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In vitro Production of Goat Embryos Using a Natural Substance as the Capacitation Agent

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ABSTRACT. *A sulfated glycoconjugate, named as S–ACT–1, extracted from a red marine algae (Gelidiella acerosa) found in the Southern coast of Sri Lanka, has been reported for improving motility of ejaculated human spermatozoa. This study was designed to assess the efficacy of S–ACT–1 on goat sperm capacitation and to establish complete in vitro maturation (IVM), fertilization (IVF) and culture (IVC) procedures for goat oocytes using S–ACT–1 as the sperm capacitation agent.*

It was found that S–ACT–1 can be used as a goat sperm capacitation agent at a concentration of 20 µg/ml. Slaughter house ovaries were used and average number of oocytes recovered from aspiration and slicing techniques were 1.9 and 5.0, respectively. Fertilized goat ova using S–ACT–1 as the sperm capacitation agent were cultured up to the blastocyst stage in synthetic oviductal fluid (SOF) culture medium. The survival rate at blastocyst stage was 25%. A significant interaction between capacitation agents and concentrations could be observed in 4–cell, 8–cell and morula stages of development.

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INTRODUCTION

A sulphated glycoconjugate extracted from a red marine algae, *Gelidiella acerosa* had reportedly induced hyper motility of immobile human spermatozoa (Premakumara, 1996). This compound has been named as "sperm activator 1" (S–ACT–1) by Premakumara (1996). However, there is no information to confirm that S–ACT–1 improves motility of spermatozoa of farm animals. Further, there is no information on naturally occurring sperm capacitation agents.

Therefore, a study was designed to assess the efficacy of S–ACT–1 on goat sperm capacitation and to establish complete *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) procedures for goat oocytes using S–ACT–1 as the sperm capacitation agent. This IVF procedure could be used to produce goat embryos of known genetic composition using slaughter house ovaries.

MATERIALS AND METHODS

The ovaries for the experiment were obtained from sexually mature female goats slaughtered at a local slaughter house. The age, breed or stage of reproductive cycle of the animals were not considered at the time of collection of ovaries. Complete reproductive tracts were removed within 30–45 min of slaughter and transported immediately to the laboratory in a thermos flask containing phosphate buffered saline (PBS) solution maintained at 20–25°C.

Recovery of immature oocytes from the ovaries was carried out under a bio–hazard cabinet, class II. Ovaries were separated from the reproductive tract, and washed once in warm (39°C) sterile PBS solution in a sterile glass beaker. Primary oocytes were aspirated from follicles (>2 mm diameter) with a 19 G needle attached to a 5 ml sterile plastic syringe containing Wash I Medium (Wahid, 1993) equilibrated at least 2 h at 39°C and 5% CO_2 under maximum humidity. After aspiration, the contents were slowly transferred to a sterile plastic culture dish (60 × 15 mm) and kept in an incubator maintained at 39°C until use. Then the ovaries were divided into two halves and put in a sterile plastic culture dish (60 × 15 mm) containing Wash I Medium. They were sliced with a sterile surgical blade to separate the oocytes from the ovarian tissues. Recovery of oocytes was performed under a stereo microscope maintained in a sterile environment at room temperature (29–30°C) using a pipette fixed to a sucking device and a mouth piece.

All oocytes in the culture dish were collected irrespective of the presence or absence of the cumulus oocyte complexes (COC) and washed twice in the Wash I Medium. Similarly all aspirated oocytes were also washed twice with the Wash I Medium. Oocytes were pooled and placed in 50 µl aliquots of Wash I Medium under light mineral oil and kept at 39° C until use.

Oocytes were washed 2–3 times in droplets of Maturation Medium (Wahid, 1993) equilibrated for 2 h at 39°C in humidified air with 5% CO_2. Approximately 10 oocytes were placed in 100 µl aliquots of Maturation Medium under light mineral oil in 35 × 10 mm sterile culture dishes. The oocytes were allowed to mature for 27 h at 39°C in humidified air with 5% CO_2.

Fresh semen was collected using an artificial vagina from two fertile Saanen bucks maintained at the Central Artificial Insemination Station (CAIS), Kundasale, Sri Lanka, and transported to the laboratory immediately. For each ejaculate, semen volume, gross motility, general motility and concentration were assessed on visual appraisal by the same personnel and graded as "good" or "poor". Samples, which were rated as "good", were used for the experiment. An aliquot of 600 µl of fresh semen was mixed in 8 ml PBS(+) containing 4 mg/ml BSA and centrifuged at 500 g for 5 minutes and the supernatant was siphoned to separate the sperm pellet. Approximately 0.25 ml of washed fresh goat semen was layered under 1.0 ml of Capacitation Medium (Gordon, 1994) in 16 × 100 mm plastic test tubes. Four such tubes were prepared and incubated for 1 h at 39°C in a water bath. Tubes were kept at an angle of 45°. After 1 h of incubation, 10 µl was taken from the top of the supernatant and checked for sperm number and motility. Approximately 0.5 ml of the supernatant was removed from the tubes, showing satisfactory sperm motility and pooled. The pooled sperm sample was centrifuged once at 200 g for 5–7 min and the sperm pellet was separated.

The sperm concentration was determined using a haemocytometer and the final dilution was made with fresh Capacitation Medium to obtain 10×10^6 spermatozoa/ml. Thereafter, different concentrations of capacitation agents (10, 20, 50, 100 and 200 µl heparin or S–ACT–1) were added and pre–incubated for 15 minutes at 39°C.

The Fertilization Medium used in this study was modified Tyrode's medium (Gordon, 1994). The medium was sterilized by filtration through a 0.22 µm Millipak–60 filter. The sperm motility stimulating mixture (Gordon, 1994); penicillamine, hypotaurine and epinephrine (PHE), were added to the

Fertilization Medium at a concentration of 40 µl/ml and equilibrated for at least 2 h before use. Fertilization droplets were prepared with 46 µl of Fertilization Medium under sterile light mineral oil (Sigma) and equilibrated for about 1 h at 39°C in humidified air with 5% CO_2. After 27 h maturation period, the oocytes were examined under an inverted microscope (25 × 10) for evidence of maturation such as expansion of cumulus cells and presence of first polar bodies in the denuded oocytes. All oocytes were removed from the maturation droplets and washed once in fresh Wash II Medium (Chandrasiri, 1997). COCs were denuded of surrounding expanded cumulus cells by mechanical stripping with a micropipette attached to a suction device using 3% sodium citrate solution. After the removal of COCs, the oocytes were washed twice in Wash II Medium.

Approximately 10 denuded matured oocytes were put in each equilibrated fertilization droplet and 5 µl of capacitated sperm suspension was added. The dishes were incubated for 24 h at 39°C in humidified air with 5% CO_2. After 24 h of incubation, the excess spermatozoa were washed by mechanical aspiration through a micropipette using Wash II Medium. Oocytes were examined under an inverted microscope (25 × 10 magnification) for any evidence of cleavage (2-cell). Oocytes were not examined for early signs of fertilization because it is very difficult to observe such signs in fresh samples. Morphologically normal healthy oocytes were used for culture. *In vitro* culture of oocytes was done in 100 µl aliquots of Synthetic Oviduct Fluid (SOF) medium (Tervit *et al.*, 1972) under light mineral oil, at 39°C and 5% CO_2 in air under maximum humidity. Embryos were examined daily for cell division and embryonic development. Oocytes were transferred to droplets of fresh SOF medium every other day of culture.

The experimental design was a 2 × 5 factorial (two capacitation agents and five concentrations) performed by taking goat oocyte cleavage rate as the dependent variable. Each treatment combination was replicated four times. The statistical software package, SAS was used for data analysis (SAS, 1985).

RESULTS

The number of ovaries used and the number of oocytes recovered using aspiration and slicing techniques are shown in Table 1. The average number of oocytes recovered using the aspiration and slicing techniques per ovary were 1.9 and 5.0, respectively.

Table 1. Recovery of follicular oocytes from slaughter house goat ovaries using two different recovery techniques.

No. of ovaries	No. of oocytes		Total	No. of oocytes/ovary
	Aspiration	Slicing		
125	235	630	865	6.92

The maturation rate was 89.75%. Before maturation, the oocytes were surrounded by several layers of intact, unexpanded cumulus cells, which dispersed or expanded after maturation.

Results of the cleavage rate of goat oocytes are shown in Table 2. With heparin, cleavage rate was maximum at the concentration of 10 µg/ml (42.2%), decreasing drastically at 50 µg/ml (3.4%) to reach the lowest rate (2.6%) at 200 µg/ml. With S–ACT–1, the cleavage rate was 14.1% at 10 µg/ml, increasing to 33.3% at 20 µg/ml and then reached 0% at 100 µg/ml.

The capacitation agents did not show a significant effect on the oocyte cleavage rate ($P = 0.14$). However, the effect of concentration was highly significant ($P = 0.0001$). There was no significant interaction between capacitation agent and concentration ($P = 0.17$).

Results of the *in vitro* embryo culture system are shown in Table 3. Development up to the 8–cell stage was easily detectable in the fresh samples. As it was difficult to differentiate 16–cell stage from the morula stage, embryos, which contained more than eight cells and not developed up to the blastocyst stage were considered as morulae.

4–cell stage

The main effects were masked by the significant ($P = 0.04$) interaction between capacitation agents and concentrations, for the survival rate of goat embryos up to the 4–cel stage of development.

Table 2. Cleavage rates of *in vitro* matured goat oocytes after incubation with various concentrations of S–ACT–1 and heparin.

Capacitation agent	Concentration (µg/ml)	No.of ova*	No. cleaved*	% cleaved (cleavage rate)
Heparin	10	90	38	42.2
	20	72	26	36.1
	50	59	02	3.4
	100	62	03	4.8
	200	76	02	2.6
S–ACT–1	10	85	12	14.1
	20	68	23	33.8
	50	63	04	6.3
	100	70	-	-
	200	82	-	-

* Values represent the sum of four replicates.

Table 3. Development of *in vitro* produced 2–cell goat embryos in SOF culture medium.

Capacitation agent	No. of 2–cell embryos	No. of embryos developing to different stages			
		4–cell	8–cell	Morula	Blastocyst
Heparin 10 µg/ml	19	16 (84.2)	16 (84.2)	12 (63.2)	04 (21.1)
S–ACT–1 10 µg/ml	06	04 (66.7)	-	-	-
Heparin 20 µg/ml	13	10 (76.9)	-	-	-
S–ACT–1 20 µg/ml	12	12 (100)	08 (66.7)	06 (50.0)	03 (25.0)

Numbers within parenthesis indicate percentages.

The survival rate of 4–cell embryos was higher (84.2%) with 10 µg/ml than with 20 µg/ml (76.9%) of heparin. Although some cleavage occurred with high concentration of heparin, the survivability of such embryos was low. On the contrary survival rate of the 4–cell embryos produced using 10 µg/ml (low conc.) was lower (66.7%) than the 20 µg/ml (high conc.) of S–ACT–1 (100%). This indicates that although the sperm capacitated with sub–optimum concentrations of S–ACT–1 produced embryos, their developmental competence was lower.

8–cell stage

Survival rate of the embryos produced with low concentration of heparin (10 µg/ml) was 84.2%. At higher concentration (20 µg/ml) the survival rate was zero. Survival rate of embryos produced with low concentration (10 µg/ml) of S–ACT–1 was zero and at a higher concentration (20 µg/ml) the survival rate was 66.7%. At eight–cell stage also embryos had a highly significant ($P = 0.0001$) interaction between capacitation agent and concentration on survival rate.

Morula stage

Survival rates of 63.2 and 50% were observed with 10 and 20 µg/ml of heparin and S–ACT–1, respectively. None of the embryos obtained in sub optimum concentration of S–ACT–1 (10 µg/ml) or higher concentration of heparin (20 µg/ml) survived. Even in the morula stage, a significant ($P = 0.003$) interaction between capacitation agents and concentration on survival rate was observed.

Blastocyst stage

Observations were similar to those of morulae stage embryos. Survival rates of 21.1% and 25% were observed with 10 and 20 µg/ml of heparin and S–ACT–1, respectively. But the interaction effect between the capacitation agent and concentration was not significant ($P = 0.13$). A gradual decrease in the overall survival rate of the embryos was observed during the culture period.

With 10 µg/ml heparin survival rate decreased from 84.2% at 4–cell stage to 21.1% at the blastocyst stage. Similarly with 20 µg/ml S–ACT–1, survival rate decreased from 100% at 4–cell stage to 25% at the blastocyst stage.

DISCUSSION

In this study, transport of ovaries in PBS at 20–25°C was done in accordance with the practice adopted by Moodie and Graham (1989), who noted highest maturation rates when the ovaries were stored at 22°C for a period not more than 4 h after slaughter. Yang *et al.* (1990) also found that storage of cattle ovaries at 22–25°C for 8 h did not significantly decrease maturation and subsequent development rates. They suggested a wide range of temperature (20–35°C) for storage of ovaries. However, First and Parrish (1987) observed adverse effects on maturation and subsequent development of oocytes collected from ovaries stored at temperatures below 30°C.

Oocytes can be recovered from slaughter house ovaries by aspiration (Bou Shorgan *et al.*, 1990), slicing (Suss and Madison, 1983) and dissection (Staigmiller and Moor, 1984). The disadvantage of aspiration is that only few oocytes can be recovered from the aspirated follicles. Lonergan (1990) found that significantly greater yields of follicular oocytes could be collected by follicular dissection. Wahid (1993) using sheep ovaries from slaughter house, recovered 4.1 oocytes/ovary by aspiration and 15.2 oocytes per ovary by slicing. The average oocyte number per ovary was 8.1. Quirke and Gordon (1971) were able to recover only 3.7 oocytes per sheep ovary.

The oocyte recovery in this experiment (6.9 oocytes/ovary) was lower than those reported in the literature. In this study, the stage of oestrous cycle was not considered at the time of collection of the ovaries and it was evident that most of the ewes were in anoestrus at the time of slaughter. Since most of the goats were from small holder farms in rural areas, anoestrus could be a reason for slaughter. Thus, recovery of small number of oocytes from goats in this study was expected.

S–ACT–1, derived from a natural product, acts as a sperm capacitating agent in goats. Although the mode of action of S–ACT–1 on goat spermatozoa is not precisely known, its' mechanism might be like that of heparin as both substances possess similar chemical properties. It was observed that the two capacitation agents behaved in a similar manner. There was a direct inverse

relationship between the oocyte cleavage rate and the concentration of the sperm capacitation agents.

With heparin (10 µg/ml) and S–ACT–1 (20 µg/ml), oocyte cleavage rates were 42.2 and 33.3%, respectively. With increasing concentrations of the capacitation agents, cleavage rate decreased rapidly. This observation is in agreement with the observations made in other experiments. Fukui *et al.* (1990) found that the fertilization rate of bull spermatozoa increased as the concentration of heparin was increased from 0 to 25 µg/ml. Premakumara (1996) also observed a dose dependent activity of S–ACT–1 on the motility of human spermatozoa.

In this experiment, the cleavage rates at concentrations below 10 µg/ml was not investigated. Future studies should investigate cleavage rates at very low concentration levels such as 2, 4, 6 and 8 µg/ml of heparin and S–ACT–1.

Reports available on the *in vitro* fertilization of sheep and goat oocytes are limited. According to Watson *et al.* (1991) *in vitro* capacitation of spermatozoa of farm animals is more difficult. The reasons are not clear. Cheng (1985) found fewer oocytes from abattoir–derived ovaries fertilized following IVM and IVF.

Ovaries of slaughtered animals are a good source of oocytes for mass embryo production. Reduction in the survival rate of embryos from slaughter house ovaries observed in this experiment could be due to many reasons. Stress conditions prior to slaughter might have contributed to poor quality of the oocytes resulting in lower penetration rates and poor subsequent developmental competence. If the welfare of the animals prior to slaughter can be improved it would be possible to obtain better quality oocytes.

The development rate (survivability) of embryos in this experiment was low. Although several factors may affect the *in vitro* development of embryos, the actual reasons for lower developmental rates cannot be clearly stated. *In vitro* matured oocytes have lower developmental competence than *in vivo* matured oocytes. Sirard and Blondin (1996) concluded that this may be due to differences in the cytoplasmic competence between *in vivo* and *in vitro* matured oocytes. Unfavourable culture conditions could be one of the reasons.

This study is a starting point to divert research interests towards testing of naturally available sperm capacitation agents.

CONCLUSIONS

Based on the results of this experiment it can be concluded that S–ACT–1 can be used as a sperm capacitation agent at a concentration of 20 µg/ml for goat spermatozoa. Fertilized goat ova using S–ACT–1 as the capacitation agent, can be cultured up to the blastocyst stage in SOF culture medium.

Further research is needed to improve the penetrability and survivability of the goat embryos produced from slaughter house ovaries using S–ACT–1 as the capacitation agent.

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