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SUPEROXIDE DISMUTASE ACTIVITY REGULATION BY SPERMINE : A NEW DIMENSION IN SPERMINE BIOCHEMISTRY AND SPERM DEVELOPMENT

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Amines are a group of highly important compounds of biological importance ; they are known promoters of cell growth, can complex with nucleic acids and can stimulate DNA-primed RNA polymerase activity. Spermine, a polyamine abundantly present in the secretions of the male accessory sex organs, has recieved no functional attributes till date. This study had been a pioneer attempt to validate the hypothesis of a metalloenzyme activity modulation by spermine and implicit correlations have been drawn.

Mammalian spermatozoa are known to possess superoxide dismutase (1), which is thought to play an important role in protecting cell against highly reactive univalently reduced oxygen i. e., $O_2^{\cdot -}$ (2). If the flux of superoxide anion radical exceeded the scavenging ability of the ambient level of superoxide dismutase, cellular damage would occur (3). Differences in the activity of superoxide dismutase and in the level of superxide anion radical generated by spermatozoa during epididymal maturation and capacitation have been studied (4). However the overall protection against oxygen radicals in the biological system seems to involve complementary defence mechanisms, the enzymic (SOD mediated) and the non enzymic (mediated mainly by thiol compounds viz., GSH, cystein) (5).

Important maturation-associated changes in spermatozoa of mammals that take place during their transit through epididymis include alterations both in head and tail, changes in surface properties and constituents and acquisition of sustained forward motility (6). Employing a biophysical technique viz., spin labeling, the plasma membrane of spermatozoa recovered from various segments of

Abbreviations,
SOD = superoxide dismutase, $O_2^{\cdot -}$ = superoxide anion radical, DTPA = Diethylene triamine pentacetic acid, HBSS = Hank's Balanced Salt Solution.

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epididymis viz., caput, corpus, cauda and after capacitation was characterized. We have already suggested that the loss of total superoxide dismutase activity and an increased superoxide anion radical generation could modulate the membrane fluidity in spermatozoa during epididymal maturation and capacitation (4). Recent work in our lab has elucidated the inhibitory effect of thiol compounds viz., reduced glutathione and cysteine on superoxide dismutase through their ligation with Cu (II) leading to the inactivation of this enzyme (5).

Polyamines appeare to be another class of active biomolecules exhibiting multifarious effects in living systems. Spermine and also spermidine and putrescine have been quantitatively determined in male accessory organs, secretions and entire seminal plasma of several mammalian species (7). Both spermine and spermidine are recognized promoters of cell growth and can serve as cations for complexing with nucleic acid. They are moreover able to stimulate the DNA primed RNA polymerase, but this type of stimulation though possibly of importance to the accessory organs is unlikely to be operative in spermatozoa since they are notoriously weak in histone proteins (8).

However, the precise role of spermine and other related bases in sperm development is still obscure. We hypothesize that these bases could act as enzyme modulators probably through mechanisms similar to those exhibited by thiol compounds. As the first step in checking the validity of this hypothesis, we have designed experiments to evaluate the effect of spermine on the activity of superoxide dismutase extracted from spermatozoa of various maturational status. A detailed doccumentation of our investigations on this aspect appears in this report.

MATERIALS AND METHODS

Reagents. Trizma HCl, Trizma Base, Diethylene triamine penta acetic acid (DTPA), N-2-hydroxyethyl piperazine-N-2-ethane sulphonic acid (HEPES), Triton X-100 and N, N'-bis [3-Aminopropyl]-1,4 butanediamine (spermine) were obtained from Sigma Chemical Company, USA. Pyrogallol was of Loba Chemie, India and all other chemicals were of reagent grades. Tris HCl buffer (50 mM, pH 8.2) was made by mixing 50 mM Trizma HCl and 50 mM Trizma base in a ratio of 1:2. 1mM DTPA (final concentration) was added to this solution and the pH was adjusted to 8.2 at $27^{0}C$.

Methods.

Sperm prepration. Adult male golden hamsters of the age group of 3-5 months housed separately in temperature ($27\pm1^{0}C$) and light (14h light :10h dark)

controlled rooms were used for experiments. Animals were sacrificed by cervical dislocation and epididymis along with vasa diferentia was excised and placed in a pertridish containing 5 ml warm (37°C) HBSS pH 7.2 (9). Adhering fatty tissues were cleared from the organs and the final washing was given in fresh buffer. All the three segments of epididymis were kept in separate watch glasses containing 1 ml of HBSS each. The spermatozoa were collected from caput and corpus epididymides by mincing which was then followed by filtering through 80u nitex screen and the sperm suspension was made upto 5 ml using fresh HBSS. The cauda spermatozoa were obtained by retrograde flushing through vas deferens using a 5ml syringe fitted with a 24 guage needle, after giving a slit over the blunt end of the cauda epididymidis. The sperm suspension thus obtained was filtered through a nitex screen (80u) and was transferred into 15 ml conical glass centrifuge tube. The final volume was made to 5 ml with fresh HBSS and sperm suspensions were centrifuged at 3,500 rpm for 10 minutes. Pellets were reconstituted in 5 ml HBSS and spermatocrits were computed.

Sperm capacitation <u>in vitro</u>. The spermatozoa were capacitated under <u>in vitro</u> conditions using Tyrode's medium (containing 10 mM sodium lactate and sodium pyruvate) (10). The spermatozoa collected from cauda epididymidis were diluted to 1 ml by adding Tyrode's buffer. This suspension was incubated under paraffin oil in a plastic tissue culture dish (Falcon Labware/USA) at 37°C for 6 hours. The spermatozoa were confirmed to have undergone capacitation by their hyperactivation. The capacitated spermatozoa thus obtained were used for experiments. All the experiments were repeated five times with fresh samles and observations were averaged.

Assay of superoxide dismutase activity of sperm homogenates. Sperm preparation was done as per earlier description, but HBSS was replaced by chilled Tris-HCl buffer (50mM pH 8.2) containing 1mM DTPA (a chelator of metal ions). Final reconstitution of the sperm pellets was made in 4 ml chilled Tris-HCl buffer, the sperm counts were made, spermatozoa were homogenized at 4°C at a speed of 13,000 rpm (2 cycles, 30 seconds each) using Polytron homogenizer with PT 10 accessory. The homogenates were treated with 1 ml of 1% Triton X-100 so that the final concentration of the detergent would be 0.2%. After treating for 30 minutes, the suspensions were centrifuged at a speed of 15,000 rpm at 4°C for 30 minutes using a Sorvoll OTD 65B Ultracentrifuge and a fixed angle rotor T-865.1. Pellets were discarded and supernatants were divided into two equal aliquots. One aliquot was analysed for superoxide dismutase activity based on the ability of the enzyme to inhibit autoxidation of pyrogallol (11). Control preparations were made by boiling the enzyme extract for 30 minutes in a water bath maintained at a temperature of 100° C. The effect of this heat inactivated extract on the rate of autoxidation of pyrogallol was also asayed.

Assay of SOD activity of sperm homogenate after spermine treatment. A fraction of each sperm sample was incubated with 10^{-3}M spermine (final concentration) for 1 hour and then subjected to superoxide dismutase assay, as described above. The effect of spermine on the pyrogallol autoxidation was also enumerated. The enzyme kinetics were monitored using an LKB Ultrospec 4050 Spectrophotometer with peripheral Apple 2e data acquistion system and Epson FX 800 printer. The software used was the Program Enzyme Kinetics (LKB Biochrom Inc, Cambridge) (12). All calculations were made on per sperm basis.

Statistical analysis. All the experiments were repeated five times and the mean of each observation along with its standard error was calculated.The results were subjected to Independent t -Test and the percentage inhibition of superoxide dismutase activity caused by spermine was compared.

RESULTS

In this study, we have assayed the total superoxide dismutase activity of the homogenates of spermatozoa from the various segments of the epididymis of

hamster, and have attempted to validate the hypothesis that this enzyme activity could be modulated by a biologically important amine, viz., spermine. All the sperm populations analyzed were found to possess superoxide dismutase activity. Whereas the enzyme activity was maximum in spermatozoa recovered from caput epididymidis of hamsters, there was a strong tendency for the loss of the superoxide dismutase activity with the advent of sperm maturation. Thus, there was a statistically significant loss of the activity of this enzyme during the passage of spermatozoa from caput to corpus ($p<0.30$), corpus to cauda ($p<0.10$) and while they undergo capacitation in vitro ($p<0.35$) (figure 1A).

The treatment of superoxide dismutase with 10^{-3}M spermine has yielded the following results. The superoxide dismutase extracted from caput and corpus spermatozoa underwent a considerable loss of its activity upon this treatment (Figure 1B). Eventhough the cauda spermatozoa were found to have relatively spermine-resistant class of superoxide dismutase, the in vitro capacitation of

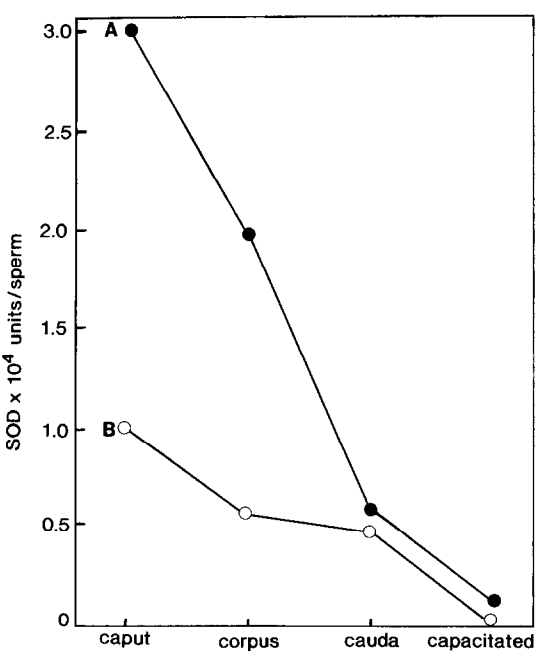

Figure 1(A). Changes in the levels of superoxide dismutase activity [●] of hamster spermatozoa during the course of epididymal maturation and capacitation. (B). Changes in the levels of superoxide dismutase activity [○] of sperm homogenates (after spermine treatment) recovered from various epididymal segments, viz.,caput, corpus, cauda and after capacitation. Data represented are the mean values ± standard error of the mean [s. e. m.] of five replicates taken. Experimental details are given under materials and methods section.

Table 1

Percentage inhibition and the significance limit of the superoxide dismutase activity after spermine action during sperm maturation and capacitation

Sample	% inhibition	Significance limit
Caput	66.12	$p<0.01$
Corpus	67.50	$p<0.15$
Cauda	28.57	$p<0.45$
Capacitated	70.00	$p<0.30$

these spermatozoa has led to its increased susceptibility to spermine action (Figure 1B). A tabular representation of the data is provided (Table 1).

DISCUSSION

In our earlier attempts to analyze some biochemical alterations during sperm maturation, we have witnessed that superoxide dismutase activity declines eventually as epididymal maturation progresses resulting in the induction of free radical species (13). High levels of oxygen radical have been shown to be involved in the maintenance of membrane fluidity (14), (15) and oxidative changes including increase in the level of unsaturation of phospholipids of sperm membrane, oxidation of thiol groups to disulphides and conversion of saturated phospholipids to unsaturated intermediates culminating the phospholipid bilayer of spermatozoon into a more fluid system, thereby facilitating the sperm membrane capable of sperm-egg interaction (16), (17).

Spermine and also spermidine and putrescine are believed to be involved in sperm maturation, even though the exact mechanism is unknown (18). It is known that when spermine is supplemented with ATP (*in vitro*) it increases the rate of fructolysis which may be due to facilitation of the sugar across the plasma membrane (19). Literature manifests the maturation favouring role of spermine in mammalian spermatozoa viz., it protects against lipid peroxidation, enhances sperm motility and inhibits conversion of proacrosin into acrosine. Spermine inhibitable microbial growth in human seminal plasma may be due to inhibitory effects on cell metabolism by oxidative products of spermine (20).

Now that we have discussed the importance of superoxide anion radical-superoxide dismutase system in sperm membrane configuration and also the available materials about the spermine biochemistry in relation to male reproductive system, the data presented herein can be aptly figured out. Once we discovered that a maturation -associated loss of superoxide dismutase is a pre-requisite for a successful sperm development, the question as to how the sperm cells achieve this floated up. Two probabilities could be envisaged: these cells could loose this very protein itself through some intricate expulsion mechanism or this protein could be retained by the sperm cells in some inactive forms. The latter speculation appeared to be more plausible since the spermatozoa dwell in an enviornment rich in biologically active amines and thiol compounds. This has led us to investigate the effect of spermine, a maturation - promoting amine present in abundance in seminal fluid, on the superoxide dismutase activity. The data presented herein indicates that spermine acts as a negative modulator of superoxide dismutase activity. This would in turn lead to elevation in the level of $O_2^{\cdot -}$ (13). But, the exact mechanism behind this action remains to be worked out. Notwithstanding the many unresolved problems it should be apparent now that the biochemistry pertinent to interactions between the accessory secretions themselves and those between the seminal plasma and spermatozoa deserves all out efforts of high research priority. As the first step, under the sequence of investigations in this direction, this study reveals a novel aspect of spermine-involvement during epididymal maturation and capacitation of mammalian spermatozoa.

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