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Effect of Fresh and Frozen Semen on *in vitro* Fertilization and Subsequent Development of Goat Embryos

Research Article

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ABSTRACT

This study was undertaken to compare the efficiency of fresh and frozen-thawed buck semen on *in vitro* fertilization (IVF) of goat oocytes. Cumulus oocytes complexes (COCs) were collected by aspiration of 2-6 mm diameter follicles, which were obtained from slaughterhouse. Upon grading, only normal quality COCs were maturated in TCM-199 for 48 hours. The percentage of COCs reached to the M-II stage was 61.41 ± 1.97%. The matured COCs were fertilized for 5 hours in Brackett and Oliphant media using fresh and frozen thawed semen separately. After fertilization the oocytes were cultured in TCM-199 for 48 hours to observe the cleavage rate. The maturation, fertilization and culture were performed in an incubator at 38.5 ˚C with 5% CO_2 in humidified air. After fertilization cleavage rates were observed to check the fitness of zygotes to be morula and blastocyst. It was observed that the rates of normal fertilization (2 PN formations) for fresh and frozen semen were 36.02 ± 2.79 and 34.73 ± 2.58%, respectively and the cleavage rates were 25.19 ± 2.5 and 21.01 ± 2.8%, respectively. No significant differences (P>0.05) was observed between fresh and frozen semen in the efficiency of *in vitro* fertilization and subsequent development of goat embryos. It can be concluded that, both fresh and frozen semen can be used for IVF and subsequent development of goat embryos.

KEY WORDS embryo, fresh semen, frozen semen, *in vitro* fertilization.

INTRODUCTION

Latest developments in gametes and embryo cellular biology, the field of molecular embryology of farm animals has been poorly explored due to the limited availability of suitable experimental materials at an acceptable cost. After a dramatic development of cellular biology over the last 10-15 years, a lot of research efforts have been moved towards the implementation of embryo technologies involving multiple ovulation and embryo transfer (MOET), *in vitro* production (IVP) of embryos, cloning and transgenesis to transfer a targeted number of embryos produced from animal having desired genetic make-up. In MOET, embryos are collected *in vivo* from super ovulated donors at the required developmental stage and transferred to a number of synchronized recipients. Goats are numerically and economically very important and promising animal genetic resources in developing country like Bangladesh and accounted for about 34.5 million heads (7.40% of the total population in Asia) (FAO, 2003). Goats significantly contribute to the GDP in Bangladesh through production of meat, milk and skin representing about 28.0, 23.0 and 28.0%, respectively (FAO, 2003). Genetic improvement of goat in Bangladesh could be made by planned artificial insemination (AI), multiple ovulation and embryo transfer (MOET) and *in vitro* production (IVP) of embryos. But

considering the poor ovulatory response in goat the application of MOET is difficult to adopt (Danilda, 2000). Nevertheless, it has not become widely used in goat due to its unpredictability (Baldassarre and Karatzas, 2004). Besides this, animal welfare concerns have more and more limited the use of animals for experimentation. For these reasons *in vitro* production (IVP) of goat embryos has received great attention and support in recent years (Holtz, 2005).

In vitro production of embryos refers to the use of laboratory system to all procedure occurred naturally for the production of embryos *in vivo.* This process usually includes retrieval of oocytes from the ovarian follicles of a female, *in vitro* maturation, *in vitro* fertilization of oocytes and *in vitro* culture of presumptive zygotes to the morula or blastocytes stage (Brackett *et al.* 1982). However, in Bangladesh, i*n vitro* techniques in goat is a recent concept but a great deal of work has been done regarding evaluation and grading of ovaries, collection of cumulus oocytes complexes (COCs) from slaughterhouse ovaries and grading of oocytes followed by IVM, IVF of the oocytes and IVC (Ferdous, 2006; Islam *et al.* 2007; Mondal *et al.* 2008; Hoque, 2009).

In most cases IVF of oocytes is done by the use of fresh semen because of its high motility rate, but frozen semen can be a useful, if proper processing could be done and can be used after the death of valuable male. Frozen semen along with *in vitro* production of embryos can be used to minimize inbreeding and the subsequent risk of expression of unfavorable or even lethal genetic traits, as well as the loss of advantageous characteristics through unnatural selection. To popularize this technique one must have to compare the *in vitro* fertilizing capacity of buck spermatozoa from fresh and frozen-thawed semen. Keeping the aforesaid reality in mind the present research was undertaken with the objectives of oocyte recovery rate from the ovaries with or without corpus luteum and compare the effect of fresh and frozen semen on *in vitro* fertilization of goat oocytes and subsequent development of goat embryo.

MATERIALS AND METHODS

The experiment was conducted at the reproductive biotechnology laboratory, department of animal breeding and genetics, Bangladesh agricultural university, Mymensingh.

Collection and processing of ovaries

A total of 195 ovaries from goats of unknown reproductive history were collected from local slaughterhouse. The ovaries were kept in collection vial containing 0.9% physiological saline in a thermo flask at 25 ˚C to 30 ˚C and transported to the laboratory within 4 to 5 hours of slaughter. The ovaries were rinsed thoroughly by physiological saline solution at 25 ˚C. In the laboratory, each ovary was trimmed to remove the surrounding tissues and overlying bursa.

Evaluation of ovary

After collection and trimming ovaries were evaluated on the basis of the corpus luteum (CL) present and absent.

Collection of oocyte and evaluation of COCs

After collection, trimming and washing of ovaries, COCs were collected by aspiration from 2-6 mm diameter follicles using 10 mL syringe with 18 gauge needle. The COCs were then classified into 4 grades as described by Khandoker *et al.* (2001). The grades: grade A) oocytes completely surrounded by cumulus cells; grade B) oocytes partially surrounded by cumulus cells; grade C) oocytes not surrounded by cumulus cells and grade D) degeneration observed both in oocytes and cumulus cells. The grade A and B were considered as normal and grade C and D as abnormal COCs.

In vitro maturation (IVM) of COCs

Normal quality COCs (A and B grade) were washed 2-3 times separately in D-PBS and finally into the maturation media (TCM-199) and placed into the droplets containing TCM-199. The droplets were then kept in a CO_2 incubator at 38.5 ˚C with 5% carbon dioxide in humidified air for 48 hours.

In vitro fertilization (IVF)

After 48 hours of maturation, the half of the matured COCs was proceed to fertilization with fresh semen and remaining half of the matured COCs was proceed to fertilization with frozen semen in Brackett and Oliphant (BO) medium in CO_2 incubator at 38.5 ˚C with 5% carbon dioxide in humidified air for 6 hours. Then the pronuclei developments were observed separately for fresh and frozen semen.

In vitro culture (IVC) and observation

After 5 hours incubation, the fertilized ova were taken from the semen drops with cumulus cells by using the appropriate micropipette. Then, they were washed three times in pre-incubated medium (TCM-199) and were transferred to other culture drop (100 µL) of TCM-199. The dish was then kept in the CO_2 incubator at 38.5 ˚C with 5% CO_2 in air. The development rates were checked after 48 hours of culture. The cleavage rates were recorded after 48 hours of culture.

Statistical analysis

The data generated from this study were entered in microsoft excel worksheet, organized and processed for analysis. Analysis was performed with the help of statistical analysis system (SAS, 1998).

RESULTS AND DISCUSSION

Ovarian types and collected COCs per ovary

Goat ovaries collected from local slaughterhouse were classified into two types, the ovaries without and with CL. The result of the number of follicles aspirated and collected COCs from two types of ovaries are summarized in Table 1. Among 195 ovaries, 150 were found as without CL and 45 as with CL. The doe destined slaughtering were usually less reproductive performer and most of them might be non-cyclic. So, there had been the possibility to get more non-cyclic ovaries from the slaughterhouse during random sampling. Hoque (2009) had also drawn a similar observation when they collected 264 ovaries of which 204 were found as without CL and 60 as with CL.

The totals of 936 follicles were observed in both types of ovary and among them 780 were obtained from ovaries without CL and 173 from with CL. The totals of 571 follicles were aspirated in both types of ovary and among them 456 were obtained from without CL and 117 from with CL ovaries. The significantly higher (P<0.05) number of follicles were aspirated per ovary in without CL than in with CL with the mean of 3.04 and 2.59 follicles per ovary, respectively. It is well established that all female mammals are born with a large store of follicles which rapidly declines as puberty approaches but whether this early losses represent a mechanism of physiological wastage is not definitely known.

Follicle growth initiated is one of the most important and least understood aspects of ovarian biology and represents a major challenge for experimental study. Changes in the local microenvironment such as the pH and hormonal concentration probably occur as the follicles evolve into the primary stage but these are probably effects rather than causes (Webb *et al*. 1999). Growth initiated of follicles has variously been attributed t: hormonal triggers (gonadotropins), ochastic process (fluctuation in the internal signal follicle) and external inhibitory control growing follicles (Webb *et al*. 1999). The balance between the gonadotropins (FSH and LH) and steroid (estrogen and progesterone) might be the important criteria in this process. The highest number of follicles that are found in ovaries without CL in the present study might reflect the optimum level of gonadotropins and steroid.

In the ovaries without CL, the negative effect of progesterone on anterior was not functional in this types of ovaries. As the ovaries are collected from slaughterhouse, it was impossible to confirm the cyclic state of the ovaries. So, there might have some discrepancies in the present result. On the other hand, when compared the collected COCs per ovary between the two types higher (P<0.01) number was found in ovaries without CL than with CL, with the mean of 2.04 and 1.50 follicles per ovary, respectively. The presence of CL in cyclic female's ovary produces a higher level of progesterone hormone that signals a negative response to anterior pituitary gland for the restriction of gonadotropin secretion and ultimately follicular degeneration occurs (Webb *et al*. 1999). But due to the absence of CL in non-cyclic female, the negative effect of progesterone might not be functional and estrogen-progesterone remains in balanced level which allows follicular growth and oocytes maturation. The highest number of COCs in CL absent group of ovaries than that of CL present group as found in this study explains the role of hormonal balance on goat folliculogenesis. The findings of CL-present group of ovaries explain the role of progesterone on goat follicular degeneration and further strengthening the previous statement.

Hafez (1993) reported that progesterone secreted by the luteal cells of the CL inhibits estrus and gives the negative feedback on the anterior pituitary to secrete FSH. As a result, the growing follicles regressed and became antretic. The effect of progesterone on follicular growth could not be investigated in the present study. But it can be assumed that the highest number of normal grade in ovaries without CL and the lowest number of normal grade in ovaries with CL might arise from the activity of CL. The results of the number COCs of different grades for different ovaries are present in Table 1. The significantly highest (P<0.01) number of normal COCs was obtained in CL-absent group than in CL-present, with a mean of 1.34 and 0.75 COCs per ovary, respectively. And the reverse trend was found in abnormal group with the mean of 0.69 and 0.72 follicles per ovary, respectively.

The negative effect of progesterone might not be effectively functional in this group. So, the highest number of normal COCs in this category than that of CL functional group may explain the role of hormonal balance.

Table 1 Types of the ovary, number of cumulus-oocyte-complexes (COCs) and types of COCs harvested

Types of ovary	Total no. of ovaries	Total no. of follicle per ovary (Mean±SE)	Number of follicle aspirated per ovary (Mean±SE)	Total no. of COC_s per ovary (Mean±SE)	No. of normal COC_s per ovary (Mean±SE)	No. of abnormal COC_s per ovary (Mean±SE)
without CL	150	5.20^{a}±0.15 (780)	3.04^{a}±0.10 (456)	2.04^{a}±0.07 (306)	1.34^{a}±0.06 (201)	0.69^{a}±0.51 (104)
with CL	45	3.84^{b}±0.51 (173)	2.59^{b}±1.16 (117)	1.5^{b}±0.98 (68)	0.75^{b}±0.63 (34)	0.72^{b}±0.63 (33)
Total	195	4.8±0.15 (936)	2.93±1.10 (571)	1.9±0.57 (371)	1.2±0.06 (234)	0.70±0.55 (137)

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
Figure in the parenthesis indicates the total number.
CL: corpus luteum.

In vitro maturation (IVM) of COCs

In this study, the collected COCs were matured in TCM-199 and the *in vitro* maturation of goat oocytes was checked. The maturation of COCs was confirmed by checking the nuclear maturation. The maturation rate is presented in Table 2.

Table 2 *In vitro* nuclear maturation of COCs cultured in TCM-199 media

Total no. of COCs cultured	Maturation rate (%) (Mean±SE)			
	M-II	M-I	GVBD	GV
76	61.41±2.32 (46)	17.63±2.8 (14)	17.53±1.1 (13)	3.98±2.15 (3)

M–II: metaphase–II stage; M-I: metaphase–I stage; GVBD: stage of germinal vesicle breakdown; GV: germinal vesicle.

The percentage of COCs matured up to M-II stage (complete maturation) was 61.41 ± 2.32 and to M-I stage was 17.63 ± 2.83, GVBD was 17.53 ± 1.19 and GV was 3.98 ± 2.15. The maturation rate was comparable to the results of Wang *et al.* (2007) who obtained 48-63% maturation rate in Boer goat. The maturation rate was also comparable to the results of Hoque (2009) who obtained 58.57% maturation rate in Black Bengal goat. The findings of the present study were comparable with those of cattle (Carolon *et al.* 1992), sheep (Wani *et al.* 2000) and also with goats (Pawshe *et al.* 1994). It showed that the maturation rate does not depend on the collection techniques. Cumulus cells expansion level might be considered as the tool of oocytes maturation. Cumulus cells expansion during *in vitro* oocytes maturation was beneficial for completion of the maturation process. The role of the cumulus cells might revolve around their ability to produce pyruvate to provide energy substrate during this period (Ball *et al.* 1984). Moreover, the culture condition of the present experiment might be optimum for *in vitro* maturation (IVM) of goat oocytes in the context of Bangladesh.

In vitro fertilization (IVF) of goat oocytes using fresh and frozen semen

After maturation the analysis of variance on the data of the fertilization rate of the COCs was made to test the variation between the types of semen. The mature COCs were incubated in Brackett and Oliphant (BO) medium with fresh and frozen buck semen for 5 hours and the fertilization rate was assessed based on pronuclei formation. The results mentioned are in Table 3. After maturation of COCs in TCM-199, half of the total COCs were fertilized with fresh and the rest with frozen buck semen. In the case of the fresh semen, the sperm motility was 76.66 ± 0.33% and the percentages of COCs reached to fertilization stage were 36.01 ± 2.79% and 1 PN, > 2 PN and no PN were 12.89 ± 4.41%, 8.51 ± 1.72% and 42.56 ± 5.32%. On the other hand, in case of frozen semen, the sperm motility was 72.33 ± 1.4% and the percentages of COCs reached to fertilization stage were 34.72 ± 2.58% and 1 PN > 2 PN and no PN were 10.77 ± 2.77%, 10.95 ± 0.51% and 43.54 ± 1.19%, respectively. It was observed that higher percentage of normal fertilization (formation of 2 pronuclei) was observed in fresh semen (36.01%) than in frozen semen (34.72%). But the result indicates that there was no significant difference (P>0.05) among fresh and frozen semen. Most reports on goat IVF are differed from ours in that only freshly ejaculated, not frozen-thawed semen was used. *In vitro* fertilization rate recorded in the present experiment was found less in number as reported by Fuki and Ono (1989). They obtained 58.2% fertilization rate when culturing bovine oocytes for 24 h at 39 ˚C under 5% CO_2 in TCM-199; while present results ranged between 27.78 to 38.23%. De *et al.* (1992) showed that 57.1% of *in vitro* matured goat oocytes were normally fertilized. Rho *et al.* (2001) presented different protocols for preparation of frozen-thawed caprine sperm for IVF. However, no papers have been found where results of IVF with fresh and frozen semen of the buck were compared. Ge Li Jun *et al.* (2009) conducted an *in vitro* fertilization (IVF) experiment with fresh and frozen boar semen to study the effect of semen quality on *in vitro* production.

The IVF of frozen semen with rather high vigor (P>0.05) was not significantly different from that of fresh semen (54.3% to 51.1%) in boar which further supports the above statement. The cumulus cells and their associated matrix were present in this experiment and they may influence the fertilization rate in a non-specific manner by just increasing the contact area between spermatozoa and the oocytes and at the same time keeping a physical relation between spermatozoa in the process of capacitation (Fuki and Ono, 1989).

The physiochemical properties of the matrix would retain non-capacitated spermatozoa and would facilitate the forward displacement of those hyper activated spermatozoa. Finally, the presence of the cumulus cells would trap more spermatozoa and would guide the displacement of hyper activated sperm to the oocyte surface. On the basis of our results, it may be concluded that differences in IVF efficiency capacitation is a crucial process that mammalian sperm must undergo in order to achieve fertilizing ability.

The fertilization rates of frozen spermatozoa may vary due to a number of reasons such as the site of semen collection, age of the animals and their use in natural or artificial breeding, methods of semen preparation, quality of semen and proper timing of insemination and also developmental competence of the oocytes. But the result indicates that there was no significant difference (P>0.05) among fresh and frozen semen in the efficiency of fertilization *in vitro* of goat oocytes, so this variation might be minimized by proper attention.

Table 3 Effect of fresh and frozen semen on i*n vitro* fertilization (IVF) of goat COCs based on pronuclei formation

Types of semen	Sperm motility (%)	Total No of COCs fertilized	Fertilization rate (%) based on pronuclei (PN) (Mean±SE)				Level of significance (P>0.05)
			2 PN	1 PN	> 2 PN	No PN	
Fresh	76.66±0.33	62	36.01±2.79 (22)	12.89±4.41 (8)	8.51±1.72 (5)	42.56±5.32 (27)	NS
Frozen	72.33±1.4	63	34.72±2.58 (21)	10.77±2.77 (7)	10.95±0.51 (7)	43.54±1.19 (28)	NS

Oocyte with 2 PN: normal fertilization; Oocyte with 1 PN: no fertilization and Oocyte with > 2 PN: polyspermia.
NS: not significant.

Polyspermy was the main abnormality detected in IVF with frozen semen and it affected almost 20% of the in-seminated oocytes (De *et al.* 1992).

In vitro embryo development

After 48 hours of culture the microscopic observations of rate of cleavage (%) are observed and the cleavage rates of zygote using two different types of semen have been shown in Table 4.

Table 4 Effect of fresh and frozen semen on *in vitro* culture (IVC) of goat COCs after 48 hours of culture

Type of semen	Total zygote	Cleavage rate (%)	Level of significance (P>0.05)
Fresh	40	26.19±4.9 (10)	NS
Frozen	40	21.42±5.9 (9)	NS

NS: not significant.

In the present study, the cleavage rate was 26.19% in case of fresh and 21.42% for frozen semen. Comparatively higher number of cleavage rate was obtained from fresh semen (26.19%) than that of frozen semen (21.42%). There was no significant difference (P>0.05) between fresh and frozen semen in the efficiency of cleavage rate of zygote on *in vitro* goat production. The similarity in cleavage rates may be attributable to survivability of frozen sperm with fresh sperm; the functional and structural parameters of sperm are impaired after the freeze-thaw process. There is now a growing amount of evidence to suggest that while male variability can influence the developmental competence of embryos, the effect is not as drastic as in the case of the oocyte quality. Further evidence confirming this statement comes from the results of the present experiment.

There are a number of factors, which play a role in successful *in vitro* embryo production, such as source of oocytes, source and preparation of semen, culture media and culture conditions, which seem to differ for different species. However, availability of viable and functional spermatozoa during the storage period is a prerequisite for AI and IVF, thus necessitating the application of optimal diluents and proper storage conditions to maintain the quality and fertilizing ability of the spermatozoa for longer periods. To our knowledge, there are no comparable studies on frozen spermatozoa with fresh in this species. Silva *et al.* (2002) carried out a similar study in mares and they concluded that embryo development rates were different (P<0.05) among groups (fresh, 9/11, 82%; cooled, 4/16, 25%; frozen, 1/12, 8%). Some studies reported that the IVF of frozen semen with rather high vigor was not significantly (P>0.05) different from that of fresh semen (48.2% to 45.4%) in embryos production.

The main reasons are that goats are less valuable asset than other species, and that the technical difficulties involved in collecting and transferring embryos are considerably larger. Nevertheless, efforts were being made to establish embryo transfer programs in goats not only for conserving and propagating valuable genetic stock, but also as a basic technology for a range of *in vitro* manipulations including the generation of transgenic animals. As IVF has become more extensively used in domestic animals, the need for the cryopreservation of semen has become apparent and semen cryopreservation can be seen as an integral part of assisted reproductive technologies. This experiment was not preceded up to blastocyst stage. But in our biotechnology laboratory, the research on this topic is still being continued in order to get the blastocyst.

CONCLUSION

Considering the effect of semen on *in vitro* fertilization and culture of goat oocytes, it can be concluded that both fresh and frozen semen can be used on *in vitro* fertilization of goat oocytes and subsequent development of goat embryos.

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