ORIGINAL ARTICLE

Determination of the antioxidant potential of goat sperm cells

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Ascorbic acid; Free radicals; Goat; Reactive oxygen species; Sperm motility

Abstract

Objective: Reactive oxygen species (ROS) such as the superoxide anion, hydroxyl radical and hydrogen peroxide are highly toxic to cells. Cellular antioxidant enzymes and free radical scavengers normally protect a cell from toxic effects of ROS. However when generation of ROS overtakes the antioxidant defense then tissue damage is caused. The present study aims to find the ascorbic acid levels in sperm cells of different parts of the epididymis and the relation of level of ascorbic acid with motility status of cauda sperm cells considering goat sperm as a model system. The antioxidant potential of motile cauda sperm cells was also revealed.

Methods: Ascorbic acid assay and antioxidant potential assay was done to measure the levels of ascorbic acid and the levels of scavenging of free radicals. Sperm motility was determined by microscopic and spectrophotometric methods.

Results: From the results of the present study it was revealed that the level of ascorbic acid is significantly less in the sperm cells of the cauda epididymis. The level of total antioxidant potential of the sperm cells of the cauda epididymis was tested. The sperms were found to be having significant amount of antioxidants. No significant relation could be concluded regarding the levels of ascorbic acid in the sperms of the cauda epididymis and their motility status.

Conclusions: Since the sperms of the cauda epididymis are mostly motile, so, less ascorbic acid in cauda sperm may be due to the consumption of the antioxidant (ascorbic acid) by the sperm cells to scavenge the free radicals produced during motility. Reduction of ROS or free radicals seems to be necessary for sperm motility.

INTRODUCTION

Reactive oxygen species (ROS) such as the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) are highly toxic to cells. Cellular antioxidant enzymes and free radical scavengers normally protect a cell from toxic effects of ROS. However when generation of ROS overtakes the antioxidant defense, tissue damage is caused. Oxidative damage of the cellular macromolecules, *i.e.* lipids, proteins and nucleic acids, occurs leading finally to various pathological conditions [1, 2].

Oxygen is vital for aerobic processes, however 5% or more of the inhaled O_2 is converted to ROS by univalent reduction [3]. When the balance between the ROS production and antioxidant defense is lost, oxidative stress is caused, which through a series of events, deregulates cellular functions leading to various pathological conditions including cardiovascular and gastroduodenal dysfunction, neurodegenerative disease, cancer and premature aging [4]. The protective antioxidant system in the semen is composed of enzymes, as well as nonenzymatic substances, which closely interact with each other to ensure optimal protection against ROS. Non-enzymatic antioxidants include vitamins A, E, C and B complex, glutathione, pantothenic acid, coenzyme Q10 and carnitine, and micronutrients such as zinc, selenium and copper. It seems that a deficiency of any of them can cause a decrease in total antioxidant status. *In vitro* and *in vivo* studies demonstrate that many antioxidants possess a beneficial effect on fertility [5]. A decreased seminal plasma antioxidant capacity could have significant role in the etiology of impaired sperm functions [6]. Oxidative stress negatively affects flagellar axonemal structure with subsequent impairment of forward progressive sperm motility [7].

Superoxide dismutase (SOD), catalase (CAT) and peroxidases constitute a mutually supportive team of defense against ROS [4, 8, 9]. Glutathione peroxidase (GPx), horseradish peroxidase, lacto-peroxidase and other mammalian peroxidases have been studied extensively [10-13]. CAT, present in almost all the mammalian cells, is localized in the peroxisomes. It protects the cells from oxidative damage by scavenging of H_2O_2 and •OH [14]. Low levels of ROS exert critical function in normal sperm physiology, such as

fertilizing ability, *i.e.* acrosome reaction, hyper-activation, capacitation and chemotaxis, and sperm motility; while increased ROS generation and/or decreased antioxidant capacity leads to the imbalance between oxidation and reduction in living systems, which is called sperm oxidative stress [15, 16]. The secretion of seminal vesicles contains ascorbic acid [17] vitamin C; ascorbic acid acts as an antioxidant, and it participates in the detoxification reaction [18].

The antioxidant ascorbic acid plays important role in various physiological processes in the body including detoxification of different toxic compounds [19]. The production of ROS by sperm was reduced by supplementation *in vitro* with ascorbate and alpha-tocopherol [20]. Ascorbic acid in concentrations below 1000 μM protects spermatozoa from free radical damage as evidenced from improvement in their motility and viability [21]. Supplementation with vitamin E and/or C reduced ROS generation, prevented loss of motility and capacity of oocyte penetration in lead (Pb)-exposed rats [22]. When vitamin C is added to the capacitation medium, a significant decrease in the percentage of capacitated spermatozoa was observed [23, 24].

Ascorbic acid deficiency reduced both sperm concentration and motility, and thus fertility, of rainbow trout. These results indicate that vitamin C is important for male fish reproduction [25]. Treatment with ascorbic acid increased ejaculate volume, sperm concentration, total sperm output, sperm motility index, total motile sperm, packed sperm volume, initial hydrogen ion concentration (pH) and semen initial fructose concentration. Abnormal and dead sperm were significantly decreased in ascorbic acid treated animals. The activities of aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase were significantly decreased, whereas glutathione S-transferase showed a significant increase in seminal plasma of treated animals compared with the controls. These reports indicated that supplementation of drinking water with antioxidant ascorbic acid, vitamin E and their combination reduced the production of free radicals and can improve rabbit semen quality [26]. *In vitro* ascorbic acid supplementation during teratozoo-spermic semen processing for assisted reproductive technology (ART) could protect against oxidative stress, and could improve ART outcome [27]. Low intake of folic acid, zinc and vitamin E were related to poor sperm concentration and motility [28]. Antioxidants generally were not beneficial except improving the percentage of motile sperm by 6-11% [29]. Co-administration with vitamin C reversed the effect of bisphenol A or methoxychlor induced oxidative stress in epididymal sperm and epididymis [30, 31].

Taken together, the present study aims to determine the ascorbic acid levels in sperm cells of different parts of the epididymis and the relation of level of ascorbic acid with motility status of cauda sperm cells considering goat sperm as a model system. The antioxidant potential of motile cauda sperm cells was also investigated.

MATERIALS AND METHODS

Chemicals

Ringer phosphate buffer, Ficoll, Folin reagent, copper sulfate, sodium potassium tartarate, sodium carbonate, sodium hydroxide, Bradford reagent, bovine serum albumin (BSA), sodium tungstate, disodium hydrogen phosphate, L-ascorbic acid, H_2SO_4, NaOH, toluene: butanol (3:1), butanol saturated with H_2SO_4, Na-acetate buffer, chloroform, Fast blue BB salt, butanol saturated with water, pyridine, glacial acetic acid, phosphate buffer, dimethylsulfoxide (DMSO), ascorbic acid.

All the analytical grade reagents were purchased from Sigma (St. Louis, MO, USA). Goat sperm was taken as the model system because of its stability in synthetic medium and the capability to withstand several treatments and centrifuge without any damage to its structure or function. Moreover, goat testis is easily available from meat shops as food material and does not require any ethical clearance for its use.

Collection of goat epididymal tissue

Goat epididymal tissue was collected from the nearby slaughterhouses. The tissue samples were taken to the research laboratory in a plastic container at the atmospheric temperature. Spermatozoa were extracted from the epididymides within 2-3 h of slaughtering [32]. The tissues were washed with RPS solution to remove the blood vessels. Then the tissues cut into small pieces with the help of scissors and kept in the ringer phosphate solution to prevent the dryness of the tissue.

Preparation of spermatozoa

Spermatozoa were obtained from goat cauda-epididymis by the procedure as standardized in the laboratory [33]. Spermatozoa were extracted at room temperature from the epididymis in a modified Ringer's solution (RPS medium: 119 mM NaCl 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, 16.3 mM potassium phosphate, pH 6.9, 50 units/ml penicillin). Numbers of spermatozoa in the sample were determined with a hemocytometer. Freshly extracted sperm preparation contains 10-20 x 10^7 cells/ml and nearly 60-80% of these cells showed form of flagellar motility. The sperm preparation should be used immediately (within 15 min) for motility assay.

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Preparation of epididymal plasma

Goat epididymal plasma (EP) was prepared by the procedure standardized in our laboratory [33]. Freshly extracted sperm suspensions were first centrifuged at 800g for 10 min, so that most of the spermatozoa were removed as a pellet. The resulting slightly turbid solution was spun at 14000g for 30 min to obtain cell-free EP. The pooled sample of EP get concentrated at 4-6ºC by the treatment of polyethylene glycol (12 mg/ml); after that this is preserved at -10ºC for the further use and studies.

Sperm motility assays

Microscopic method: Plasma membrane and its fractions were estimated by evaluating forward motility (FM) of spermatozoa using hemocytometer as the counting chamber. To eliminate the possibility of artifact due to sperm adhesion to glass, motility assays were carried out in presence of EP (1.2 mg-protein/ml) that contained adequate anti-sticking activity to cause nearly 100% inhibition of sperm adhesion to glass surface [34]. Spermatozoa (0.5×10^6 cells) were incubated with EP (0.6 mg-protein/ml) in the absence or presence of specified amounts of test samples (cauda sperm plasma membrane) at room temperature (32 ± 1°C) for 2 min in a total volume of 0.5 ml of RPS medium. A portion of the cell suspension was then placed in the hemocytometer and the FM sperm and total number of sperm cells were counted under phase contrast microscope at 400x magnification. The percentage of FM sperm was then calculated [35]. The calculated percentages of FM cells are given as the mean ± SEM of at least three experiments.

Spectrophotometric method: FM of spermatozoa was estimated quantitatively. The microscopic method of motility assay described above takes into consideration only the numbers of cells with forward progression but not their velocity whereas the spectrophotometric method is based not only on the motile cell numbers but also their velocity. The method consists of layering 50 µl of freshly extracted cauda-epididymal spermatozoa (7×10^6 cells) suspended in RPS medium (1.3 ml) containing 1% Ficoll-400 at the bottom of a standard optical cuvette (3 ml capacity), which was sufficient to cover the entire width of the light beam. Vigorously motile spermatozoa that moved upwards into the light beam were registered continuously as an increase of absorbance at 545 nm with a spectronic spectrophotometer equipped with a recorder [36]. After reaching maximal absorbance or absorbance at equilibrium (A_{Eq}) the contents of the cuvette were mixed and the absorbance for all the cells or total absorbance (A_T) was noted. The percentage of cells that showed vigorous FM was calculated as $A_{Eq}/A_T \times 100$ (Fig.1).

Protein estimation

Unless otherwise specified, the protein contents of the samples were estimated according to Lowry *et al* [37], using bovine serum albumin (BSA) as standard.

Ascorbic acid assay

A simple method has been developed for determination of ascorbic acid by using acid phosphotungstate. The reagent has been found to be specific and sensitive to ascorbic acid. At first the spermatozoa from different segments of epididymis were collected in RPS medium and epididymal plasma was discarded by centrifugation. The forward and total motility of the cauda spermatozoa was counted under microscope by the procedure developed earlier in our laboratory [35]. The sperm was diluted to 200×10^6/ml in such a way so that with 500 µl volume 100×10^6 cells could be applied in the assay system.

Ascorbic acid was estimated by a spectrophotometric method as described earlier [38]. Color reagent was prepared by dissolving 20 g sodium tungstate and 10 g disodium hydrogen phosphate in 30 ml water with warming. To this warm solution a mixture of 15 ml water and 5 ml H_2SO_4 was added slowly and the mixture was boiled gently for 2 h under reflux to avoid evaporation. Then the golden yellow colored solution was cooled to room temperature to get the final color reagent. For standard, 1 mg/100 ml ascorbic acid was dissolved in the modified Ringer phosphate buffer. Water was taken as blank. 500 µl of blank, standard and samples with duplicates were taken in separate tubes and 500 µl of color reagent was added to them. Then the tubes were incubated at room temperature (in dark) for 30 min and after that centrifuged at 3000 rpm for 15 min. Then the optical density (OD) was taken at 700 nm. The result obtained was expressed as µM of ascorbic acid.

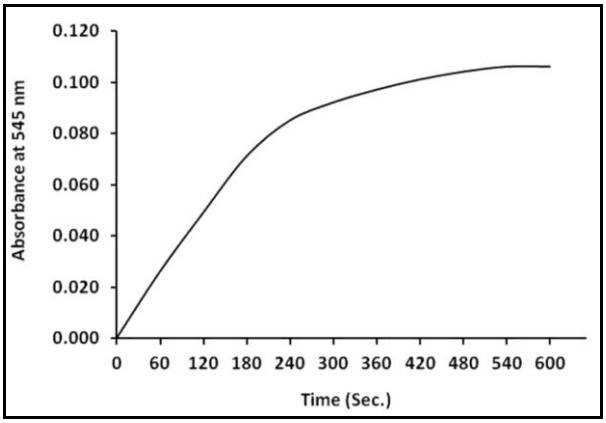

Figure 1. Increase in absorbance at 545 nm with time as the sperm cells move upward from the bottom of the optical cuvette into the light beam of the spectrophotometer.

Assay of antioxidant potential of sperm cells from cauda epididymis

The cauda epididymal sperm cells were diluted to 1000 x 10^6/ml so that 50 x 10^6 cells and 100 x 10^6 cells could be applied in the assay system with 50 µl and 100 µl. Water was taken as blank and DMSO as standard for the assay. The antioxidant potential of the sperm cells is estimated in competition with DMSO that is added to the sample test tubes also. 50 µl of 1 M PO_4 buffer, 200 µl of DMSO, 100 µl of 10 mM ascorbic acid and 20 µl of 10 mM Cu^{2+} were added to all the tubes except the DMSO is not added in the blank. Then, in the sample tubes, 50 and 100 µl of sperm solution were added. The volume was made upto 1 ml with water in all the tubes. Then all the tubes were vortexed and incubated for 1 h at 37ºC.

Color reagent was prepared by dissolving 40 mg Fast blue BB salt in 2 ml water (50 mM), then 2 ml of chloroform was added to it. The tube was vortexed for 1 min wrapped in aluminum foil and the upper golden yellow colored layer is taken as the final color reagent.

To each tube 1 ml of distilled water was added, then 0.2 ml 10 N H_2SO_4 was added and after that 2 ml (toluene:butanol, 3:1) was added; then all the tubes were vortexed for 1 min and all the tubes were centrifuged at 1000 rpm for 5 min; after centrifugation upper layer was discarded. To the lower layer 4 ml of butanol saturated with 1 M H_2SO_4 was added and all the tubes were vortexed for 1 min. Then all the tubes were centrifuged at 1000 rpm for 5 min. Fresh glass tubes were taken into which 1 ml fresh 1 M sodium acetate and 1 ml distilled water was added. The upper layer of the previous centrifuged tubes are poured in to the sodium acetate containing tubes and all these tubes were vortexed for 1 min and the centrifugation is done at 1000 rpm for 5 min. After centrifugation the upper layer was discarded and to the lower layer 0.1 ml of colour reagent was added; these are kept in dark for 15-20 min. Then 1.5 ml of toluene:butanol was added and all the tubes were vortexed for 1 min, then centrifuged at 1000 rpm for 5 min. Lower layer was discarded and to the upper layer 2 ml butanol saturated with water was added. All the tubes were vortexed for 1 min and centrifuged at 1000 rpm for 5 min. After centrifugation, the upper layer was collected and to that 0.1 ml of pyridine-glacial acetic acid solution was added and vortexed for 15 seconds. Then OD was read at 425 nm [39].

Statistical analysis

All experiments were repeated at least three times or more. The data are presented as the mean ± SEM. Significance of difference (wherever required) was analyzed by Student's *t*-test.

RESULTS

The main objective of the ascorbic acid assay experiment was to find the levels of ascorbic acid in the sperm cells of different parts of the epididymis and also to find out if there is any correlation between ascorbic acid (antioxidant) level of the sperm cells and sperm motility.

Four sets of experiments were done with each set containing three tissues (combined). From these experiments it was found that the levels of ascorbic acid in the sperms of caput and corpus section of the epididymis were more or less same with no significant difference. But the sperm cells of the cauda section contain significantly less ascorbic acid levels, in spite of having higher motility status than the other two sections (Fig.2).

The experiments regarding the motility status of the cauda sperm cells in correlation with ascorbic acid levels did not reveal any significant correlation. Both the FM and total motility (TM) were measured along with the ascorbic acid levels in four groups of tissues with each group containing three tissues. But the ascorbic acid levels did not go hand in hand with the motility status (Fig.3)

Experiments were performed to assay the antioxidant potential of the cauda epididymis sperm cells by assaying the amount of methyl sulfenic acid formed. DMSO standard (*i.e.* in absence of sperm cells) gave around 0.27 absorbance at 425 nm, but when competed with sperm cells, the absorbance was reduced showing that the antioxidants of sperm cells are scavenging the hydroxyl radicals in competition with DMSO and thus reducing the level of methane sulfinic acid formation. Application of 50 million cells reduced the absorbance by about 50% and application of 100 million cells further reduced the absorbance by around 66% (Fig.4). These experiments reveal that the mature sperm cells do have antioxidant potential.

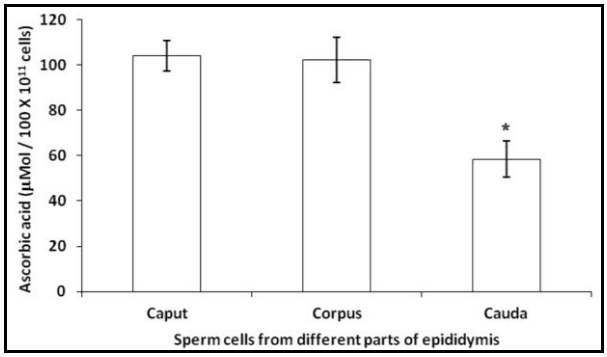

Figure 2. Ascorbic acid levels in the sperm cells isolated from caput, corpus and cauda parts of the epididymis expressed in µM/10^{11} cells. Data shows mean ± SEM of four experiments. *****P < 0.05 compared to caput and/or corpus.

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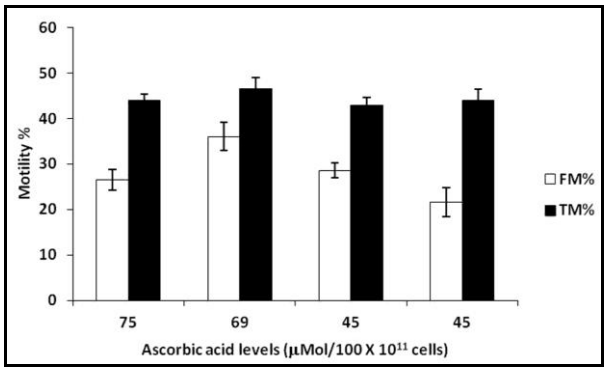

Figure 3. Motility status given as forward motility (FM) and total motility (TM) percentage along with ascorbic acid levels ($\mu M/10^{11}$ cells). Data showing mean ± SEM of three motility counting experiments for each sample.

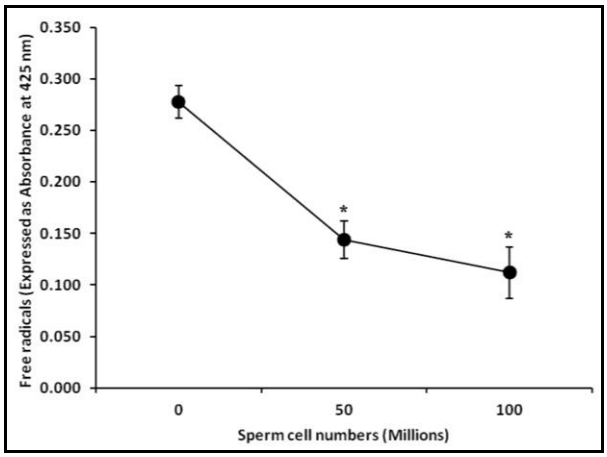

Figure 4. Estimation of the antioxidant potential of cauda sperm cells by estimation of methane sulfinic acid formation using DMSO as the molecular probe for 50 and 100 million cells. Data showing mean ± SEM of three experiments. *****$P < 0.05$ compared to 0.

DISCUSSION

Cellular antioxidant enzymes and free radical scavengers normally protect a cell from toxic effects of ROS. But when generation of ROS overtakes the antioxidant defense and the balance between the ROS production and antioxidant defense is lost, then a series of events deregulate cellular functions and oxidative stress is caused. Thus there is tissue damage, leading finally to various pathological conditions [1, 4]. Small amounts of ROS are required for normal sperm functioning; however, disproportionate levels can negatively impact the quality of spermatozoa and impair their overall fertilizing capacity [40].

It is known that the secretion of seminal vesicles combines ascorbic acid (vitamin C) [17]. Vitamin C acts as an antioxidant and participates in the detoxification reactions; in concentrations below 100 mM, it protects spermatozoa from free radical damage as evidenced from improvement in their motility and viability [22]. It has been evidenced that treatment with ascorbic acid increases ejaculate volume, sperm concentration, total sperm output, total motile sperm, sperm motility index, packed sperm volume, initial pH, and semen initial fructose concentration. Abnormal and dead sperm were significantly decreased in ascorbic acid-treated animals [26].

Thus from the results of the present study it is revealed that the level of ascorbic acid is significantly less in the sperms of the cauda part of the epididymis. As the sperms of the cauda epididymis are mostly motile, so, this may be due to the consumption of the antioxidant (ascorbic acid) by the sperm cells to scavenge the free radicals produced during motility. From the four sets of experiments performed, no significant relation could be concluded regarding the levels of ascorbic acid in the sperms of the cauda epididymis and their motility status. The level of total antioxidant potential of the sperm cells of the cauda epididymis has been tested by our experiments. The sperm cells were found to be having significant amount of antioxidants as evidenced in their antioxidant potential results.

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COMPETING INTERESTS

The authors declare that they have no conflict of interest.

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