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A Comparative Study on Fresh and Frozen Embryo Transfer after Superovulation in Black Bengal Goats (*Capra-hircus*)

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SUMMARY

The experiment was divided into two phases. In phase-I fresh embryos were transferred and in phase-II frozen embryos were transferred. Embryos were collected by using Dulbecco's phosphate buffered saline. In phase-I total of 65 ova were collected out of 107 ovulation in 18 goats. Recovery of ova was 60.74%, of which 51 (78.46%) was fertilized. Sixteen embryos were transferred to 10 recipient goats and kidding was observed in 6 goats, that produced 10 kids. Thus, 62.50% embryo survival and 60% kidding were achieved in phase-I. In phase-II of the experiment, 17 regular cyclic Black Bengal goats were used. The main purpose was to study the viability of caprine embryos after cryopreservation. In this phase the embryos were collected and frozen using Bio-cool freezers. A two step addition of cryoprotectants (5% glycerol and 10% glycerol) and three-step dilution of cryoprotectants with 1mole (M) sucrose was used. Embryos were preserved for 10 to 45 days. Out of 27 embryos preserved, 18 were recovered after freezing and thawing (37°C water bath) with 33.33% embryonic loss. Seventeen frozen and thawed embryos were transferred in 9 recipient goats, out of which kidding was observed in 6 goats and 7 kids were produced, giving a 66.66% kidding and embryo survival of 41.17%. The technique utilized for fresh and frozen embryo transfer can be successfully utilized to produce goats of superior genetic merits. The protocol used for addition of cryoprotectant, freezing, thawing and dilution was found suitable for caprine embryo freezing.

(Key words: embryo transfer, frozen embryo transfer, goat and superovulation)

INTRODUCTION

In recent years the technique of embryo transfer has been modified and refined so that the fresh embryos can be preserved in a frozen state under ultra low temperature of liquid nitrogen. It has also been found possible to bisect the embryos and utilized each half for successful production of young ones. Embryo sexing is another landmark, which enables production of desired sex. Recently greater emphasis has been made on *in vitro* maturation and *in vitro* fertilization of oocytes.

Caprine embryos like other mammalian embryos cannot maintain viability at room temperature for more than a few hours. Embryo freezing and its preservation is the only alternative to store viable embryos ("Embryo Bank") to utilize for transfer as and when required during the normal estrous cycle. Bilton and Moore (1976) reported first successful

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attempt for cryopreservation of goat embryos. Cryopreservation of embryos and its success for producing viable offspring is an important landmark in the history of cryobiology. Compatible freezing and thawing techniques and the judicious selection of embryos prior to freezing has enabled the establishment of a general procedure for preservation of embryos for goats (Bilton and Moore, 1976; Trouson and Pugh, 1982). Embryos of high -producing goats can be stored as a future genetic resource and goats that have been dead for years can become genetic parents. Such embryos collected from superior females can now be marketed to breeders throughout the world (Amoah and Gelaye, 1991). Warwick et al. (1934) reported the first embryo transfer in sheep and goats. When Hunter et al. (1955) transferred 19, 2 ~ 16 cell embryos to 18 recipient ewes, eight lambs were born. Successful recovery and transfer of embryos have been reported with surgical and non-surgical methods (Agrawal and Bhattacharyya, 1982; Armostrong and Evans, 1983; BonDurant et al., 1984; Kiessling et al., 1986).

In case of the goat it may be argued that embryo transfer has limited utility possibly due to lack of superior genetic merit for milk and meat production. Extensive genetic improvement of the goat, both male and female will have to be exploited simultaneously. Genetic exploitation of female appears to be possible only through multiple ovulation, embryo transfer and cryopreservation of embryos.

In retrospect the present investigation aimed at developing suitable technique for fresh and frozen embryo transfer with following objectives.

1. To study the superovulatory response, fertility and kidding behavior of Black Bengal goats after embryo transfer.
2. To study the viability of caprine embryo after cryopreservation.

MATERIALS AND METHODS

Forty-two adult (between the age of 1.5 to 3.5 years of age) Black Bengal female goats were utilized from the Goat Unit of the Department of Physiology, Ranchi College of Veterinary Scienceand Animal Husbandry, Birsa Agriculture University, Ranchi (Jharkhand). The goats were maintained on a normal balanced ration having following composition, Maize −42.250kg/quintal, Wheat bran −37.00 kg/quintal, Ground Nut Cake −18.50 kg/quintal, Mineral mixture −2.00 kg/quintal, Common salt −0.250 kg/quintal and Vitamin AD_3 was mixed at 20 gm/quintal The goats were allowed to graze 3 ~4 hours daily and green fodder was made available ad lib during the period of investigation. Diet contained 16% crude protein and 70% total digestible nutrient. During phase-I, the experimental goats were divided into two groups. Group-1 (n=24) served as recipients for embryo transfer and group-2 (n=18) were subjected to the superovulatory treatment and used as donors for embryo collection. Goats of both the groups were synchronized by the injection Prostaglandin $F_{2\alpha}$ (Dinofertin, Alved Pharmaceutical, Chennai) 5 mg/goat intramuscularly. Goats of group-2 received Pregnant Mare Serum Gonadotrophin (PMSG), (Folligon, Intervet, Boxmeer, Holland) 750 IU/goat intramuscular on day 10 th of appearance of estrus to promote follicular development. Prostaglandin $F_{2\alpha}$ (dinofertin) 5 mg/goat IM was given on the next day of the PMSG treatment to ensure luteolysis and human chorionic gonadotrophin (hCG) (Chorulon, Intervet, Boxmeer, Holland) 500 IU/goat on the day of return of estrus following the above treatment to induce multiple ovulation and luteal development (Wani and Golderman, 1987). The superovulated animals of group-2 were mated twice at an interval of 12 hours during estrus with a fertile buck available. Laparotomy was performed in the animals of both

groups on third day following the appearance of estrus as per the method of Dziuk (1971). Number of corpora lutea and follicles present in each ovary were counted. For collection of embryos modified Dulbecco's phosphate buffered saline (DPBS) was used. The total number of embryos and ova collected from each goat were recorded. Embryos were then aspirated into a 1.5-mm diameter polythene straw and kept at 37℃ until their transfer to recipients. Embryo transfer was carried out in recipients in group-2 with a synchronous stage of estrus comparable to the donor whose luteal stage matched with age of embryos as set out by Agrawal et al. (1982) with slight modifications. The embryos were loaded in 0.25-ml French mini straw and were inserted carefully through the fimbriated end of fallopian tube. Embryos were placed in the upper two third portion of fallopian tubes lumen after giving air pressure in the straw with the help of syringe. One to two embryos were placed in ipsilateral fallopian tube having corpus luteum.

In Phase-II, seventeen adult, non-pregnant (between the age of 1.5 to 3.5 years of age) regular cyclic Black Bengal female goats and crosses were selected. Group-A (n=9) goats served as recipients and group-B (n=8) animals served as donor and were subjected to the superovulatory treatment as discussed in phase-I. Donors were mated and embryos were collected and evaluated as discussed in Phase-I.

1. Cryopreservation of Embryos

Embryos collected from the animals of group-B animals were separated according to their developmental stage. Approximately half of the collected embryos were preserved for 45 days and another half were preserved for 10 days in liquid nitrogen. In this phase the embryos were collected. These embryos were loaded in 0.25-ml French mini straws and frozen using Bio-cool freezer (FTS - 16, model No. - T.P. C. 44). Two step addition of cryoprotectant (5% and 10% Glycerol) was used and three-step dilution of cryoprotectant with 1M sucrose was used. A starting temperature of cooling was 20℃. Freezing rate was −1.5℃/min. Seeding was done at −6℃. After that cooling was done at the rate of 0.3℃ /min. up to −30℃ and then embryos were plunged into liquid nitrogen at −196℃. Thawing was done after placing the straw in a water bath having temperature of 37℃ for 55 to 65 seconds and cryoprotectant was removed using 4-step process. The morphology of the embryos was evaluated. Embryos were kept at 37 ℃ in an incubator until transferred. Frozen thawed embryos were transferred into the fallopian tube of the recipient of group-A as discussed in Phase-I. One or two embryos were transferred to each recipient whose luteal stage coincided with the age of embryo (i.e. third day after standing heat). Percentage of success was recorded on the basis of pregnancy established or kidding in the recipient. Comparison was made between percentage success and between fresh and frozen embryo transfer. Statistical analysis was done as per Snedecor and Cochran (1989).

RESULTS

1. Phase-I

The purpose of the experiment was to study the superovulatory responses in Black Bengal goats on the basis of number of corpora lutea and follicles (different sizes) on the ovarian surface. Also to determine the fertility and kidding behavior of Black Bengal goats after embryo transfer.

1) Superovulatory Response

During the process of embryo collection in goats ovulatory response were compared between the donors and the recipients. Statistical analysis showed significant ($P<0.01$) effects of treatment but

non-significant effects due to position of ovary (i.e. left and right ovary) on average number of follicles. Significantly more average number of follicles per ovary per goat were 4.16±0.17 in the recipients compared with 8.27±0.37 ($P<0.01$) in the donor group. However, non-significant difference was observed regarding average number of follicles present on left and right ovary 5.57± 0.37 vs 6.07 ±0.41($P>0.01$). One way analysis of variance was also done to see the significant difference in the number of follicles due to different sizes (i. e. small, medium and large). Average number of corpora lutea in donor group (2.93±0.86) was significantly ($P<0.01$) higher than in the recipient group (0.74± 0.04).

2) Embryo Collection

Embryos were collected from the donor group after doing mid ventral laparotomy. Out of 107 corpora lutea counted in 18 goats, 65 ova were collected, 51 were fertilized ova (embryos) and 14 was unfertilized (oocytes). Percentage of ova recovery was 60.74% and fertilization percentage was 78.46 (Table 1). Out of 51 embryos 13 were 2-cell stage, 9 were 4-cell stage, 22 were 8-cell stage and 7 were 16-cell stage (Table 2). Average number of ova collected per goat were 3.61±0.51 (65 out of 18 goats).

3) Embryo Transfer

Only 16 embryos out of 51 embryos were

Table 1. Superovulatory response and kidding percentage due to fresh embryo transfer in goats in Phase-I

Parameters	Response/Phase I
Total no. of goats superovulated	18
Total no. corpora lutea/ovulation point	107
Total no. of ova collected	65
Percentage of ova recovery (%)	60.74 (65/107)
Total no. unfertilized i.e. oocyte collected	14
Total no. of fertilized ova i.e. embryos collected	51
Fertilization %	78.46 (51/ 65)
Total no. of embryo transferred	16
Total no. of recipients	10
Total no. of goats kidded	6
Total no. of kid born	10
Kidding %	60
Embryo transfer success % (embryo survival)	62.50 (10/16)

Table 2. Effect of cell stage on success of embryo transfer

Cell stage	Number of embryos collected	Number of embryos transferred	Number of kids born	Success rate (Percentage)
2	13	4	3	75
4	9	3	2	66
8	22	5	4	80
16	7	4	1	25
Total	51	16	10	62.5

transferred due to the non-availability of suitable recipients at the time of embryo collection. A total number of 10 kids were born (4 male and 6 female) when 16 embryos were transferred in 10 goats with a success of 62.5%. Four 2-cell stage, three 4-cell stage, five 8-cell stage and four 16-cell stage embryos were transferred and 3,2,4 and 1 kid were born, respectively, with a success rate of 75%, 66%, 80% and 25%, respectively (Table 2).

2. Phase-II

The main purpose of the experiment was to study the viability of caprine embryos after cryopreservation. Kidding and fertility behavior in 9 recipient goats was studied after of transferring seventeen frozen thawed embryos. Results of different parameters are given below-

1) Embryo Collection

Thirty-five ova were collected against 48 corpora lutea present on the ovarian surface of 8 goats. Recovery was 72.91% (35/48). Out of the 35 ova, 8 were unfertilized (oocytes) and 27 were fertilized (embryos). Fertility percentage was 77.14 (Table 3). Among 27 embryos, 7 were 2-cell stage, 5 were 4-cell stage, 14 were 8-cell stage and 1 was 16-cell stage. All embryos were morphologically normal (Table 4).

2) Embryo Cryopreservation

Twenty-seven frozen embryos of different cell stage (2 to 16) were preserved in liquid nitrogen at −196℃ for 10 to 45 days. Out of 27 embryos preserved, 18 embryos were recovered after successful thawing (33.33 % loss). Four 2-cell stages, five 4-cell stages and nine 8-cell stages, embryos were recovered after thawing out of seven 2-cell stage, five 4-cell stage, fourteen 8-cell stage and one 16-cell stage embryos frozen(Table 4). One 8-cell stage embryo was transferred after 45 days of preservation. Two 2-cell stages and two 4-cell stage embryos were transferred after 25 days of preservation. Two 8-cell and two 4-cell stage embryos were transferred after 10 days of pres-

Table 3. Superovulatory response and kidding % due to frozen embryo transfer in goats in Phase-II

Parameters	Response/Phase-II
Total no. of goats superovulated	8
Total no. of corpora lutea/ ovulation point	48
Total no. of ova collected	35
Percentage of ova recovery	72.91 (35/48)
Total no. of unfertilized ova i. e. oocyte collected	8
Total no. of fertilized ova i.e. embryo collected	27
Fertilization %	77.14 (27/35)
Total no. of embryo frozen	27
Total no. of embryo recovered after freezing and thawing	18
Total no. of embryo losses during freezing and thawing	9
Percentage of loss during freezing and thawing	33.33 (9/27)
Total no. of embryo transfer	17
i. Total no. of recipient	9
ii. Total no. recipient kidded	6
Total no. of kids born/ pregnancy established	7
i. Kidding %	66.66
Percentage of success after embryos transfer	41.17

Table 4. Effect of cell stage on success of embryo transfer after freezing

Cell stage	Number embryos frozen	Number embryos recovered after freezing & thawing	Number embryos transferred	Number kids born or pregnancy established	Success rate (%)
2	7	4	4	1	25
4	5	5	5	2	50
8	14	9	8	4	50
16	1	0	0	0	0
Total	27	18	17	7	41.17%

ervation. Two 2-cell, five 8-cell and one 4-cell stage embryos were transferred after 17 days of preservation.

3) Frozen Embryos Transferred

Only 17 embryos out of 18 embryos recovered after freezing and thawing were transferred to 9 suitable recipients. 16 embryos were transferred paired and 1 was transferred single in each recipient. 7 kids were born out of 17 embryos transferred (3 aborted during pregnancy period). Percentage of success after embryos transferred was 41.17%, on the basis of kidding result or pregnancy established. Four 2-cell, five 4-cell and eight 8-cell stage frozen and thawed embryos were transferred and 1, 2 and 4 kids were born, respectively, with a success rate of 25%, 50% and 50% (Table 4).

Comparison was also made between the percentage of success of fresh and frozen embryos transferred in the term of kid born/ pregnancy established (Table 5).

Table 5. Comparision of superovulatory response and success between fresh and frozen embryo transfer

	Response	
	Phase -I	Phase -II
Total no. of goats superovulated	18	8
Total no. of corpora lutea/ ovulation point	107	48
Total no. of ova collected	65	35
Percentage of ova recovery	60.74(65/107)	72.91(35/48)
Total no. of unfertilized ova i.e. oocyte collected	14	8
Total no. of fertilized i.e. embryo collected	51	27
Fertilization %	78.46(51/65)	77.14 (27/35)
Total no. of embryo frozen	-	27
Total no. of embryos recovered after freezing and thawing	-	18
Total no. of embryo losses during freezing and thawing	-	9
Percentage of loss during freezing and thawing	-	33.33 (9/27)
Total no. of embryos transferred	16	17
Total no. of recipients	10	9
Total no. of kids born / pregnancy established	10	7
Percentage of success after embryos transferred	62.5	41.17

DISCUSSION

1. Superovulatory Response

In the present studies the average corpora lutea were 6 in the donor group as against 1.5 in the recipient group. Black Bengal goats being prolific usually have two ovulation and the ovulation number varies between 1 to 3 with an average ovulation rate of 1.5 as revealed in the recipient group. Superovulatory treatment gave an improvement of about 4.5 ovulation per animal. Ahmed and Maurya (1981) who recorded a mean ovulation rate 12.54 (9~15) in 24 goats. Tervit et al. (1985) who obtained overall average ovulation rate of 9.1 using PMSG. The results for Angora goats (5.3) were lower than simultaneously treated Saanen goats (29.3). Jain and Madan (1986) recorded 5 to 8 follicles in six goats (Alpine X Beetal and Saanen X Beetal). Pande (1988) recorded ovulation ranged from 7.00±0.57 to 12.00±0.81. Khumbhakar (1991) reported ovarian response of Black Bengal goats after the administration of PMSG on day − 12 followed by hCG at ensuing estrus. He recorded 9.5±2.17 ovulation.

2. Embryo Collection

The average ova recovery per donor was 3.60 and embryo recovery per donor was 2.8. Moore and Eppleston (1979) reported an average of embryo recovery of 5.78 per donor and the fertilization was 79% in equine FSH treated does and 46% in PMSG treated does. Vandnere and Mani (1986) reported a success rate of 60 to 75% in embryo collection. Khumbhakar (1991) reported 69.75% recovery rate out of which 75% were fertilized. Taneja et al. (1991) reported an average embryo recovery of 10.17 to 13.5 per donor and the fertilization was 95.06% to 98.36%. Khumbhakar and Prasad (2001) recorded a total of 32 (69.56) recoveries out of 46 ovulation, 8 and 24 were ova and embryos, respectively. The success of fertilization was 75% (24/32) 11 of 8-cell and 13 were of 4-cell.

3. Embryo Transfer

Although a total of 51 embryos were collected from 18 donors only 16 embryos varying between 2 cell to 16-cell stage were transferred to 10 recipients. Four embryos were transferred singles and 6 were transferred in pairs. A total 10 kids (4 males + 6 females) were produced. It was further revealed that only one kid was produced (25%) out of the 4 embryos transferred single, while 9 kids were produced (75%). Out of 12 embryos transferred in pairs (i.e. 6 pairs). Overall success rate was 62.5% (10 kids from 16 embryos). In the present studies the success rate (62.5%) was higher and hence not in agreement with Soma and Sugi (1971), who could get only 5 kids out of 16 embryos transferred giving a kidding percentage 31.25%. Moore (1974) transferred 275 embryos in 158 Angora goats but only 47 recipients could produce 68 kids.

Present findings are similar and hence in agreement with Moore and Eppleston (1976) who

Table 6. Ovarian responses, embryo transfer and kidding behavior in goats

Donor	Average No. of ovulation	Ova recovered		No. of embryos transferred	No. of kids born/ pregnancy established	Percentage of success
		Total	Fertilized			
Fresh 10	69	39	32	16	10	62.50
Frozen 8	60	35	27	17	7	41.17

recorded 59% success rate when majority of 8 cell to morula stage embryos were transferred to uterine horns. Armstrong et al. (1983) who recorded 50%, 69% and 67.6% success rate when 1～4 cell, 5～8 cell and 8 cell embryos were transferred to oviducts, respectively. Agrawal et al. (1982) reported embryo survivability of 60.0% and 33.33 %, when transferred to the fallopian tube as against transferred in the uterus, respectively. Pandiya and Rathor (1986), Selgrath et al. (1990) recorded 43.0 % and 42.0%, respectively. While Sarmah et al. (1992) transferred 29 embryos in 9 goats and 5 goats conceived. Khumbhakar and Prasad (2001) recorded 33.33% survival rate after transfer of 11 embryos in 6 recipients.

4. Cryopreservation and Transfer of Frozen Embryo

Mani and Vandnere (1988) reported 25% and 50% survival in DMSO vs glycerol. Rong and Guangya (1989) transferred 13 frozen thawed caprine embryos in 5 goats out of which 4 became pregnant and produced 7 kids. Li et al. (1990) reported 60% and 64% pregnancy rate for frozen embryos using DMSO and glycerol, respectively. Puls et al. (1992) compared one step vs three-step equilibration with glycerol using morulae and blastocyts of goats and recorded no difference in pregnancy rate and embryo survival for morulae between one-step vs three step. Blastocysts were found to be more suitable for cryopreservation than morulae and one step equilibration was superior to three-step procedure.

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