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Qualities of goat semen in Tris-Citrate-Glucose extender containing glutathione

B. Mishra[1], M. G. S. Alam[*], M. A. M. Y. Khandokar[2], S. Mazumder[3] and M. N. Munsi[4]
Departments of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

Abstract

Glutathione (GSH) 0 (control), 2, 4 and 8 mM was used in the preservation of chilled goat semen. Treated and control samples were kept at 4 – 5°C up to seven days. Sperm motility and acrosome abnormality were assessed daily under phase contrast microscope. The sperm motility was significantly ($P<0.01$) higher in the semen treated with 8 mM GSH. Optimum sperm motility (≥50%) for artificial insemination was retained for three days with 2 and 4 mM GSH and up to four days with 8 mM GSH. Acrosomal damage was significantly ($P<0.01$) reduced to ≤ 1.0% after addition of 8 mM GSH. It is suggested that GSH may be used as an antioxidant for better preservation of goat semen for artificial insemination. (*Bangl. vet*. 2010. Vol. 27, No. 2, 46 – 55)

Introduction

Breeding bucks are scarce in Bangladesh, because bucks are castrated at 5-11 weeks for better quality of meat and skin. Selective breeding to improve the species requires an artificial insemination (AI) program using semen from males with high genetic merit (Roberts and Foote, 1989). Buck ejaculates are small in volume with high concentrations of spermatozoa (Mann *et al*., 1980). Unlike bulls, buck inseminates should contain high number of spermatozoa with a suitable semen extender for preservation (Evans and Maxwell, 1990).

An extender increases the volume of semen and should prolong the life of spermatozoa with fertilizing capacity. At chilling temperature, the fertilizing ability of spermatozoa reduces with time (Shamsuddin *et al*., 1987; Shamsuddin *et al*., 2000) and the motility and morphology of spermatozoa deteriorate after two days (Alam *et al*., 2005). During storage of mammalian spermatozoa phospholipids undergo peroxidation, the formation of toxic fatty acid peroxides causing structural damage to

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[1] Laboratory of Veterinary Physiology, Department of Veterinary Medicine, Faculty of Agriculture, Iwate University, Morioka, Japan
[2] Department of Animal Breeding and Genetics, Faculty of Animal Husbandry, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
[3] Department of Agricultural Statistics, Faculty Agricultural Economics and Rural Sociology, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
[4] Goat and Sheep Production Division, Bangladesh Livestock Research Institute, Savar, Dhaka, Bangladesh
* Corresponding author. E-mail: mgsalam52@yahoo.com

the sperm cell accompanied by decreased motility (Jones and Mann, 1977; Mann *et al*., 1980). Longer preservation at 5°C may be achieved with the addition of antioxidants to semen diluents (Beconi *et al*., 1993; Bilodeau *et al*., 2000). The antioxidant system in the sperm of different species is weak, so spermatozoa can readily undergo lipoperoxidation (Foote *et al*., 2002). Improved motility of spermatozoa and increased preservation time are achieved in other species by incorporating GSH in chilled or frozen semen (Gupta and Tripathi, 1984; Slaweta and Laskowska, 1987), but there are no reports of its available in goats. Therefore, the aims of the present study were to determine the effect of different concentrations of GSH on the quality of chilled goat semen.

Materials and Methods

General description and management of bucks

The semen was collected from four Black Bengal bucks (*Capra hircus*) aged 18 – 24 (20.5 ± 1.5) months, weighing 19 - 27 (21.6 ± 1.9) kg reared separately from does and with scrotal circumference 19.5 - 22.0 (20.6 ± 0.5) cm. Bucks were allowed natural grazing for six to seven hours daily under supervision. Each buck was fed 0.5 to 0.8 kg concentrate mixture (maize grit, rice bran, wheat bran, wheat polish, soybean crush, common salt) daily in two meals. In addition, each buck was supplied green grass (2 – 3 kg fresh weight) each evening with *ad libitum* drinking water. Bucks were vaccinated against *Peste des petits ruminant* and tetanus. The animals were treated three times a year with morantel citrate (Deminth® Renata Animal Health Ltd., New DOHS, Mohakhali, Dhaka, Bangladesh) @ 10 mg/kg orally. Ivermectin (Vermic® Techno Drugs, Segunbagicha, Dhaka, Bangladesh) @ 0.2 mg/kg body weight was injected subcutaneously for controlling ectoparasites when requires.

Preparation of extender and reagents

The semen diluent was prepared by dissolving Tris (3.8g), citric acid (2.2g), and glucose (0.6g) in 100 ml distilled water (Shamsuddin *et al*., 2000). The stock solutions were sterilized by filtration (0.22 µm) and preserved at + 4 to + 7°C for a maximum of two weeks. On the day of semen collection, fresh egg yolk was added (2.5%, v/v) with penicillin (1000 iu/ml) and streptomycin sulfate (1 mg/ml).

Three different concentrations of GSH solution, 2, 4 and 8 mM were prepared weighing 6.1, 12.2 and 24.4 mg of GSH (SIGMA® Glutathione, Product of Japan, SIGMA-ALDRICH CO., P.O. Box 14508 St. Louis) in 10 ml of egg-yolk tris-citric acid – glucose extender (EYTCG).

Buffered formol saline was prepared by dissolving disodium hydrogen phosphate (6.2g), potassium dihydrogen phosphate (2.5g), sodium chloride (5.4g) and concentrated formaldehyde (175 ml) in 1000 ml of distilled water.

Stock solution-I for Williams' stain was prepared by dissolving 10g of basic fuchsin in 100 ml of 95% alcohol. Stock solution-II was prepared by dissolving a saturated solution of bluish eosin in 95% alcohol. Stock solution-III was prepared by

mixing 10 ml of stock solution-I with 170 ml of 5% phenol solution. The final working solution contained 25 ml of stock solution-II and 50 ml of stock solution-III. The stain was left for at least two weeks for stabilization, and filtered before use.

To prepare hematoxylin-eosin solution 2g hematoxylin in 100 ml 95% alcohol, 2.5g potassium alum in 100 ml distilled water, 100 ml glycerin and 10 ml glacial acetic acid were mixed thoroughly. The eosin solution was prepared by dissolving 1g eosin (yellowish) in 100 ml 95% alcohol. The hydrochloric acid-alcohol solution was prepared by mixing 280 ml-distilled water, 720 ml 95% alcohol and 10 ml 25% hydrochloric acid.

Semen collection and evaluation

The bucks were trained to ejaculate in an artificial vagina (AV) at homosexual mount (Williams, 1920). Briefly, AV consists of an outer casing (15 cm × 5.5 cm) with good insulation properties containing an inner liner of thin rubber. The liner was extended at least 2 - 3 cm beyond the end of the outer casing and folded back and secured with rubber bands to form a watertight jacket. The jacket was two-thirds filled with water at 50°C (to achieve 45°C inside AV) through a tap on the side of the AV and inflated by blowing air through, which was then closed. The penis end of the AV was lubricated with non-spermicidal gel (# x B640, L'Aigle Cedex, France). At the outer end of the AV, a plastic cone with a calibrated plastic tube was fixed. Before collection, the prepuce of the buck was wiped clean to reduce contamination. An ovariectomized doe was secured in a collection bail and her rear end was cleaned. The operator crouched or knelt at the right of the doe and held the AV in the right hand along its flank and with the open end facing towards the male and downwards at an angle of 45°C. When the male mounted, the erect penis was directed into the open end of the AV to vigorous upward and forward thrust. The buck was allowed to withdraw his penis immediately after ejaculation. The graduated tube was separated from the cone and its mouth closed with a plastic cap and labeled.

The tube was immediately placed in a beaker containing lukewarm water (37°C). Eight ejaculates were collected from each buck over a period of 28 days.

The routine evaluation of fresh semen was done immediately (Shamsuddin *et al.*, 2000). The volume of the ejaculate was measured using a micropipette.

Colour

The colour of the semen was assessed in the collecting tube immediately by naked eye.

Mass activity (Wave motion)

To evaluate mass activity, a drop (20 µl) of semen was placed on a pre-warmed slide (37°C) without a cover slip and examined under phase-contrast optics (100×; CH-2, Olympus, Tokyo, Japan). The mass activity was scored: 1 = no perceptible

motion, 2 = weak motion without waves, 3 = small, slow moving waves, 4 = vigorous movement with moderately rapid waves and 5 = dense, very rapidly moving waves.

Motility

A drop (10 µl) of both fresh and diluted (EYTCG) semen was placed on a clean warmed slide and covered with a cover slip. The proportion of spermatozoa moving actively forward was estimated separately by two independent investigators at 400× magnifications.

Concentrations

The concentration of the spermatozoa (million/ml) was determined using a hemocytometer (Shamsuddin *et al*., 2000). Semen samples were diluted with distilled water (1 : 200), a drop was placed on the counting chamber, and sperm were allowed to settle for 5-6 min before placing the chamber on the stage of the microscope. The spermatozoa were counted in five large squares; four at corners and one in the centre of 25 large squares. The number of heads in the large squares was recorded. The concentration of the spermatozoa per milliliter of semen was calculated by multiplying the total number in large squares and expressed as million per milliliter.

Evaluation of acrosome, mid-piece and tail morphology

The acrosome, mid-piece and tail of spermatozoa was examined in semen fixed with buffered formol-saline (1:200) under phase-contrast optics (1000×); at least 200 spermatozoa were counted (Hancock, 1957).

Evaluation of sperm head morphology

The sperm were examined after staining thin air-dried smears with carbol-fuchsin (Williams, 1920); at lease 500 spermatozoa were examined using phase-contrast microscope (1000×).

Evaluation of cells other than spermatozoa

The cells other than sperm were examined using thick smear stained with Ehrlich hematoxylin and evaluated at 1000× (Williams, 1920).

Preservation of semen in the GSH solution

Three different concentrations, 2, 4 and 8 mM of GSH solution were kept at 37°C. Semen samples (100 µl) were taken in a glass vial and diluted with 15 µl EYTCG. GSH solution was added to it and kept at 37°C for 15 min.

Samples of chilled semen containing 0 (control), 2, 4 and 8 mM GSH were evaluated 24 hours after treatment and daily up to 7 days by the same operator for motility and acrosome damage.

Statistical analyses

Analysis of variance (for one-way and two-way classification) was done with the help of computer package MSTAT and SPSS. For the percentage data, the analysis of variance was performed after transforming the data by arcsin transformation using the formula $Y = \sin^{-1}\sqrt{X}$ (Zaman *et al*., 1982). The treatments mean values were compared by least significant difference (LSD) adjusted by Duncan's new multiple range tests (Gomez and Gomez, 1984). A regression analysis was performed to evaluate the effect of different concentrations of GSH on sperm motility with advancement of time (Petrie and Watson, 1999).

Results and Discussion

Evaluation of fresh buck semen

The characteristics of fresh semen are depicted in Table 1.

Table 1. Characteristics of fresh buck's semen (mean ± SE)

Buck no.	Volume (ml)	Concentration ($\times 10^6$/ml)	Motility (%)	Mass activity	Normal acrosome, mid-piece, tail (%)	Normal head (%)
1	0.7 ± 0.1	292.8 ± 15.8	85.0 ± 4.1	3.8 ± 0.2	96.8 ± 1.0	99.3 ± 0.3
2	0.7 ± 0.1	288.3 ± 21.3	87.5 ± 2.9	3.8 ± 0.2	96.0 ± 1.9	99.0 ± 0.2
3	0.6 ± 0.1	284.3 ± 13.3	82.5 ± 6.5	3.7 ± 0.2	94.8 ± 0.7	98.8 ± 0.3
4	0.5 ± 0.1	285.5 ± 17.9	77.5 ± 2.9	3.7 ± 0.2	95.5 ± 1.2	99.0 ± 0.3
	**	-	*	-	-	-

** Significant difference (P<0.01); * Significant difference (P<0.05)

Effects of GSH on the sperm motility

The mean progressive motility of spermatozoa in control and GSH-treated semen is shown in Table 2. The mean motility of spermatozoa was significantly (P<0.01) higher for the group treated with 8 mM GSH than that of others after Day 4. Optimum sperm fertility for AI was retained for three days with 2 and 4 mM GSH and up to four days with 8 mM GSH (≥ 50%). Sperm motility decreased progressively in each group with time. In semen treated with 2, 4 and 8 mM GSH, the sperm motility decreased by 6%, 5%, and 4%, respectively, per day, but by 8% each day in the control group.

Similarly, the coefficient of determination $R^2_1 = 0.96$, $R^2_2 = 0.99$, $R^2_3 = 0.96$ and $R^2_4 = 0.95$ indicated that 96%, 99%, 96% and 95% of total variation in sperm motility occurred with time in control and in semen containing 2, 4 and 8 mM GSH, respectively.

Effects of GSH on acrosome damage

The measures of acrosomal damage are presented in Tables 3 and 4. Pooled data of four bucks showed that the mean percentage of acrosomal abnormality (1.1 ± 0.3)

was significantly (P<0.01) lower in the semen with 8 mM GSH than with 2 (2.2 ± 0.2) or 4 (1.6 ± 0.2) mM GSH or control group (2.4 ± 0.2).

Table 2. Motility of buck sperm with different concentrations of GSH (mean values after transformation of percentage data)

Concentration of GSH	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
Control	61.5	52.1^{b}	43.7^{c}	36.2^{d}	28.8^{d}	19.7^{d}	13.2^{d}
2 mM	61.5	56.2^{a}	49.7^{b}	44.3^{c}	37.8^{c}	30.2^{c}	21.4^{c}
4 mM	61.5	56.7^{a}	52.4^{a}	47.8^{b}	42.1^{b}	35.8^{b}	25.3^{b}
8 mM	61.5	57.0^{a}	53.7^{a}	50.4^{a}	46.5^{a}	39.8^{a}	29.5^{a}
CV (%)	00.0	02.6	01.7	02.0	01.5	03.0	04.6
Level of significance	-	**	**	**	**	**	**
LSD	-	3.289	1.887	2.041	1.342	2.176	2.337

** Significant difference (P<0.01); Any two mean values having different superscripts differ significantly (P<0.01)

Table 3. Acrosomal abnormality (%) in chilled buck's semen with different concentrations of GSH (mean ± SE)

Concentrations of GSH	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
Control	2.4 ± 0.3	2.3 ± 0.4	2.6 ± 0.6	2.8 ± 0.2	2.6 ± 0.2	2.9 ± 0.3	2.7 ± 0.2
2 mM	2.5 ± 0.8	2.8 ± 0.9	2.7 ± 0.6	2.9 ± 0.3	2.7 ± 0.2	2.8 ± 0.1	2.6 ± 0.2
4 mM	1.8 ± 0.4	2.0 ± 0.2	1.5 ± 0.4	1.6 ± 0.7	1.9 ± 0.3	2.0 ± 0.2	1.8 ± 0.3
8 mM	1.5 ± 0.4	1.7 ± 0.6	1.3 ± 0.6	1.0 ± 0.4	0.8 ± 0.4	0.9 ± 0.3	0.8 ± 0.4

Table 4. Acrosomal abnormality of buck's sperm with different concentrations of GSH (mean values after transformation of percentage data)

Concentrations of GSH	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
Control	$12.1b^{c}$	15.2^{b}	17.1^{c}	20.0^{c}	22.5^{c}	22.5^{ac}	22.9^{c}
2 mM	11.3^{d}	15.6^{c}	16.9^{d}	19.1^{b}	20.5^{b}	26.1^{d}	28.7^{d}
4 mM	11.5^{c}	16.7^{c}	18.8^{b}	21.4^{cd}	21.7^{cd}	28.2^{c}	29.3^{b}
8 mM	12.6^{ab}	17.6^{b}	20.6^{a}	23.1^{a}	24.7^{a}	29.3^{a}	30.4^{a}
CV %	2.0	2.6	3.1	2.4	2.6	3.0	4.5
Level of Significance	*	*	*	*	*	*	*
LSD	0.502	0.845	1.173	1.065	1.316	1.601	1.910

Significant difference (P<0.01); Any two mean values having different superscripts differ significantly (P<0.01)

The present study indicated that addition of 8 mM GSH to buck semen kept at 5°C enhanced the keeping quality of semen. In this experiment, at least 50% sperm motility was maintained up to four days at 5°C using 8 mM GSH and than declined by 4% a day. Similarly, progressive sperm motility declined by 5%, 6% and 8% in semen containing 4, and 2 mM GSH and control, respectively. In this study, semen treated with 2 and 4 mM GSH maintained at least 50% motility for up to three days. On the other hand, after addition of 8 mM GSH 50% motility remained up to Day 4 with only 1% acromosal damage. In chilled bull semen, optimum sperm motility remained up to two days (Alam *et al*., 2005), but with 0.5 mM GSH, lower than in the present study, sperm motility remained at least 40% with 12% acrosome abnormality (Munsi *et al*., 2007). No comprehensive data are available on goats semen preserved with GSH, but information on chilled goat's semen preserved with different diluents is reported elsewhere (Shannon *et al.,* 1983; Sinha *et al*., 1996; Shamsuddin *et al*., 2000).

In this investigation, a lower percentage (0.8 – 1.7%) of acrosome abnormality of sperm was found in semen treated with 8 mM GSH. This may be the reason for better motility in semen treated with 8 mM GSH, which is in agreement with another report (Slaweta and Laskowska, 1987). GSH helps maintain the integrity of the acrosome in the sperm head (Sinha *et al*., 1996). It is reported that 1-buthionine-S, R-sulfoximine, a GSH synthesis inhibitor causing depletion of antioxidant enzyme activities in the cauda epididymis, adversely affects sperm motility (Zubkova and Robaire, 2004). Moreover, GSH may stabilize the plasmolemma of spermatozoa as judged by their increased motility.

It is suggested that glutathione plays an active role in sperm fructolysis (Slaweta and Laskowska, 1987). It is a co-enzyme of 1, 3-diphosphoglyceric-aldehyde dehydrogenase that leads to oxidation of triose phosphate to phosphoglyceric acid, which is later reduced to pyruvic acid and then to lactic acid. Mammalian spermatozoa are highly sensitive to lipid peroxidation. Lipid peroxidation is also affected by hydrogen peroxide produced as a result of oxidative deamination of amino acids in semen. Toxic oxygen metabolites are emerging as a final common pathway of cell injury as well as cellular aging (Cotran *et al*., 1989). Intracellular oxygen, in conjunction with a number of oxidative enzymes, produces partially reduced toxic oxygen molecules such as superoxide, hydrogen peroxide and hydroxyl radicals. These free radicals must be destroyed by the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase that damages the cell membrane by lipid peroxidation during preservation (Cotran *et al*., 1989). Lipid peroxidation is further initiated by hydroxyl radicals, which in turn react quickly with oxygen to form lipid peroxides. The lipid peroxides then act as free radicals initiating an autocatalytic chain reaction, resulting in further damage to the cell membrane (Cotran *et al*., 1989).

It is suggested that lipid peroxidation and subsequent membrane damage is at its peak during thawing (Singh *et al*., 1995). Although, the exact mechanism is not clear, there is some evidence that the free radicals are toxic oxygen metabolites produced at low levels in cells with a restricted supply of oxygen but there is an

increase in their production on restoration of oxygen supply to the cell. Hence, it is speculated that the sudden increase in oxygen utilization by the spermatozoa during thawing might be responsible for increased production of free radicals, leading to increased lipid peroxidation and thus membrane damage.

GSH plays an important role in scavenging reactive oxygen intermediates and other free radicals such as hydrogen peroxide and hydroxyl radical with the help of glutathione reductase (Meister and Anderson, 1983). GSH might protect the sperm from membrane damage by inhibiting lipid peroxidation.

It is suggested that spermatozoa of many species have a weak antioxidant system and can readily undergo lipoperoxidation, particularly in the presence of oxygen (Foote *et al*., 2002). Addition of antioxidant has been beneficial to spermatozoa, particularly in media devoid of a mixture of macromolecules. The beneficial effect occurred with spermatozoa stored at 5°C and minor effects during pre-freeze processing might have some beneficial effect on sperm after insemination that is not detectable by evaluation of sperm motility *in vitro*.

In this study, air-dried semen smears stained with hematoxylin and eosin showed no cells other than sperms.

Further studies could include use of physiological concentrations of GSH, tests of membrane integrity, studies of frozen semen, and effects on pregnancy rate.

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