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Effect of Malus Domestica (Apple Peel) Extract on Peripheral Blood and Bone Marrow Cells of Prednisolone Treated Mice

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ABSTRACT

Introduction: Apple peel is rich in many bioactive compounds and has been studied for its beneficial effects and protective role in multiple disorders, but its effect on blood cells and bone marrow needs to be investigated. **Aims & Objectives:** To determine the effects of apple peel extract on peripheral blood and bone marrow cells of prednisolone treated mice. **Place and duration of study:** Pharmacology Department of Post Graduate Medical Institute and Pathology Department of Fatima Jinnah Medical University, Lahore from June to September, 2016. **Material & Methods:** Forty Swiss albino mice were randomized into five equal groups. Normal control group was given distilled water. Model group was given prednisolone 4 mg/kg orally. Experimental low, medium, and high dose groups were given 25 mg/kg, 50mg/kg and 100 mg/kg apple peel extract respectively along with prednisolone for 14 consecutive days by oral route. On 14th day, blood was analyzed for red blood cell, platelet, total leukocyte, and differential leukocyte count whereas, bone marrow was analyzed for erythropoiesis, megakaryocytes, myelopoiesis, lymphocyte and plasma cell count. **Results:** Prednisolone treatment group exhibited significantly raised neutrophil and decreased lymphocyte percentage, whereas anon-significant rise in RBC, WBC, and platelet count was noted as compared to the normal group. Bone marrow smear megakaryocytes, myelopoiesis, lymphocytes, and cellularity: fat ratio also increased non-significantly by prednisolone treatment. Low dose of apple peel extract enhanced these effects, making significant difference, while medium and high doses negated these effects in accordance with their dose. **Conclusion:** Apple peel extract in low dose complemented the effect of prednisolone on hematological parameters of mice peripheral blood and bone marrow, while high dose nullified these effects in a dose dependent manner.

Key words: *Malus domestica*, Prednisolone, Peripheral blood cell count.

INTRODUCTION

Blood cells and others constituents of plasma play many key physiological roles in body. Red blood cells (RBC) are necessary to maintain homeostasis apart from their classic function of oxygen transport.[1] Leukocytes interact in complex ways to confer immunity to infectious agents, resist or destroy invading organisms, produce the inflammatory response, and destroy and remove foreign materials and dead cells.[2] Granulocytes and monocytes phagocytose bacteria and other organisms, migrate to the sites of infection or inflammation and to areas containing dead tissue, and also participate in the enzymatic breakdown and removal of cellular debris. Neutrophils are the most important cellular component of the innate immune system.[3] Lymphocytes are concerned with the development of immunity. Acquired resistance to specific microorganisms is in part attributable to antibodies, proteins that are formed in response to the entry into the body of a foreign substance. Platelets not only play important role in hemostasis, but their granules also contain growth factors required for tissue repair.[4]

Bone marrow contains hematopoietic and adipose tissue. Stem cells in the hematopoietic tissue mature into different blood cell lines, i.e., red blood cells, mononuclear and polymorphonuclear leukocytes and platelets.[5] Bone marrow microenvironment provides appropriate support for T cells to develop

in the absence of the thymus.[6,7] Lymphoid follicles in the bone marrow are increased during infections, inflammation, and autoimmunity. Antigen-specific antibody producing plasma cells are also largely found in the bone marrow. Thus, bone marrow is a nest for function, migration and selective retainment of innate and adaptive immune cells. Considering the need, the stem cells in bone marrow differentiate into committed cell lines.[5]

Prednisolone is a synthetic glucocorticoid which reduces inflammation by suppressing the migration of polymorphonuclear leukocytes and reverses increased capillary permeability. It is used to treat a wide range of health problems including allergies, blood disorders, skin diseases, infections, certain cancers and prevents organ rejection after a transplant due to its immunosuppressive action[8]. Corticosteroids are used in blood disorders like idiopathic thrombocytopenic purpura, autoimmune haemolytic anaemia, and autoimmune neutropenia. Along with beneficial effects on platelet, RBC, and neutrophil count, prednisolone decreases the number of lymphocytes, macrophages, monocytes, eosinophils, and basophils,[8] which reduces immunity. There is a need for an agent which can ameliorate immunosuppressant effect of corticosteroids, while preserving beneficial effect on other cell lines.

Apples are generally considered "healthy food"; one of the most important features of apples, the one that makes them interesting for researchers, is their polyphenol content, especially flavan-3-ols, phenolic acids, flavanols, dihydrochalcones and anthocyanins.[9] Apple peel contains a higher polyphenolic content and antioxidant capacity than apple flesh.[10] Studies have revealed immunostimulatory effect of apple peel in vitro[11] and enhanced production of IgG antibodies in vivo.[12] In the light of these pharmacological activities of apple peel, it may be considered to antagonize immunosuppressant effect of corticosteroids, but it is not known whether it will also antagonize the beneficial effect on blood cells or not. The current study was conducted to evaluate effects of co-administration of prednisolone and different doses of apple peel extract on hematological parameters of mice peripheral blood and bone marrow.

MATERIAL AND METHODS

Materials used in this research were Apples (red delicious variety obtained from local market of Lahore),[13] injection Ketamine (Indus Pharma Pvt. Ltd), Prednisolone tablets (Pfizer Pharma Pvt. Ltd), Electronic balance (Wuhan Panscale Hardware Co. Ltd, Model no. DH-V300A), Hematology analyzer (Sysmex Model no: KX-21, serial no. B 3483), Light microscope (Olympus CHT, Japan), EDTA blood vacutainer (Bio- Vac Stars Pakistan), Whatman filter paper. No.1 (Whatman International Ltd, Maidstone, UK), Giemsa stain (Diachem, China), Hematoxylin & Eosin stains (Diachem, China), Disposable syringes 3 &5ml (BD syringe Becton Dickinson, Pakistan), Disposable examination gloves (Max Pluss-100, Malaysia).

The study was conducted at Pharmacology Department of Post Graduate Medical Institute (PGMI) and Pathology Department of Fatima Jinnah Medical University from June to September 2016. It was approved from Ethical Committee for Basic Sciences of PGMI. Forty healthy male adult Swiss albino mice, 7-8 weeks of age weighing 25-35g were included in the study. Apparently unhealthy-looking mice were excluded. Mice were purchased from the University of Veterinary and Animal Sciences (UVAS), Lahore and kept in the animal house of PGMI under hygienic condition. The temperature was maintained in a range of 19-22°C, with a natural day and night cycle. Before the onset of study, all mice were kept for a week to acclimatize and they were provided with diet and water *ad libitum*.

Red delicious variety of local apples was selected because it contains the richest proportion of anti-oxidants among all the locally produced varieties.[13] Apples were washed with plain running water, air dried and peeled carefully so that peel may not contain flesh. The collected peel was spread and allowed to dry in shade for 2 weeks. The partially dried peels were then put in hot air oven at a temperature of 60^0C for 3 hours. The completely dried peel was then coarsely ground with pestle and mortar. The powdered peel was soaked in 80% of ethanol (1:10, w/v**)** at room temperature for 3 days with daily shaking. The filtration of solution was done by filtering it through Whatman filter paper No.1 and was separated from the liquid extract. The excess of solvent was evaporated and concentrated (semisolid) extract was stored at 4°C.[13,14] Weight of dried peel was 60g giving 43.75% yield of extract as brownish gummy paste.

Animals were randomized by lottery method into five groups of eight mice each, which were given drugs as described in the Table-1. Normal control group was given distilled water (4ml/kg). All other groups were given prednisolone in a dose of 4mg/kg[15] dissolved in 4 ml of distilled water. After 30 minutes of receiving prednisolone (4mg/4ml/kg), one prednisolone treated group (model group) was

given distilled water (4ml/kg), while experimental low, medium and high dose groups were given 25 mg/kg, 50mg/kg and 100 mg/kg apple peel extract,[16] respectively dissolved in 4ml of distilled water. All doses were given as a single morning dose by oral route for 14 consecutive days.

Groups	1st Drug by oral route (once daily dose from day 1 to 14)	2nd Drug by Oral route (once daily dose from day 1 to 14)
Normal Control	Distilled water 4ml/kg	Distilled water 4ml/kg
Model Group	Prednisolone 4mg/4ml/kg	Distilled water 4ml/kg
Experimental Low Dose Group (25 mg/kg)	Prednisolone 4mg/4ml/kg	Apple peel extract (25mg/kg) 25 mg/4ml/kg
Experimental Medium Dose Group (50 mg/kg)	Prednisolone 4mg/4ml/kg	Apple peel extract (50mg/kg) 50 mg/4ml/kg
Experimental High Dose Group(100mg/kg)	Prednisolone 4mg/4ml/kg	Apple peel extract (100 mg/kg) 100 mg/4ml/kg

Table-1: Summary of drugs administered to all groups

Sampling:

1. Blood

Twenty-four hours after the last dose, the mice were anesthetized with ketamine which was administered via single intraperitoneal injection in a dose of 100 mg/kg into the left lower quadrant of abdomen. The mice were dissected afterwards to expose the heart and blood was withdrawn directly from the right ventricle of heart with the help of 23-gauge needle and 3 ml disposable syringe. The blood was collected in EDTA vacutainer and analyzed within an hour by hematology analyzer.[14]

2. Bone Marrow

Bone Marrow Aspirate Smear: The already dissected mice were euthanized by giving a single sharp cut at neck using surgical scalpel and further dissected to obtain the right femur bone. The contents of right femur were aspirated into 0.2ml of ice-cold phosphate buffer saline by using 23-gauge needle and 10 ml syringe. The aspirate was then spread over slide and smear was prepared. Once the smear was air dried it was dipped into methanol solution to fix the specimen over the slide. Finally, the slide was stained with Geimsa stain, washed with plain running water and cover slip was applied.[14]

Bone Marrow Biopsy: The left femur of already dissected mouse was obtained and preserved in 10% formalin solution. Tissue was then processed in histopathology lab of PGMI and slides were prepared and stained with Haematoxylin and Eosin stain.[14]

Study Variables:

1. Blood Cells Count

Blood was analyzed for red blood cell, platelet, total leukocyte, and differential leukocyte counts. RBC, total leukocyte, and platelet counts were analyzed by hematology analyzer (Sysmex Model no. KX-21) and DLC was analyzed manually on Giemsa-stained slides under oil immersion lens. [14]

2. Bone Marrow Aspirate Smear

Smears were analyzed for myelopoiesis, erythropoiesis, megakaryocytes, lymphocytes, and plasma cells. Entire length of the tissue was scanned and viewed on 100X of light microscope (Olympus CHT, Japan). 200 cells were counted and relative percentage of cells was entered in the proforma.[17]

3. Bone marrow Biopsy:

Assessment and examination of the bone marrow biopsy was done under light microscope (40X). The sections were examined for cellularity and presence of fat and were graded on a 6-point scale.[14]

0=absent
1=very slight
2=slight
3=moderate
4=marked
5=very marked

Statistical analysis:

After proper collection, the data was transcribed into GraphPad Prism version 7.0. As data was found to be normally distributed by Shapiro Wilk test, analysis of variance (ANOVA) and *post hoc* Tukey's test were applied to see which group mean differs. The *p*-value of this hypothesis test was <0.05 (level of significance).

RESULTS

The comparison of means of red blood cell, leukocyte, and platelet counts; polymorph neutrophil and lymphocyte percentage in blood sample and myelopoiesis, megakaryocyte, erythropoiesis, cellularity: fat ratio by ANOVA revealed a significant difference between the groups.

Bone marrow lymphocytes and plasma cells did not show significant difference.

Prednisolone treatment raised blood RBC, WBC, and platelet count non-significantly but raised neutrophil percentage and lowered lymphocyte percentage significantly from normal control group.

In bone marrow smear megakaryocytes, myelopoiesis, lymphocytes, and cellularity: fat ratio increased non-significantly by corticosteroid treatment. Low dose of apple peel extract augmented these effects while medium and high doses reversed its effects in a dose dependent manner.

Red blood cell count was significantly higher in experimental low dose group as compared to normal control group and other experimental groups. Erythropoiesis was significantly higher in experimental low and medium dose groups as compared to high dose group (Fig-1).

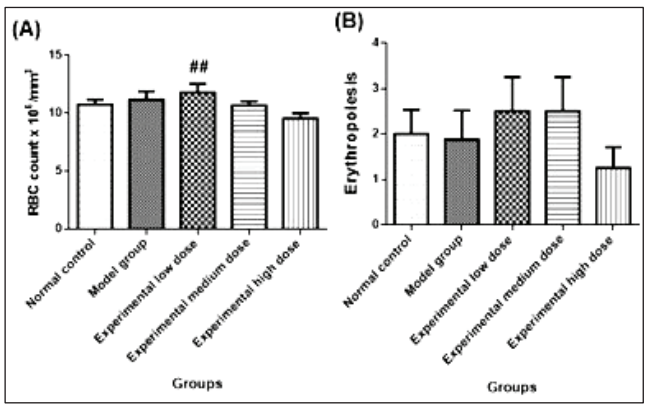

Fig-1: Effect of apple peel extract on red blood cell count (A) and bone marrow erythropoiesis (B) of prednisolone treated mice. Data represents mean ± SD (n=8);
##*p* value ≤ 0.01 vs Normal Control, Experimental low, medium and high dose.

Platelet count and the number of megakaryocytes were significantly higher in experimental low dose group as compared to normal control and other experimental groups. (Fig-2)

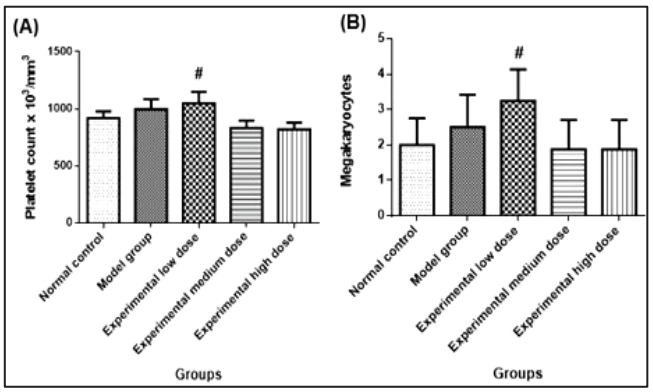

Fig-2: Effect of apple peel extract on blood platelet count (A) and bone marrow megakaryocytes (B) of prednisolone treated mice. Data represents mean ± SD (n=8);
#*p* value ≤ 0.05 vs Normal Control, Experimental low, medium and high dose.

Total leukocyte count was non-significantly higher in model group verses normal control but significantly higher in experimental low dose group only. It was 5763±1831, 6775±787, 8050±854, 6613±779, and 5738±1857 in normal control, model group, experimental low, medium, and high dose respectively.

Polymorph neutrophils percentage and the myelopoiesis in bone marrow smear were significantly higher in experimental low dose group as compared to normal control and other experimental groups (Fig-3).

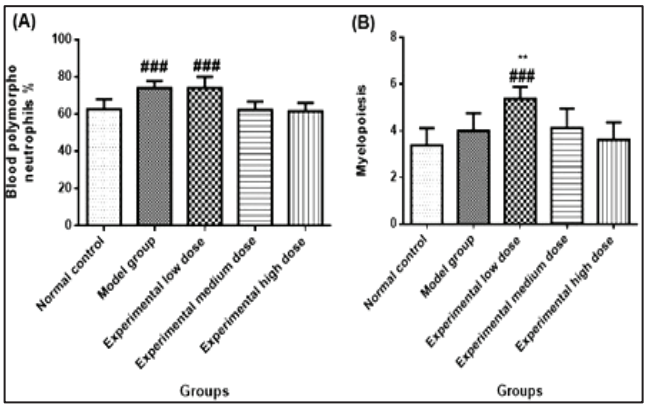

Fig-3: Effect of apple peel extract on blood poly morpho neutrophils % (A) and bone marrow myelopoiesis (B) of prednisolone treated mice. Data represents mean ± SD (n=8); ** *p* value ≤ 0.01 vs Model group, ### *p* value ≤ 0.01 vs Normal Control, Experimental low, medium and high dose.

Experimental medium and high dose groups had significantly higher percentage of lymphocytes in the blood as compared to model group and near to normal control group and non-significantly higher number of lymphocytes were seen in the bone marrow with experimental low dose group (Fig-4).

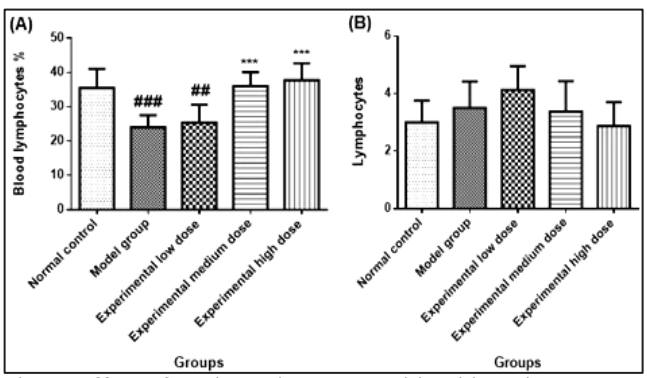

Fig-4: Effect of apple peel extract on blood lymphocytes % (A) and bone marrow lymphopoiesis (B) of prednisolone treated mice. Data represents mean ± SD (n=8);
***p* value ≤ 0.01 vs experimental medium and high dose group,
##*p* value ≤ 0.01 vs Experimental low dose.
p value ≤ 0.01 vs Model group.

Plasma cells in bone marrow smear were lower in model group but difference between groups was not significant. Count was 1.75±0.46, 1.25±0.46, 1.75±0.46, 1.62±0.51 and 1.25±0.46 in normal control, model group, experimental low, medium and high dose respectively.

Cellularity: fat ratio was significantly higher in low and medium dose groups as compared to normal control and model groups (Fig-5).

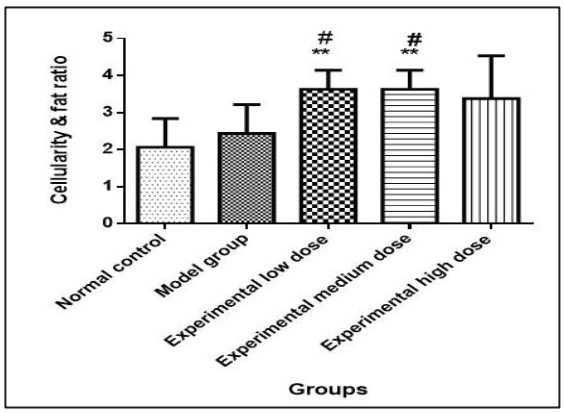

Fig-5: Effect of apple peel extract on cellularity: fat ratio in prednisolone treated mice. Data represents mean ± SD (n=8);
#*p* value ≤ 0.05 vs Normal Control
***p* value ≤ 0.05 vs Model group.

DISCUSSION

The corticosteroids used clinically for many hematological disorders leads to many adverse effects.[18] It was postulated that combination of a nutraceutical extract may lower the dose requirement of corticosteroid, thereby reducing the adverse effects. In the current study, *Malus domestica* (apple peel) extract is used to observe its effect on hematological changes induced by prednisolone. It contains a number of anti-inflammatory and antioxidant polyphenols like flavonoids, phenolic acids, catechins and epicatechins.[19] Results of this study indicated that low dose of extract enhanced the effects of prednisolone on blood and bone marrow parameters whereas, high dose extract reversed these effects and medium dose has intermediate and variable action.

Corticosteroids are known to stimulate erythropoiesis.[20] Results of this study indicate that this effect is not nullified by apple peel extract; rather red blood cells are significantly higher in low dose treated group as compared to normal control. Erythropoiesis is also numerically higher with low and medium dose. The marked reduction of erythropoiesis by high dose is of much importance.

Blood platelet count and megakaryocytes in bone marrow are also significantly higher in low dose group versus normal and other treated groups. Corticosteroids stimulate platelet production in majority of patients.[21] In this study these counts are only numerically higher with prednisolone treatment as compared to normal control. Higher dose and/or longer duration of study might have produced significant effect, but low dose of extract has potentiated this effect while higher dose has nullified it. Apple peel extract of same variety has shown to increase platelet count and megakaryocyte percentage of carboplatin treated mice at dose of 25 mg/Kg and 50 mg/Kg.[13]

In DLC, this study shows that the poly morpho neutrophils percentage is significantly higher in model and experimental low dose groups as compared to other groups. The possible explanation of an increase in polymorph neutrophil count in model group is that steroids shift poly morpho neutrophils from bone marrow to circulation and decrease their movement from blood circulation to the sites of inflammation.[22] They are also shown to stimulate myelopoiesis.[23] Apple peel extract in low dose has enhanced this effect on myelopoiesis leading to significant difference from other groups.

Lymphocyte percentage in blood of model group is significantly lower than normal control. Steroids cause movement of lymphocytes from blood vessels towards lymphoid tissue[18] as evidenced in this study from higher lymphocytes in bone marrow smear. Low dose of apple peel extract has maintained this effect, while medium and high doses have caused rise in blood lymphocyte percentage and lowered the bone marrow lymphocytes.

Plasma cells in bone marrow are numerically lower in model group and equal to normal in experimental low dose group. Plasma cells are responsible for antibody production and B lymphocyte differentiation into plasma cells is inhibited by corticosteroids.[24] A study using same strain of mice, prednisolone and apple peel extract in same doses for similar duration has shown higher IgG level with extract which is not dose dependent,[12] but present study failed to increase number of plasma cells above normal. May be production is stimulated without increase in number of cells. Had the IgG and plasma cell number estimated in same animals, picture would have been clearer. Presently no other study is available on effect of apple peel on plasma cells for comparison.

The study shows that bone marrow cellularity is significantly higher in experimental low and medium dose groups as compared to both normal and model groups, suggesting overall proliferative

effect of apple peel extract. No similar study is available to compare the results of present study. To the best of our knowledge this is first study to show agonistic action of prednisolone and apple peel extract in low dose and antagonistic action in high dose.

Present research is limited to effect of apple peel extract on hematological parameters only but opens avenues of future research on this topic. Results of this study indicate the need to investigate combined effect of different doses of corticosteroid with low dose apple peel extract for prolonged period as their long-term use is associated with metabolic adverse effects while apple peel has shown to have beneficial effect in metabolic disorders.[25] It is recommended that the hematological parameters, immune function, and metabolic affects should also be observed in same subjects to assess magnitude of benefit.

CONCLUSION

Apple peel extract in low dose has complemented the effect of prednisolone on hematological parameters of mice peripheral blood and bone marrow, while high dose has nullified these effects.

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