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THE EFFECT OF MINOCYCLINE ON OXIDATIVE STRESS AND MEMORY DEFICITS IN AGED RATS

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ABSTRACT
Alzheimer's disease (AD) is a progressive, neurological and psychiatric age associated disorder. Ageing and age-related neurodegenerative disorders like AD are associated with Oxidative-nitritive stress that may cause impairment of learning and memory in animals and human beings. These losses reflect the failure of cellular processes that encode memory or disturbances in events that retrieve it. The oxidative stress play a major role on the aging process and associated cognitive decline, therefore antioxidant treatment may alleviate age-related impairment in spatial memory. Age-related morphological alterations of hippocampus and other areas associated with memory formation may be a reason for Cognitive impairment. The aim of this study was to examine the relationship between the effects of Minocycline, a tetracycline derivative on spatial memory in aged male rats. In this study 24 months old male rats were used. Rats were divided into control and Minocycline groups and injected intraperitoneally (ip) for 25 days. Learning experiments were performed using Morris water maze. Spatial learning was significantly better in Minocycline treated rats compared to Aged rats. Minocycline treatment elicited a significant decrease of lipid peroxidation and nitrittive stress. A significant increase of glutathione peroxidase, catalase and SOD activity was also observed in Minocycline treated rats compared with aged animals. In conclusion, we demonstrated that Minocycline increases spatial memory performance in aged male rats and this increase may be related to suppression of lipid peroxidation and other oxidative stress parameters. The results of such studies may be useful in pharmacological modification of aging process.

Keywords: Minocycline, Alzheimer's disease, Aging, Spatial memory, Lipid peroxidation and Antioxidant enzymes

INTRODUCTION

Natural aging is known to deteriorate memory in human beings[1]. The brain, for long has been studied in terms of its bioelectric properties and anatomical connectivity. It has been recognized as a complex target tissue for the genomic effects of drugs which bring about long lasting alterations in brain structure and neurochemistry as well as changes in behavior and endocrine functions. Memory decline associated with normal aging, often referred to as AAMI (age-associated memory impairment), greatly reduces the quality of life and affects at least 50% of individuals in their 60s according to estimations[2]. Oxygen free radicals, the harmful by products of oxidative metabolism are known to cause organic damage to the brain because the brain is believed to be particularly vulnerable to oxidative stress due to a relatively high rate of oxygen free radical generation without commensurate levels of antioxidative defences[3], which may be responsible for the development of dementia or Alzheimer's disease in elderly[4]. Functional impairments of the CNS are associated with increased susceptibility to develop many neurodegenerative diseases such as Alzheimer's diseases (AD). While a number of genetic and environmental factors have been demonstrated to be linked with the development of

AD, the single greatest risk factor is aging[5]. Aging affect learning and memory in rodents, and in humans. Compared with young rats, aged rats exhibit learning and memory deficits in the Morris water maze task, radial arm maze task, tunnel maze task, and the delayed non-matching to place task in water[6] and aging is often associated with a storage decrease of cognitive functions.

Cognitive decline in old age has been linked to changes in brain anatomy, morphology, volume, and functional deficits[7]. Among the available animal models usable to examine potential therapies for age related cognitive impairments like in AD, the aged rat has the advantage of being a naturally occurring model, i.e. one without a genetic or lesion manipulation. Such model seems especially appropriate for testing therapeutics that may act via multiple pathways or mechanisms[8].

Aged rats utilized in this study are a more appropriate model for age-related mental decline seen in human's too[9]. Thus aged rats associated changes may be considered as an appropriate model to investigate changes associated with AD.

MATERIALS AND METHODS

Animals: Male Wistar rats, 24 to 25 month old weighing 180-200 g, were considered as aged rats [10-12] and used in the study. These rats were obtained from animal house of Onkar College of Pharmacy, Sajuma, Punjab, (India) and were housed two to a cage in the animal room where the temperature was maintained approximately at 24-25^{o}C and relative humidity of 60- 65%, with 12 hours dark light cycle (lights on 06.00 - 18.00 h).

The food in the form of dry pallets and water was made available *adlibitum*. All behavioural experiments were carried out between 10 AM and 4 PM. All experiments were performed as per the norms of ethical committee and the studies were approved and clearance obtained by the Institutional review board. Animals were acclimatized to laboratory conditions prior to experimentation.

Drugs and Chemicals: Minocycline Hydrochloride (Ranbaxy, India) was used and the drug solutions were prepared by suspending them in one or two drops of Tween 80 in normal saline and administered intraperitoneally. All other chemicals and reagents used were of analytical grade (AR). Minocycline was administered at the dose of 10, 20 and 40 mg/kg ip.

PARAMETERS EVALUATED

1. Behavioural Parameters

Morris water maze test: Aged rodents show impairments in learning and memory, especially on tests requiring the use of spatial orientation to escape from stressful situation. Aged rats impaired on these tests may be unable to effectively use spatial orientation to learn to escape. Hence, it is reasonable to expect differences in the aged brains in regions processing spatial orientation. Because the hippocampal formation is involved with spatial learning age associated loss of integrity of its neurons may be involved in impairment of learning in aged rats. In this study, spatial learning and memory of animals were tested in a Morris water maze[13].The test was performed on day 20-23 and a probe trial was performed on day 24.

It consisted of a circular water tank (180 cm diameter, 60 cm height) filled with water (25±1 °C) to a depth of 40 cm. A non-toxic water dispersible emulsion was used to render the water opaque. Four equally spaced locations around the edge of the pool (North, South, East, and West) were used as start points, which divided the pool into 4 quadrants. An escape platform (10 cm in diameter) was placed in the pool 2 cm below the surface of water. The escape platform was placed in the middle of one of the randomly selected quadrants of the pool and kept in the same position throughout the entire experiment (north-east for this study). Before the training started, the rats were allowed to swim freely into the pool for 120 s without platform. Animals received a training session consisting of 4 trials per session (once from each starting point) for 4 days, each trial having a ceiling time of 120 s and a trial interval of approximately 30 s. After climbing onto the hidden platform, the animals remained there for 30 s before commencement of the next trial. If the rat failed to locate the hidden platform within the maximum time of 120 s, it was gently placed on the platform and allowed to remain there for the same interval of time. The time taken to locate the hidden platform (latency in seconds) was measured. Twenty four hours after the acquisition phase, a probe test (day 24) was conducted by removing the platform. Rats were allowed to swim freely in the pool for 120 s and the time spent in target quadrant, which had previously contained the hidden platform, was recorded. The time spent in the target quadrant indicated the degree of memory consolidation which had taken place after learning.[11, 14]

2. Estimation of biochemical parameters

All the biochemical parameters were measured in the brain homogenate on day 25 following 1st Minocycline Dose.

***Brain homogenate preparation*:** Animals were sacrificed by decapitation and brains were removed and rinsed with ice-cold isotonic saline. Brain tissue samples were then homogenized with ice-cold 0.1 M phosphate buffer (pH7.4) in a volume 10 times the weight of the tissue. The homogenate was centrifuged at10, 000×g for 15min and aliquots of supernatant separated and used for biochemical estimation.

***Protein estimation*:** Protein was measured in all brain samples by the method of Lowry[15] using bovine serum albumin (BSA) (1 mg/ml) as a standard.

***Estimation of malondialdehyde (MDA)*:** The quantitative measurement of malondialdehyde (MDA) – end product of lipid peroxidation – in brain homogenate was performed according to the method of Wills.[16] The amount of MDA was measured after its reaction with thiobarbituric acid at 532 nm using spectrophotometer (Shimadzu, UV-1700). The concentration of MDA was determined from a standard curve and expressed as nmol per mg protein.

Estimation of reduced glutathione (GSH): GSH is the antioxidant in the body and usually be the biomarker for measuring the severity of oxidative stress. If the GSH markedly decrease as compared to the normal physiological level, the oxidative stress will be indicated. Reduced glutathione in brain was estimated according to the method described by Ellman[17].One ml supernatant was precipitated with 1 ml of 4% sulfosalicylic acid and cold digested at 40C for 1h.The samples were centrifuged at 1200×g for 15min. To 1 ml of the supernatant, 2.7 ml of phosphate buffer (0.1M, pH 8) and 0.2 ml of 5,5 dithiobis (2-nitrobenzoic acid) (DTNB) were added. The yellow colour that developed was measured immediately at 412 nm using a spectrophotometer. The concentration of glutathione in the supernatant was determined from a standard curve and expressed as μmol per mg protein.

Estimation of nitrite: *T*he accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO), was determined by a colorimetric assay using Greiss reagent (0.1% N-(1- naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid) as described by Green[18]. Equal volumes of supernatant and Greiss reagent were mixed, the mixture incubated for 10 min at room temperature in the dark and the absorbance determined at 540 nm spectrophotometrically. The concentration of nitrite in the supernatant was determined from sodium nitrite standard curve and expressed as μmol per mg protein.

Catalase Activity: Catalase activity was assessed by the method of Luck,[19] wherein the breakdown of hydrogen peroxide is measured. Briefly, the assay mixture consisted of 3mL of H_2O_2 phosphate buffer and 0.05Ml of the supernatant of the tissue homogenate. The change in absorbance was recorded for 2 minutes at 30-second interval at 240nm spectrophotometerically. The results were expressed as micromoles of H_2O_2 decomposed per minute per mg protein.

Superoxide Dismutase Activity: Superoxide dismutase (SOD) activity was assayed by the method of Kono[20]. The assay system consisted of EDTA 0.1mM, sodium carbonate 50mM and 96mM of nitro blue tetrazolium (NBT). In the cuvette, 2mL of the above mixture, 0.05mL of hydroxylamine and 0.05mL of the supernatant were added, and the auto-oxidation of hydroxylamine was measured for 2 minutes at 30-second interval by measuring the absorbance at 560nm using Perkin Elmer Lambda 20 spectrophotometer.

RESULTS

Effect of Minocycline on memory performance in Morris water maze task in aged rats: The latencies to reach the submerged platform decreased gradually in experimental animals of all the groups during 4 days of training in Morris water maze (MWM) task (Fig. 1A) except control group wherein the mean latencies were found to be still significantly prolonged indicating poorer learning performance of this group of animals. Amongst the doses of Minoocycline investigated in the present study, the 40 mg/kg ip was found to be the most effective in ameliorating associated spatial memory deficit.

During the probe trial(Day,24), with the platform removed, aged rats failed to remember the precise location of the platform, spending significantly less time in the target quadrant than Mionocycline [pb0.001, Fig. 1B]. But the mean percent time spent in the target quadrant by aged rats treated with MInocycline was significantly increased as compared to aged control group indicating improved consolidation of memory.

Effect of Minocycline on brain malondialdehyde (MDA) and Nitrite levels in aged rats: The level of MDA and nitrite was significantly higher in aged control rats as. But the treatment of aged rats with minocycline significantly decreased MDA and nitrite levels in a dose dependent manner as compared with those of control rats. (Fig. 2 & 3)

Effect of Minocycline on brain glutathione (GSH), catalase and SOD levels in i.c.v. streptozotocin infused rats: The levels of GSH, catalase and SOD were found to be significantly depleted in aged control rats ($P<0.001$). Chronic treatment of aged rats with Minocycline was able to raise these levels significantly compared with those of control group animals (Fig.4,5,6).

DISCUSSION

Aging associated functional impairments of the CNS are associated with increased susceptibility to develop many neurodegenerative diseases such as AD.Beside other changes, aging is associated with a decline in cognitive performance, and is the biggest risk factor for the development of Alzheimer's disease (AD)[5].

Numerous neuronal and behavioral changes during aging which include decrements in calcium homeostasis[21] and in the sensitivity of several receptor systems, most notably, adrenergic,[22] dopaminergic,[23] muscarinic [24] and opioid.[25] These decrements can be expressed, ultimately, as alterations in both motor and cognitive behaviours. Beside other mechanisms, most important among them may be enhanced vulnerability to oxidative stress shown by the CNS, which may increase further in aging. The increased production of toxic species and free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) have the capacity to modify proteins, lipids, and nucleic acids to develop or enhance age related manifestations.

Several antioxidant defence mechanisms have evolved to protect cell components from the attacks of ROS and RNS. Two major groups of endogenous antioxidants are low molecular weight antioxidants compounds (eg, vitamins C and E, lipoic acid and ubiquinones), and antioxidant enzymes (eg, superoxide dismutases [SOD], superoxide reductases, catalase, glutathione peroxidises, and many heat-shock proteins. In oxidative stress there is imbalance resulting from the production of ROS and RNS that exceed the capacity of these antioxidant defense systems. The CNS possesses: (1) high content of peroxidizable unsaturated fatty acids, (2) high oxygen consumption per unit weight, (3) high content of lipid peroxidation key ingredients: iron and ascorbate, and (4) the scarcity of antioxidant defenses systems[26] [Floyd,1999]. Therefore, the CNS is more susceptible to oxidative stress than other tissues. Free radical-associated oxidative stress induces physiological alterations in the CNS, which consequently causes age related dementia.

Lipid peroxidation, a hallmark of oxidative tissue injury, has been found to be elevated in the AD brain.[27] Furthermore, Generation of MDA in brains increase as a function of age. In aging brain, peroxidation of arachidonic acid (AA) forms malondialdehyde (MDA)[28] which then is hydrolyzed by lysine or arginine to form formic acid or acetaldehydes. Levels of arachidonic acid (AA) are decreased in brains of aged rats with impaired ability to sustain long-term potentiation (LTP).[29] The ability to sustain LTP is usually used as a parameter of cognitive function and oxidative depletion of AA levels may relate to the cognitive deficit in rats. Furthermore, MDA is a highly reactive genotoxic compound, because it induces DNA damage by reacting with nucleic acids to form adducts that disrupts DNA base pairing. Researchers have found that the basal MDA level was significantly elevated (19%) in hippocampus of old rats[30]. In the present study too MDA was found to be elevated and the levels were reversed by Minocycline.

Glutathione is one of the major antioxidant enzymes in CNS and GSH metabolism plays an important role in diseases such as cancer, neurodegenerative diseases, and aging. Reduced glutathione (GSH) is the most prevalent non protein thiol in animal cells. Its *de novo* and salvage syntheses maintain a reduced cellular environment. Consequently, ROS are frequently removed by GSH in both spontaneous and catalytic reactions[31]. Glutathione and glutathione-related enzymes play a key role in protecting the cell against the effects of reactive oxygen species. In addition to protection against ROS, glutathione is an excellent scavenger of lipid peroxidation products such as 4- hydroxyl-2-nonenal (HNE) and acrolein, both of which have been found to bind proteins, inhibiting their activities. In the present study the age associated decline in GSH was reversed effectively by the test drug, Minocycline.

Activities of many other enzymes decline during brain aging including antioxidant enzymes: superoxide dismutase, catalase, glutathione peroxidase, g-glutamylcysteine synthetase, glutathione reductase, glutathione-S-transferase, and g-glutamyl transpeptidase[32- 34]. In the present study

too, there was significant decrease in the level of these antioxidant enzymes indicating age related decline of antioxidant defence. Minocycline effectively reverses these changes in dose dependent manner.

Consistent with previous behavioral findings [35-37] we found that the aged rats had poor memory in Morris water maze and shows increased oxidative stress. Aged rats had longer escape latencies and they also spent less time in the correct quadrant. The learning and memory process in Minocycline treated aged rats are very faster than control aged rats. As well as the percent of time that rats spent in goal quarter during probe trial was increased significantly in Minocycline treated aged rats. These results imply that Minocycline increased spatial memory performance in aged rats. These findings have indicated that Minocycline enhanced spatial memory in aged male rats thereby protecting the central nervous system from the memory impairment. The probable mechanism of memory enhancement in aged rats may be attributed to its antioxidant properties shown in the study.

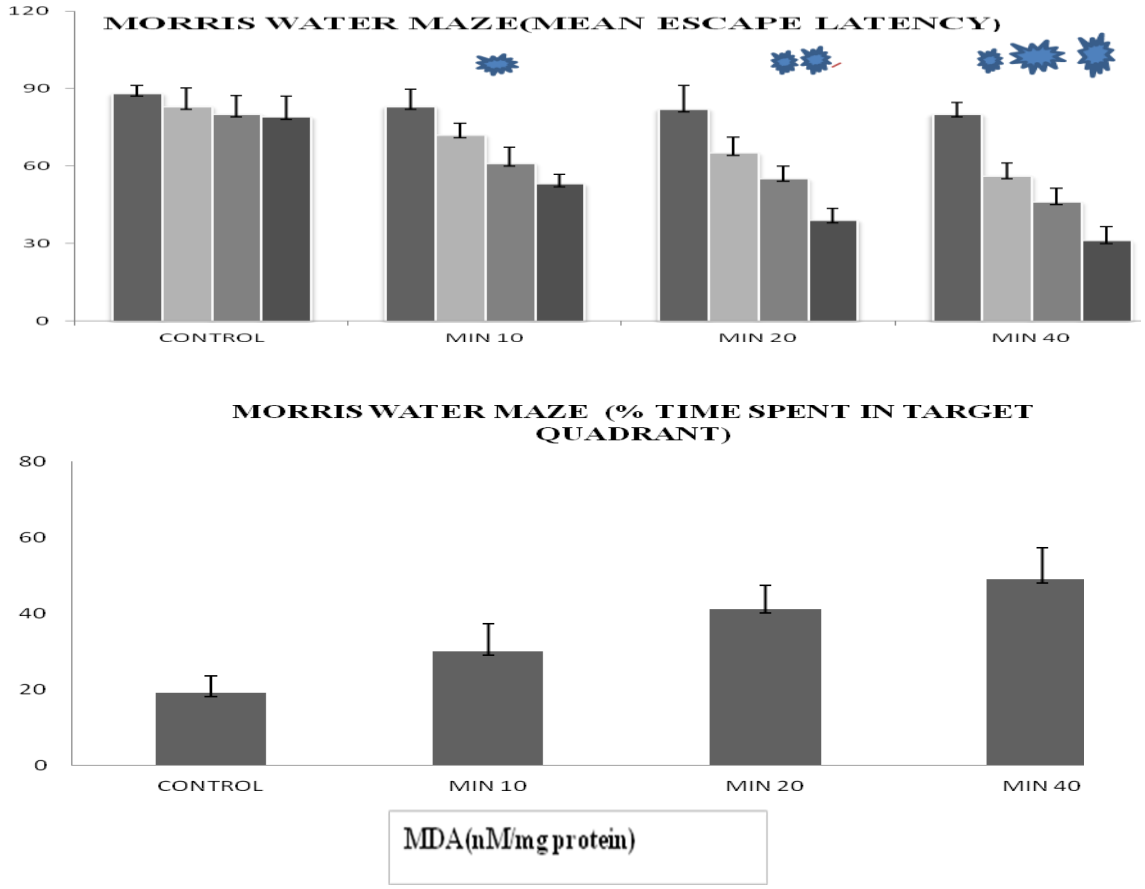


Fig.1 (A) **Effect of Minocycline on memory performance in Morris water maze task in aged rats**. Effect of Minocycline on escape latency to find the hidden platform in Morris water maze test in aged rats, mean escape latency to locate the hidden platform was recorded on days 20,21,22 and 23 to a maximum of 120 s.The mean escape latency (days 20-23) to find the hidden platform was significantly prolonged in aged rats compared with minocycline group (*P<0.05 vs aged rats). Minocycline significantly reversed age induced learning deficit compared with control group.(B) **Effect of Minocycline on percentage of time spent in target quadrant in aged rats.** The percentage of time spent in target quadrant (day24) was significantly lesser in aged rats compared with Minocycline treated rats.

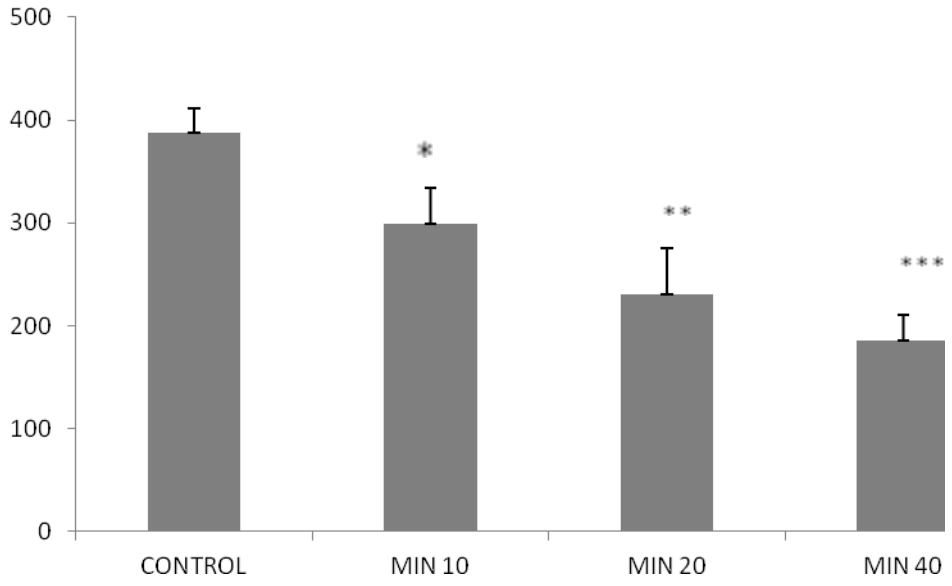


Fig. 2.Levels of MDA measured in aged (control) and aged (minocyline treated) rats. The levels of MDA were significantly increased in aged control group compared with aged rats treated with Minocycline. Minocycline significantly decreased age induced increase in malondialdehyde levels.

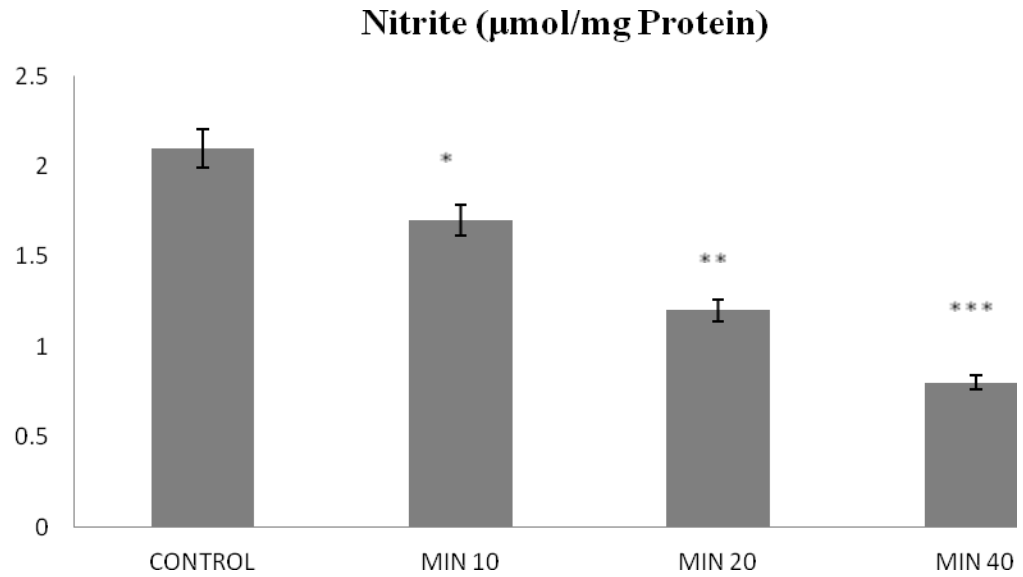


Fig. 3 Levels of Nitrite measured in aged (control) and aged (minocyline treated) rats. The levels of nitrite were significantly increased in aged control group compared with aged rats treated with Minocycline. Minocycline significantly decreased age induced increase in malondialdehyde levels.

Fig.4 Levels of Glutathione measured in aged (control) and aged (minocyline treated) rats. The levels of glutathione were significantly increased in aged control group compared with aged rats treated with Minocycline. Minocycline significantly decreased age induced increase in glutathione levels.

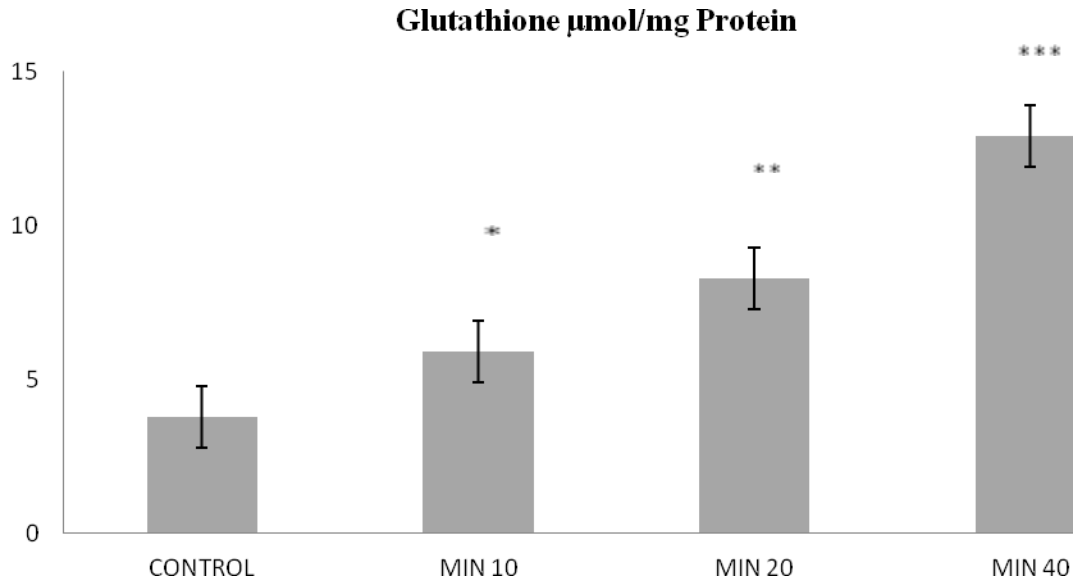


Fig.5 Levels of SOD measured in aged (control) and aged (minocyline treated) rats. The levels of SOD were significantly increased in aged control group compared with aged rats treated with Minocycline. Minocycline significantly decreased age induced increase in SOD levels.

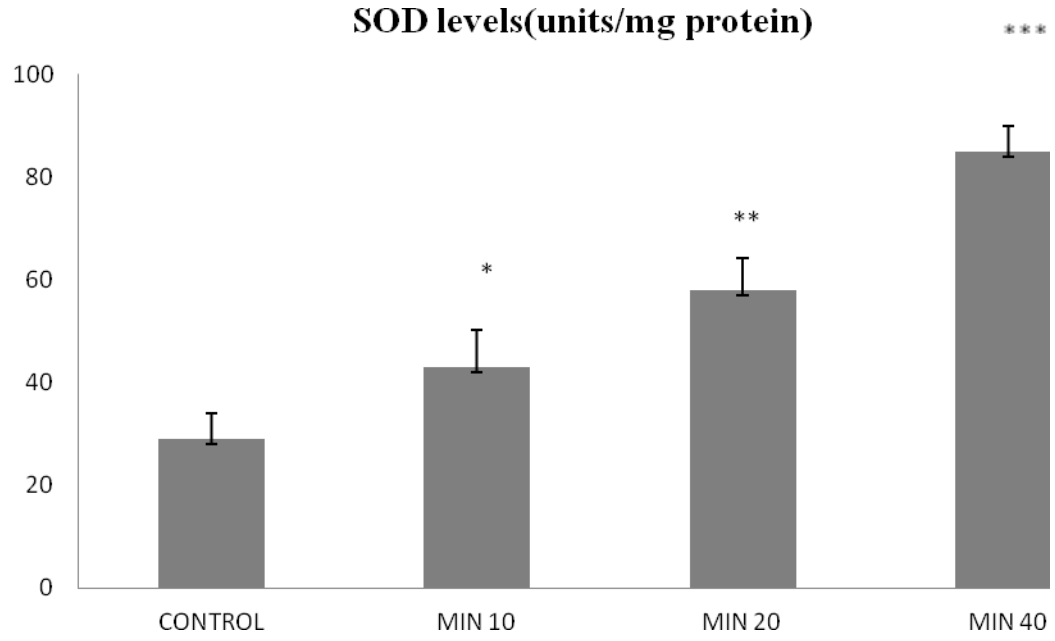
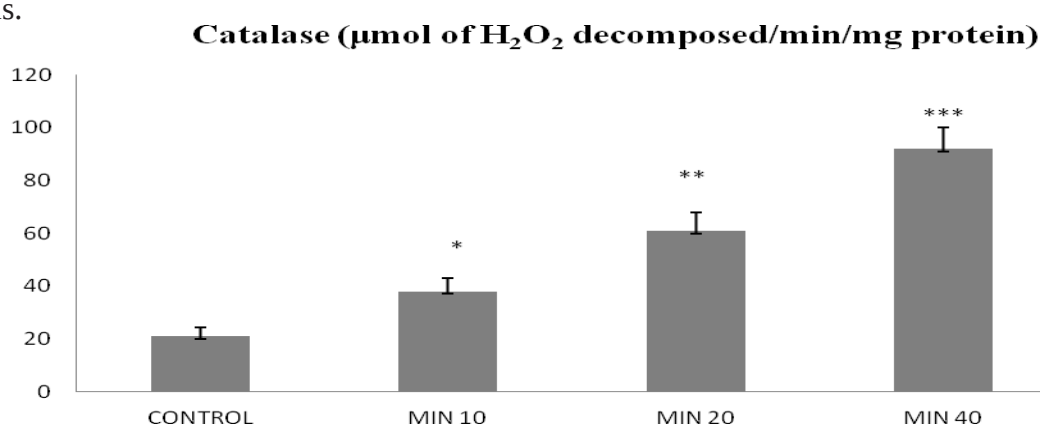


Fig.6 Levels of catalase measured in aged (control) and aged (minocyline treated) rats. The levels of catalase were significantly increased in aged control group compared with aged rats treated with Minocycline. Minocycline significantly decreased age induced increase in catalase levels.



GROUPING OF ANIMALS AND TREATMENT SCHEDULE

No.	Group	Treatment
1	Control	Old rats (24 month old) weighing 180- 200g were treated as control rats
2	MIN 10	Old rats (24 month old) weighing 180-200g were treated with minocycline 10 mg/kg ip for 25 days
3	MIN 20	Old rats (24 month old) weighing 180- 200g were treated with minocycline 20 mg/kg ip for 25 days
4	MIN 40	Old rats (24 month old) weighing 180-200g were treated with minocycline 40 mg/kg ip for 25 days

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