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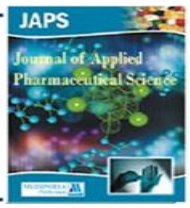

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In-vitro effect of nimbolide, an isoprenoid of neem leaf, on antioxidant system of rat cauda epididymal spermatozoa: A dose dependent study

Shashidhar B. Kumbar, Umadevi C. Jadaramkunti and Ravindranath H. Aladakatti

Shashidhar B. Kumbar,
Umadevi C. Jadaramkunti
Department of Zoology,
Government First Grade College, Sira city,
Karnataka, India.

Ravindranath H. Aladakatti
Central Animal Facility,
Indian Institute of Science,
Bangalore city,
Karnataka, India.

For Correspondence
Ravindranath H. Aladakatti
Email:
ravindranath@caf.iisc.ernet.in

ABSTRACT

The present in vitro study aims to address the possible evaluation of nimbolide, a major component of neem leaves, on the antioxidant system of rat spermatozoa to promote oxidative stress in a dose-dependent manner. To assess the effect of the nimbolide on activities of superoxide dismutase (SOD), catalase, Glutathione reductase (GR), Glutathione peroxidase (GPx), α-Glucosidase, production of Hydrogen peroxide (H_2O_2) and level of lipid peroxidation (LPO) in rat spermatozoa, increasing quantities 0.5 mM, 1 mM, 1.5 mM and 2.0 mM per ml of the nimbolide was added to the cultured medium prior to the addition of cauda epididymal spermatozoa. The spermatozoa were observed at 6^{th} h post-culture and the expressions of enzyme activities and production of H_2O_2, level of LPO were recorded. The activities of antioxidant enzymes decreased significantly while the levels of H_2O_2 generation and LPO increased significantly in nimbolide treated spermatozoa in a dose dependent manner when expressed in terms of milligram protein and milligram DNA. The activity of α-glucosidase, a negative control against antioxidant enzymes, did not show any significant change at any of the doses. The results suggest that graded doses of nimbolide elicit depletion of antioxidant defense system in sperm, indicating nimbolide-induced oxidative stress in the epididymal sperm of rats.

Keywords: Nimbolide, Antioxidant enzymes, Cauda epididymal spermatozoa Oxidative stress, Lipid peroxidation and Rats

INTRODUCTION

In recent years, great interest in finding natural antioxidants from plant materials has been drawn more and more attention. Plant material and products thereof are rich sources of a variety of biologically active compounds such as antioxidant and radical scavenging activities. Regarding literature, foods containing phytochemicals such compounds have potential protective effects against many diseases (Rice-Evans *et al.*, 1997). Indigenous plants have been the traditional source of raw materials for the manufacture of medicines. The diverse culture of our country is a rich source of traditional medicines, many of which one of plant origin scientific data on such plant derivatives could be of clinical importance (Gupta, 1994).

The trend of using natural products has increased and the active plant extracts are frequently screened for new drug discoveries (Das *et al.,* 1999). Indian neem tree *Azadirachta indica* (Syn: Melia azadirach: Meliaceae) is recognized since long for its unique properties (Nadkarni, 1954) and elaborates a vast array of bioactive phytochemicals that exhibit potent medicinal properties (Biswas *et al.,* 2002). A number of studies have indicated that extracts and purified fractions of different parts of *A. indica* plant may possess antioxidant nature (Arivazhagan *et al.,* 2004, Gupta *et al.,* 2004, Raji *et al.,* 2009).

Neem products or their ingredients are applied for different purposes. A number of chemical components have been isolated from neem plant and many of the secondary compounds of the neem have been identified, purified and some have been tested for their effects on mammals (Van der Nat *et al.,* 1991; Atawodi and Atawodi, 2009). Nimbolide (Fig.1) is an isoprenoid shown to be present in neem leaves and seed extract. This compound has been shown to have some biological activities such as anti-feedent (Suresh *et al.,* 2002), anti-malarial (Rochanakij *et al.,* 1985) and antimicrobial activities (Rojanpo *et al.,* 1985). It has also exhibited significant anti-cancer activity (Kigodi *et al.,* 1989; Cochen *et al.,* 1996). However, the toxicity of a compound has always become an issue in therapeutic use. It has been shown that nimbolide, when given through an intragastric route to experimental animals (Glinsukon *et al.,* 1986), did not show any toxicity. Preliminary study shown that nimbolide exerted detrimental effects at high dose on biochemical parameters of male reproductive functions in rats following oral administration (Aladakatti *et al.,* 2011).

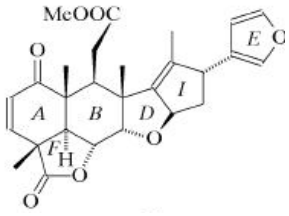

Fig. 1: chemical structure of nimbolide (adapted from Cohen *et al.,*)

Several studies have been reported that antioxidants can protect sperm DNA from free radicals and increase blood testis barrier stability (Jedlinska-krakowska *et al.,* 2006). Evidence suggests that certain phytochemicals found and play a major role in treating or retarding chronic diseases, including anti-oxidative, anti-carcinogenic, cardiovascular protective, neuro-protective, bone health promotion and anti-inflammatory diseases. Antioxidants protect DNA and other important molecules from oxidation and damage and can improve sperm quality and consequently increase fertility rate in men (Rajeev *et al.,* 2006). Therefore, the role of nutritional and biochemical factors in reproduction and sub- fertility treatment is very important. Since lack of literature on active principles of *A.indica* leave's on antioxidant system of rat spermatozoa, the present study was undertaken to evaluate the effect of nimbolide on the antioxidant system of rat cauda epididymal spermatozoa to promote oxidative stress in a dose-dependent manner.

MATERIALS AND METHODS

Animals

Colony bred healthy adult male albino rats (Wistar strain) weighing 200 g were utilized for experiments. All animals were proven fertility and obtained from the rat colony maintained in the department. They were housed at a temperature of 22 ± 2^0 C with 12:12 light and dark cycle. They were maintained on a standard rat pellet diet and water was given *ad libitum.* The animals were acclimatized to the laboratory conditions before conducting experiments and the care of the laboratory animals was taken as per the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) regulations. Necessary approval from the Institutional Animal Ethics Committee (IAEC) was obtained before undertaking animal experimentation.

Chemicals and Reagents

Reagents were obtained as follows: Technical Nimbolide from SPIC Ltd., Chennai, India). A stock solution of nimbolide (5 mM) was prepared in dimethyl sulfoxide (DMSO). Thiobarbituric acid and malondialdehyde, NADPH and glutathione oxidized were obtained from Sisco Research Laboratories (Mumbai, India). Deoxyribonucleic acid (DNA) and pyrogallol were obtained from Himedia Laboratories (Mumbai, India). Castanospermine and p-nitrophenyl a-glucopyranoside were obtained from Sigma Chemical Company (St. Louis. MO., USA). All other chemicals were of analytical grade and purchased from local commercial sources.

Spermatozoa Collection

Animals are euthanized by cervical dislocation and are wiped with absolute alcohol on the dorsal surface of the animal. The cauda epididymis was removed and spermatozoa were obtained by an adapted method of Holloway *et al.* (1990). Briefly, a small portion of the cauda epididymis was dissected out and placed in 60 mm dish containing 600 µl of culture medium and incubated at 37°C in 5% CO_2 in air for approximately 5 min, to allow sperm to "swim out" into the medium. The cauda tissue was removed from the medium and a 10 µl aliquot of sperm suspension approximately 1 x $l0^6$ (sperm/ml) was then taken and transferred into culture dishes 35 mm dish containing 400 µl of culture medium and assessed for motility under a Olympus Stereo Zoom Microscope.

Sperm Support Medium

The medium was adapted from method of Toyoda and Chang (1974), containing 94.6 mM NaCl, 4.78 mM KCl, 1.71 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25.07 mM $NaHCO_3$, 21.58 mM Na lactate, 0.5 mM Na pyruvate, 5.56 mM glucose, 4.0 mg/ml BSA, 50 µg/ml streptomycin sulphate, and 75 µg /ml potassium penicillin G. Phenol red (2 mg/l) was added to the medium as a pH indicator. Further, supplemented with 27 mg caffeine, 50 g hypotaurine, and 1.0 mg heparin per 100 ml, filtered through a 0.22 µm filter, and equilibrated with 5% CO_2 in air at

37°C to pH 7.4. Osmolarity was 280 to 300 mOsm as described by modification method of Woods and Garside (1996).

Incubation of spermatozoa with Nimbolide

A 10µl aliquot of sperms is taken from here, diluted in a 1:20 ratio in sperm dilution buffer 0.6 M $NaHCO_3$, 40% HCHO, (pH 7.2-7.4) and added to the counting chamber of a Neubauer haemocytometer. The number of sperms is counted and appropriate volumes of the sperm suspension are added to medium to a final concentration of 1 x 10^6 sperm per ml and incubated in a humidified atmosphere of 5% CO_2 in air at 37 ºC. In this study, stock nimbolide (5 mM) used to the culture medium in appropriate doses by adjusting pH to 7.4. The *in vivo* situation was simulated as far as possible, and it was, therefore, necessary to adapt some of the procedures for the *in vitro* studies. The concentration of nimbolide was used *in vitro*, i.e., 0.5, 1.0, 1.5 and 2.0 mM were calculated as described from the study of Glinsukon *et al*. (1986). Equivalent volumes of 50% DMSO are added to all control dishes. Aliquots are obtained from the sperm suspensions at 6^{th} h post-culture to analyze the expressions of enzyme activities, production of H_2O_2 and level of lipid peroxidation. All concentrations were carried out in triplicate.

Biochemical studies

The epididymal sperm were centrifuged at 225 g for 10 min at 4°C and the pellet was resuspended in the normal saline. The sperm pellet were homogenized with the help of a glass-Teflon homogenizer for a few seconds and centrifuged at 800 g for 10 min at 4°C. The supernatant was used for 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)

Superoxide dismutase (EC.1.15.1.1) was assayed 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 uL of 0.2 mM pyrogallol (0.2mM) and 300 uL enzyme source. The increase in absorbance was measured immediately at 420 nm against a blank containing all the components except the enzyme source and pyrogallol at 10 s intervals for 3 min on a Systonics Spectrophotometer. The enzyme activity was expressed as nanomoles pyrogallol oxidised per minute per milligram protein or milligram DNA at 32°C.

Catalase

Catalase (EC. 1.11.1.6) was assayed by the method of Claiborne (1985). Briefly, the assay mixture contained 2.4 mL phosphate buffer (50 mM, pH 7.0), 10 uL of 19 mM hydrogen peroxide and 50 uL enzyme source. The decrease in absorbance was measured immediately 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 per minute per milligram protein or milligram DNA at 32°C.

Glutathione reductase (GR)

Glutathione reductase (EC. 1.6.4.2) was assayed by the method of Carlberg and Mannervik (1985). Briefly, the assay mixture contained 1.75 mL phosphate buffer (100 mM, pH 7.6), 100 uL of 200 mM NADPH, 100 uL of 10 mM EDTA, 50 uL of 20 mM oxidised glutathione, and 50 uL enzyme source. Disappearance of NADPH was measured immediately at 340 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 nanomoles of NADPH oxidised per minute per milligram protein or milligram DNA at 32°C

Glutathione peroxidase (GPx)

Glutathione peroxidase (EC.1.11.1.9) 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), 100uL of 10 mM EDTA, 100 uL sodium azide, 50 uL glutathione reductase, 100uL reduced glutathione, 100 uL of 200 mM NADPH, 10 uL hydrogen peroxide, and 10 uL enzyme source. Disappearance of NADPH was measured immediately at 340 nm against a blank containing all the components except the enzyme source at 10 s intervals for 3 min on a Systronics Spectrophotometer. The activity of enzyme was expressed as nanomoles NADPH oxidised per minute per milligram protein or milligram DNA at 32°C.

Hydrogen peroxide (H_2O_2) generation assay

Hydrogen peroxide 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 uL horse radish peroxidase (8.5 units/mL), 30uL of 0.28 nM phenol red, 165 uL of 5.5 nM dextrose, and 600 uL of enzyme source, incubated at 32°C for 30 min. The reaction was terminated by the addition of 60 uL of 10 N sodium hydroxide. The 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 generated per minute per milligram protein or milligram DNA at 32°C. For construction of a standard curve, known amounts of H_2O_2 and all the above reagents except enzyme source were incubated for 30 min at 32°C before addition of 60 ul of sodium hydroxide (10 N) and optical density was read at 610 nm.

Lipid peroxidation (LPO)

A breakdown product of lipid peroxidation, thiobarbituric acid reactive substance (TBARS), was measured by the method of Ohkawa *et al.* (1979). Briefly, the stock solution contained equal volumes of 15% (w/v) trichloroacetic acid in 0.25 N hydrochloric acid and 0.37% (w/v) 2-thiobarbituric acid in 0.25 N hydrochloric acid. 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 absorbance of the supernatant was measured at 532 nm against

a blank containing all the reagents except test sample. The value was expressed as micromoles malondialdehyde formed per minute per milligram protein or milligram DNA. A standard curve was constructed by extrapolating the amount of commercially obtained product malondialdehyde to the measured absorbance.

α-Glucosidase

The activity of a-glucosidase (EC 3.2.1.20) was assayed by the method of Cooper *et al.* (1988). Briefly, the assay mixture containing 10 ul enzyme source, 16 ul castanospermine (1 mM) and 200 ul *p*-nitrophenyl a-glucopyranoside was incubated at 32°C for 2 h. The reaction was terminated by addition of 2 ml sodium carbonate (100 mM). Corresponding set of controls were maintained without the addition of castanospermine. Absorbance was read at 405 nm within 60 min, against a reagent blank, on a Systronics spectrophotometer. A standard curve was prepared using graded concentrations of *p*-nitrophenol. The activity of enzyme was expressed as micromoles *p*-nitrophenol produced per minute per milligram protein or milligram DNA at 32°C.

Statistical analyses

Data were analyzed using one way analysis of variance (ANOVA) using the Graph Pad Prism software method, followed by Dunnet test by comparing all treated groups against controls. Values represented are mean ± SEM (n=3). $P \leq 0.01$ is considered to indicate a significant difference between experimental and controls.

RESULTS

In order to study the in vitro effect of the nimbolide on activities of superoxide dismutase (SOD), catalase, Glutathione reductase (GR), Glutathione peroxidase (GPx), α-Glucosidase, production of Hydrogen peroxide (H_2O_2) and level of lipid peroxidation (LPO) in rat spermatozoa, increasing quantities 0.5 mM, 1 mM, 1.5 mM and 2.0 mM per ml of the nimbolide was added to the cultured medium prior to the addition of cauda epididymal spermatozoa. The spermatozoa were observed at 6^{th} h post-culture and the expressions of enzyme activities of SOD, catalase, GR, GPx, α-Glucosidase and production of H_2O_2, level of LPO were recorded.

Super oxide dismutase (SOD)

The data showed that there was a Dose dependent effect nimbolide on the activity of SOD of rat spermatozoa which expressed as nanomoles pyrogallol oxidised /minute/ milligram protein or milligram DNA at 32°C (Fig.2). The activities of SOD of rat spermatozoa (minute/ milligram protein) decreased significantly ($P \leq 0.01$) at graded concentrations (1.0 mM, 1.5mM and 2.0 mM per ml of nimbolide respectively). However, 0.5 mM of nimbolide exhibits no changes in SOD when compare to controls. Whereas, activities of SOD (minute/ milligram DNA) decreased significantly ($P \leq 0.01$) in all concentrations of nimbolide treated spermatozoa against to controls.

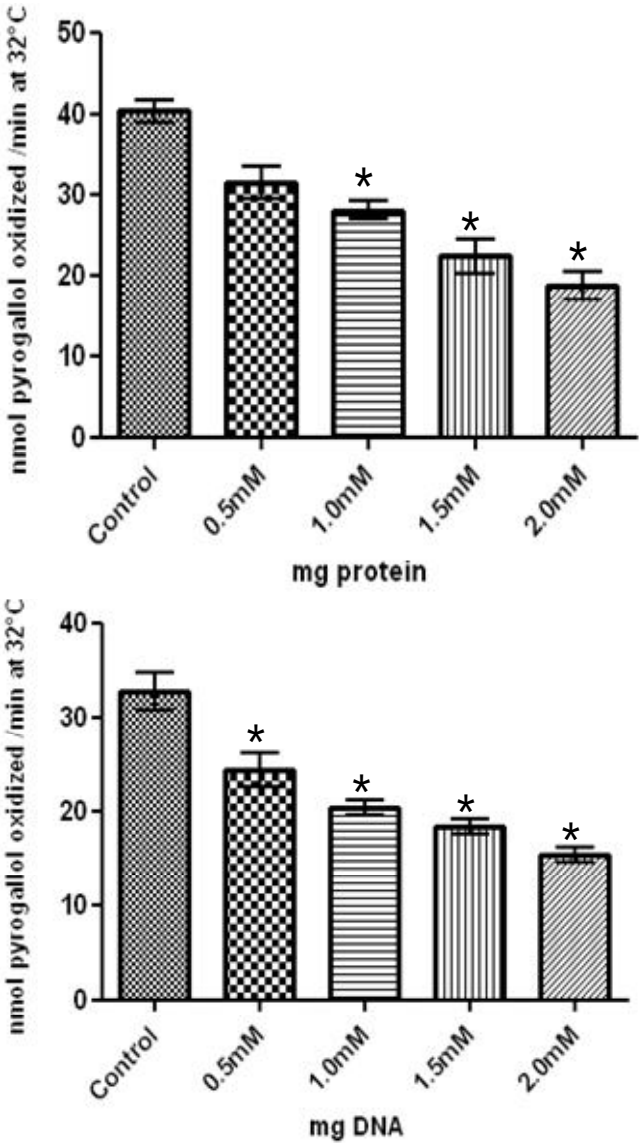

Fig. 2: Effect of nimbolide on the superoxide dismutase activity of rat cauda epididymal spermatozoa. Spermatozoa of control, nimbolide of increasing quantities of 0.5mM, 1.0 mM, 1.5 mM and 2.0 mM per ml respectively, were cultured and assessed for a period of 6h. The enzyme activity is expressed as nanomoles pyrogallol oxidised /minute/ milligram protein or milligram DNA at 32°C. Values are mean ± SEM (n=3). $P \leq 0.01$ (asterisks) is considered to indicate a significant difference between experimental and controls.

Catalase

The data showed that there was a dose dependent effect nimbolide on the activity of catalase of rat spermatozoa which expressed as micromoles H_2O_2 consumed per minute and milligram of protein or milligram DNA at 32°C (Fig.3). The activities of catalase of rat spermatozoa (minute/ milligram protein) decreased significantly ($P \leq 0.01$) at the higher concentration of 2.0 mM per ml of nimbolide. However, rest of the graded concentrations (0.5 mM, 1.0 mM and 1.5mM per ml of nimbolide respectively) exhibit no changes in catalase when compare to controls. Whereas, activities of catalase (minute/ milligram DNA) decreased significantly ($P \leq 0.01$) in all concentrations of nimbolide treated spermatozoa against to controls.

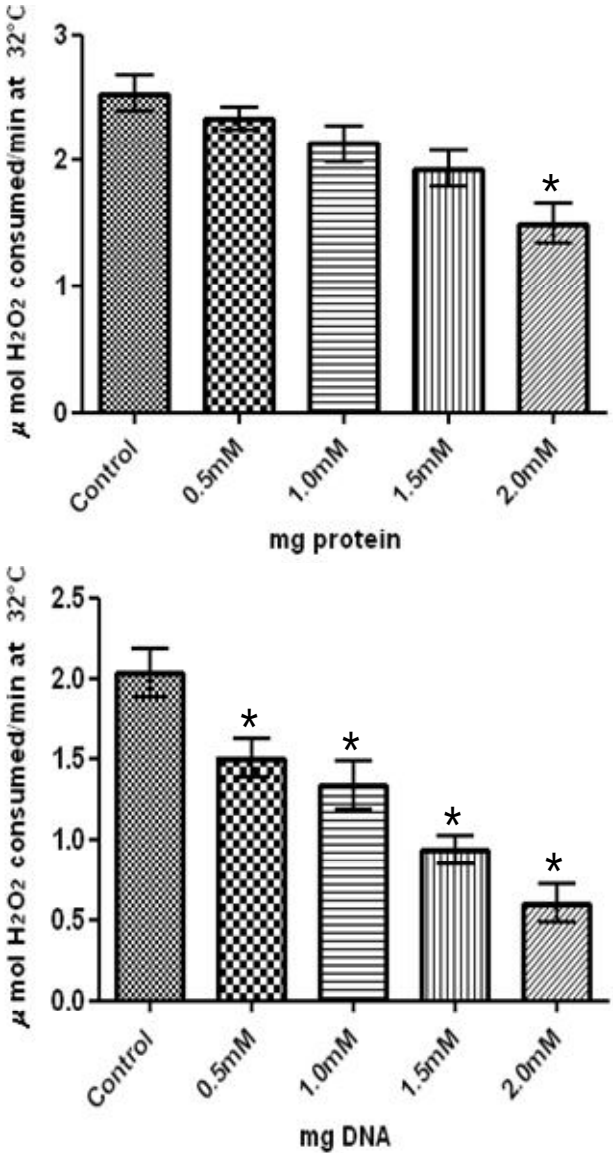

Fig. 3: Effect of nimbolide on the catalase activity of rat cauda epididymal spermatozoa. Spermatozoa of control, nimbolide of increasing quantities of 0.5mM, 1.0 mM, 1.5 mM and 2.0 mM per ml respectively, were cultured and assessed for a period of 6h. The enzyme activity is expressed as micromoles H_2O_2 consumed per minute and milligram of protein or milligram DNA at 32°C. Values are mean ± SEM (n=3). $P \leq 0.01$ (asterisks) is considered to indicate a significant difference between experimental and controls.

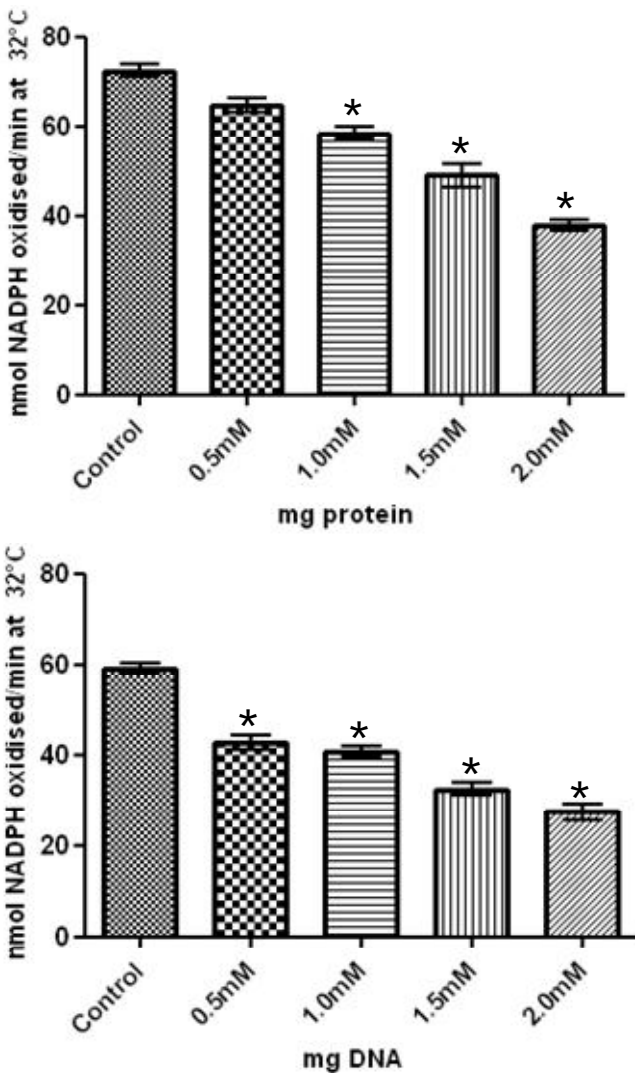

Fig. 4: Effect of nimbolide on the glutathione reductase activity of rat cauda epididymal spermatozoa. Spermatozoa of control, nimbolide of increasing quantities of 0.5mM, 1.0 mM, 1.5 mM and 2.0 mM per ml respectively, were cultured and assessed for a period of 6h. The enzyme activity is expressed as nanomoles NADPH oxidised /minute/ milligram protein or milligram DNA at 32°C. Values are mean ± SEM (n=3). $P \leq 0.01$ (asterisks) is considered to indicate a significant difference between experimental and controls.

Glutathione reductase (GR)

The data showed that there was a dose dependent effect nimbolide on the activity of GR of rat spermatozoa which expressed as nanomoles NADPH oxidised /minute/ milligram protein or milligram DNA at 32°C (Fig.4). The activities of GR of rat spermatozoa (minute/ milligram protein) decreased significantly ($P \leq 0.01$) in 1.0 mM, 1.5mM and 2.0 mM per ml of nimbolide concentration respectively). However, this enzyme activity at the lower concentration (0.5 mM per ml of nimbolide) did not show any significant changes when compare to controls. Whereas, activities of GR (minute/ milligram DNA) decreased significantly ($P \leq 0.01$) in all concentrations of nimbolide treated spermatozoa against to controls.

Glutathione peroxidase (GPx)

The data showed that there was a dose dependent effect nimbolide on the activity of GPx of rat spermatozoa which expressed as nanomoles NADPH oxidised /minute/ milligram protein or milligram DNA at 32°C (Fig.5). The activities of GPx of rat spermatozoa (minute/ milligram protein) decreased significantly ($P \leq 0.01$) at the higher concentration of 2.0 mM per ml of nimbolide. However, rest of the graded concentrations (0.5 mM, 1.0 mM and 1.5mM per ml of nimbolide respectively) exhibit no changes in GPx when compare to controls. The activities of Glutathione peroxidase (minute/ milligram DNA) decreased significantly ($P \leq 0.01$) in 1.5mM and 2.0 mM per ml of nimbolide respectively, whereas, rest of the lower concentrations (0.5 mM, 1.0 mM and 1.5mM per ml of nimbolide respectively) did not show any changes in GPx when compare to controls.

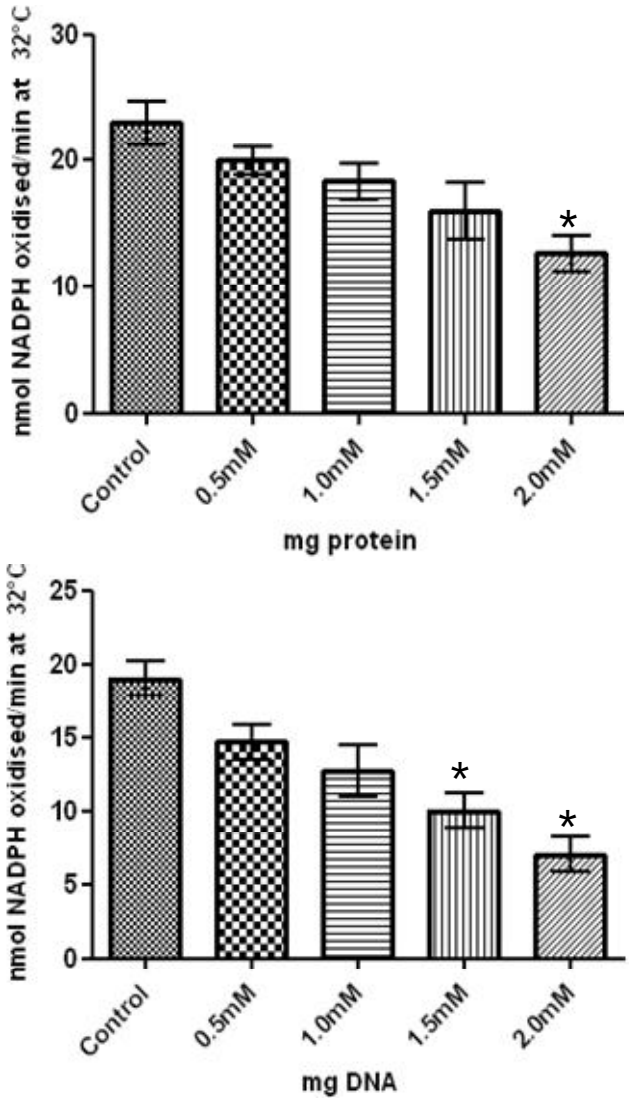

Fig. 5: Effect of nimbolide on the glutathione peroxidase activity of rat cauda epididymal spermatozoa. Spermatozoa of control, nimbolide of increasing quantities of 0.5mM, 1.0 mM, 1.5 mM and 2.0 mM per ml respectively, were cultured and assessed for a period of 6h. The enzyme activity is expressed as nanomoles NADPH oxidised /minute/ milligram protein or milligram DNA at 32°C. Values are mean ± SEM (n=3). $P \leq 0.01$ (asterisks) is considered to indicate a significant difference between experimental and controls.

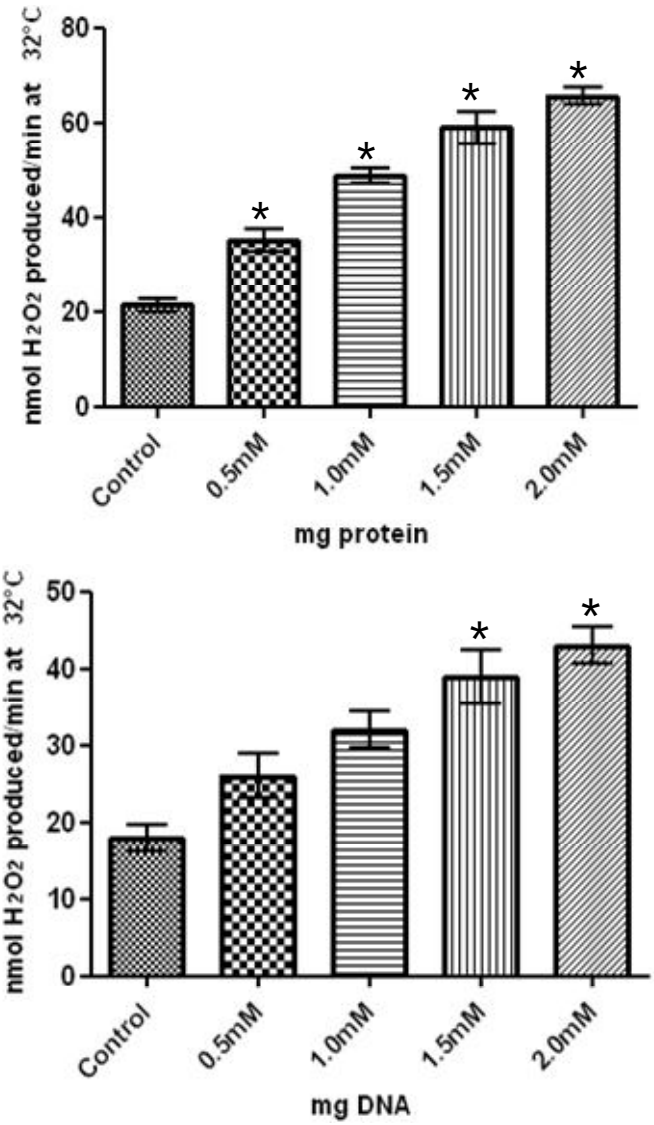

Fig. 6: Effect of nimbolide on hydrogen peroxide generation in the rat cauda epididymal spermatozoa. Spermatozoa of control, nimbolide of increasing quantities of 0.5mM, 1.0 mM, 1.5 mM and 2.0 mM per ml respectively, were cultured and assessed for a period of 6h. The enzyme activity is expressed as nanomoles H_2O_2 generated per minute per milligram protein or milligram DNA at 32°C. Values are mean ± SEM (n=3). $P \leq 0.01$ (asterisks) is considered to indicate a significant difference between experimental and controls.

Hydrogen peroxide (H_2O_2) generation assay

The data showed that there was a dose dependent effect nimbolide on the production of H_2O_2 of rat spermatozoa and the quantity of H_2O_2 produced was expressed as nanomoles H_2O_2 generated per minute per milligram protein or milligram DNA at 32°C (Fig.6). The generation of H_2O_2 of rat spermatozoa (minute/ milligram protein) decreased significantly ($P \leq 0.01$) in all concentrations of nimbolide treated spermatozoa against to controls. Whereas, generation of H_2O_2 (minute/ milligram DNA) decreased significantly ($P \leq 0.01$) at the higher concentrations of 1.5mM and 2.0 mM per ml of nimbolide respectively. However, rest of the lower concentrations (0.5 mM and 1.0 mM per ml of nimbolide respectively) exhibit no changes in production of H_2O_2 when compare to controls.

Lipid peroxidation (LPO)

The data showed that there was a dose dependent effect nimbolide on the value was expressed as micromoles malondialdehyde formed per minute per milligram protein or milligram DNA (Fig.7). Activities of LPO of rat spermatozoa (minute/ milligram protein) decreased significantly ($P \leq 0.01$) in all concentrations of nimbolide treated spermatozoa against to controls. However, LPO activity (minute/ milligram DNA) decreased significantly ($P \leq 0.01$) at the higher concentrations of 1.5mM and 2.0 mM per ml of nimbolide respectively and rest of the lower concentrations (0.5 mM and 1.0 mM per ml of nimbolide respectively) exhibit no changes in activities of LPO when compare to controls.

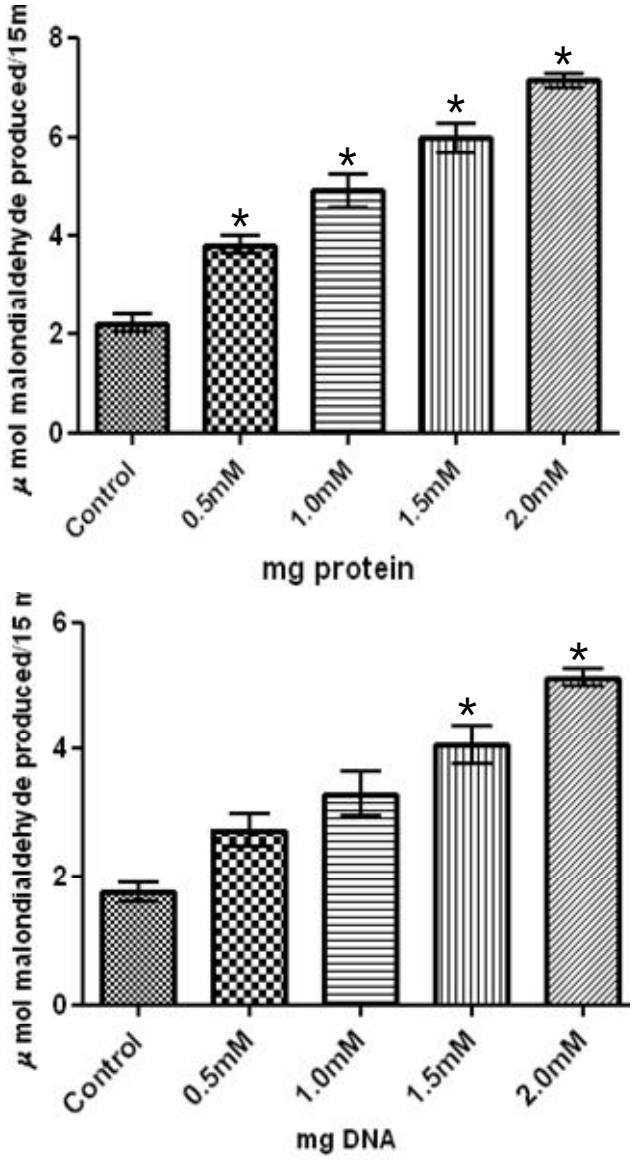

Fig. 7: Effect of nimbolide on the lipid peroxidation of rat cauda epididymal spermatozoa. Spermatozoa of control, nimbolide of increasing quantities of 0.5mM, 1.0 mM, 1.5 mM and 2.0 mM per ml respectively, were cultured and assessed for a period of 6h. The enzyme activity is expressed as micromoles malondialdehyde formed per minute per milligram protein or milligram DNA. Values are mean ± SEM (n=3). $P \leq 0.01$ (asterisks) is considered to indicate a significant difference between experimental and controls.

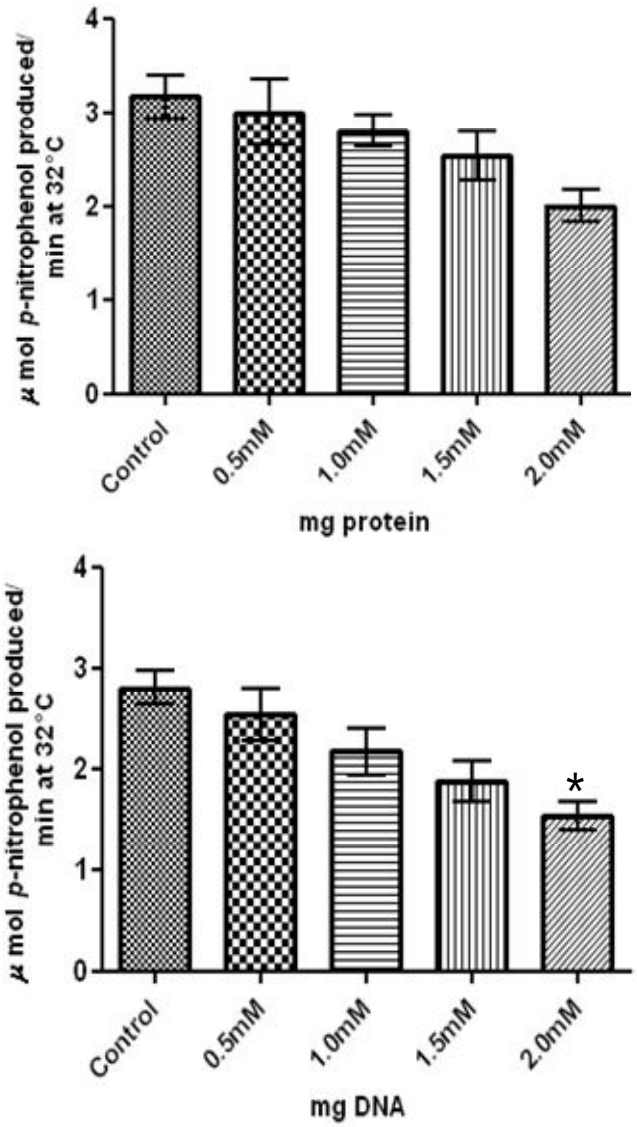

Fig. 8: Effect of nimbolide on the a-glucosidase of rat cauda epididymal spermatozoa. Spermatozoa of control, nimbolide of increasing quantities of 0.5mM, 1.0 mM, 1.5 mM and 2.0 mM per ml respectively, were cultured and assessed for a period of 6h. The unit of a-glucosidase activity is expressed as micromoles p-nitrophenol produced per minute per milligram protein or milligram DNA at 32°C. Values are mean ± SEM (n=3). $P \leq 0.01$ (asterisks) is considered to indicate a significant difference between experimental and controls.

α-Glucosidase

The data showed that there was a dose dependent effect nimbolide on the activity of α-Glucosidase of rat spermatozoa which expressed as micromoles *p*-nitrophenol produced /minute/ milligram protein or milligram DNA at 32°C (Fig.8). The activities of α-Glucosidase of rat spermatozoa (minute/ milligram protein) did not show any changes in all concentrations of nimbolide treated spermatozoa against to controls. However, activities of α-Glucosidase (minute/ milligram DNA) decreased significantly ($P \leq 0.01$) at the higher concentration of 2.0 mM per ml of nimbolide. However, rest of the graded concentrations (0.5 mM, 1.0 mM and 1.5mM per ml of nimbolide respectively) exhibit no changes in catalase when compare to controls.

DISCUSSION

Spermatozoa have been shown to be able to produce reactive oxygen species (ROS) (Aitken *et al*., 1989). Research, however, revealed that oxygen radical generating systems are present in a wide variety of cell types including endothelial cells (Sundquist, 1991), adipocytes, and germ cells (embryos). The ROS production is implicated in the etiology of disparate pathological conditions as well as in such physiological cell functions as signal transduction and second messenger penetration (Koshio *et a!.*, 1988). In some pathologies the imbalance between pro- and antioxidant levels, called oxidative stress, may initiate several metabolic and functional disregulations, eventually leading to cell

death (Aitken, 1989). Such situations may arise either from increased exposure to radicals/oxidants or may be a result of decreased antioxidant capacity. The epididymis has been shown to possess antioxidant defence system to protect spermatozoa from oxidative injury by elaborating scavengers of ROS (Zini and Schlegel, 1997). Cytoplasm of spermatozoa is extremely limited in volume and localization, thus the polyunsaturated fatty acids bound to the sperm plasma membrane are very susceptible to ROS attack. To counteract the effects of ROS, spermatozoa are equipped with antioxidant defense systems namely, glutathione peroxidase, superoxide dismutase and catalase to prevent cellular damage (Ochsendorf *et al.,* 1998). Catalase and GPx/GR system catalyse the degradation of H_2O_2 and lipid hydroperoxides by using reduced glutathione (Alvarez and Storey 1989). In the present of in vitro studies, dose dependent decreased the activities of SOD, catalase, GR, GPx, α-Glucosidase and concomitantly increased the levels of production of H_2O_2 and LPO in nimbolide treated cauda epididymal sperm of rats in terms of both protein and DNA.

Mitochondrial respiration is the main biological source of superoxide anion radicals in physiological conditions. SOD is considered the first line of defense against deleterious effects of oxyradicals in the cell by catalyzing the dismutation of superoxide radicals to H_2O_2 and molecular oxygen. A reduction in the activity of SOD causes a rise in the level of superoxide anion, which is known to inactivate catalase activity (Kono and Fridovich 1982). Catalase or GPx fails to eliminate H_2O_2 from the cell, and the accumulated H_2O_2 has been shown to cause inactivation of SOD (Sinet and Garber 1981). Griveau and Lannou (1997) reported that ROS such as H_2O_2 appear to be key agents in the cytotoxic effects in spermatozoa, and in addition to their direct effect on the cellular constituent, to produce oxidative stress by decreasing the enzymatic defenses. The antioxidant enzymes catalase and peroxidase protect SOD against inactivation by H_2O_2. Reciprocally, the SOD protects catalase and peroxidase against inhibition by superoxide anion. Thus, balance of this enzyme system may be essential to eliminate superoxide anion and peroxides generated in epididymal sperm. GPx, a selenium-containing antioxidant enzyme, removes peroxyl radicals from various peroxides including H_2O_2, whereas GR regenerates reduced glutathione from its oxidized form (Sikka, 2001).The reduction in catalase activity reflects the inability of spermatozoa to eliminate H_2O_2 produced or to enzyme inactivation caused by excess ROS production in epididymal sperm (Pigeolet *et al.*, 1990). In the present study, the reduction in the activities of SOD, catalase, GPx and GR and enhanced in the levels of LPO and H_2O_2 could reflect the adverse effect of nimbolide on the antioxidant enzymes in epididymal sperm.

Studies have reported that ROS induce LPO, and the toxicity of lipid peroxides play a key role in the inhibition of sperm function and the pathophysiology of male infertility (Alvarez *et al.,* 1987). When ROS concentrations are high, pre damaged spermatozoa are exposed to LPO by polyunsaturated fatty acids. ROS cause damage to sperm and other cytoplasmic organelle membrane structures through peroxidation of phospholipids, proteins, and nucleotides, thereby altering sperm motility (Ichikawa *et al.,* 1999). Report has been shown that ROS such as H_2O_2 appear to be key agents in the cytotoxic effects in spermatozoa. In addition to their direct effect on cellular constituents, ROS produce oxidative stress by decreasing enzymatic defenses (Griveau and Lannou (1997). The sperm membranes undergo permeability changes following enhanced LPO and glutathione depletion (Chance *et al.,* 1979). ROS modulate sperm function as they play an important part in the defence mechanisms against pathological conditions, and have been produced by spermatozoa themselves (Kessopoulou *et al.*, 1992). However, excessive generation of ROS has been shown to impair spermatozoa function. Thus, in the present study, decreased activity of antioxidant enzymes and increased levels of LPO reveals that nimbolide disrupts the pro-oxidant/antioxidant balance and increases the formation of ROS, thereby causing oxidative stress in epididymal sperm of rats.

Sperm cells utilize the metabolic pathways for the production of energy. Since, lack of literature on effects of nimbolide on metabolic enzymes of spermatozoa, in the present study, α-glucosidase, a metabolic enzyme, was used as an indicator of energy metabolism and served as negative control against the antioxidant enzymes of epididymal sperm. α-Glucosidase has been shown to be involved in hydrolysis of glycosidic linkages and to facilitate the transport of hydrolysate from seminal fluid to spermatozoa for energy production (Mann 1964). The epididymis and prostate have been shown to secrete α-glucosidase, which is unlikely to have antioxidant activity (Cooper *et al.,* 1988). In the present study, excluding of higher concentration of 2.0 mM nimbolide treated cauda epididymal sperm of rats in terms of DNA, no significant changes in the activity of α-glucosidase, indicating that nimbolide, at lower concentration, did not affect metabolic pathway in spermatozoa. The activity of α-glucosidase in sperm has been shown to remain unchanged during induced oxidative stress in HIV-seropositive men (Umapathy *et al.*, 2001).

The data from other sources indicate that abnormal sperm carry a much higher probability of producing ROS than motile sperm suspensions from fertile individuals (Aitken, 1989). As a consequence, in the present study, decreased concentrations of antioxidant enzymes in nimbolide treated spermatozoa may be caused by increased levels of ROS. It should be emphasized that all the graded treatments of nimbolide studied exhibited elevated levels of malonaldehydes produced per minute per milligram protein or milligram DNA, which can be proof for membrane LPO due to high levels of ROS production. But the mechanism of action of nimbolide on the production of ROS remains unclear. Hence, from these in vitro observations studies, it suggest that the graded doses of nimbolide elicit depletion of the antioxidant defense system in spermatozoa and its adverse effect on the membrane system, indicating nimbolide induced oxidative stress in the epididymal sperm. This may lead to disruption of the functional integrity of cell organelles and in the onset of sperm damage under nimbolide-induced pathologic conditions. Further, analyses of this compound have some significance in the evaluation of male

infertility. This in turn raises the possibility for potential application of the antioxidants for therapeutic purposes. From this study, these observations provide important implications for our understanding of the fundamental cellular mechanisms regulating sperm function and the development of novel contraceptive strategies targeting the male gamete.

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