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## Document heading

## Age associated changes in antioxidant and antioxidative enzymes in human neutrophil of different aged people

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## ABSTRACT

**Objective:** To evaluate the level of lipid–protein damage and antioxidant status in neutrophils of healthy individuals to correlate between oxidative damage with the aging process. **Method:** Twenty healthy individuals of each age group (11–20; 21–30; 31–40; 41–50; and 51–60 years) were selected randomly. Blood samples were drawn by medical practitioner and neutrophils were isolated from blood samples. Superoxide ( $O_2^-$ ) generation anion, malondialdehyde (MDA), protein carbonyls (PC) level were evaluated to determine the lipid and protein damage in neutrophils. Superoxide dismutase (SOD), catalase (CAT), glutathione and glutathione dependent enzymes were estimated to evaluate the antioxidant status in the neutrophils. **Results:** Our results demonstrated that increased MDA and PC levels strongly support the increased oxidative damage in elderly subject than young subjects. **Conclusion:** The results indicate the balance of oxidant and antioxidant systems in neutrophil shifts in favour of accelerated oxidation during aging.

## 1. Introduction

During the last decades, much research has been directed towards establishing correlations between oxidative damage, antioxidant defense systems and aging. Aging a complex phenomenon is an inevitable biological process that is generally characterized by the declining ability to respond to stress, increasing homeostatic imbalance and increase risk of disease. Because of this death is an ultimate consequence of aging. The aging process includes the accumulation of changes with time and a decline of the organism response to these changes. It is well known that with aging, even during healthy aging there is a decrease of the immune response, this is termed as Immunosenescence. A general feature of the aging or senescence process is a progressive physiological deterioration with time leading to an impairment of the homeostasis, vulnerability to disease and ultimately to death of the organism[1]. The free radical theory of aging proposes that age–dependent deterioration in cell function is related to accumulation molecular

oxidative damage, caused by reactive oxygen. An increase in production of reactive radicals, particularly as a result of mitochondrial dysfunction has been recognized as a major cause of oxidative stress. However age–associated alterations in the antioxidant status could also contribute to increased oxidative stress in aging. Reactive oxygen species (ROS) are implicated in aging and in various degenerative disorders[1,2]. ROS such as hydrogen peroxide, superoxide anion, hydroxyl radical, and nitric oxide, are formed in the body as a consequence of aerobic metabolism, damaging all intracellular components, including nucleic acids, proteins, and lipids. To attenuate such a rampant attack, appropriate anti–oxidant defenses arise to protect against damage from ROS. The first line anti–oxidant system includes enzymes, such as superoxide dismutase (SOD), glutathione (GSH) peroxidase and catalase, which are re–enforced by nonenzymatic anti–oxidants, GSH, protein–SH, vitamin C, E,  $\beta$ –carotene, and uric acid. During normal oxygen metabolism the concentration of free radicals is controlled by various antioxidants and a balance exists between prooxidant and antioxidant process[2]. Changes in antioxidant capacities like hydrophilic radical scavengers (ascorbate, urate and glutathione), lipophilic radical scavengers (tocopherols, carotenoids), metal chelators and antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase activities have been

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demonstrated in aging<sup>3</sup>. Several studies have explored the effects of aging on total antioxidant capacity of plasma and tissues. In the present study, we attempted to document the effects of aging on the neutrophil redox status by quantifying lipid peroxidation, protein oxidation and various antioxidant enzymes activities in healthy individuals, and possible relationship between antioxidant potential and markers of lipid peroxidation and protein oxidation.

## 2. Materials and methods

### 2.1. Selection of human subjects

This study was carried out in 100 healthy subjects who were divided according to five different age groups; Group A (11–20 yrs), Group B (21–30 yrs), Group C (31–40 yrs), Group D (41–50 yrs) and Group E (51–60 yrs). All subjects enrolled in this study were asymptomatic and none of them had abnormality on physical examinations and routine laboratory tests. Groups had similar socioeconomic status and dietary habits. All the subjects were from same geographical area and same economic status, non-smokers and non-alcoholic and having same food habit. These subjects received no medication, including vitamin E and vitamin C. All subjects gave informed consent. The selection excluded not only individuals with acute infections or chronic diseases, but also excluded healthy individuals undergoing supplementation with antioxidative substances. The study protocol was in accordance with the declaration of Helsinki, and was approved by the ethical committee of Vidyasagar University.

### 2.2. Chemicals and reagents

Sodium dodecyl sulfates, DTNB, standard reduced glutathione, glutathione reductase, NADPH Na<sub>4</sub>, oxidized glutathione were purchased from Sigma Chemical Co., USA. Sodium chloride, ethylene diamine tetra acetate, tryptic soy broth, sodium azide were purchased from Himedia, India. Tris-HCl, Tris buffer, KH<sub>2</sub>PO<sub>4</sub>, K<sub>2</sub>HPO<sub>4</sub>, sodium hydroxide, O-phenylenediamine, alcohol and other chemicals were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. All other chemicals were from Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest grade available.

### 2.3. Collection of blood samples and separation of neutrophils

Fasting blood samples were collected from all groups of individuals satisfying the Helsinki protocol. Neutrophils were separated by sequential sedimentation in Dextran T-500 (Pharmacia LKB, Biotechnology, Piscataway, NJ) in 0.9% sodium chloride, centrifugation on a Fycoll-Hypaque cushion (specific gravity 1077, Pharmacia) and hypotonic lysis of erythrocytes, as described in our previous laboratory report<sup>1</sup>. The preparation was more than 98% pure and 98% of the cells were viable judged by Trypan blue exclusion.

## 2.4 Biochemical assays

### 2.4.1. Superoxide anion (O<sub>2</sub><sup>-</sup>) generation

The superoxide production was measured by the SOD-inhibitable reduction of acetylated cytochrome c<sup>4</sup>. Samples were resuspended in phosphate buffer saline (PBS) supplemented with 1 mM L-NMMA (N-methyl-L-arginine, to avoid sequestering of O<sub>2</sub><sup>-</sup> by nitric oxide) at 2×10<sup>6</sup> cells/mL. 0.1 μg/mL phorbol 12-myristate 13-acetate (PMA; Sigma), a potent stimulant, and 20 μM horse cytochrome c (Sigma) were added to neutrophils suspensions. Cytochrome c reduction by generated superoxide was then monitored spectrophotometrically at 550 nm wavelength with or without the addition of 3 μM SOD. Results are expressed as nmol/min/10<sup>6</sup> neutrophil.

### 2.4.2 Determination of lipid peroxidation (MDA)

Lipid peroxidation of neutrophil was estimated by the method of Gautam *et al*<sup>1</sup>. Briefly, the reaction mixture contained Tris-HCl buffer (50 mM, pH 7.4), tetra-butyl hydroperoxide (BHP) (500 μM in ethanol) and 1 mM FeSO<sub>4</sub>. After incubating the samples at 37 °C for 90 min, the reaction was stopped by adding 0.2 mL of 8% sodium dodecyl sulfate (SDS) followed by 1.5 mL of 20% acetic acid (pH 3.5). The amount of malondialdehyde (MDA) formed during incubation was estimated by adding 1.5 mL of 0.8% TBA and further heating the mixture at 95 °C for 45 min. After cooling, samples were centrifuged, and the TBA reactive substances (TBARS) were measured in supernatants at 532 nm by using 1.53×10<sup>5</sup> M<sup>-1</sup>·cm<sup>-1</sup> as extinction coefficient. The levels of lipid peroxidation were expressed in terms of nmol/mg protein.

### 2.4.3. Determination of protein oxidation (PC)

Neutrophil PC levels were measured based on spectrophotometric detection of the reaction of 2, 4 dinitrophenylhydrazine with protein carbonyl to form protein hydrazones<sup>1</sup>. Briefly, after precipitation of protein with an equal volume of 1% trichloroacetic acid (TCA), the pellet was resuspended in 10 mM DNPH in 2 N HCl. Next, after the washing procedure with 1:1 ethanol/ethyl acetate, the final pellet was dissolved in 6 M Guanidine. The carbonyl group was determined from the absorbance at 370 nm. The results were expressed as nmoles of carbonyl groups per milligram of protein with molar extinction coefficient of 22 000 M<sup>-1</sup>·cm<sup>-1</sup>.

### 2.4.4. Determination of reduced glutathione (GSH) level

Reduced glutathione estimation in neutrophil was performed by the method of Gautam. The required amount of sample was mixed with 25% of trichloroacetic acid and centrifuged at 2 000×g for 15 min to settle the precipitated proteins. The supernatant was aspirated and diluted to 1 mL with 0.2 M sodium phosphate buffer (pH 8.0). Later, 2 mL of 0.6 mM DTNB was added. After 10 min the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman's reagent) was measured at 405 nm. A standard curve was obtained with standard reduced glutathione. The levels of GSH were expressed as μg of GSH/mg protein.

#### 2.4.5. Determination of oxidized glutathione (GSSG) level

The oxidized glutathione level in neutrophil was measured after derevatization of GSH with 2-vinylpyridine according to the method of Gautam. In brief, with 0.5 mL sample, 2  $\mu$  L 2-vinylpyridine was added and incubates for 1 h at 37 °C. Then the mixture was deprotonized with 4% sulfosalicylic acid and centrifuged at 1 000 $\times$ g for 10 min to settle the precipitated proteins. The supernatant was aspirated and GSSG level was estimated with the reaction of DTNB at 412 nm in spectrophotometer and calculated with standard GSSG curve.

#### 2.4.6. Determination of super oxide dismutase (SOD) activity

SOD activity of neutrophil was determined from its ability to inhibit the auto-oxidation of pyrogallol. The reaction mixture considered of 50 mM Tris (hydroxymethyl) amino methane (pH 8.2), 1 mM diethylenetriamine penta acetic acid, and 20–50  $\mu$  L of sample. The reaction was initiated by addition of 0.2 mM pyrogallol, and the absorbance measured kinetically at 420 nm at 25 °C for 3 min. SOD activity was expressed as unit/mg protein.

#### 2.4.7. Determination of catalase (CAT) activity

Catalase activity of neutrophil was measured by the method of Gautam. The final reaction volume of 3 mL contained 0.05 M Tris-buffer, 5 mM EDTA (pH 7.0), and 10 mM H<sub>2</sub>O<sub>2</sub> (in 0.1 M potassium phosphate buffer, pH 7.0). About 50  $\mu$  L of sample was added to the above mixture. The rate of change of absorbance per min at 240 nm was recorded. Catalase activity was calculated by using the molar extinction coefficient of 43.6 M<sup>-1</sup>·cm<sup>-1</sup> for H<sub>2</sub>O<sub>2</sub>. The level of CAT was expressed in terms of  $\mu$  mol H<sub>2</sub>O<sub>2</sub> consumed/min/mg protein.

#### 2.4.8. Determination of glutathione peroxidase (GPx) activity

The GPx activity of neutrophil was measured by the method of Gautam. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1 U glutathione reductase and 1 mM reduced glutathione. The sample, after its addition, was allowed to equilibrate for 5 min at 25 °C. The reaction was initiated by adding 0.1 mL of 2.5 mM H<sub>2</sub>O<sub>2</sub>. Absorbance at 340 nm was recorded for 5 min. Values were expressed as n mol of NADPH oxidized to NADP by using the extinction coefficient of 6.2 $\times$ 10<sup>3</sup> M<sup>-1</sup>·cm<sup>-1</sup> at 340 nm. The activity of GPx was expressed in terms of n mol NADPH consumed/min/mg protein.

#### 2.4.9. Determination of glutathione reductase (GR) activity

The GR activity of neutrophil was measured by the method of Gautam. The tubes for enzyme assay were incubated at 37 °C and contained 2.0 mL of 9 mM GSSG, 0.02 mL of 12 mM NADPH, Na<sub>2</sub>S<sub>2</sub>O<sub>4</sub>, 2.68 mL of 1/15 M phosphate buffer (pH 6.6) and 0.1 mL of sample. The activity of this enzyme was determined by monitoring the decrease in absorbance at 340 nm. The activity of GR was expressed in terms of n mol NADPH consumed/min/mg protein.

#### 2.4.10. Determination of glutathione-s-transferase (GST) activity

The GST activity of neutrophil was measured by the method of Gautam. The tubes of enzyme assay were incubated at 25 °C and contained 2.85 mL of 0.1 M potassium phosphate (pH 6.5) containing 1 mM of GSH, 0.05 mL of 60 mM 1-chloro-2,4-dinitrobenzene and 0.1 mL of sample. The activity of this enzyme was determined by monitoring the increase in absorbance at 340 nm.

#### 2.4.11. Protein estimation

Protein content (mg/g tissue) was estimated by following the method of Lowry *et al* using BSA as the standard[5].

### 2.5. Statistical analysis

The data were expressed as mean  $\pm$  SEM,  $n=20$ . Comparison of the means of Group A with Group B, C, D and Group E were made by one way ANOVA with multiple comparison  $t$ -test,  $P<0.05$  as a limit of significance.

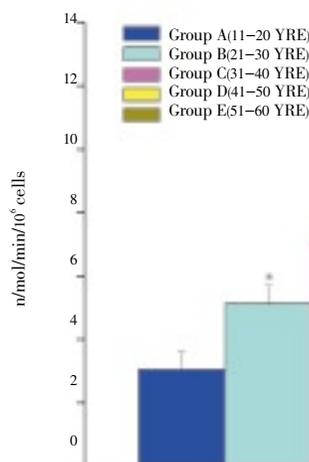
## 3. Results

In the present study, we investigated the age related changes in neutrophils redox status. The neutrophil antioxidant capacity was assessed by measuring the enzymatic and non enzymatic antioxidants activity.

### 3.1. Superoxide radical generation in neutrophils

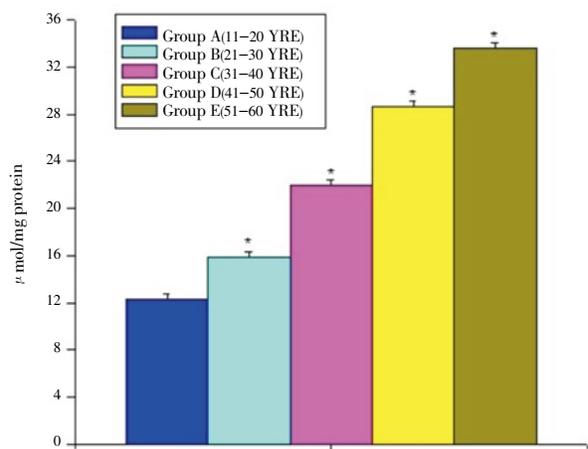
Superoxide radical generations were observed in all the different age groups of neutrophils. The superoxide radical generation was increased significantly ( $P<0.05$ ) 15.89%, 21.99%, 28.62%, & 33.63% with aging in group B, C, D & E respectively, when compared to group A (Figure 1).

### 3.2. Lipid peroxidation and protein oxidation level in neutrophils

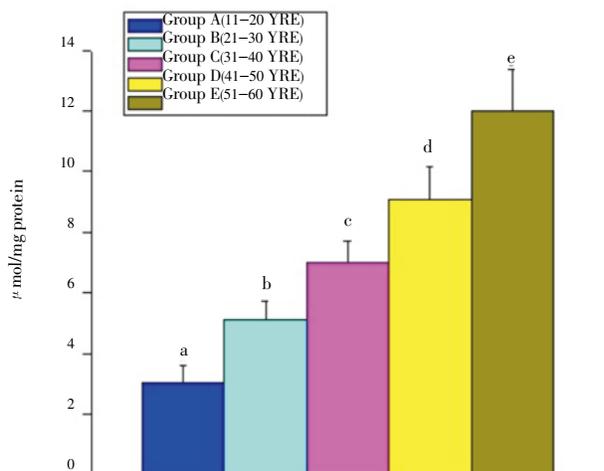


**Figure 1.** Superoxide radical ( $O_2^-$ ) generation in different age group of neutrophils. Data are expressed as mean  $\pm$  SEM,  $n=20$ . \*Indicates the significant difference ( $P<0.05$ ) compared with Group A by the one way ANOVA with multiple comparison  $t$ -test.

MDA levels were significantly ( $P<0.05$ ) increased in neutrophils of Group B, Group C, Group D and Group E by 15.89%, 21.99%, 28.62% and 33.63% respectively as compared to group A (Figure 2). PC levels were significantly ( $P<0.05$ ) increased in neutrophils of Group B, Group C, Group D and Group E by 5.13%, 7.02%, 9.05% and 12.02% respectively as compared to group A (Figure 3).



**Figure 2.** Lipid peroxidation (MDA level) in different age group of neutrophils. \* $P<0.05$ .

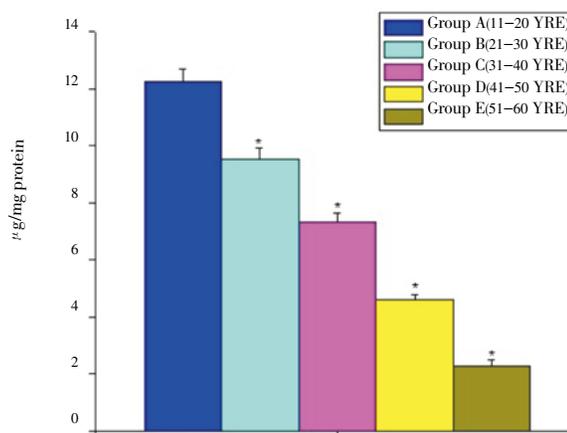


**Figure 3.** Protein oxidation (PC content) in different age group of neutrophils. \* $P<0.05$ .

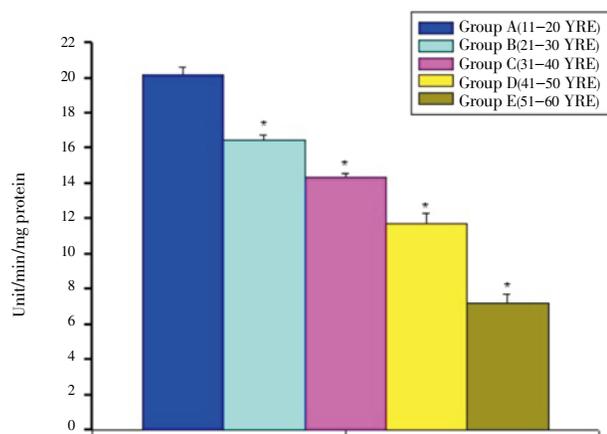
### 3.3. Glutathione level in neutrophils

Reduced GSH level was declined with age. There was significantly ( $P<0.05$ ) decreased 21.36%, 14.45%, 10.53% and 6.88% in GSH activity of Group B, Group C, Group D and Group E respectively, when compared to group A (Figure 4). The oxidized glutathione (GSSG) levels in neutrophils were decreased significantly ( $P<0.05$ ) by 9.53%, 7.34%, 4.6% and 2.28% in Group B, Group C, Group D and Group E respectively, when compared to group A (Figure 5).

### 3.4. Superoxide dismutase and catalase activity in neutrophils

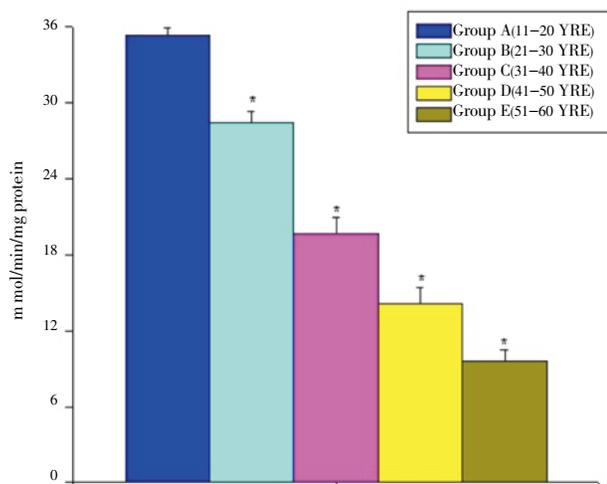


**Figure 5.** Oxidized glutathione (GSSG) level in different age group of neutrophils. \* $P<0.05$ .



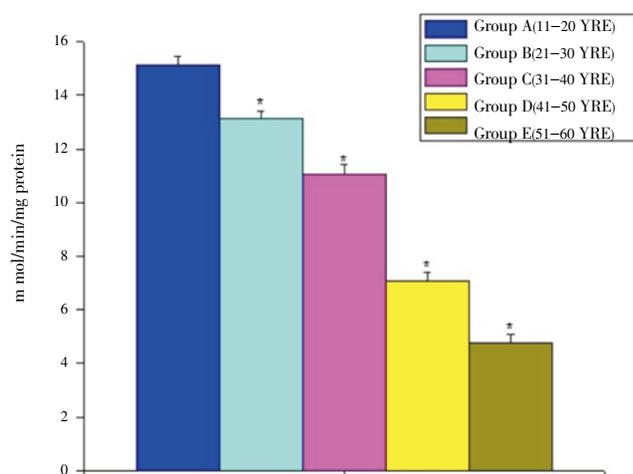
**Figure 6.** Super oxide dismutase (SOD) activity in different age group of neutrophils. \* $P<0.05$ .

SOD activity was observed in all the different age groups of neutrophils. The SOD activity decreased significantly ( $P<0.05$ ) 16.5%, 14.34%, 11.73% and 7.23% with aging in group B, C, D & E respectively, when compared to group A (Figure 6). Catalase activity was observed in all the different age groups of neutrophils. The catalase activity decreased significantly ( $P<0.05$ ) 28.45%, 19.66%, 14.16% and 9.59% with aging in group B, C, D & E respectively, when compared to group A (Figure 7).

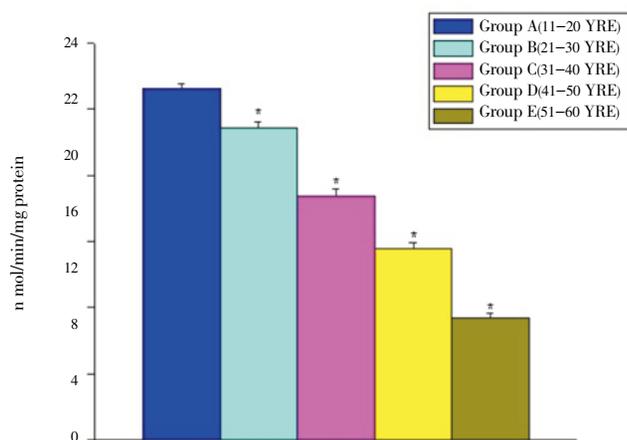


**Figure 7.** Catalase (CAT) activity in different age group of neutrophils. \* $P<0.05$ .





**Figure 8.** Glutathione peroxidase (GPx) activity in different age group of neutrophils. \* $P < 0.05$ .



**Figure 9.** Glutathione reductase (GR) activity in different age group of neutrophils. \* $P < 0.05$ .

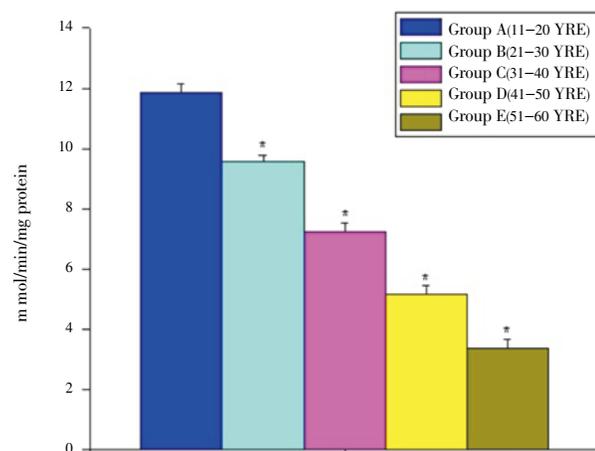
### 3.5. Glutathione dependent enzymes activity in neutrophils

Glutathione peroxidase (GPx) activity was observed in all the different age groups of neutrophils. The GPx activity decreased significantly ( $P < 0.05$ ) by 13.15%, 11.04%, 7.06% and 4.77% in Group B, Group C, Group D and Group E respectively, when compared to group A (Figure 8). Glutathione reductase (GR) activity were significantly ( $P < 0.05$ ) decreased in neutrophils of Group B, Group C, Group D and Group E by 18.89%, 14.76%, 11.58% and 7.38% respectively as compared to group A (Figure 9). Glutathione-s-Transferase (GST) activity were significantly ( $P < 0.05$ ) decreased in neutrophils of Group B, Group C, Group D and Group E by 9.55%, 7.22%, 5.16% and 3.37% respectively as compared to group A (Figure 10).

## 4. Discussion

When a disturbance in the pro-oxidant antioxidant balance (redox status) occurs in favor of the former, the potential for tissue & cell damage due to oxidative stress ensues[6]. Aging and age-related diseases are associated with oxidative stress from the over-production of ROS. In the present study, we investigated the redox status of human neutrophils during aging. Our study found age-related decline in activities of

antioxidant enzymes in neutrophils from five different age groups. Decreased content of GSH and decline in activities of catalase, SOD, GR, GPx and GST enzymes were the prominent alterations observed in the present investigation with aging. The destruction of reactive oxygen intermediates and of free radicals involves the activities of SOD, Catalase, GR, GPx and GSH as well as supply of NADPH. Our results depict a parallel decline in GST activity and GSH content with aging. A major factor that affects glutathione homeostasis is its utilization by conjugation primarily via



**Figure 10.** Glutathione-s-transferase (GST) activity in different age group of neutrophils. \* $P < 0.05$ .

GST. The ability of GST to alter levels of cellular glutathione in response to production of reactive oxygen species has been implicated in protection of cells from reactive oxygen species inducing agent[7]. There are several conflicting reports on GR activity with aging. Some studies reported a decline in GR activity that accompanies the age-related decrease in GSH content, others have indicated that GR activity either does not change or even increase with age[8,9]. Glutathione is an important cellular reductant, which offers protections against free radicals, peroxide and toxic compounds. It is reformed from GSSG by donation of hydrogen from NADPH, the reaction being catalyzed by glutathione reductase (GR)[9]. Adequate concentrations of GSH are required for a variety of immune functions, it has been suggested that oxidative stress and deficiency of thiol compounds may play an important pathogenic role in the development of immuno deficiency. The present study reports a decline in GSH content in neutrophils from elder group like Group C, D and E as compared to younger groups like Group A, B. Depletion of intracellular GSH inhibits T cell function[10]. Our findings indicate that total thiol levels in elderly group like C, D and E are significantly lower than those observed in young. Decreased glutathione levels and glutathione peroxidase activity are coupled to increased oxidative damage to DNA, lipids and proteins. If the level of reduced GSH is high, the oxidative stress is low and the level of lipid peroxidation is low, but with age the level of GSH is falling down and this is seen as raising amounts of MDA. Free radicals can also react with proteins and DNA, in addition to lipids. Lipid peroxidation is one of the important phenomena and has been implicated in a number of deleterious effects such as increased osmotic fragility, decreased membrane rigidity and cellular deformation[11], thus, we have measured the lipid peroxidation in all groups and it has been observed that the LP level increased significantly with age. Current studies on cellular injury

implicate peroxidation of polyunsaturated fatty acids (PUFA), leading to the degradation of phospholipids as an index of cellular deterioration<sup>[11]</sup>. Our results show a significant increase in neutrophils MDA levels with aging. Recent report by our laboratory suggested that, increased lipid peroxidation and decreased antioxidant enzyme status can be indicator of disease progression of oral cavity cancer patient. PC formation has been proposed to be an early marker for protein oxidation. However, available knowledge on PC formation as a function of age is limited in humans with aging. Some investigators have reported that PC content increased in neutrophils, muscle, fibroblasts and eye lens of elderly subjects<sup>[11]</sup>. In this study, we detected a significant increase in neutrophils PC levels in elderly subjects from Group B, C, D and E, as previously reported. The highest level is in group E. Oxidative modification of proteins may lead to the structural alteration and functional inactivation of many enzyme proteins, as evidenced by the decreased activity of different antioxidant enzymes like SOD, CAT, GPx, GR and GST. The results strongly suggest that a shift in the redox balance occurs between pro-oxidant and anti-oxidant in favor of the former during aging. To further support this possibility, we assessed neutrophils glutathione system levels, a major contributing factor to the maintenance of the redox state, particularly in the leukocytes. Aerobic cells contain various amounts of two main antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT). SODs rapidly dismutate superoxide anion ( $O_2^{\cdot-}$ ) to less dangerous  $H_2O_2$ , which is further degraded by CAT and glutathione peroxidase (GSH-Px) to water and oxygen<sup>[11]</sup>. Decreased CAT activity may compromise the overall antioxidant enzyme defense system. The results of the present study showed a significant fall in SOD activities, in the elderly groups like Group B, C, D and E. The depletion in SOD activity may be due to dispose off the free radicals, produced due to aging induced oxidative stress.  $H_2O_2$  produced by dismutation of superoxide anion, may have been efficiently converted to  $O_2$  by CAT but the enzyme activities showed a marked reduction<sup>[12]</sup>. The depletion of antioxidant enzyme activity may be due to inactivation of the enzyme proteins by ROS generation with aging, depletion of the enzyme substrates, and/or down-regulation of transcription and translation processes. In summary, the present experiments demonstrate that the neutrophils redox status undergoes increased oxidative stress with age, reflected by a decreased anti-oxidant capacity and an associated increased of biomolecule oxidation. Our results strongly support the presence of increased oxidative stress in elderly subjects.

### Conflict of interest

We declare that we have no conflict of interest.

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