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In Vitro Time Dependent Nicotine-Induced Free Radical Generation and Status of Glutathione Cycle in Murine Peritoneal Macrophage.

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Abstract: Nicotine is an alkaloid and the precursor to many tobacco carcinogens present in various tobacco products. The aim of the present study was to evaluate the free radical generation and status of the glutathione cycle in murine peritoneal macrophage during in vitro nicotine exposure with different time interval. Peritoneal macrophages were treated with 10mM nicotine in vitro for different time interval (3, 6, 9, 12, 15, 18, & 24h) and super oxide anion generation and components of glutathione cycle were analyzed. Super oxide anion generation, and NADPH oxidase activity got peak at 12hr, indicates maximum free radical generation through activation of NADPH oxidase in murine peritoneal macrophages during nicotine treatment. Reduced glutathione level, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase activity were decreased significantly ($p<0.05$) with increasing time of nicotine treatment. But the oxidized glutathione level was time dependently increased significantly ($p<0.05$) in murine peritoneal macrophages. The redox ratio also decreased significantly ($p<0.05$) at all time of nicotine treatment. All the changes in peritoneal macrophages after 12hr in vitro nicotine treatment had no significant difference. From this study, it may be summarized that nicotine not only generates excess free radical but also affect the glutathione cycle in murine peritoneal macrophage.
Key words: Nicotine, free radical, glutathione, peritoneal macrophage.

Introduction

Cigarette (smoking) is a risk factor for many diseases and has been increasingly prevalent in economically developing regions of the world. Smoking has been identified as the second leading risk factor for death from any cause worldwide [1-2]. Nicotine is a pharmacologically active substance and major toxic component present in smoke and smokeless tobacco products and has long been recognized to result in oxidative stress by inducing the generation of reactive oxygen species (ROS). Macrophages are ubiquitous mononuclear phagocytes in mammalian tissues. The peritoneal macrophages, representative of other macrophage populations [3], are easily available in mice in greater amounts than blood phagocytes (monocytes or neutrophils). The immune cells use ROS for carrying out their normal functions. But an excess amount of ROS can alter the oxidant/antioxidant balance and lead to oxidative immune cell damage. In our previous in vitro study, it is clearly established that nicotine can generate oxidative stress in lymphocytes and peritoneal macrophages through excess super oxide radical generation and depletion of antioxidant status [4-5]. Glutathione (GSH), a tripeptide consisting of γ-glutamic acid, cysteine, and glycine, is the most prevalent nonprotein intracellular thiol present

in high concentrations (0.5 to 10mM) in almost all living cells [6]. GSH is characterized by its reactive thiol group –SH and its γ-glutamyl bond that makes it resistant to normal peptidase activity. Reaction of GSH with oxidants converts it to either glutathione disulfide (GSSG), an oxidized glutathione form, or to a mixed disulfide (RSSG) [7]. Thus, three forms of GSH may exist in a cell. More over, the reduced glutathione/oxidized glutathione (GSH/GSSG) complex is the major buffer in the cell [6]. Glutathione is an important biomolecules present in the immune cell, protects erythrocytes and eye lens cells against oxidative damage by its reaction with radicals and peroxides [8-9]. This reaction proceeds spontaneously but is greatly accelerated by the enzyme glutathione peroxidase. The product of this reaction, the disulfide GSSG, is reduced again to GSH with NADPH in the glutathione reductase reaction**.** For several reasons, this redox system is believed to be also important in protecting phagocytic leukocytes against oxidative damage. First, during phagocytosis, these cells release large amounts of super oxide, hydrogen peroxide, and possibly other highly reactive oxygen radicals [10-12]. Thus, these cells can be under heavy oxidative stress. Second, glutathione, as well as glutathione peroxidase and glutathione reductase, is present in high amounts in the cytosol of cells [13]. Third, deficiencies in this redox system have been found that lead to dysfunctions in the bactericidal capacities of these cells, probably owing to oxidative damage [14-15]. And fourth, glutathione is an important protectant of microtubule synthesis in neutrophils [13,16-17]. So, glutathione determination in murine peritoneal macrophages has a great impact on immune cell damage. In our previous lab report, we observed that, nicotine toxicity leads to the decreased glutathione level in rat tissues [18], and dose dependently alter the glutathione system in murine peritoneal macrophages [5]. The aim of this present investigation was to focus on the time dependent nicotine toxicity through assays the free radical generation and status of glutathione cycle.

Materials and Methods

2.1. Chemicals and reagents: Hydrogen tartarate salt of nicotine, phorbol mirested aceted (PMA), horse heart cytochrome-c, sodium dodecyl sulfate (SDS), 5', 5'-dithio (bis)-2-nitrobenzoic acid (DTNB), standard reduced glutathione, NADPH Na_4, oxidized glutathione (GSSG) were obtained from Sigma, USA. RPMI 1640, fetal bovine serum (FBS), heparin, ethylene diamine tetra acetate (EDTA) were purchased from Himedia, India. All other chemicals were from Merck Ltd., SRL Pvt. Ltd. Mumbai and were of the highest grade available.

2.2. Animals: Experiments were performed using Swiss male mice 6-8 weeks old, weighing 20-25g. The animals were fed standard pellet diet with vitamins, antibiotic and water were given ad libitum and housed in polypropylene cage (Tarson) in departmental animal house with 12h light:dark cycle, and the temperature of 25±2ºC. The animals were allowed to acclimatize for one week. The animals used did not show any sign of malignancy or other pathological processes. Animals were maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, and approved by the ethical committee of Vidyasagar University.

2.3. Isolation of the peritoneal macrophages and cell culture: All efforts were made to minimize animal suffering and to reduce the number of animals used. Macrophages were isolated by peritoneal lavage from male Swiss mice, after 24hrs injection of 2ml of 4% starch according to our previous lab report by Kar Mahapatra et al [5]. Washing the peritoneal cavity with ice cold phosphate buffer saline (PBS) supplemented with 20U/ml heparin and 1mM EDTA performed lavage. Care was taken not to cause internal bleeding while collecting macrophages in the exudates. The cells were then cultured in 60mm petridishes in RPMI-1640 media supplemented with 10% FBS, 50µg/ml gentamycin, 50µg/ml penicillin and 50µg/ml streptomycin for 24h at 37ºC in a humidified atmosphere of 5%CO_2 - 95% air in CO_2 incubator. Non-adherent cells were removed by vigorously washing three times with ice-cold PBS. Differential counts of the adherent cells used for the experiments were determined microscopically after staining with Giemsa and the cell viability evaluated by Trypan blue exclusion was never below 95% [19, 20].

2.4. Preparation of nicotine : Hydrogen tartarate salt of nicotine, obtained from Sigma was dissolved in normal saline (0.9%NaCl) to get the required concentration. Then pH of the nicotine solution was adjusted to 7.4 by NaOH [5].

2.5. Experimental design and sample preparation : The peritoneal macrophages were divided into 7 groups for seven different duration dependent experiments as follows: Group I: 3hr; Group II: 6hr; Group III: 9hr; Group IV: 12hr; Group V: 15hr; Group VI: 18hr and Group VII: 24hr. Each group is divided into two sub-groups, one is control and another is for 10mM nicotine treatment. Each sub-group contained 6 petridishes (4 X 10^6 cells in each). The cells of each petridishes were maintained in RPMI 1640 media supplemented with 10% FBS, 50µg/ml gentamycin, 50µg/ml penicillin and 50µg/ml streptomycin at 37ºC in a 95% air/5% CO_2 atmosphere in CO_2 incubator. The concentration of nicotine was selected according to our previous lab report by Kar Mahapatra et al [5]. After the treatment schedule the cells were collected from the petridishes separately and centrifuged at 2200 rpm for 10 min at 4°C. Then the supernatant was collected in separate micro centrifuge tube and the cells were washed twice with 50 mM PBS, pH 7.4. The pallets were lysed with hypotonic lysis buffer (10 mM TRIS, 1 mM EDTA and Titron X-100, pH 8.0) for 45 min at 37°C and then processed for the biochemical estimation [21]. Intact cells were used for superoxide anion generation and NADPH oxidase activity.

2.6. Biochemical estimation :

2.6.1. Assessment of superoxide anion ($O_2^{\cdot-}$) generation : Superoxide anion generation was determined by a standard assay [22]. Briefly, 0.1µg/ml of PMA (Sigma), a potent macrophage stimulant, and 0.12mM horse heart cytochrome-c (Sigma) were added to cell suspensions after treatment schedule and washing with PBS. Cytochrome-c reduction by generated super oxide was then determined by spectrophotometric absorbance at 550nm wavelength. Results are expressed n mol of cytochrome-c reduced/min, using the extinction coefficient 2.1×10^4 M^{-1} cm^{-1}.

2.6.2. NADPH oxidase activity: After the treatment schedule the macrophages of different groups prewarmed in krebs ringer buffer (KRB) with 10mM glucose at 37 °C for 3 min. PMA (0.1µg/ml) prewarmed at 37°C for 5 min was added, and the reaction was stopped by putting in ice. Centrifugation was carried out at 400 g for

5min and the resultant pellet was resuspended in 0.34M sucrose. The cells were then lysed with hypotonic lysis buffer. Centrifugation was carried out at 800 X g for 10min and the supernatant used to determine enzyme activity. NADPH oxidase activity was determined spectrophotometrically by measuring cytochrome c reduction at 550nm. The reaction mixture contained 10mM phosphate buffer (pH 7.2), 100mM NaCl, 1mM $MgCl_2$, 80µM cytochrome c, 2mM NaN_3 and 100µl of cell supernatant (final volume 1.0ml). A suitable amount of NADPH (10-20µl) was added last to initiate the reaction [23]. Results are expressed as n mol/min/ 2 X 10^6 cells.

2.6.3. Determination of lipid peroxidation (MDA): Lipid peroxidation was estimated by the method of Ohkawa et al. in cell lysate [24]. Briefly, the reaction mixture contained Tris-HCl buffer (50mM, pH 7.4), tert-butyl hydroperoxide (BHP) (500µM in ethanol) and 1mM $FeSO_4$. After incubating the samples at 37°C for 90min, the reaction was stopped by adding 0.2ml of 8% sodium dodecyl sulfate (SDS) followed by 1.5ml of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5ml of 0.8% TBA and further heating the mixture at 95°C for 45min. After cooling, samples were centrifuged, and the TBA reactive substances (TBARS) were measured in supernatants at 532nm by using 1.53×10^5 M^{-1} cm^{-1} as extinction coefficient. The levels of lipid peroxidation were expressed in terms of µ mol/mg protein.

2.6.4. Determination of reduced glutathione (GSH): Reduced glutathione estimation in the cell lysate was performed by the method of Moron et al [25]. The required amount of the cell lysate was mixed with 25% of trichloroacetic acid and centrifuged at 2000×g for 15min to settle the precipitated proteins. The supernatant was aspirated and diluted to 1ml with 0.2M sodium phosphate buffer (pH 8.0). Later, 2.0ml of 0.6mM DTNB was added. After 10min the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman's reagent) was measured at 405nm. A standard curve was obtained with standard reduced glutathione. The level of GSH was expressed as µg/mg protein.

2.6.5. Oxidized glutathione level (GSSG): The oxidized glutathione level was measured after derevatization of GSH with 2-vinylpyidine according to the method of Griffith [26]. In brief, with 0.5ml cell lysate, 2.0µl of 2-vinylpyidine was added and incubates for 1.0h at 37º C. Then the mixture was deprotenized with 4% sulfosalicylic acid and centrifuged at 1000×g for 10min to settle the precipitated proteins. The supernatant was aspirated and GSSG level was estimated with the reaction of DTNB at 412nm in spectrophotometer and calculated with standard GSSG curve. The level of GSSG was expressed as µg/mg protein.

2.6.6. Activity of glutathione peroxidase (GPx): The GPx activity was measured by the method of Paglia and Valentine [27]. The reaction mixture contained 50mM potassium phosphate buffer (pH 7.0), 1mM EDTA, 1mM sodium azide, 0.2mM NADPH, 1U glutathione reductase, and 1mM reduced glutathione. The sample, after its addition, was allowed to equilibrate for 5min at 25°C. The reaction was initiated by adding 0.1ml of 2.5mM H_2O_2. Absorbance at 340 nm was recorded for 5min. Values were expressed as n mol of NADPH oxidized to NADP by using the extinction coefficient of 6.2 X $10^3M^{-1}cm^{-1}$ at 340nm. The activity of GPx was expressed in terms of n mol NADPH consumed/min/mg protein.

*2.6.7. Activity of glutathione reductase (GR) :*The GR activity was measured by the method of Miwa [28]. The tubes for enzyme assay were incubated at 37^0C and contained 2.0ml of 9mM GSSG, 0.02ml of 12mM NADPH, Na_4, 2.68ml of 1/15M phosphate buffer (pH 6.6) and 0.1ml of cell lysate. The activity of this enzyme was determined by monitoring the decrease in absorbance at 340nm. The activity of GR was expressed in terms of n mol NADPH consumed/min/mg protein.

2.6.8. Activity of glutathione-s-transferase (GST): The activity of GST activity was measured by the method of Habig et al [29]. The tubes of enzyme assay were incubated at 25^0C and contained 2.85ml of 0.1M potassium phosphate (pH 6.5) containing 1mM of GSH, 0.05ml of 60mM 1-chloro-2, 4-dinitrobengene and 0.1ml cell lysate. The activity of this enzyme was determined by monitoring the increase in absorbance at 340nm and expressed as m mol/mg protein.

2.6.9. Protein estimation : Protein was determined according to Lowry et al. [30] using bovine serum albumin as standard.

2.7. Statistical analysis : The data were expressed as mean ± SEM, n=6. Comparisons between the means of control and nicotine treated group of each time interval were made by one-way ANOVA test (using a statistical package, Origin 6.1, Northampton, MA 01060 USA) with student's t-tests, $p< 0.05$ as a limit of significance.

Results

3.1. Superoxide radical generation: In vitro time dependent superoxide anion generation due to nicotine toxicity in peritoneal macrophages is presented in fig. 1.

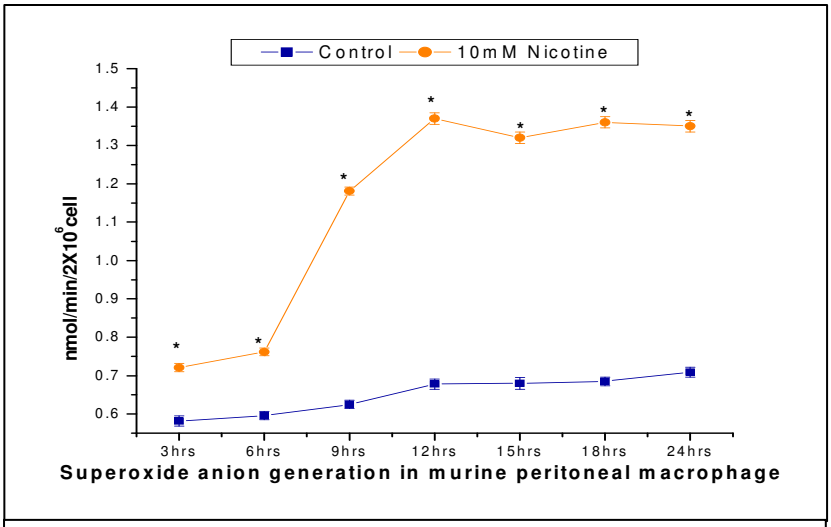

Fig. 1. : Superoxide anion generation in peritoneal macrophages of control and nicotine treated group. Values are expressed as mean±SEM, n=6. * Significant difference (P<0.05) in nicotine treated group compared to control group at respective time interval.

Significant ($p<0.05$) increases in superoxide anion generation, as indicated by reduction of cytochrome-c, were observed compare to control levels at all respective time interval. Highest amount of radical generation was found at 12h

treatment schedule and it was 102.06% high than control group in this time period. After that, it is gradually decreased up to 90.04% than control group at 24h.

3.2. NADPH oxidase activity: NADPH oxidase activation is the key factor to

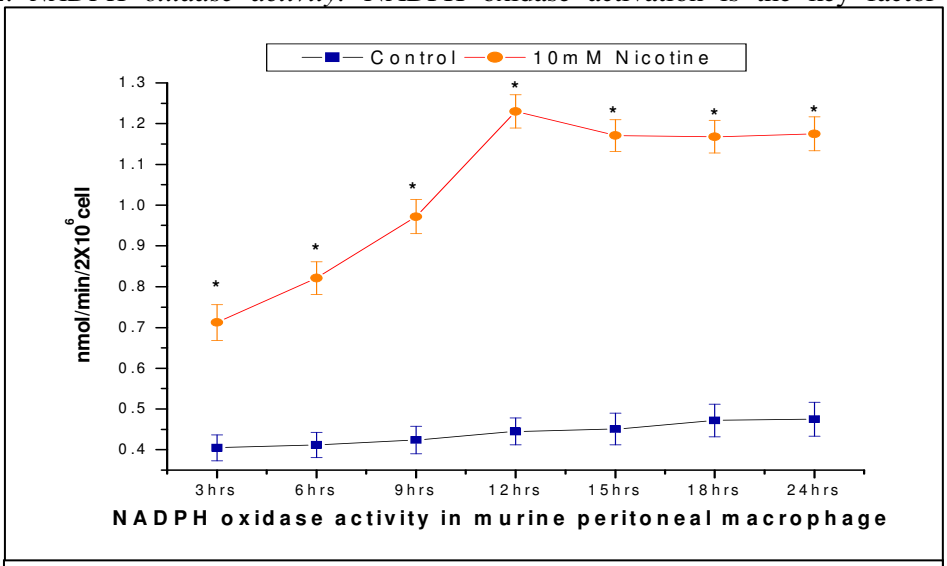

Fig. 2. : NADPH oxidase activity in peritoneal macrophages of control and nicotine treated group. Values are expressed as mean±SEM, n=6. * Significant difference (P<0.05) in nicotine treated group compared to control group at respective time interval.

generate the superoxide anion in cell. NADPH oxidase activity was also time dependently increased significantly (p<0.05) from 3 to 12h nicotine treatment in macrophage comparison to their respective control group and highest level of activity of this enzyme was noted at 12h. After that the activation was slightly decreased. It was also found that, there was no significant difference in NADPH oxidase activity at 12, 15, 18, 24h nicotine treated macrophages (Fig. 2.).*3.3. Lipid peroxidation :* Lipid peroxidation in terms of malondialdehyde (MDA) is an important determination of cellular injury. MDA level in macrophages of each group are shown in fig. 3. MDA

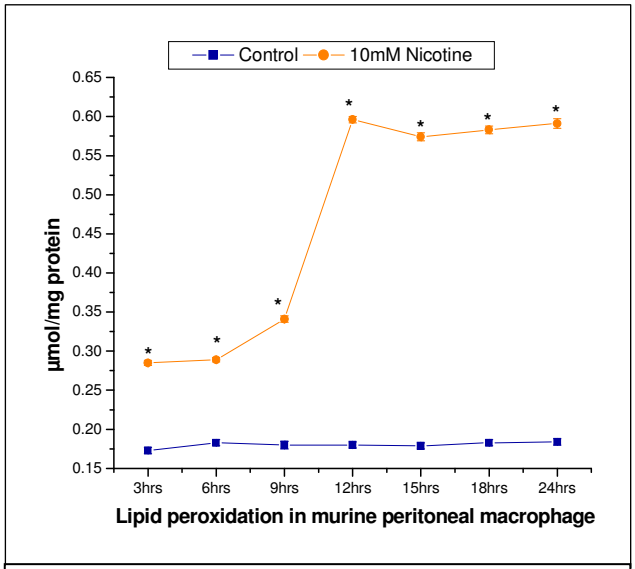

Fig. 3. : Lipid peroxidation (MDA) level in peritoneal macrophages of control and nicotine treated group. Values are expressed as mean±SEM, n=6. * Significant difference (P<0.05) in nicotine treated group compared to control group at respective time interval.

levels were increased significantly ($p<0.05$) in time dependent manner in nicotine

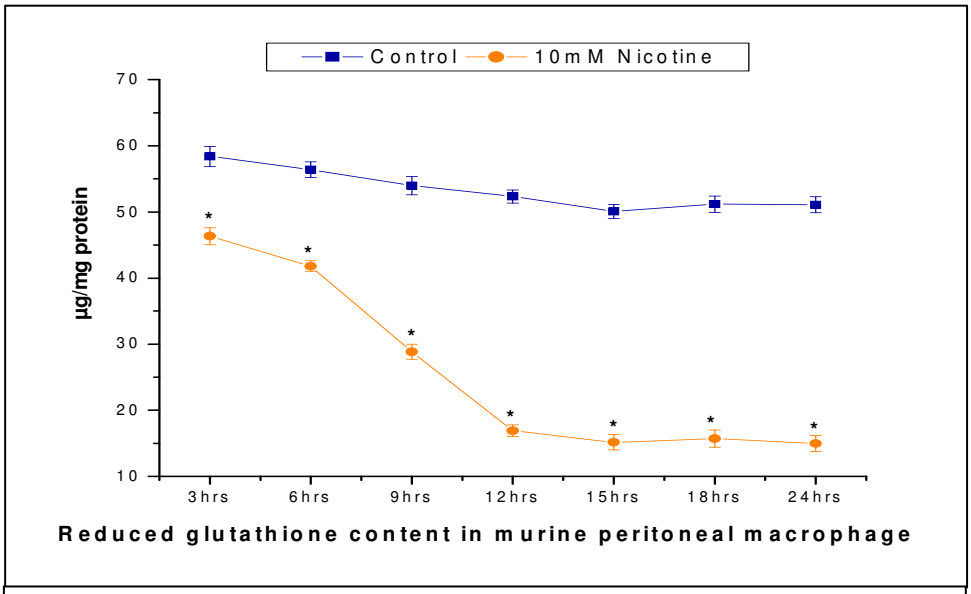

Fig. 4. : GSH level in peritoneal macrophages of control and nicotine treated group. Values are expressed as mean±SEM, n=6. * Significant difference (P<0.05) in nicotine treated group compared to control group at respective time interval.

exposed macrophage compare to respective control. Starting with 64.74% increase of MDA level at 3h in macrophage, it gone up to 221.19% at 24h nicotine treatment but highest degree of damage was found at 12h (231.85%) relative to there respective control group.

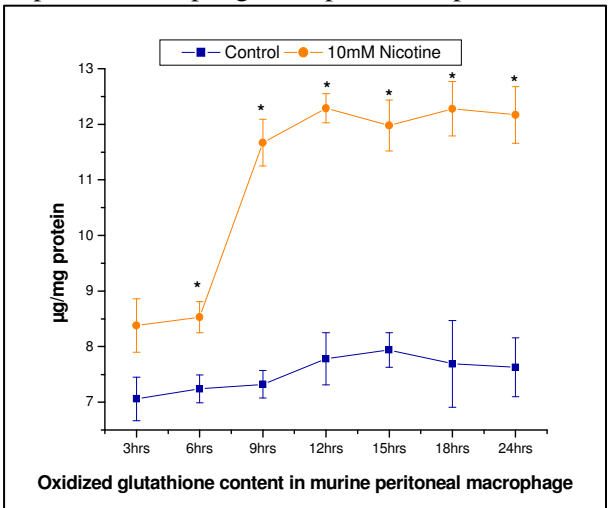

Fig. 5. : GSSG level in peritoneal macrophages of control and nicotine treated group. Values are expressed as mean±SEM, n=6. * Significant difference (P<0.05) in nicotine treated group compared to control group at respective time interval.

3.4. Glutathione cycle : The reduced glutathione (GSH) levels were decreased significantly ($p<0.05$) at all duration of nicotine treatment; 20.64%, 25.86%, 46.54%, 67.67%, 69.66%, 69.26%, and 70.68% GSH levels were decreased due to in vitro 10mM nicotine treatment for 3h, 6h, 9h, 12h, 15h, 18h, and 24h duration, respectively, in peritoneal macrophages compared with respective control cells (Fig. 4). Maximum decreased GSH level was found at 24h nicotine treatment. The oxidized glutathione (GSSG) levels in

peritoneal macrophages were increased significantly ($p<0.05$) in time dependent manner and found 59.89% increased GSSG level compare to control at 15h treatment schedule (Fig. 5). The redox ratio (GSH/GSSG) was increased significantly ($p<0.05$) up to 24h nicotine treatment, as GSH level gradually decreased upto 24hr treatment (Fig. 6.).

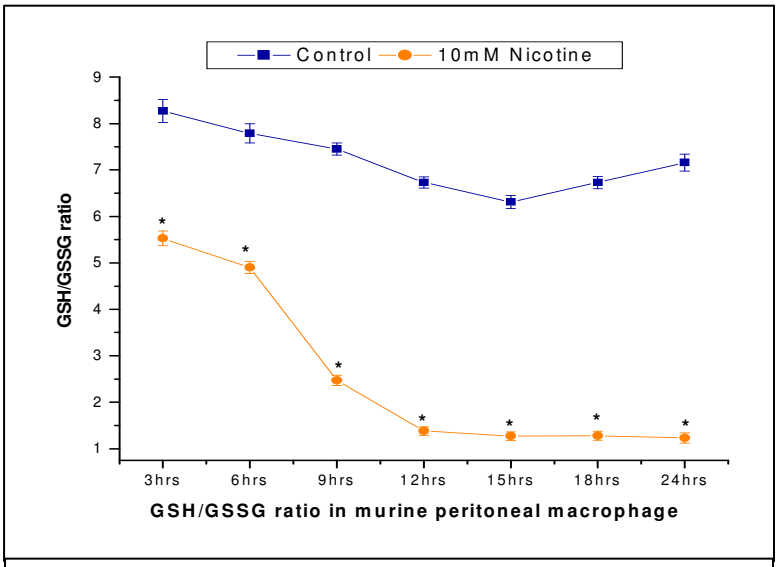

Fig. 6. : GSH/GSSG ratio in peritoneal macrophages of control and nicotine treated group. Values are expressed as mean±SEM, n=6. * Significant difference (P<0.05) in nicotine treated group compared to control group at respective time interval.

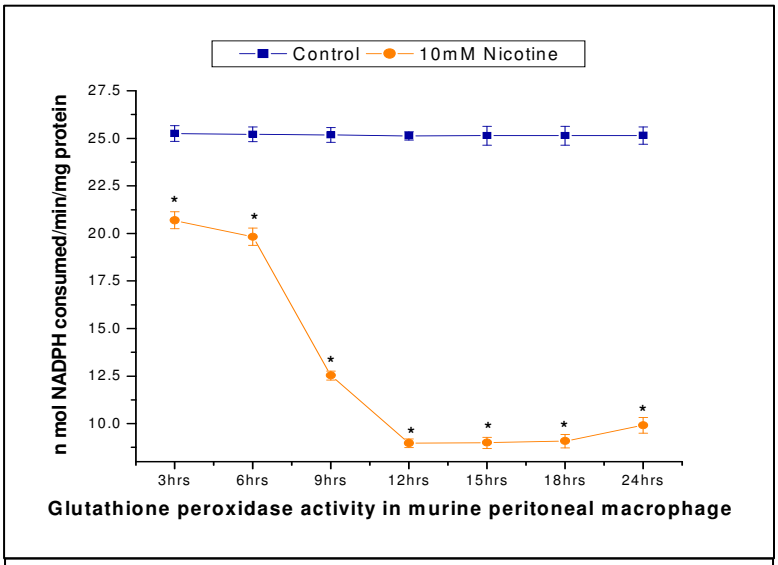

Fig. 7. : GPx activity in peritoneal macrophages of control and nicotine treated group. Values are expressed as mean±SEM, n=6. * Significant difference (P<0.05) in nicotine treated group compared to control group at respective time interval.

GPx and GR activity were decreased significantly ($p<0.05$) at all duration of the nicotine treatment (Fig. 7 & 8). GPx activity decreased maximum (64.31% decreased

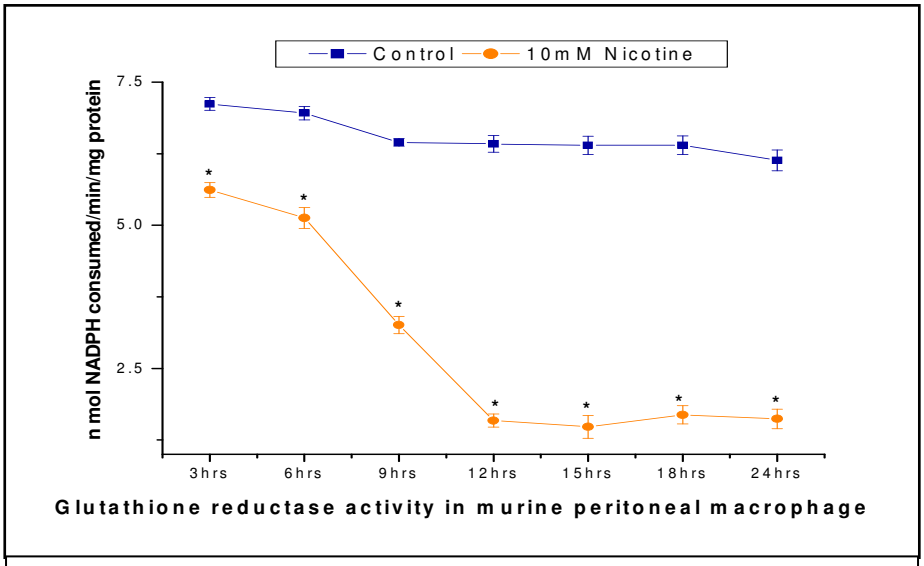

Fig. 8. : GR activity in peritoneal macrophages of control and nicotine treated group. Values are expressed as mean±SEM, n=6. * Significant difference (P<0.05) in nicotine treated group compared to control group at respective time interval.

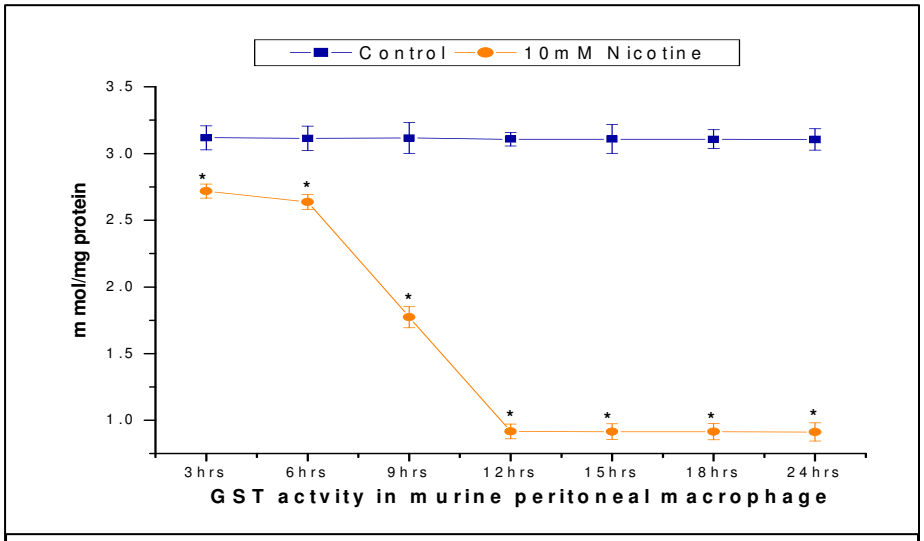

Fig. 9. : GST activity in peritoneal macrophages of control and nicotine treated group. Values are expressed as mean±SEM, n=6. * Significant difference (P<0.05) in nicotine treated group compared to control group at respective time interval.

activity than control) at 12h. But GR activity was decreased maximum at 15h and at this time it was 76.87% lower than control group. GST activity was found decreased significantly ($p<0.05$) starting from 6h (15.34% lower than control group) and upto 24h (70.6% lower than control group) in vitro nicotine treatment (Fig. 9.).

Discussion

Nature has provided cells with very strong biological antioxidant defense mechanisms. These include a variety of enzymatic and non-enzymatic molecules with enormous capabilities to mitigate the deleterious and potentially harmful effects of ROS and other free radicals. One of the primary antioxidant defense mechanisms is the GSH redox system. The enzymes of this system provide a formidable protective shield against oxidative damage. Alterations in their activities ultimately may result in irreversible manifestation of cellular damage. In the present study, in vitro nicotine can generate excess amount of super oxide anion through excess activation of NADPH oxidase in time dependent manner (Fig. 1 & 2). The activated NADPH oxidase transports electrons from NADPH on the cytoplasmic side of the membrane to oxygen in the extra cellular fluid to form $O_2^{\cdot-}$ [31]. This excess $O_2^{\cdot-}$ leads to oxidative damage of macromolecules, lipid, and lowers antioxidant system in cell. As a result, increased lipid peroxidation (MDA level) was found in time dependent manner at all duration of the in vitro nicotine treatment (Fig. 3.). Glutathione system is an important antioxidant system in cell and involved in protection against free radicals, peroxides, and toxic compounds and their reactive intermediates formed intracellularly either spontaneously or enzymatically in cellular systems [32]. The enzymatic conjugation of these toxic substances by GSH is catalyzed by a group of cytosolic enzymes, glutathione-S-transferases (GST) [33]. The capacity of GSH to act as a reductant is prompted by the enzyme glutathione peroxidase (GPx) that converts toxic peroxides to less toxic hydroxyl derivatives. In this reaction, GSH itself is oxidized to form GSSG. GSSG is again reduced to GSH by another enzyme, glutathione reductase (GR), which utilizes NADPH. In our present study, GSH levels were decreased (Fig. 4) and GSSG levels were increased (Fig. 5) with the increasing duration of nicotine toxicity. The decreased GSH levels represent its increased utilization with the increasing time interval of nicotine treatment. But at the duration of nicotine treatment more than 12h, there was not enough scope to increase further GSH utilization. So, there was a plateau observed at 12h onwards nicotine treatment. On the other hand, decreasing GSH level may be due to increasing level of lipid oxidation products which may be associated with less availability of NADPH required for the activity of glutathione reductase (GR) to transform GSSG to GSH [34], due to the increasing production of ROS at a rate that exceeds the ability to regenerate GSH in macrophages with increasing time of nicotine toxicity. In our present study, the increasing levels of GSSG and decreasing GR activity (Fig. 8) along with increasing duration of nicotine treatment may hold up the explanation. GPx activity was also decreased in time dependent manner during nicotine toxicity in macrophage may be due to less availability of substrate, as GSH level was decreased time dependent manner (Fig. 4 & 7). GPx works non-specifically to scavenge and decompose excess hydro peroxides including H_2O_2, which may be prevalent under oxidative stress [35, 36]. Glutathione-S-transferase (GST) mainly detoxifies electrophilic compounds [37], and has a well-established role in protecting cells from mutagens and carcinogens as a free radical scavenger along with glutathione. In the present study, the decreasing level of GSH and decreased activity

of GSH-dependent enzymes, i.e. GPx, GR, and GST (Fig. 7-9) in peritoneal macrophages on in vitro treatment with increasing time of nicotine treatment (3h to 24h) may be due to increased utilization to scavenge the free radical generation. But it was observed that duration of the nicotine treatment higher that 12h, the scope of further utilization of these enzymes remains the same. A decrease in the level of antioxidant status can lead to the excessive availability of super oxide and peroxyl radicals which in turn generate hydroxyl radicals, resulting in the initiation and propagation of lipid peroxidation during nicotine toxicity. Lipid peroxidation is known to cause cellular injury by inactivation of membrane enzymes and receptors, depolymerisation of polysaccharide, as well as protein cross-linking and fragmentation. Since membrane lipids are vital for the maintenance and integrity of cell function, the breakdown of membrane phospholipids and lipid peroxidation due to the generation of free radicals are expected to change membrane structure, fluidity, transport and antigenic properties, all of which play an important role in the pathogenesis of organ disorders [38]. In summery, the present study clearly established that nicotine can time dependently increase the superoxide anion generation through the activation of NADPH oxidase in murine peritoneal macrophage, which reduce the important antioxidant system i.e. glutathione system in it.

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