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Short Paper

Study of TALP and TRIS citrate medium on caprine sperm capacitation and subsequent *in vitro* embryo production

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Summary

The aim of the present investigation was to compare Tyrode's albumin lactate pyruvate (TALP) medium and TRIS citrate medium for capacitation of caprine sperm. In experiment 1 capacitation was assessed by chlortetracycline assay and in experiment 2 with *in vitro* fertilization and embryo development. In experiment 2, cumulus oocyte complexes (COCs) recovered by slicing the caprine ovaries were matured in maturation medium for 27 h in humidified atmosphere at 38.5°C with 5% CO_2. After 27 h of culture a total of 2480 *in vitro* matured oocytes were selected and randomly divided into two groups. Group 1 (n=1124) matured oocytes were fertilized by the spermatozoa capacitated in TALP medium and in group 2 (n=1356) matured oocytes were fertilized by the spermatozoa capacitated in TRIS citrate medium. The results of experiment 1 indicated a comparatively more number of sperms with Chlortetracycline (CTC) Pattern B in TRIS citrate than TALP medium (55.32 ± 0.91% vs 47.96 ± 0.20%). In experiment 2, the cleavage rate and blastocyst production were higher following capacitation of spermatozoa in TRIS citrate than TALP medium. In conclusion, TRIS citrate can be used as an alternative and effective media for sperm capacitation to get higher cleavage rate and blastocyst production in goat.

Key words: Blastocyst, Capacitation, Caprine spermatozoa, TRIS citrate medium

Introduction

Numerous laboratories have investigated techniques for the production of viable caprine embryos. *In vitro* fertilization is a well-established technology with a variety of applications in basic and applied sciences (Bavister, 2002). Experimentation in *in vitro* fertilization (IVF) and *in vitro* culture (IVC) of caprine oocytes has led to marked improvements in the production of embryos *in vitro* and the birth of kids (Kharche *et al*., 2011). These advances have allowed for progress in the study of early fertilization events so that the efficiency of embryo production *in vitro* can be improved. One such process is sperm capacitation (Bedford, 1983), which involves modifications in membrane composition and fluidity, increases in intracellular cAMP, induction of tyrosine phosphorylation events, and the expression of hyperactivated motility (Aitken and Nixon, 2013). Sperm undergo these changes within the female reproductive tract or, *in vitro*, when incubated in a medium that supports capacitation.

To date, several media additives, of both synthetic and animal origin, have been successfully used to support *in vitro* capacitation of small ruminants spermatozoa. In caprine, the media employed for sperm capacitation and fertilization are modified Tyrode's albumin lactate pyruvate medium (TALP, Kharche *et al*., 2011), modified Defined Medium (mDM, Crozet *et al*., 1995), TCM-199 (Slavik and Fulka, 1992), Brackett and Oliphant (BO) and modified Synthetic Oviductal Fluid (mSOF) (Kharche *et al*., 2011).

To the best of our knowledge, despite advances in researches regarding additives that may support mammalian sperm capacitation *in vitro*, the capacitation of sperm in TRIS citrate has not been reported in any species. TRIS citrate diluent at pH = 6.8 is commonly used as diluting medium for semen cryopreservation and artificial insemination (Kharche *et al*., 2013; Gangwar *et al*., 2015). Therefore the present investigation was undertaken with the objectives being to determine the optimal medium between TALP (pH = 7.2-7.4) and TRIS citrate (pH = 6.8) for sperm capacitation and subsequent embryo development of *in vitro* fertilized caprine oocytes.

Materials and Methods

Experiment 1. Assessment of *in vitro* capacitation

Fresh semen was collected using the artificial vagina from six adult Sirohi bucks. The first and second seminal ejaculates were examined for volume, color, consistency

and gross sperm and progressive motility. Each collected semen sample was divided into two groups for capacitation of spermatozoa. Washing of semen samples was done as per Kharche *et al*. (2016). In group 1, washing was done with sperm TALP medium while for group 2 semen washing TRIS citrate medium was used. Sperms were then kept for capacitation in a CO_2 incubator in humidified atmosphere of 5% CO_2 at 38.5°C for 1 h. The capacitation status of fresh buck semen was assessed by chlortetracycline staining as per the method of Elkhawagah *et al*. (2013). Semen sample from each buck was assessed and at least 180-200 spermatozoa per slide were evaluated and classified into one of these three Chlortetracycline (CTC) staining patterns described by Fraser *et al*. (1995).

Experiment 2. *In vitro* embryo production

Collection of ovaries, recovery of oocytes and *in vitro* maturation (IVM) was done as per Kharche *et al*. (2016). Briefly, a total of 2666 cumulus oocyte complexes (COCs), recovered by slicing the 721 caprine ovaries, were matured in TCM199 supplemented with 10% fetal bovine serum (FBS) + 10% follicular fluid + FSH (5 µg/ml) + LH (10 µg/ml) + estradiol (1 µg/ml) + EGF (10 ng/ml) + BSA (3 mg/ml) for 27 h in humidified atmosphere at 38.5°C with 5% CO_2 in CO_2 incubator. After 27 h of IVM, matured oocytes were denuded with hyaluronidase enzyme (0.1%), after denuding oocytes with even granular cytoplasm, with polar body extrusion cone or with polar body were selected and washed 3-4 times in research vitro cleave (RVCL) media (COOK Australia) medium for removal of residual cumulus cells.

Washing of group 1 and group 2 semen samples were performed as described in experiment 1. Sperms were then kept for capacitation in a CO_2 incubator in humidified atmosphere of 5% CO_2 at 38.5°C for 1 h. *In vitro* fertilization was carried out as per the method described by Kharche *et al*. (2011) with slight modifications. Denuded oocytes from both groups were washed separately 8-10 times with Fert-TALP medium containing 10% FBS, 8 mg/ml fatty acid free BSA and 50 µg/ml heparin. Approximately 15-20 matured oocytes were transferred in each 50 µL drop of Fert-TALP medium and the drops were inseminated with 15-20 µL of the final diluted semen so as to obtain a sperm concentration of 1-2 × 10^6 sperm/ml.

After 18 h of sperm-oocytes co-incubation, oocytes were washed in embryo development medium to remove sperm cells adhered to zona pellucida. Oocytes of both groups were finally transferred into 50 µL drops of RVCL media supplemented with 1% BSA from Cook Medical, Australia for 48 h in humidified atmosphere of 5% CO_2 at 38.5°C in CO_2 incubator. After 48 h of post insemination, fertilized oocytes were evaluated under phase contrast microscope for cleavage rate. Cleaved oocytes were cultured in embryo development media for a further 8-10 days.

Statistical analysis

CTC patterns, cleavage rates and embryo development between the different treatment groups were compared using the chi square test. The level of significance was recorded at the 5% level of confidence (Snedecor and Cochran, 1989).

Results

Because of the simplicity and reliability of CTC method, this was used to differentiate between capacitated and non-capacitated sperms. However, in group 2 a comparatively more number of sperms showed CTC pattern B than group 1 (55.32 ± 0.91% vs 47.96 ± 0.20%). Furthermore, CTC pattern F and CTC pattern acrosome reacted (AR) in group 1 (13.78 ± 0.36 and 37.24 ± 0.26, respectively) was higher than that of group 2 (10.15 ± 0.44 and 32.99 ± 0.19, respectively).

The cleavage rate of *in-vitro* matured caprine oocytes in group 1 and group 2 was 35.93 ± 3.17% and 39.96 ± 3.04%, respectively. The blastocyst production was comparatively higher in group 2 (9.81 ± 1.99%) than that of group 1 (5.82 ± 1.68%), respectively (Figs. 1A-D). The cleavage rate and blastocyst production were comparatively higher following capacitation of spermatozoa in TRIS citrate as compared to TALP medium.

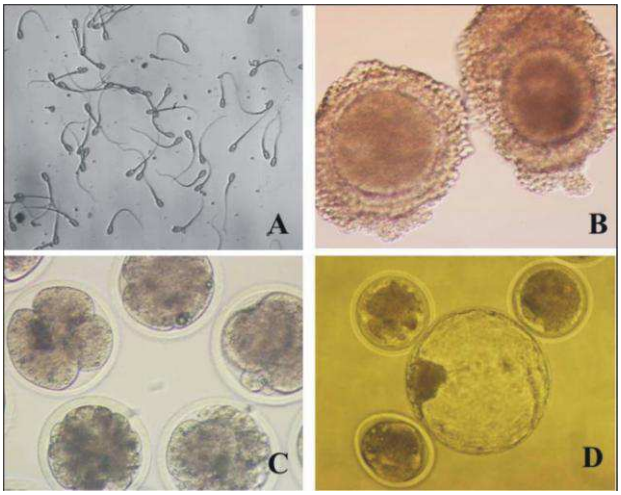

Fig. 1: Sperm, oocytes and embryos used in the experiments. **A**: Goat spermatozoa, **B**: Immature oocytes, **C**: Cleaved oocytes, and **D**: Blastocyst

Discussion

The improvement of caprine IVF technology is highly desirable in terms of the production of preimplantation stage embryos, both for biotechnological studies and for the embryo transfer industry (Wu *et al*., 2015). One contributing factor to this poor understanding is that many culture systems employ the same medium for capacitation as well as for fertilization (Kharche *et al*., 2011; Kharche *et al*., 2016).

To the best of our knowledge, despite advances in knowledge regarding additives that may support mammalian sperm capacitation *in vitro*, capacitation of sperm in TRIS citrate has not been reported in any

species. The main purpose of the diluent is to extend fresh semen to increase total volume of the semen ejaculated from a particular buck at a specific time and its extended use over a large number of does to get more kids (Kharche *et al*., 2007; Kharche *et al*., 2008a, b; Kharche *et al*., 2013). However, diluents used for buck semen should have similar properties to that of fresh semen in terms of hydrogen ion concentration (pH), tonicity and energy source. TRIS citrate diluent at pH 6.8 is commonly used as diluting medium for semen crypreservation and artficial insemination (AI) (Gangwar *et al*., 2015). Since, the needs and the metabolic activity of the male and female gametes are not the same (First and Parrish, 1987) thus it is necessary to use one medium for sperm capacitation and another for oocyte insemination. Because of the simplicity and reliability of CTC method, this was used to differentiate between capacitated and non-capacitated sperms in TALP and TRIS citrate medium. Our results demonstrated higher caprine capacited spermatozoa (pattern B) in both TALP (47.96 ± 0.20%) and TRIS citrate medium (55.32 ± 0.91%) as compared to that of ram spermatozoa (13.0 ± 1.0%) in SOF medium (Perez *et al*., 1996). Similarly, the result of incapacitated caprine spermatozoa (pattern F) was lower in both TALP and TRIS medium than that of ram spermatozoa (78.0 ± 2.0%) whereas AR caprine spermatozoa were higher in both TALP and TRIS citrate than that of ram spermatozoa in SOF medium (Perez *et al*., 1996). After CTC staining, three types of fluorescent patterns were visible under the microscope and were similar to those reported for bovine (Elkhawagah *et al*., 2013). A high percentage of spermatozoa exhibited CTC pattern B (capacitated spermatozoa) in both groups (TALP and TRIS citrate).

Our result of cleavage rate following spermatozoa capacitated in TALP is in agreement with that reported by Younis *et al*. (1991) in TALP (26.7%), mDM (33.3%), and mH-M199 (35.7%) and Kharche *et al*. (2011) in BO medium (32.84%), and mSOF medium (36.21%) in caprine oocytes. whereas it was comparatively higher following spermatozoa capacitated in TRIS citrate medium.

Capacitation has been shown to be correlated with changes in sperm intracellular ion concentrations, plasma membrane fluidity, metabolism, and motility (Yanagimachi, 1994). Although these changes have been known for many years to accompany the process of capacitation, the reason for embryo development underlying these events is poorly understood. Since in our study, the cleavage rate and blastocyst production of *in-vitro* matured caprine oocytes in group 2 was comparatively higher than group 1 (39.96 ± 3.04% vs 35.93 ± 3.17%, respectively) and (9.81 ± 1.99% and 5.82 ± 1.68%, respectively). It is well documented that the first two embryonic cell divisions are primarily controlled by the maternal genes (Braude *et al*., 1988) and paternal effects commence at the 4-cell stage. Further, the detrimental effects of sperm DNA damage are more prominent during the later stages of embryo development (Dar *et al*., 2013).

In conclusion, TRIS citrate can be used as an alternative and effective media for sperm capacitation to get higher cleavage rate and blastocyst production.

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Conflict of interest

On behalf of all co-authors, the corresponding author indicates that there is no conflict of interest involved in publishing this research paper.

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