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Hematopathology in Sprague–Dawley Rats Following Sub-Chronic Topical Application of Para-Phenylenediamine

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Abstract The aim of the present study was to analyze the hematological profile of male SD rats treated topically with aqueous solution of para-phenylenediamine (PPD), a component of almost all hair dye formulations. The rats were painted with different concentration of PPD (0, 1, 2 and 3 mg $Kg^{-1}Day^{-1}$) for 90 days and then sacrificed. The hematological profile indicated severe anemia characterized by significant ($p < 0.05$, 0.001) reduction of total RBC count (59%), packed cell volume (PCV, 50%) and haemoglobin level (70%) in the peripheral blood of PPD treated animals when compared to control group. The leucocytes profile exhibited an overall elevation of around twofold as compared to the control group with significant lymphocytosis (44.4%) and a higher percentage of blast cells (8.5%) as well as smudge (10.3%) and hairy cells (6.2%) in the peripheral blood of treated animals. Histopathological examination of spleen from treated rat's exhibit red pulp congestion, expansion of the germinal centre, hyperplasia of the membrane capsule and extensive accumulation of hemosidderin pigments in the red pulp of the spleen. Overall this study indicated an abnormal pathophysiological condition indicating adverse effect of PPD in the treated animal groups. The risk assessment of hair dye formulation needs to be reviewed in view of widespread usage of paraphenylenediamine in almost all hair dye formulation.

Keywords Para-phenylenediamine · Topical exposure · Hematology · Spleen histology

The henna and hair dye is one of the luxurious items that have been found to dominate the present world from ancient time owing to its ability as a hair coloring agent. Millions of consumers use hair dye for personal enhancement. Henna has been used for more than 4,000 years as a cosmetic by Mediterranean, Middle Eastern, and Asian cultures. In many circumstances, the dye is applied over extensive areas of the body to create a variety of designs, and is frequently applied to newborns for ceremonial purposes (Kandil et al. 1996). Para-phenylenediamine (PPD; 1, 4 Diaminobenzene; CAS: 106:50:3) is a colourless/slightly pink, grey or yellow crystalline solid. On oxidation, usually through exposure to air, it turns red, brown then finally black and forms trinuclear dye called bandrowski base (N, N-bis (4-aminophenyl)-2, 5-diamino-1, 4-quinone-diimine). The formation of bandrowski base is the critical step known to be involved in PPD mediated allergic contact dermatitis (Krasteva et al. 1993). PPD is widely used in almost all hair dye formulations (Corbett and Menkert 1973). This compound is also used as photographic developing agent and as an intermediate in the manufacture of azo dyes, antioxidants, and as 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.

Epidemiological studies indicated an association between long time usage of hair dye containing PPD and

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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). Para-phenylenediamine is known to induce DNA fragmentation (Chye et al. 2008) as well as apoptotic cell death in cultured cells (Chen et al. 2010). Previous report indicated that topical exposure to aqueous solution of PPD causes histopathological changes in liver of rat accompanied by elevation of serum marker (ALP, ALT and AST) of hepatic injury (Bharali and Dutta 2009).

In most of the hair dye formulation PPD is often mixed with hydrogen peroxide to accelerate the process of oxidation of PPD (SCCP 2005). Since PPD is auto oxidized in the presence of air, the present experiment was designed to evaluate the potential hematopathological changes in SD rats after topical application.

Materials and Methods

Para-Phenylenediamine was purchased from Merck, Germany and all other chemicals used in the experiments were of analytical grades. Male Sprague–dawley rats were used in the present experiment. All experiments were done in accordance with institutional animal care and ethical guidelines. In the experimental set up the animals (n = 20) were randomly divided into four groups, each consisting of five animals per group. The test chemical i.e. para-phenylenediamine is dissolved in double distilled water and applied daily over an area of 1.5 × 1.5 cm on the dorsal side clipped free of fur at the dose of 0, 1, 2 and 3 $mgKg^{-1}$ body weight. The exposure paradigm was continued for 90 consecutive days and then the animals were sacrificed. The body weights of the group of animals were noted after every 30 days till termination.

Blood samples were collected from heart by cardiac puncture after ketamine hydrochloride anaesthesia (10 mg/kg). About 1.0–1.5 ml of blood samples was collected from each animal and clotting was prevented by addition of EDTA. The hematological parameters (TEC, TLC, Hb, PCV and reticulocytes) were examined manually soon after blood collection. Blood film was prepared immediately from fresh blood, stained with wright giemsa and observed under high power magnification for enumeration of lymphocytes and abnormal or atypical cells in the peripheral circulation.

The spleen was fixed in neutral buffered formalin, dehydrated, embedded in paraffin and 5 μm sections were stained with H & E for histopathological observation. A few sections were stained for detection of iron pigments according to Sayre et al. (2004).

All data generated from experiment are presented as mean ± SEM. The significance of mean differences in the numerical data of both control and test group of population were subjected to ANOVA (Analysis of Variance, one way) followed by post hoc tukey test. The mean differences between control and treated animals were considered significant at p value not less than 0.05 ($p < 0.05$). All statistical procedures were computed with SPSS 10.0 software.

Results and Discussion

Topical application of PPD seems to retard the growth of the experimental animals when compared with the control untreated group. But significant variation were observed only in 3 $mgKg^{-1}Day^{-1}$ group after 90 days of treatment (Fig. 1, $p < 0.05$).

Peripheral blood smear from PPD treated animal indicated severe morphological changes in the erythrocytes compared to the control group (Fig. 2 upper panel). A significant and dose dependent decrease in total erythrocyte count and hemoglobin content were also observed in the peripheral blood of the experimental animals after PPD treatment (Fig. 2, $p < 0.05$, 0.001). Significant reduction in packed cell volume (PCV) was also noted in PPD treated animals (Fig. 2, $p < 0.05$, 0.001). The peripheral blood of PPD treated rat also exhibited significant increases in the

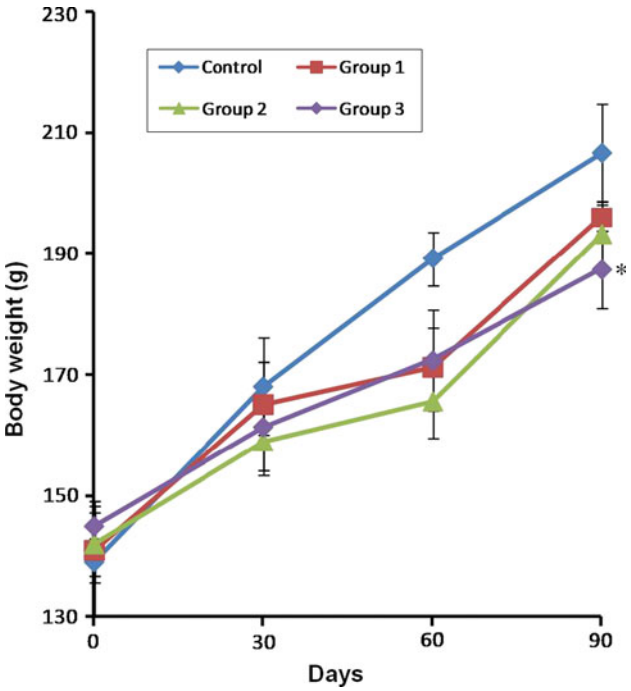

Fig. 1 Body weight gain by control and PPD treated animals during 90 days treatment protocol

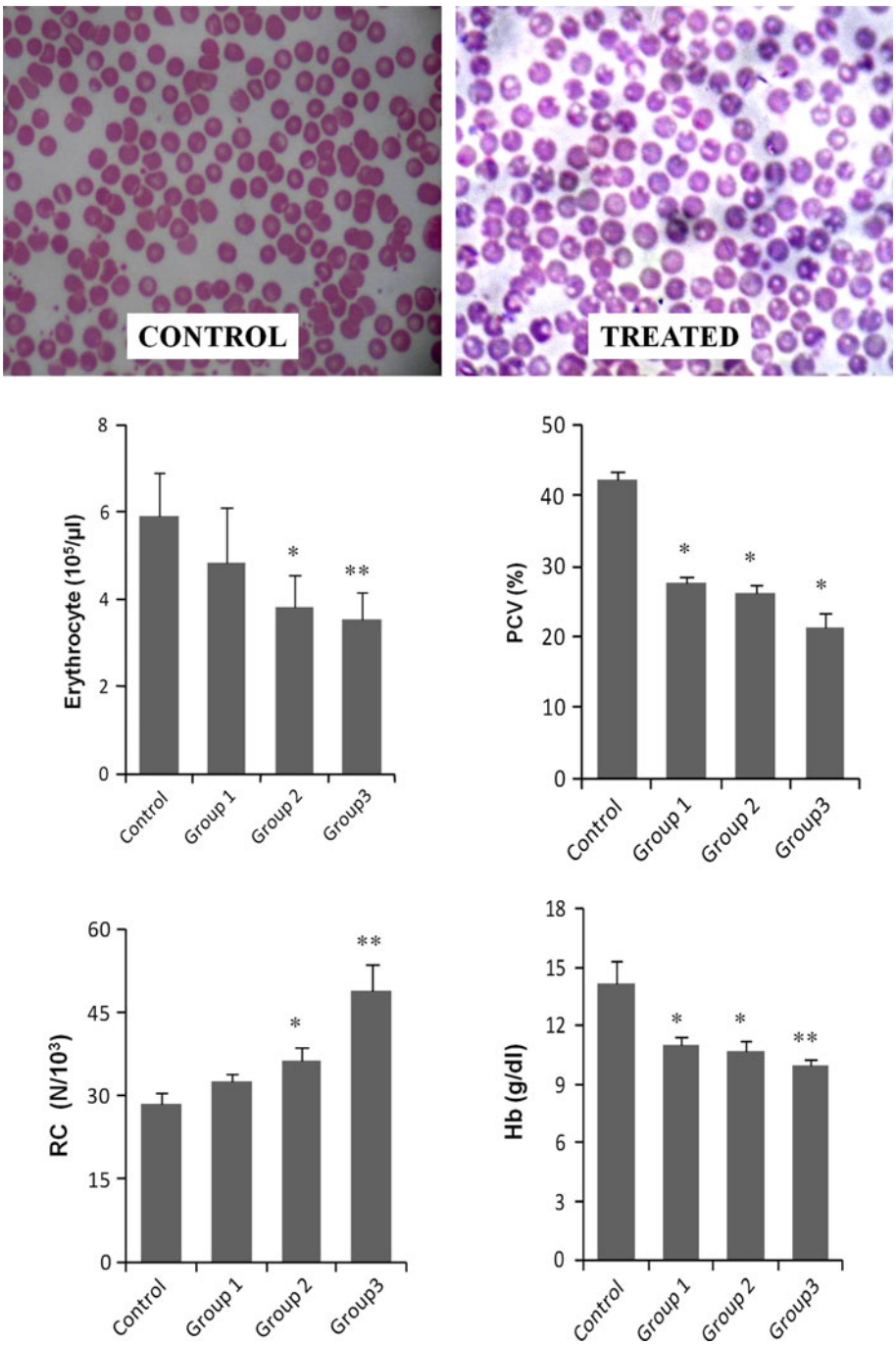

Fig. 2 *Upper Panel* Representative peripheral blood smear from the control and PPD treated animal groups. PPD treatment leads to hemolytic changes in the erythrocytes of rats. *Lower panel* Total erythrocyte count, Packed cell volume, Reticulocytes and Hemoglobin content of the peripheral blood in the control and PPD treated animal groups after 90 days of treatment. PPD treatment significantly reduced the total erythrocyte count, PCV, and hemoglobin content where as percentage of reticulocytes significantly increased after the treatment. $^{*}p < 0.05$, $^{**}p < 0.001$, magnification $\times 100$

no. of reticulocytes compared to the control group (Fig. 2, $p < 0.05$, 0.001).

The peripheral blood picture of PPD treated rats exhibited significant leucocytosis (Fig. 3, $p < 0.05$, 0.001) and higher percentage of atypical leucocytes comprising smudge cells, hairy cells and immature blood cells (Fig. 3).

The spleen from PPD treated rats displayed treatment related hypertrophy, expansion of germinal follicles, congestion, hyperpigmentation of the red pulp and focal hyperplasia of the splenic capsule (Fig. 4d–f). No such histopathological abnormalities were recorded in control group of animals (Fig. 4a–c). Pearl's Prussian blue staining of the spleen section from treated rats exhibited extensive

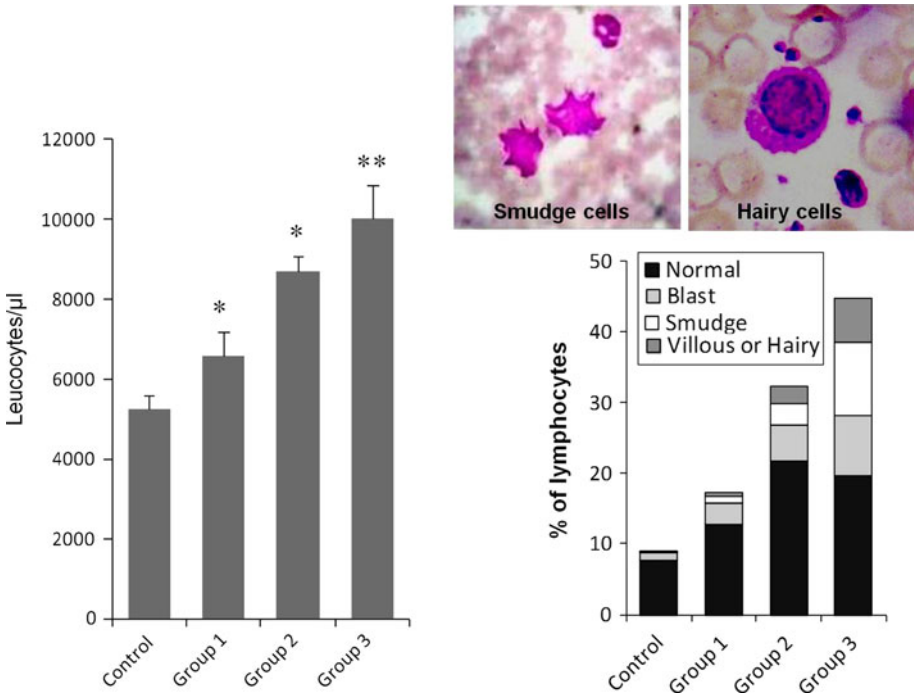

Fig. 3 PPD treatment significantly elevated the total leucocytes count and percentage of lymphocytes in the rats. Abnormal lymphocytosis was accompanied by increased in the percentage of blast cells, smudge cells and hairy cells or villous lymphocytes. *$p < 0.05$, **$p < 0.001$, magnification ×100

staining for hemosidderin pigments when compared to the control group (Fig. 5) suggesting treatment related increase in the accumulation of iron pigments within splenic tissues.

The present study was conducted to investigate the effect of sub chronic topical application of PPD, a component of all hair dye formulation on the hematology of SD rats. The data generated during the study confirmed that topical application of PPD causes severe disturbances in the physiology of the treated animals. PPD treatment caused hemolytic anemia in the rats as it was evident from morphological changes in the erythrocytes, reduced erythrocyte count, packed cell volume, low hemoglobin concentration, higher percentage of reticulocytes in the peripheral blood. The decline in total erythrocyte count and PCV associated with depletion in hemoglobin concentration in the PPD treated group of animals has occurred due to destruction of large numbers of RBCs which was evident from the morphological changes of red blood cells in the peripheral circulation. Similar hematological disorders have been reported in rats exposed to aniline (Pauluhn 2004). Erythrocytes are more sensitive to oxidative stress due to their oxygen rich environment. PPD is known to cause oxidative stress in vitro as was reported earlier (Chen et al. 2010). In normal circumstances erythrocytes can prevent oxidative damage due to the presence of many cellular antioxidant like reduced glutathione, catalase etc. But during repeated exposure scenario the oxidative stress overwhelm the cellular antioxidant status and thus causing cellular injury and hemolytic death of the RBCs.

The leucocytosis and lymphocytosis observed in the PPD treated animals accounted for the ongoing inflammatory reactions within the body. Increase percentage of the smudge cells, a well defined marker of chronic lymphocytic leukemia (Nowakowski et al. 2007), with bone marrow infiltration of lymphocytes and vilous lymphocytes, a marker of splenic lymphoma (Melo et al. 1987) in the peripheral circulation of treated rats indicated the possible hematopoietic malignancies. These also explain the causal association already being reported between hair dye usage and occurrence of leukemia and lymphoma during epidemiological studies (Rauscher et al. 2004; Zhang et al. 2008).

The major function of the spleen is to remove damaged or senescent erythrocytes. Increased sequestration of RBCs and vascular congestion in the red pulp of the spleen of PPD treated animals indicated the active involvement of the organ in the process of blood filtration. The abundance of macrophages in the spleen of the treated animals indicated that they are involved in the scavenging activites in order to remove the damaged RBCs. This process also enhances the deposition of large amount of iron from the damaged RBCs in the splenic

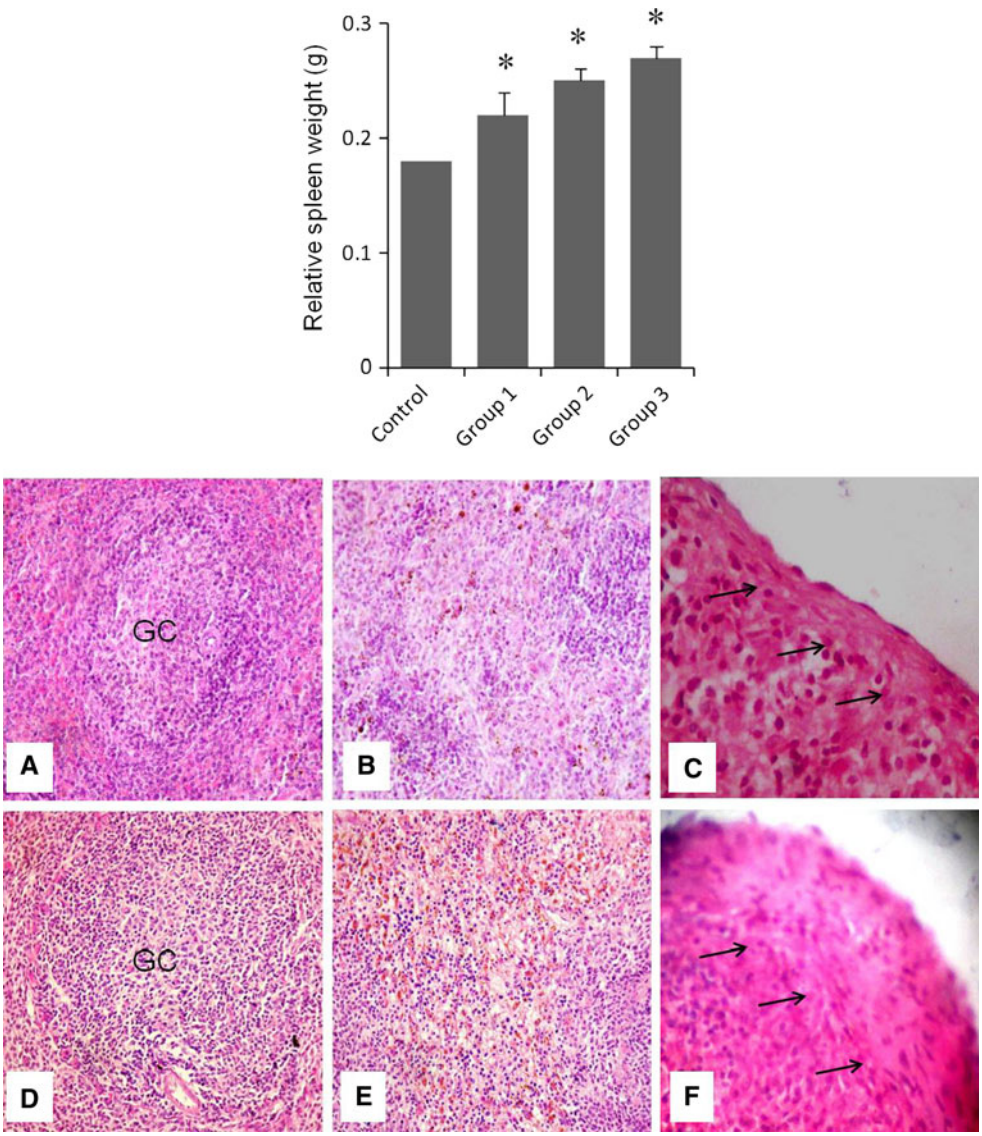

Fig. 4 *Upper panel* Relative spleen weight in control and PPD treated group of animals. Significant dose-dependent increased in spleen weight has been observed. *Lower panel* Representative sections of spleen from control (**a**–**c**) and PPD treated rats (**d**–**f**). Spleen sections showing **a** germinal centre, **b** *red pulp* and **c** capsular histology in control group of rats. Spleen sections showing **d** expansion of germinal centre, **e** *red pulp* with hyper pigmentation compared to control group and **f** capsular histology demonstrating fibrosis and hyperplasia after PPD treatment. Magnification ×40, H & E stain (Color figure online)

pulp as was evident from the Pearl's Prussian blue staining reaction. Similar dose- and time-dependent accumulation of iron in the spleen of aniline treated rats was reported earlier (Khan et al. 1997). The germinal centres of splenic follicles are mainly involved in the productions of mature B-lymphocytes. Increased expansion of the germinal centres in the PPD treated animals indicated hyperproliferation of B-lymphocytes due to sensitization reaction by the treatments. This is well correlated with the peripheral blood leucocytosis especially lymphocytosis. A remarkable feature observed in the spleen of PPD treated group was focal hyperplasia and fibrosis of the capsular membrane. This characteristic histopathological feature was also reported earlier during aniline induced toxicity (Khan et al. 1997). It should be noted that aniline was a regular component of hair dye formulation which is now a day's prohibited due to its toxicity.

Overall the study suggest that topical application of PPD leads to hemolytic anemia due to intravascular hemolysis and increased sequestration of damaged erythrocytes within the splenic sinuses. This sequestration events lead to increased deposition of the heme proteins which triggers histopathological changes in the spleen. The hematopathology of PPD treated rats indicated a shift towards lymphocytosis, suggesting the possible role of PPD in epidemiological observation of lymphoma and leukemia in chronic hair dye users.

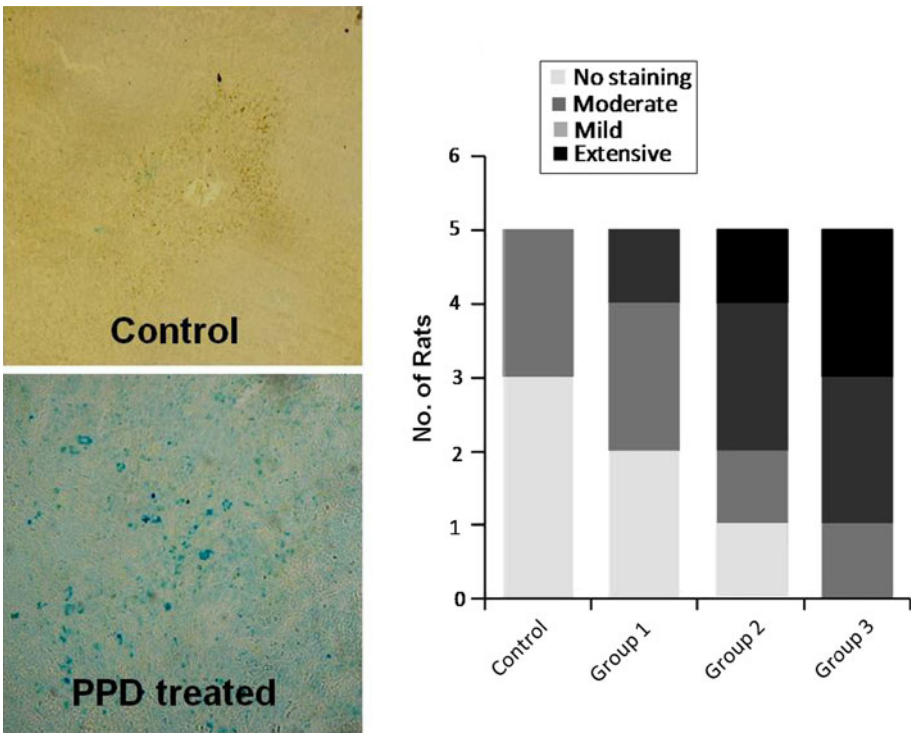

Fig. 5 **a** Representative section of spleen stained with Pearl's Prussian blue for detection of hemosidderin pigmentation. PPD treatment increased the deposition of hemosidderin pigmentation in the red pulp of spleen compared to the control group of animal. **b** Quantitative analysis of iron positive spleen from control and PPD treated group. Magnification ×40

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