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Research Article

IN VITRO FERTILIZABILITY OF OOCYTES USING FRESH, FROZEN AND EPIDIDYMAL SPERMATOZOA FROM CROSSBRED BULLS IN THE TROPICS

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Abstract

The objective of the study was to examine the fertilizability of bovine follicular oocytes from abattoir ovaries using fresh, frozen and epididymal spermatozoa from crossbred bulls in the tropics. Oocytes were cultured in maturation media TCM-199 containing 25mM HEPES, 1mM glutamine L, 2.2mg/mL sodium bicarbonate, antibiotics, 22 µg/mL pyruvate, 1µg/mL estradiol-17β, 0.5µg/mL FSH and 0.06 IU hCG and supplemented with 20% heat inactivated estrus cow serum (serum collected in early estrum) at 39°C temperature, 5% CO2 tension with maximum humidity for 24 hours. Oocytes with maximum cumulus expansion were used for *in vitro* fertilization. Fresh, frozen and epididymal semen from crossbred bulls was used for in vitro fertilization as treatment 1(n =1690), treatment 2(n = 1620) and treatment 3(n = 1710) respectively. Control group of oocytes (1680) were treated in the same protocol along with each treatment group of oocytes in separate *in vitro* fertilization drop without sperm injection. The *in vitro* fertilization medium consisted of Fert-TALP medium supplemented with 1µM epinephrine, 10µM hypotaurine, 20µM pencillamine and 0.56µg/ml heparin. Culture conditions set for IVF were 39°C temperature, 5% CO2 tension with maximum humidity. Oocytes showing sperm penetration evidence like presence of enlarged sperm head, male pronuclei with its accompanying sperm tail in the cytoplasm, oocytes with two pronuclei and a clear second polar body but without a sperm tail were considered as fertilized. None of the oocytes in control group showed cleavage due to parthenogenetic activation. Significantly higher results of fertilization rate (p<0.05) were observed when oocytes inseminated with epididymal spermatozoa than fresh ejaculated semen followed by frozen semen.

Key words: cattle; epididymal spermatozoa; fresh semen; frozen semen; *in vitro* fertilizability; Oocyte

Introduction

In vitro reproduction biotechnology has wide range applications with decisive advantages. *In vivo* methods requiring cumbersome procedures with high costs to recover the oocytes/early embryos, the *in vitro* techniques use abattoir ovaries which are easily available, inexpensive can provide a large number of oocytes in a short span to support various researches in the field of *in vitro* reproductive biotechnologies. In combination with ultrasound guided ovum pick up from live cows, this technology can maximize the number of progenies from elite animals and thereby to support the breeding bull production and genetic improvement through large-scale artificial insemination. Use of sexed semen in conjunction with *in vitro* embryo production is a potentially efficient means of obtaining offspring of predetermined sex (Romo *et al.*, 2014). Progenies can be produced even from pre-pubertal and dead animals by IVF technique. Conservation of endangered species and rare livestock breeds are possible through *in vitro* fertilization techniques (Nicholls, 2012). *In vitro* production of embryos coupled with embryo transfer is a solution to the problem of low fertility of lactating dairy cows during hot periods of the year (Barusellia *et al.*, 2011). More over the *in vitro* technology has relevance to assessing bull fertility (Morado *et al.*, 2015).

The key to a successful *in vitro* fertilization is to promote sperm capacitation and to induce the acrosome reaction *in vitro* (Debby *et al.*, 2012). Mammalian spermatozoa undergo a series of physiological events in the female reproductive tract prior to acquiring the fertilization capability (Austin, 1951; Chang, 1951). There are differences in the capacitation ability of ejaculated and epididymal spermatozoa. While capacitation of bovine epididymal spermatozoa can be achieved in a simple salt solution (Ball *et al.*, 1983), ejaculated bovine spermatozoa do not undergo capacitation unless stimulated by capacitating agents (Parrish *et al.*, 1986). Decapacitation factors of seminal plasma (Chang, 1951; Chung *et al.*, 2011) may be responsible for these differences. However, freshly ejaculated and deep frozen bull spermatozoa do not differ in their capacitation ability *in vitro* (Essam *et al.,* 2015). The objective of the present study was to examine the fertilizability of bovine follicular oocytes retrieved from

abattoir ovaries using fresh, frozen and epididymal spermatozoa.

Materials and Methods

Oocyte Retrieval and Processing

Ovaries from crossbred cattle of Kerala were used for the study. Ovaries were dissected out from animals within 30-60 minutes of slaughter and transported to the laboratory within 30-60 minutes in freshly prepared Dulbecco's phosphate buffered saline fortified with 100 IU/ml Benzyl penicillin and 100µg/ml Streptomycin sulphate maintained at 36-38ºC. The ovaries were washed repeatedly in Dulbecco's buffered saline fortified with 100 IU/ml Benzyl penicillin and 100µg/ml Streptomycin sulphate maintained at 36-38ºC, after trimming off the extraneous tissue. The final washing was done with Dulbecco's phosphate buffered saline supplemented with 0.5% BSA and maintained at 36-38ºC.

Oocytes were retrieved from ovaries by applying aspiration method. Surface follicles measuring 2-8 mm in size were aspirated with a sterile 18 gauge disposable needle connected to a 10 ml disposable syringe .The retrieval process was carried out in COC handling media prepared with Dulbecco's phosphate buffered saline supplemented with 5% Day 0 estrus cow serum and 0.5% BSA and maintained at 37ºC. A total of 810 ovaries were used for oocyte retrieval in the study. After the isolation of oocytes by aspiration method, the medium containing oocytes were examined under the zoom stereomicroscope at 10x magnification for identification of oocytes. Identified oocytes were transferred into 35 mm petri dish containing COC handling media maintained at 37ºC. Oocytes were examined under 40x magnification of zoom stereomicroscope and grade I and gradeII categories were selected based on the number of layers of cumulus cells and ooplasm character (Grade I: More than 5 complete layers of cumulus cells and uniform granulation of ooplasm; Grade II: 3-5 complete layers of cumulus cells and uniform granulation of ooplasm). Grade I and Grade II oocytes were combined together to form a new class namely culture grade oocytes and used for the study. Grade I and Grade II oocytes were put separately in labeled maturation media and further processing for *in vitro* maturation and *in vitro* fertilization study was done separately.

In vitro Maturation

Medium used for maturation of oocytes was freshly prepared Hepes modified TCM-199 (25 mMHepes) enriched with 22µg/ml sodium pyruvate, 2.2mg/ml sodium bicarbonate and antibiotics penicillin G sodium and streptomycin 0.5mg/ml each. This medium was further supplemented with hormones, 1µg/ml estradiol-17β, 0.5µg/ml FSH, 0.06 IU hCG and 20% heat-inactivated estrus cow serum prior to employing it for IVM. COCs were washed twice in maturation medium without hormones and transferred in groups of 5-10 into 100µl drops of maturation medium under silicone oil and allowed to complete incubation for a period of 24 h without any disturbance in the culture conditions. Culture conditions set for this study were 39ºC temperature, 5% CO_2 tension with maximum humidity. After 24 h culture in maturation medium, all the oocytes in the culture drops were examined under zoom stereomicroscope for maturation changes such as expansion and mucification of cumulus cells. Oocytes after 24 h culture were classified based on the degree of cumulus cell expansion as per the classification of Hunter and Moore (1987) and oocytes with maximum degree of cumulus expansion (Grade 1) were selected for further *in vitro* fertilization studies.

In vitro Fertilization

At the end of *in vitro* maturation, the selected COCs were rinsed twice in Fert-TALP medium (Parrish *et al.*, 1988). The *in vitro* fertilization medium consisted of Fert-TALP medium supplemented with 1µM epinephrine, 10µM hypotaurine, 20µM pencillamine and 0.56µg/ml heparin (Khurana and Niemann, 2000).

Fresh ejaculated semen was obtained from healthy crossbred bulls and 50µl of fresh semen was layered over percoll density gradients and separated the motile spermatozoa. The frozen semen dose of crossbred bull was thawed in a 37ºC water bath. The semen sample was applied on the top of the percoll gradient and motile spermatozoa were separated. Sperms from cauda epididymis of crossbred bulls were collected by gentle slicing and squeezing of the cauda epididymis and motile spermatozoa were separated by percoll density gradient separation. Semen was layered on a percoll gradient prepared by adding 2 ml of 45% percoll on 2ml of 90% percoll in a 15 ml centrifuge tube and centrifuged the semen overlaid on percoll gradient for 20 minutes at 700 x g. The recovered sperm pellet was resuspended in sperm-TALP and again centrifuged at 300 x g for 10 minutes. After removal of the supernatant, spermatozoa were resuspended in IVF medium (Khurana and Niemann, 2000). Concentration of the sperms in IVF medium was determined with haemocytometer and adjusted the concentration at 2 x 10^6/ml using IVF medium. The final concentration in fertilization droplet was adjusted to 15,000 to 20,000 sperms per oocytes.

Each category of matured oocyte (Grade I and Grade II Oocytes) after washing was loaded gently into separate *in vitro* fertilization drops and allowed to complete incubation for a period of 24 h without any disturbance in the culture conditions. Depending upon the availability, 5-10 matured oocytes were allowed into each fertilization drop. Culture conditions set for IVF were 39ºC temperature, 5% CO_2 tension with maximum humidity (Khurana and Niemann, 2000).

Assessment of Fertilization

Oocytes after 24 hour culture in fertilization medium were denuded by repeated pipetting and evaluated for evidence of sperm penetration. Oocytes showing sperm penetration evidence like presence of enlarged sperm head, male pronuclei with its accompanying sperm tail in the cytoplasm, oocytes with two pronuclei and a clear second polar body but without a sperm tail were considered as fertilized. Oocytes with more than two pronuclei and decondensed sperm heads were evaluated as polyspermic. Oocytes were examined under bright field of inverted phase contrast microscope and then stained with aceto orcein for the assessment of fertilization. The whole mount fixation of oocytes were carried out as per Chian *et al.* (1995) and fixed in acetic alcohol (acetic acid: ethanol, 1:3 v/v) for 48-72 h at room temperature. The slide taken out from fixative was examined under 20 X magnification of zoom stereo microscope to locate the oocytes. Oocytes were stained with 1% orcein in 45% acetic acid. All the oocytes stained as above were examined under bright field of inverted phase contrast microscope at 200 X- 400 X magnifications for evidence of sperm penetration.

Experimental Design

Culture grade oocytes matured *in vitro* were subjected to *in vitro* fertilization using fresh, frozen and epididymal spermatozoa in IVF medium as per the protocol of Khurana and Niemann(2000) and assessed the fertilization rates in the three groups separately. The effect of sources of spermatozoa on the yield of fertilized oocytes was assessed. Data on fertilization rate was statistically analyzed with one way ANOVA. P-value of less than 0.05 was considered statistically significant.

Results

Effect of Sources of Spermatozoa on Fertilization Rate

The fertilization rate obtained with total culture grade oocytes when fresh ejaculated, frozen and epididymal semen used for *in vitro* fertilization was 37.86±0.47 (640/1690), 27.72±0.89 (450/1620) and 44.51±0.57 (760/1710) per cent respectively. There was significant difference observed between these three treatment groups (P<0.01). *In vitro* fertilization using epididymal spermatozoa showed significantly higher results when compared to IVF using fresh ejaculated semen, followed by IVF using frozen semen(P<0.01). The fertilization percentage obtained with grade I oocytes was 36.52±1.68, 28.65±0.76 and 46.53±1.32 per cent when fresh, frozen and epididymal spermatozoa was used for *in vitro* fertilization. IVF using epididymal spermatozoa yielded significantly higher fertilization rate and IVF using frozen semen yielded lowest among the group when grade I oocytes alone considered for IVF(P<0.01). Grade II oocytes yielded 45.00±5.63, 23.89±3.03 and 35.20±3.03 per cent when fresh, frozen and epididymal spermatozoa were used for IVF. There was significantly higher results observed with fresh semen than with frozen semen in the yield of fertilized oocytes (P<0.01). But grade II oocytes alone are considered, there was no significant difference observed between epididymal source of spermatozoa and fresh semen or with frozen semen (P>0.05).

Table 1: Effect of sources of spermatozoa on the fertilization percentage and yield of fertilized oocyte per ovary (Morphological grade wise)

Source of Spermatozoa	Morphological grade of oocytes					
	Grade I		Grade II		Total Culture grade oocytes	
	Percentage oocytes showed fertilization signs	Mean number of oocytes per ovary showed fertilization signs	Percentage oocytes showed fertilization signs	Mean number of oocytes per ovary showed fertilization signs	Percentage oocytes showed fertilization signs	Mean number of oocytes per ovary showed fertilization signs
Fresh	36.52±1.68[a]	1.87±0.06[a]	45.00±5.63[a]	0.51±0.07[a]	37.86±0.47[a]	2.38±0.05[a]
Frozen	28.65±0.76[b]	1.43±0.04[b]	23.89±3.03[b]	0.31±0.05[b]	27.72±0.89[b]	1.73±0.07[b]
Epididymal	46.53±1.32[c]	2.33±0.05[c]	35.20±3.03[a]	0.39±0.06[a]	44.51±0.57[c]	2.72±0.03[c]
Total	37.24±1.91	1.87±0.09	34.70±3.24	0.40±0.04	36.70±1.71	2.28±0.10

Values with different superscripts (a, b, c) in same column differ significantly (P<0.01)

Effect of Sources of Spermatozoa on the Yield of Fertilized Oocytes per Ovary

The mean number of oocytes fertilized per ovary was 2.38±0.05 (640/1690), 1.73±0.07 (450/1620) and 2.72±0.03 (760/1710), when fresh, frozen and epididymal spermatozoa were used for *in vitro* fertilization. There was significant difference observed between these three groups (P<0.01). Mean number of fertilized oocytes per ovary was significantly higher with epididymal spermatozoa than with fresh ejaculated semen. Similarly fresh ejaculated semen yielded significantly higher number of fertilized oocytes per ovary than with frozen semen. The mean number of fertilized oocytes per ovary obtained from grade I premature oocyte was 1.87±0.06, 1.43±0.04 and 2.33± 0.05 when fresh, frozen and epididymal spermatozoa was used for IVF. From grade II premature oocytes the yield of fertilized oocytes per ovary obtained was 0.51±0.07, 0.31±0.05 and 0.39±0.06 when fresh, frozen and epididymal spermatozoa was used for IVF. A significant difference was observed between the source of spermatozoa with grade I premature oocytes (P<0.01). Significantly higher results obtained with epididymal source than with fresh semen, followed by frozen semen. But no significant difference was observed between these three groups with grade II premature oocytes (P>0.05).

Discussion

To the best of my knowledge, this is the first study carried out to compare simultaneously the effects of fresh, frozen and epididymal spermatozoa on fertilizability of crossbred bovine follicular oocytes. Fertilization rate obtained in the present study using fresh, frozen and epididymal spermatozoa was comparable to the results of many scientists and even better than the results of some workers (Saha *et al.*, 2014). However, the overall results obtained was lower than the results of many workers, which might be due to multiple reasons like laboratory environment, breed and nutritional status of donor animals, level of expertise etc. Multiple factors like cytotoxic materials, water quality, gas composition, temperature, hydrogen ion concentration and osmolality of media and light exposure might be critical to the outcome of in vitro fertilization and embryo culture (Parrish, 2014).

There was significant difference observed between these three sources of spermatozoa on fertilization rate of bovine oocytes *in vitro*. Significantly higher results obtained with epididymal source than with fresh semen, followed by frozen semen when grade I and total culture grade oocytes were used for *in vitro* fertilization. There were significantly higher results observed with fresh semen than with frozen semen but there was no significant difference observed between fresh and epididymal sperm if grade II oocytes alone considered. The number of observations made with grade II oocytes was lower when compared to grade I and total culture grade oocytes and this might be the major reason for this variation. Fresh bull semen showed significantly higher fertilization rate when compared to frozen semen in the present study which is in agreement with the results of De Los Reyes *et al.* (2009) in canine. They reported that, fresh dog spermatozoa showed significantly higher penetration rate with both immature and in *vitro* matured canine oocytes in comparison to chilled or frozen sperm. Nagai *et al.* (1988) obtained significantly higher percentage of penetrated porcine oocytes *in vitro* with frozen epididymal spermatozoa when compared to the fertilization rate with frozen ejaculated spermatozoa which is also in agreement with the results of the present study in bovines.

Many studies were conducted on the effect of seminal plasma on fertilizability of bull spermatozoa *in vitro* (Katska *et al.*, 1996; Leahy and de Graaf, 2012). Katska *et al.* (1996) studied the effect of seminal plasma on the *in vitro* fertilizability of bull spermatozoa. In their study, the *in vitro* embryo production efficiency with frozen-thawed epididymal spermatozoa was higher for fertilization, cleavage rate and blastocysts yield, when compared to the results with fresh ejaculated semen. Moreover, the seminal plasma deprived ejaculated sperm showed significantly more oocytes fertilized, cleaved and developed to the blastocyst stage when compared to ejaculate sperm. They opined that, the absence of seminal plasma during capacitation has appeared favorable to embryo production efficiency *in vitro.* The reversible antifertility effect of seminal plasma has been suggested as being due to inhibition of various acrosomal enzymes. Reports on several species including cattle suggest that seminal plasma contains factors that may influence male fertility (Leahy and de Graaf, 2012). Generally, these factors are believed to inhibit sperm capacitation, the acrosome reaction, or acrosomal enzymes and ultimately interfere with fertilization (Leahy and de Graaf, 2012). On the other hand, in many studies the positive role of seminal plasma in capacitation of bull sperm especially increasing the number of binding sites for heparin has been emphasized (Parrish, 2014; Leahy and de Graaf, 2012).

Contrary to these findings, (Henault *et al.*, 1995) reported positive role of seminal plasma inducing the acrosome reaction in epididymal spermatozoa. The effect of seminal plasma on epididymal spermatozoa has been suggested as being due to heparin-binding proteins potentiating the ability of epididymal spermatozoa to be capacitated by heparin and to undergo acrosomal exocytosis at frequencies similar to those of epididymal sperm. Many scientists recommended the use of frozen cauda epididymal spermatozoa for mass embryo production of unknown genetic value (Katska *et al.*, 1996; El-Badry *et al.*, 2015).

Conclusion

In conclusion, the present data indicate that epididymal spermatozoa retrieved from abattoir bulls could be

efficiently used for *in vitro* fertilization of *in vitro* matured bovine oocytes equal to or even better than fresh ejaculated or frozen thawed semen. The *in vitro* fertilizing ability of cauda epididymal spermatozoa can be utilized for *in vitro* production of embryos from wild animals, endangered species and other animals in which collection of ejaculated semen is a difficult task.

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