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# HEMATOTOXIC AND GENOTOXIC EFFECT OF PARA-PHENYLENEDIAMINE AFTER SUBCHRONIC TOPICAL APPLICATION IN RATS

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**ABSTRACT**
Dermal exposure and subsequent toxicity evaluation is a routine trend in toxicological research after it has been realized that skin also act as a route of exposure for xenobiotics. Para-Phenylenediamine (PPD/1, 4 diaminobenzene), a monocyclic arylamine, is widely used in almost all hair dye formulation. Systemic exposure to PPD and PPD containing hair dye has been shown to induce hypersensitivity in sensitized individual only. But in others PPD or hair dye containing PPD doesn't elicit any negative response, thus allowing continuous use of such hair dye products containing PPD. Epidemiological studies indicated higher risk of leukemia, lymphoma and bladder cancer among user of hair dye containing PPD. In vitro studies involving PPD and its various derivatives have also been shown to cause DNA fragmentation in lymphocytes. The present study was conducted to investigate the hematological and genotoxic effect of three different doses of PPD (1, 2, 3 mg/kg) after repeated topical application for 30 days. The results of the present investigation clearly indicated hematoxicity of PPD as was evident from reduced RBC count, PCV value, Hemoglobin and increased reticulocytes in the peripheral circulation and significant changes in the cytomorphology of the blood cells. The comet assay of the peripheral blood lymphocytes of PPD treated rat's shows significant amount of DNA damage compared to the control animal group, suggesting that PPD is genotoxic in nature.

## INTRODUCTION

Dermal exposure and subsequent toxicity evaluation is a routine trend in toxicological research after it has been realized that skin also act as a route of exposure for xenobiotics. The cytomorphological changes and alteration in the total number of blood cells, both erythrocytes and leucocytes including platelets are of considerable significance as a measure of reaction of the body to different physio-chemical agents. In many cases these alterations give useful indication of disease processes that may be seen not only in acute infection but also in many chronic ailments and toxicological stress (Henry, 1984).

The assessment of genotoxicity of a chemical is one of the important step during safety evaluation of drug or chemicals intended to use in day to day life and that having probability of finding its way into the natural system. SCGE or Comet assay was described for the first time by (Ostling and Johanson, 1984) and then Singh *et al*. (1988) introduced alkaline conditions to this technique. This assay is extremely versatile, and is used extensively in Biology, Medicine and Toxicology, due to its capacity and sensitivity in demonstrating both single and double strand DNA breaks, and alkali-label sites (Fairbairn *et al.*, 1995; Sardas *et al.*, 1998, Stavreva *et al.*, 1998).

Para Phenylenediamine (PPD/ 1,4 Diaminobenzene) is widely used in almost all hair dye formulation (Corbett and Menkert, 1973), photographic developing agent and as an intermediate in the manufacture of azo dyes, antioxidants, and accelerators for rubber vulcanization (Hansen *et al.*, 1993). The main purpose of using PPD as hair dye ingredients is to fasten the process of dyeing as compared to traditional henna. Available scientific data indicated an association between long time usage of hair dye containing PPD and the risk of leukemia and other blood disorders (Czene *et al.*, 2003; Rauscher *et al.*, 2004) as well as bladder cancer (Henley and Thun, 2001). Previously we have demonstrated that topical exposure to aqueous solution of PPD causes histopathological changes in the liver of rat accompanied by elevation of serum marker (ALP, ALT and AST) of hepatic injury (Bharali and Dutta, 2009).

The objective of the present investigation was to examine the effect of subchronic topical exposure to PPD on the hematology and DNA fragmentation of the lymphocytes in male Sprague Dawley rats.

## MATERIALS AND METHODS

### Animals and treatment

Only male Sprague Dawley rats were used during present investigation. The animals (n = 20) weighing approximately 130 ± 5 gm were procured from stock animal facility of Department of Zoology, Gauhati University and randomly divided into four groups (Control, Group 1, Group 2 and Group 3), five rats per group. The animals were acclimatized

to the laboratory condition prior to treatment and given food and water ad libitum throughout the experiment period. All the procedures of animal experimentation were approved by the Institutional animal care and use committee. The test substance was dissolved in double distilled water and applied to the dorsal side of each animal (clipped free of fur) except the control animal, where they were painted with distill water only. The treatment was continued for 30 days and maximum daily exposure per animal was 0 mg/kg, 1 mg/kg, 2 mg/kg and 3 mg/kg body weight in control, Group 1, Group 2 and Group 3 respectively. After 30 days of continuous exposure, rats were sacrificed by exsanguination under Ketamine hydrochloride anaesthesia, blood collected in EDTA tubes for hematology and lymphocytes from buffy coat were subjected to Comet assay or single cell gel electrophoresis (SCGE). The body weight of all animals at the start of the experimentation (day 0) and at the time of termination of experiment (day 30) were recorded.

**Hematology**

Blood collected in EDTA tubes were used for total count of RBC, WBC, hemoglobin (Hb), packed cell volume (PCV) and reticulocyte count. All the hematology procedure were done manually and completed within the same day of blood collection. Total count of RBC, WBC, PCV and Hb were done according to Dacie and Lewis (2008). Reticulocytes counting were done after staining with new methylene blue stain and counting at least 1000 RBCs per slide subjected to 2 slides per animal per group.

**Comet assay**

Lymphocytes isolated from the buffy coat were subjected to comet assay according to Singh *et al*., (1988). Briefly 100 $\mu$l of the lymphocytes cell suspension was mixed with 100 $\mu$L of 1% LMPA at 37ºC was loaded onto two microscope slides pre-coated with 1% NMPA. The cover slip was placed gently to allow even spreading of gels. The slides were kept on ice for 5 min to allow the gel to solidify. The cover slip was removed and a third layer of 0.5% LMPA was added onto the slide and allowed to solidify over ice for 10 min. Finally the cover slip was removed and slides were immersed in freshly prepared and chilled lysing solutions containing 2.5M NaCl, 100mM EDTA, 10mM Tris (pH 10) with 1% Triton X- 100 being added just before use. The slides were kept for overnight at 4ºC. Slides were placed side by side in a electrophoresis buffer (1mM EDTA, 300mM NaOH, pH > 13) and left for 20 min to allow unwinding of DNA and electrophoresis was performed at 0.7V/cm and 300mA at room temperature for 30 min. Slides were then removed from the electrophoresis buffer and Tris-buffer (pH 7.4) was added drop wise to neutralize the excess alkali. The slides were allowed to sit for 5 min and the procedure repeated for 3 times. Slides were then dipped in chilled distill water and dried in methanol for 5 min and stored in a humidified slide box until silver staining. The silver staining of the comet gels were performed according to Nadin *et al*., (2001).

One hundred (100) comets on each slide subject to three slides per animal were scored visually according to the relative intensity of silver stains in the tail. An intensity score from class 0 (undamaged) to class 4 (severely damaged) was assigned to each cell, based on the procedures described by Visvardis *et al.,* (1997). The DNA damage was quantified by visual classification of cells in to five categories 'comets' corresponding to the tail length: undamaged, Type 0; low level of DNA damage, Type 1; medium level damage, Type 2; high level DNA damage, Type 3; complete DNA damage, Type 4, as demonstrated in Fig 2. From arbitary values assigned to the different categories the percentage of damaged cell and genetic damage index was calculated for each subject following Palus *et al*., (1999) and Piturque *et al*., (1999) respectively.

**Statistisics**

All data were presented as means ± SEM. Statistical analysis were performed using one way analysis of variance (ANOVA). A p-value < 0.05 were taken into consideration for determining significance. All statistical procedures were computed using SPSS 10.0 software.

## RESULTS

**Effect of PPD on body weight**

The body weight recorded at the onset of the experiment and at termination was presented in Table 1. The body weight gain was found to be less in PPD treated animals as compared to control group of animal. The difference in mean body weight gain among the groups was however not found to be significant.

**Table 1: Body weight gain by the experimental animals during 30 days study period, value were presented as Mean ± SEM, *p < 0.05, **p < 0.001**

| Groups | Dose | Initial bodyweight (g) | Final bodyweight (g) | Body weightgain (g) |
|---|---|---|---|---|
| Control | 0 mg/kg | 128.6 ± 4.73 | 168 ± 8.03 | 39.4 ± 5.26 |
| Group 1 | 1 mg/kg | 129.8 ± 3.72 | 165 ± 7.00 | 35.2 ± 3.77 |
| Group 2 | 2 mg/kg | 130.4 ± 2.73 | 159 ± 5.69 | 28.6 ± 3.85 |
| Group 3 | 3 mg/kg | 132.4 ± 3.50 | 159 ± 7.35 | 28.6 ± 4.32 |

**Table 2: Total count of RBC, WBC and (Lower panel) PCV, Hb and RC after 30 days topical application of PPD, value were presented as Mean ± SEM, *p < 0.05, **p < 0.001**

| Groups | Total count of RBC (million/$\mu$L) | PCV (%) | HB (g/dl) | RC (N/$10^3$) | WBC (N x $10^3$/ $\mu$L) |
|---|---|---|---|---|---|
| Control | 4.75 ± 0.52 | 34.0 ± 1.14 | 14.52 ± 0.90 | 25.0 ± 2.11 | 5.90 ± 0.47 |
| Group 1 | 4.04 ± 0.75 | 28.8 ± 1.77* | 14.42 ± 0.22 | 24.8 ± 1.78 | 4.47 ± 0.70 |
| Group 2 | 3.46 ± 1.02* | 23.8 ± 1.85* | 13.94 ± 0.04 | 28.0 ± 1.34 | 3.49 ± 0.26* |
| Group 3 | 3.20 ± 0.58** | 23.0 ± 2.19* | 12.26 ± 0.36* | 31.0 ± 2.10* | 4.18 ± 0.23* |

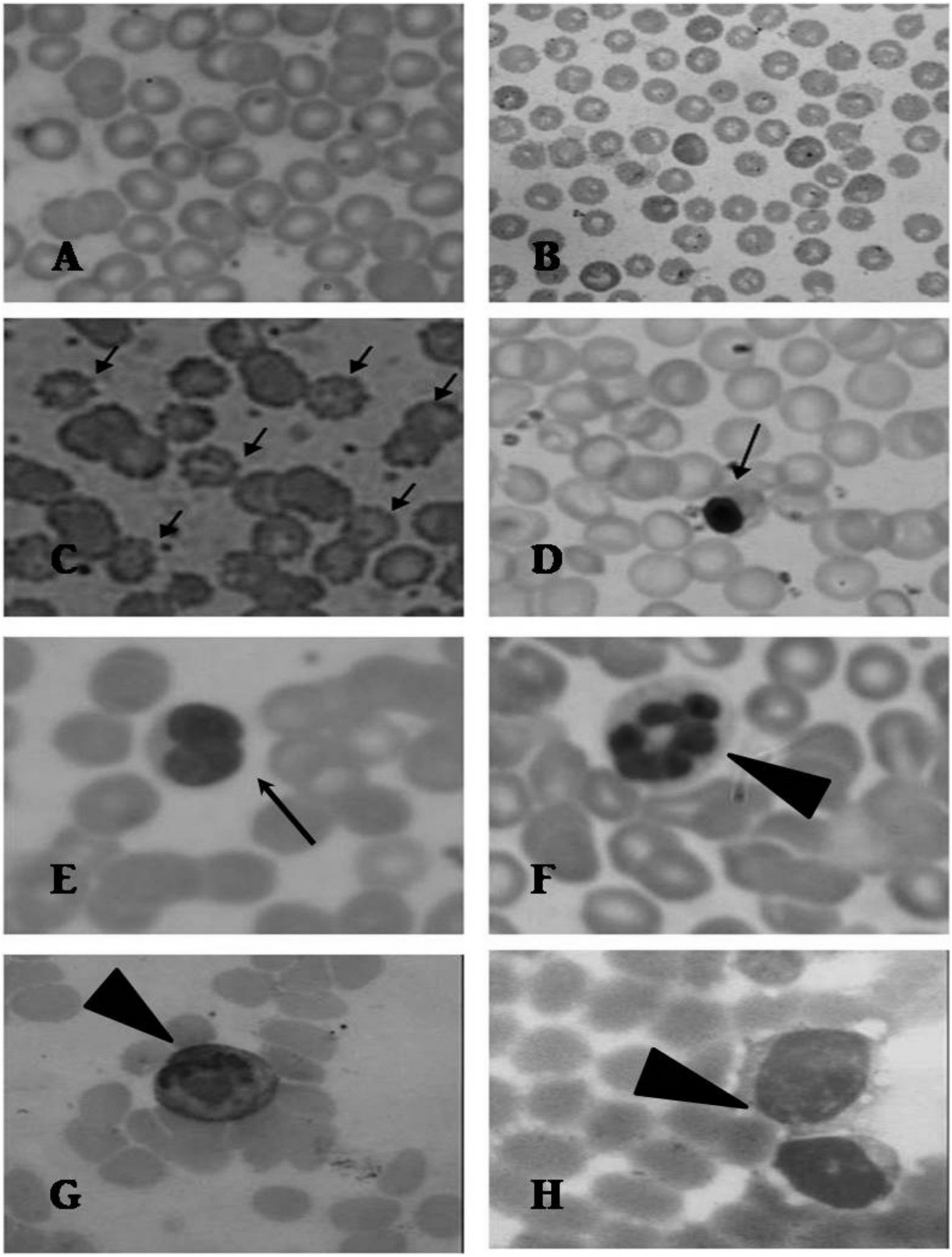


**Figure 1: Haematological picture from control (A) and PPD treated animals (B-H). (B) polychrmoasia of peripheral blood in treated rats. (C) Echinocytic transformation of normal discocyte RBC in treated rats. (D) Occurrence of nucleated RBC in peripheral blood of treated rats. (E) Presence of mitotic figures, (F) Nuclear pycnosis and hyper segmentation in neutrophil, (G) Presence of band or immature neutrophil, (H) Cytoplasmic vacuolation in the leucocytes**

### Effect of PPD on hematology

PPD causes significant changes in cytomorphology of circulating RBC and WBC of peripheral blood in treated rats and are presented in Fig. 1. The important morphological alterations observed includes changes in shape and staining pattern of RBC (polychromasia, echinocytosis, nucleated RBC), presence of mitotic figures, nuclear pycnosis, toxic vacuolation, band neutrophil in peripheral blood smear.

The effect of PPD on hematology of the experimental rats was presented in Table 2. Topical exposure to PPD causes significant reduction in the numbers of circulating RBC ($p < 0.05$, 0.001) and WBC ($p < 0.05$) in group 2 and 3. The decline in the total RBC count of the peripheral blood was also found to accompanied by significant reduction of the packed cell volume (PCV, $p < 0.05$) and increase in the reticulocyte count in the peripheral blood in the PPD treated group ($p < 0.05$). Treatment related decline in hemoglobin concentration were also recorded but found to be significantly different from control group only in group 3 ($p < 0.05$).

### Effect of PPD on DNA fragmentation

PPD causes significant ($p < 0.05$) increases in DNA fragmentation of the lymphocytes in group 2 and 3 rats as was

**Table 3: Effect of topical exposure to PPD on DNA fragmentation as measured by SCGE or Comet assay in the peripheral lymphocytes of rats. Values are mean ± SEM, *p < 0.05, **p < 0.001**

| Group and doses | PROPORTION OF DAMAGED NUCLEI | | | | | % of damaged cells [a] | GDI [b] |
|---|---|---|---|---|---|---|---|
| | Type 0 | Type I | Type II | Type III | Type IV | | |
| Control (0 mg/Kg) | 73 | 18.66 | 4.33 | 4 | 0 | 8.33 ± 2.33 | 0.39 ± 0.04 |
| Group 1 (1 mg/Kg) | 72 | 14.66 | 7.66 | 5.66 | 0 | 13.33 ± 2.84 | 0.47 ± 0.05 |
| Group (2 mg/Kg) | 68.66 | 14.33 | 10.66 | 6.33 | 0 | 17 ± 4 | 0.54 ± 0.05 * |
| Group 3 (3 mg/Kg) | 62.33 | 12.66 | 12 | 10 | 0 | 22 ± 1.52 | 0.68 ± 0.02 * |

Type IV = 0, complete DNA damage was not observed during present study; [a] % of damaged cells = Type II + Type III + Type IV (Palus *et al.,* 1999); [b] Genetic Damage Index (GDI) = (Type I + 2 x Type II + 3 x Type III + 4 x Type IV) / (Type 0 + I + II + III + IV); [b] (Piturque *et al.,* 1999)

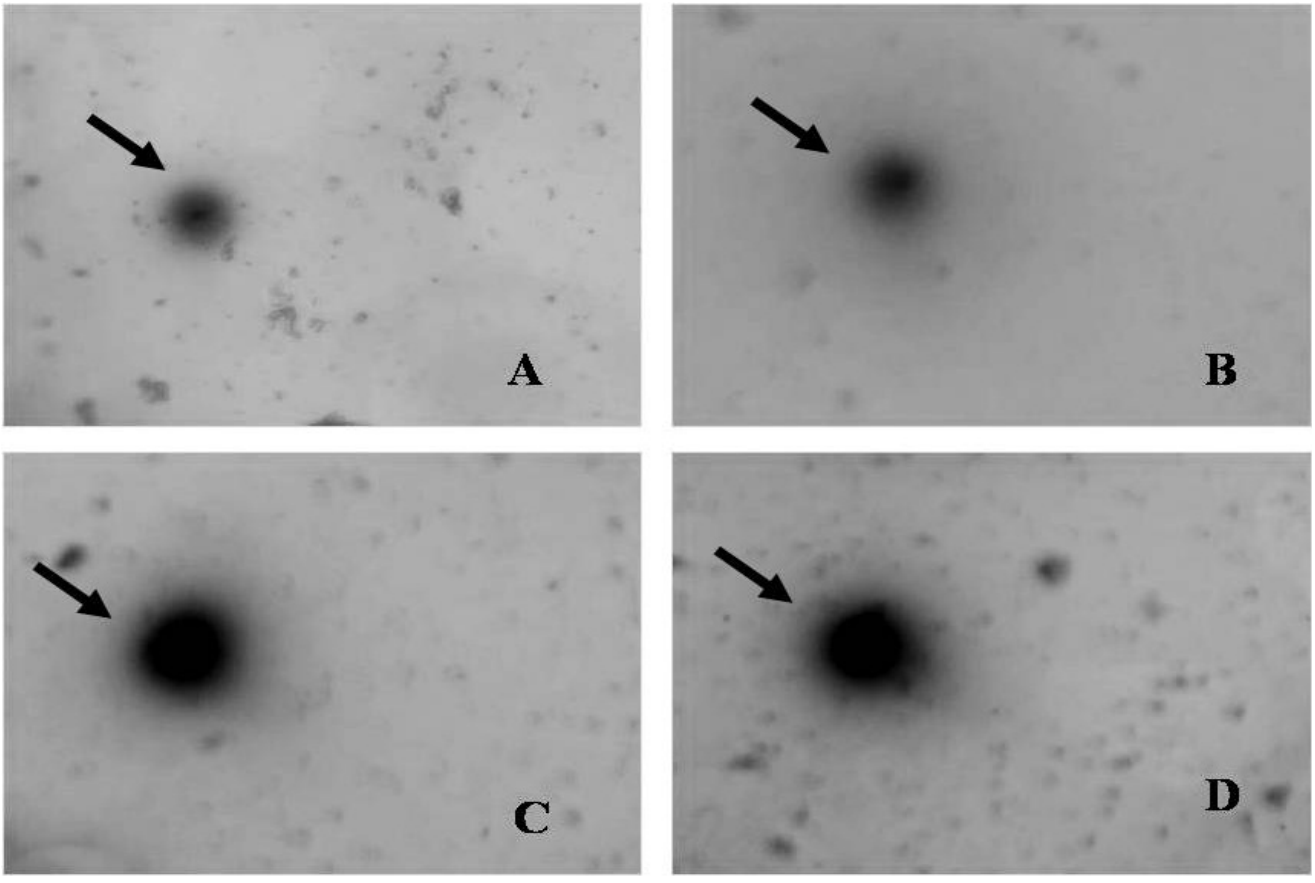


**Figure 2: Different forms of comet formed during present study and DNA damage scoring criteria according to tail length as was proposed by Visvardis *et al,* 1997. A: Type 0 = 0 (zero DNA damage), B: Type I = 1 (low DNA damage), C: Type II = 2 (moderate DNA damage), D: Type III = 3 (high DNA damage), Tye IV = 4, complete DNA damage not observed in the present study**

observed in the present study (Fig. 2, Table 3). The amount of DNA fragmentation was found to be dose dependent and a high positive correlation (r=0.989766) has been observed between dose of PPD applied and amount of DNA fragmentation.

## DISCUSSION

Present study shows that topical application of PPD causes significant physiological disturbances which retards body weight gain compared to control animals. Earlier studies reported that PPD can reach systemic circulation during topical application after transcuteneous absorption (Hueber-Becker *et al.,* 2004; Dressler and Appelqvist, 2006) thus posing a risk of systemic toxicity. Therefore it is expected that topical application of the chemical *i.e.* PPD may lead to absorption and subsequently may cause systemic toxicity. The importance of body weight gain/loss as a clinical sign during toxicological evaluation was discussed earlier (Andersen *et al.,* 1999; Kim and Carlson, 1986). The less body weight gain by treated animals compared to the control animals may be due to inhibition of food intake secondary to the physiological disturbances caused by chemical treatment. The chemical induced stress is also reported to activate adrenal gland and resulted in the reduction of food intake (Wandhwa *et al.,* 1991).

The gradual changes observed in the cytomorphology of RBC (normal discocytes to echinocytes), decline in total RBC count in peripheral blood, low Hb concentration and PCV value in the PPD treated animal indicated the hemolytic activity of the chemical and subsequent development of compensatory anemia evidenced by increases in reticulocyte count. Similar association between increased reticulocytosis of peripheral blood and chemical induced hemolytic anemia has been reported during aniline/ 1, 4 Diaminobenzene toxicity (Pauluhn, 2004). The PPD mediated intravascular hemolysis may be attributed to its oxidative potential, as it has already known to increase oxidative stress in keratinocytes (Picardo *et al*., 2006). Mature RBCs are more prone to oxidative damage since they are constantly being exposed to a higher concentration of oxygen than other tissues and have a limited antioxidant capacity (Lee *et al.,* 2003). Thus repeated application of henna formulation mixed with PPD may cause severe hemolytic anemia in such individual and in normal human beings with compromised antioxidant status.

The significant alteration of leucocytes morphology and gradual decline in total count of WBC indicated the physiological disorders caused by topically applied PPD. The occurrence of cytoplasmic vacuolation in peripheral blood leucocytes in acute alcoholism (Davidson and McPhie, 1980) and their significance in diagonsis of metabolic disorders has been reported (Anderson *et al.,* 2005). The role of leucocytes especially neutrophil during inflammation and subsequent leucopenia of blood has also been well documented (Claudine *et al.,* 2003).The leucopenia observed in treated rats during present investigation may suggest the ongoing inflammatory reactions within the body, which recruited these cells to the site of inflammation. Another factor for observed leucopenia may be frequent removal of the leucocytes with compromised genetic condition from the peripheral circulation, as PPD caused DNA fragmentation in the peripheral lymphocytes (Table 3).

The findings of the present study demonstrate mild genotoxicity of PPD and it causes DNA fragmentation in a dose dependent manner. In vitro genotoxic effect of PPD on human lymphocytes has been reported earlier (Chye *et al.,* 2008). Whether PPD or its metabolite is genotoxic is an area that needs further study. The reported incidence of excess risk of hematoloigcal cancer among chronic hair dye users may be attributed to the genotoxic potential of PPD. Long term and continuous usage of hair dye containing PPD therefore must be avoided as far as possible, as DNA fragmentation often increases the risk of cancer (Emily, 2004).

## CONCLUSION

Present study shows that topical application of PPD causes significant physiological disturbances in the rats. Repeated topical application of PPD leads to hemolytic anemia and subsequent DNA fragmentation in the lymphocytes of rats. Lawsone, present in pure Henna is known as a hemolytic agent, but its effect confined only to G6PD deficient people. Therefore henna formulation mixed with PPD may cause severe hemolytic anemia in such individual and in normal human being. PPD is genotoxic in nature and causes DNA fragmentation in a dose dependent manner. Long term and continuous usage of hair dye containing PPD must be avoided, as DNA fragmentation often increases the risk of cancer.

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