Effect of nimbolide on rat spermatozoa under *in-vitro* condition: A study on sperm functional events and antioxidants during capacitation

Chaitra NT,[1] Ravindranath H. Aladakatti [2]* and Nandeshwarappa B. P[3]

Abstract

However, little evidence has been documented to evaluate the clear specific effects of neem-active ingredients in a rat model. This study aims to evaluate the in vitro effects of nimbolide (tetranortriterpenoids group, one of the main components of neem leaves), particularly on sperm functional studies, and biochemical and molecular events during capacitation in a dose-dependent manner. The results showed a dose- and time-dependent decrease in the functional consequence of capacitation process i.e., motility score, percentage of motile spermatozoa, Sperm Motility Index (SMI) and levels of molecular events in sperm followed by declined spontaneous acrosome reaction (AR), which subsequently leads to lesser binding of cauda epididymal sperm to the Zona pellucida (ZP). Biochemical studies have shown that the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase, Glutathione reductase (GR) and Glutathione peroxidase (GPx) decreased significantly while the levels of Hydrogen peroxide (H_2O_2) generation and lipid peroxidation (LPO) increased significantly in the treated groups indicating nimbolide-induced oxidative stress in rat epididymal sperm. This study confirmed that nimbolide convincingly inhibited sperm motility in rats by blocking certain biochemical pathways, such as energy utilization, and showed that sperm capacitation was associated with a reduction in AR, with changes in antioxidant enzymes reflecting a reduction at the levels of molecular events. This will lead us to produce a special product as a source of a new male contraceptive.

Keywords: Nimbolide, Spermatozoa, Motility, ATP content, Biochemical, AR and Rat

***Correspondence:** Dr. Ravindranath H. Aladakatti, Principal Research Scientist, Central Animal Facility, Indian Institute of Science (IISc), Bengaluru-560012, Karnataka, India. Email: ravindranath@.iisc.ac.in

[1]Department of Life Science, Bangalore University, Bengaluru-560056, Karnataka, India.

[2]Central Animal Facility, Indian Institute of Science, Bengaluru-560012, Karnataka, India.

[3]Department of Studies in Chemistry, Davangere University, Shivagangotri, Davangere - 577 007, Karnataka, India.

To cite: Aladakatti et al., (2024)) Effect of nimbolide on rat spermatozoa under *in-vitro* condition: A study on sperm functional events and antioxidants during capacitation, JLAS,7(1) pp10-23

Received - 05-09-2023 Revised -16-09-2023 Accepted - 20-09-2023

Introduction

Over the years many natural compounds of plant origin have been tested for their ability to inhibit male fertility. Many preparations and compounds with therapeutic effects and benefits have been introduced, providing sufficient support for researchers to search for more information on medicinal plants. The neem tree (*Azadirachta indica,* Syn: Melia azadirach: Meliaceae) has long been recognized for its unique properties. Phytochemical studies on the activities of biological and medicinal compounds obtained from different parts and extracts of this plant (Devi and Babu, 2023). The investigation of the compounds obtained from the neem plant, which is rich in antioxidant and free radical scavenging activity, has attracted the attention of the pharmaceutical industry, especially because of its perceptible success in the treatment of many diseases and its credible applications as contraceptive agents. Many secondary compounds of neem have been identified, purified, and tested for their effects on animals, some of which are biologically active such as nimbolide, azadirachtin, nimbinin, Zafaral, nimbin, solannin, nimbidinin, ascorbic acid, n-hexacosanol, and nimbiol (Kumar et al., 2018).

Nimbolide (5,7,4′-trihydroxy-3′, 5′-diprenylflavanone, Figure.1) is a tetranortriterpenoid, originally obtained from neem leaves and flower extract. This bioactive compound belongs to the limonoid group and has a classic limonoid skeleton with an α, β-unsaturated ketone system and δ-lactone nucleus (Anitha et al.,2006). Collected information on this compound and evidence suggests that nimbolide has many remedial uses, including antimalarial, antimicrobial, antifibrotic, anti-inflammatory, antioxidant, hepatoprotective, cardioprotective, and anticancer activities (Nagini et al., 2021). However, the toxicity of the compounds is questionable in therapeutic use; nimbolide has been shown to be no/less toxic in experimental animals (Glinsukon et al., 1986). Moreover, nimbolide has been shown to have antioxidant and free radical scavenging activities. Nimbolide has been shown to be a potent antioxidant compared to ascorbic acid (vitamin C) and azadirachtin (Priyadarsini et al., 2009). Earlier *in vivo*/*in vitro* studies of nimbolide showed changes in biochemical aspects of male reproductive function (Aladakatti et al., 2011); act as a spermicidal (Kumbar et al., 2012a), elicit depletion of the antioxidant defense system in spermatozoa (Kumbar et al., 2012b), and demonstrated its effect against carbon tetrachloride (CCl_4)-induced hepatotoxicity in rats (Baligar et al., 2014).

Although there is much information on the biological and pharmacological activity of bioactive compounds purified from neem in various animal models, there is no information on the effects of nimbolide on rat sperm from *in vitro* studies. Therefore, this study aims to investigate the *in vitro* effects of nimbolide on cultured rat spermatozoa, specifically on functional studies, total sperm ATP levels, sperm capacitation, AR, sperm-egg interaction, and antioxidant enzymes. In addition, it is intended to execute a pilot effort to evaluate the *in vitro* effects of nimbolide on molecular events important to the sperm capacitation process, including the determination of cyclic adenosine-3′,5′-monophosphate (cAMP) and calcium (Ca^{2+}) levels in sperm cultures for different time periods.

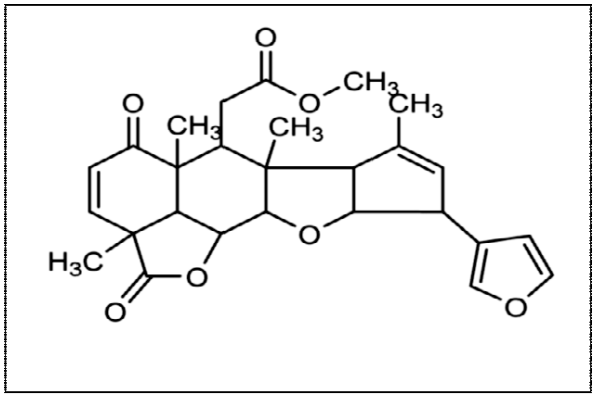

Figure 1. Chemical structure of nimbolide (5,7,4′-trihydroxy-3′, 5′-diprenylflavanone) (Adopted from Cohen et al., 1996)

Materials and methods

Animals

Healthy adult Wistar albino rats, approximately 3 months old, weighing 200 g, were used in the experiment. All animals were proven fertility and were maintained in individual polypropylene cages in the animal house facility with a 12:12h light: dark schedule. The temperature in the animal house was maintained at 23 ± 2°C and the relative humidity was between 50% and 70% throughout the study period. They were fed with a standard rat pellet diet and provided with water as required. Animals were acclimated to the laboratory conditions before experiments and were cared for in accordance with Committee for the Control and Supervision on Experiments on Animals (CCSEA) guidelines. Obtained necessary approval from the Institutional Animal Ethics Committee (IAEC) before performing animal experiments.

Chemicals and Reagents

Reagents were obtained as follows: Technical Nimbolide (purity ≥97%) purchased from SPIC Science Foundation, Tuticorin, India. Bioluminescent somatic cell ATP assay kit, [^{3}H] cAMP kit (Amersham, UK. Arsenazo III), Ca^{2+} estimation kit, equine chronic gonadotropin (eCG), and human chorionic gonadotropin (hCG) from Sigma diagnostics. Thiobarbituric acid and malondialdehyde, NADPH and glutathione oxidized (Sisco Research Laboratories), Deoxyribonucleic acid (DNA) and pyrogallol (Himedia Laboratories) and Castanospermine and p-nitrophenyl a-glucopyranoside (Sigma Chemical Company) were obtained from the laboratory of Division of Biological Science, IISc. All the other reagents used in the experiments were analytical reagents obtained commercially.

In vitro studies on rat spermatozoa

Sperm Support Medium

For *in vitro* studies, the culture medium was adapted (Toyoda and Chang method, 1974a) based on Krebs Ringer Bicarbonate, composed of 4.8 mM KCl, 1.2 mM KH_2PO_4, 95mM NaCl, 5.56 mM glucose, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 0.4% bovine serum albumin (BSA), and 2 mM pyruvate. Then filtered through a 0.22 µm filter and equilibrated with 5% CO_2 in air at 37°C to pH 7.4. Phenol red (2 mg/l) was added to the medium as a pH indicator Osmolarity was measured on 10 µl of medium repeated using an osmometer ranging from 280 to 300 mOsm (Hoos and Garside, 1996).

Spermatozoa collection

After animals were euthanized by cervical dislocation, cauda epididymis was excised and then spermatozoa were collected according to a modified method (Holloway et al., 1990). In brief, a small puncture was made in the distal portion of the cauda and a drop of the dense mass of sperm was placed in the master dish (60 mm) containing culture medium, incubated at 37°C in 5% CO_2 in air for approximately 5 min, and then allowed sperm to disperse and swim up the medium. Tissue fragments were removed from the medium, then 10µl aliquot of sperm suspensions, approximately one million spermatozoa (1 x $l0^6$ sperm/ml) were collected and shifted into 35 mm dishes containing 400µl of culture medium and evaluated for sperm motility. During sperm motility assessment, only those sperm presenting vigorous motility in suspensions were

employed using Olympus Stereo Zoom Microscope.

Stock solution of nimbolide

Nimbolide (5 mM) solution was dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C, then diluted with culture medium to the desired concentration before use.

Incubation of spermatozoa with nimbolide

10µl aliquot of sperm suspension was in use, diluted in a 1:20 ratio in sperm dilution buffer (0.6M $NaHCO_3$, 40%HCHO, pH 7.2-7.4) and placed in the counting chamber of Neubauer hemocytometer. At this time, a number of sperms were counted, approximately 1x $l0^6$ (sperm/ml) were added to culture medium dishes and then incubated in a humidified environment of 5% CO_2 in air at 37ºC. To simulate the situation in vivo as much as possible, it is necessary to adapt some *in vitro* research methods. Hence, for *in vitro* sperm functional studies and further studies, mM concentrations of nimbolide were designed and employed according to the Glinsukon et al, (1986) method. In this study, nimbolide (5mM) was added to the culture medium at an appropriate concentration (0.5, 1.0, 1.5, and 2.0 mM/ ml) by correcting pH to 7.4. In contrast, an equal amount of 50% DMSO was added to those culture medium dishes served as a control. Aliquots of control and treatment of each sperm suspension were obtained at different time points for motility, biochemical parameters, and other analyses. All experiments were repeated each time, and each concentration was carried out in five different experiments.

Assessment of sperm morphology and viability

Sperm suspensions from both control and graded concentrations of nimbolide treated were then observed individually within 90-120 secs after the addition of the 10µl stain and observed under a phase contrast microscope using eosin-Y stain to trace any change in the sperm morphology (Jeyendran *et al.,* 1984, WHO,1999). Correspondingly, for sperm viability evaluation, one drop of each sperm suspension mixture, control and treated, was mixed with two drops of 1% eosin Y. Following 30 sec, three drops of 10% nigrosin solution were added and mixed. A drop of each of the control and treated sperm eosin-nigrosin mixture was observed under a microscope. Unstained spermatozoa are considered live sperm, and stained spermatozoa are considered dead spermatozoa (WHO,1999).

Sperm motility analysis

According to the method described by Bavister and Andrews (1988) and the modified method by Ain et al. (1999), the proportion of sperm motility and the quality of motility of control and treated samples were evaluated for each 1h for the period of 5h under the Olympus Stereo Zoom Microscope. The quality of sperm motility is graded as follows: 0- if not moving; 1- if is twitching or weak in moving forward; 3- is good in moving forward; 4- is vigorous, rapid without hyperactivation, and 5- is a hyperactivation. The sperm motility index (SMI) is defined as the percentage of motile sperm X [quality score, 0-5]^2. An SMI value of ≥1000 is an indicator of sperm capacitation.

Determination of ATP content of spermatozoa

Aliquots of 50µl, equivalent to 0.05 million spermatozoa each, were obtained from the cultures of control and treated samples at intervals of 1, 3, and 5 h, and their total ATP content was measured using a bioluminescence assay kit by the manufacturer's instructions. Briefly, to hydrolyze an endogenous ATP, 100µl of the ATP assay mix was taken in an Eppendorf tube (vial A) and allowed to stand for about 3 min. At the same time, 50µl of Milli-Q water and 50µl of the sample were added to 100µl of ATP-releasing reagent in a separate tube (vial B). From this vial B, 100µl of this mix was taken and added to vial A (the ATP assay mix). After spinning briskly, the amount of light emitted was measured with a luminometer (TD-20/20, Turner Designs, Sunnyvale, CA). By repeating this procedure using an internal ATP standard, the amount of ATP released was measured and expressed in terms of ATP per million sperm.

Determination of Ca2+ and cAMP levels in spermatozoa

Aliquots were obtained from the cultures of control and treated samples at intervals of 1, 3, and 5 h and centrifuged at 3000 rpm for 15 min. To determine Ca^{2+} levels, the sperm pellets were lysed in 0.01 M phosphate-buffered saline buffer (PBS) containing 4% sodium dodecyl sulphate (SDS), at 4 °C for 30 min, and then sonicated for 60 seconds on ice. Homogenate was centrifuged at 3000 g for 10 min. Ca^{2+} was estimated in the supernatants using the Arsenazo III kit, where the intensity of the purple complex formed with the reagent was read at 600 nm in a spectrophotometer. The protein content of the pellet was estimated by the modified method of Lowry (1951).

For sperm cAMP level estimation, the cAMP estimation kit (Amersham) was used. Briefly, the sperm pellet was washed three times with 0.05 M Tris- Ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.4) and 10% trichloroacetic acid (TCA), prepared in the same buffer, then sonicated for 60 seconds on ice. Centrifuged the extract at 3000 g for 15 minutes and the supernatant was washed with four volumes of water-saturated ether to remove TCA (Hammerstedt and Hay, 1980). The neutralized samples (0.6-1 mg protein) were assayed for cAMP. The samples and standards (0.2-16 pmoles) were incubated with [^{3}H] cAMP at 4 °C for 15h. The charcoal extracted, [^{3}H] cAMP bound to a specific binding protein, was counted in a β counter using Bray's scintillation fluid. TCA-precipitated proteins in the residues were estimated following Lowry's method (1951).

Biochemical analysis

Control and nimbolide treated aliquots of each sperm suspension were obtained at different time points, centrifuged at 225 g for 10 min at 4°C and the pellet was resuspended in the normal saline. Homogenized the sperm pellet with the help of a glass-Teflon homogenizer for a few seconds and centrifuged at 800 g at 4 °C for 10 mins. The supernatant was used for further biochemical studies. Protein was estimated by the method of Lowry et al. (1951) and DNA by the method of Burton (1956). The antioxidant parameters were estimated by the following methods.

Superoxide dismutase (SOD): SOD was analyzed by the method of Marklund and Marklund (1974). Briefly, the assay mixture contained 2.4 ml of 50 mM Tris HCl (50mM) buffer containing 1 mM EDTA (pH 7.6), 300 µl of 0.2 mM pyrogallol (0.2mM), and 300 µl enzyme source. The increase in absorbance was measured instantly at 420 nm against a blank containing all the components except the enzyme source and pyrogallol at 10-second intervals for 3 min on a Systonics Spectrophotometer. The enzyme activity was expressed as nanomoles pyrogallol oxidized /min/mg protein or mg DNA at 32°C.

Catalase: Catalase was assayed by the method of Claiborne (1985). Briefly, the assay mixture contained 2.4 ml phosphate buffer (50 mM, pH 7.0), 10 µl of 19 mM hydrogen peroxide, and 50 µl enzyme source. The decrease in absorbance was measured instantly at 240 nm against a blank containing all the components except the enzyme source at 10 s intervals for 3 min on a Systronics Spectrophotometer. The enzyme activity was expressed as micromoles H_2O_2 consumed /min/mg protein or mg DNA at 32°C.

Glutathione reductase (GR): GR was determined by the method of Carlberg and Mannervik (1985). Briefly, the assay mixture contained 1.75 ml phosphate buffer (100 mM, pH 7.6), 100 µl of 200 mM NADPH, 100 µl of 10 mM EDTA, 50 µl of 20 mM oxidised glutathione, and 50 µl enzyme source. Loss of NADPH was measured instantly at 340 nm against a blank containing all the components except the enzyme source at 10-second intervals for 3 min on a Systronics Spectrophotometer. The enzyme activity was expressed as nanomoles of NADPH oxidised /min/mg protein or mg DNA at 32°C.

Glutathione peroxidase (GPx): GPx was assayed by the method of Mohandas et al. (1984). Briefly, the assay mixture contained 1.59 ml phosphate buffer (100 mM, pH 7.6), 100 µl of 10 mM EDTA, 100 µl sodium azide, 50 µl glutathione reductase, 100 µl reduced glutathione, 100 µl of 200 mM NADPH, 10 µl hydrogen peroxide, and 10 µl enzyme source. Loss of NADPH was measured instantly at 340 nm against a blank containing all the components except the enzyme source at 10-second intervals for 3 min on a Systronics Spectrophotometer. The activity of the enzyme was expressed as nanomoles NADPH oxidised /min/mg protein or mg DNA at 32°C.

Hydrogen peroxide (H_2O_2) generation assay: H_2O_2 generation was assayed by the method of Pick and Keisari (1981). Briefly, the incubation mixture contained 1.641 ml phosphate buffer (50 mM, pH 7.6), 54 µl horse radish peroxidase (8.5 units/ml), 30µl of 0.28 nM phenol red, 165µl of 5.5nM dextrose, and 600µl of enzyme source, incubated at 32°C for 30 min. The reaction was stopped by the addition of 60µl of 10N sodium hydroxide. Absorbance was read at 610 nm against a reagent blank on a Systronics Spectrophotometer. The quantity of H_2O_2 produced was expressed as nanomoles H_2O_2 produced /min/mg protein or mg DNA at 32°C. To make the standard curve, known amounts of H_2O_2 and all the above reagents except the enzyme source were incubated for 30 min at 32°C before the addition of 60 µl of NaOH (10 N), and optical density was read at 610 nm.

Lipid peroxidation (LPO): Thiobarbituric acid reactive substance (TBARS), a breakdown product of lipid peroxidation, was determined by the method of Ohkawa et al. (1979). Briefly, the stock solution contained equal volumes of 15% (w/v) TCA in 0.25 N HCl and 0.37% (w/v) 2-thiobarbituric acid in 0.25 N HCl. One volume of the test sample and two volumes of stock reagent were mixed in a screw-capped centrifuge tube, vortexed, and heated for 15 min in a boiling water bath. After cooling on ice, the precipitate was removed by centrifugation at 1000 g for 15 min, and the absorbance of the supernatant was measured at 532 nm against a blank containing all the reagents except the test sample. The value was expressed as micromoles malondialdehyde (MDA) produced/min/mg protein or mg DNA at 32°C. A standard curve was created by adding the amount of MDA in the product to the absorbance measurement.

α-Glucosidase: The activity of α-Glucosidase was assayed by the method of Cooper et al. (1988). Briefly, the assay mixture containing 10µl enzyme source, 16µl castanospermine (1 mM), and 200 µl p-nitrophenyl a-glucopyranoside was incubated at 32°C for 2 h. The reaction was stopped by the addition of 2 ml sodium carbonate (100 mM). Corresponding set of controls was maintained without the addition of castanospermine. The absorbance at 405 nm was read at 60 min against a blank reagent on a Systronics spectrophotometer. A standard curve was prepared using graded concentrations of p-nitrophenol. Enzyme activity is expressed as micromoles p-nitrophenol / min/mg protein or mg DNA at 32°C.

Assessment of AR of spermatozoa

Eosin-Y staining (0.5% wt/vol) was performed for viable spermatozoon by mixing 10µl cultured spermatozoa with 10µl of the stain on a microscope slide, covered with a cover slip and analyzed by light Microscope at 400X magnification. During the evaluation, three populations of spermatozoa were distinguished as acrosome intact and live, acrosome reacted, and live and dead spermatozoa from the different points of culture. The proportion of acrosome-reacted spermatozoa, which were viable during the process, was assessed at different time points for the presence or absence of an acrosomal cap. The results were presented as the proportion of acrosome-reacted live sperm in five different experiments.

Egg Preparation

Immature female rats were superovulated by an injection (intraperitoneal) of 10 international units (IU) of equine chronic gonadotropin (eCG), followed by an injection (subcutaneous) of 25 IU of human chorionic gonadotropin (hCG) 54 h later. Ovulated oocytes were obtained from the oviducts 13–14 h after hCG administration. The cumulus cells were removed by incubating the oocyte-cumulus complex for 3 min in a medium containing 0.1% hyaluronidase type IV. Zona-intact oocytes were thoroughly washed in a culture medium and distributed among treatment groups.

Sperm-Egg Binding Assay

Cauda epididymal sperm (2×10^6 cells/ml) were incubated with oocytes with the ZP in drops of 100 µl fertilization

medium in tissue culture dishes under mineral oil at 37°C in an atmosphere of 5% CO_2 in air. Incubation was performed in the absence or presence of graded concentrations of nimbolide (0.5 to 2.0 mM/ml, respectively) for 5h and the average number of oocytes with sperm bound to the ZP was analyzed according to Toyoda and Chang (1974b) protocol. After incubation, eggs were removed and transferred serially through several drops of fresh fertilization medium to remove loose sperm attached to the egg surface. After washing, oocytes were mounted on slides. The corners of the coverslips were coated with a Vaseline/paraffin mixture and the number of sperm attached to an egg was scored under a phase contrast microscope at 400 X magnification.

Statistical analysis

All statistical analyses were performed with analysis of variance (ANOVA) followed by Dunnett's post hoc test to compare all treatments with the control group. For the AR study, two-way analysis of variance (ANOVA) was used for comparisons within multiple groups, followed by the Bonferroni test. Results are expressed as the mean ± SEM of five different experiments. $P \leq 0.01$ was considered to indicate a significant difference. All tests were performed using Graph Pad Prism version 5.00 for Windows (Graph Pad Software, San Diego, CA, USA).

Results

Effect of nimbolide on sperm morphology and viability

To confirm that the effects of nimbolide on sperm capacitation were not due to any toxic substance, morphology, and viability assessments were carried out in the presence of graded concentrations of nimbolide. No changes in sperm morphology were observed in all nimbolide groups (Figure.2A and B); however, the percentages of viable cells of the control vs. treated groups were comparable (Figure.2C). At higher nimbolide concentrations of 1.5 and 2.0mM, nimbolide showed significant effects on sperm viability in all-time course of culture periods when compared to controls. 0.5 and 1.0mM treatments did not show significant effects till the 3h of incubation, however during the later time points, showed significant effects on viability.

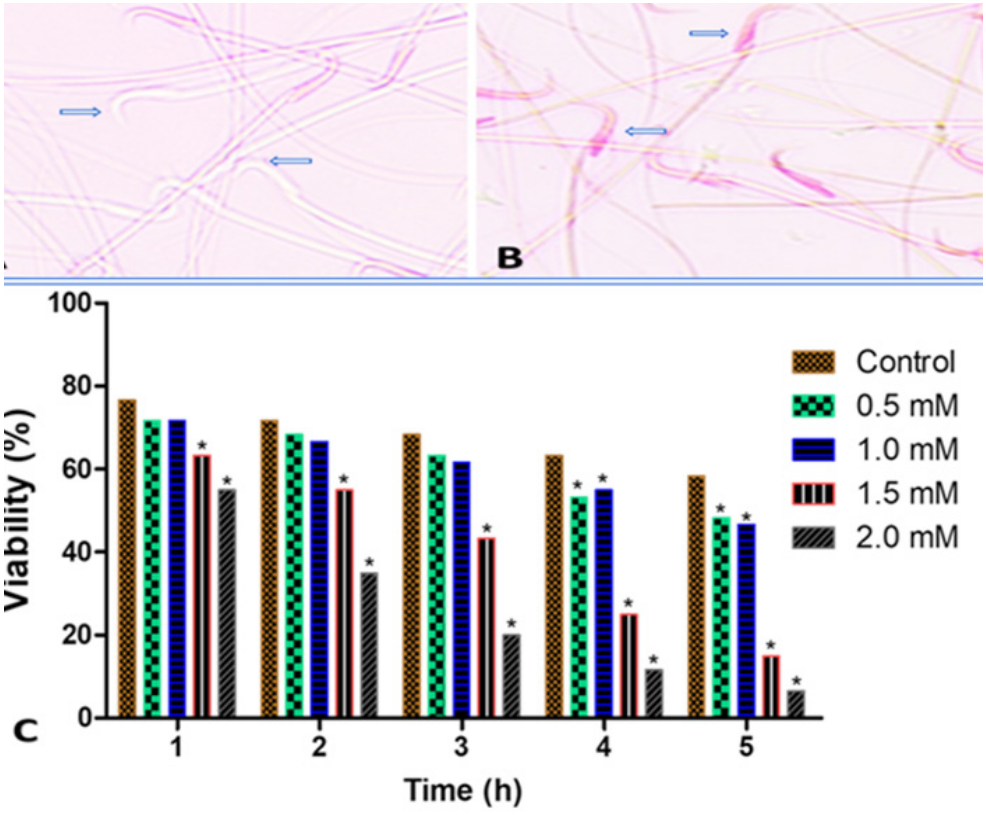

Figure 2A-C. Effect of nimbolide on morphology and viability of rat spermatozoa. Control and graded concentrations of nimbolide (0.5 to 2.0 mM/ml, respectively) treated sperm were incubated separately in sperm culture medium and assessed for a period of 5h. The percentage of viable spermatozoa was assessed by 0.5% eosin-Y staining method (C). Sperm Viability: Viable (A) and Dead (B) Sperms (X 400 magnification). Values are mean ± SEM (n=5). A significant difference ($P \leq 0.01$) is indicated (asterisks).

Effect of Nimbolide on capacitation in cultured rat spermatozoa

To evaluate the effect of nimbolide on rat sperm capacitation in rats, graded concentrations of nimbolide (0.5 to 2.0 mM/ml, respectively) were supplemented to the cultured medium before the adding of cauda epididymal spermatozoa. The sperms were examined each 1 h for the period of 5h and the percentage of motile sperm, motility score, and SMI were recorded. There was a grade-dependent effect on the number of motile spermatozoa and the quality of motility in treated spermatozoa (Figure 3A and 3B). Both motile sperm percentage and motility score with time resulted in reduction in the SMI values (Figure.3C) of higher concentrations ($P \leq 0.01$). However, 1.5 mM caused a significant ($P \leq 0.01$) inhibition of sperm motility after 4h post-culture. Whereas 2.0 mM caused an absolute inhibition of sperm motility (SMI = 0) after the 4h post-culture.

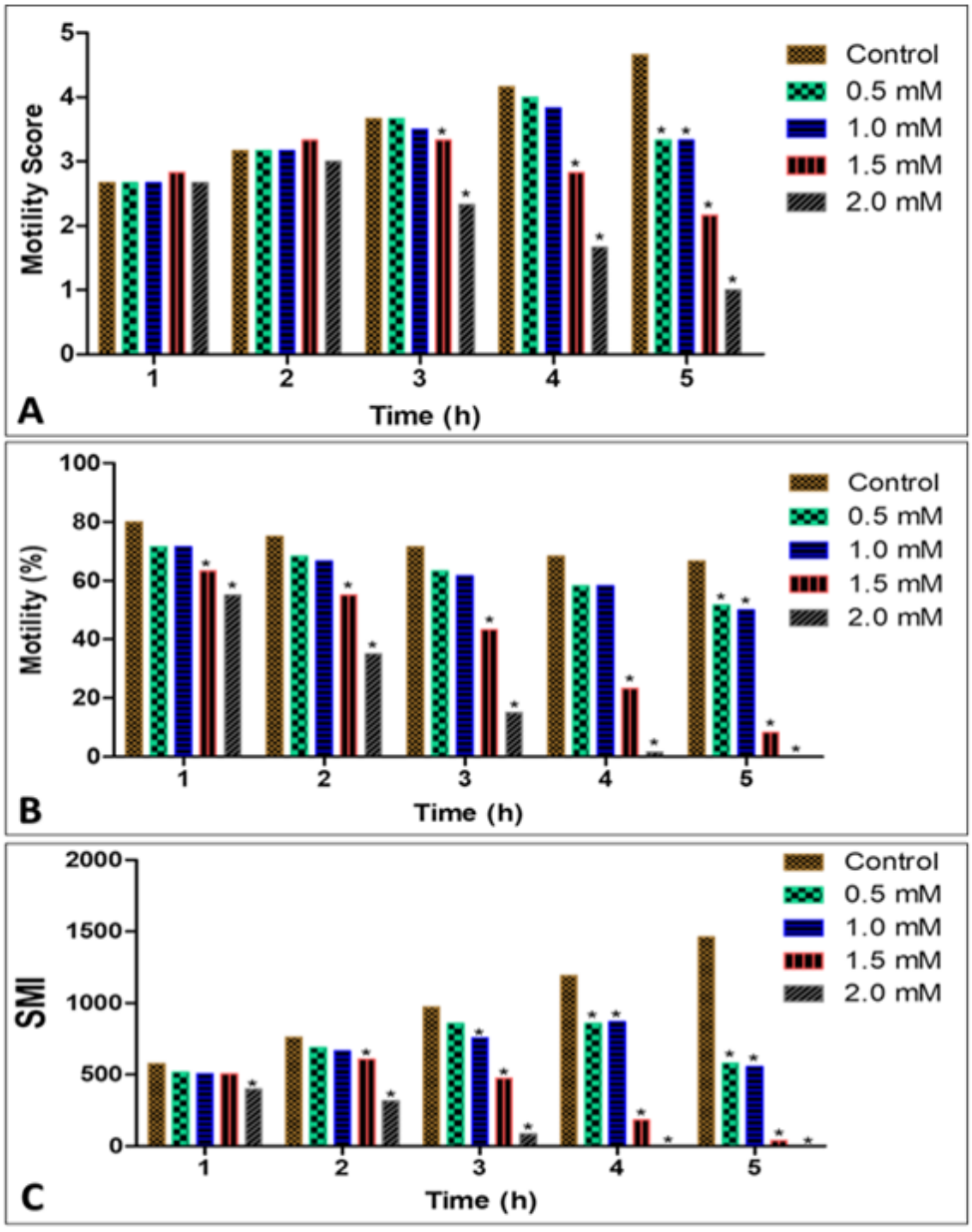

Figure 3A-C. Effect of nimbolide on rat sperm motility score (A), percentage motility (B), and sperm motility index (SMI) in rats. Control and graded concentrations of nimbolide (0.5 to 2.0 mM/ml, respectively) treated sperm were incubated separately in sperm culture medium and assessed for a period of 1 to 5h. Values are mean ± SEM (n=5). A significant difference ($P \leq 0.01$) is indicated (asterisks).

0.5 mM and 1.0 mM nimbolide groups did not show any effect on sperm motility and underwent hyperactivation/capacitation during the 1-3h of sperm culture. However, after 4h post-culture onwards, spermatozoa show inhibition of sperm motility and do not hyperactivate/capacitate, resulting in a reduction in the percentage of motile sperms and scores when compared to control. Whereas in 1.5 mM nimbolide, sperm motility did not show inhibition until 2h of incubation, but later, there was an extreme decline in the percentage of motile sperms and scores and did not undergo capacitation. However, at 2.0 mM there was a significant inhibition (P≤0.01) on the percentage of sperm motility within the 1h of culture and incapacitation, resulting in a significant reduction (P≤0.01) in SMI values.

Effect of nimbolide on ATP content in cultured rat spermatozoa

To ensure the decline of sperm viability, motility (score and percentage), and SMI values in nimbolide treatment thereby leading to an energy-deficient state of spermatozoa, the total ATP content of spermatozoa was evaluated. Luminometric assay of ATP levels indicated that in the control spermatozoa, it was in the range of 2.5-3.8 nmoles/10^6 cells. During early 1h, sperm groups treated with graded concentrations of nimbolide (0.5 to 1.5 mM/ml, respectively) did not affect total sperm ATP content, and 2.0 mM nimbolide was lower than those in the control sample ($P \leq 0.01$). However, from 3h of incubation onwards, all graded concentrations exhibited a significant decline in total sperm ATP content when compared to controls ($P \leq 0.01$) during the *in vitro* sperm capacitation study(Figure 4A).

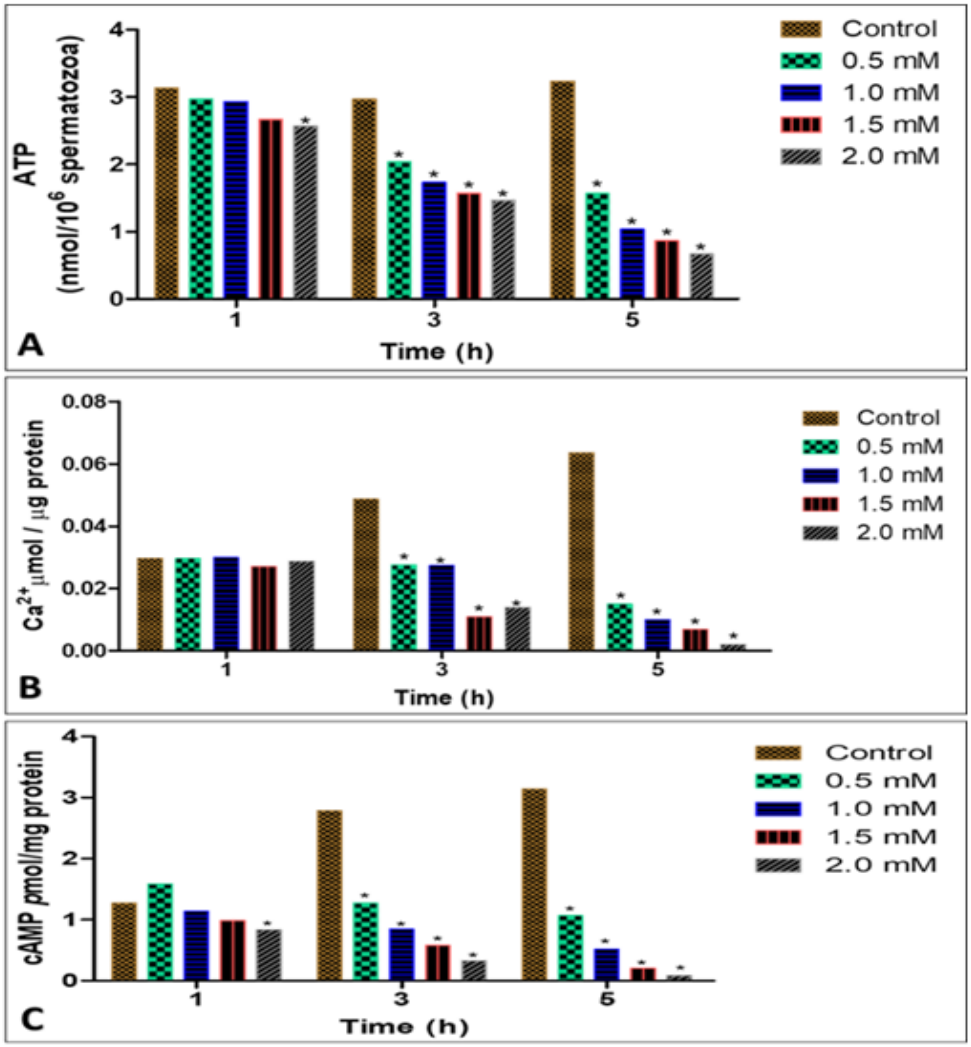

Figure 4A-C. Effect of nimbolide on ATP content, Ca2+ and cAMP levels of rat spermatozoa. Control and graded concentrations of nimbolide (0.5 to 2.0 mM/ml, respectively) treated sperm were incubated separately in sperm culture medium and assessed for a period of 1h, 3h, and 5h respectively. The values are represented as ATP content/106 spermatozoa for ATP (A) and Ca2+ and cAMP levels (B and C), the values are represented as Ca2+ content/106 spermatozoa. Values are mean ± SEM (n=5). A significant difference ($P \leq 0.01$) is indicated (asterisks).

Effect of nimbolide on Ca2+ and cAMP levels in cultured rat spermatozoa

In this study, nimbolide showed a clear dose-dependent inhibition of these molecular events during sperm capacitation and the levels of sperm Ca^{2+} and cAMP expressed as µmol/µl protein and *p*mol/µg protein respectively. 3h of incubation onwards, the levels of Ca^{2+} (Figure.4B) and cAMP (Figure.4C) of rat spermatozoa decreased significantly ($P \leq 0.01$) at higher concentrations (0.5 to 1.5 mM/ml, respectively) when compared to controls. However, during early 1h, the cAMP levels were lower than those in the control sample ($P \leq 0.01$) during the in vitro sperm capacitation study.

Effect of nimbolide on antioxidant parameters in cultured rat spermatozoa

To study the *in vitro* effect of nimbolide on the activities of SOD, catalase, GR, GPx, α-Glucosidase, H_2O_2 production, and LPO levels in rat spermatozoa, graded concentrations of nimbolide (0.5 to 2.0 mM/ml, respectively) were supplemented to the cultured medium before the adding of cauda epididymal spermatozoa. Sperm were analysed after 5h culture and expression of antioxidant parameters were recorded.

SOD activity: Effect of nimbolide on the SOD activity of rat spermatozoa which expressed as nanomoles pyrogallol oxidized min/mg protein or mg DNA at 32°C (Figure.5A and B). The SOD activity (min/mg protein) decreased significantly

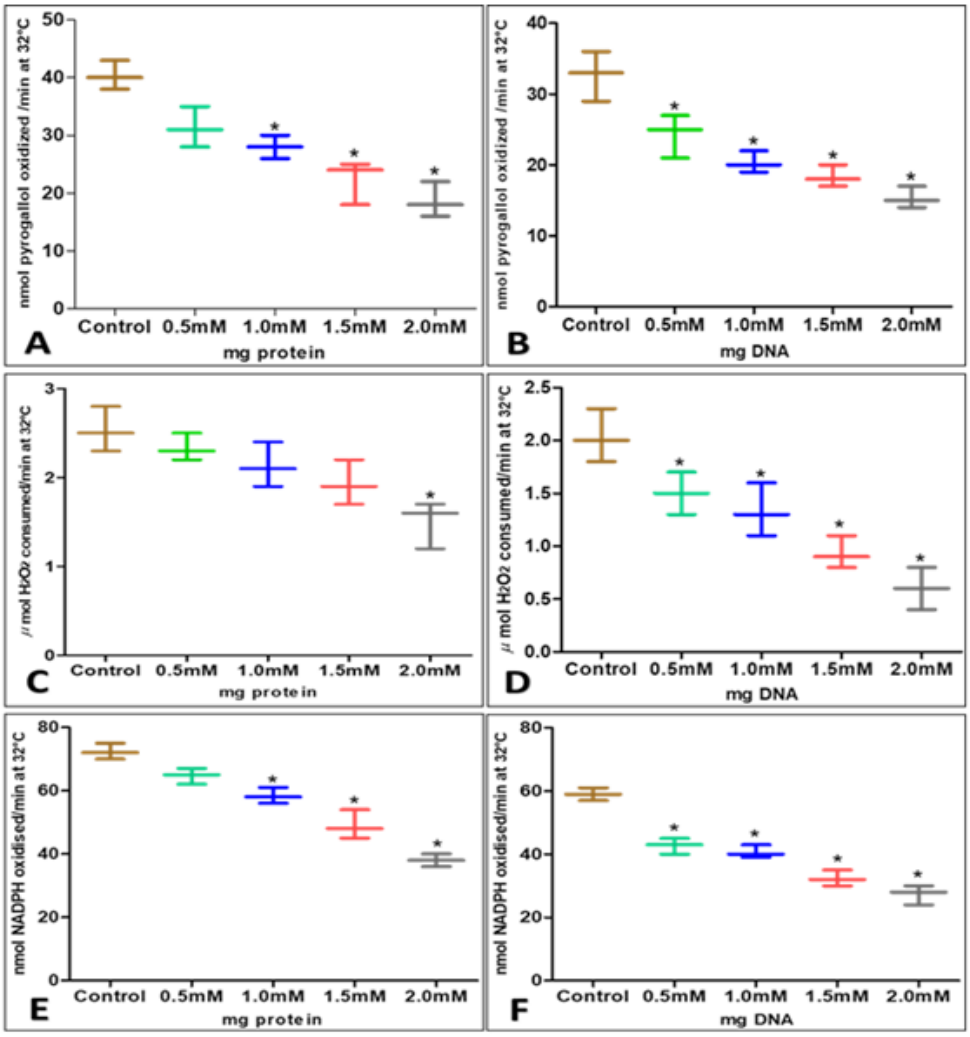

Figure 5A-F. Effect of nimbolide on the SOD, Catalase and GR activities of rat spermatozoa. Control and graded concentrations of nimbolide (0.5 to 2.0 mM/ml, respectively) treated sperm were cultured and assessed for a period 5h. SOD enzyme activity (5A and B) is expressed as nanomoles pyrogallol oxidised/min/mg protein or mg DNA at 32°C. Catalase enzyme activity(5C and D) is expressed as micromoles H_2O_2 consumed / min/ mg of protein or mg DNA at 32°C and GR enzyme activity(10E and F) is expressed as nanomoles NADPH oxidised /min/ mg protein or mg DNA at 32°C. Values are mean ± SEM (n=5). A significant difference ($P \leq 0.01$) is indicated (asterisks).

($P \leq 0.01$) at higher levels of nimbolide (1.0 to 2.0 mM/ml, respectively). However, there was no change in SOD at 0.5 mM nimbolide compared to control. Whereas SOD activity (min/mg DNA) decreased significantly ($P \leq 0.01$) in all concentrations of nimbolide-treated spermatozoa against control.

Catalase activity: Effect of nimbolide on the catalase activity of rat spermatozoa which expressed as micromoles H_2O_2 consumed min/mg protein or mg DNA at 32°C (Figure 5C and D). The catalase activity of rat spermatozoa (min/mg protein) decreased significantly ($P \leq 0.01$) at the high concentration of 2.0 mM of nimbolide. However, the rest of the graded concentrations (0.5 to 1.5 mM/ml, respectively) did not show any change in catalase compared to the control. Whereas catalase activity (min/mg DNA) decreased significantly ($P \leq 0.01$) in all concentrations of nimbolide-treated spermatozoa against to control.

GR activity: Effect of nimbolide on the GR activity of rat spermatozoa which expressed as nanomoles NADPH oxidized min/mg protein or mg DNA at 32°C (Figure 5E and F). The GR activity of rat spermatozoa (min/mg protein) decreased significantly ($P \leq 0.01$) at higher levels of nimbolide (1.0 to 2.0 mM/ml, respectively). However, this enzyme activity at the lower concentration (0.5 mM/ml) did not show any change when compared to control. Whereas GR activity (min/mg DNA) decreased significantly ($P \leq 0.01$) in all concentrations of nimbolide-treated spermatozoa against control.

GPx activity: Effect of nimbolide on the GPx activity of rat spermatozoa which expressed as nanomoles NADPH oxidised min/mg protein or mg DNA at 32°C (Figure.5G and H). GPx activity of rat spermatozoa (min/mg protein) decreased significantly ($P \leq 0.01$) at the higher concentration of 2.0 mM of nimbolide. However, the rest of the graded concentrations (0.5 to 1.5 mM/ml, respectively) exhibit no changes in GPx when compared to controls. The GPx activity (min/mg DNA) decreased significantly ($P \leq 0.01$) at 1.5mM and 2.0 mM of nimbolide respectively, whereas the rest of the lower concentrations showed no changes in GPx when compared to controls.

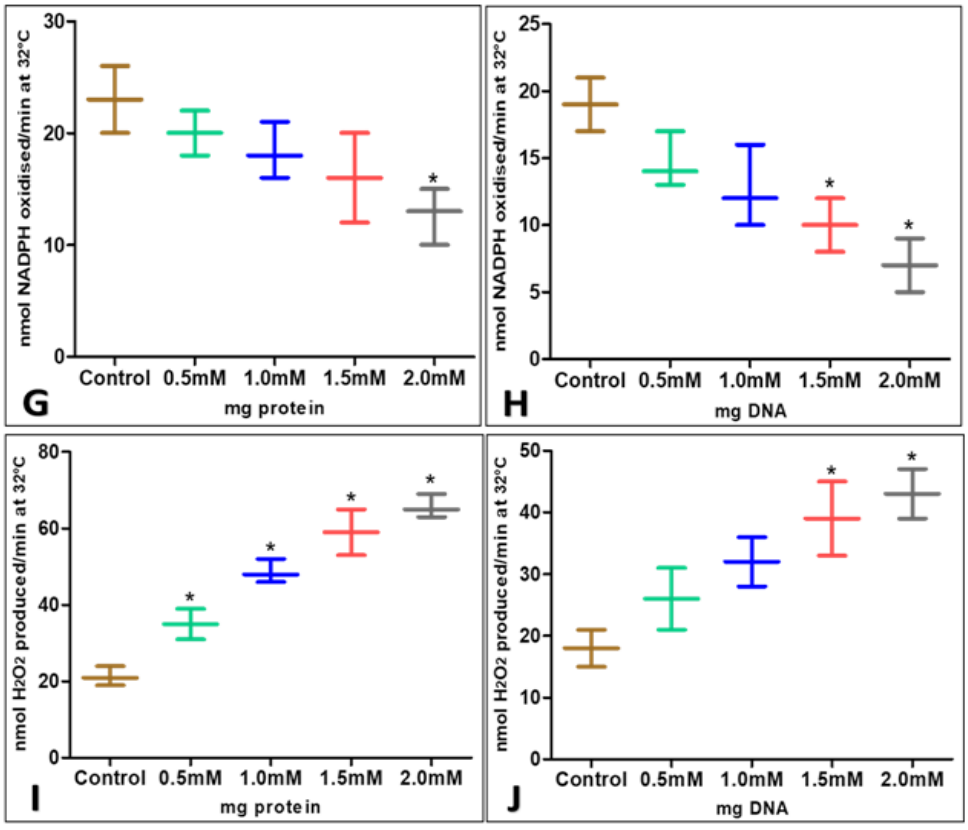

Figure 5G-J. Effect of nimbolide on the GPx activity and H_2O_2 generation of rat spermatozoa. Control and graded concentrations of nimbolide (0.5 to 2.0 mM/ml, respectively) treated sperm were cultured and assessed for a period of 5h. GPx enzyme activity (5G and H) is expressed as nanomoles NADPH oxidised /min/ mg protein or mg DNA at 32°C and for H_2O_2 generation assay (5I and J), enzyme activity is expressed as nanomoles H_2O_2 generated / min/mg protein or mg DNA at 32°C. Values are mean ± SEM (n=5). A significant difference ($P \leq 0.01$) is indicated (asterisks).

H_2O_2 generation assay: Effect of nimbolide on the H_2O_2 production of rat spermatozoa and the quantity of H_2O_2 produced was expressed as nanomoles H_2O_2 generated min/mg protein or mg DNA at 32°C (Figure.5I and J). The H_2O_2 generation of rat spermatozoa (min/mg protein) increased significantly ($P \leq 0.01$) in all concentrations of nimbolide-treated spermatozoa against to controls. Whereas H_2O_2 generation (min/mg DNA) increased significantly ($P \leq 0.01$) at the higher dose levels of nimbolide (1.5mM and 2.0 mM/ml, respectively). However, the rest of the lower concentrations showed no changes in the production of H_2O_2 when compared to controls.

LPO activity: The effect of nimbolide on the LPO activity and value was expressed as micromoles malondialdehyde (MDA) produced min/mg protein or mg DNA at 32°C (Figure 5K and L). LPO activity of rat spermatozoa (min/mg protein) increased significantly ($P \leq 0.01$) in all concentrations of nimbolide-treated spermatozoa against controls. However, LPO activity (min/mg DNA) increased significantly ($P \leq 0.01$) at the higher dose levels of nimbolide (1.5 mM and 2.0 mM/ml, respectively). Whereas the rest of the lower concentrations showed no changes in LPO activity when compared to controls.

α-Glucosidase activity: Effect of nimbolide on the α-Glucosidase activity of rat spermatozoa which expressed as micromoles p-nitrophenol produced min/mg protein or mg DNA at 32°C (Figure.5M and N). The α-Glucosidase activity of rat spermatozoa (min/mg protein or min/mg DNA) did not show any changes in graded concentrations (0.5 to 1.5 mM/ml, respectively) of nimbolide-treated spermatozoa against controls. However, this enzyme activity (min/mg protein or min/mg DNA) decreased significantly ($P \leq 0.01$) at the higher concentration of 2.0 mM of nimbolide when compared to controls.

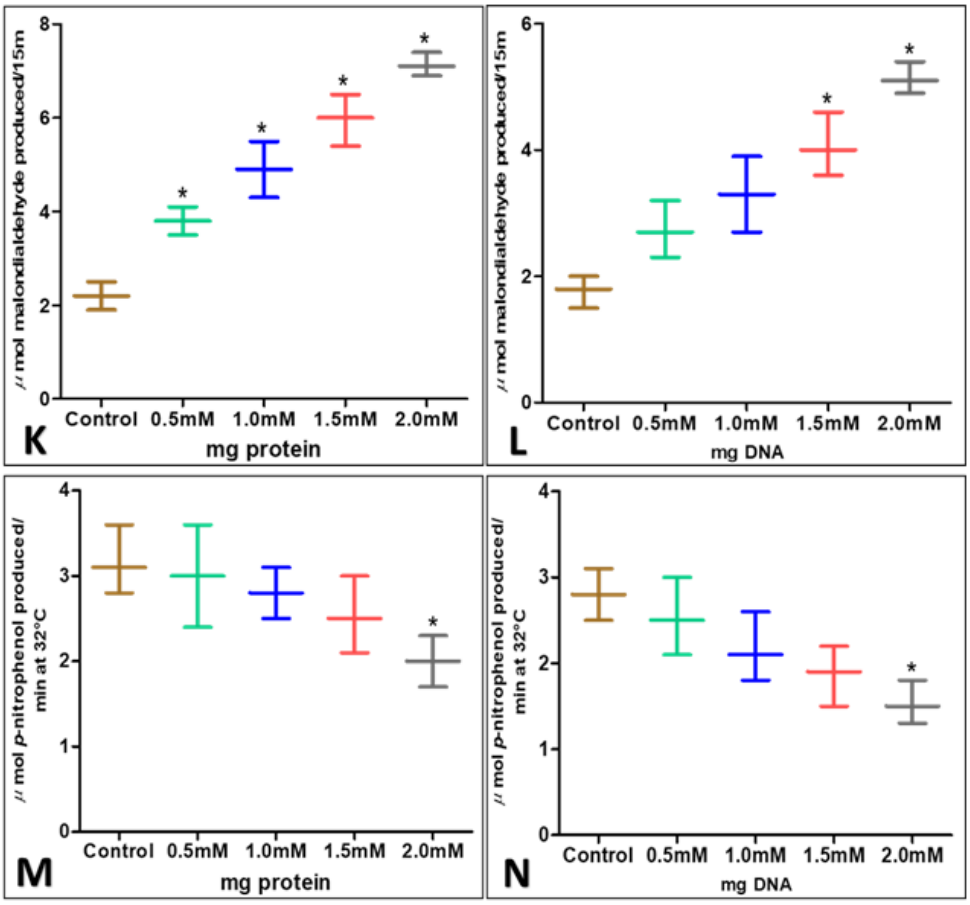

Figure 5K-N. Effect of nimbolide on the LPO and α-Glucosidase activities of rat spermatozoa. Control and graded concentrations of nimbolide (0.5 to 2.0 mM/ml, respectively) treated sperm were cultured and assessed for a period of 5h. LPO enzyme activity (5K and L) is expressed as micromoles MDA formed /min /mg protein or mg DNA at 32°C and α-Glucosidase enzyme activity (5M and N) expressed as micromoles p-nitrophenol produced/ min/mg protein or mg DNA at 32°C. Values are mean ± SEM (n=5). A significant difference ($P \leq 0.01$) is indicated (asterisks).

Effect of nimbolide on acrosomal status in cultured rat spermatozoa

To evaluate the nimbolide supplement on AR in viable sperm during different point of culture, graded concentrations of nimbolide treated and control sperm were taken and analyzed (Figure.6A). 0.5 mM and 1.0 mM of nimbolide exhibited no inhibitory effect on AR of viable spermatozoa during 1- 4h of *in vitro* sperm cultures and underwent hyperactivation/capacitation, whereas in 5h culture onwards, spermatozoa of 1.0 mM of nimbolide treated exhibited significant inhibition ($P \leq 0.01$). 1.5 mM and 2.0 mM groups did not show any inhibitory effect on the status of the percentage of acrosome reacted sperms in the 1-2h of sperm cultures and underwent hyperactivation/capacitation. However, at 3h (2.0 mM) and 4h (1.5 mM) of cultures onwards and at 5h (1.0 mM) cultures, there was a significant decline ($P \leq 0.01$) in the percentage of acrosome reacted sperms, respectively, and did not undergo hyperactivation/capacitation when compared to controls. This clearly shows that nimbolide, at higher concentrations, can have inhibitory effects on the AR in rat spermatozoa.

Effect of nimbolide on Sperm-Egg Interaction

To test this possibility, caudal epididymal spermatozoa were incubated for 5h in the presence or absence of nimbolide and allowed to attach to cells. The average number of oocytes containing sperm bound to ZP at 5h was significantly different in graded concentrations (1.0 to 2.0 mM/ml, respectively) of nimbolide-treated groups. Whereas in 0.5 mM of nimbolide, the number of oocytes with sperm bound to the ZP was lower than that in the control group (Figure 6B).

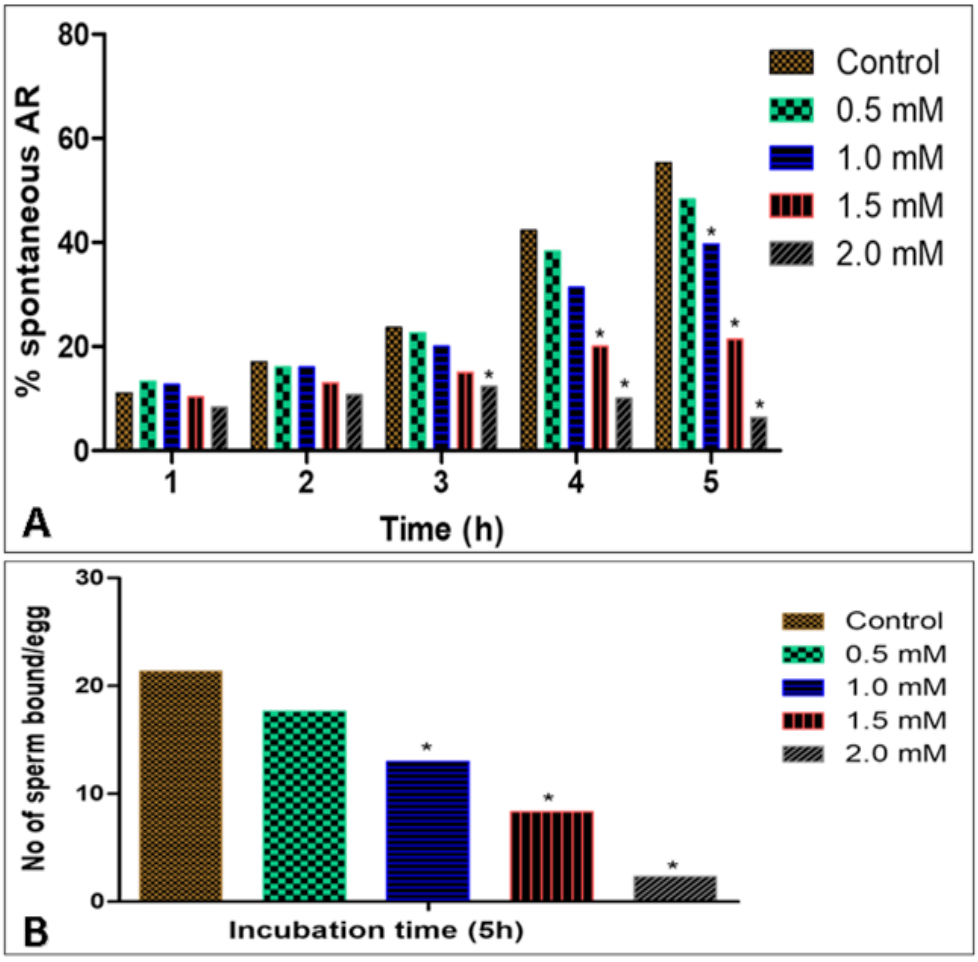

Figure 6A-B. Effect of nimbolide on AR of rat spermatozoa and sperm-egg interaction. Control and graded concentrations of nimbolide (0.5 to 2.0 mM/ml, respectively) treated sperm were incubated separately in sperm culture medium and evaluated for a time of 1 to 5h. Sperm that went through an AR was evaluated by the method of eosin-Y staining (A). Incubated sperm with ZP-bound oocyte medium for 5 h in the presence or absence of nimbolide and recorded the average number of oocytes with sperm bound to the ZP. Values are mean ± SEM (n=5). A significant difference ($P \leq 0.01$) is indicated (asterisks).

Discussion

In this study, the results of the *in vitro* effects of nimbolide on various parameters of sperm function in rats showed that nimbolide is a potent inhibitor of sperm motility. Nimbolide inhibited motility patterns at low concentrations (mM), and the inhibition process of sperm motility was similarly affected by sperm immobilization. Previous findings have shown that this constituent is a potent inhibitor of sperm motility, even at low doses, affecting antioxidant defense and thus negatively affecting the function of membrane systems and organelles (Kumbar et al., 2012a). Another study found that nimbolide, the minimum effective concentration of 3.50 µg/million sperm, has the effect of immobilizing sperm by directly controlling the structure and function of the plasma membrane or by interacting with certain impairment of some biochemical track as a synergistic effect (Kumbar et al., 2012b). Hence, it is anticipated that this active constituent may cause the above events by changing the surface composition and could predict that inhibition may be responsible for plasma membrane damage, which may be the first result of the interaction of the active constituent with sperm, and then protease activity can be performed when this constituent enters the cytoplasm. Reports on sperm have shown that plasma membrane content is important for the movement of sperm to the fertilization site and that plasma membrane function is an important factor in sperm motility, sperm AR, capacitation, metabolism, and binding to ZP (Agarwal et al., 2017). Therefore, the results on sperm function indicate that nimbolide may affect the mitochondrial sheath component, which is important for early sperm motility or may have resulted in unbalanced metabolism. Therefore, further studies should be done to confirm this hypothesis.

Sperm capacitation and hyperactivation are considered prerequisites for successful fertilization. This process is triggered by an influx of Ca^{2+} and bicarbonate ions. Both Ca^{2+} ions and ROS are involved in the initiation of the capacitation to cause the activation of adenylate cyclase which results in the production of cAMP (Aitken et al., 1998, Du Plessis et al., 2015). Ca^{2+}, cAMP, and ATP have received great attention as potential host regulators of sperm motility in various species (Steegborn, 2014, Schlingmann et al., 2007). ATP is the main energy source for sperm motility and ATP production levels are reduced, resulting in poor energy or poor sperm motility (Rodriguez-Miranda et al., 2008). In our study, the decrease in total ATP content of later time-points of culture indicates that the results are non-physiological, possibly due to inhibition of some sperm enzymes required for ATP regeneration. Additionally, the occurrence of SMI in this study is partially explained by decreased sperm ATP content attributable to AR inhibited by nimbolide. It has been reported that Ca^{2+} is the most important signaling molecule in the regulation of sperm motility (Suarez, 2008) and that increasing the Ca^{2+} level can activate soluble adenylyl cyclase and thus increase the cAMP concentration in sperm (Du Plessis et al., 2015). The role of cAMP in the enhancement of sperm motility is an important regulator of capacitation and AR (Beltran et al., 2007). The reduction in the levels of Ca^{2+}, cAMP, and ATP suggests that nimbolide stimulates cAMP-dependent motility-related protein substrates by directly affecting the activation of motility-related enzymes, resulting in a reduction in forward motility (Aitken et al., 1998).

Sperm are very sensitive to high ROS levels due to their limited antioxidant capacity. ROS regulate sperm function as they play an important role in the defense mechanisms against pathological conditions and are produced by the spermatozoa themselves (O'Flaherty and Scarlata, 2022). However, overproduction of ROS alters sperm motility by damaging the membrane structure of spermatozoa and other cytoplasmic organelles through the peroxidation of phospholipids, proteins, and nucleotides (Baskaran et al., 2021). Intracellular ROS concentration is determined by the stability between the rate of ROS generation and elimination through various antioxidant defense mechanisms. (Thompson et al., 2013). Studies have reported that ROS induces LPO and that the toxicity of lipid peroxides plays an important role in the inhibition of sperm function and the pathophysiology of male infertility (Agarwal et al., 2017). A proper level of H_2O_2 plays an important role in sperm function including sperm maturation, chromatin stability, capacitation, hyperactivation of sperm, and acrosome reaction, and increases the rate of sperm-oocyte fusion (Aitken et al., 1995). To counteract the effects of ROS, spermatozoa are equipped with antioxidant compounds such as GPx, SOD, GSH, and catalase resulting in disruption of membrane permeability, and thus efflux of ATP, impairing flagellar movement (Agarwal et al., 2017). Reports show that GSH-supplemented freezing extender improved sperm motility by reducing intracellular ROS levels, and improved sperm quality was associated with reduced LPO and increased antioxidant levels (Yánez-Ortiz et al., 2021). Therefore, the decrease in antioxidant enzyme activity and the increase in LPO and H_2O_2 levels in this study show that nimbolide increases ROS production by disrupting the prooxidative/antioxidative balance, thus causing oxidative stress in rat epididymal spermatozoa in terms of protein and DNA. The report has shown that α-Glucosidase is a metabolic enzyme used as an indicator of energy metabolism and a positive correlation between this enzyme activity and sperm motility percentage with good forward progression (Viljoen et al., 2009). Excluding sperm protein or DNA (min/mg) treated with a higher concentration of nimbolide, there was no significant change in α-Glucosidase activity; this shows that nimbolide at lower concentration did not affect sperm metabolic pathways. The decreased activity of antioxidant enzymes in nimbolide-treated sperm caused an increase in malonaldehyde production; this may influence the increased production of membrane LPO due to ROS production. However, the mechanism of action of nimbolide on ROS production is still unclear. *In vitro* studies show that nimbolide causes depletion of antioxidant defense in spermatozoa and its effects on membranes; this confirms that nimbolide induces oxidative stress in epididymal spermatozoa leading to impairment of sperm functional events.

AR is an important factor indicating the success of fertilization, and the percentage of acrosome-reacted sperm is closely related to the ability of sperm to bind to the oocyte (Du Plessis et al., 2015). A decrease in the rate of spontaneous AR was observed at higher nimbolide concentrations in this study. In contrast, the rate increased over time and reached its maximum value in the 5h of the incubation period in the control group. This time point corresponds to the point at which sperm begin to enter the egg in *vitro* fertilization studies (Toyoda and Chang, 1974b). However, at higher nimbolide doses of later time-points of culture, there was a time-dependent decrease in the

percentage of spontaneous acrosome-reacted sperms with low progression may have led to the impairment of the sperm's ability and capacity to generate energy (Woods and Garside, 1996). The sperm's ability to fertilize depends not only on its motility but also on other functions. Sperm motility and high velocity are important in terms of sperm binding and entry into ZP to ensure fertilization (He et al., 2022). The ability of the sperm membrane to selectively allow the transport of molecules is important for the regulation of sperm motility, stimulation of AR, and other important events possibly associated with fertilization (Puga Molina et al., 2018). In this study, the significant difference in the number of sperm fertilizing the egg at higher doses is because of the inhibitory effect of nimbolide on acrosome integrity and the ability of the sperm to fertilize the egg.

This study showed a dose- and time-dependent reduction in the functional outcome of the capacitation process associated with a reduction in the levels of molecular events. This was followed by resultant changes in the activity of antioxidant enzymes reflecting a decline in AR, which subsequently led to less cauda epididymal sperm binding to the ZP. We can conclude that the presence of nimbolide causes inhibition of sperm motility in rats by blocking some biochemical processes, may lead to the impairment of the ability and capacity of sperm to generate energy, or may directly interfere with the activation of motility-related enzymes, causing oxidative stress in rat epididymal sperm, which leads to a decrease in AR and less binding of sperm to the ZP. Currently, the results could provide a vision of how nimbolide can cause inhibition of sperm motility status, perhaps further research in this aspect is expected to clarify this mechanism.

References

1. Agarwal A, Sharma R, Gupta S, Harlev A, Ahmad G, du Plessis SS, Esteves SC, Wang SM, Durairajanayagam D (2017). Oxidative stress in human reproduction: shedding light on a complicated phenomenon. eds. New York City, NY, USA: Springer Publishing.
2. Ain R, Uma Devi K, Shivaji S, Seshagiri PB (1999). Pentoxifylline-stimulated capacitation and acrosome reaction in hamster spermatozoa: involvement of intracellular signalling molecules. *Mol Hum Reprod.* 57:618-626.
3. Aitken RJ, Harkiss D, Knox W, Paterson M, Irvine DS (1998). A novel signal transduction cascade in capacitating human spermatozoa characterized by a redox-regulated, camp mediated induction of tyrosine phosphorylation. *J Cell Sci.* 111:645-656.
4. Aitken RJ, Paterson M, Fisher H, Buckingham DW, van Duin M (1995). Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. *J Cell Sci*.108:2017-2025.
5. Aladakatti RH, Sukesh B, Jadaramkunti UC, Hiremath M (2011). Effect of graded doses of nimbolide (a major component of *Azadirachta indica* leaves) on biochemical and sperm functional parameters in male albino rats. *J Lab Animal Sci.* 1: 24-30.
6. Anitha G, Josepha LRJ, Narasimhan S, Anand SK, Rajan SS (2006). Nimbolide and isonimbolide. *J Asian Nat Prod Res*. 8(5):445–449.
7. Baligar NS, Aladakatti RH, Mukhtar Ahmed, Hiremath MB (2014). Hepatoprotective activity of neem based constituent azadirachtin-A in carbon tetrachloride intoxicated albino rats. *Can J Physiol Pharmacol.* 92: 267-277.
8. Baskaran S, Finelli R, Agarwal A, Henkel, R (2021). Reactive oxygen species in male reproduction: A boon or a bane? *Andrologia*.53: e13577.
9. Bavister BD, Andrews JC (1988). A rapid sperm motility bioassay procedure for quality- control testing of water and culture media. *J In Vitro Fert Embryo Transf.* 52:67-75.
10. Beltran C, Vacquier VD, Moy G, Chen Y, Buck J, Levin LD, Darszon A (2007). Particulate and soluble adenylyl cyclases participate in the sperm acrosome reaction. *Biochem Biophys Res Commun.* 358:1128–1135.
11. Burton K (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *J Biochem.* 62: 316–320.
12. Carlberg I, Mannervik BJ (1985). Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *J Biol. Chem.* 250:5474–5480.
13. Claiborne A (1985). Catalase activity. Ed: R. Greenwald, CRC Handbook of methods for oxygen radical research. CRC Press, Boca Raton, Fla, pp: 283-284.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This project is supported by CPDA and PRJIE/REDA grants from IISc. The authors thank the laboratory of Division of Biological Science, IISc for supporting research facilities and wish to duly acknowledge the Department of Studies in Chemistry, Davangere University for technical support.

Authors' Contribution

Chaitra NT conducted experimental work and drafted manuscript, Nandeshwarappa B. P contributed to the implementation of the research and analysis of the results and, Ravindranath H. Aladakatti involved in planning and supervised the experimental work and reviewed manuscript. All authors have read and agreed to the final version of the manuscript.

Funding

The authors declare this research was supported by faculty research support allowances of Cumulative Professional Development Allowance (CPDA) and Institute of eminence/ revenue development Allowance (REDA) from Indian Institute of Science

14. Cohen E, Quistad GB, Casida JE (1996). Cytotoxicity of nimbolide, epoxyazadiradione and other limonoids from neem insecticide. *Life Sci.* 58: 1075–1081.
15. Cooper TG, Yeung CH, Nashan D, Nieschlag E (1988). Epididymal markers in human infertility. *J Androl.* 9:91–101.
16. Devi J, Babu R (2023). Sharma Medicinal Importance of *Azadirachta indica*: An Overview. *J Drug Delivery Therapeutics*. 13(6):159-165.
17. Du Plessis SS, Agarwal A, Halabi J, Tvrda E (2015). Contemporary evidence on the physiological role of reactive oxygen species in human sperm function. *J Assist Reprod Genet.* 32: 509-520.
18. Glinsukon T, Somjaree R, Piyachaturawat P, Thebtaranonth Y (1986).Acute toxicity of nimbolide and nimbic acid in mice, rats and hamsters. *Toxicol Lett.* 30: 159–166.
19. Hammerstedt RH, Hay SR (1980). Effect of incubation temperature on motility and cAMP content of bovine sperm. *Arch Biochem Biophys.*199: 427–437.
20. He Z, Xie M, Li QQ, Duan J, Lu X (2022). Research Progress on the Microregulatory Mechanisms of Fertilization: A Review. *In Vivo.* 36(5): 2002–2013.
21. Holloway AJ, Moore HDM, Foster PMD (1990). The use of in vitro fertilization to detect reductions in the fertility of spermatozoa from males exposed to ethylene glycol monomethyl ether. *Reprod Toxicol.* 4:21-27.
22. Jin S, Yang W (2017). Factors and pathways involved in capacitation: how are they regulated? *Oncotarget.* 8:3600-3627.
23. Kumar R, Mehta S, Pathak SR (2018). Bioactive constituents of neem. In: Synthesis of medicinal agents from plants. Elsevier, pp. 75- 103.
24. Kumbar SB, Jadaramkunti UC, Aladakatti RH (2012a). In vitro spermicidal efficacy of nimbolide, an isoprenoid of neem leaf, in albino rats. *J Phytother Pharmacol.* 1: 1-13.
25. Kumbar SB, Jadaramkunti UC, Aladakatti RH (2012b). In vitro effect of nimbolide, an isoprenoid of neem leaf, on antioxidant system of rat cauda epididymal spermatozoa: A dose dependent study. *J Appl Pharmaceutical Sci.* 2: 84-93.
26. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with folin phenol reagent. *J Biol Chem.* 193: 265–275.
27. M. H. Viljoen, M. S. Bornman, M. P. van der Merwe, D. J. du Plessis (2009). Alpha-glucosidase activity and sperm motility. *Andrologia.* 22(3):205-208.
28. Marklund S, Marklund G (1974). Involvement of superoxide anion radical in antioxidation of pyrogallol and a constituent assay for superoxide dismutase. *Eur. J Biochem.* 47:469–474.
29. Mohandas J, Marshall JJ, Duggin GG, Horvath JS, Tiller DJ (1984). Low activities of glutathione-related enzymes as factors in the genesis of urinary bladder cancer. *Cancer. Res.* 44: 5086–5091.
30. Nagini S, Nivetha R, Palrasu M, Mishra R (2021). Nimbolide, a Neem Limonoid, Is a Promising Candidate for the Anticancer Drug Arsenal. *J Medicinal Chem.* 64 (7): 3560-3577.
31. O'Flaherty C, Scarlata E (2022). Oxidative stress and reproductive function: The protection of mammalian spermatozoa against oxidative stress. *Reproduction.* 164: F67–F78.
32. Ohkawa H, Ohishi N, Yagi K (1979). Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* 95:351–358.
33. Pick E, Keisari Y (1981). Superoxide anion and H_2O_2 production by chemically elicited peritoneal macrophages-induced by multiple nonphagocytic stimuli. *Cell. Immunol.* 59:301–318.
34. Priyadarsini RV, Manikandan P, Kumar GH, Nagini S (2009). The neem limonoids azadirachtin and nimbolide inhibit hamster cheek pouch carcinogenesis by modulating xenobiotic-metabolizing enzymes, DNA damage, antioxidants, invasion and angiogenesis. *Free Radical Research.* 43(5):492-504.
35. Puga Molina LC, Luque GM, Balestrini PA, Marín-Briggiler CI, Romarowski A, Buffone MG (2018). Molecular Basis of Human Sperm Capacitation. *Front. Cell Dev. Biol.* 6:Article 72(1-23).
36. Rodriguez-Miranda E, Buffone MG, Edwards EE, Ord TS, Lin K, Sammel MD, Gerton GL, Moss SB, William C (2008). Extracellular adenosine 5′-triphosphate alters motility and improves the fertilizing capability of mouse sperm. *Biol Reprod.* 79:164–171.
37. Schlingmann K, Michaut MA, McElwee JL, Wolff CA, Travis AJ, Turner RM (2007). Calmodulin and CaMKII in the sperm principal piece: evidence for a motility-related calcium/ calmodulin pathway. *J Androl.* 28: 706–716.
38. Steegborn C (2014). Structure, mechanism, and regulation of soluble adenylyl cyclases-similarities and differences to transmembrane adenylyl cyclases. *Biochim Biophys Acta (BBA)-Mol Basis Dis*. 1842: 2535–2547.
39. Suarez SS (2008). Control of hyperactivation in sperm. *Hum Reprod Update.* 14: 647–657.
40. Thompson A, Agarwal A, Du Plessis SS (2013). Physiological role of reactive oxygen species in sperm function: a review. In: Parekattil SJ, Agarwal A, edn. Antioxidants in male infertility: a guide for clinicians and researchers. New York, USA: Springer Science and Business Media, pp. 69–89.
41. Toyoda Y, Chang MC (1974a). Capacitation of epididymal spermatozoa in a medium with high K/Na ratio and cyclic AMP for the fertilization of rat eggs in vitro. *J Reprod Fertil.* 36:125-134.
42. Toyoda, Y and Chang, MC (1974b). Fertilization of rat eggs *in vitro* by epididymal spermatozoa: development of eggs following transfer. *J Reprod Fertil.* 36:9–22.
43. WHO (1999). Laboratory manual for the examination of human semen and sperm cervical mucus interaction. 4th ed. Cambridge: Cambridge University Press.
44. Woods J, Garside DA (1996). An in vivo and in vitro investigation into the effects of alpha- chlorohydrin on sperm motility and correlation with fertility in the Han Wistar rat. *Reprod Toxicol.* 103:199-207.
45. Yánez-Ortiz I, Catalán J, Delgado-Bermúdez A, Carluccio A, Miró J, Yeste M (2021). Addition of reduced glutathione (gsh) to freezing medium reduces intracellular ros levels in donkey sperm. *Vet Sci.* 8:302.