THE EFFECTS OF PROSTAGLANDIN $F_{2\alpha,}$ OXYTOCIN AND GONADOTROPIN RELEASING HORMONE ON EJACULATE CHARACTERISTICS IN THE DOG

by Milan Hess

Virginia Polytechnic Institute and State University

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Approved by: Beverly Purswell, DVM, PhD, DACT
Chairperson of Supervisory Committee

Niki Parker, DVM, MS, DACT

John Dascanio, VMD, DACT, DABVP

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Veterinary Medical Science

Abstract

Prostaglandin $F_{2\alpha}$ (PGF$_{2\alpha}$), oxytocin and gonadotropin releasing hormone (GnRH) have been used in bulls, rams, boars, stallions or rodents to increase sperm numbers in the ejaculate. Improving sperm quantity in the canine ejaculate would benefit all assisted reproductive techniques used in this species. The purpose of the present study was to evaluate the effects of PGF$_{2\alpha}$, oxytocin and GnRH on canine ejaculate characteristics.

Eight, mature, medium size (25-30 kg), mixed breed dogs were randomly assigned to one of four treatment groups (N=2 dogs each); each group received one treatment per week for four weeks. Treatments were assigned based on a Latin Square design. A two-week training period was used to acclimate the dogs to manual semen collection. Treatments were 0.1 mg/kg PGF$_{2\alpha}$ 15 minutes prior to collection, 2.5 units/dog oxytocin 10 minutes prior to collection, 50 μg/dog GnRH 60 minutes prior to collection, or 1.0 ml of saline 30 minutes prior to collection. An

evaluator that was blinded to treatment analyzed ejaculate characteristics. Samples were evaluated for semen volume, concentration of spermatozoa per milliliter, motility, morphology, total sperm number and total morphologically normal motile sperm number (TNMS). In addition, a subjective ease of collection score was assigned following each collection (Scale 1-9, 1 being easiest to manually ejaculate).

Semen concentration, motility and morphology were not different between treatments. Semen volume was greater for dogs treated with $PGF_{2\alpha}$ or oxytocin compared to saline. Total sperm number and TNMS were greater when dogs were treated with $PGF_{2\alpha}$ compared to oxytocin, GnRH and saline ($p<0.05$). The subjective ease of collection score was lower for dogs receiving $PGF_{2\alpha}$ compared to GnRH or saline ($p<0.05$).

Treatment	Volume (ml)	Total Sperm Number (x 10^6)	TNMS (x 10^6)	Ease of Collection
$PGF_{2\alpha}$	1.94[a]	449.25 [a]	321.30 [a]	2.37 [b]
Oxytocin	1.93 [a]	236.44 [b]	175.90 [b]	3.00 [a, b]
GnRH	1.77 [a, b]	229.13 [b]	158.68 [b]	4.37 [a]
Saline	1.16 [b]	165.63 [b]	117.04 [b]	4.75 [a]

[a, b] Means with the same letter are not statistically different

In summary, administration of $PGF_{2\alpha}$ or oxytocin prior to semen collection increased semen volume and $PGF_{2\alpha}$ increased total sperm number in the ejaculate of the dog. It did not appear that treatment with GnRH had an effect on semen parameters evaluated in this study.

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ACKNOWLEDGMENTS

LIST OF ABBREVIATIONS

Artificial Vagina   AV

Computer-Aided Semen Analysis   CASA

Daily Sperm Output   DSO

Daily Sperm Production   DSP

Follicle Stimulation Hormone   FSH

Gonadotropin Releasing Hormone   GnRH

Gonadotropin Releasing Hormone Agonist   GnRH-A

Human Chorionic Gonadotropin   hCG

Idiopathic Hypogonadotrophic Hypogonadism   IHH

International Unit   IU

Intramuscular   IM

Intravenous   IV

Kilogram   kg

Luteinizing Hormone   LH

Luteinizing Hormone Releasing Hormone   LHRH

Micrograms	μg
Milligrams	mg
Milliliter	ml
Milliunit	mU
Milli International Unit	mIU
Minute	min
Prostaglandin E_2	PGE_2
Prostaglandin $F_{2\alpha}$	$PGF_{2\alpha}$
Subcutaneous	SC
Total Normal Motile Sperm	TNMS

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INTRODUCTION

Increasing use of assisted reproductive techniques in the dog has uncovered a
need for additional aids to help veterinarians optimize the ejaculates obtained
from their canine patients. Specifically, improving upon the number of
spermatozoa obtained during semen collection would benefit most areas of
assisted reproduction, including semen cryopreservation and artificial
insemination. Although some dogs consistently give high quality semen
samples when collected for semen evaluation, shipment, freezing or
insemination, other dogs are more difficult to collect and give ejaculates with
low numbers of sperm and other suboptimal semen characteristics.

The use of sexual preparation in conjunction with semen collection has been
shown to optimize the number of spermatozoa in the ejaculate of the rabbit and
bull[1-3]. Typically, sexual preparation in the dog includes the use of a bitch in
estrus or the use of pheromones in the form of vaginal discharge from a bitch in
estrus preserved on bedding or other absorbent materials. Unfortunately, the
availability of teaser bitches and pheromones is frequently limited and clinicians
are therefore forced to collect semen from dogs without sexual preparation.

Collections obtained in this manner often have a decreased volume and total sperm number compared to collections obtained with sexual preparation. This decrease in sperm cell quantity without sexual preparation is seen in multiple species including the rabbit and bull[2,3]. For this reason a study was undertaken to determine the effect of a variety of drugs on semen parameters in the hope of finding a drug which will increase sperm output in the ejaculate when sexual preparation is not possible.

Research conducted in bulls, buffalo, rams, rabbits and stallions has identified two hormones that when administered prior to semen collection will improve ejaculate quality by increasing total sperm number in the ejaculate. The first hormone, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), increases the total number of spermatozoa in the ejaculate of bulls, rabbits, rams and stallions[1,4-11]. Total sperm numbers are increased as a function of greater spermatozoa concentration, ejaculate volume or both. In the boar, total sperm cell numbers per ejaculate are not affected by $PGF_{2\alpha}$ administration, but there is an increase in the concentration and volume of the sperm rich portion of the ejaculate[12]. The proportion of sperm in each ejaculate fraction is redistributed such that there is more sperm in the sperm rich fraction and less sperm in the remaining fractions. This is of use to producers in porcine artificial insemination programs as the sperm rich portion of the boar ejaculate is typically the only portion collected.

It has been demonstrated in rabbits, bulls, buffalo and stallions that while $PGF_{2\alpha}$ will increase total sperm numbers in the first ejaculate following administration, semen parameters are typically unchanged from that of controls in the second ejaculate following $PGF_{2\alpha}$ administration[5,8-10].

The mechanism behind the increase in ejaculate volume and/or concentration in response to $PGF_{2\alpha}$ is not fully understood. It is thought that $PGF_{2\alpha}$ acts directly on the contractile tissues of the testicular capsule and epididymis causing an increased rate of sperm passage from the epididymis to the deferent ducts. The use of $PGF_{2\alpha}$ prior to collection may optimize the number of sperm in a collection by enhancing sperm movement from the epididymis to the deferent duct where they are available for ejaculation. The dosages and intervals from $PGF_{2\alpha}$ administration to collection have varied widely between reports. Response to treatment has also varied between reports and with method of collection. In addition, one report in the bull cites the additive benefit of $PGF_{2\alpha}$ and sexual preparation on increasing total sperm numbers in the ejaculate[6]. Other research has not shown an additive benefit to drug administration and sexual preparation[5].

The second hormone, oxytocin, has also been shown to increase sperm output in buffalo and rams[4,13-15]. The increase in sperm number following oxytocin administration has been attributed to an increase in smooth muscle contraction

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surrounding the epididymis, which enhances spermatozoa movement into the deferent duct[13]. Oxytocin is also thought to influence the secretion rate of the male accessory glands, which, may account for the increased volume seen in the ejaculate of rams and buffalo following administration[4,15].

Semen collected from bulls, buffalo, rams, stallions and boars is frequently cryopreserved for future use. Techniques that increase the number of spermatozoa in the ejaculate can be beneficial in that they increase the number of straws frozen. However, such techniques are only useful as long as they are not detrimental to the ability of the semen to undergo cryopreservation. Because of this, researchers evaluated the post-thaw motility of cryopreserved ejaculates collected after $PGF_{2\alpha}$ or oxytocin administration. Ibrahim, Marshall and Hafs found that the post-thaw motility of cryopreserved buffalo and bull ejaculates collected after $PGF_{2\alpha}$ or oxytocin administration was not different from that of untreated controls[4,6].

Gonadotropin releasing hormone (GnRH) is also utilized to improve ejaculate quality in the dog. Gonadotropin releasing hormone causes a surge in luteinizing hormone (LH) and testosterone, 30 and 60 minutes, respectively, after administration in the dog[16-18]. Gonadotropin releasing hormone has been used clinically prior to collection to improve the performance of dogs with poor libido[18]. The testosterone surge following GnRH administration is the proposed

cause for the improvement in libido. Increases in total sperm number appear to be independent of increases in plasma testosterone concentration seen following prostaglandin administration in some species. In rabbits, $PGF_{2\alpha}$ administration caused a testosterone surge and 50% increase in ejaculate concentration while prostaglandin E_2 (PGE_2) administration resulted in a decreased plasma testosterone concentration and an 84% increase in ejaculate concentration[9]. It remains to be seen how GnRH administration prior to semen collection will affect ejaculate characteristics in normal dogs.

The purpose of the following study was to evaluate the effect of $PGF_{2\alpha}$, oxytocin and GnRH on ejaculate characteristics in the dog collected without sexual preparation. If a drug were found to enhance ejaculate quality, it would be of great benefit to practitioners in the field of canine reproductive. For the drug to be of maximal benefit, it must not affect the ability of semen to be frozen or cooled. Therefore, the second goal of this study is to evaluate the effect of $PGF_{2\alpha}$, oxytocin and GnRH on the motility of cooled semen as well as thawed frozen semen.

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BACKGROUND

Sperm transport in the testicle

Sperm transport through the testicle from the site of spermiation in the
seminiferous tubules through the rete testis and into the epididymis is mediated
by actions not inherent to the spermatozoa. Sperm cells do not gain the ability
to propel themselves forward until they have traveled through the epididymis[19].
Transport of non-motile spermatozoa is governed primarily by contraction of
smooth muscle surrounding the seminiferous tubules[20,21] and contraction of
smooth muscle within the testicular capsule[22-24]. Sperm transport is also
assisted by secretion of fluid by the seminiferous tubules, which supplies a
medium, and pressure gradient that promotes movement. Obstruction of fluid
flow at the level of the efferent duct will reduce sperm flow distal to the point of
obstruction. A significant increase in testicular fluid accumulation following
obstruction indicates that fluid flow in the testicle is an important mediator of
sperm transport[25,26].

Contractile cells are found in the testicular capsule of all mammals that have been investigated[23]. Among the species studies, contractile cells are most prominent in the testicular capsule of the dog, rabbit and boar[23,27]. Contraction of the testicular capsule occurs at a frequency and amplitude that varies between species. Contractions are rhythmic in the dog and the rabbit, and spontaneous and phasic in the boar[21]. Control of testicular capsule contractility appears to be mediated by both neuronal and non-neuronal factors. Testicular capsule preparations contract in response to sympathomimetic agents *in vitro*[22]. Stimulation of the perivascular nerve increases intratesticular pressure in the dog due to contraction of the testicular capsule indicating innervation of the smooth muscle[28].

Smooth muscle cells of the seminiferous tubules are not innervated by any known nerve supply. Other factors including tubular pressure gradients and hormones are involved in regulating contractility of seminiferous tubules[29]. Supporting evidence for this is found in mice where it was discovered that differentiation of contractile cells and initiation of seminiferous tubule contractile activity was dependent on maturation of the pituitary[30]. Additionally, seminiferous tubule preparations from newborn rats require an androgen source for normal development[31]. These findings, along with the lack of known nervous innervation suggest that hormonal mediators are responsible for

seminiferous tubule contractility. The testicular capsule and seminiferous tubule respond similarly to stimulation with exogenous hormone preparations although the response is not necessarily similar between species[20,21]. Voglmayr catheterized the rete testis and deferent duct of rams to determine the effect of oxytocin on seminal fluid flow[13]. Following an intravenous priming injection of 12 mU/kg of oxytocin, rams were given oxytocin by constant rate infusion of 1.5 mU/kg/min for one hour. At this dosage, semen was discharged in distinct waves lasting 30-40 seconds and recurring every 1-3 minutes. The volume collected was approximately twice what was collected during control studies without oxytocin. The volume of fluid collected from the catheterized rete testis was also increased slightly during the oxytocin infusion compared to pretreatment values. The Leydig cells produce endogenous oxytocin[32]. Depletion of testicular oxytocin by destruction of the Leydig cells can be induced by ethane dimethane sulfate. The destruction of Leydig cells is associated with a decrease in spontaneous seminiferous tubule contractility. Contractility can be restored by the administration of exogenous oxytocin[33].

In a similar experiment, Free and colleagues determined that intravenous administration of $PGF_{2\alpha}$ to rats would increase the flow of fluid from the rete testis 2-3 fold over a period of 20-40 minutes compared to untreated controls[34]. Free noted that an intact testicular capsule was not necessary for basal sperm

transport through the seminiferous tubules in rats[34]. However, if the testicular

capsule were removed, the response to $PGF_{2\alpha}$ was eliminated or diminished.

Farr and Ellis determined that $PGF_{2\alpha}$ stimulated seminiferous tubule contraction

frequency *in vitro* while PGE_2 inhibited the size and frequency of the

contractions[35]. Furthermore, spontaneous contraction of the testicular capsule

and seminiferous tubules is significantly inhibited by indomethacin, a potent

prostaglandin synthesis inhibitor[36,37].

Sperm transport in the epididymis and ductus deferens

Once the non-motile spermatozoa have passed from the seminiferous tubules,

through the efferent duct and rete testis, they enter the proximal epididymis.

Transport of sperm cells through the epididymis is dependent on peristaltic

contractions of the smooth muscle layers surrounding the epididymal wall.

Smooth muscle composition of the epididymis increases from proximal to distal

regions[38]. Correspondingly, sympathetic innervation of the epididymal duct

walls increases from proximal to distal with the cauda epididymis and ductus

deferens richly innervated[38]. Not surprisingly then, the tissue composition of

the epididymis changes from proximal to distal. Bartke and Koerner

demonstrated that the prostaglandin composition of the distal epididymis in rats

and mice was significantly greater than the proximal portions[39]. Similarly, the

concentration of prostaglandins in the lumen of the ram epididymis is 15-20

times greater in the distal regions compared to the proximal regions[40]. The concentration of $PGF_{2\alpha}$ in the cauda epididymis fluid is eight times that of the rete testis fluid in bulls[41]. Oxytocin receptors have also been identified in the ovine epididymis and ductus deferens[42]. Unlike prostaglandin, endogenous oxytocin exists in the epididymis in similar concentrations in all regions[43].

There is abundant evidence for the inherent contractile ability of the epididymis *in vitro*. Spontaneous contraction of the epididymis *in vitro* can be inhibited or accelerated in the same manner in which contraction of the testicular capsule and seminiferous tubules is altered. Norepinipherine, acetylcholine and testosterone have a stimulating effect on caput epididymis contractions, and this effect appears to be modulated by prostaglandins[44,45]. Indomethacin reduced spontaneous contractions of the epididymis *in vitro.* Oxytocin has also been shown to increase contractions of the epididymis *in vitro*[46].

There is convincing *in vivo* support for the effect of $PGF_{2\alpha}$ and oxytocin on epididymal contractility. In a series of experiments, Hafs *et al* used anesthetized rabbits to demonstrate that exogenous $PGF_{2\alpha}$ significantly increased the movement of sperm from the epididymis to the deferent duct[47]. In the first experiment, one testicle and associated epididymis and deferent duct were removed. The second testicle, epididymis and deferent duct were removed 10, 30 or 60 minutes following injection of 5 mg $PGF_{2\alpha}$ into the tunica

vaginalis surrounding the remaining testicle. The second experiment was similar to the first except that $PGF_{2\alpha}$ was administered subcutaneously (SC) 10 or 30 minutes prior to removal of the testicle, epididymis and deferent duct. The number of spermatozoa in the deferent duct, cauda epididymis and corpus-caput epididymis were determined. Following peritesticular $PGF_{2\alpha}$ injection, the number of spermatozoa in the deferent duct was more than twice that of the controls. Thirty minutes after SC injection of $PGF_{2\alpha}$ the number of spermatozoa in the deferent duct was 2.5 times greater than that of controls. In a non-anesthetized study, rabbits were given 10 mg $PGF_{2\alpha}$ SC three times at 20-minute intervals. The rabbits were then killed and the distribution of spermatozoa in the epididymis and deferent was determined. As with the anesthetized rabbits, the number of spermatozoa in the deferent duct of treated rabbits was almost 3 times greater than saline treated controls.

Using anesthetized rams, Nicholson et al demonstrated that 10 and 100 μg of oxytocin significantly increased the output of fluid and number of spermatozoa from the cauda epididymis[48]. By cannulating the cauda epididymis and collecting luminal fluid every 10 minutes, Nicholson and colleagues were able to show that treatment with oxytocin caused an increase in sperm number and fluid volume starting 10 minutes after treatment. Values did not return to that of controls until 40 minutes after treatment. Administration of an oxytocin

antagonist (des Gly-NH$_2$d(CH$_2$)$_5$-[-Tyr^2,Thr^4]OVT) had no immediate effects on sperm numbers or fluid flow but there was a significant reduction in both values 40-50 minutes after treatment. Melin also demonstrated the *in vivo* effects of oxytocin[49]. Using anesthetized rabbits, Melin showed that administration of 20 mIU of oxytocin caused a significant increase in the amplitude of contractions in the distal epididymis and proximal ductus deferens.

Factors affecting sperm output

Johnson defines daily sperm output (DSO) as the total number of spermatozoa released in the ejaculate daily by a pair of testes[50]. Daily sperm output is closely related to daily sperm production (DSP) and it has been determined that DSP can be estimated by quantifying sperm numbers in the ejaculate once epididymal reserves have been stabilized through frequent collection[51]. In the dog, stabilization of spermatozoa output can be accomplished by collecting semen for 5-6 consecutive days[52]. Once gonadal reserves have been stabilized, DSO is an accurate measure of DSP with 79% of DSP accounted for by DSO in the dog[52]. Factors affecting sperm output include testicular size, season, age, testicular disease, genetics, and environment[53]. Factors affecting total sperm number in the ejaculate include those that affect sperm output, as well as frequency of collection or ejaculation; conditions imposed at the time of

collection such as sexual preparation, method of semen collection and
exogenous drug administration[5,53].

Frequency of collection does not alter the rate of sperm production[51]. However,
frequency of collection does effect the composition of the reservoir of sperm
stored in the cauda epididymis. The first ejaculate obtained after a period of
rest will have more sperm than subsequent ejaculates[54]. Eventually, with
frequent collection, the number of spermatozoa obtained in the ejaculate
reflects the daily sperm output. At this point, the epididymal reserves have
been stabilized. In the dog, 4-5 days between ejaculates is required to build up
epididymal reserves and maximize the number of spermatozoa in each
ejaculate[52].

Sexual preparation prior to semen collection in bulls by active restraint and a
series of false mounts has been shown to significantly increase the number of
spermatozoa in the ejaculate[1,2,51]. Likewise, in rabbits, sexual preparation in
the form of one false mount and a three-minute delay before semen collection
yielded 278% of the sperm collected without sexual preparation[3]. Three false
mounts resulted in an additional 40% increase in sperm numbers compared to
one false mount. Hafs *et al* determined that the magnitude of sexual
preparation is directly correlated with the increase in sperm in the ejaculate[1].

Voglmayr noted that the sperm output from a catheterized testis increased 62% during exposure to estrus ewes for 24 hours[13].

The method of collection and equipment used to obtain ejaculates also has a significant effect on the number of spermatozoa recovered. Ejaculates obtained by artificial vagina (A.V.) had significantly more sperm than those collected using electroejaculation in the bull[55]. In addition, the temperature of the A.V. has an effect on the bull and stallion's stimulus to ejaculate[56,57].

Administration of oxytocin prior to semen collection increased the total sperm number obtained in the ejaculate in the ram, and bull[14,15,58]. The sperm cell number was increased as a function of increased concentration, semen volume or both. The findings and oxytocin treatment regimes from the referenced studies are summarized in Table 1.

Administration of $PGF_{2\alpha}$ increases sperm numbers in the ejaculate of the rabbit, bull, stallion, buffalo, and ram[4-7,9,10]. Like oxytocin, the increase in sperm number in the ejaculate is a function of greater sperm cell concentration, volume or both. Table 2 summarizes the findings of researchers investigating the effects of $PGF_{2\alpha}$ on ejaculate parameters in various species.

Table 1. Summary of results obtained by researchers investigating the effects of oxytocin on ejaculate characteristics in rams and bulls.

Author(s)	Species	Dose	Route	Treatment-Collection Interval	Sperm/ ejaculate	Sperm concentration	Semen volume
Knight & Lindsay[14]	Ram	7 I.U.	I.V.	5 min	↑	unchanged	↑
Knight[15]	Ram	10 I.U.	I.V.	10 min	↑	↑	↑
Berndtson & Igboeli[58]	Bull	50 I.U.	I.V.	10 min	↑	↑	unchanged

In the boar, $PGF_{2\alpha}$ increased the number of sperm in the sperm rich fraction of the ejaculate as well as the duration of ejaculation of the sperm rich fraction. However, there was no effect seen on total ejaculate sperm numbers, which led the authors to postulate that $PGF_{2\alpha}$ caused a redistribution of sperm into the sperm rich fraction of the ejaculate[12].

Table 2. Summary of results obtained by researchers investigating the effects of PGF$_{2\alpha}$ on ejaculate characteristics in various species.

Author(s)	Species	Dose	Route	Treatment-Collection Interval	Sperm/ ejaculate	Sperm concentration	Semen volume
Ibrahim[4]	Buffalo	0.5 -2.0 mg	I.M.	10 min	↑	Unchanged	↑
Marshall and Hafs[6]	Bull	40 and 2x40 mg	S.C.	1 hour; 1 and 2 hours	↑	↑	Unchanged
Reichard et al[9]	Rabbit	5 mg	S.C.	2 and 4 hours	↑	↑	Unchanged
Cornwell et al[10]	Stallion	10 mg	I.M.	1 hour	↑	↑(Not significant)	↑(Not significant)
Mekonnen et al[7]	Ram	1.0 and 2.0 mg	I.M.	30 min	↑	Unchanged	↑
Hafs et al[5]	Rabbit	2.5 mg	S.C.	2 hours	↑	↑	Unchanged
Hafs et al[5]	Bull	40 or 80 mg	I.M.	30 min	↑	↑	Unchanged

The increase in spermatozoa and fluid flow from the rete testes, epididymis and deferent duct following oxytocin or PGF$_{2\alpha}$ administration does not appear to be the result of an increase in spermatogenesis. Spermatogenesis is disturbed with chronic administration of high doses of PGF$_{2\alpha}$ in the rat and mouse and with oxytocin in the ram as evidenced by alterations in the populations of

ejaculated spermatozoa and changes in the histology of the seminiferous tubules[14,59,60]. Other researchers have determined that long-term oxytocin administration had no effect on spermatogenesis in the bull[58]. In addition, those studies that have documented an increase in sperm numbers in the ejaculate following $PGF_{2\alpha}$ or oxytocin administration have shown that the effect is limited to the first of two successive ejaculates[5,6,10,58,61]. Knight determined that while administration of oxytocin prior to semen collection in the ram would increase sperm numbers in the ejaculate by 45%, the time interval between treatment and collection was critical[15]. Optimal increases in sperm number occurred between 5 and 10 minutes after intravenous oxytocin injection and the effect was absent if the rams were collected 90 minutes after treatment.

Physiologic effects of oxytocin, $PGF_{2\alpha}$ and GnRH

In addition to increased sperm numbers in the ejaculate following $PGF_{2\alpha}$ administration, some researchers noted that treated animals had more libido at the time of semen collection[7,8,11]. Libido was assessed using quantifiable observations, such as time to initial false mount and time to ejaculation in buffalo, and time for collection in rams. Initially, the effects on reaction time and collection time were attributed to a surge in testosterone following $PGF_{2\alpha}$ administration. In yearling beef bulls, serum testosterone increased two-fold following $PGF_{2\alpha}$ and remained elevated for over four hours[62]. Prostaglandin

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$F_{2\alpha}$ treatment caused a release of testosterone in the bull that became apparent in 40-50 minutes and persisted for at least 8 hours[63]. Reichard *et al* reported that $PGF_{2\alpha}$ treatment caused a testosterone surge in rabbits that peaked at two hours and was gone after four hours[9]. In the same study, Reichard reported that administration of PGE_2 prior to semen collection increased sperm numbers in the first ejaculate, but depressed serum testosterone secretion. The increase in testosterone following $PGF_{2\alpha}$ treatment was thought to be due to direct stimulation of testicular steroidogenesis. Prostaglandin $F_{2\alpha}$ stimulates cyclic AMP production in the testicle; cyclic AMP then stimulates testosterone synthesis[9,63].

Use of $PGF_{2\alpha}$ and oxytocin to increase sperm numbers in the ejaculate is only beneficial if the resulting sperm have normal fertilizing and cryopreservation ability. In the rabbit it has been reported that cauda epididymal sperm incubated with $PGF_{2\alpha}$ have normal fertilizing ability[64]. Treatment of intact male hamsters with 50μg $PGF_{2\alpha}$ once daily for 10 days had no effect on fertilizing ability *in vivo*[65]. Post-thaw motility of cryopreserved buffalo[4,8] and bull[6] semen was unaffected by administration of $PGF_{2\alpha}$ and oxytocin prior to semen collection and freezing.

Gonadotropin releasing hormone is produced by the hypothalamus and signals the release of LH and follicle stimulation hormone (FSH) from the anterior

pituitary. Luteinizing hormone acts as a trigger for testosterone release by the

Leydig cells in the testicle. Administration of exogenous GnRH to male dogs

induces a dose dependent release of LH immediately following treatment[17].

Increases in testosterone concentration are measurable starting 10 minutes

after GnRH administration, peak in 50-60 minutes and remain elevated above

control values for 4-24 hours depending on the GnRH preparation used in the

study[17,18].

Normal hypothalamic-pituitary-testicular axis function is critical for reproductive

health; for this reason it has been studied and manipulated in the hope of

finding a way to treat individuals with reproductive dysfunction. In humans,

oligospermia can be associated with elevated FSH concentrations. The

condition, known as idiopathic hypogonadotrophic hypogonadism (IHH) is

postulated to be due to low LH pulse frequency resulting in high FSH

concentrations.   This hypothesis was supported by experiments in

hypothalamic-lesioned female rhesus monkeys treated with subnormal GnRH

pulse frequencies. The monkeys displayed normal LH concentrations but high

FSH concentrations[66]. Furthermore, treatment of infertile men with pulsatile

GnRH lowers FSH concentrations[67] and Wagner *et al* recommended the use of

GnRH as a treatment for oligospermia associated with elevated FSH

concentrations in men[68]. However, that recommendation was not supported by

Bals-Pratsch and colleagues who determined that pulsatile GnRH therapy had

no effect on the semen parameters of infertile men with elevated FSH concentrations[67]. Gonadotropin releasing hormone has been suggested as a treatment for reproductive dysfunction in stallions resulting from IHH[69]. Although Brinsko acknowledges that IHH is not well documented in stallions, he reports that GnRH therapy has improved fertility in a limited number of horses[69].

In the dog, improvements in semen quality have been reported after a single injection of human chorionic gonadotropin (hCG)[16,70]. Human chorionic gonadotropin has LH-like activity in most mammals. In the first study, Kawakami *et al* reports that one intramuscular (IM) injection of 1,000 IU hCG temporarily improved the semen quality of an oligospermic beagle between 3 and 4 weeks post treatment[16]. In the second study, the semen quality of a collie with idiopathic oligospermia improved 2 weeks after treatment with 1000 IU hCG I.M.[70]. The use of hCG in male dogs with normal semen parameters was reported to slightly decrease semen volume and increase serum testosterone concentration without affecting other semen parameters[71].

In addition to using hCG for reproductive dysfunction in the dog, Kawakami *et al* also evaluated the effects of a GnRH agonist (GnRH-A), as well as a luteinizing hormone releasing hormone analog (LHRH). Treatment of a collie with idiopathic oligospermia with one IM injection of 1 μg/kg GnRH-A was reported

to markedly increase semen volume, sperm number, sperm motility and viability as well as decreasing the percentage of morphologically abnormal sperm[70]. These changes were apparent 4 weeks after GnRH-A treatment. Treatment with two injections of 400 μg LHRH given 3 hours apart, improved the semen quality of 3 male dogs with idiopathic oligospermia and improved the fertility of one of the dogs[72]. The reported changes occurred 20-40 days after treatment and were attributed to the temporary rise in plasma LH and testosterone measured 30 and 90 minutes, respectively, after LHRH treatment. The use of hCG in male dogs with normal semen parameters was reported to slightly decrease semen volume and increase serum testosterone concentration without affecting other semen parameters[71].

C h a p t e r   3

MATERIALS AND METHODS

Eight adult male dogs (20-35 kg) were housed in individual runs in the animal

containment facilities at the Virginia-Maryland Regional College of Veterinary

Medicine and Center for Molecular Medicine and Infectious Diseases. Water

was available ad libitum and dry dog food was provided twice daily. All dogs

used in the project were screened to ensure palpably normal reproductive

anatomy, a generally agreeable disposition and normal physical examination

findings. Prior to onset of the study, the dogs were acclimated for 14 days in

the animal containment facilities. During the acclimation period, semen was

manually collected from the dogs twice weekly[73,74]. No sexual preparation was

used prior to or during semen collection. Semen obtained during the

acclimation period was evaluated under a light microscope to ensure viable

spermatozoa were collected. No other analysis was performed on the

ejaculates during the acclimation period.

This experiment was designed to determine the effects of $PGF^a_{2\alpha}$, oxytocin[b] and GnRH[c] on ejaculate characteristics in the dog. The first and second ejaculate fractions were collected from all dogs twice weekly, two days apart, for four weeks. The first weekly collection was evaluated for the presence of spermatozoa and served to ensure all dogs had similar epididymal spermatozoa stores prior to the second collection each week, which occurred two days later. No treatment was given prior to the first collection each week. Prior to the second collection, dogs were treated with either $PGF_{2\alpha}$ 0.1 mg/kg SC 15 minutes prior to semen collection; oxytocin 2.5 IU/dog IM 10 minutes prior to semen collection; GnRH 50 μg/dog IM 60 minutes prior to semen collection; or saline 1.0 ml/dog SC 30 minutes prior to semen collection. Drug dosage and time interval from administration to ejaculate collection were selected based on known dosages in the dog. The dose of $PGF_{2\alpha}$ and oxytocin was extrapolated from dosages used in the bitch to cause uterine contraction[75,76]. The time interval was chosen based on the amount of time required for the drug to exert a maximal response[77]. The dosage of GnRH and interval to collection was based on previous reports[18].

Two individuals that were trained in manual semen collection were involved in collecting semen from the dogs during the project; however, the same person

collected the same dogs each time. Collectors were aware of the treatment

each dog received, however, an evaluator blinded to treatment performed the

semen analyses.

The treatments were assigned based on Latin square design such that all dogs

received all treatments. Four different treatment orders were designed to

ensure that treatment orders were not repeated. Dogs were assigned a letter

A-H for identification and were then randomly assigned to a treatment order.

Table 3 summarizes the treatment schedule for the four-week experiment.

Table 3. Treatment schedule prior to second semen collection each week.

Order / Dog	Week 1	Week 2	Week 3	Week 4
G, D	$PGF_{2\alpha}$	Oxytocin	GnRH	Saline
C, H	Oxytocin	Saline	$PGF_{2\alpha}$	GnRH
E, A	GnRH	$PGF_{2\alpha}$	Saline	Oxytocin
B, F	Saline	GnRH	Oxytocin	$PGF_{2\alpha}$

Aliquots of ejaculates obtained from the first collection each week were

evaluated under a light microscope to confirm the presence of spermatozoa.

No other analysis was performed. Semen samples obtained during the second

collection each week were evaluated for volume (ml), concentration ($x10^6$/ml),

motility, percent normal morphology, total sperm number ($x10^6$/ejaculate) and

total normal motile sperm number (TNMS)[73,74,78]. Volume was measured using a graduated conical tube. Concentration was measured by counting the number of spermatozoa present on both sides of a hemocytometer after the semen had been diluted 1:99 in formalin and then multiplying by a dilution factor. Concentration measurements were repeated if counts from the two sides of the hemocytometer varied by more than 10%. Motility was assessed using a computer-aided semen analyzer (CASA)[d]. If the CASA was unable to measure motility due to high concentration, the sample was diluted with equal parts 37°C physiologic saline and semen until the motility was obtained. When an ejaculate sample required dilution for the CASA, the dilution was performed for every ejaculate from that dog. The percentage of morphologically normal spermatozoa was determined by staining an aliquot of each ejaculate with eosin-nigrosin stain[e] and counting 100 sperm cells under oil immersion. Total sperm number was determined as a product of sperm concentration and semen volume. Total normal motile sperm number was a product of total sperm number, percent motility and percent normal morphology.

Following each ejaculate collection, every dog was assigned an ease of collection score. The score was based on observable criteria such as length of time to obtain ejaculate, the number of attempts required to obtain ejaculate, and presence of mounting and thrusting behavior. A minimum score of 1 was assessed if the dog was extremely easy to collect, required only one collection

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attempt, ejaculated in less than one minute and displayed mounting and thrusting behavior. A maximum score of 9 was assessed if the dog was impossible to collect, required more than 3 collection attempts, took greater than 5 minutes to collect, and displayed no mounting or thrusting behavior.

Table 4. Ease of collection score criteria.

Score	Behavior
1	Ejaculate collected in 1 attempt, dog exhibiting thrusting and mounting behavior. Total time for collection less than 1 minute.
2	Ejaculate collected in 1 attempt, dog exhibited mounting or thrusting behavior. Total time for collection less than 1 minute.
3	Ejaculate collected in 1 attempt, dog exhibited no mounting or thrusting behavior. Total time for collection less than 1 minute.
4	Ejaculate collected after 2 attempts, dog exhibited mounting and thrusting behavior. Total time for collection less than 2 minutes.
5	Ejaculate collected after 2 attempts, dog exhibited mounting or thrusting behavior. Total time for collection less than 2 minutes.
6	Ejaculate collected after 2 attempts, dog exhibited no mounting or thrusting behavior.
7	Ejaculate collected after 3 attempts, dog exhibited mounting and/or thrusting behavior. Total time of collection less than 5 minutes.
8	Ejaculate collected after 3 attempts, no mounting or thrusting behavior. Total time of collection less than 5 minutes.
9	Ejaculate not collected after 3 attempts, dog displayed no mounting or thrusting behavior. Collection time exceeded 5 minutes.

Experiment 2

This experiment was designed to assess whether administration of $PGF_{2\alpha}$, oxytocin or GnRH, prior to ejaculate collection, had an effect on the motility of semen that had been cooled and stored for 24 and 48 hours. Aliquots of each ejaculate were extended 1:3 in commercial semen cooling extender[f] and placed in a prefabricated semen cooling and shipping container[g]. The samples were then evaluated at 24 and 48 hours to determine the motility. Motility was assessed using the CASA.

Experiment 3

This experiment was designed to assess whether administration of $PGF_{2\alpha}$, oxytocin or GnRH, prior to ejaculate collection, had an effect on the motility of semen that had been frozen and thawed. Aliquots of each ejaculate were centrifuged within 10 minutes of collection at 400G for 10 minutes to separate the sperm from the seminal plasma. The seminal plasma was discarded and the sperm pellet was re-extended in canine semen freezing extender[h]. After a 2-hour acclimation period at $5°C$, freezing extender was added again to make a final dilution of 1 part sperm pellet and 4 parts extender. Following another 1-hour acclimation at $5°C$, the extended semen was dropped into shallow wells in a dry ice block and was frozen in pellets. Once the pellets were frozen, they were dropped into liquid nitrogen to complete the freezing process. Twelve to

24 hours after the freezing process was complete, one pellet from each sample was thawed in 0.75 ml sterile saline at 37°C for 45 seconds. Post-thaw motility was assessed using the CASA.

<u>Statistical Analysis</u>

The results are given in terms of mean. Comparisons were statistically evaluated using an ANOVA of Latin Square. In addition, multiple comparisons were made using Duncan's multiple range test[79].

C h a p t e r   4

RESULTS

Side effects were noted following $PGF_{2\alpha}$ administration in 7 of 8 dogs. Side

effects were considered mild. Seven of eight dogs consistently displayed

panting within 5 minutes of administration. Hypersalivation was noted to occur

approximately 25% of the time. One dog regurgitated within 10 minutes of

$PGF_{2\alpha}$ treatment and one dog defecated within 10 minutes of treatment. All

side effects resolved within 20 minutes of administration. No side effects were

noted following oxytocin, GnRH or saline administration.

Experiment 1

Tables 5 and 6 show the results for the second ejaculate collected each week

from the eight dogs. Although there were variations between dogs in the

various parameters measured, definite effects of $PGF_{2\alpha}$ and oxytocin were

observed. Semen volume was greater following treatment with $PGF_{2\alpha}$ or

oxytocin than saline. Total sperm number and TNMS were greater following

$PGF_{2\alpha}$ treatment than other treatments. The ease of collection score was lower

for dogs treated with $PGF_{2\alpha}$ than GnRH or saline, indicating it was easier to

obtain an ejaculate from $PGF_{2\alpha}$ treated dogs. In addition, although not

statistically significant, there was a trend towards greater concentration in

samples collected following $PGF_{2\alpha}$ administration.

Table 5. Effects of treatment on semen volume, concentration, motility and
morphology.

Treatment	Volume (ml)	Concentration $(x10^6/ml)$	% Motility	% Normal Morphology
$PGF_{2\alpha}$	$1.94^a \pm 1.19$	$273.75^a \pm 168.05$	$91.00^a \pm 5.29$	$74.25^a \pm 12.38$
Oxytocin	$1.93^a \pm 1.55$	$188.75^a \pm 178.40$	$84.00^a \pm 12.90$	$65.85^a \pm 18.83$
GnRH	$1.76^{a,b} \pm 1.51$	$173.12^a \pm 155.03$	$79.87^a \pm 16.36$	$63.66^a \pm 26.12$
Saline	$1.15^b \pm 1.09$	$185.00^a \pm 160.35$	$80.37^a \pm 24.89$	$66.85^a \pm 11.72$

[a,b] Means with the same letter are not significantly different

This non-significant increase in concentration combined with the significant

increase in volume served to make the differences in total sperm number and

TNMS following $PGF_{2\alpha}$ administration markedly greater than any of the other

compounds tested. There was a significant effect of dog on semen volume,

concentration, total sperm number, and TNMS.

Table 6. Effects of treatment on total sperm number, TNMS and ease of collection.

Treatment	Total sperm number (x10^6)	TNMS (x10^6)	Ease of collection
PGF$_{2\alpha}$	449.25^a ± 209.46	321.30^a ± 185.15	2.37^b ± 0.91
Oxytocin	236.43^b ± 218.19	175.89^b ± 161.81	3.0^{b,a} ± 1.51
GnRH	229.12^b ± 255.83	158.68^b ± 148.71	4.37^a ± 2.32
Saline	165.62^b ± 158.90	117.04^b ± 119.97	4.75^a ± 2.60

^{a,b} Means with the same letter are not significantly different

Experiments 2 and 3

There was no observable effect of treatment or dog on the ability of ejaculate samples in this study to undergo cooling or cryopreservation. The motility of all samples was similar to saline treated control values during each analysis. Table 7 contains the motility data for semen that was extended and cooled for 24 and 48 hours, as well as the post-thaw motility of semen that had been frozen.

Table 7. Effects of treatment on semen cooling and post-thaw motility

Treatment	24 hour % Motility	48 hour % Motility	Post-Thaw % Motility
PGF$_{2\alpha}$	71.87[a] ± 7.52	50.62 [a] ± 20.43	59.37 [a] ± 4.17
Oxytocin	61.25[a] ± 25.46	50.0 [a] ± 29.03	64.37 [a] ± 13.47
GnRH	61.87[a] ± 9.61	48.12 [a] ± 25.62	58.75 [a] ± 13.82
Saline	58.75[a] ± 23.56	54.37 [a] ± 28.46	56.87 [a] ± 21.86

[a,] Means with the same letter are not significantly different

C h a p t e r   5

DISCUSSION

Results of this study indicate that administration of $PGF_{2\alpha}$ 15 minutes prior to semen collection in the dog can improve not only the ejaculate quality, but also the ease of sample collection. Improvement in ejaculate quality following $PGF_{2\alpha}$ administration was observed most significantly as increased total sperm number, TNMS number and volume. Semen concentration was also noticeably greater in samples from $PGF_{2\alpha}$ treated dogs, although the increase was not statistically different from other treatments. The mechanism behind these increases is almost certainly due to redistribution of sperm from the cauda epididymis to the deferent duct during smooth muscle contraction stimulated by $PGF_{2\alpha}$. The significant effect of dog on semen volume, concentration, total sperm number and TNMS is consistent with the observed variation seen between normal dogs[73,74].

The increase in sperm number in the ejaculate following $PGF_{2\alpha}$ administration is not due to an increased rate of spermatogenesis. Spermatogenesis is unaffected by collection frequency or short term $PGF_{2\alpha}$ administration[6,51]. Rather, $PGF_{2\alpha}$ increases the rate of passage of spermatozoa from the cauda epididymis to the deferent duct. Contraction of smooth muscle surrounding the epididymis occurs in response to $PGF_{2\alpha}$ in other species[45]. Prostaglandin receptors in the epididymis are most plentiful in the distal segments[39] making these areas more sensitive to changes in $PGF_{2\alpha}$ concentration. It is, therefore, reasonable to speculate that endogenous prostaglandins exert more of an

effect in the cauda epididymis, the portion of the epididymis that acts as a site of storage for mature spermatozoa. When the cauda epididymis contracts in response to $PGF_{2\alpha}$, mature spermatozoa are moved into the deferent duct where they are available for ejaculation.

Hafs *et al* clearly showed that $PGF_{2\alpha}$ administration to anesthetized rabbits caused a redistribution of spermatozoa from the epididymis to the deferent duct[47]. Hafs and colleagues then went on to demonstrate that semen collected from rabbits after $PGF_{2\alpha}$ administration had significantly more sperm than non-treated controls[5]. Prostaglandin $F_{2\alpha}$ only increases the sperm number in the first of multiple ejaculates if the ejaculates are collected within a short period[5,6,8-10,61,62]. This is a logical finding, as $PGF_{2\alpha}$ causes redistribution of semen from cauda epididymis to deferent duct, not increased sperm production. This finding is also useful in practice as dogs presented for reproductive evaluation, ejaculate collection for artificial insemination or for cryopreservation are typically collected only once daily. Results of this study indicate that $PGF_{2\alpha}$ could be used to enhance sperm numbers in the ejaculate after only a 48-hour period of sexual rest. The increases in sperm number may have been even greater if a full 4 to 5 days elapsed between collections. Four to 5 days is the length of time determined to be necessary for the dog to build up maximal epididymal reserves[52].

In addition to the effects that $PGF_{2\alpha}$ has on smooth muscle of the epididymis, the testicular capsule also contracts in response to $PGF_{2\alpha}$[34,37,38]. The dog is known to have a prominent supply of contractile cells in the testicular capsule[23,27]. Although not specifically addressed in this study, it is likely that contraction of the testicular capsule in response to $PGF_{2\alpha}$ plays a role in increasing the number of spermatozoa available for ejaculation.

Lack of serious or significant side effects following $PGF_{2\alpha}$ administration was notable, as the purpose of the experiment was to find a drug that could be used in the clinical setting to improve the canine ejaculate. The presence of significant side effects would have limited the application of $PGF_{2\alpha}$ use in routine assisted reproduction in the dog.

Experiment 1 also evaluated the effects of oxytocin on canine ejaculate characteristics. Oxytocin was administered 10 minutes prior to ejaculate collection, a time interval chosen based on time for maximal response to the drug. Oxytocin increased semen volume in this study without affecting other ejaculate characteristics.

Serum oxytocin concentrations increase in the male during copulation[80] and in some species the testicular capsule, seminiferous tubules, and epididymis contain contractile tissue sensitive to oxytocin treatment[20,21]. If oxytocin release is blocked at the level of the hypothalamus using methallibure, sperm numbers in the ejaculate are reduced[81]. The reduction can be overcome by exogenous oxytocin administration. It is evident that in some species oxytocin plays a significant role in regulating physiologic sperm transport.

The increase in semen volume following oxytocin administration in the current study is consistent with studies that reported increases in ejaculate volume in the ram and bull[14,15,58]. However, these reports also demonstrated an increase in sperm numbers in the ejaculate as well. The lack of significant increase in sperm number following oxytocin administration in the current study may have been due to inadequate dosage or route of administration. Other studies consistently administered relatively high doses of oxytocin intravenously, while it was administered subcutaneously in this study. Subcutaneous administration may have delayed absorption and resulted in a

longer duration of low-dose oxytocin which was inadequate to have an effect on ejaculate characteristics.

Administration of GnRH had no effect on any of the semen parameters evaluated in the current study. This is contradictory to reports from clinicians who feel that libido and semen quality are improved one hour following GnRH administration in the dog (Purswell, personal communication). The resulting surge in LH and subsequent testosterone release is thought to influence the behavioral response during semen collection, in essence by improving libido. Multiple studies have confirmed that GnRH administration does result in testosterone release one hour later[17,18]. Although GnRH has not been shown to improve semen quality in the immediate post-administration period, it has been shown to improve semen quality over longer periods[16,70]. In retrospect, the lack of response to GnRH in the current study is understandable. Reichard *et al* have shown that, in rabbits, administration of either $PGF_{2\alpha}$ or PGE_2 will increase sperm numbers in the ejaculate, but serum testosterone concentration is increased by $PGF_{2\alpha}$ and decreased after PGE_2[9]. Therefore, at least with prostaglandins, increases in sperm number are independent of serum testosterone concentrations and the same may be true with GnRH.

During Experiment 1, an ease of collection score, a 1 (easy) – 9 (difficult) grading scale, was assigned to each dog following each collection. One of the more significant findings in this report was the decrease in the ease of collection score in dogs treated with $PGF_{2\alpha}$ indicating $PGF_{2\alpha}$ treated dogs were collected more readily than dogs receiving other treatments. This finding is important, as it can be difficult to collect semen from dogs without preceding or accompanying sexual preparation. It is uncertain how sexual preparation works to increase sperm numbers in the ejaculate[1,3].

One possible explanation is that sexual preparation involves release of $PGF_{2\alpha}$, which stimulates contraction of the male excurrent duct system, increasing sperm numbers in the ejaculate. In support of this thought, Drs. Root Kustritz and Traas found that $PGF_{2\alpha}$ administration in combination with sexual preparation in the form of an estrus teaser bitch does not result in significantly greater sperm numbers than ejaculates obtained after sexual preparation only (Personal communication, Dr. Root Kustritz, University of Minnesota). Hafs *et al* reported that although $PGF_{2\alpha}$ administration to bulls prior to ejaculation increased sperm number in the ejaculate by 33%, this figure was 30% less than that after sexual preparation in the same bulls[5]. It could be that sexual preparation elicits a maximal response and additional $PGF_{2\alpha}$ has no further effect on sperm numbers in the ejaculate.

Macmillan and Hafs reported that the intensity of sexual stimulation was related to sperm numbers and seminal volume[3]. Rabbits ejaculated after more intense sexual preparation had greater seminal volume than less intense sexual preparation. In addition, the average fructose concentration of the ejaculate increased with increasing sexual preparation indicating that sexual preparation affected accessory sex gland secretion as well as epididymes. The magnitude of the increase in fructose indicated that the epididymes responded more strongly than the accessory sex glands. Voglmayr reported that the concentration of $PGF_{2\alpha}$ in the cauda epididymis plasma was 8-10 times greater than that of peripheral plasma, rete testis fluid and seminal plasma[41]. These studies indicate a link between sexual preparation and $PGF_{2\alpha}$ that warrants further investigation.

Experiments 2 and 3 indicate that the administration of $PGF_{2\alpha}$ oxytocin or GnRH prior to semen collection in the dog do not affect the motility of semen

that has been cooled or frozen. Others have reported that treatment with PGF$_{2\alpha}$ has no effect on the fertilizing capability of the sperm[64,65]. This is important because clinical application of these findings require that the semen not be adversely affected by the treatments. The results of this study indicate that PGF$_{2\alpha}$ can be given to a dog presented for semen collection for artificial insemination or cryopreservation when a teaser bitch is unavailable with the expectation that sperm numbers will be improved and semen cooling and freezing will be unaffected.

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Endnotes

[a] Lutalyse® (5 mg/ml dinoprost tromethamine; Upjohn, Kalamazoo, MI)

[b] Oxytocin (20 IU/ml Phoenix Pharmaceutical, Inc. St Joseph, MO)

[c] Cystorelin® (50 µg/ml gonadorelin diacetate tetrahydrate; Abbott Laboratories, North Chicago, IL)

[d] Hamilton Thorne Version 10 IVOS semen analyzer (Hamilton Thorne Research, Beverly, MA)

[e] Morphology stain (Lane Manufacturing, Inc, Denver, CO)

[f] E-Z Mixin® "CST" (Glucose, non-fat dry milk solids, amikacin sulfate. Animal Reproduction Systems, Chino, CA)

[g] Equitainer™ I (Hamilton Thorne Research, Inc., South Hamilton, MA)

[h] Center for Reproductive Excellence Using Advanced Technology and Endocrinology frozen semen extender (1.21 Gm tris, 0.63 Gm citric acid monohydrate, 0.45 Gm glucose, 10 ml egg yolk, 0.05 ml of 500 IU/ml penicillin K+, 0.05 Gm of 1000µg/ml streptomycin sulfate, 2 ml glycerol, 38 ml deionized water. Virginia-Maryland Regional College of Veterinary Medicine, CREATE Laboratory, Blacksburg, VA)

Education   **Bachelor of Science**
1990 – 1993 Colorado State University
Ft. Collins, Colorado

Doctor of Veterinary Medicine
1994 – 1998 Colorado State University
Ft. Collins, Colorado

Master of Science
1999 – 2002 Virginia-Polytechnic and State University
Blacksburg, Virginia

Professional   Small Animal Intern
experience   1998 – 1999 Alameda East Veterinary Hospital
Denver, Colorado

Theriogenology Resident
1999 – 2002 Virginia-Maryland Regional College of
Veterinary Medicine
Blacksburg, Virginia

Professional   American Association of Equine Practitioners
memberships
American Veterinary Medical Association

Society for Theriogenology

Virginia Veterinary Medical Association