cortisol (100%), 11 deoxycortisol (7.6%, corticosterone (3.3%), deoxycorticosterone (0.3%), and 17-hydroxyprogesterone (0.2%). Cross-reactivity with estrogen, progesterone, and testosterone was determined to be less than 0.1%. The results are expressed as the ratio of the cortisol concentration to the cross-reacting substance concentration at 50% inhibition of maximal binding.

Progesterone in semen was analyzed by a validated radioimmunoassay procedure (Eiler and Graves, 1981). Statistical evaluation was completed by Duncan’s multiple-range test and student t-test. Probabilities are given at the 0.05 level of significance.

RESULTS

The volume of semen obtained per ejaculate per bull by EE fluctuated between 4.0 and 5.1 ml, and that obtained by AV, between 3.7 and 4.5 ml.

Cortisol in semen

During period one, the ejaculate number did not affect either progesterone or cortisol secretion (Figure 2-1). The concentrations (mean ± SEM) of cortisol in seminal plasma obtained by EE (9.1 ± 0.7 ng/ml) and AV (8.0 ± 0.6 ng/ml) at 0 time were not significantly (P > 0.05) different. Concentrations of cortisol in seminal plasma
obtained by EE at 20, 60, 120 and 180 minutes after cortisol-progesterone was injected were 146 ± 45, 39 ± 11, 12 ± 1, and 11 ± 3 ng/ml, respectively, whereas those in seminal plasma obtained by AV were 207 ± 64, 105 ± 20, 47 ± 5, and 30 ± 8 ng/ml, respectively. In both collection methods, an abrupt decrease was noted in cortisol concentrations and in total cortisol transferred into the ejaculate between 20 and 180 minutes. Total cortisol in seminal plasma obtained by EE at 20, 60, 120, and 180 minutes after cortisol-progesterone was injected was 840 ± 260, 197 ± 58, 75 ± 18, and 67 ± 21 ng, respectively, whereas total cortisol in seminal plasma obtained by AV was 765 ± 236, 326 ± 60, 130 ± 16, and 84 ± 23 ng, respectively.

**Progesterone in semen**

The concentrations (mean ± SEM) of progesterone in seminal plasma obtained by EE (1.0 ± 0.1 ng/ml) and by AV (1.4 ± 0.1 ng/ml) at 0 time were not different (Figure 2-2). Concentrations of progesterone in seminal plasma obtained by EE at 20, 60, 120, and 180 minutes after cortisol-progesterone was injected were 5.1 ± 0.4, 1.8 ± 0.6, 1.5 ± 0.5, and 1.2 ± 0.2 ng/ml, respectively, whereas concentrations of progesterone in semen obtained by AV were 9.0 ± 1.0, 5.2 ± 0.6, 3.2 ± 0.4, and 2.3 ± 0.4 ng/ml, respectively. The total progesterone per ejaculate obtained by EE (5.9 ± 0.3 ng) was not significantly (P > 0.05) different from that in ejaculate obtained by AV (4.3 ± 0.6 ng) at 0 time collections. Total
progesterone in semen obtained by EE at 20, 60, 120 and 180 minutes was 25 ± 2.0, 8.2 ± 2.0, 8.2 ± 1.9, and 7.8 ± 4.0 ng, respectively, whereas total progesterone in ejaculate obtained by AV was 23 ± 4.0, 14 ± 2.0, 9 ± 0.5, and 6 ± 0.7 ng, respectively.

**Progesterone-cortisol ratio**

The passage of cortisol into the ejaculate was significantly (P < 0.001) larger than that of progesterone. Total cortisol and total progesterone in ejaculate obtained by AV and EE are shown in Figure 2-3.

Progesterone-cortisol molar ratio values in the ejaculate obtained by AV at 0, 20, 60, 120 and 180 minutes were 0.167, 0.030, 0.042, 0.069, and 0.071, respectively. This indicates that approximately 6, 33, 23, 14, and 14 times more total cortisol than total progesterone was excreted at the respective collection times.

**DISCUSSION**

The present study showed that the concentrations of markers (cortisol and progesterone) in the electroejaculate were significantly less than those in the AV collection. However, when the total excretion of markers was calculated, significant difference between the two procedures was not found. When selected samples were analyzed for water content, the dry weight of the electroejaculate was less than that of the AV collection (data not presented). This indicates that EE may have caused more passage of water into the
ejaculate due to overstimulation of the accessory glands than during AV collection (Furman et al., 1975; Aalbers, 1966). This indicates that when testing the excretory capability of the male reproductive tract, the measurement of total excretion per ejaculate may provide additional information, compared with the single measurement of marker concentration in the ejaculate.

The ratio of cortisol to progesterone excreted into the semen was not affected by the collection procedure. Although cortisol and progesterone were injected in equimolar concentrations, cortisol excretion into semen was approximately 14 times (180 minutes) to 33 times (20 minutes) higher than that of progesterone, depending on sampling time (Figure 2-3). This means that about 14 to 33 times more cortisol than progesterone was recovered regardless which semen collection technique was used. This is in agreement with other studies (Eiler and Graves, 1981; Graves and Eiler, 1977).

The reason for the greater passage of cortisol into semen than that of progesterone is unknown, and is beyond the scope of the present study. The half-time elimination of cortisol in blood (60 minutes) and progesterone in blood (80 minutes) is relatively similar, as calculated from other experiments in the bull (Eiler and Graves, 1977; Graves and Eiler, 1977). Both steroids share the cortisol-binding globulin. However, the affinity of cortisol-binding globulin for cortisol is greater than that for progesterone in man (Pugeat et al., 1981). Electroejaculation causes transient increases in blood cortisol and progesterone concentrations (Welsh and Johnson, 1981). However, the EE process caused no increase in either seminal
cortisol or seminal progesterone concentrations in the bull (Eiler and Graves, 1977; Graves and Eiler, 1977).

The present study of the excretory profile of the ejaculate, when an exogenous marker is injected IV, hypothetically offers the possibility of testing several steps involved in the transfer of a given marker into the ejaculate from blood, including membrane permeability (Abbaticchio et al., 1981), patency of excretory ducts (Wetterauer and Heite, 1980), and the conversion rate of a given marker to its metabolite (Hammerstedt and Amann, 1976). The major difficulty of this approach is to identify a suitable marker specific to a given accessory gland. Regarding the validity of obtaining semen by EE for the excretion study, further research is needed for each specific marker to be used. In the present work, the concentrations of the markers in the electroejaculate were significantly less than those when the semen was obtained via the AV. However, the total excretion of markers was identical in both collection techniques. It is concluded that total excretion and not just concentration of markers in semen should be accounted for when conducting excretion studies.
LITERATURE CITED


APPENDIX
Figure 2-1  Cortisol excretion in the ejaculate. Points represent the mean value for six bulls. Dispersion values are given in the results section. Means marked with "s" or "n" indicate a significant or nonsignificant differences (P > 0.05) between artificial vagina (AV) and electroejaculation (EE) collection procedures at a given time. Total cortisol per ejaculate is given for AV T and EE T collections; cortisol concentrations (ng/ml) are also shown. Notice the logarithmic scale of the axis.
Figure 2-2 Progesterone excretion in the ejaculate. Points represent the mean value for six bulls. Dispersion values are given in the results section. Means marked with "s" or "n" indicate significant or nonsignificant differences (P > 0.05) between artificial vagina (AV) and electroejaculation (EE) collection procedures at a given time. Total progesterone per ejaculate is given for AV T and EE T collections; cortisol concentrations (ng/ml) are also shown. Notice the logarithmic scale of the axis.
Figure 2-3 Total cortisol and progesterone excretions in the ejaculate. Points represent the mean value of six bulls. Dispersion values are given in the results section. Means marked "s" or "n" indicate significant or nonsignificant (P > 0.05) difference between artificial vagina (AV) and electroejaculation (EE) collection procedures. There was a significant (P < 0.001) difference between total cortisol and total progesterone (P₄) throughout the experiment, as indicated by superscript X. Notice the logarithmic scale of the axis.
Table 2-1. Concentration of Progesterone and Cortisol in semen of six bulls following intravenous administration of equimolar concentrations of Progesterone and Cortisol collected by electroejaculation (EE) or artificial vagina (AV).*

<table>
<thead>
<tr>
<th>Time (min) Post Injection</th>
<th>PROGESTERONE (ng/ml)</th>
<th>CORTISOL (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EE</td>
<td>AV</td>
</tr>
<tr>
<td>0</td>
<td>1.0 ± 0.1&lt;sup&gt;x&lt;/sup&gt;</td>
<td>1.4 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>5.1 ± 0.4&lt;sup&gt;y&lt;/sup&gt;</td>
<td>9.0 ± 1.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>60</td>
<td>1.8 ± 0.6&lt;sup&gt;x&lt;/sup&gt;</td>
<td>5.2 ± 0.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>120</td>
<td>1.5 ± 0.5&lt;sup&gt;x&lt;/sup&gt;</td>
<td>3.2 ± 0.4&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>180</td>
<td>1.2 ± 0.2&lt;sup&gt;x&lt;/sup&gt;</td>
<td>23 ± 0.4&lt;sup&gt;a,d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Values are mean ± SEM for six bulls. Analysis of Progesterone and Cortisol by Radioimmunoassay.

<sup>a,b,c,d</sup> Different and similar superscripts indicate significant (P < 0.05) and nonsignificant differences within each column.
PART III

PASSAGE OF THYROID HORMONES INTO BOVINE SEMEN
SUMMARY

The metabolic effects of thyroxine (T4) and triiodothyronine (T3) on spermatozoa metabolism and male anatomy have been demonstrated. The metabolic effects of T3 and T4 could affect the physiologic characteristics of the spermatozoa. Little data is available on the passage of T4 and T3 into the ejaculate from blood. The passage of exogenous T4 and T3 from the blood into semen was measured after T4 (45 mg) or T3 (37.5 mg) was injected IV into 8 bulls. Blood and electroejaculate were obtained simultaneously at 0, 20, 40, 60, 120, and 180 minutes and 24 hours after bulls were injected to determine T3 and T4 concentrations compared with baseline values. Blood T3 and T4 concentrations were increased (P < 0.05) at 20 minutes after bulls were injected (1.1 ± 0.25 to 598 ± 76.3 ng/ml and from 75.2 ± 5.3 to 1,288 ± 105 ng/ml, respectively). Seminal concentrations of T4 were unchanged until 120 minutes after bulls were injected, when they increased (P < 0.05) from < 1.2 ng/ml to 4.7 ± 1.9 ng/ml. However, seminal concentrations of T3 were increased (P < 0.05) from < 0.1 ng/ml to 0.5 ± 0.2 ng/ml at 20 minutes and to 12.5 ± 2.9 ng/ml at 120 minutes after bulls were injected. It was concluded that exogenous thyroid hormones passed into the ejaculate from blood, with T3 passing faster than T4.

INTRODUCTION

The effects of thyroxine (T4) and triiodothyronine (T3) on the sperm metabolism in vitro are significant and well established. The
uptake of thyroid hormone by the spermatozoa (Sullivan and Menge, 1965) and the presence of T3 (0.4 ng/ml) and T4 (15 ng/ml) in human semen (Landau et al., 1983) have been shown. Furthermore, the regulatory effects of T3 and T4 on oxygen consumption (Carter, 1930; Lardy and Phillips, 1943; Schultze and Davis, 1948), fructose metabolism (Gassner and Hopwood, 1955), activation of sperm adenyl cyclase (Casillas and Hoskins, 1970) and fertility rate (Carter, 1932; Schultze and Davis, 1949) have been reported. In these metabolic studies, T3 was the most active hormone. However, the dose of T3 and T4 was critical in some of these experiments (Schultze and Davis, 1948; Gassner and Hopwood, 1955; Casillas and Hoskins, 1970). Thus, the passage of T3 and T4 into the ejaculate may affect sperm physiologic characteristics. However, Landau and workers (1983) failed to show an increase in seminal concentrations of T3 and T4 when men were given thyroid hormones once a day. This was attributed by the authors to the inability of thyroid hormones to cross the tissue barrier of the reproductive tract in man.

The objective in the present research was to determine to what extent the concentrations of thyroid hormones in semen can be changed by administration of T3 and T4 into the bull. This information is important in determining the availability of these two hormones, T3 and T4, in the ejaculate and in defining the excretory capability of the bull reproductive tract.
MATERIALS AND METHODS

Semen and blood collection

Indwelling jugular catheters were placed in eight 18-month-old virgin Angus bulls (406 to 526 kg) from the same herd and calving season for blood collection. Semen was obtained by electroejaculation. All bulls were evaluated clinically and by routine laboratory semen analysis. All bulls were classified to be satisfactory potential breeders, as defined by the Society for Theriogenology (Ball et al., 1983). During a control period, prior to the experiment, when nothing was injected, base-line blood and semen samples were obtained at 0, 20, 40, 60, 120, and 180 minutes and at 24 hours.

After a 72-hour rest, four bulls was injected IV with 45 mg of L-thyroxine (sodium salt, pentahydrate, crystalline) (Sigma Chemical Co., St. Louis, Mo.) double distilled water (pH adjusted to 8.2 with sodium hydroxide). The T4 was injected over a three-minute period. Blood and semen samples were obtained at 0, 20, 40, 60, 120, and 180 minutes and at 24 hours after T4 injection was completed.

A different group of four bulls were subjected to the same experimental procedure, but these bulls were injected IV with 37.5 mg of 3,3', 5 triiodo-L-thyronine (sodium salt) dissolved in 300 ml of double distilled water (pH adjusted to 8.2). Thus, T3 and T4 were given at approximately equimolar concentrations.
Semen and blood samples were centrifuged (4,000 x g, 20 minutes, 5°C). Semen-plasma and blood plasma were frozen (-20°C) until analysis. Semen-plasma and blood-plasma will be referred to as semen and blood, respectively.

Thyroxine and T3 were measured by radioimmunoassay (RIA) in the semen and blood of bulls injected with T4. Only T3 was measured in the semen and blood of bulls injected with T3. The total amount of hormone per ejaculate (ng) was calculated as the product of hormone concentration and ejaculate volume.

**Hormone assays**

The T4 was assayed by a RIA (T4 RIA kit procedure, Amerlex, Amersham Co, Arlington Heights, Ill.) validated for bovine semen (plasma) and bovine blood (plasma). In the assay, the samples and standards were incubated for 45 minutes with [125I]T4 tracer and antibody bound to polymer particles of uniform diameter. Separation of the antibody-bound fraction was accomplished by centrifugation and decanting the supernatant. Thimerosal was used as a thyroxine-binding globulin blocking agent. The recovery of T4 (60 ng/ml) added to semen (base-line sample) fluctuated between 73% and 86%. The recovery of T4 (60 ng/ml) added to blood (base-line sample) fluctuated between 78% and 87%. Linearity for the assay of blood was tested by assaying 100, 200, and 300 µl of plasma (base-line sample). Compared with a 100 µl sample, the final concentration of T4 in a 200 µl sample was 107.9%, and in the 300 µl sample, 140%. Subsequently,
the sample volume was kept constant at 100 μl. When further dilution was needed, base-line plasma was used, and T4 concentrations were subtracted. Linearity in semen data was tested by assaying 100, 200, and 300 μl of semen (plasma) obtained 24 hours after bulls were injected with T4. Compared with the 100 μl sample, the final concentration of T4 in the 200 μl sample was 112%, and in the 300 μl sample, 94%. In the present study, 100 μl of semen was assayed. The within-assay coefficient of variation for semen T4 analysis fluctuated between 2.4% and 18%. The within-assay coefficient of variation for blood T4 analysis fluctuated between 2.3% and 10.3%. The percentage of cross-reactivity with T3 by weight was less than 5%. The sensitivity of the T4 assay was 1.2 ng/ml.

The analysis of T3 was completed by a RIA kit validated for both semen (plasma) and blood (plasma). The principle of this assay was identical to that of T4 assay. The recovery of T3 (2.0 ng/ml) added to blood fluctuated between 78% and 88% and between 76% to 84% when added to semen. Linearity was tested in semen (base-line sample), in which 1 ng of T3/ml was added. The final concentration of T3 in semen was proportional to the volume of semen tested, within 16% error. When the volume of blood was increased from 10 μl to 25 μl and 50 μl, the final blood T3 concentration was 91% and 80%, respectively, compared with a calculated value for 10 μl. Subsequently, the sample volume was kept constant at 10 μl. Within-assay coefficient of variation fluctuated between 1.5% and 10.2% in the base-line semen sample, where 1 ng of T3/ml was added. The overall within-assay coefficient of variation for blood samples
the base-line semen sample, where 1 ng of T3/ml was added. The overall within-assay coefficient of variation for blood samples fluctuated between 6% and 14%. The sensitivity of the assay was 0.1 ng/ml. The percentage of cross-reactivity of the T3 RIA with T4 by weight was less than 0.3%.

Analysis of variance was done to determine effect of treatment on the response variables. When significant differences among treatment means were identified by an F test, mean separation was accomplished by Duncan's multiple-range test. Significance was reported at the 0.05 level.

RESULTS

Effect of EE number on T4 concentrations during the control period

The concentration of T4 in either blood or semen was not affected by the ejaculate number during the control period. Overall mean ± SEM blood T4 concentration was 66 ± 5 ng/ml. Seminal T4 concentration was 1.2 ng/ml (assay sensitivity).

Concentrations of T4 in blood and semen of bulls injected with T4

Mean blood concentrations of T4 increased (P < 0.05) from 75.2 ± 6.3 ng/ml at 0-time to 1,286 ± 105 ng/ml at 20 minutes and decreased steadily during 180 minutes after T4 was injected (Figure 3-1). Twenty-four hour blood samples were not available for T4 analysis in bulls injected with T4. At 180 minutes, the mean blood concentration
of T4 was 734 ± 126 ng/ml. Semen concentrations of T4 increased from either 1.2 ng/ml (assay sensitivity) to 4.7 ± 1.9 ng/ml at the 120-minute ejaculate and remained increased thereafter. At 24 hours after T4 was injected, the mean concentration of T4 in the semen was 3.4 ± 0.9 ng/ml (Figure 3-1). Total T4 (ng) obtained per ejaculate was significantly increased at 120 to 180 minutes and 24 hours after T4 was injected (Figure 3-2). In general, the semen/blood ratio value of T4 was smaller than that of T3 (Figure 3-3).

**Concentrations of T3 in blood and semen of bulls injected with T4**

Mean blood concentration of T3 increased (P < 0.05) from 0.3 ± 0.14 ng/ml (0 time) to 3.73 ± 0.11 ng/ml at 20 minutes (Figure 3-1) and remained essentially unchanged through 180 minutes (Figure 3-1). Triiodothyronine was not detectable in semen between 0-time and 180 minutes after T4 was injected. T3 concentrations in the 24-hour semen and blood samples were not available for analysis in bulls injected with T4.

**Effect of EE number on T3 concentrations during the control period**

The concentration of T3 in either blood or semen was not affected by the ejaculate number during the control period. The overall mean ± SEM blood T3 concentrations was 1.1 ± 0.25 ng/ml. Seminal T3 concentration was < 0.1 ng/ml (assay sensitivity).
Concentrations of T3 in blood and semen of bulls injected with T3

Mean blood concentration of T3 increased (P < 0.05) from 1.3 ± 0.32 ng/ml (O-time) to 598 ± 76.3 ng/ml at 20 minutes (Figure 3-4) and decreased steadily thereafter. At 180 minutes after T3 was injected, the blood T3 concentration was 217 ± 35 ng/ml, and at 24 hours, 67.3 ± 40 ng/ml (Figure 3-4). Semen concentrations of T3 were not detectable (< 0.1 ng/ml) at O-time. At 20 minutes after injection was done, T3 was detectable (0.5 ± 0.2 ng/ml) in all semen samples. Further, T3 increased to 6.2 ± 2.1 ng/ml at 40 minutes and to 12.5 ± 2.9 ng/ml at 120 minutes. At 24 hours after injection was done, the concentration of T3 in semen was < 0.1 ng/ml (Figure 3-4). The semen/blood molar ratios of T3 are shown in Figure 3-3. Total T3 (ng) per ejaculate was significantly (P < 0.05) increased at 20 to 180 minutes from the nondetectable base-line value (Figure 3-2). At 120 minutes, the ejaculate value of T3-total was approximately 3.8-times greater than that of T4-total. O-time T3 and T4 totals were not shown since T3 was nondetectable at O-time.

DISCUSSION

The present study showed that seminal concentration of either T3 or T4 can be increased by injecting the native hormone systemically. The seminal concentration of T3 and T4 was low or undetectable by the assay system used in the present study. However, the injection of equimolar amounts of T3 and T4 into the bulls resulted in significant excretion of T3 and T4 into the ejaculate.
Support for the finding that the passage of T3 into the ejaculate was greater than that of T4 is threefold. First, there were larger excretions of T3-total than T4-total (Figure 3-2). Second, T3 concentration in semen were increased at 20 minutes after T3 was injected, whereas T4 concentration were increased at 120 minutes after T4 was injected. Third, after T4 was injected, semen T4 concentration increased to 4.7 ng/ml, whereas semen T3 concentration increased to 12.5 ng/ml in the T3-injected bulls. This indicated that T3 maximal concentrations in semen were 2.6-times those of T4. This was probably the result of a less-ionized state of T3 in the blood (Hagen et al., 1974; Robbins, 1981). However, no increase in semen T3 concentration was found in the T4-injected bulls, although blood T3 concentration was increased. This increase in blood T3 concentration after T4 was injected was expected and was probably due in part to the peripheral conversion of T4 to T3 (Chopra, 1977; DiStefano et al., 1982).

The preferential passage of T3 into the ejaculate found in the present study was consistent with results of a distribution study of radioactive T3 and T4 in the brain and cerebrospinal fluid (Hagen and Solberg, 1974) and in other tissues of the ruminant (Miralles-Garcia et al., 1981; Fisher et al., 1972).

Contrary to results in the bulls in the present study, the oral administration of thyroid hormones to men did not increase seminal concentrations of either T3 or T4 (Landau et al., 1983). This difference between man and the bull may be due, in part, to the use
of different experimental procedures in the two experiments and species differences (Refetoff et al., 1970).

Whether the physiologic characteristics of either the sperm or accessory glands can be modified by the administration of T3 and T4 to the bull cannot be answered from the present experiment. Calculations of data from the present study and data published by others indicate that T3 and T4 concentrations found in semen in the present experiment were between 1/7 (Gassner and Hopwood, 1955) and 1/50 (Calillas, 1970) the dose needed to cause an increase in oxygen uptake and cyclic adenosine-3',5'-monophosphate accumulation, respectively, when thyroid hormones were added to the sperm in vitro. However, the value of the described dose-response comparison is limited, because the composition of the incubation media significantly affected the uptake of thyroid hormone by the sperm (Sullivan and Menge, 1965), and different incubation media have been used by different investigators. It is also known that the tissue metabolism (Eiler et al., 1964; Menezes-Ferreira et al., 1983) can be affected different ways other than oxygen uptake and cyclic adenosine-3',5'-monophosphate metabolism. Specific differences may also exist for response of sperm or accessory glands to T3 or T4.

The presence of prostatic thyroid-releasing hormone (Bhasin et al., 1984) and thyroid hormones binding proteins (Abbaticchio et al., 1981) in semen is also puzzling from a physiologic viewpoint. It is pointed out that along with the metabolic effects found on the sperm, the carbohydrate metabolism in the epididymus was also increased by treatment with thyroid hormones in the rat (Pereira et al., 1983;
1983b). This indicates that thyroid hormones may exert an indirect effect on the sperm metabolism through altering the composition of secretions of the male reproductive tract.

Furthermore, the present experiment showed that exogenous T3 and exogenous T4 are two hormones which can be added to the list of substances (Mann and Lutwak-Mann, 1982) that are readily excreted in the ejaculate. Antibiotics (Armstrong et al., 1968; Hessel and Stamey, 1971; Eliasson and Malmborg, 1976), chemicals (Eliasson and Dornbusch, 1980; Taylor et al, 1980), and steroidal hormones (Eiler and Graves, 1977; 1981; Graves and Eiler, 1979) also are excreted in the ejaculate without much difficulty.
LITERATURE CITED


Figure 3-1 Mean blood and semen thyroxine (T4) and triiodothyronine (T3) concentrations after thyroxine was injected IV (arrow). Notice the logarithmic scale of the y axis. For dispersion (SEM) values, see Table 3-1. Similar and different superscripts (a and b) indicate nonsignificant and significant (P < 0.05) differences, respectively, compared with 0-time. Semen concentration of triiodothyronine was below assay sensitivity (0.1 ng/ml).
Figure 3-2  Total ng of triiodothyronine (T3) and thyroxine (T4) per ejaculate in the semen of bulls injected IV with either T3 or T4, respectively. Dots and squares indicate mean values. The coefficient of variation of T3-total throughout the experiment was 22% to 63%, whereas that of T4-total was 31% to 74%. For dispersion (SEM) values of T3 and T4, see Table 3-1. Similar and different superscripts (a and b) indicate nonsignificant and significant (P < 0.05) differences, respectively, compared with 0-time.
Figure 3-3  Semen-blood ratio values (means) of triiodothyronine (T3)-injected bulls and thyroxine (T4)-injected bulls. Consistently larger values were found in T3-injected bulls that in T4 injected bulls. The T3 passed into the ejaculate faster and to a greater degree than did T4.
Figure 3-4 Mean blood and semen triiodothyronine (T3) concentrations after bulls were injected IV with T3 (arrow). Notice the logarithmic scale of the y axis. For dispersion values see Table 3-1. Similar and different superscripts (a and b) indicate nonsignificant and significant (P < 0.05) differences, respectively, compared with 0-time. T3 was not detected in semen at 0-time or at 24 hours. At 20 minutes, T3 was 0.5 ± 0.4 ng/ml (off scale) in the semen.
Table 3-1. Concentration of Thyroxine (T4) and Triiodothyronine (T3) in blood and Semen of eight bulls following intravenous administration of T4 and T3*.

<table>
<thead>
<tr>
<th>Time (min) Post Injection</th>
<th>T4 (ng/ml)</th>
<th>T3 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
<td>Semen</td>
</tr>
<tr>
<td>0</td>
<td>75.2 ± 5.3</td>
<td>\ 1.2a</td>
</tr>
<tr>
<td>20</td>
<td>1277 ± 105</td>
<td>1.24 ± 0.025</td>
</tr>
<tr>
<td>40</td>
<td>1286 ± 45</td>
<td>0.68 ± 0.22</td>
</tr>
<tr>
<td>60</td>
<td>1149 ± 44</td>
<td>1.6 ± 0.33</td>
</tr>
<tr>
<td>120</td>
<td>1154 ± 43</td>
<td>4.7 ± 1.91</td>
</tr>
<tr>
<td>180</td>
<td>734 ± 126</td>
<td>5.7 ± 0.99</td>
</tr>
<tr>
<td>1440</td>
<td>NA</td>
<td>3.4 ± 0.9</td>
</tr>
</tbody>
</table>

* Values are mean ± SEM for eight bulls.

NA= Not Available

a Assay sensitivity
PART IV

PASSAGE OF HUMAN CHORIONIC GONADOTROPHIN

INTO BOVINE SEMEN
This study was done in order to determine if human chorionic gonadotrophin (HCG), a complex glycoprotein, is able to pass into the bovine ejaculate following an intravenous dose of HCG. Six Angus bulls, 18 months old, were injected with 30,000 IU of HCG intravenously, following collection of baseline blood and semen. Semen was collected by electroejaculation. Semen and blood specimens were collected at 20, 40, 60, 120, 180 minutes and 24 hrs following HCG injection. Blood and seminal plasma were analyzed for HCG and androgens by radioimmunoassay.

No HCG was detected in blood or semen baseline samples. Plasma concentrations of HCG were maximal at 20 minutes post injection and gradually decreased over 24 hours. Mean concentrations of HCG in semen were maximal at 20 minutes post injection. At 60 minutes there was a decrease in HCG concentrations. From 60 to 180 minutes, seminal HCG concentrations remained unchanged. A high correlation was found between the concentration of HCG in blood and in semen at 20, 40, and 60 minutes post injection.

Blood concentrations of androgens were significantly increased at 40 minutes post HCG injection and remained elevated at the 24 hour sample. Androgen concentrations in the semen remained unchanged during the experimental period.
INTRODUCTION

The elucidation of the type of substance that can be passed into the semen and the understanding of the mechanism of transfer of different substances into the ejaculate from the blood is of physiological, diagnostic and therapeutic relevance. The homeostasis of the lumen of the seminiferous tubule is guarded in part by the existence of the blood-testis barrier (Setchell et al., 1975). This protection is also extended to the epididymis by the existence of the blood-epididymal barrier (Setchell et al., 1975?). Paradoxically different types of exogenous substances such as gonadal steroids (Eiler et al., 1977; 1981; 1987), adrenal steroids (Graves et al., 1979), thyroid hormone (Eiler et al., 1987), antibiotics (Armstrong et al., 1968; Eliasson et al., 1976; Hessl et al., 1971) and chemicals (Eliasson et al., 1980; Taylor et al., 1980) are found in the ejaculate when administered to the male.

The objective of this work was to determine if HCG, which has a relatively more complex structure (glycoprotein) than substances studied so far, passes into the ejaculate. This experiment will provide evidence whether or not the ejaculate is composed of blood born substances along with accessory gland secretions and testicular secretions.

MATERIALS AND METHODS

All bulls assigned to the present study were evaluated clinically and by routine semen analysis. All bulls were classified
as satisfactory potential breeders as defined by the Society for Theriogenology (Ball et al., 1983).

Six virgin Angus bulls, 18 months old were used in this study. Semen was collected by electroejaculation. Peripheral blood (10 ml) was collected by an indwelling jugular catheter. The volume of semen collected was 5.2 ± 0.28 ml (mean ± SEM). There was no statistically significant (P > 0.05) difference in the volume of the electroejaculate within and between bulls. During the control period semen was collected three times with a 20 minute interval between collections. Seventy-two hours after the control period, the bulls were ejaculated and immediately injected intravenously with 30,000 IU of human chorionic gonadotrophin (Follutein, Squibb and Sons Inc., Princeton, NJ). Blood and semen were simultaneously collected at 20, 40, 60, 120, 180-minutes and 24-hours post HCG injection. Samples were centrifuged at 3,000 g for 15 minutes. Blood plasma (designated plasma) and seminal plasma (designated semen) were maintained at -20°C until analysis for HCG and testosterone were conducted by radioimmunoassay procedure.

**Testosterone Radioimmunoassay**

The analysis of testosterone was completed in plasma and semen samples by a previously validated radioimmunoassay (RIA) procedure (Eiler and Graves, 1981). Since plasma and semen samples were not chromatographed, results were expressed as androgens. In previous
assay validation, 76% and 96% of the androgen in semen and plasma, respectively, were testosterone.

**Human Chorionic Gonadotrophin Radioimmunoassay**

The analysis of human chorionic gonadotrophin (HCG) was completed by RIA kit procedure (Clinical Assays, Cambridge, Mass. 02139) validated in our laboratory for both bovine semen and bovine plasma. Plasma and semen were analyzed directly. No extraction procedure was used. Quantities of 150 µl of semen and 150 µl of plasma were assayed in duplicate. When needed, samples were diluted with either 0-time plasma or 0-time semen. HCG was not detected in control samples. The sensitivity of the assay was 5 mU/ml. Intra-assay error, expressed as the coefficient of variation, was 15.7% and 4.8% for plasma and semen respectively. Inter-assay error was not measured because all samples were assayed in a single assay.

The effects of plasma volume on mean (n=3) concentrations of HCG was studied. HCG concentrations in plasma were 12,333, 11,315 and 13,752 mU/ml in 150, 200 and 250 µl respectively. HCG concentrations in semen were 580, 575 and 620 mU/ml in 150, 200 and 250 mU/ml respectively. These results indicated satisfactory parallelism values. When 50 and 100 mU HCG were added to semen samples, 115% and 109%, respectively were recovered. The plasma concentrations of HCG and the clearance slopes observed after HCG injection were what were expected for this molecule. This biological clearance of HCG from the blood was used as a biologic validation of the HCG assay. The
cross reactivity of this assay was LH, 1.2%; FSH, 0.3%; and TSH, 0.6% when tested at 50% binding as reported by the manufacturer.

**Human Chorionic Gonadotrophin Bioassay**

The Galli Magnini pregnancy test was used to test the bioactivity of HCG found in the semen. Different groups of 5 frogs (*Rana climatans*) each were injected with 1 and 2 ml of seminal plasma collected at 0, 20 and 60 minutes post HCG injection. The frogs were injected in the dorsal lymph sacs. Cloacal fluid was examined under the microscope for sperm. The reactivity of the frogs to HCG treatment was investigated in a different group of frogs injected with semen plus 20 IU HCG.

Statistical analysis system was used to perform regression analysis using the general linear model procedures. In addition, Duncan's multiple range test and student's t-test were used for mean separation.

**RESULTS**

Figure 4-1 shows plasma and semen concentrations of HCG as a function of time. No HCG was detected in either 0-time plasma or semen samples. As expected, plasma concentrations of HCG were maximal at 20-minutes, then gradually decreased until 24-hours. The concentrations of HCG in the plasma were not significantly different from each other at 20, 40, 60 or 120-minutes after injection.
However, concentrations of HCG in the plasma at 180-minutes and 24 hours were significantly different from the earlier times.

The half-life of HCG in plasma was calculated to be approximately 180-minutes during the first 3-hours of the experiment. Concentrations (mean ± SEM) of HCG in plasma were 3070 ± 247, 2740 ± 201, 2850 ± 238, 2378 ± 224, 1550 ± 191 and 259 ± 8 mU/ml at 20, 40, 60, 120, 180-minutes and 24-hours respectively. For statistical evaluation see Figure 4-1 and Table 4-1.

Mean concentrations of HCG in semen was maximal at 20-minutes post injection. At 60-minutes there was a significant fall in HCG concentrations. Between 60 and 180-minutes HCG concentrations in semen remained unchanged. At 24-hours, HCG in the semen was still detectable. Semen concentrations of HCG were 674 ± 109, 490 ± 103, 189 ± 138, 131 ± 18, 133 ± 32 and 33 ± 15 mU/ml at 20, 40, 60, 120, 180-minutes and 24-hours respectively. For statistical evaluation see Figure 4-1 and Table 4-1.

Since there was not a difference in the volume of semen collected between bulls and within bulls, the change in total HCG collected per bull was due to the change in HCG concentration in each sample. Total output of HCG per ejaculate (concentration x ejaculate volume) was 3322 ± 537, 2786 ± 745, 847 ± 601, 743 ± 196, 677 ± 175 and 418 ± 185 mU at 20, 40, 60, 120, 180-minutes and 24-hours respectively.

The semen blood ratio value was elevated at 20 and 40- minutes. However, the ratio value decreased at 60, 120 and 180 minutes. This was mainly due to decreased HCG concentrations in semen, while plasma
concentrations of HCG remained elevated. The clearance rate of HCG from the blood was found to be similar for all bulls. However the clearance rate of HCG from the semen was not similar.

There was a high correlation (0.96, 0.98, 0.92) between the concentration of HCG in the blood and semen at 20, 40, and 60 minutes post injection, respectively. This corresponds to the period in which HCG was at its highest concentrations in the blood and semen. Semen blood ratio values were 0.21, 0.17, 0.06, 0.05, 0.08 and 0.12 at 20, 40, 60, 120, 180-minutes and 24-hours respectively.

The prediction equation for the clearance of HCG from blood was

\[ Y = -4875.37 \pm 6506.12X - 1381.68X^2 + 80.12X^3 \]

Where \( Y \) equals the concentration of HCG in the blood in \( \mu \text{g/ml} \) and \( X \) equals time after injection in minutes. The R-SQUARE value for the prediction equation was 0.70. All values in the equation were significant at the 0.05 level.

The prediction equation for the clearance of HCG from the semen was

\[ Y = -667.13 \pm 963.21X - 261.16X^2 + 19.81X^3 \]

Where \( Y \) equals the concentration of HCG in the semen in \( \mu \text{g/ml} \) and \( X \) equals the time after injection in minutes. All values in the equation were significant at the 0.01 level. The R-SQUARE value for this equation was 0.43.

Frogs injected with seminal plasma released no sperm into the cloaca. This was interpreted as being a negative response. Seventy percent of the frogs injected with semen plus the HCG standard reacted positively within 24-hours of the test.
Figure 4-2 shows the androgen concentrations of both plasma and semen of bulls injected with HCG. Plasma concentrations of androgens were significantly (\(P < 0.05\)) increased in the 40-minute post injection sample. Plasma androgens remained elevated at 24-hours, while androgen concentrations in semen remained unchanged during the experimental period. Plasma concentrations of androgens were 2548 ± 435, 3925 ± 453, 7039 ± 1021, 7935 ± 1281, 8194 ± 1235, 7815 ± 1052 and 7713 ± 1062 pg/ml at 0-time, 20, 40, 60, 120, 180-minutes and 24 hours respectively. Androgen concentrations in semen were 561 ± 161, 508 ± 217, 769 ± 336, 559 ± 195, 819 ± 395, 750 ± 213 and 565 ± 168 pg/ml at 0-time, 20, 40, 60, 120, 180-minutes and 24-hours respectively. For statistical evaluation see Figure 4-2.

DISCUSSION

The results have shown that HCG, which is a relatively complex glycoprotein with a molecular weight of 32,000 and composed of at least two subunits, was quickly transferred into the semen. The bioactivity of HCG found in the semen was not confirmed by the bioassay used (frog assay). This was probably due in part to the relatively small concentration of HCG in the semen of the bulls. In this work, 5.0 international units of HCG standard caused a positive reaction in 70% of the frogs tested. This indicated that 30% of the frogs did not react to 5.0 IU of HCG. The HCG injected into the bulls was bioactive. This was confirmed by the significant elevation of plasma androgens that followed HCG injection. The elevation in
plasma androgen that followed HCG injection did not affect the concentrations of androgen in the semen. However, it has been shown that the injection of a relatively large dose of testosterone caused elevation of androgens in the semen of bulls (Eiler et al., 1981).

This work provides evidence that the ejaculate contains not only accessory gland and testicular secretions, but also contains blood-born substances. The fact that HCG does not exist in the bull facilitated this conclusion. This is the main physiological implication of these findings.

The anatomical location of the transfer of HCG is unknown. Possible sites of passage of HCG include the accessory glands, ductus deferens, pampiniform plexus, epididymis, and less probable, the seminiferous tubules.

The pharmacokinetic study indicated that the HCG half-life was approximately 180-minutes. Assuming a first-order reaction, 93.7% of the HCG should have been eliminated in 12-hours. However, at 24-hours, plasma concentrations of HCG were 259 mU/ml indicating a longer half-life than 3 hours.

The semen-blood ratio values were 0.21 and 0.17 at 20 and 40-minutes. This indicated significant transfer of HCG into the ejaculate during the distribution phase. Between 60 and 180-minutes, the passage of HCG into semen decreased significantly, while plasma concentrations of HCG remained elevated. The reason for this decreased transfer of HCG is unknown.

The fact that the semen concentrations of HCG remained unchanged between 60 and 180 minutes, while plasma concentration of HCG
decreased at 180-minutes, suggested that a constant amount (zero order kinetics) of HCG present in blood was ejaculated per unit of time. Whether or not zero order kinetics indicates unequivocal saturations of the transport system of the reproductive tract of the bull needs further investigation.
LITERATURE CITED


Figure 4-1  Clearance profile of HCG from blood-plasma and seminal-plasma of bulls as analyzed by RIA at various times following intravenous injection. Dispersion bars indicate SEM for each mean point. Notice the logarithmic scale of the y-axis. HCG was not detectable in blood or semen at 0-time. For actual values, see Table 4-1. Similar and different superscripts (a,b) indicate nonsignificant and significant differences between means.
Figure 4-2 Androgen concentrations in blood-plasma and seminal-plasma of HCG injected bulls as analyzed by RIA at various times following intravenous injection. Dispersion bars indicate SEM for each mean point. Notice the logarithmic scale of the y-axis. For actual values, see Table 4-1. Similar and different superscripts (a,b) indicate nonsignificant and significant differences between means.
Table 4-1. Concentration of HCG and Androgens in Blood and Semen of bulls following intravenous injection of HCG.*

<table>
<thead>
<tr>
<th>Time (in minutes) after HCG injection</th>
<th>HCG pg/ml</th>
<th>Androgens pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood</td>
<td>Semen</td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>20</td>
<td>3070 ± 247^a</td>
<td>674 ± 109^a</td>
</tr>
<tr>
<td>40</td>
<td>2740 ± 201^a</td>
<td>490 ± 103^a</td>
</tr>
<tr>
<td>60</td>
<td>2850 ± 238^a</td>
<td>189 ± 138^ab</td>
</tr>
<tr>
<td>120</td>
<td>2378 ± 224^a</td>
<td>131 ± 18^b</td>
</tr>
<tr>
<td>180</td>
<td>1550 ± 191^b</td>
<td>133 ± 32^b</td>
</tr>
<tr>
<td>1440</td>
<td>259 ± 8^b</td>
<td>33 ± 15^b</td>
</tr>
</tbody>
</table>

ND = Not Detectable
Values are mean ± SEM for six bulls.
Analysis for HCG and Androgens by RIA.

Different and similar superscripts indicate significant (P < 0.05) and nonsignificant differences respectively within the same column.
PART V

PASSAGE OF CALICIVIRUS INTO BOVINE SEMEN
SUMMARY

It is important to determine if a virus, a small virus, is able to pass directly from the blood into the seminal fluid. If it is able to do so, a disease having a transient or persistent viremic stage could possibly be transmitted through the semen (AI or natural). This would also give us a better idea of the molecular size able to be directly transferred into seminal fluid.

In this experiment, calicivirus (10 million MW) was injected intravenously into three 18 month old bulls. Blood and semen were collected at 0, 20, 40, 60, and 180 minutes after the injection. Virus isolation was performed on seminal plasma, red blood cells and blood plasma.

Calicivirus was isolated from the 20- and 40-minute blood specimen of all bulls. No virus was isolated from the seminal plasma of any bulls. It is likely the virus particles were cleared by the mononuclear phagocyte system quite rapidly (within 40 minutes). Also the viral size is much larger than the glycoprotein hormone HCG which is one of the largest found to pass immediately into the semen.

INTRODUCTION

The passage of many chemicals, drugs, hormones and infectious agents into the semen has been documented in various species. Research has shown that the very small steroid hormones, some antibiotics and the large protein hormone HCG can be recovered from the semen of bulls when administered intravenously (Mann et al.,
1982). Recently, caffeine has been found to pass directly into semen after oral administration in humans (Beach et al., 1982).

Viruses that have been isolated from bovine semen include infectious bovine rhinotracheitis, bovine virus diarrhea, bluetongue virus (Schultz et al., 1982; Kupferschmied et al., 1986; Howard et al., 1985), paravaccinia virus (Johnston et al., 1971), and in humans, HTLV-III (a lentivirus) (Ho et al., 1984), type B hepatitis virus (Ayoola et al., 1980; Scott et al., 1980), cytomegalovirus (Biggar et al., 1983) and papillomavirus DNA (Ostrow et al., 1986), have all been identified in the semen of men. Transmission of the hepatitis virus is documented as being transferred via the semen (Ayoola et al., 1980). This could be a mechanism of transmission for these other viruses also.

Transfer of viral agents via semen, which has not been shown for most viral diseases, has the potential to be an important method of disease perpetuation. For example, ephemeral fever virus may be found in the semen of some bulls; foot and mouth disease virus may be excreted in the semen of some bulls before the onset of clinical disease; and IBR virus may contaminate the semen of bulls as a result of genital infection. Bluetongue virus has also been isolated from the semen of latently infected bulls (Phillips et al., 1986).

The presence of these viruses in the semen suggests that this may serve as another mode of transmission of their disease. The virus may be directly passed through from the blood into the semen as is the case with the mentioned drugs and chemical agents. In most cases, a systemic viremia followed by a localized infection of the
reproductive and accessory organs would precede viral shedding in the semen. With BTV, the virus is only shed from 7-28 days after infection. This is the time of viremia. The virus can be cultured from blood in high concentrations at this time (Howard et al., 1985).

It is important to determine if a virus is able to pass directly from the blood into the seminal fluid. If it is able to do so, a disease having a transient or persistent viremic stage could possibly be transmitted through the semen (AI or natural). This would also provide a better idea of the molecular size able to be directly transferred into seminal fluid.

This experiment was undertaken to determine if intravenously injected calici virus could be recovered from the semen of bulls. Calicivirus was chosen for this experiment because of its small size and noninfectivity to man and bull, thus being less of a health risk in both cases.

**MATERIALS AND METHODS**

**Virus culture**

The calicivirus was grown up on CRFK cells in tissue culture to a titer of $1 \times 10^9$ PFU/ml. The virus was then harvested and frozen in tissue culture media at -20°C until the experiment.
Protocol

In this experiment, three 18-month-old virgin Angus bulls (360 kg) were utilized. The bulls were evaluated clinically and by routine semen analysis. All bulls were classified as satisfactory potential breeders as defined by the Society for Theriogenology (Ball et al., 1983). Each bull was fitted with a jugular catheter and injected intravenously with 60 ml of Calici virus (1 x 10^9 PFU/ml) and then given 40 cc of Calici virus at a rate of 2 cc per minute over the next 20 minutes. Blood was collected in heparinized test tubes and semen obtained via electroejaculation at 0, 20, 40, 60, and 120 minutes after the initial bolus injection. Blood and semen were kept on ice the duration of the experiment.

Viral culture from specimens

Viral isolation was performed on seminal plasma, red blood cells and blood plasma. Semen specimens (3.5 ml) were centrifuged at low speed to remove sperm and debris. The seminal plasma was then pelleted in the ultracentrifuge (24,000 rpm) or (70,000 x g) for 60 minutes at 4°C. The pellet was then resuspended in 1-2 ml cleaning fluid. Blood plasma was treated like seminal plasma and pelleted. Red blood cells were washed in a medium with antibiotics and then lysed with filtered double distilled water. The lysed red blood cells were then plated.

All samples were plated on CRFK cells for a 90 minute incubation. The plates were rinsed and fed with D-MEM (minimal
essential medium) + 2% FBS (fetal bovine serum). The plates of CRFK cells were then incubated at 37°C in a 5% CO₂ oven. Plates were observed daily for viral destruction of cells. When a cell monolayer in a well was totally destroyed, the fluid was passed into a larger one liter sized flask of CRFK cells. These were grown up to use in EM identification of the virus. CRFK cells were also grown on microscope slides and infected for use in the fluorescent antibody test.

Validation

Virus was added to blood plasma, seminal plasma and red blood cells to run along with the samples as positive controls and make sure the virus could infect the CRFK cells from these media. Samples of blood plasma, seminal plasma and red blood cells known not to be contaminated with virus (baseline samples) were run as negative controls.

Positive and negative virus controls were run on blood plasma, seminal plasma and red blood cells to insure that the virus could infect CRFK cells from these mediums. In addition, semen was tested to determine if there was a substance within it that inactivated the virus and prevented its attachment to the CRFK cells. To do this, semen or media were mixed 1:1 with the virus and incubated one hour at 5°C. Semen and media were then clarified by centrifuging. The supernatants were ultracentrifuged one hour at 70,000 X g. The pellet of each tube was then resuspended in 2 ml medium. Twenty-four
well plates of CRFK cells were incubated with 10 fold serial dilutions (to $1 \times 10^8$) of both seminal fluid and media resuspensions. Following a one hour incubation, the fluid was removed and CRFK cells fed with D-MEM + 2% FBS. Plates were run in duplicate. Plates were observed for signs of cell rounding up or plaque formation.

**Virus Identification**

Virus morphology was studied from virus which was grown from the tissue culture samples of seminal plasma, red blood cells and blood plasma using negative staining techniques for electron microscopy.

Indirect Immunofluorescent Antibody Test for calicivirus was performed on the CRFK cells grown up on the slides and infected with specimens showing signs of viral lysis.

**RESULTS**

Calicivirus was isolated from the 20- or 40-minute blood plasma sample of all bulls. No virus was isolated from the seminal plasma of any of the bulls.

Validation was done to determine if a substance was present in the semen which inactivated or was toxic to the virus. Semen with virus added to it was then cultured under the same conditions as the other specimens. Cell monolayers of CRFK cells from all dilutions of semen and culture medium were destroyed by the virus. This rules out the possibility that the virus was inactivated in the semen.
Although it could have been inactivated prior to entry into the semen.

The bulls were examined one week to 30 days after the experiment and remained healthy, showing no signs of calicivirus infection.

Calicivirus was morphologically identified by electron microscopy from tissue cultures of CRFK cells which had been incubated with the red blood cells or blood plasma.

DISCUSSION

Calicivirus was isolated from the blood plasma of all bulls. This indicated that the injected virus was viable and not inactivated by a factor in the blood, since it infected the CRFK cells in vitro. It also indicates that the isolation procedures utilized in the laboratory to culture and isolate the virus were also valid.

Calicivirus was not isolated from the semen of bulls in this experiment. In comparison, Bluetongue virus is only excreted into the semen when the bull is viremic. This ranges from 10 to 28 days (Bowen et al., 1985). Seminal shedding of Bluetongue virus is confined to a period of peak viremia. In Bowen’s study, the virus was never isolated from semen of any bull after termination of viremia. The present study tried to mimic the viremic stage (as in bluetongue) by a constant infusion of calicivirus and a large initial dose in an attempt to get viral excretion into the semen. Although a very large dosage of virus was administered as a bolus and the infusion continued for 20-minutes, calicivirus was not isolated from
the bull semen in this experiment. However, the virus was isolated from the blood at this time.

It was not totally unexpected to find that calicivirus was not isolated from the semen. The virus particles were probably cleared by the mononuclear phagocyte system quite rapidly. Also, their size is much larger than the glycoprotein hormone HCG, which is one of the largest found to pass immediately into the semen. The intercellular pores which the virus must pass through to get into the semen also may have been too small to facilitate its passage. It is more likely that viruses get into semen by being carried through intracellularly.

It is possible that the calicivirus passed into the semen and was inactivated. However, semen did not inactivate the virus in an in vitro control experiment.

In this experiment, bulls were observed and examined for 3 weeks post-calicivirus injection for signs of an infection. In retrospect, it would have been a good idea to take both blood and semen specimens at this time also. Calicivirus' has been known to be species specific and non-infective to the bovine species until recently when a calicivirus was isolated from calves (Smith et al., 1983).

In other cited cases of seminal virus shedding, e.g. Hepatitis B virus, BVD, Paravaccinia, IBR and HTLV-III, the animal was known to be infected or to have been infected (titer) with the virus. The virus which was shed into the semen had replicated in the tissue of the reproductive tract (e.g. endothelium of the urethra, prostate, seminal vesicles, epididymis, or testes) and, therefore shed at ejaculation into semen and fluids.
Efforts to isolate calicivirus from semen were burdened by technical problems not associated with isolation from other inocula. Semen alone has a cytotoxic effect on cell culture and may also contain virus neutralizing substances. Methods utilized in this study to avoid these problems include centrifugation, ultracentrifugation, dilution, a validation study, and a short 90-minute incubation period of semen with cell culture. Results of the semen validation tests indicated that the virus added to semen remained viable and was infective to the CRFK-cells.


PART VI

PASSAGE OF PENICILLIN AND OXYTETRACYCLINE INTO BOVINE SEMEN
SUMMARY

This study was conducted in order to determine if penicillin and/or oxytetracycline pass into the bovine ejaculate when administered to bulls. Two groups of four 18-month-old bulls were used for the study. One group was injected intravenously with 50 million units of penicillin G potassium, and the other group was injected with 6 grams oxytetracycline. Blood and semen specimens were collected at 0, 20, 40, 60, 120, 180 minutes and 24 hours after injection. Semen was collected by electroejaculation. The volume of semen collected was not significantly different between groups. The concentrations of the respective antibiotics in blood and semen specimens were determined by bioassay using the principles of inhibition of bacterial growth to measure antibiotic concentrations.

Blood concentrations of both penicillin and oxytetracycline were highest at 20 minutes post injection and steadily declined over the sampling period. Penicillin was present in the semen of bulls at 20 minutes post injection (32 ± 29 U/ml). It increased to a peak of 293 ± 47 U/ml at 120 minutes and steadily declined to nondetectable levels at 24 hours. Oxytetracycline was present in the semen of bulls at 20 minutes post injection (3.0 ± 2.6 µg/ml). It increased to its highest levels at 120 and 180 minutes (10 to 14 µg/ml) and was still detectable at 24 hours. These results indicate that both penicillin and oxytetracycline gain rapid entry into the bovine ejaculate. Penicillin is rapidly cleared from the semen as it was not detected at 24 hours post injection. However, oxytetracycline
was still detectable (2.0 ± 0.13 μg/ml) in semen at 24 hours post-injection.

INTRODUCTION

In Man, evidence is available that indicates that the for accessory sex glands are involved in the transfer of antibiotics into semen. Evidence suggests (Malmburg et al., 1975) mainly prostatic transfer. The blood-testis barrier protects the immature spermatocytes from these antibiotics and their effects. But the effects of antibiotics or drugs secreted by the accessory glands on mature spermatozoa in the ejaculate are unknown.

In man, sulfamethoxazole levels in the prostate are found to reach plasma levels. Trimethoprim levels are found to be two to three times higher in the prostate than they are in plasma (Oosterlinck, 1975). Tetracyclines also have been found to pass into prostatic fluid, some in therapeutic levels 0.5-1.5 μg/ml. Green and Green (1985) report a female patient who developed urticaria due to an allergy to the penicillin in her partner's semen.

Although much work has been done in man and dogs regarding antibiotic passage into semen and the minimum inhibitory concentration in the prostate, little work has been done to determine if and where antibiotic passage into semen occurs in the bovine. In contrast to primarily prostatic accessory gland secretions in dogs, the bovine has three accessory glands; seminal vesicles, prostate and
and bulbourethral glands. This may affect seminal antibiotic concentration by allowing more sites of antibiotic transfer.

The following study was conducted in order to determine if penicillin and/or oxytetracycline (OTC) pass into semen when administered to bulls and if inhibitory concentrations against gram-negative bacteria are achieved and to gain understanding of the bovine excretory function in the male reproductive tract.

**MATERIALS AND METHODS**

All bulls assigned to the present study were virgin and were evaluated clinically and by routine semen analysis. The bulls were classified as satisfactory potential breeders as defined by the Society for Theriogenology. Indwelling jugular catheters were placed into two groups of four 17-19 month-old Angus bulls for blood collection. Following collection of baseline blood from the catheter and baseline semen by electroejaculation, Group I bulls were injected intravenously with 50 million units of Penicillin G potassium (E.R. Squibb & Sons, Inc., Princeton, N.J.), and Group II bulls were intravenously injected with 6 gm oxytetracycline hydrochloride (Liquamycin, Pfizer Inc., Clifton, N.J.) 50:50 in saline. Blood and semen were collected at 20, 40, 60, 120, 150, 180-minutes and 24-hours after injection of the respective antibiotic.

Blood and semen samples were kept on ice for the duration of the experiment. They were then centrifuged and blood plasma (referred to as blood) and seminal plasma (referred to as semen) were collected
and frozen at -60°C until analysis by bioassay for the respective antibiotic.

The volume of semen collected in Group I was 4.8 ± 0.13 ml (mean ± SEM) and in Group II it was 4.5 ± 0.21 ml. There was no difference (P < 0.05) between the volume of semen collected in Group I and Group II bulls.

The design of this study was based on a time-course relationship. Statistical evaluation was completed by Student's t-test and regression analysis using SAS. Probabilities are given at the 0.05 level of significance.

**Penicillin Bioassay**

The penicillin G potassium bioassay (Barr, 1983) utilized principles of inhibition of bacterial growth to measure antibiotic concentrations. A culture of *Sarcinia lutea* (ATCC 9341) was grown up overnight on a BHI plate at 35-37°C. The growth was washed from the surface of the plate with sterile saline. A solution was made by diluting this culture with sterile saline to obtain 50% light transmission of a wavelength of 550 nm on a coleman spectrophotometer (model 6/20). Three milliliters of this inoculum were used per liter of seed agar.

Plates for this assay were poured in two layers. The base layer consisted of 30 ml of Antibiotic medium B (BBL Microbiology systems, Cockeysville, MD) poured into 150 x 25 mm disposable petri dishes. When this layer had solidified, a seed layer consisting of 15 ml
Antibiotic medium A (BBL) inoculated with *Sarcinia lutea* was poured over the base layer.

The standard curve was prepared using concentrations of 0.1, 0.5, 1.0, 2.5, and 10 U/ml penicillin in antibiotic free plasma or semen. When samples were too concentrated, they were diluted appropriately in antibiotic free plasma or semen to obtain values within the range of the standard curve.

Twenty-five μl of each sample or standard was pipetted onto each sensi-disk (BBL) and the disks were then placed on the plates. Six disks (three of the internal reference standard and three of the sample or standard) were alternately arranged on each plate. Each sample was run on three plates for a total of nine measurements per sample. The plates were inverted and incubated 20 hours at 35-37°C in a humidified oven. Zones of inhibition were measured and means calculated for each sample.

A standard curve was constructed by plotting the diameter of the zone of inhibition (mm) on the X-axis against the concentration of penicillin (U/ml) on the Y-axis on semi-log paper. Penicillin concentrations were read from the curve using the zone of inhibition.

**Oxytetracycline bioassay**

A culture of *Bacillus subtilis* (ATCC 6633) was grown overnight on a BHI plate at 35-37°C. The organism was then transferred into 10 ml sterile saline using a cotton swab. The mixture was vortexed until a homogeneous solution was obtained. The plates of AK agar
were inoculated with 1-2 ml of the *B. subtilis* solution. Plates were inverted and incubated at 35-37°C for 72 hours. The growth was washed from the surface of the plates into 50 ml sterile saline using a cotton swab. It was then incubated in a 80°C waterbath one hour to kill all except the spores. The solution was centrifuged, the pellet was saved and washed three times in 30 ml sterile saline. This final solution of *B. subtilis* spores in saline can be stored at 4°C for up to one year.

Plates for this assay were poured in two layers. The base layer of 30 ml antibiotic assay medium E (BBL) was poured into 150 x 25 mm disposable petri dishes. After solidification of this layer, a seed layer of 15 ml of antibiotic assay medium A (BBL), inoculated with the *B. subtilis* spore solution, was poured over the base layer. The amount of *B. subtilis* spore solution used to inoculate the seed solution will vary with the amount of growth in preparation of the solution. Different amounts of the *B. subtilis* spore solution were added to the seed agar to titrate the correct amount needed to get a smooth growth covering the plate. This amount was kept constant throughout the oxytetracycline assay.

A standard curve was prepared by using concentrations of 0.625, 1.25, 2.5, 5.0 and 10 µg/ml OTC in antibiotic free plasma or semen. When samples were too concentrated, they were diluted appropriately with antibiotic free plasma or semen in order to obtain values within the range of the standard curve.

Twenty-five µl of each sample or standard was pipetted onto each sensi-disk (BBL) and the disks were then placed on the plates. Six
disks (three of the internal reference standard and three of the sample or standard) were alternately arranged on each plate. Each sample was run on three plates for a total of nine measurements per sample. The plates were inverted and incubated 15 hours at 35-37°C in a humidified oven. Zones of inhibition were measured and means calculated for each sample.

A standard curve was constructed by plotting the diameter of the zone of inhibition (mm) on the X-axis against the concentration of OTC (μg/ml) on the Y-axis on semi-logarithmic paper. Oxytetracycline concentrations were read from the curve using the zone of inhibition.

**RESULTS**

**Penicillin bioassay**

Blood concentrations of penicillin in the Group I bulls were highest at 20-minutes post injection (430 ± 74 U/ml) and steadily declined over the 180-minute sampling period. At 180-minutes after injection, the mean blood concentration was 4.3 ± 0.7 U/ml. Blood concentrations of penicillin (mean ± SEM) were 430 ± 74, 251 ± 150, 139 ± 63, 31 ± 22, 7 ± 0.06, 4.3 ± 0.7 and 0 U/ml at 20, 40, 60, 120, 150, 180-minutes and 24 hours after penicillin injection (see Figure 1).

Penicillin was present in the semen of Group I bulls at 20-minutes after injection (32 ± 29 U/ml). Concentrations of penicillin in the semen steadily increased to a peak of 293 ± 47 U/ml at 120-
minutes and then declined to 28 ± 9 U/ml at 180-minutes. Semen concentrations of penicillin were 32 ± 29, 146 ± 135, 123 ± 74, 293 ± 47, 101 ± 55, 28 ± 9 and 0 U/ml at 20, 40, 60, 120, 150, 180-minutes and 24 hours after penicillin injection (see Figure 6-1). Penicillin was not detectable in either blood or semen at 24-hours after injection.

The clearance profile of penicillin from the blood plasma and seminal plasma of bulls is also given in Figure 6-1. At 20-minutes, blood plasma concentrations were significantly higher (P < 0.05) than seminal plasma values. Concentrations were not different (P > 0.05) at 40, 60 and 150-minutes. Seminal plasma concentrations of penicillin were significantly higher than blood plasma concentrations at 120 and 180-minutes.

The clearance of penicillin from the blood was predicted by the regression equation

\[ Y = -1.62X \pm 278.53 \]

where Y equals the concentration of penicillin in U/ml and X equals the time after injection. Both parameters were significant at the 0.001 level. The R-SQUARE value for the prediction equation was 0.437. The clearance of penicillin from the semen was predicted by the regression equation

\[ Y = -0.04X^2 \pm 8.23X - 138.77 \]

Where Y equals the concentration of penicillin in U/ml and X equals the time after injection. The parameters of time were significant at the 0.01 level. The R-SQUARE value for the prediction equation was 0.348.
**Oxytetracycline bioassay**

Blood concentrations of OTC in the Group II bulls were highest at 20-minutes (29.5 ± 3.3 µg/ml) after injection and steadily declined over the 180-minute sampling period. By 180-minutes post injection, the mean blood concentrations had dropped to 1.42 ± 0.48 µg/ml. Blood concentrations of OTC were 29.5 ± 3.3, 14.6 ± 0.89, 9.2 ± 0.59, 6.9 ± 0.97, 7.5 ± 0.03, 6.4 ± 0.81, 1.4 ± 0.48 and 1.42 ± 0.48 at 20, 40, 60, 120, 150, 180-minutes and 24 hours after OTC injection (see Figure 6-2).

OTC was present in the semen of Group II bulls at 20-minutes (3.0 ± 2.6 µg/ml) after injection. Concentrations of OTC in the semen were highest at 120 and 180-minutes after injection. Semen concentrations of OTC were 3.0 ± 2.6, 7.4 ± 6.8, 3.0 ± 2.2, 10.1 ± 3.9, 2.8 ± 2.2, 14 ± 7.7 and 2.0 ± 0.13 µg/ml at 20, 40, 60, 120, 160, 180-minutes and 24-hours after OTC injection (see Figure 6-2).

The clearance of OTC from the blood was predicted by the regression equation of

\[ Y = -2.91X^3 + 0.01X^2 - 1.21X + 49.24 \]

where Y equals the concentration of penicillin in µg/ml and X equals the time after injection (in minutes). All parameters were significant at the 0.001 level. The R-SQUARE value for the prediction equation was 0.897.

Due to the large amount of variation, in seminal concentrations of OTC, a significant prediction equation for OTC was not found.
The clearance profile of OTC from the blood plasma and seminal plasma of bulls is also shown in Figure 6-2. At 20 and 60-minutes, blood plasma concentrations were significantly higher ($P < 0.05$) than seminal plasma concentrations. There were no significant differences ($P > 0.05$) between blood plasma and seminal plasma concentrations of OTC 40, 120, 150 and 180-minutes after injection. Seminal plasma concentrations were not significantly higher than blood plasma concentrations of OTC as they were in the penicillin experiment.

DISCUSSION

The results indicate that penicillin, injected intravenously, can gain rapid entry into the semen of bulls. Penicillin was found in the semen at the first sampling period which was 20-minutes after injection and reached a peak concentration at 120-minutes.

The dosage of penicillin given (5 million U/100 kg) in this experiment was high enough to maintain therapeutic levels for the duration of the experiment. The minimum inhibitory concentration (MIC) of most penicillin susceptible organisms is 1 to 10 U/ml. Concentrations of penicillin in the semen reached 300 U/ml at 120-minutes and were around 30 U/ml at 180-minutes.

Our results are in agreement with the trend found by Malmborg (1975). He found ampicillin in all seminal samples taken from one to five hours after administration in man. He also found that concentrations of ampicillin at three hours were closely related to zinc content which suggested ampicillin was mainly excreted through
the prostate. Other researchers (Armstrong, et al., 1968) have found if ampicillin is given for a longer period of time (24-48 hours) seminal ampicillin concentrations were equal to or greater than serum ampicillin concentrations.

Winningham and others (1968) measured prostatic secretions in dogs which had been injected with penicillin G or ampicillin. Neither antibiotic was present in the prostatic fluid of dogs (less than 0.2 μg/ml) even when plasma concentrations reached 54 μg/ml (ampicillin) and 62 μg/ml (penicillin G). This is in contrast to our present work in bulls and the previously discussed experiments by Malmborg and others (1975) which were conducted in man. It seems that the penicillins do not pass into semen via the prostate in dogs, whereas they do in man and in the bull.

The results of the OTC experiment showed that OTC, injected intravenously, also gains rapid entry into the semen of bulls similarly to penicillin. Oxytetracycline was present in the semen at the first sampling period which was 20-minutes after injection, and reached a peak concentration at 180-minutes.

The results of the present OTC experiment in bulls are not consistent with work by Winningham and others (1968) in dogs. They found no OTC in prostatic fluid (less than 2.0 μg/ml) of dogs given OTC when plasma concentrations were between 10 and 22 μg/ml. This can be explained by the dog only having one accessory gland, the prostate and the bull having a prostate plus two additional accessory glands.
Armstrong and others (1968) found tetracycline levels in human semen to be equal to serum concentrations. Work by Eliasson and Malmborg (1976) and Malmborg and others (1975) have found that doxycycline is excreted in human seminal plasma, and by using split ejaculate techniques, they have determined that it is mainly excreted through the prostate. This was confirmed by Oosterlink and others (1976) who found therapeutic levels of doxycycline in enucleated prostate tissue in man. Hessl and Stamey (1971) studied four tetracyclines and their concentrations in the prostatic fluid of dogs. Their work was in agreement with Winningham and others (1968). Of the four tetracyclines studied, only OTC did not pass into the prostatic fluid of dogs.

In comparing antibiotic passage into the semen acrossed species, it seems that passage in the bovine follows trends found in man in this respect.
LITERATURE CITED


Figure 6-1  Concentration (mean) of oxytetracycline (OTC) in blood and semen of bulls following IV injection of OTC. Notice the logarithmic scale of the y-axis. Dispersion values are given in the text. Points with different and similar superscripts (a,b,c,x) are significantly (P < 0.05) and not significantly different, respectively.
Figure 6-2  Concentration (mean) of penicillin in blood and semen of bulls following IV injection of penicillin. Notice the logarithmic scale on the y-axis. Dispersion values are given in the text. Points with different and similar superscripts (a,b,x,y) are significantly (P < 0.05) and not significantly different, respectively.
PART VII

PASSAGE OF GENTAMICIN INTO THE BOVINE SEMEN:
EFFECT OF VASECTOMY
This experiment was conducted in order to determine if gentamicin is able to pass into the ejaculate of bulls and using vasectomy, determine the area of passage. Comparison of radioimmunoassay (RIA) and bioassay techniques for gentamicin were also conducted.

The passage of gentamicin into the semen from the blood was measured after four 18-month old bulls were injected intravenously with 3.0 grams of gentamicin. Blood and semen specimens were collected at 20, 40, 60, 120, 160, and 180 minutes after injection. Semen was collected by electroejaculation (EE). Bulls were vasectomized to remove the contribution of the testis and epididymis from the ejaculate, and the experiment was repeated.

In both, the RIA and bioassay, blood levels of gentamicin were highest at 20 minutes after injection and slowly declined over the 180 minute period. Concentrations of gentamicin in the semen were apparent at 20 minutes and reached maximal concentrations at 120 minutes. Concentrations were back to baseline at 20 hours. Concentrations of gentamicin in the semen of vasectomized bulls were not significantly different from intact bulls. Results from the bioassay and RIA were very similar.

It appears that gentamicin injected intravenously can gain rapid entry into the ejaculate of bulls. Vasectomy did not significantly decrease the amount of gentamicin in the ejaculate, suggesting
passage via the accessory glands rather than in the testis or epididymis.

**INTRODUCTION**

There is some evidence for accessory gland (mainly the prostate) involvement in the transfer of antibiotics from blood into the semen. In work done in dogs, erythromycin and oleandomycin were found to readily cross the prostate epithelium into the prostatic fluid producing two to three times higher concentrations in prostatic fluid than in plasma (Winningham et al., 1968; Stamey et al., 1970). However, in these same studies, antibiotics such as penicillin G, kanamycin and oxytetracycline were not detectable in prostatic fluid of dogs. Other researchers have found doxycycline in the seminal plasma of man (Mathisen et al., 1975; Gnarpe and Friberg, 1972). Malmborg and others (1975) established, using split-ejaculate techniques, that doxycycline was mainly excreted through the prostate.

Other compounds such as steroidal hormones (Eiler and Graves, 1977; 1981; Graves and Eiler, 1977), thyroid hormones (Eiler et al., 1982), and chemicals (Taylor et al., 1980; Gerber and Lynn, 1978; Swanson et al., 1978) have also been found to be transferred into the semen when administered to males. Knowledge of the kinetics of those antibiotics capable of reaching inhibitory concentrations in the reproductive tract of the bull will facilitate treatment of
reproductive tract infections such as seminal vesiculitis and other accessory gland diseases.

In order to investigate whether or not antibiotics in the semen come from accessory gland excretions, bulls were vasectomized and antibiotic concentrations of the ejaculate determined. It is of interest to learn whether or not the antibiotic molecules are inactivated as they are excreted. For this reason, samples were analyzed by both radioimmunoassay and bioassay. Gentamicin was chosen for this study because of the commercially available RIA procedure and a known bioassay procedure.

The objective of this study was to determine if gentamicin passes into the ejaculate and if gentamicin concentrations excreted from the accessory glands were inhibitory for bacterial growth and secondly to gain a basic understanding of the secretory capability of the accessory glands.

**MATERIALS AND METHODS**

Four 18-month-old virgin Angus bulls were assigned to the present study and were evaluated clinically and by routine semen analysis. All bulls were classified as satisfactory potential breeders as defined by the Society for Theriogenology (Ball et al., 1983):

Following collection of baseline blood from jugular catheters and baseline semen by electroejaculation, 3.5 grams of gentamicin sulfate (Gentocin, Schering Corporation, Kenilworth, NJ, 07033) was
injected intravenously into each bull. Blood and semen samples were taken at 20, 40, 60, 120, 160, 180 minutes and 24 hours after gentamicin injection. Another group of six bulls was vasectomized and the experiment repeated.

Semen samples were checked for the presence of spermatozoa in the control group of bulls to insure that fluids were being collected from the proximal portion (testis, epididymis) of the male reproductive tract and not just the accessory glands. All semen samples collected in the control group were positive for spermatozoa. Two weeks after vasectomy, semen samples collected from the vasectomized bulls did not contain spermatozoa. Vasectomies were confirmed by histological sections of the removed portion of the ductus deferens and by postmortem examination at slaughter.

Blood and semen samples were kept on ice for the duration of the experiment. They were then centrifuged and blood plasma and seminal plasma collected. Samples were frozen (-20°C) until analysis by RIA and bioassay procedures for gentamicin.

The volume of semen collected in the control group was 4.7 ± 0.14 ml (mean ± SEM). There was no significant difference (P > 0.05) in the volume collected within or between bulls. The volume of ejaculate collected in the vasectomized group of bulls was 5.2 ± 0.18 ml. There was no significant difference (P > 0.05) in the volume of ejaculate collected within or between the control group and vasectomized group of bulls.

The design of this study was based on a time-course relationship. Statistical evaluation was completed by Students t-
Regression analysis was conducted by general linear models procedure. Probabilities are given at the 0.05 level of significance.

Radioimmunoassay

The analysis of gentamicin was performed by a gentamicin radioimmunoassay kit procedure (Gammacoat, Clinical Assays, Cambridge, Mass. 021239). The unknown samples and standards were incubated with $^{125}$I gentamicin tracer in antibody-coated tubes where the antibody was immobilized onto the lower inner wall of the tube. After incubation, the contents of the tube were aspirated or decanted and the tube was counted. A standard curve was prepared with five serum standards ranging from 1.0 to 16 $\mu$g/ml. Unknown values were obtained from the standard curve by interpolation. The entire assay, except for dilution of the sample and controls, was performed in the coated tube. This procedure was validated for both bovine blood plasma and bovine seminal plasma. The recovery of added gentamicin (4 $\mu$g/ml) to blood plasma (0-time) was 86%. The recovery of added gentamicin to bovine seminal plasma (40-minute) was 91 ± 3%. Linearity in blood plasma was determined by assaying 50, 100 and 150 $\mu$l of blood plasma (180 minute). As compared to the 100 $\mu$l sample, the final concentration of gentamicin in the 50 $\mu$l sample was 59.2% and in the 150 $\mu$l sample 133.3%. In this assay, the sample volume, 100 $\mu$l was kept constant. When blood plasma samples were too
concentrated and further dilution was needed, 0-time blood plasma was used to dilute the sample.

Linearity in seminal-plasma was tested by assaying 50, 100 and 150 \( \mu l \) of seminal plasma (120-minute). As compared to the 100 \( \mu g \) sample, the final concentration of gentamicin in the 50 \( \mu l \) sample was 57% and in the 150 \( \mu l \) sample it was 130%. In this assay, 100 \( \mu l \) of seminal plasma was used. The sensitivity of this gentamicin assay was 1 \( \mu g/ml \). The within assay coefficient of variation for blood plasma gentamicin fluctuated between 3 and 8.7%. The within assay coefficient of variation for gentamicin in seminal plasma fluctuated between 2 and 10.8%. Interassay error was less than 15%.

The crossreactivity of the assay antibody as reported by the manufacturer was 43.2% with sinsomicin (a fermentation product not used in a clinical setting) and less than 0.1% with amikacin, ampicillin, bacitracin, chloramphenicol, kanamycin sulfate, oxytetracycline, penicillin G, streptomycin and tobramycin. A crossreactivity study was not conducted in our laboratory because the bulls received no other treatment except gentamicin.

**Bioassay**

The bioassay (Barr, 1984) consisted of plating disks containing known amounts of gentamicin or the unknown samples onto Neomycin assay agar which had *Staphylococcus epidermis* (ATCC) incorporated into the seed (top) layer. Plates were inverted and incubated 20 hours at 35-36\(^\circ\)C in a humidified oven. Zones of inhibition were
measured around each disk. A standard curve was calculated from known concentrations of gentamicin. Samples were diluted appropriately with antibiotic-free plasma or semen so that their values fell within the range of the standard curve. Concentrations of the unknown samples were read from the standard curve by using the zone of inhibition measurement. Samples of blood plasma and seminal plasma from control bulls were analyzed for gentamicin by both bioassay and RIA. Samples from vasectomized bulls were analyzed by RIA only.

RESULTS

Radioimmunoassay

Blood concentrations of gentamicin in nonvasectomized bulls were highest 20 minutes after injection (60.75 ± 8.67 µg/ml) (mean ± SEM) and slowly declined over the 180-minute sampling period. At 180-minutes post injection the mean plasma concentration of gentamicin was 12.2 ± 3.5 µg/ml. Blood concentrations of gentamicin were 60.7 ± 8.7, 36.3 ± 8.7, 32 ± 5.7, 20.3 ± 3.8, 12.2 ± 3.5 and 0 µg/ml at 20, 40, 60, 120, 180-minutes and 24 hours after gentamicin injection (see Figure 7-1).

Gentamicin was present in the semen of control bulls 20-minutes after injection (11.5 ± 11.5 µg/ml) (see Figure 7-4 and 7-5). Concentrations of gentamicin in the semen steadily increased to a peak of 43.1 ± 10 µg/ml at 120 minutes (see Figure 7-2) and then
declined to $4.8 \pm 1.7 \text{ \mu g/ml}$ at 180 minutes. Semen concentrations of gentamicin were $11.5 \pm 11.5$, $12.8 \pm 9.2$, $29.7 \pm 18.1$, $43.2 \pm 10$, $24.2 \pm 7.6$, $4.8 \pm 1.7$ and $0 \text{ \mu g/ml}$ at 20, 40, 60, 120, 160, 180-minutes and 24 hours after gentamicin injection. Gentamicin was not detectable in either blood plasma or seminal plasma at 24 hours after injection.

Blood concentrations of gentamicin in vasectomized bulls are illustrated in Figure 7-3 and 7-6. Gentamicin was highest at 20 minutes ($25.6 \pm 2.7 \text{ \mu g/ml}$) and decreased over the 180-minute testing period ($7.0 \pm 0.92 \text{ \mu g/ml}$). It was not present in the blood at 24-hours after injection. Blood concentrations of gentamicin in vasectomized bulls were $26.5 \pm 2.7$, $16.6 \pm 1.7$, $14.8 \pm 1.2$, $9.7 \pm 1.7$, $7.0 \pm 0.9$ and $0 \text{ \mu g/ml}$ at 20, 40, 60, 120, 180-minutes and 24-hours after gentamicin injection. Although the shape of the slopes of the clearance of gentamicin from the blood of vasectomized and nonvasectomized bulls are similar, the values were slightly, but significantly different ($P < 0.05$). For statistical evaluation, see Figure 7-3.

The concentration of gentamicin in the semen of vasectomized bulls is illustrated in Figure 7-4 and 7-6. Gentamicin was present in the semen at 20 minutes ($3.4 \pm 2.5 \text{ \mu g/ml}$), increased until it peaked out at 120 minutes ($23.8 \pm 12.1 \text{ \mu g/ml}$) and then decreased. Gentamicin was not present in the semen of vasectomized bulls at 24 hours after injection. There were no significant differences ($P > 0.05$) between gentamicin concentrations in the semen of vasectomized and control bulls. For statistical evaluation see Figure 7-4. Semen concentrations of gentamicin in vasectomized bulls were $3.4 \pm 2.5$, \ldots
20.5 ± 15.7, 23.8 ± 12.1, 10.2 ± 4.0, and 0 µg/ml at 20, 40, 60, 120, 180-minutes and 24-hours respectively.

**Bioassay**

Plasma concentrations of gentamicin were highest 20 minutes after injection (47.5 ± 10.2 µg/ml) and slowly declined over the 180 minute sampling period. At 180 minutes post injection, the mean plasma concentration of gentamicin was 11.5 ± 0.05 µg/ml. Blood concentrations of gentamicin were 47.5 ± 10.2, 32.2 ± 6.9, 27.7 ± 4.6, 20.2 ± 3.0, 14.5 ± 1.9, 11.5 ± 0.5 and 0 µg/ml at 20 40, 60, 120, 180-minutes and 24-hours after gentamicin injection respectively (see Figure 7-1).

Gentamicin was present in the semen of the bulls only 20 minutes after injection (4.3 ± 2.9 µg/ml). Concentrations of gentamicin in the semen steadily increased to reach a peak of 52.5 ± 7.6 µg/ml at 120-minutes after injection (see Figure 7-2) and then declined to 14.8 ± 11.0 µg/ml at 180-minutes. Semen concentrations of gentamicin were 4.3 ± 2.9, 19.2 ± 14.2, 30.1 ± 23.8, 52.5 ± 7.6, 18.5 ± 7.5, 14.8 ± 11.0 and 0 µg/ml at 20, 40, 60, 120, 180-minutes and 24-hours after gentamicin injection respectively.

The results from the RIA and bioassay were similar (see Figure 7-1 and 7-2). There were no significant differences (P > 0.05) between the two assays for concentrations at any time period in plasma or semen.
The clearance profile of gentamicin from the blood plasma and seminal plasma of control and vasectomized bulls is given in Figure 7-5. At 20-minutes, blood plasma concentrations of control bulls were significantly higher ($P < 0.05$) than seminal plasma values. Concentrations were not different at 40 and 60-minutes, but seminal plasma concentrations of gentamicin became significantly higher than blood plasma at 120-minutes in control bulls.

Blood plasma concentrations of vasectomized bulls were found to be significantly higher ($P < 0.05$) than seminal plasma concentrations at 20-minutes. Concentrations were not significantly different between blood plasma and seminal plasma at other time periods for vasectomized bulls.

The clearance of gentamicin from the blood of control bulls was predicted by the regression equation of:

$$Y = 59.93 - 0.36X$$

where $Y$ equals the concentration of gentamicin in $\mu g/ml$ and $X$ equals the time after injection in minutes. Both the intercept and time parameter in the model were significant at the 0.005 level. The $R^2$ square value for the model was 0.51.

The clearance of gentamicin from the blood of vasectomized bulls was predicted by the regression equation of:

$$Y = 14.33 - 6.39X$$

where $Y$ equals the concentration of gentamicin in $\mu g/ml$ and $X$ equals the time after injection in minutes. Both the intercept and time parameter in the model were significant at the 0.0001 level. The $R^2$ square value for the model was 0.59.
DISCUSSION

The results of this experiment showed that gentamicin injected intravenously can gain rapid entry into the semen of bulls. Gentamicin was found first in the semen 20-minutes after injection and peaked at 120-minutes (Figure 7-5 and 7-6).

The concentrations of gentamicin as determined by RIA and bioassay were not significantly different when measured in semen or in blood (Figure 7-1 and 7-2). The fact that the bioassay results matched the RIA results indicates that the gentamicin being measured from the blood and semen was bioactive.

Since there was no change in the output of antibiotic in the semen between the vasectomized and nonvasectomized bulls, it would appear that gentamicin passed from the blood into the semen via the accessory glands, and not through the testis or epididymis. The relevance of this finding lies in the possibility that if gentamicin is able to accumulate in the accessory glands in a high enough concentration, it could inhibit bacterial growth and aid in the treatment of reproductive tract infections such as seminal vesiculitis. Experiments by Winningham and others (1968) may shed some light on the accessory gland passage of antibiotics. In their experiment, dogs ureters and vas deferentia were ligated so that pure prostate secretions could be collected. Different continuously infused antibiotics were tested in this study. One of them was kanamycin, an aminoglycoside similar to gentamicin. No kanamycin (less than 0.2 μg/ml) was found in prostatic fluid when blood plasma
levels were 31-42 μg/ml. Therefore in dogs, it seems that aminoglycosides do not pass or pass in very limited amounts via the prostate.

The present experiment used a similar aminoglycoside, gentamicin. Passage was determined to be through the accessory glands, but the specific gland(s) of passage is still unknown. From the work by Winningham and others in dogs (1968), it would seem that the antibiotic is limited in passing through the prostate. The bull has two additional accessory sex glands (seminal vesicles and bulbourethral glands) when compared to the dog. This may be where transfer of gentamicin from the blood into the ejaculate occurs.

Work done in human males by Armstrong and others (1968) investigated kanamycin levels in semen. Semen concentrations of kanamycin were 6.64 μg/ml (plasma level 25.41 μg/ml at 24-hours) up to 17.38 μg/ml (plasma level 5.73 μl/ml at 72-hours). In this experiment, kanamicin was administered intramuscularly, 500 mg, twice a day for 3 days. His work showed that in men, gentamicin does pass into the semen and can be concentrated to three times serum concentrations. Several of the other sixteen antibiotics in this study were not only found to pass into the semen, but they were present in the semen at equal to ten times the serum concentrations. This observation becomes important when the diseased states of the male reproductive tract are considered.
LITERATURE CITED


Figure 7-1  Comparison of analysis by RIA and bioassay. Concentrations of gentamicin in the blood plasma of control bulls at various times following intravenous injection of gentamicin. Points with similar superscripts are not significantly different (P > 0.05) for the given time period.
Figure 7-2 Comparison of analysis by RIA and bioassay. Concentrations of gentamicin in the seminal plasma of control bulls at various times following intravenous injection of gentamicin. Points with similar superscripts are not significantly different (P > 0.05) for the given time period.
Figure 7-3  Effect of vasectomy on concentrations of gentamicin in the blood plasma of control (RIA-C) and vasectomized (RIA-V) bulls at various times following intravenous injection of gentamicin. Points with different and similar superscripts are significantly and not significantly different (P > 0.05) from each other for a given time period.
Figure 7-4 Effect of vasectomy on concentrations of gentamicin in the seminal plasma of control (RIA-C) and vasectomized (RIA-V) bulls at various times following intravenous injection of gentamicin. Points with similar superscripts are not significantly different (P > 0.05) for the given time period. The broken line gives bioassay results for control bulls.
Figure 7-5  Clearance profile of gentamicin from blood plasma (circles) and seminal plasma (squares) of intact bulls as analyzed by RIA at various time periods following intravenous injection. Points are the mean of six bulls. Points with different and similar superscripts (a,b), (x,y) are significantly and not significantly different (P > 0.05), respectively.
Figure 7-6  Clearance profile of gentamicin from blood plasma (circles) and seminal plasma (squares) of vasectomized bulls as analyzed by RIA at various time periods following intravenous injection. Points are the mean of six bulls. Points with different and similar superscripts (a,b), (x,y) are significantly and not significantly different (P > 0.05), respectively.
Table 7-1. Effect of an intravenous injection of Gentamicin on blood and semen concentrations of Gentamicin in nonvasectomized and vasectomized bulls.

<table>
<thead>
<tr>
<th>Time (in minutes) Post Gentamicin Injection</th>
<th>Concentration of Gentamicin ( \mu g/ml )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NONVASECTOMIZED</td>
</tr>
<tr>
<td></td>
<td>BLOOD</td>
</tr>
<tr>
<td>20</td>
<td>60.7 ± 8.7(^a)</td>
</tr>
<tr>
<td>40</td>
<td>36.3 ± 8.7(^a)</td>
</tr>
<tr>
<td>60</td>
<td>32 ± 5.7(^a)</td>
</tr>
<tr>
<td>120</td>
<td>20.3 ± 3.8(^a)</td>
</tr>
<tr>
<td>180</td>
<td>12.2 ± 3.5(^a)</td>
</tr>
</tbody>
</table>

|                                           | VASECTOMIZED                          |
|                                           | BLOOD         | SEMEN         |
| 20                                        | 26.5 ± 2.7\(^b\) | 3.4 ± 2.5\(^a\) |
| 40                                        | 16.6 ± 1.7\(^a\) | ---           |
| 60                                        | 14.8 ± 1.2\(^b\) | 20.5 ± 15.7\(^a\) |
| 120                                       | 9.7 ± 1.7\(^b\) | 23.8 ± 12.1\(^a\) |
| 180                                       | 7.0 ± 0.9\(^a\) | 10.2 ± 4.0\(^a\) |

Numbers are mean ± SEM for 4 bulls
Analysis for Gentamicin by RIA.

\( a,b \) Different superscripts indicate significant \( (P < 0.05) \) difference in the same row while the same superscript indicates non-significance when comparing blood to blood or semen to semen.
PART VIII

PASSAGE OF CARNITINE INTO BOVINE SEMEN:

EFFECT OF VASECTOMY AND COLLECTION TECHNIQUE
SUMMARY

The effects of carnitine injection, vasectomy and electroejaculation collection procedure were investigated on the concentrations of carnitine in bovine ejaculate. Intravenous injection of L-carnitine (4.2 gram) increased plasma carnitine to 100 - 130 nMol/ml from 9 - 37 nMol/ml at 20 minutes post injection. Blood elimination half-life was 2.2 - 2.3 hours.

Vasectomy decreased the concentration of carnitine in semen from 180 nMol/ml to 37 nMol/ml, when the ejaculate was collected by natural mating. Concentrations of carnitine in the electroejaculate (3.0 nMol/ml) were significantly less than natural mating, and were not affected by vasectomy.

Carnitine injection caused no increase in the concentration of carnitine in semen, whether semen was collected by natural mating or electroejaculation.

It was concluded that 79% of carnitine in the semen originated in the epididymis (and possibly the testis) and 21% originated from the other accessory sex glands. Injection of carnitine did not affect the concentration of carnitine in the semen.

INTRODUCTION

Although there is a large concentration of carnitine in the epididymal wall and in the epididymal fluid (Brooks, 1980; Casillas, 1972; Hinton and Hernandez, 1985), carnitine is not synthesized by the epididymis. It is derived from food and from liver biosynthesis
Carnitine is preferentially absorbed by the epididymal cauda from blood, and concentrated in the epididymal fluid to about 2000-times the blood level (Hinton and Setchell, 1980; Hinton and Hernandez, 1985).

The relative uniqueness of carnitine to the epididymis suggests that the study of carnitine in semen, along with citrate and fructose, could be of diagnostic value in epididymal pathology and obstructive azoospermia (Wetterauer, 1980; Casano et al., 1987; Tomamichel et al., 1986; Soufir et al., 1984). In men, vasectomy has been shown to cause a decrease, but not a disappearance of carnitine from the ejaculate (Golan et al., 1983; Wetterauer, 1980; Tomamichel et al., 1986). It has been estimated that in men about 58% (Frenkel et al., 1974), 85% (Casano et al., 1987), and 95% (Wetterauer et al., 1980) of seminal carnitine originated in the epididymis. Although early studies on epididymal carnitine were conducted in bulls (Casillas, 1972), the kinetics of carnitine excretion in the bull is unknown.

Electroejaculation has been a useful technique to collect semen in the bovine, but it has also been shown to affect the excretion of markers into the ejaculate (Eiler et al., 1987).

The objective of this work was to study the effects of intravenous administration of carnitine, vasectomy and electroejaculation collection on the carnitine content of bull ejaculate.
MATERIALS AND METHODS

Electroejaculation, carnitine injection, and vasectomy

Indwelling jugular catheters were placed in six 18-month-old virgin Angus bulls (430 to 518 kg), from the same herd and calving season. Bulls were evaluated clinically and by routine semen analysis. All bulls were classified to be satisfactory potential breeders, as defined by the Society of Theriogenology. Blood from catheter and semen collected by electroejaculation were collected simultaneously at 0, 20, 60, and 1440 minutes to study the effect of consecutive ejaculations on the carnitine content of the semen.

Three days later, bulls were injected (IV) with 4.2 grams of DL-carnitine (Sigma Co.) in saline (30 cc) over a three minute period, and samples (blood and semen) were collected at 0, 20, 60, 120, 180 minutes and 24 hours. Bulls were vasectomized and two weeks later, the experiment was repeated.

Semen and blood samples were centrifuged within 30 minutes of collection, and stored at -20°C until analysis. Carnitine was determined in seminal plasma and blood plasma (from now on called semen and blood respectively). Carnitine and its derivatives were analyzed according to a radioisotopic procedure of Cederblad and Lindstedt (1972) with minor modifications (Sachan et al., 1984). Free carnitine was consistently present in semen, therefore it was analyzed for in all samples.
Natural ejaculation, carnitine injection, and vasectomy

In a follow-up experiment a different group of five bulls was used. Ejaculates were collected at 0, 20, 60, 120, and 180 minutes after carnitine injection using the artificial vagina technique. Three days later, bulls were injected (IV) with carnitine (4.2 grams in saline) and the experiment was repeated. Semen was collected at 0, 20, 60, 120, and 180 minutes post-carnitine injection. Then, bulls were vasectomized and the experiment was repeated two weeks later. Semen samples were treated as in experiment one.

The blood-plasma concentrations of carnitine were fit to a one-compartment model using an automated curve-stripping procedure followed by nonlinear least squares regression (SAS Institute, Cary N.C.). Analysis of variance was conducted to determine the effect of treatment on the response variables. When significant differences among treatment means were identified by an F-test, mean separation was accomplished by Newman Keull's test. Significance was reported at the 0.05 level.

RESULTS

The effect of intravenous injection of carnitine on blood concentration of carnitine is shown in Figure 8-1 and Table 8-1. The pre-injection (0-time) concentration of carnitine in blood was significantly ($P < 0.05$) less in the vasectomized bulls than in nonvasectomized bulls. However, at 20-minutes post injection, blood concentrations of carnitine were not statistically different between
the two groups. In both groups, blood concentrations of carnitine declined gradually until 180 minutes post-injection. At 24 hours post injection, plasma concentrations of carnitine were back to pre-injection values. Calculations showed a relatively similar elimination-half-life, blood clearance and volume of distribution before and after vasectomy (see Table 8-1).

**Effect of electroejaculation**

The concentration of carnitine in the semen of bulls collected by electroejaculation before (intact bulls) and after vasectomy is shown in Table 8-2 and in Figure 8-2. Concentrations of carnitine in the electroejaculate were significantly smaller than those in semen collected by natural mating. The mean seminal concentration of carnitine was not significantly different between vasectomized and nonvasectomized bulls prior to carnitine injection. Carnitine injection did not cause significant elevation in the concentration of carnitine in the electroejaculate.

**Effect of natural ejaculation**

The concentration of carnitine in the semen of bulls collected by an artificial vagina, before (intact bulls) and after vasectomy is shown in Table 8-3 and Figure 8-3. The mean concentration of carnitine in the semen of intact bulls was roughly 4.8-times that of vasectomized bulls. Vasectomy caused a drop of seminal carnitine to 21% of prevasectomy levels. Therefore, 79% of seminal carnitine
originated in the epididymis (and possibly the testis). However, carnitine injection caused no significant elevation in the mean concentration of carnitine in semen of either nonvasectomized or vasectomized bulls that were collected using an artificial vagina.

**DISCUSSION**

Carnitine found in semen is derived from food, synthesized in the liver, and secreted into the ejaculate preferentially by the epididymis, (Brooks, 1980). Factors that regulate the transfer of carnitine into the semen are unknown. A positive correlation between the concentration of carnitine in seminal plasma and blood has been reported in men (Rosecrans et al., 1987). This was not confirmed in our experiment, in the bull.

The dose of carnitine injected intravenously in this work was about 10 times the size of the carnitine blood pool. This caused an acute increase in the blood concentration of carnitine of approximately 4 to 11 times the baseline value. However, there was not a significant elevation in seminal carnitine during this time. This suggests that, in the bull, the blood concentrations of carnitine are not critical in determining the net secretion of carnitine into the semen. The influx/absorption rates of carnitine into the epididymis, as determined under different experimental conditions (Hinton and Hernandez, 1985) cannot be evaluated in this experiment.
In general, a variety (Mann and Lutwak-Mann, 1982) of substances including small sized endogenous molecules have been shown to pass readily into the ejaculate, when injected intravenously (Eiler et al., 1987). However, carnitine did not increase in the semen when an intravenous dose of 4.2 grams was given to bulls. This could be explained in part, because carnitine, unlike other substances, is actively reabsorbed from the lumen of the tail of the epididymis as concentrations increase (Hinton and Setchell, 1980; Hinton and Hernandez, 1985). Whereas other substances are not reabsorbed into the blood, but remain in the lumen.

Regardless of species differences in metabolism, the elimination-half life of carnitine in the blood of bulls (2.3 hr.) (this work) is in agreement with the half life for humans (1.5 hr.) (Cederblad, 1984).

Carnitine found in semen is not exclusively originated in the epididymis. This is supported by the fact that approximately 21% of carnitine persisted in the ejaculate of bulls, after vasectomy. Vasectomy eliminated the epididymal contribution to the semen. The relative contribution of each accessory gland including ampulles, is unknown in the bovine. It has been estimated that in men, the prostate contributes around 5% and the other accessory glands around 10% of the carnitine in the semen (Menchini-Fabris et al., 1984). It has also been determined that, in man, about 58% (Frenkel et al., 1974), 85% (Casano et al.,1987), and 95% (Wetterauer et al., 1980) of seminal carnitine originates in the epididymis. Our results indicate that, in the bull, 79% of the carnitine was originated in the
epididymis. This percentage is relatively consistent with that reported in men. The above estimates are not totally accurate since the testicular contribution of carnitine, probably negligible was not investigated.

The concentration of carnitine in the semen of bulls collected by electroejaculation (Table 8-2) was significantly (P < 0.05) smaller than that collected by natural mating (Table 8-3 and Figure 8-3). The reason for this difference cannot be explained in this experiment, since two different groups of bulls were used. However, in previous research we have found that the concentrations of exogenous progesterone and cortisol in semen collected by electroejaculation, was significantly smaller than in semen collected by natural mating (Eiler et al., 1987). This was probably due to overstimulation of accessory glands, that resulted in a relatively larger amount of water in the electroejaculate than in the natural ejaculate (Seidel and Foote, 1970) causing a dilution effect.

One should bear in mind the lack of organ specificity for carnitine excretion, and the dilution effects of electroejaculation when attempting to interpret the analysis of carnitine in semen of infertile bulls for diagnostic purposes such as congenital lack of ductus deferens, ductus deferens obstruction and epididymal pathology.
LITERATURE CITED


Figure 8-1 Concentrations of carnitine in the blood following intravenous injection of 4.2 grams of carnitine in bulls. For dispersion values and probabilities, see Table 8-1.
Figure 8-2 Concentrations of carnitine in the semen of vasectomized (top) and nonvasectomized (bottom) bulls collected by EE before (control period) and after intravenous injection of 4.2 grams of carnitine (arrow). For dispersion values and probabilities, see Table 8-2. The control period was three days prior to carnitine injection.
Figure 8-3 Concentrations of carnitine in the semen of vasectomized (bottom) and nonvasectomized (top) bulls collected by AV before (circles) and after (squares) intravenous injection of carnitine. For dispersion values and probabilities, see Table 8-3. The control period (circles) was three days prior to carnitine injection.
Table 8-1. Effect of an intravenous injection of carnitine (4.2 g) on plasma concentrations of carnitine before (intact) and after vasectomy.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Intact (nMol/ml)</th>
<th>Vasectomized (nMol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>37 ± 6.7&lt;sup&gt;a,y&lt;/sup&gt;</td>
<td>9 ± 1.6&lt;sup&gt;a,z&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>130 ± 15.9&lt;sup&gt;b,y&lt;/sup&gt;</td>
<td>100 ± 5.0&lt;sup&gt;b,y&lt;/sup&gt;</td>
</tr>
<tr>
<td>60</td>
<td>97 ± 12.8&lt;sup&gt;b,y&lt;/sup&gt;</td>
<td>62 ± 3.2&lt;sup&gt;b,z&lt;/sup&gt;</td>
</tr>
<tr>
<td>120</td>
<td>77 ± 6.3&lt;sup&gt;b,y&lt;/sup&gt;</td>
<td>46 ± 2.3&lt;sup&gt;b,z&lt;/sup&gt;</td>
</tr>
<tr>
<td>180</td>
<td>70 ± 12.0&lt;sup&gt;b,y&lt;/sup&gt;</td>
<td>39 ± 1.8&lt;sup&gt;b,z&lt;/sup&gt;</td>
</tr>
<tr>
<td>1440 (24h)</td>
<td>38 ± 9.6&lt;sup&gt;b,y&lt;/sup&gt;</td>
<td>13 ± 1.8&lt;sup&gt;a,z&lt;/sup&gt;</td>
</tr>
<tr>
<td>elimination T&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>2.3 ± 0.3&lt;sup&gt;y&lt;/sup&gt;</td>
<td>2.2 ± 0.2&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>Vol Distribution (l/kg)</td>
<td>62 ± 7.0&lt;sup&gt;y&lt;/sup&gt;</td>
<td>86 ± 5.5&lt;sup&gt;z&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blood Clearance (ml/kg/min)</td>
<td>313 ± 39.7&lt;sup&gt;y&lt;/sup&gt;</td>
<td>403 ± 76.2&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for 6 bulls

<sup>a,b</sup> Different superscript indicates significant (P < 0.05) difference under the same column, while the same superscript indicates no difference (P > 0.05).

<sup>y,z</sup> Different superscript indicates significant (P < 0.05) difference between mean on the same row, while the same superscript indicates no difference (P > 0.05).
Table 8-2. Carnitine concentrations in semen of intact and vasectomized bulls collected by electroejaculation at different times before and after an intravenous injection of carnitine.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>INTACT</th>
<th></th>
<th>VASECTOMIZED</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-carnitine (nMol/ml)</td>
<td>Post-carnitine (nMol/ml)</td>
<td>Pre-carnitine (nMol/ml)</td>
<td>Post-carnitine (nMol/ml)</td>
</tr>
<tr>
<td>0</td>
<td>3.1 ± 2.0(^a)</td>
<td>3.0 ± 1.7(^a)</td>
<td>7.7 ± 5.5(^a)</td>
<td>5.5 ± 3.3(^a)</td>
</tr>
<tr>
<td>20</td>
<td>1.9 ± 1.8(^a)</td>
<td>27.4 ± 10.9(^a, z)</td>
<td>2.8 ± 1.8(^a, y)</td>
<td>5.4 ± 2.3(^a)</td>
</tr>
<tr>
<td>60</td>
<td>4.5 ± 2.1(^a, y)</td>
<td>21.6 ± 5.6(^a, z)</td>
<td>4.9 ± 3.6(^a, y)</td>
<td>30.1 ± 14.7(^a, y)</td>
</tr>
<tr>
<td>120</td>
<td>ND</td>
<td>26.3 ± 14.0(^a)</td>
<td>ND</td>
<td>25.0 ± 7.6(^a)</td>
</tr>
<tr>
<td>180</td>
<td>ND</td>
<td>13.7 ± 7.4(^a)</td>
<td>ND</td>
<td>20.1 ± 13.2(^a)</td>
</tr>
<tr>
<td>1440 (24h)</td>
<td>2.8 ± 0.9(^a, y)</td>
<td>3.1 ± 1.2(^a, y)</td>
<td>6.4 ± 2.4(^a, y)</td>
<td>5.9 ± 2.9(^a, y)</td>
</tr>
<tr>
<td>Mean</td>
<td>3.0 ± 1.7(^y)</td>
<td>18.4 ± 7.8(^y)</td>
<td>5.5 ± 3.3(^y)</td>
<td>17.3 ± 8.1(^y)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for six bulls.

\(^a, b\) The same superscript indicates no significant difference (\(P > 0.05\)) between means under the same column. Different superscript indicates significant difference.

\(^y, z\) The same superscript indicates no significant difference (\(P > 0.05\)) between means on the same row. Different superscript indicates significant difference.

ND = Not Determined

Notice carnitine injection caused no elevation in the concentrations of carnitine in semen.
Table 8-3. Carnitine concentrations in semen of intact and vasectomized bulls collected by artificial vagina at different times before and after an intravenous injection of carnitine.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>INTACT</th>
<th></th>
<th>VASECTOMIZED</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-carnitine (nMol/ml)</td>
<td>Post-carnitine (nMol/ml)</td>
<td>Pre-carnitine (nMol/ml)</td>
<td>Post-carnitine (nMol/ml)</td>
</tr>
<tr>
<td>0</td>
<td>260 ± 29&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>180 ± 40&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>74 ± 32&lt;sup&gt;az&lt;/sup&gt;</td>
<td>37 ± 14&lt;sup&gt;az&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>185 ± 31&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>181 ± 40&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>56 ± 20&lt;sup&gt;az&lt;/sup&gt;</td>
<td>46 ± 17&lt;sup&gt;az&lt;/sup&gt;</td>
</tr>
<tr>
<td>60</td>
<td>146 ± 47&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>224 ± 19&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>20 ± 10&lt;sup&gt;az&lt;/sup&gt;</td>
<td>41 ± 19&lt;sup&gt;az&lt;/sup&gt;</td>
</tr>
<tr>
<td>120</td>
<td>147 ± 44&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>139 ± 35&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>22 ± 4&lt;sup&gt;az&lt;/sup&gt;</td>
<td>25 ± 13&lt;sup&gt;az&lt;/sup&gt;</td>
</tr>
<tr>
<td>180</td>
<td>160 ± 47&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>164 ± 48&lt;sup&gt;ay&lt;/sup&gt;</td>
<td>13 ± 3&lt;sup&gt;az&lt;/sup&gt;</td>
<td>35 ± 12&lt;sup&gt;az&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mean</td>
<td>180 ± 40&lt;sup&gt;y&lt;/sup&gt;</td>
<td>177 ± 36&lt;sup&gt;y&lt;/sup&gt;</td>
<td>37 ± 14&lt;sup&gt;z&lt;/sup&gt;</td>
<td>37 ± 15&lt;sup&gt;z&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SEM for five bulls.

<sup>a,b</sup> The same superscript indicates no significant difference (P > 0.05) between means under the same column. Different superscript indicates significant difference.

<sup>y,z</sup> The same superscript indicates no significant difference (P > 0.05) between means on the same row. Different superscript indicates significant difference.

Notice vasectomy caused carnitine concentration to drop to about 20%, while carnitine injection did not affect seminal carnitine.
This dissertation has revised that the passage of various substances from the body into the semen of man and animals does occur. Many of these substances potentially have a detrimental effect on reproduction, whether it be on male fertility by affecting spermatozoa maturation and viability, or have an action on the ovum and sperm interaction and fertilization. Many of the substances acquire entry into the semen by unknown mechanisms. Still, the precise anatomical location of transfer of these substances is also unknown.

A protocol for a systemic approach to studying the time-course relationship of substances as they relate to blood and semen was provided by this work.

This dissertation has shown that several drugs and antibiotics used in veterinary practice today can gain rapid access into the semen from blood in bulls. The passage of these substances depended on their blood concentrations, plasma binding and molecular complexity.

Notice this study has expanded the scientific literature on carnitine excretion into the ejaculate to include the bull. The epididymal contribution of carnitine was found to be 79% while 21% of seminal carnitine came from the other accessory sex glands of the bull. The experiment with carnitine also demonstrated that intravenous injection of exogenous carnitine did not increase the carnitine content in the ejaculate.

In the course of this work, three different microbial bioassay techniques were developed to quantitate the concentration of
antibiotics (gentamicin, penicillin, oxytetracycline) in blood and semen. The gentamicin bioassay was determined to be as sensitive as the gentamicin radioimmunoassay for both blood and semen.

As researchers, we must become increasingly aware and cognizant of the influences and effects drugs, chemicals antibiotics, environmental toxicants and microorganisms can have on animals in terms of fertility.

Further research is needed to develop testing procedures to aid in determining the compartment of the male reproductive tract which is dysfunctioning and to determine the anatomical location of passage into the ejaculate.
VITA

Cindy Sue Backus was born in Union City, Indiana on December 20, 1957. She attended Saint Mary's Catholic Elementary School and graduated from Greenville Senior High, Greenville, Ohio, in June 1976. The following September, she entered The Ohio State University, Columbus and in June 1980 received a Bachelor of Science degree in Agriculture with a major in Animal Science. In the Autumn of 1980, she accepted a graduate research assistantship at The University of Tennessee, Knoxville and began graduate study in Physiology. In August, 1982 she received the degree Master of Science with a major in Animal Science from The University of Tennessee, Knoxville. Remaining at The University of Tennessee, she continued her graduate study in Reproductive Physiology until Autumn, 1985 when she entered the University of Tennessee Veterinary Medicine program. In June, 1988 she received the degree Doctor of Veterinary Medicine. In June, 1988 she joined a mixed animal veterinary practice in Union City, Indiana with Dr. Bruce Sickels. She completed her graduate work in Reproductive Physiology and in May, 1989 she received the degree Doctor of Philosophy with a major in Animal Science from The University of Tennessee, Knoxville.