EFFECTS OF CAFFEINE CITRATE AND PROSTAGLANDIN (PGF_2 alpha) ON MOTILITY, PENETRATION RATE IN OESTROUS MUCUS AND FERTILITY OF FROZEN SPERMATOZOA OF HOLSTEIN FRIESIAN AND JERSEY BULLS.

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RAJENDRA AGRICULTURAL UNIVERSITY, BIHAR
PUSA (SAMASTIPUR)-848125

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Dr. Dhirendra Kumar Thakur

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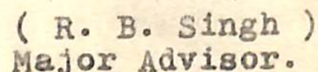
C E R T I F I C A T E - I

This is to certify that the thesis entitled "EFFECTS OF CAFFEINE CITRATE AND PROSTAGLANDIN (PGF_2 alpha) ON MOTILITY, PENETRATION RATE IN OESTROUS MUCUS AND FERTILITY OF FROZEN SPERMATOZOA OF HOLSTEIN FRIESIAN AND JERSEY BULLS" submitted in partial fulfilment of the requirement for the Degree of Master of Veterinary Science (Veterinary Gynaecology) of the Faculty of Post-graduate studies, Rajendra Agricultural University, Bihar, is the record of bonafide research carried out by Dr. Dhirendra Kumar Thakur under my supervision and guidance. No part of the thesis has been submitted for any other Degree or Diploma.

It is further certified that such help or information received during the course of this investigation and preparation of the thesis have been dully acknowledged.



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C E R T I F I C A T E - II

We, the undersigned, members of the Advisory Committee of Dr. Dhirendra Kumar Thakur, a candidate for the Degree of Master of Veterinary Science with major in Veterinary Obstetrics and Gynaecology have gone through the manuscript of the thesis and agree that the thesis entitled "EFFECTS OF CAFFEINE CITRATE AND PROSTAGLANDIN (PGF_2 alpha) ON MOTILITY, PENETRATION RATE IN OESTROUS MUCUS AND FERTILITY OF FROZEN SPERMATOZOA OF HOLSTEIN FRIESIAN AND JERSEY BULLS" may be submitted by Dr. Dhirendra Kumar Thakur in partial fulfilment of the requirements for the Degree.

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C E R T I F I C A T E - III

This is to certify that the thesis entitled "EFFECTS OF CAFFEINE CITRATE AND PROSTAGLANDIN (PGF_2 alpha) ON MOTILITY, PENETRATION RATE IN OESTROUS MUCUS AND FERTILITY OF FROZEN SPERMATOZOA OF HOLSTEIN FRIESIAN AND JERSEY BULLS" submitted in partial fulfilment of the requirements for the Degree of Master of Veterinary Science (Veterinary Gynaecology) of the faculty of Post-graduate Studies, Rajendra Agricultural University, Bihar, was examined and approved on 25. 6. 1992.

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A C K N O W L E D G E M E N T S

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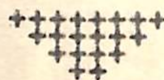

INTRODUCTION

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The objectives behind artificial insemination are to improve the genetic make-up of the indigenous cattle by using exotic germplasm on a large scale in a short period for bringing White Revolution and thereby improving the economic conditions of the poor and marginal farmers. Over a few decades, the frozen semen has largely supplanted liquid semen for artificial insemination of cattle, but the reduced motility exhibited by the spermatozoa after cryostorage and the concomitant loss of fertilizing potential is a major problem confronting artificial insemination programmes by frozen semen. Since semen is deposited in the genital tract during oestrous which coincides with the maximal secretion of mucus, the sperms have to exercise their both motility and vigour in penetrating the oestrous mucus and ovum to complete the process of fertilization. Literature shows positive correlation between mucus penetration capacity of sperm and fertilizing ability in human beings (Insler *et al*., 1979). According to Akhtar *et al*. (1980), the penetration rate of sperms in bovine cervical mucus was always higher in those animals which conceived as compared to those that did not conceive. Besides, the incidence of different types of sperm abnormalities have also direct bearing on conception rate. Thus, motility and penetration rate of sperms in the oestrous mucus and morphology of the sperms have been considered as very important

criteria for assessment of the quality of the semen samples in relation to fertility.

In the past, attempts have been made to improve the motility of spermatozoa, as well as, fertility of semen by using various stimulating agents. Glycine, fructose, chelating agents (like EDTA, DEDTC, cysteine hydrochloride and glutathione) to yolk and milk-based diluents have been tried with variable results (Sengupta et al., 1969; Tomar et al., 1979). In contrast to above agents, amino acids and enzymes have been used with much advantage (Hafs, 1968; Kirton and Hafs, 1965; Kirton et al., 1968; Hafs, 1971; Foulkes and Goody, 1980 and Bhavsar et al., 1989).

In recent past, there are many reports regarding incorporation of caffeine and prostaglandin F_2 alpha as additives in the fresh, chilled or frozen thawed semen samples of the different livestock, laboratory animals and human beings. Some of these reports have indicated a stimulatory effect, but others have obtained contradictory results. With the advent of recent technology for extensive use of frozen semen for insemination, these contradictory reports make it difficult to establish whether treatment of bovine semen samples with these agents prior to deep freezing would improve the sperm motility and oestrous mucus penetration rate. Further, it would also necessitate to assess whether such additives have detrimental effects on morphology of the sperms and thereby augumenting the

fertility. As there is a relationship between the sperm fertility and concentration of prostaglandins in the semen (Howkins, 1968), adequate concentration of prostaglandin in diluted semen is essential for optimum fertility. Normally prostaglandins are diluted to a great extent when semen is extended for artificial insemination. Hence, prostaglandin F_2 alpha may not be in adequate concentration be sufficient to augument sperm fertility inspite of it being in fair quantity in seminal plasma.

Keeping these aims in view, the present study was undertaken with the following objectives :

1. To study the effects of caffeine citrate and prostaglandin F_2 alpha on post thaw motility of frozen semen of Holstein Friesian and Jersey bulls.
2. To measure the penetration rate of frozen semen samples of Holstein Friesian and Jersey bulls supplemented with caffeine citrate and prostaglandin F_2 alpha into the oestrous mucus of cows.
3. To find out the morphological changes in the sperms of the frozen semen of Holstein Friesian and Jersey bulls treated with caffeine citrate and prostaglandin F_2 alpha.
4. To evaluate the fertility rate of frozen semen samples of Holstein Friesian and Jersey bulls supplemented with caffeine citrate and prostaglandin F_2 alpha.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Numerous tests to evaluate the quality of semen in relation to fertility have been conducted extensively. No single test can be regarded as an accurate predictor of the fertility of individual ejaculate. However, when several tests are carefully selected and combined, semen samples may have presumably higher fertility potential. Hence, the review of literature has been delt with the following four important widely used semen parameters along-with their response to caffeine and prostaglandins as additives :

1. Caffeine, prostaglandins and sperm motility.
2. Caffeine, prostaglandins and sperm penetration into the cervical mucus.
3. Caffeine, prostaglandins and sperm morphology.
4. Caffeine, prostaglandins and sperm fertility.

<u>Caffeine, prostaglandin and sperm motility</u> :

Garbers <u>et al</u>. (1971) observed that bovine epididymal spermatozoal motility and respiration were stimulated by methylxanthines and dibutyryl - 3:5 - cyclic adenosine monophosphate. However, dibutyryl - 3:5 - cyclic adenosine monophosphate was found to be more effective due to its resistance to hydrolysis by phosphodiesterases and greater lipid solubility/membrane permeability.

Caffeine was found to maintain bovine epididymal spermatozoa in an ejaculated - like state (Hoskins, 1973). The motility of sperms was stimulated by the cyclic nucleotides and phosphodiesterase inhibitors through faster degradation of adenosine triphosphate.

Addition of caffeine to human ejaculated semen was recorded to increase the percentage of motility by initiating the movement of non-motile spermatozoa and also accelerating the mean motility rate. More effective results in the presence of glucose was also obtained (Haesungcharern and Chulavatnatol, 1973).

Schoenfeld et al. (1973) reported that caffeine greatly increased the motility of ejaculated human spermatozoa. Both the motility percentage and the grade of motility increased and were maintained in the presence of 6 mM caffeine for atleast 5 hours at 37°C.

Hoskins et al. (1974) showed that motility could be induced upto 20 per cent in caput spermatozoa by caffeine or theophylline with a simultaneous increase in cyclic adenosine monophosphate level.

Supplementation of ram semen with prostaglandin F_2 alpha (unknown concentration) was found to have no adverse effect on post thaw motility or survival of the spermatozoa (Carbo et al., 1975).

Norman and Gombe (1975) observed the stimulatory effect of the lysosomal stabilizer, chloroquine on the respiration and motility of fresh and aged bovine spermatozoa in vitro. Similar effects of the phosphodiesterase inhibitors, theophylline and caffeine suggested that enhancement of sperm activity by chloroquine may be mediated by cyclic adenosine monophosphate.

Dougherty *et al*. (1976) observed no stimulatory effect of caffeine on human sperm motility.

Homonnai *et al*. (1976) reported that caffeine, the phosphodiesterase inhibitor , was the most potent drug in enhancing the sperm motility.

The addition of even five times the normal concentration of the prostaglandin F_2 alpha in sheep semen , no significant difference in sperm motility could be observed by Marley *et al*. (1976).

Abbitt *et al*. (1977) studied the effect of tris (hydroxymethyl) animo methane salt of prostaglandin F_2 alpha on the post thaw motility of bovine spermatozoa. This salt of prostaglandin F_2 alpha was added to the glycerol fraction of the extender to provide 37.5, 112.5 and 337.5 μg of active prostaglandin F_2 alpha per 0.5 ml straw. The straws were frozen to about $-180^{\circ}C$ for two minutes. With the three salt concentrations progressive motility was found to be 17.8, 13.6 and 5.8 per cent respectively against 19.3 for straws frozen without the prostaglandin F_2 alpha salt. Thus addition of the salt of prostaglandin F_2 alpha was found to depress the post-thaw progressive motility.

Dimov and Georgiev (1977) reported that the addition of prostaglandin E_1 and F_2 alpha to diluted ram semen equivalent to the total amounts of these prostaglandins in one ejaculate showed no correlation between the concentration of prostaglandins and spermatozoal motility.

Tamblyn and First (1977) found that Brij-35 (polyoxyethylene alcohol, a non-ionic surfactant) treated bovine spermatozoa lost their motility but were reactivated with adenosine triphosphate and the reactivated motility was stimulated by caffeine.

Addition of cyclic adenosine monophosphate to motile demembranated preparations of bull spermatozoa was observed to produce measured increase in motility. The stimulation was of the same relative magnitude as that obtained by the addition of caffeine to intact spermatozoa (Lindemann, 1978).

Caffeine was not found to stimulate the motility of spermatozoa in oligozoospermic semen samples of human beings (Read and Schnieden, 1978).

After addition of 6 mM caffeine in oligospermic and asthenospermic semen samples of human beings, Harrison (1978) observed increased motility of the spermatozoa.

According to the experimental results of Guillory _et al_. (1979) addition of prostaglandin F_2 alpha tended to increase the progressive motility of bull spermatozoa relative to that in semen in which prostaglandin F_2 alpha was not added. Sperm survival also increased in the presence of prostaglandin F_2 alpha.

Caffeine was found to increase and maintain the motility index of bull spermatozoa incubated with glucose, fructose or

pyruvate for about five hours at 37°C and the maximum effect achieved with 10 mM caffeine. In the presence of 7 to 10 per cent glycerol both prefreezing and post thaw motility indices were not as good in caffeine treated spermatozoa as in untreated ones. On the other hand, the addition of caffeine to frozen thawed spermatozoa in the presence of glycerol was found to enhance the sperm motility index and longevity as compared to the controls without caffeine (Miyamoto and Nishikawa, 1979).

Makler et al. (1980) investigated the effects of caffeine, cyclic adenosine 3:5 monophosphate, prostaglandin E, triiodothyronine and insulin on spermatozoal motility. No significant differences were found between experimental and control specimens with the exception of caffeine. Caffeine immediately increased the percentage of motile spermatozoa by about 30 to 50 per cent of initial values in approximately two third of the cases. No influence on sperm velocity was detected during this period. Non-motile live spermatozoa were activated by caffeine without any effect on the percentage of dead sperm.

Caffeine was reported to produce a dose-dependent increase in human sperm motility whereas imipramine produced a dose-dependent decrease (Robert et al., 1980).

Memon (1981) supplemented pooled semen of 6 rams with different doses of prostaglandin E_1 and F_2 alpha before freezing and found no effect on post thaw motility upto about 300 μg/ml, but higher levels caused a decrease in sperm motility.

Varnavskii (1981) found no effect of commercial prostaglandin F_2 alpha on the post thaw motility, resistance and survival rate of ram spermatozoa, compared with semen processed without the prostaglandin.

Aitken *et al*. (1983) observed a significant increase in the percentage of motile spermatozoa when three doses of caffeine (2, 5 and 10 mM) were added to frozen thawed human semen.

Moussa (1983) studied the effect of six different doses of caffeine on human spermatozoal motility, swimming speed, progressive sperms and progressive swimming speed by using videomicrography. In doses of 3 and 6 mM per ml caffeine significantly increased the percentage of motile sperms but had no influence on sperm velocity. In doses more than 6 mM per ml, caffeine exhibited a slight non-significant stimulation while in a dose of 60 mM per ml exerted an inhibitory effect on all parameters of sperm motility. Caffeine was found to cause complete immobilization of human spermatozoa with a dose of 120 mM per ml.

Stoyanov (1984) used prostaglandin F_2 alpha in the amounts of 4, 10 and 20 μg to 0.5 ml of the 2.8 per cent sodium citrate thawing solution for semen pellets. Sperm motility after thawing was 3.9, 3.1 and 6.0 per cent units higher for thawing medium with three concentrations of prostaglandin F_2 alpha than for

the control medium without prostaglandin and sperm survival time at 46°C was 76, 88 and 89 minutes vs 69 minutes for the control medium respectively.

Ramana (1986) found better motility with lower prostaglandin concentration in the buffalo bull semen preserved at 4°C.

Gehlaut and Srivastava (1987) diluted buffalo bull semen samples in different diluents with and without 6 mM caffeine citrate. The diluted semen samples were frozen in liquid nitrogen (-196°C), thawed at 50°C for 2 minutes after 0 and 20 days of storage and thereafter found that the post thaw motility of spermatozoa was the same as on 0 and 20 days of storage. Caffeine citrate increased the post thaw motility of the spermatozoa in different diluents from 25 to 100 per cent. Highly significant stimulation due to caffeine citrate was observed in samples with low post thaw motility, irrespective of the diluents used. The mean percentage motility observed in post thawed control and caffeine treated semen were 5.0 ± 0.00 and 10.00 ± 0.00 respectively in tris diluent showing 100 per cent increase in case of caffeine citrate treatment.

Schoff and Lardy (1987) reported that the putative phosphodiesterase inhibitor caffeine (2.5 mM) restored motility and increased the respiration in fluoride treated

spermatozoa and even in untreated control spermatozoa. Caffeine did not change the internal pH of spermatozoa. It increased the intracellular cyclic adenosine monophosphate titres by about 35 per cent.

Shannon and Curson (1987) observed that caffeine increased the motility score at 6 hours of incubation of spermatozoa diluted in dialysed egg yolk buffers and at 24 hours of incubation of spermatozoa diluted in whole egg yolk or dialysed egg yolk with K-ion buffers. It increased the duration of motility of spermatozoa diluted in a dialysed egg yolk buffer by 16 hours but depressed the duration of motility when spermatozoa were diluted in whole egg yolk or dialysed egg yolk buffers with K-ions by 10 hours.

In frozen buffalo bull semen with supplementation of caffeine in tris diluent, Hukeri (1989) reported significantly higher post thaw motility.

Muralinath et al. (1989) studied the effect of prefreeze addition of prostaglandins on post thaw motility of cryopreserved buffalo bull semen. The lower dose combination of prostaglandins resulted in improved motility and the response was bull specific with individual bull differences.

EL-Gaafary (1989) reported depression of sperm motility with increase in prostaglandin F_2 alpha concentration in rabbit semen.

Daader et al. (1989) observed stimulatory effect of caffeine on tris diluted chilled bull spermatozoa. They found reduced fructose content at 20 mM level and increased seminal pH at 10 mM compared with controls and concluded that 10 to 20 mM caffeine per 100 ml gives the highest motility and metabolic activity of spermatozoa.

Bhosrekar et al. (1990) showed significantly higher post thaw forward motility in diluent containing detergent mixture when compared to caffeine puris or control.

EL-Gaafary et al. (1990) observed higher bull sperm motility in tris-based diluent than in citrate-based. Supplementation with caffeine at 10 mM and 20 mM per 100 ml diluent was shown to increase the percentage of motile spermatozoa irrespective of the diluents compared to controls.

Kusunoki et al. (1990) reported that caffeine maintained the goat sperm motility above 50 per cent for only one hour.

Muralinath et al. (1990a) found significantly improved post thaw motility in frozen buffalo bull semen supplemented with lower concentrations of prostaglandin E_2 and F_2 alpha after zero hour of incubation at $37^{o}C$ compared with higher concentrations of prostaglandins or no prostaglandins.

Caffeine, prostaglandins and sperm penetration into the cervical mucus :

Memon (1981) measured migration distance of frozen sperms of ram in the pooled cervical mucus of ewes after 15, 30, 60, 120 and 180 minutes of semen-mucus contact and found increased sperm migration distance with time, but with no difference between prostaglandin F_2 alpha supplemented and control semen samples.

Aitken et al. (1983) suggested that the addition of caffeine to frozen thawed human spermatozoa increased the number of spermatozoa penetrating the cervical mucus in unit time, by increasing the frequency rather than the success of collisions between spermatozoa and the cervical mucus interface.

Memon and Gustafsson (1984) found that the penetration distance of ram sperm in cervical mucus after 15 and 180 minutes of exposure was not significantly affected by prostaglandin treatment.

EL-Gaafary et al. (1990) studied the effect of 0, 10, 20, 40 and 80 mM caffeine for 100 ml diluent on cervical mucus penetration characteristics of Friesian bull semen and found increased penetrative ability of spermatozoa into the cervical mucus in the presence of caffeine.

Muralinath et al. (1990a) reported that the addition of prostaglandins at lower concentration significantly improved the penetrated sperm motility of frozen buffalo

bull semen after 30 minutes of initial semen-mucus contact at 10 millimeter distance compared to higher concentration and untreated controls, this difference was not seen at 2, 4 or 6 hours after incubation.

Muralinath et al. (1990b) studied the cervical mucus penetration characteristics of frozen Murrah buffalo bull semen supplemented with lower concentrations of prostaglandin E_2 and F_2 alpha and observed no cognisable effect either on sperm penetration distance or penetrated sperm count. However, sperm progression speed was significantly improved in the presence of prostaglandins.

Caffeine, prostaglandins and sperm morphology :

According to Carbo et al. (1975) addition of prostaglandin F_2 alpha in ram semen was not found to cause any adverse effect on morphology of the spermatozoa.

The inclusion of five times the normal semen concentrations of the five prostaglandins including prostaglandin F_2 alpha, Marley et al. (1976) observed no significant differences in sperm acrosomal morphology after the incubation or chilling of diluted ram semen.

Harrison (1978) showed that the in vitro caffeine treatment of fresh semen for artificial insemination with husband's semen of 34 men, of whom 14 had normal semen analysis and the other 20 were oligozoospermic or asthenozoospermic, or both, did not positively influence the conception rate of their wives as the caffeine damaged the spermatozoa.

Harrison *et al.* (1980a) found that there was disruption of the normal shape of the sperm head in the asthenozoospermic semen of men after caffeine incubation.

Harrison *et al.* (1980b) studied the surface morphology of human sperm after 2 hours of incubation with 6 mM caffeine and found more disruption of the sperm head and swelling of the mid piece.

In vitro caffeine treatment of fresh proven donor semen showed no morphological changes (Barkay *et al.*,1984). It was concluded by them that *in vitro* caffeine treatment of fertile donor semen did not cause any damage to the different organelles of the spermatozoal heads and tails even after prolonged incubation.

Meizel and Turner (1984) found adverse effect of prostaglandins, especially prostaglandin E_2 on the acrosomal maintenance of hamster spermatozoa during chilled storage.

AL-JuBuri *et al.* (1989) observed a lower percentage of abnormal sperms with the prefreeze addition of prostaglandin F_2 alpha (0 to 300 μg/ml) in frozen washed spermatozoa than frozen whole semen of Awassi ram.

Muralinath *et al.* (1989) reported that the prostaglandins had no effect on the percentage of live spermatozoa of buffalo bull semen after cooling, but significantly reduced the percentage of spermatozoa with intact acrosomes from

67.46 ± 0.9 in control diluent to 64.64 ± 0.7 and 65.3 ± 0.9 per cent with the lower and higher concentrations respectively, while the percentage of abnormal and acrosome - intact spermatozoa was not affected by prostaglandins on cryopreservation.

Caffeine, prostaglandins and sperm fertility :

Bygdeman et al. (1970) found that the percentage of semen samples with a low concentration of prostaglandin E compounds (below 11 μg/ml) constituted 0 per cent in fertile men group, 17 per cent in noninvestigated infertile marriage group and 41 per cent in infertile marriage group with no clinical or laboratory abnormal findings. The groups were found to differ statistically significantly. The high number of cases in the last group with a low concentration of prostaglandin E strongly pointed out that these compounds were of importance in the reproductive process.

Spilman et al. (1973) found that in rabbits ligation of utero-tubal junction 2.5 to 3 hours after insemination improved the fertilization rates by adminstration of prostaglandin F_2 alpha either with the rabbit semen (100 μg) or as a series of four subcutaneous injections of 1 mg per kg body weight. This improvement was hypothesised to be the result of a beneficial effect of prostaglandin F_2 alpha on sperm transport.

The addition of prostaglandins to sheep semen prior to deep freezing was observed to improve the fertility rate (Zhiltsov *et al.*, 1974 and Gustafsson *et al.*, 1975).

Dimov and Georgiev (1976) studied the effect of primary diene prostaglandins on conception rate of ewes. They diluted ram semen with glucose phosphate buffer diluent with and without 5 mg prostaglandin F_2 alpha, 50 mg prostaglandin E_2 or 5 mg prostaglandin F_2 alpha + 50 mg prostaglandin E_2 per dose. For the 3 groups of prostaglandin additives the conception rate of 52, 51 and 35 ewes inseminated was 67.3, 72.5 and 82.8 per cent vs 62.5, 58.6 and 68.4 for 48, 41 and 91 ewes respectively inseminated without prostaglandin additives. For groups of 35, 47 and 43 ewes inseminated with semen diluted with the above diluent to which had been added 5 mg prostaglandin F_2 alpha + 50 mg prostaglandin E_2, the conception rate was 82.8, 78.7 and 74.4 per cent vs 86.7, 91.3 and 72.7 for groups of 28, 23 and 22 ewes inseminated with semen diluted with the above diluent to which had been added 10 mg prostaglandin F_2 alpha + 100 mg prostaglandin E_2. For 2 rams, the conception rate of ewes inseminated without a prostaglandin additive to diluted semen was 59.7 and 36.8 per cent vs 60.0 and 91.7 for ewes inseminated with 50 mg prostaglandin E_2 added diluted semen.

Dimov and Georgiev (1977) observed that the addition of prostaglandin F_2 alpha to diluted ram semen in amounts comparabl

to the total amounts of these prostaglandins in one ejaculate increased the fertility of rams by more than 15 per cent.

Harrison (1978) carried out 400 cervico-vaginal artificial insemination using husband's semen for a variety of clinical indications (oligospermia, asthenospermia etc.). Before insemination, to one half of the samples caffeine was added to a final concentration of 6 mM. No pregnancies were achieved by the artificial insemination with subnormal semen even in the presence of caffeine.

Fraser (1979) found accelerated mouse sperm penetration and capacitation when the fertilizing ability of sperm suspensions was tested after preincubation, for a period of time insufficient to permit full capacitation under standard conditions, in the presence or absence of caffeine. When eggs were fixed after one hour, the majority (76.7 per cent) in the caffeine containing medium were fertilized compared with only 32.1 per cent in the caffeine free medium. In the absence of caffeine, early stages of nuclear development were predominant, while in the presence of caffeine, a significant proportion of eggs reached terminal stages of activation.

Rogers and Garcia (1979) found that the caffeine increased the fertilizing ability of guinea pig spermatozoa by enhancing their penetration rate.

Barkay and Zuckerman (1980) studied the effect of 7.2 mM caffeine on fertilizing capacity of frozen human sperms <u>in vitro</u>

and found 10 to 15 per cent increase in their fertilizing ability.

Varnavakii (1981) inseminated 96 ewes with frozen semen to which prostaglandin E_1 or F_2 alpha had been added before freezing; the lambing rates were 41.4 and 47.3 per cent respectively. When prostaglandins were added to thawed semen, the lambing rates for 13 and 7 ewes were 61.7 and 57.0 per cent respectively against 60.0 inseminated with fresh semen.

Aitken <u>et al</u>. (1983) observed no improvement in the fertilizing ability of the spermatozoa when the caffeine stimulated frozen thawed human spermatozoa washed free of seminal plasma. However, when 2 mM caffeine added to washed suspensions of capacitated spermatozoa, significant enhancement in the fertilizing ability of the spermatozoa was evident.

Barkay <u>et al</u>. (1984) studied the influence of <u>in vitro</u> caffeine treatment on human sperm fertilizing capacity. Sixty women (with infertile husbands) underwent artificial insemination by donor with frozen thawed semen over a period of 12 months, using randomised addition of 7.2 mM caffeine in alternate months. Fourteen women became pregnant during the 6 months they received caffeine treated semen, whereas only 7 pregnancies occurred during the 6 months for the women who received semen without caffeine. It seemed to improve the fertilizing capacity by 10 to 15 per cent.

Stoyanov (1984) found that the conception rate of 826 cows inseminated with semen containing 4 μg Enzaprost (prostaglandin F_2 alpha) in an insemination dose was 60 per cent vs 59 for 244 cows inseminated with semen containing 20 μg Enzaprost per dose and 40 to 50 per cent for control inseminated with semen without prostaglandin.

Niwa <u>et al</u>. (1988) studied the effect of caffeine in media for pretreatment of frozen thawed sperm on <u>in vitro</u> penetration of cattle oocytes. In the first experiment, thawed semen was washed with modified Kreb's Ringer Bicarbonate solution or modified Tyrode's solution with or without 10 mM caffeine, then preincubated at 39°C for 5 hours in the two media with or without 5 mM caffeine, before incubation with oocytes for 20 to 22 hours. In the second experiment, semen was washed in Tyrode's solution without caffeine and then incubated with 0, 1, 3, 5 or 10 mM caffeine, before incubation with oocytes. For semen in Tyrode's solution in the first experiment, 80, 30 and 78 and 23 per cent of oocytes were penetrated by spermatozoa when semen was washed and preincubated, washed and not preincubated, not washed and preincubated, and neither washed nor preincubated with caffeine respectively. Corresponding figures for Kreb's Ringer solution were 35, 21, 45 and 17 per cent. In the second experiment 27, 48, 60, 94 and 85 per cent of oocytes were penetrated at concentration of 0, 1, 3, 5 and 10 mM caffeine respectively. Polyspermy occurred in 0 to 29 and 0 to 8 per cent of ova in

Tyrode's and Kreb's Ringer solution respectively, and was not correlated with the presence of caffeine in the washing and/or preincubation media.

Bird et al. (1989) observed that the bovine frozen thawed spermatozoa penetrated the zona-free hamster oocytes following treatment with calcium ionophore in the absence of bovine serum albumin (BSA) and in the presence of 10 mM caffeine. Sperm velocity was also found to be stimulated with concentration of caffeine less than 2.5 mM following dilution with medium containing BSA.

EL-Gaafary (1989) studied survival rate and fertility of rabbit sperm supplemented with prostaglandin F_2 alpha (600 μg/ml). The percentage fertility after insemination was 81.3 and 50.0 for untreated control and prostaglandin treated semen respectively.

Burov and Kolomoets (1990) found 19.6 per cent increased conception rate in cows with the addition of 0.2 ml Enzaprost (prostaglandin F_2 alpha) to diluted bull semen.

Radoslavov et al. (1990) inseminated 307 and 335 cows, using cloprostenol in the semen diluent (5 mg per dose) and 300 and 317 cows inseminated without cloprostenol. The conception rate was 41.7, 43.6, 33.3 and 36.9 per cent respectively to first insemination and 56.0, 57.3, 45.7 and 47.3 per cent overall.

Niwa *et al.* (1991) studied penetration of bovine oocytes during maturation *in vitro* by frozen spermatozoa using caffeine and heparing in medium. They found 100 per cent penetration of oocytes incubated for 0 to 4 hours, and had a decondensing sperm head, indicating no requirement of maturation of oocytes for *in vitro* sperm penetration into the vitellus, or for *in vitro* decondensation of the sperm nucleus.

M A T E R I A L S   A N D   M E T H O D S

MATERIALS AND METHODS

The present study was conducted on semen samples collected from 6 bulls, 3 Holstein Friesians and 3 Jerseys within the range of age between 4 and 7 years. All these bulls were maintained on similar ration schedule and isomanagemental conditions at Frozen Semen Bank, Patna. All the bulls were of documented fertility.

Collection and evaluation of semen :

Semen samples were collected by artificial vagina method and schedule of two collections per week was followed for each bull. Immediately after collection, different attributes of the semen were evaluated. Volume and colour of each semen sample were recorded by visual examinations. Mass activity was determined by taking a small drop of semen on prewarmed clear and dry glass slide under phase contrast microscope with 10 objective and 10 phase combination having biotherm. Activity of the semen sample was assessed on the basis of vigour of wave motion and the mass movement of spermatozoa. It was graded grossly into 6 categories ranging from +5 to 0 along with the following distinctive and differential features (Tomar, 1976).

I +5 very quick wave motion with formation of eddies.

II +4 the swirls and eddies were comparatively not so rapid as in +5 grade but they were observed to move towards extremeties.

III	+3	swirls were slow and scattered in the field.
IV	+2	swirls were absent and individual movement of sperms were evident in the field.
V	+1	no wave motion was observed.
VI	0	the spermatozoa were not motile.

Ten semen samples from each bull showed mass activity of grade +4 and above and ejaculate volume 3 ml and above were only included for freezing.

The initial motility was graded in percentage spermatozoa having progressive motility. This was done on dilution with dilutor in the ratio of 1:10 in small test tubes before the semen was finally diluted. A thin drop of diluted semen was kept on a warm slide and seen under phase contrast microscope with 20 phase. An approximate idea of dead or immotile or oscillating or circular motile and progressively motile spermatozoa were estimated in a particular field under microscope and the percentage of motile spermatozoa was determined.

The concentration of spermatozoa was determined by Corning Photoelectric Colorimeter 252. 30 ml of the 2.9 per cent sodium citrate buffer was taken into a small sized measuring cylinder and to it 0.2 ml neat semen was added and mixed thoroughly. In colorimeter tube buffer and semen mixture was taken upto the mark and the transmittance recorded with violet filter No. 430. Sperm concentration ($x10^9$ per ml) was noted from the

chart already prepared for each degree of transmittance.

Preparation of tris dilutor :

Tris dilutor was prepared as follows according to Raju and Rao (1983) :

Tris (hydroxy methyle) aminomethane (Qualigens Fine Chemicals)	-- --	12.11 gms.
Citric acid (Nice)	-- --	6.80 gms.
Fructose (B.D.H.)	-- --	5.00 gms.
Glycerol (Qualigens Fine Chemicals)	-- --	32.00 ml.
Double glass distilled water	-- --	368.00 ml.
Egg yolk	-- --	100.00 ml.

After thorough mixing the prepared dilutor was filtered with Whatman's filter paper No. 41 in a sterilized conical flask. Benzyl penicillin and streptomycin sulphate were incorporated at the concentration of 1000 I.U. and 1.0 mg per milliliter dilutor respectively.

Dilution of semen :

Dilution rate of the representative semen sample was determined by the prepared chart corresponding to the reading of concentration and initial motility of the spermatozoa, to keep about 25 to 30×10^6 spermatozoa per dose. Dilution of semen was done at a single step at room temperature.

Addition of additives :

Caffeine citrate (Mol. Wt. 386.33, M/S Golden Fibre and Dressing Syndicate, Goregaon, Bombay) and Dinoprost (5 mg Prostaglandin F_2 alpha per ml, Alved Products, Madras) were dissolved in tris buffer. The diluted semen samples were splitted in three equal aliquots. Two aliquots were supplemented with caffeine citrate and dinoprost solutions, to make final concentration of 7.2 mM (Barkay *et al.*, 1984) and 4 μg per ml diluted semen respectively, while third aliquot was being left without any addition. The concentration of Prostaglandin F_2 alpha was choosen on the basis of pilot trial with 1 to 10 μg per ml diluted semen, the 4 μg per ml yielded better result on the sperm motility, as such the same concentration was used throughout the experiment.

Packing and equilibration of diluted semen :

Diluted semen of each aliquot was packed and sealed in plastic minitubes (0.25 ml) by automatic filling and sealing machine (Minitub, Gm bH. Abfull-und Labortechnik Tiefenbach, West Germany). The filled straws were arranged horizontally on the freezing racks. Equilibration was done in refrigerator (5°C) for 4 to 5 hours.

Deep freezing and storage of semen :

After equilibration the racks containing straws were placed on grill 4" above the liquid nitrogen kept in

thermocol box, so that they get static vapour. Then the straws were dipped in liquid nitrogen after 15 minutes. Afterwards straws were filled in the precooled plastic goblets and transferred in the liquid nitrogen vessel.

Thawing of frozen semen :

The straw was taken out from the liquid nitrogen vessel with the help of a long forceps and individually thawed in warm water at 35°C for 12 seconds. Then straw was soaked with towel and rotated under palm to ensure thorough mixing of the semen.

Post thaw progressive sperm motility :

Examination of post thaw progressive sperm motility was done within 2 hours of storage by haemocytometer method. Washed and dried haemocytometer chamber was focussed under phase contrast microscope with 20 phase having biotherm. The thawed semen sample to be tested for progressive motile spermatozoa was taken in a prewarmed small watch glass. The semen was sucked in WBC pipette upto 0.5 mark and diluted with tris buffer (1:20). Thoroughly mixed the semen with beaded ball of pipette under palm. Few drops were discarded. Then prefocussed chamber of haemocytometer was charged carefully to prevent overflowing of the diluted semen. Five large squares, namely left upper, right upper, right lower, left lower and middle ones were utilized for counting the dead and weakly motile spermatozoa. Then chamber was kept in refrigerator at 0°C for approximately one hour to kill all the live spermatozoa.

Again all dead spermatozoa of five large squares were counted and by the following formula percentage of progressively motile spermatozoa was calculated :

$$\frac{\text{Total dead spermatozoa after freezing} - (\text{dead} + \text{weakly motile spermatozoa})}{\text{Total dead spermatozoa after freezing}} \times 100$$

= percentage of progressively motile spermatozoa.

<u>Penetration rate of sperm into the oestrous mucus</u> :

The normal cyclic cows of mid-oestrous period brought at the out-door clinics of Gynaecology Department, Bihar Veterinary College, Patna for artificial insemination were utilized for the collection of mucus. Proper detection of oestrum was done on the basis of signs and symptoms. The hind quarter and the external genitalia were washed with distilled water. A sterilized metallic vaginal speculum was introduced into the vaginal canal. About 20 to 30 ml of only clear and transparent oestrous mucus outflow was collected in a 100 ml capacity beaker. Immediately after collection the collected oestrous mucus was brought to the laboratory for the test.

Determination of sperm penetration rate was carried out according to Akhtar <u>et</u> <u>al</u>. (1980) with slight modification. Plain capillary tubes of 75 mm length having diameter of 1.0 mm to 1.2 mm were used for the purpose. One end of the capillary tube was fitted in the narrow corresponding translucent plastic connecting tube. This connective tube was again fitted into another slightly longer connecting

plastic tube. The connecting tube was fitted with the nozzle of the SPROJTE DL Syringe (DANSK LABORATORIES UDSTYRA/SRYESGADE 32200 KOSEN HAVN, N TIE (01) 392600) of 5 ml capacity. There was a metallic screw arrangement to push ahead the piston of the syringe. The screw was tightened to push the piston upto the mark 2 to 3 ml of the syringe. The open end of the plain capillary tube was dipped into the mucus sample. The screw was then loosened gradually. The mucus was pipetted in the plain capillary tube. One end of the capillary tube was sealed with the ordinary soft wax (plasticin). The tube was then fitted in a specially designed slide. Two small semicups made up of plastic were fitted above the border of the slide. A thick band of soft wax was arranged about 3 cm above the semispherical cups. One open end of the capillary tube filled with mucus sample was fitted in such a manner that the open end dipped in the cup of the slide. A moist filter paper was kept in a petri-dish. The slide was then placed on the moist filter paper of the petri-dish. The petri-dish was kept in the incubater for 20 minutes at 37°C for the equilibration of the mucus sample inside the capillary tube in humidified atmosphere. The frozen semen samples having atleast 40 per cent post thaw motile spermatozoa were thawed at 35°C for 12 seconds. Then one to two small drops of thawed semen were kept in the cup. Again petri-dish along with mucus filled capillary tube which was in contact with the semen was vertically incubated for 20 minutes. Then the capillary tubes were taken out, wiped clean and examined under microscope at a magnification of 100X.

Spermatozoa were studied while swimming in the interior mid plane of the tube, and also in plane just below the upper surface of the tube. The distance travelled by two most progressively motile spermatozoa in the oestrous mucus inside the capillary tube was measured in millimeter by means of stage micrometer.

Morphological characteristics of spermatozoa :

The morphological studies of spermatozoa were done by eosin-nigrosin staining technique.

Preparation of staining solution - The compound stain was prepared as follows :

Eosin Y (Water soluble, Hi-Media)	-- --	1 gm.
Nigrosin (Water soluble, Hi-Media)	-- --	5 gm.
Sodium citrate dihydrate (Hi-Media)	-- --	3 gm.
Sterilized double glass distilled water	-- --	100 ml.

The solution was warmed in water bath for 30 minutes and filtered after cooling with Whatman's filter paper No. 41. Prepared stain was stored in refrigerator to prevent bacterial growth, but warmed to room temperature before it was used for staining the spermatozoa.

Preparation of smear - One large drop of compound stain was poured in a clean and dry watch glass and a small drop of

frozen thawed semen was put in the watch glass (Swanson and Beardon, 1951). It was mixed thoroughly by gently blowing air through a pipette and was allowed to stand for one and half minute. Thin smear was drawn on a clean grease free microslide and was dried in air. Semen smears were examined under oil immersion lens at a magnification of 1000X. Not less than two hundred sperms were counted at random in different fields and the percentage of sperm abnormalities calculated in order to find out the morphological variation among treated and untreated frozen sperms of both the breeds.

Assessment of fertility rate :

The unsupplemented as well as supplemented frozen semen samples were used for insemination. Inseminations were carried out in cows of normal oestrous cycle brought to the Gynaecology Department, Bihar Veterinary College, Patna. Before insemination, the cows were clinically identified for signs of oestrous such as oedematous swelling of the valvae, congestion in the vaginal mucosa, condition of cervix, tone, turgidity of the uterus, mucus discharge and the presence or absence of follicles and corpus luteum in either left or right ovary by means of visual examination of the external genitalia and rectal palpation of the internal genital organs. Thawing of the straw was done in warm water (35°C) for 12 seconds. Insemination was done accordingly. Records, including date of insemination, number and breed of bull, date of freezing the semen, date of pregnancy diagnosis and other relevant informations were maintained in a register. The fertility rate was calculated

on the basis of pregnancy diagnosis by per rectum palpation of the gravid cornua after 60 to 90 days of first insemination.

The details of the insemination done for the fertility rate are presented in table - 1.

Table - 1.

Showing number of cows inseminated with frozen semen of different breed of bulls under different treatment groups.

Breeds \ Treatments	Number of cows inseminated		
	Control	Caffeine citrate	Prostaglandin F_2 alpha
Friesian			
HF-153	51	54	53
HF-648	46	49	52
HF-662	54	49	49
Total :	151	152	154
Jersey			
J-17/87	58	53	52
J-254	51	50	51
J-24/87	53	51	52
Total :	162	154	155

R E S U L T

RESULT

The observations of present study are summerized into the following four subheadings :

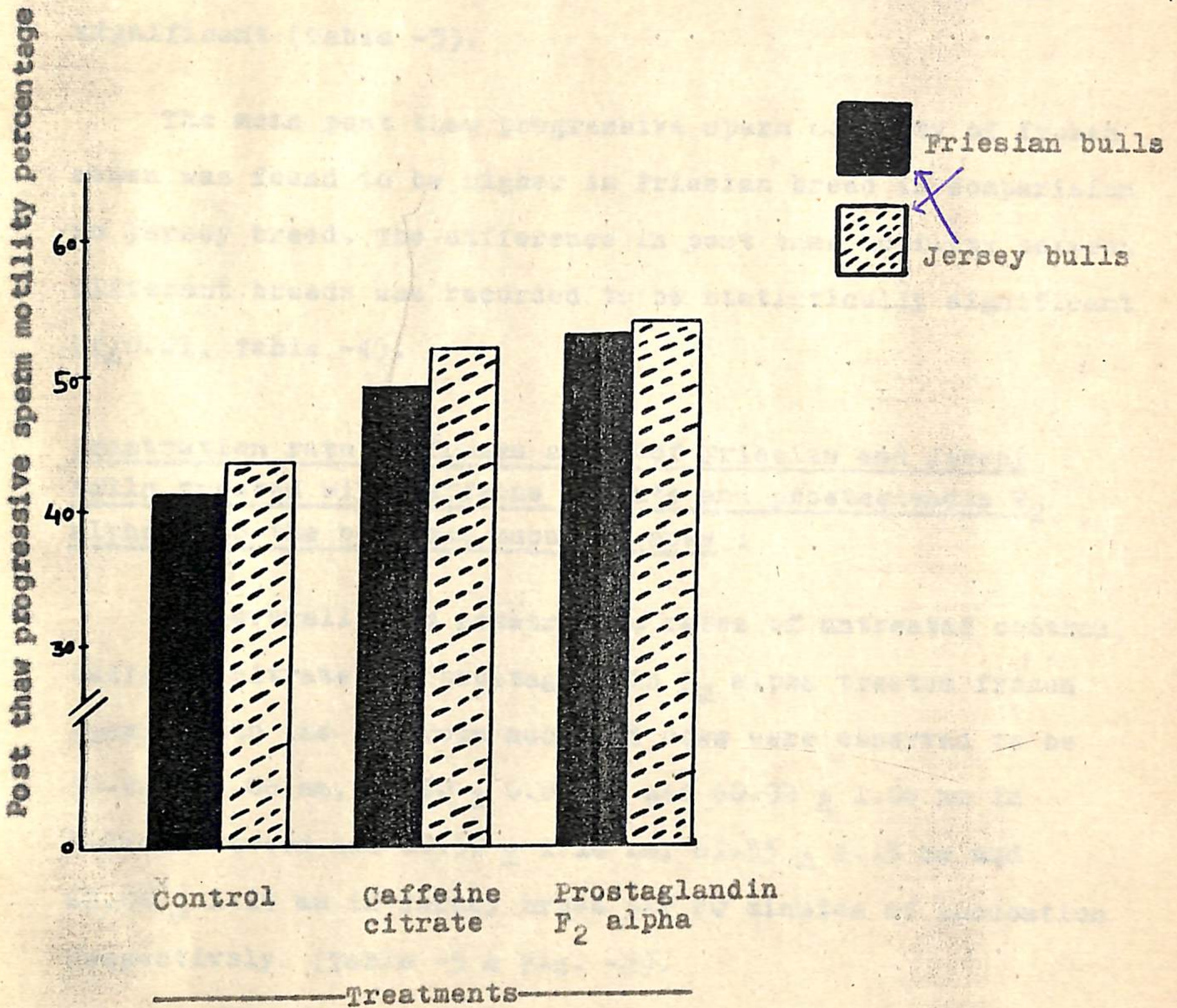
1. Post thaw progressive sperm motility of frozen semen of Friesian and Jersey bulls treated with caffeine citrate and prostaglandin F_2 alpha.
2. Penetration rate of frozen sperm of Friesian and Jersey bulls treated with caffeine citrate and prostaglandin F_2 alpha into the oestrous mucus of cows.
3. Abnormal sperm percentage in frozen semen of Friesian and Jersey bulls treated with caffeine citrate and prostaglandin F_2 alpha.
4. Fertility rate of frozen semen of Friesian and Jersey bulls treated with caffeine citrate and prostaglandin F_2 alpha.

Post thaw progressive sperm motility of frozen semen of Friesian and Jersey bulls treated with caffeine citrate and prostaglandin F_2 alpha :

The overall mean of post thaw progressive sperm motility of untreated control, caffeine citrate and prostaglandin F_2 alpha treated frozen semen was observed to be 42.96 $\pm$ 0.73, 52.12 $\pm$ 0.53 and 53.84 $\pm$ 0.96 per cent in Friesian breed and 41.04 $\pm$ 1.07, 49.28 $\pm$ 0.60 and 53.37 $\pm$ 0.60 per cent in Jersey breed respectively (Table -2 & Fig.-1). From this it was evident that in both breeds the mean percentage of post thaw progressive sperm motility was highest in prostaglandin F_2 alpha treated group followed by caffeine citrate treated group

Fig. -1.

Histogram showing the mean post thaw progressive sperm motility of frozen semen of Friesian and Jersey bulls under different treatment groups.



and lowest in untreated frozen semen. The difference in the mean percentage of post thaw progressive sperm motility between different treatments of frozen semen was found to be statistically significant ($P < 0.01$, Table -3).

Analysis of variance revealed significant ($P < 0.05$) difference in the mean percentage of post thaw progressive sperm motility between the bulls of Jersey breed while between the bulls of Friesian breed it turned out to be non-significant (Table -3).

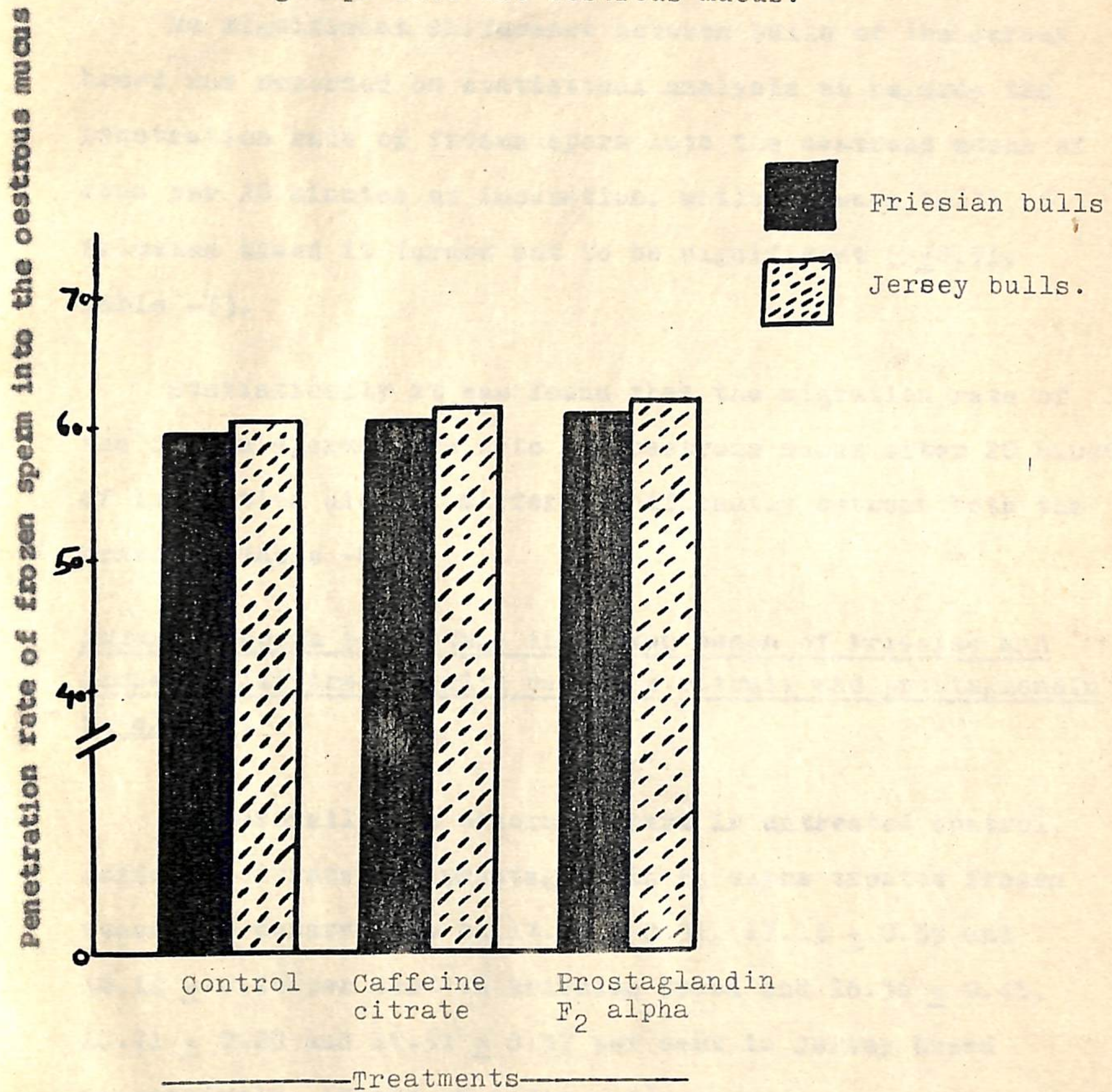
The mean post thaw progressive sperm motility of frozen semen was found to be higher in Friesian breed in comparision to Jersey breed. The difference in post thaw motility between different breeds was recorded to be statistically significant ($P < 0.01$, Table -4).

<u>Penetration rate of frozen sperm of Friesian and Jersey bulls treated with caffeine citrate and prostaglandin F_2 alpha into the oestrous mucus of cows</u> :

The overall mean penetration rates of untreated control, caffeine citrate and prostaglandin F_2 alpha treated frozen sperms into the oestrous mucus of cows were observed to be 59.88 $\pm$ 1.08 mm, 60.60 $\pm$ 0.01 mm and 60.92 $\pm$ 1.06 mm in Friesian breed and 60.50 $\pm$ 1.16 mm, 61.55 $\pm$ 1.15 mm and 62.04 $\pm$ 1.16 mm in Jersey breed per 20 minutes of incubation respectively (Table -5 & Fig. -2).

Fig. - 2.

Histogram showing the mean penetration rate of frozen sperm of Friesian and Jersey bulls under different treatment groups into the oestrous mucus.



Analysis of variance revealed no significant difference between different treated groups of frozen sperm in their penetration rate into the oestrous mucus after 20 minutes of incubation (Table -6).

No significant difference between bulls of the Jersey breed was recorded on statistical analysis as regards the penetration rate of frozen sperm into the oestrous mucus of cows per 20 minutes of incubation, while between bulls of Friesian breed it turned out to be significant ($P < 0.01$, Table -7).

Statistically it was found that the migration rate of the frozen spermatozoa into the oestrous mucus after 20 minutes of incubation did not differ significantly between both the breeds (Table -8).

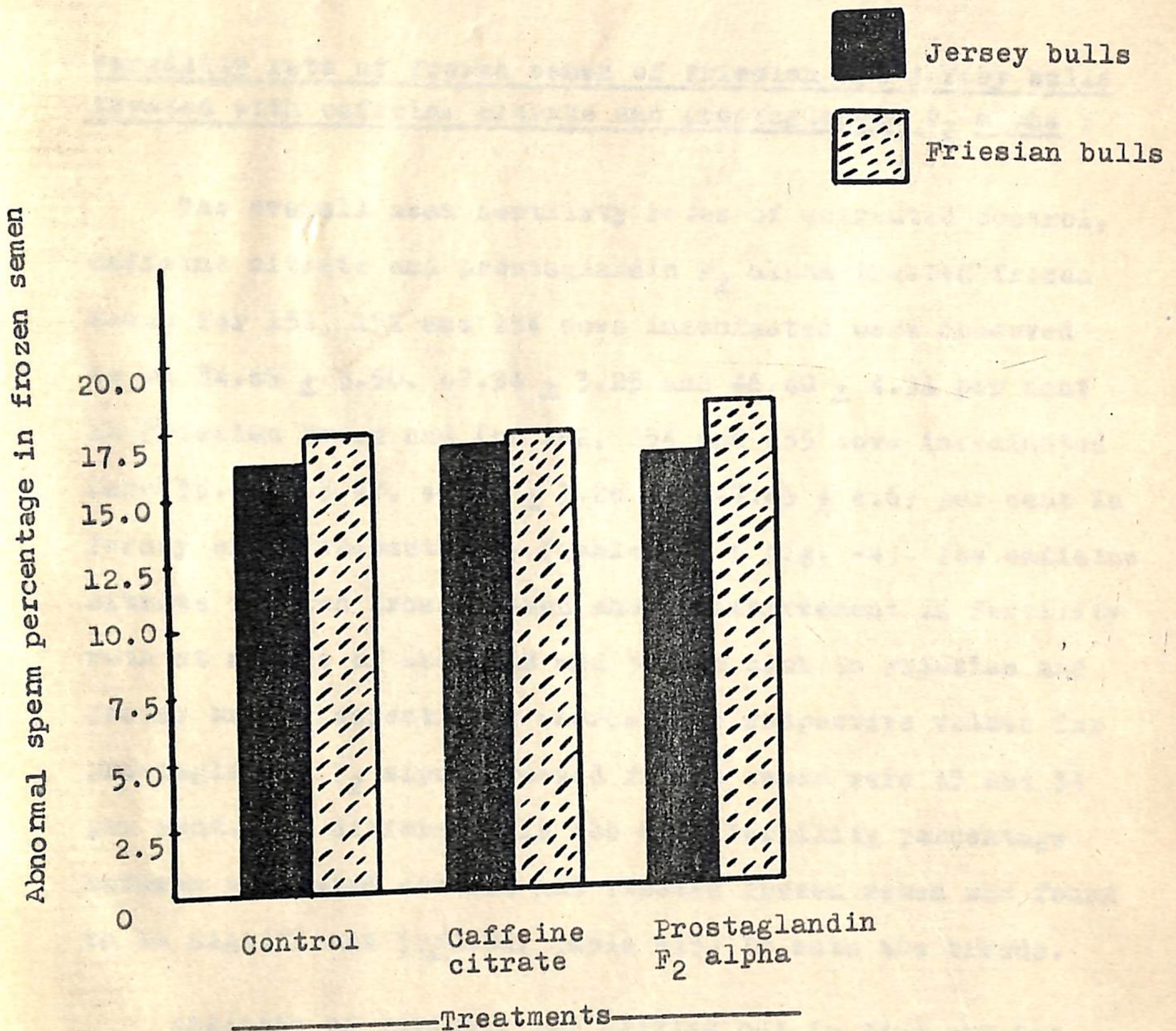
Abnormal sperm percentage in frozen semen of Friesian and Jersey bulls treated with caffeine citrate and prostaglandin F_2 alpha :

The overall mean abnormal sperm in untreated control, caffeine citrate and prostaglandin F_2 alpha treated frozen semen was observed to be 17.33 ± 0.31, 17.13 ± 0.39 and 18.11 ± 0.28 per cent in Friesian breed and 16.36 ± 0.45, 16.71 ± 0.29 and 16.31 ± 0.37 per cent in Jersey breed respectively (Table -9 & Fig. -3).

On statistical analysis no significant difference was observed in the percentage of abnormal sperm between untreated and treated frozen semen. The bull to bull difference was also

Fig. -3.

Histogram showing the mean abnormal sperm percentage in frozen semen of Friesian and Jersey bulls under different treatment groups.



found to be statistically non-significant in both the breeds (Table -10).

The mean abnormal sperm concentration in the frozen semen revealed higher percentage in Friesian as compared to Jersey, thereby indicating a breed to breed variation. The difference in percentage of abnormal sperm between breeds was recorded to be statistically significant ($P \angle 0.01$, Table -11).

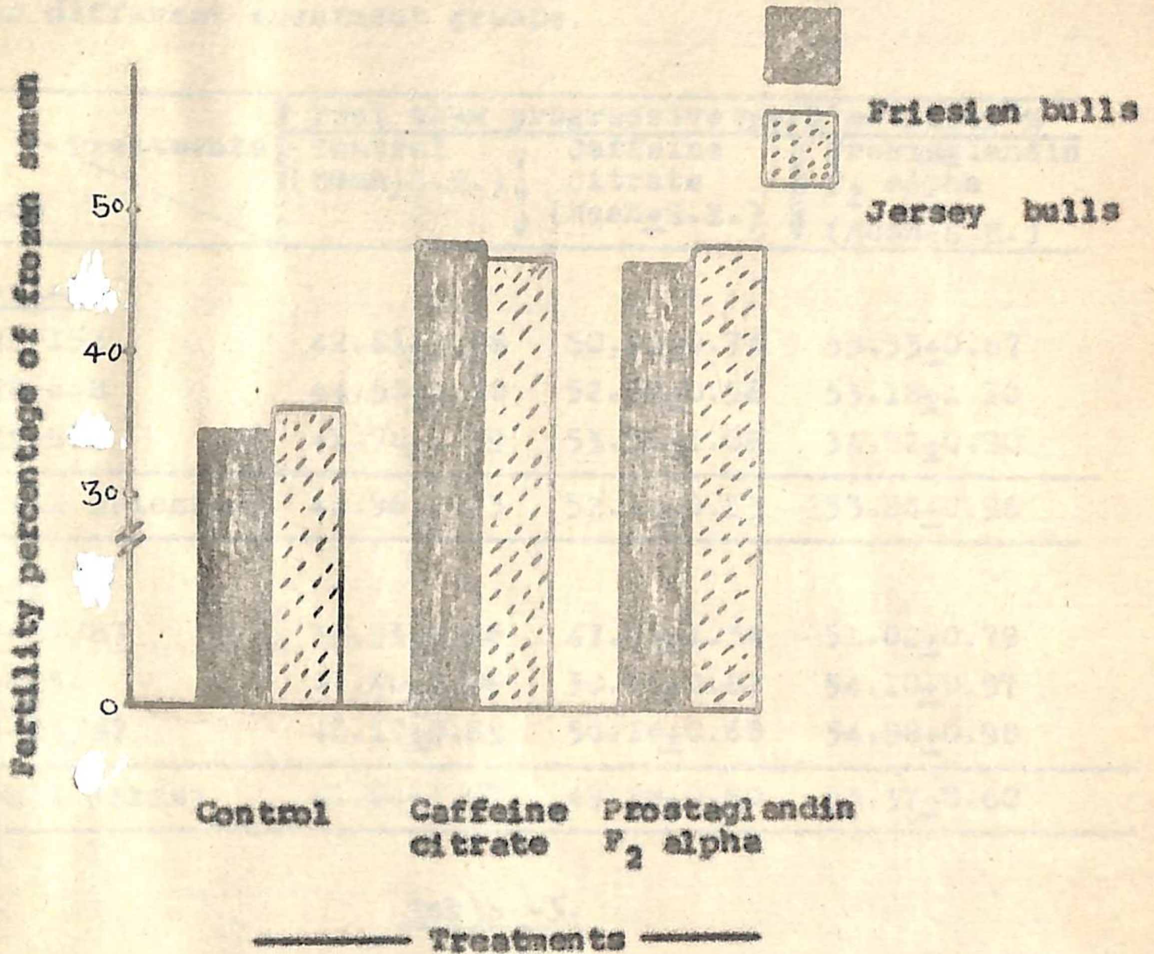
Fertility rate of frozen semen of Friesian and Jersey bulls treated with caffeine citrate and prostaglandin F_2 alpha :

The overall mean fertility rates of untreated control, caffeine citrate and prostaglandin F_2 alpha treated frozen semen for 151, 152 and 154 cows inseminated were observed to be 34.69 $\pm$ 3.50, 47.94 $\pm$ 3.25 and 46.40 $\pm$ 4.34 per cent in Friesian breed and for 162, 154 and 155 cows inseminated were 35.99 $\pm$ 3.97, 46.81 $\pm$ 3.26 and 47.89 $\pm$ 4.69 per cent in Jersey breed respectively (Table -12 & Fig. -4). The caffeine citrate treated frozen semen showed improvement in fertility rate at a tune of about 38 and 31 per cent in Friesian and Jersey breed respectively whereas the respective values for prostaglandin F_2 alpha treated frozen semen were 35 and 34 per cent. The difference in the mean fertility percentage between untreated control and treated frozen semen was found to be significant ($P \angle 0.01$, Table -13) in both the breeds.

Analysis of variance was carried out to find out the effect of bulls as well as breeds on the percentage of fertility rate. It was found that bulls and breeds have no

Fig.- 4

Histogram showing the mean fertility percentage of frozen semen of Friesian and Jersey bulls under different treatment groups.



significant effect on the fertility rate of frozen semen (Table -13 & 14).

Table -2.

Showing the mean post thaw progressive sperm motility percentage in frozen semen of different breed of bulls under different treatment groups.

Breeds \ Treatments	Post thaw progressive sperm motility %		
	Control (Mean±S.E.)	Caffeine Citrate (Mean±S.E.)	Prostaglandin F_2 alpha (Mean±S.E.)
Friesian			
HF-153	42.61±1.08	50.22±0.73	53.53±0.87
HF-648	44.50±1.30	52.58±0.58	53.18±1.10
HF-662	41.78±1.39	53.56±1.08	54.82±0.90
Overall Friesian	42.96±0.73	52.12±0.53	53.84±0.96
Jersey			
J-17/87	39.55±2.36	47.44±1.34	51.02±0.79
J-254	41.40±2.08	50.25±0.87	54.10±0.97
J-24/87	42.17±0.89	50.14±0.60	54.98±0.98
Overall Jersey	41.04±1.07	49.28±0.60	53.37±0.60

Table -3.

Analysis of variance table showing effect of bulls and treatments on post thaw progressive sperm motility percentage in frozen semen of different breed.

Sources of variation	Post thaw progressive sperm motility %			
	Friesian		Jersey	
	d.f.	M.S.	d.f.	M.S.
Between bulls	2	16.52^NS	2	82.44*
Between treatments	2	1025.61**	2	1181.90**
Error	85	11.12	85	17.27
Total :	89		89	

* P/0.05; ** P/0.01; NS = Non-significant.

Table -4

Analysis of variance table showing effect of breeds on post thaw progressive sperm motility percentage in frozen semen.

Source of variation	Post thaw progressive sperm motility %	
	d.f.	M.S.
Between breeds	1	2353.19**
Within breeds	178	27.02
Total :	179	

** $P < 0.01$.

Table -5.

Showing the mean penetration rate of frozen sperm of different breed of bulls under different treatment groups into the oestrous mucus.

Treatments / Breeds	Penetration rate into the oestrous mucus, mm/20 minutes		
	Control (Mean±S.E.)	Caffeine citrate (Mean±S.E.)	Prostaglandin F_2 alpha (Mean±S.E.)
Friesian			
HF-153	61.29±1.30	61.86±1.24	62.29±1.15
HF-648	63.20±0.92	64.20±1.07	64.60±0.87
HF-662	54.50±1.41	55.00±1.58	55.13±1.55
Overall Friesian	59.88±1.08	60.60±0.81	60.92±1.06
Jersey			
J-17/87	65.00±1.16	65.75±0.88	66.37±1.25
J-24/87	61.00±1.46	62.83±1.66	63.00±1.67
J-254	55.63±1.49	56.38±1.49	57.00±1.24
Overall Jersey	60.50±1.16	61.55±1.15	62.04±1.16

Table -6

Analysis of variance table showing effect of treatments on the penetration rate of frozen sperm of different breed of bulls into the oestrous mucus.

Source of variation	Penetration rate of frozen sperm into the oestrous mucus			
	Friesian		Jersey	
	d.f.	M.S.	d.f.	M.S.
Between treatments	2	7.09^{NS}	2	13.68^{NS}
Within treatments	72	27.65	63	188.09
Total :	74		65	

NS = Non-significant.

Table -7

Analysis of variance table showing effect of bulls on the penetration rate of frozen sperm of different breed into the oestrous mucus.

Sources of variation	Penetration rate of frozen sperm into the oestrous mucus			
	Friesian		Jersey	
	d.f.	M.S.	d.f.	M.S.
Between bulls	2	581.40**	2	537.68^{NS}
Within bulls	72	11.69	63	171.46
Total :	74		65	

NS = Non-significant, ** P/0.01.

Table -8

Analysis of variance table showing effect of breeds on the penetration rate of frozen sperm into the oestrous mucus.

Source of variation	Penetration rate of frozen sperm into the oestrous mucus	
	d.f.	M.S.
Between breeds	1	28.24^{NS}
Within breeds	139	21.87
Total :	140	

NS = Non-significant.

Table -9

Showing the mean abnormal sperm percentage in frozen semen of different breed of bulls under different treatment groups.

Treatments / Breeds	Abnormal sperm percentage in frozen semen		
	Control (Mean±S.E.)	Caffeine citrate (Mean±S.E.)	Prostaglandin F_2 alpha (Mean±S.E.)
Friesian			
HF-153	17.37±0.19	16.06±0.25	18.18±0.34
HF-648	16.28±0.29	19.07±0.18	18.64±0.44
HF-662	18.29±0.46	17.25±0.43	17.37±0.14
Overall Friesian	17.33±0.31	17.13±0.39	18.11±0.28
Jersey			
J-17/87	16.68±0.38	15.85±0.23	15.84±0.32
J-254	15.21±0.22	16.27±0.41	16.13±0.17
J-24/87	17.10±0.75	18.43±0.14	17.19±0.53
Overall Jersey	16.36±0.45	16.71±0.29	16.31±0.37

Table -10

Analysis of variance table showing effect of bulls and treatments on the abnormal sperm percentage in frozen semen of different breed.

Sources of variation	Abnormal sperm percentage in frozen semen			
	Friesian		Jersey	
	d.f.	M.S.	d.f.	M.S.
Between bulls	2	0.19^{NS}	2	0.41^{NS}
Between treatments	2	0.32^{NS}	2	2.98^{NS}
Error	85	0.47	85	1.14
Total :	89		89	

NS = Non-significant.

Table -11

Analysis of variance table showing effect of breeds on the abnormal sperm percentage in frozen semen.

Sources of variation	Abnormal sperm percentage in frozen semen	
	d.f.	M.S.
Between breeds	1	83.67**
Within breeds	178	0.55
Total :	179	

** $P < 0.01$.

Table -12

Showing the mean fertility percentage of frozen semen of different breed of bulls under different treatment groups.

Treatments / Breeds	Fertility percentage of frozen semen		
	Control (Mean±S.E.)	Caffeine citrate (Mean±S.E.)	Prostaglandin F_2 alpha (Mean±S.E.)
Friesian			
HF-153	35.08±3.41	52.44±3.05	47.54±4.41
HF-648	35.55±3.41	47.78±3.36	48.76±5.02
HF-662	33.46±3.69	43.61±3.34	42.89±3.58
Overall Friesian	34.69±3.50	47.94±3.25	46.40±4.34
Jersey			
J-17/87	36.76±5.51	46.53±2.46	47.33±5.07
J-254	35.10±3.08	45.45±4.22	47.59±3.98
J-24/87	36.11±3.33	48.44±3.10	48.76±5.02
Overall Jersey	35.99±3.97	46.81±3.26	47.89±4.69

Table -13

Analysis of variance table showing effect of treatments and bulls on fertility percentage of frozen semen in different breeds.

Sources of variation	Fertility percentage of frozen semen			
	Friesian		Jersey	
	d.f.	M.S.	d.f.	M.S.
Between treatments	2	1492.87^{**}	2	1297.40^{**}
Between bulls	2	284.37^{NS}	2	22.33^{NS}
Error	85	107.85	85	160.54
Total :	89		89	

** $P < 0.01$, NS = Non-significant.

Table -14

Analysis of variance table showing effect of treatments, bulls and breeds on fertility percentage of frozen semen (pooled).

Sources of variation	Fertility percentage of frozen semen	
	d.f.	M.S.
Between treatments	2	2761.26^{**}
Between bulls	5	84.44^{NS}
Between breeds	1	8.89^{NS}
Error	171	133.69
Total :	179	

** $P < 0.01$, NS = Non-significant.

Table -13

Analysis of variance table showing effect of treatments and bulls on fertility percentage of frozen semen in different breeds.

Sources of variation	Fertility percentage of frozen semen			
	Friesian		Jersey	
	d.f.	M.S.	d.f.	M.S.
Between treatments	2	1492.87**	2	1297.40**
Between bulls	2	184.37NS	2	22.33NS
Error	85	107.85	85	160.54
Total :	89		89	

** P/0.01, NS = Non-significant.

Table -14

Analysis of variance table showing effect of treatments, bulls and breeds on fertility percentage of frozen semen (pooled).

Sources of variation	Fertility percentage of frozen semen	
	d.f.	M.S.
Between treatments	2	2761.26**
Between bulls	5	84.44NS
Between breeds	1	8.89NS
Error	171	133.69
Total :	179	

** P/0.01, NS = Non-significant.

D I S C U S S I O N

DISCUSSION

The frozen semen generally yields low post thaw sperm motility. Although the quantitative relation of sperm motility to fertilization has not been conclusively assessed, poor motility appears to be correlated with lowered fertility. However, various workers have used caffeine (Miyamoto and Nishikawa, 1979; Daader *et al*., 1989; EL-Gaafary *et al*., 1990) and prostaglandins (Guillory *et al*., 1979; Ramana, 1986) as additives in liquid bovine semen in order to improve the quality as well as motility of the spermatozoa. The present study has been conducted on 10 frozen semen samples each from 6 exotic bulls supplemented with caffeine citrate (7.2 mM) and prostaglandin F_2 alpha (4 μg/ml) to assess their influences on certain characteristics and fertility of the spermatozoa. The results of this investigation are discussed under the specific headings :

Post thaw progressive sperm motility of frozen semen of Friesian and Jersey bulls treated with caffeine citrate and prostaglandin F_2 alpha :

The mean post thaw progressive sperm motility in caffeine citrate and prostaglandin F_2 alpha treated frozen semen was 52.12 ± 0.53 and 53.84 ± 0.96 per cent in Friesian whereas the respective values in Jersey were found to be 49.28 ± 0.60 and 53.37 ± 0.60 per cent against 42.96 ± 0.73

and 41.04 ± 1.07 per cent in untreated control frozen semen. The results of improvement in post thaw sperm motility are in conformity with the observations of Gehlaut and Srivastava (1987), Hukeri (1989) and Muralinath et al. (1989 & 1990a) in frozen semen of buffalo bulls.

The post thaw progressive sperm motility of treated frozen semen varied from untreated control which on statistical analysis was observed to be significant ($P < 0.01$). The significant variation observed among the Jersey bulls as regards the post thaw motility due to prostaglandin F_2 alpha treatment is also corroborated with the findings of Muralinath et al. (1989). The individual bull variation is suggestive of difference in the initial seminal concentration of prostaglandin F_2 alpha. Thus the effect of added prostaglandin may depend on the endogenous prostaglandin level. A considerable variation in responsiveness to caffeine was observed between individual bull semen samples which might be correlated with the amount of motility lost as a result of freezing. However, the motility of a semen sample could not be stimulated beyond the values observed before freezing.

The results of present study are at variance with the findings of Abbitt et al. (1977), Miyamoto and Nishikawa (1979) and Bhosrekar et al. (1990). This might be attributed to many factors like difference in breed, use of different dilutors of varying composition, glycerolization, higher thawing temperature and time and inoptimal concentration of the additives, besides inaccuracy of motility evaluation based

on subjective estimation from a simple slide preparation.

In the present study the post thaw motility of spermatozoa was stimulated significantly with 7.2 mM dose level of caffeine in frozen semen having 41 to 43 per cent post thaw motility in control, whereas Gehlaut and Srivastava (1987) found similar result with 6 mM caffeine in frozen semen having only 39 per cent post thaw motility in control. Bhosrekar et al.(1990) did not find stimulatory effect of 7 mM caffeine on post thaw motility of spermatozoa in frozen semen having about 60 per cent motility in control. Thus, the variable response indicates that the level of caffeine in semen as well as the sperm plasma membrane permeability to substrate may determine the degree of stimulation of post thaw sperm motility.

The dose level of 4 μg/ml prostaglandin F_2 alpha in the present investigation revealed significant increase in post thaw motility of bovine spermatozoa. Muralinath et al. (1989) observed similar result with 1.5 ng/ml dose level of prostaglandin F_2 alpha while with 3 ng/ml found non-significant increase in post thaw motility of spermatozoa in buffalo bull frozen semen. Memon (1981) and Varnavskii (1981) reported no stimulatory effect of prostaglandin F_2 alpha upto the dose level of 300 μg/ml in frozen ram semen. From this it may be construed that there is species variation whereby bovine sperms are more resistant to prostaglandin F_2 alpha than buffalo bull sperms. On the contrary bovine spermatozoa are more sensitive

than that of ram sperms with prostaglandin F_2 alpha.

The mechanism by which caffeine stimulates sperm motility is thought to involve the inhibition of phosphodiesterase activity and the subsequent accumulation of cyclic adenosine -3':5' - monophosphate (cAMP) within the sperm cells (Garbers et al., 1971). The precise way in which elevated intracellular cAMP levels induce an increase in motility is not clearly understood. Although Peterson et al.(1979) considered it to be due to the control of calcium ion fluxes across the sperm plasma membrane. The elevated levels of intracellular calcium certainly appeared to have a detrimental effect on sperm motility, and cAMP - associated calcium pumps in the plasma membrane appeared to be critically important in regulating the content of this ion within the sperm cell. Since considerable damage is inflicted on the sperm plasma membrane during cryostorage, it is possible that the loss of motility observed after freezing and thawing spermatozoa is related to the inability of the damaged plasmalemma to control the levels of calcium within the sperm cells. Caffeine may compensate for the resultant loss of motility by stimulating the activity of an outwardly - directed plasma membrane calcium pump through the mediation of cAMP (Aitken et al., 1983). However a large proportion of the calcium in semen is either complexed with substances such as citric acid or protein. In this respect, there is a specific protein component in bovine seminal plasma that interacts with the sperm surface and decreases the permeability of the plasma

membrane to calcium (Babcock *et al.*, 1979). On the other hand Haesungcharern and Chulavatnatol (1973) advocated that cAMP may increase energy production by accelerating the glycolysis and tricarboxylic acid cycle and their utilization by the motile apparatus of the spermatozoa. More extensive studies may pin point the mechanism by which caffeine accelerates motility of sperms.

The physiologic functions of the prostaglandins in semen are still not well understood because of their wide-spread distribution in various organs and tissues. On their mechanism of action and pharmacologic effects, many hypotheses have been advanced. It is believed that prostaglandins exert their effect on sperms by interacting with the adenyl cyclase - cyclic adenosine -3':5' - monophosphate systems (Bygdeman *et al.*, 1970).

<u>Penetration rate of frozen sperm of Friesian and Jersey bulls incorporated with caffeine citrate and prostaglandin F_2 alpha into the oestrous mucus of cows</u> :

The average penetration rate of caffeine citrate and prostaglandin F_2 alpha incorporated frozen sperm into the oestrous mucus was 60.60 $\pm$ 0.01 mm and 60.92 $\pm$ 1.06 mm per 20 minutes of incubation in Friesian where as it was 61.55 $\pm$ 1.15 mm and 62.04 $\pm$ 1.16 mm per 20 minutes in Jersey against 59.88 $\pm$ 1.08 mm and 60.50 $\pm$ 1.16 mm per 20 minutes for un-treated control frozen sperm respectively. There was no significant change in the rate of penetration of spermatozoa

due to caffeine treatment. Inspite of the scarcity of literature, the present results are in conformity with the finding of Aitken <u>et al</u>. (1983) on human spermatozoa. They reported increased number of spermatozoa penetrating the cervical mucus in unit time, by increasing the frequency rather than the success of collisions between spermatozoa and the cervical mucus interface after the addition of caffeine to frozen thawed human semen. No cognizable effect of prostaglandin F_2 alpha on penetration rate of frozen bull sperm has been also observed in present study which is in close agreement with the result of Muralinath <u>et al</u>.(1990b). The result of the present investigation resembled with that of Memon (1981) in ram semen. They observed increased sperm migration distance in pooled cervical mucus of ewes with time but no difference between prostaglandin F_2 alpha treated and control frozen semen of ram. Similar result was obtained by Memon and Gustafsson (1984) with liquid ram semen too.

The lack of treatment effect observed in this study is in close conformity with the findings of Kumar (1980) who reported Friesian and Jersey bull frozen sperm penetration rate of 62.82 and 61.33 mm per 20 minutes in cervical mucus collected during middle to end of oestrous.

The mean penetration rate of frozen sperm in the mucus filled capillary tubes after 20 minutes of initial semen - mucus contact did not differ significantly between different treatment groups which might be due to absence of their

influence on the sperm velocity (Makler et al., 1980; Aitken et al., 1983). The significant variation in the penetration rate of frozen sperm was observed among the Friesian bulls which is in close agreement with the findings of Muralinath et al. (1990b). This might be due to individual variation in their sperm penetration rate irrespective of the treatments.

The sperm penetrated distance in the present study is higher than that observed by Roark and Herman (1950), Moeller and VanDemark (1955) and Akhtar et al. (1980). This might be due to their study on liquid semen. Thus, it is conceivable that the metabolic activity of the frozen semen samples are stopped thereby conserving the energy to be utilized after thawing. As such they were more vigerous in penetrating the mucus samples. The chilled sperm might have lost their energy and vigour as the metabolic activity is not stopped during their course of preservation. However, the sperm penetrated distance observed by Muralinath et al. (1990b) in cervical mucus by frozen buffalo bull sperm was much lower in comparison to the present findings. This might be due to the species variation, their study on cervical mucus collected at different stages of oestrous, storage of mucus at 4°C and difference in the levels of electrolytes in oestrous mucus. There exists correlation between the penetration rate of spermatozoa and levels of sodium and potassium in the oestrus mucus. The high level of sodium and low level of potassium increased the penetration rate of spermatozoa

whereas the low level of sodium and high level of potassium of oestrous mucus decreased the penetration rate (Kumar,1980).

<u>Abnormal sperm percentage in the frozen semen of Friesian and Jersey bulls supplemented with caffeine citrate and prostaglandin F_2 alpha</u> :

The mean abnormal sperms in caffeine citrate and prostaglandin F_2 alpha supplemented frozen semen was 17.13 ± 0.39 and 18.11 ± 0.28 per cent in Friesian whereas the respective values in Jersey were found to be 16.71 ± 0.29 and 16.31 ± 0.37 per cent against 17.33 ± 0.31 and 16.36 ± 0.45 per cent in unsupplemented frozen semen. Thus both the additives have no adverse effect on the morphology of the spermatozoa in both breeds. The reports on bovine species regarding the effects of caffeine and prostaglandin F_2 alpha on the morphology of frozen spermatozoa are apperently lacking. However, the results of present study are in close conformity with the observations of Carbo *et al*. (1975) and Marley *et al*. (1976) in liquid semen of sheep; Barkay *et al*. (1984) in fresh human semen and Muralinath *et al*. (1989) in frozen semen of buffalo bulls.

Memon (1981) observed that higher dose of prostaglandins adversely affected sperm survival and acrosomal morphology of ram frozen semen, while lower doses had no harmful effects. In present experiment only lower dose of prostaglandin F_2 alpha was used with no marked detrimental effects on morphology of the bull spermatozoa. Barkay *et al*. (1984)

carried out a quantitative electron microscopic scanning on the fresh semen samples from fertile donors and suggested that 7.2 mM caffeine did not cause any damage to the different organelles of sperm heads and tails even after prolonged incubation while morphologic analysis of the caffeine treated frozen thawed semen was inconclusive because of the freezing technique and the masking effect of the protective medium.

Contrary to the present finding, Harrison (1978) claimed that caffeine causes damage to the human sperms. Harrison et al. (1980) found disruption of normal shape of the sperm heads in the asthenozoospermic men after caffeine (6 mM) incubation. This might be due to teratozoospermic semen samples and the modified Ringer's solution used as the dilution medium (Barkay et al., 1984). AL-JuBuri et al. (1989) observed a lower percentage of abnormal sperms with the prefreeze addition of prostaglandin F_2 alpha in frozen washed semen than frozen whole semen of Awassi ram. This might be due to the compositional factors of seminal plasma.

The percentage of abnormal sperms in the unsupplemented frozen semen as observed in the present study is in accordance with the findings of Seiciu et al.(1975), Bujarbaruah et al. (1982) and Chalapathy and Rao (1982), but higher than that observed by Rao and Kotayya (1974), Saxena and Tripathi (1979), Raju and Rao (1982) and Singh and Pangawkar (1990) and less than that recorded by Rao and Rao (1975) in bull semen. These differences among the above workers might be due to variation in climatic conditions, managemental practices, feeding

schedules, frequency of semen collection and the method of semen preservation.

<u>Fertility rate of frozen semen of Friesian and Jersey bulls supplemented with caffeine citrate and prostaglandin F_2 alpha :</u>

The average fertility rate of caffeine citrate and prostaglandin F_2 alpha supplemented frozen semen was 47.94 $\pm$ 3.25 and 46.40 $\pm$ 4.34 per cent in Friesian whereas the corresponding figures in Jersey were recorded to be 46.81 $\pm$ 3.26 and 47.89 $\pm$ 4.69 per cent against 34.69 $\pm$ 3.50 and 35.99 $\pm$ 3.97 per cent for untreated control frozen semen respectively. The variation in fertility rate between untreated and treated frozen semen either with caffeine citrate or prostaglandin F_2 alpha was found to be statistically significant ($P \angle 0.01$) in both Friesian and Jersey breed. Under similar experimentation no report is available in bovine species. However the results of the present study corroborate with the observations of Spilman <u>et al</u>. (1973) in rabbit semen; Zhiltsov <u>et al</u>. (1974) and Gustafsson <u>et al</u>. (1975) in frozen semen of sheep; Dimov and Georgiev (1976 & 1977) in liquid ram semen; Barkay <u>et al</u>. (1984) in frozen thawed human semen; Stoyanov (1984) in frozen thawed bull semen and Burov and Kolomoetes (1990) and Radoslavov <u>et al</u>. (1990) in liquid bull semen. Minor variations in fertility rate observed by them may be attributed to difference in breed, levels of additives in semen during insemination, timing of insemination, timing of ovulation, semen processing technologies,

thawing temperature and time, climatic conditions, unidentified pathology of the female genital tract and harmonal balance of the female animals.

Fraser (1979) and Niwa et al. (1988) observed more than 100 per cent improvement in the fertilizing ability of the sperms in the presence of caffeine in vitro, which is comparatively much higher than the findings of present study in vivo. This might be due to many factors like absorption of the caffeine by female genital tract, blind interval between the insemination and ovulation and lack of adequate contact of caffeine with sperms during their capacitation.

Interestingly the improved fertility due to the addition of caffeine or prostaglandin F_2 alpha observed in present study does not correspond with the findings of Harrison (1978) on human ejaculated semen, Varnavskii (1981) on frozen semen of sheep and EL-Gaafary (1989) on rabbit semen. This may be ascribed to the use of oligospermic or asthenospermic semen samples and detrimental effect of higher concentration of prostaglandin F_2 alpha on the sperm.

In order for a cell to produce cAMP from ATP, the enzyme adenyl cyclase is required and adenyl cyclase have been demonstrated in the spermatozoa for a number of mammals, including man (Hicks et al., 1972). The activity of adenyl cyclase in capacitated hamster spermatozoa was found to be greater than in uncapacitated spermatozoa, based on production of cAMP per unit time (Morton and Albagli, 1973), suggesting

that during capacitation there may be an increase in the concentration of intracellular cAMP. The participation of cAMP in the capacitation of spermatozoa has also been suggested by Reyes <u>et</u> <u>al</u>. (1978), thus raising the possibilities that caffeine and prostaglandin F_2 alpha, infact, might have an effect on capacitation. The cAMP appears to bind to the surface membranes of intact spermatozoa and induces metabolic and surface changes similar to those necessary to the process of spermatozoal capacitation (Delgado <u>et</u> <u>al</u>.,1976). Caffeine has not been shown to induce capacitation but to accelerate the rate of capacitation when spermatozoa were exposed in an environment permitting capacitation. Hence the penetrative ability of spermatozoa into the egg is increased as suggested by Fraser (1979), indicating that cAMP may be a key factor in sperm capacitation. As prostaglandin F_2 alpha has also contractile effect on the smooth muscles of the uterus and fallopian tubes, their increased contraction might facilitate transport of spermatozoa in the large scale to the site of fertilization (Mandle,1972; Chang <u>et</u> <u>al</u>., 1973; Edqvist <u>et</u> <u>al</u>., 1975; Gustafsson <u>et</u> <u>al</u>., 1977).

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

The present study was conducted on three Holstein Friesian and three Jersey bulls of documented fertility aged 4 to 7 years belonging to the Frozen Semen Bank, Patna. The bulls were maintained on isomanagemental conditions and semen samples were collected twice weekly by artificial vagina method. Immediately after collection of semen, different attributes like volume, colour, mass activity, initial motility and concentration of spermatozoa were determined. Ten semen samples from each bull having ejaculate volume of 3 ml with mass activity of grade +4 and above were only included in the present investigation. The semen samples after dilution were subjected to tests to eveluate the effects of caffeine citrate and prostaglandin F_2 alpha supplementation. The fertility rate of frozen semen was determined by the pregnancy diagnosis after 60 to 90 days of first insemination.

In the caffeine citrate treated frozen semen of Friesian and Jersey bulls the mean post thaw progressive sperm motility was found to be 52.12 ± 0.53 and 49.28 ± 0.60 per cent respectively against 42.96 ± 0.73 and 41.04 ± 1.07 per cent in untreated frozen semen. The difference in post thaw motility between untreated and treated frozen semen was significant ($P < 0.01$). The penetration rate of treated frozen semen of Friesian and Jersey bulls into the oestrous mucus was observed to be 60.60 ± 0.01 mm and 61.55 ± 1.15 mm per 20 minutes respectively against the control values of 59.88 ± 1.08 mm and 60.50 ± 1.16 mm per 20 minutes, which was statistically in-significant. The mean abnormal sperms in treated frozen

semen of Friesian and Jersey bulls were recorded to be 17.13 ± 0.39 and 16.71 ± 0.29 per cent respectively against 17.33 ± 0.31 and 16.36 ± 0.45 per cent in control. Statistically the difference in abnormal sperm percentage was insignificant. The mean fertility rate of treated frozen semen of Friesian and Jersey bulls was recorded to be 47.94 ± 3.25 and 46.81 ± 3.26 per cent respectively against 34.69 ± 3.50 and 35.99 ± 3.97 per cent for untreated frozen semen. The fertility rate in the treated frozen semen improved significantly ($P<0.01$) in both the breeds.

In the prostaglandin F_2 alpha treated frozen semen of Friesian and Jersey bulls the mean post thaw progressive sperm motility was found to be 53.84 ± 0.96 and 53.37 ± 0.60 per cent respectively against 42.96 ± 0.73 and 41.04 ± 1.07 per cent in control. Statistically there was significant ($P<0.01$) increase in post thaw motility of treated frozen sperm in both the breeds. The penetration rate of treated frozen sperm of Friesian and Jersey bulls into the oestrous mucus was observed to be 60.92 ± 1.06 mm and 62.04 ± 1.16 mm per 20 minutes respectively against 59.88 ± 1.08 and 60.50 ± 1.16 mm per 20 minutes in control. The abnormal sperms in treated frozen semen of both the breeds was recorded to be 18.11 ± 0.28 and 16.31 ± 0.37 per cent against 17.33 ± 0.31 and 16.36 ± 0.45 per cent in untreated frozen semen. There was no significant change in penetration rate and abnormal sperm percentage. The fertility rate of treated frozen semen of Friesian and Jersey bulls was found

to be 46.40 ± 4.34 and 47.89 ± 4.69 per cent respectively against 34.69 ± 3.50 and 35.99 ± 3.97 per cent in control. On statistical analysis there was significant ($P \angle 0.01$) increase in fertility rate .

Analysis of variance revealed significant difference ($P \angle 0.05$) between the bulls of Jersey breed as regards the post thaw motility whereas the difference between Friesian bulls was non-significant. The frozen semen of both breeds differed significantly ($P \angle 0.01$) in the post thaw motility. The penetration rate of frozen sperm of individual Friesian bull was found to differ significantly ($P \angle 0.01$) while the same was non-significant in case of Jersey bulls. There was no significant effect on the penetration rate of frozen sperm of both the breeds. The difference in abnormal sperm percentage between the bulls of both breed was non-significant whereas the breed effect was significant ($P \angle 0.01$) in pooled data. The fertility rate of frozen semen did not differ significantly either due to bulls or breeds in the present study.

In conclusion the caffeine citrate (7.2 mM) and prostaglandin F_2 alpha (4 μg/ml) can safely be supplemented before freezing the semen for improving the post thaw progressive sperm motility as well as fertility rate of the frozen bovine semen. However, further studies on fertility are required to substantiate the findings for recommending for routine use in the field.

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