

**INTRAUTERINE INSEMINATION USING FRESH
AND FROZEN-THAWED CHITAL DEER (*Axis axis*) SEMEN**

**INSEMINASI SECARA INTRAUTERIN MENGGUNAKAN SEMEN SEGAR SEMEN BEKU PADA RUSA
TOTOL (*AXIS AXIS*)**

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ABSTRACT

The objective of the study was to achieve pregnancy through laparoscopic insemination, using fresh and frozen-thawed semen in chital deer (*Axis axis*). Oestrus and ovulation of 20 mature chital hinds were synchronised by inserting progesterone impregnated intravaginal devices (CIDR) for 14 days. To optimise the result of oestrus synchronisation, the CIDRs were replaced by day 7 of insertion. The 60×10^6 live spermatozoa, in fresh or frozen-thawed form, were inseminated laparoscopically, 48 and 60 hours following CIDR removal respectively. The pregnancy rates of 28.5% and 25% were achieved with insemination using fresh and frozen semen respectively.

Key words: chital deer, laparoscopic insemination, fresh and frozen-thawed semen

ABSTRAK

Tujuan penelitian ini adalah mengevaluasi inseminasi buatan menggunakan laparoskop untuk menghasilkan kebuntingan, menggunakan semen segar dan semen beku pada rusa totol (*Axis axis*). Estrus dan ovulasi dari 20 rusa totol dewasa distimulasi menggunakan *progesterone impregnated intravaginal devices* (CIDR) selama 14 hari. Untuk mencapai kesentakan birahi yang optimal, CIDR diganti dengan yang baru pada hari ke 7 setelah dipasang. Sebanyak 60×10^6 spermatozoa hidup baik dalam bentuk segar maupun setelah dicairkan dari kondisi beku di inseminasikan menggunakan laparoskop. Semen segar diinseminasikan 48 jam setelah CIDR diambil, sedang semen beku di inseminasikan 60 jam setelah CIDR diambil. Hasil kebuntingan menggunakan semen segar mencapai 28.5% dan menggunakan semen beku mencapai 25%.

Kata kunci: rusa totol, inseminasi secara laparoscopik, semen segar dan beku

INTRODUCTION

Laparoscopic insemination has been reported in red deer (Fennessy and Mackintosh, 1988; Haigh and Bowen, 1991), in fallow deer (Mulley *et al*, 1988; Asher *et al*, 1990; Mylrea *et al*, 1991; Jabbour *et al*, 1992), in eld's deer (Monfort *et al*, 1993). By using this technique, the semen is injected into the lumen of the uterus. There are some advantages that can be obtained by injecting semen into the uterus, such as; 1. elimination of the barrier of cervix penetration by spermatozoa; 2. minimized transportation of spermatozoa to the fertilisation site; 3. utilisation of fewer of spermatozoa or lesser quality, to produce pregnancy. Consequently, the results of pregnancy rates were reported as comparable to those of natural mating, (Boland and Gordon, 1978) or 10 to 20% higher than that of intravaginal or intracervical insemination in red deer (Fennessy and Mackintosh, 1988) and in fallow deer (Asher *et al*, 1991). The success of artificial insemination to produce higher pregnancy rate would be an effective technique, to introduce or exchange the genetic materials between parks and zoos across International boundaries, to preserve endangered or rare species of deer.

The aim of the study was to compare the pregnancy results of laparoscopic insemination using fresh and frozen-thawed semen 48 and 60 hours, following progesterone devices removal, respectively.

MATERIALS AND METHODS

Chital deer were farmed and accustomed to enter yards and crush. They were allowed to graze in the paddock, with hay and pellet supplementation, during summer and winter, as necessary. Four stags and 20 mature chital hinds were used in this study. The hinds were divided randomly into 2 groups, 12 and 8 animals, to evaluate the pregnancy rate of 48 and 60 hours insemination after CIDRs removal using fresh and frozen semen respectively.

Chital semen was collected using an electro-ejaculator (Colorado ejaculator) fitted with a ram probe (Australian Livestock Genetics, Narromine, Aust), under general anaesthetic, as described by Mylrea, (1992) and Haigh *et al*, (1993). Four mg/kg BW Xylazine hydrochloride (Rompun, Bayer Aust. Ltd, Botany, NSW, Australia) combined with 4 mg/kg Ketamine hydrochloride (Ketamine, Parnell Laboratories Aust. Pty Ltd, Silverwater, NSW, Australia) per Kg BW were injected intramuscularly, with the stags restrained in a crush. The stags were then allowed to stay in a darkened pen until they fell into lateral recumbency. The penis was then pulled out from the prepuce, by pushing the upper leg forward, and, to prevent the penis from going back into the prepuce, a piece of woven bandage was used to hold it. A bell test tube was warmed by hand and held in front of the penis. The lubricated electro-ejaculator probe

was inserted into the anus, with longitudinal electrodes on its ventral surface. The stimulation of the electro-ejaculator was applied for 5 seconds, with rest intervals, by turning off the electro ejaculator for 5 seconds. Once the semen was ejaculated, or after two minutes of stimulation, electric induction was stopped. Reverzine (Yohimbin HCl, Parnell Laboratories Aust. Pty Ltd, Silverwater, NSW, Australia), 0.4mg/kg BW was injected intramuscularly, after semen collection, to reverse the effect of anaesthesia. Then the stags were allowed to recover in a darkened pen, before being returned to their paddocks.

Semen was evaluated as soon as collected, by the following criteria: volume, wave motion, morphology, live sperm and concentrations of sperm, as described (Mylrea, 1992; Haigh *et al*, 1993). After collection, the volume of ejaculate was estimated from the bell test tube. The rate of motility was studied by dropping fresh semen (0.05 ml) onto a warmed slide, under a microscope with 40x magnification. The wave motion was scored at the edge of the drop, with a scoring system from 0 to 5 being used. Examination of morphology was done to estimate the percentage of numbers of normal cells, immature (protoplasmic droplet), abnormality during spermatogenesis (head) and post-spermatogenesis, as well as artefacts of the sperm. Two ml of semen was added to 10 ml of Nigrosin Eosin stain and smeared onto a warmed (36°C) and dried slide. The Nigrosine Eosin stain used in this study, was made of Eosin (water soluble) 1.67 gr, Nigrosine (water soluble) 10.00 gr, in 100 ml distilled water. These chemicals were mixed, filtered and warmed to 36°C before being used. Sperm morphology was examined under the microscope at 100x magnification. This staining was also used to estimate the ratio of live and dead spermatozoa. The sperm concentration was counted by using a haemocytometer pipette and counting chamber, under the microscope, after the samples were settled for 5 minutes.

After evaluation, the semen was diluted using Triladyl (Minitab, America ;Bovine semen, Aust. Pty. Ltd, Bundanon, NSW, Australia) in a 120x10⁶ live motile sperm per 0.25 ml. This semen was then loaded into the straws and sealed, then stored frozen for three weeks. Freezing method was done by holding straws 6 cm above LN₂ level for 20 minutes, before immersing. It was expected that at least 60x10⁶ live motile spermatozoa would be recovered after thawing. Fresh semen was collected as described by Mylrea, (1992); Haigh *et al*, (1993), one hour before insemination. It was then evaluated and diluted with ultra heat treated (UHT) milk (Evans and Maxwell, 1987) and stored at 30°C waterbath (60x10⁶ spermatozoa per 0.25 ml) until insemination was completed.

The chital hinds were treated with progesterone by inserting CIDRs (Controlled internal drug release,

goat, Riverina Artificial Breeder, Albury, NSW) intravaginally. The CIDRs were inserted for 14 days and they were replaced 7 days after insertion. Oestrus detection was studied using a vasectomised stag fitted with a crayon harness. The hinds were determined to be in oestrus when crayon marks were found on the hinds' rumps during observation of oestrus or at the time of insemination.

Insemination was done laparoscopically 48 hours and 60 hours, following the CIDR removal, using fresh and frozen semen respectively under general anaesthetic. General anaesthetic, intravenous 2.5 mg Xylazine mixed with 2.5 mg Ketamine per kg BW, was injected into the hinds when they were restrained in the crush. Immediately after injection, the hinds were allowed to fall in a darkened pen. The anaesthetised hinds were then placed in an insemination cradle, in dorsal recumbency with their heads tilted-down at 30° to 45° angle. Following fixation of the legs, the abdomen was then shaved and swabbed with 70 % ethanol and 1 % Sodium iodide solution. Frozen semen was thawed by immersing the straws in a 35°C water bath for 30 seconds. After the straws' seals were cut, the semen was injected into the test tube in 30°C waterbath, to allow the laparoscopic insemination needle to aspirate the 0.25 ml frozen-thawed semen. The laparoscopic insemination was done as described by Killeen and Caffery, (1982), by making incision of the skin and puncturing the abdominal wall using two trocars. The laparoscopic trocar (7 mm diameter) and insemination trocar were stabbed on either side of midline around 10 cm anterior mammary gland. The abdomen was then inflated with CO₂ to allow laparoscopic visualisation of the reproductive tract. The semen in the insemination pipette was then injected into the lumen of the uterus. One stitch was placed in each side of incision to close the skin. Finally, the long acting antibiotics (Terramycin,) were injected intramuscularly to prevent infection. Following insemination, the hinds were given Yohimbine (0.4 mg/kg BW), injected intramuscularly, and allowed to recover in darkened pens, before being returned to their paddocks.

The hinds were diagnosed to be pregnant by ultrasound (5.0 MHz rectal transducer, type 2, Ausonic Aust Pty Ltd, Lanecove, Australia.) examination 75 days after insemination, when the

foetuses or placentomes were visualised on the ultrasound screen.

RESULTS AND DISCUSSION

Progesterone impregnated intravaginal devices (CIDR) was used to induced oestrus in the present study. Progesterone treatment was reported to be more effective in synchronising oestrus; it produced better conception rates than that of prostaglandin treatments (Asher *et al*, 1991). CIDR (goat) has been successfully used in chital deer, to maintain progesterone concentration as high as that during the mid cycle of an oestrous cycle, until 13 days of insertion (Mylrea, 1992). The high level of progesterone during treatment was maintained by replacing CIDRs a week apart, to improve the tightness of the synchrony (Fennessy *et al*, 1990). However, it was reported that there was no improvement of the conception rate (Bowen, 1989). However oestrus exhibition in the present study could not be evaluated as during oestrus detection, the vasectomised stag was irritated by the crayon harness fitted on his chest. When the stag was sitting on the ground, the crayon was often covered by dirt and one occasion the crayon fell off. Consequently, there was no mark left on the hinds'rumps and the oestrus exhibition could not be examined.

Artificial insemination techniques in deer were developed by modification of techniques in small ruminants (sheep and goats). It was reported that, following progesterone device removal, the onset of oestrus in sheep was 24 - 36 hours (Maxwell, 1986) and ovulation occurred between 54 - 60 hours (Maxwell, 1986) or 24 - 26 hours after onset of oestrus (Hunter and Nichol, 1983). It was estimated that the spermatozoa remained alive in the uterus up to 28 hours (Holt, 1989) after insemination, and there was evidence that 12 to 30 thousand spermatozoa were detected 24 hours after insemination using fresh semen (Holt, 1992). By using fresh semen, laparoscopic insemination was recommended to be completed at 36 hours following progesterone device removal (Heyward, 1993). Insemination 48 hours after the progesterone device removal, was found to reduce fertility of the superovulated ewes (Holt, 1989). However, by using frozen thawed semen, the time of insemination recommended was 48 hours after progesterone device withdrawal (Heyward, 1993). In line with these recommendations, it was reported that chital deer

Table 1. Laparoscopy insemination using fresh and frozen semen in chital deer

No	Day	Treatment
1	1	CIDR insertion
2	7	Replace CIDR
3	13	CIDR out (9-10 pm) for group I
4	14	CIDR out (9-10 am) for group II
5	16	(9-10 am) Laparoscope insemination using fresh semen 48 hours after CIDR removal (Group II) and using frozen semen 60 hours after CIDR removal (Group I).
6	91	Ultrasound examination for pregnancy diagnosis.

Table 2. Semen evaluation before freezing, to study artificial insemination using frozen semen.

No	Volume (ml)	Conc x10 ⁷	Score	Abnormalit %	Death %	Normal/live %	Straws*
1	0.2	110	4	6	4	90	2
2	0.7	285	5	2	3	95	8
3	1.5	140	5	4	4	92	6

* An 0.25 ml straw contained 120x10⁷ before freezing.

showed oestrus behaviour 24 to 48 hours, and ovulation after 72 hours, following progesterone device withdrawal (Mylrea, 1992). Consequently it was hypothesised in the present study that the optimum time for laparoscopic insemination using fresh and frozen semen should be 48 hours and 60 hours following progesterone device withdrawal respectively.

Triladyl and UHT milk were used as extenders for frozen and fresh semen respectively. Triladyl has been used to preserve chital semen successfully (Haigh *et al*, 1993). UHT milk was distinguished as a simple extender for fresh semen (Evans, 1989). This sterile milk can be used straight from the container. Three ejaculates were collected, evaluated (Table 2) and preserved in sixteen of 0.25 ml straws, containing 120x 10⁶ live spermatozoa before freezing. One ejaculate was collected and evaluated an hour before insemination. This ejaculate was then diluted, using UHT milk into twenty six doses, each 0.25 ml containing 60x10⁶ live spermatozoa (Table 3).

The dose of spermatozoa per insemination used in the present study was 60 million live sperm per uterine horn either using fresh or frozen semen. Recently, acceptable (40%-70%) conception rates have been achieved by insemination laparoscopically of 50 million spermatozoa in red deer (Fennessy *et al*, 1987), 25 million (Jabbour *et al*, 1993), 12.5-25 million in fallow deer (Asher *et al*, 1991), 7.5 -10 million (Monfort *et al*, 1993) in eld's deer.

One hind had large uterine horns when insemination was done, and was excluded from the trial. Finally, seven hinds were inseminated 48 hours after CIDR removal, using fresh semen. Twelve hinds

were inseminated 60 hours following CIDR removal, using frozen semen. The pregnancy rates following insemination, and the live weight of the two groups of hinds, are presented in Table 4.

The total result of pregnancy in the present study was 26.3 %, consisting of 28.5% using frozen and 25% from fresh semen (Table 4). These results of pregnancy were relatively low compared to the other species of deer; eld's deer (Monfort *et al*, 1993) produced 45% pregnancy; red deer (Fennessy *et al*, 1987; Haigh and Bowen, (1991) produced 51% pregnancy. The reason for the low results of pregnancy rates in the present study was not understood. One factor which might be considered regarding to the the low result of pregnancy was the small number of animals used in the present study. By using a small number of animals, the result does not represent the actual results in the population. Finally, it can be concluded that laparoscopic insemination is an efficient technique to produce pregnancy. Transportation semen in fresh or frozen would be an advantage to exchange the genetic materials of small populations deer between countries.

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Table 3. Semen evaluation before dilution using UHT milk (as a fresh semen for insemination).

No	Volume (ml)	Conc x10 ⁷	Score	Abnormalit %	Death %	Normal/live %	Dose/uterine
1	1.2	144	5	2	5	93	26+

+ A dose of 60x 10⁶/ 0.25 ml/ uterine horn.

Table 4. Pregnancy examination using ultrasonography following fixed time laparoscopic insemination using frozen and fresh semen.

	Life weight of hinds (means ± SEM kg)	Number of hinds	Pregnancy (%)
Frozen semen	46.25 ± 0.97	7	28.5
Fresh semen	46.40 ± 1.23	12	25
Total	46.35 ± 0.81	19	26.3

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