Original Article

Protective Effect of Malus Domestica (apple) Peel Extract on Carboplatin Induced Fall in Blood Platelet Count and Bone Marrow Megakaryocyte Percentage in Mice

Abdul Mudabbir Rehan, Rabia Naseer Khan, Sehrish Zaffar, Mahwash Malik, Sadia Chiragh and Amtul Hafeez

Objective: To evaluate the protective effect of Malus domestica (apple) peel extract on carboplatin induced fall in blood platelet count in mice and also fall in bone marrow megakaryocyte percentage in mice.

Methods: Forty adult male mice were divided into 5 groups. Group A was negative control and only distilled water was given to mice. Group B was disease control and mice in this group received carboplatin intraperitoneally in a dose of 125 mg/kg. Group C, D & E were experimental groups and carboplatin along with the apple peel extract in three different doses of 25, 50 & 75 mg/kg was given. After 7 days, blood & tissue sampling was done.

Results: The results revealed that apple peel extract significantly prevented the fall in platelet count and megakaryocyte percentage in all experimental groups treated with carboplatin.

Conclusions: Apple Peel Extract effectively prevented carboplatin induced fall in platelet count and megakaryocyte percentage and produced m,ore significant results in a dose of 50 mg/kg.

Keywords: thrombocytopenia, carboplatin, apple peel extract, bone marrow aspirate.

Introduction

Thrombocytopenia refers to decrease in platelet count <150,000/μL in blood (normal count is 150,000 to 450,000/ μL).[1] Thrombocytopenia is usually asymptomatic and it is picked up on a routine full blood count or peripheral blood smear examination. The individuals may experience external bleeding such as nosebleeds or bleeding gums. Women can experience heavier or longer periods or breakthrough bleeding. Bruising, particularly purpura can occur under the skin in forearms, whereas, Petechiae may occur on feet and legs.[2] Platelet counts of 75,000/ μL to 150,000/ μL are defined as grade-I thrombocytopenia, 50,000 to 75,000/μL as grade-II, 25,000 to 50,000/ μL as grade-III, and below 25,000/ μL as grade IV thrombocytopenia.[1] Platelets maintain vessel wall integrity, whereas thrombocytopenia is associated with a defect of primary hemostasis. The most common causes of thrombocytopenia are megalo- blastic anemia, idiopathic thrombocytopenia, infections, hematological malignancies, aplastic anemia, pregnancy, alcoholism and cancer chemotherapeutic drugs. Thrombocytopenia in cancer patients can result from chemotherapy, radiation treatment or from the underlying disease itself, but it is most frequently observed due to myel-osuppressive side effect of cancer chemotherapy and most of chemotherapeutic agents cause myelosuppression in a dose dependent manner.[3] Thrombocytopenia creates a number of problems in the care of a cancer patient. At platelet count<10,000/μL, spontaneous bleeding is increased and radiations/ chemotherapy are administered with caution for fear of worsening the thrombocytopenia and increasing risk of bleeding. At platelet counts <50,000/μL, surgical procedures are complicated by bleeding.[4] Therapeutic and prophylactic platelet transfusions create the additional risk of infusion complications. Clinicians' responses to thrombocytopenia in a cancer patient vary. Reduction of the dose intensity of chemotherapy or radiation is common and for some patients, treatment of the underlying cause of thrombocytopenia (e.g. stopping therapy with the offending drug) may work. Platelet transfusion is often the only readily available treatment. The discovery of thrombopoietin in 1994 generated great expectations and the 1st generation recombinant thrombopoietin agonists reduced chemotherapy-related thrombocytopenia in early clinical trials, but their subsequent development was halted due to antibody formation against endogenous thrombopoietin.[5] Whereas, two 2nd generation thrombopoietin receptor agonists have now been developed, but neither has yet been tailored for treating thrombocytopenia in cancer patients.[6] Carboplatin is an anticancer drug which belongs to alkylating class of cancer chemotherapeutic drugs. It is used effectively to treat many cancers. Experimentally, carboplatin has been used to induce myelosuppression in mice.[7] Carboplatin induces myelosuppression by increasing overall oxidative stress and decrease in the glutathione content inside

bone marrow.[8] It also cross links the DNA and generates oxidative stress products such as malondialdehyde.[9] It is studied now that various substances like Glutathione,[10] Squalene[11] and L-Carnitine[8] have protective effects on carbo- platin induced myelotoxicity in animal models.

It can be concluded that toxicities of carboplatin are due to increase in the overall oxidative stress inside bone marrow and if this abrupt rise in oxidative stress could somehow be prevented then the serious untoward effects can be avoided. Apples are the most widely consumed fruit worldwide and contain five major polyphenols. Apple peel contains 3 to 6 times more flavonoids as compared to apple flesh that's why, apple peel extracts have more antioxidant potential than apple flesh extracts.[12] The aim of study is to observe the protective effects of apple peel extract on carboplatin induced thrombocy- topenia. Carboplatin suppresses the bone marrow production of various cells by interfering with redox reactions inside marrow cavity. Carboplatin shifts the redox equilibrium towards oxidative side, and apple peel extract by virtue of its antioxidant property will restore the redox equilibrium by reduction and will improve the bone marrow suppression.

Methods

It was an experimental study conducted at Postgraduate Medical Institute Lahore. Forty adult male mice were divided into 5 groups containing 8 mice in each group. Mice were kept in animal house of Postgraduate Medical Institute Lahore inside cages under hygienic conditions. They were given humane and veterinary care according to the criteria outlined in the "Guide for the care and use of laboratory animal" (Council, 2010). 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 for acclimatization and were provided with diet and water ad libitum. A single intraperitoneal injection of carboplatin (Injection Carpsol 150mg/15ml by Pfizer pharma) in a dose of 125 mg/kg was given to induce myelosuppression in mice.[7] 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.[12] The filtration of solution was done by filtering it through What man filter paper No. 1 and was separated from the liquid extract. The excess of solvent was evaporated and concentrated extract was stored at 4 °C.[13] The five study groups were given drugs as described in the table below. The drug given to each mice in all groups via intraperitoneal route was administered once on day 0, whereas the drug given to each mice in all groups via oral route was administered in the morning as a single dose for 7 days starting from day 0.

Mice Groups	Drug by Intra peritoneal route (single dose on day 0 only)	Drug by Oral route (once daily\ dose from day 0 to 7)
A	Sterile water	Distilled Water
Normal Control	1.25mg/kg	4ml/kg
B	Carboplatin	Distilled Water
Disease Control	125mg/kg	4ml/kg
C	Carboplatin	Apple peel extract (25mg/kg)
Experimental 25mg/kg	125mg/kg	25mg/4ml/kg
D	Carboplatin	Apple peel extract (50mg/kg)
Experimental 50mg/kg	125mg/kg	50mg/4ml/kg
E	Carboplatin	Apple peel extract (75mg/kg)
Experimental 75mg/kg	125mg/kg	75mg/4ml/kg

Sampling:

Blood: On day 7 the mice were anesthetized with ketamine which was administered via single intraperitoneal injection in a dose of 100mg/kg 14 into the left lower quadrant of abdomen. The mice were dissected afterwards to expose the heart and 1.5ml 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.

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 andCover slip was applied.[15]

Parameters:

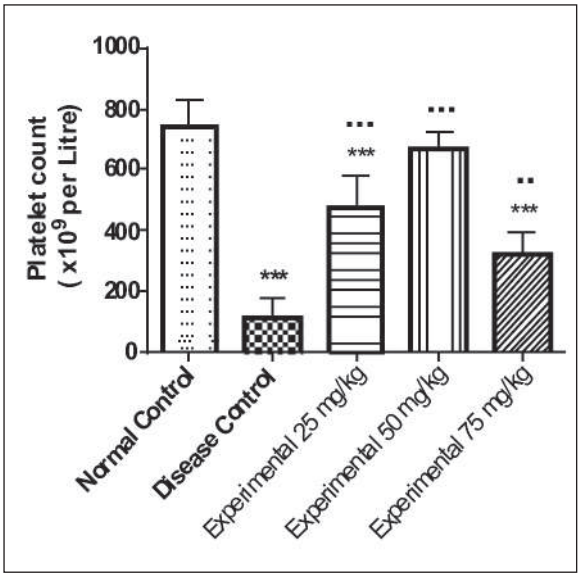
Platelet Count: The platelet count was analyzed by hematology analyzer in hematology lab of PGMI.

Bone Marrow Aspirate Smear: Differential cell count was done under oil immersion lens of light microscope. 200 cells were counted and relative percentage of platelets was entered in the proforma only.[15]

Results

1. Platelet Count:

The mean platelet count ± standard deviation of all the groups are illustrated in **Fig-1.** The comparison of means of all groups by ANOVA revealed a significant difference between the group means with a p value of <0.001.



****=p value <0.001 (vs Normal Control), …= p value <0.001 (vs Disease Control), .. = p value <0.01 (vs Disease Control)*

Fig-1: Effect of carboplatin and carboplatin with 3 different doses of APPE on platelet count (M.ean ±SD) in mice (n=8).

The post hoc Tukey's test was applied to analyze platelet count difference between the groups. The disease control group had markedly lower platelet count as compared to normal control group, whereas all experimental groups had significant higher platelets count as compared to disease control group. All experimental groups had lower platelets count as compared to normal control, but the difference was insignificant with 50 mg/kg dose of APPE. When the experimental group were compared with each other, then the group with 75mg/kg dose had significant lower platelets count as compared to the groups with 25 & 50 mg/kg dose.

Table-1: Comparison of effect of carboplatin and carboplatin with 3 different doses of APPE on platelet count in mice by post hoc Tukey's test (n=8)

Post hoc Tukey's multiple comparison test		Mean Difference	Significance
Group A Normal Control	Disease control	622.80	***
	Experimental (25 mg/kg)	261.40	***
	Experimental (50 mg/kg)	73.38	ns
	Experimental (75 mg/kg)	414.60	***
Group B Disease Control	Experimental (25 mg/kg)	-361.40	***
	Experimental (50 mg/kg)	-549.40	***
	Experimental (75 mg/kg)	-208.10	**
Group C Experimental (25mg/kg)	Experimental (50 mg/kg)	-188.004	**
	Experimental (75 mg/kg)	153.30	*
Group D Experimental (75mg/kg)	Experimental (75 mg/kg)	341.30	***

2. Megakaryocyte Percentage:

The mean megakaryocyte percentage±standard deviation of all groups are given in **Table-2.** The post hoc Tukey's test was applied to analyze the megakaryocyte percentage difference between the groups. The disease control group had markedly lower megakaryocyte percentage as compared to normal control group, whereas all experimental groups had significant higher megakaryocyte percentages as compared to disease control group.

Table-2: Comparison of effect of carboplatin & carboplatin with 3 differents doses of APPE on megakaryocyte percentage in bone marrow aspirate smear by post hoc Tukey's test (n=8).

Post hoc Tukey's multiple comparison test		Mean Difference	Significance
Group A Normal Control	Disease control	7.87	***
	Experimental (25 mg/kg)	2.87	***
	Experimental (50 mg/kg)	2.12	**
	Experimental (75 mg/kg)	3.87	***
Group B Disease Control	Experimental (25 mg/kg)	-5.00	***
	Experimental (50 mg/kg)	-5.75	***
	Experimental (75 mg/kg)	-4.00	***
Group C Experimental (25mg/kg)	Experimental (50 mg/kg)	-0.75	ns
	Experimental (75 mg/kg)	1.00	ns
Group D Experimental (75mg/kg)	Experimental (75 mg/kg)	1.75	*

*ns = not significant, * = p value < 0.05, ** = p value < 0.01, *** = p value <0.001*

All experimental groups had significant lower megakaryocyte percentage as compared to normal Control. The group with 75 mg/kg dose had significant lower megakaryocyte percentage as

compared to the group with 50 mg/kg dose.

Discussion

Cancer is the 2nd most common leading cause of death worldwide which causes severe morbidity and mortality in all age groups. Multiple preventive and treatment modalities like cancer screening, chemotherapy, radical surgeries & radiotherapy are available now a days, but among all these modalities chemotherapy is the prime option for initial medical management of various cancers. However, it is the acute or cumulative toxicity of chemotherapeutic agents which impairs treatment.[16] The adverse effects are many, but myelosuppression is the only treatment limiting toxicity in almost all of the cancer patients.[3] That is why adjuvants, neo-adjuvants and adjunct therapies are used along with the principal therapeutic strategy to abate cancers. The GM-CSF has been used in cancer patients to improve peripheral neutropenia,[17] and thrombopoietin to treat thrombocytopenia.[6] However, the indications of the above regimens are limited because of their adverse effects and high costs.[18] The ultimate solution to chemotherapy (CP) induced myelosuppression lies in the natural compounds which can prevent chemotherapy induced myelosuppression by virtue of their antioxidant capacity. We selected APPE of red delicious variety due to its maximum antioxidant potential[13] to observe the antioxidant effect on oxidative stress driven by carboplatin on bone marrow of mice. The statistical analysis revealed a significant decrease in peripheral blood platelet count which was accompanied by a significant decrease in megakaryocyte percentage in bone marrow aspirate smear of disease control group. The platelet count and the megakaryocyte percentage increased significantly in all experimental groups as compared to disease control group and the maximum increase was observed in group which received 50 mg/kg dose No similar study of effect of APPE is available for comparison, but studies utilizing other herbal preparations containing polyphenols/antioxidants have demonstrated similar results. A recent study conducted by (Tahir and her colleagues in 2014)[7] demonstrated the effect of Carica papaya leaf juice in preventing the fall in platelet count in mice induced by carboplatin. The Carica papaya leaf juice contains polyphenols which serve as antioxidants and reduce oxidative stress inside bone marrow of carboplatin treated mice. The increase in platelet count in all experimental groups is a remarkable finding because carboplatin has predominant effect on the decrease in platelet cell count[19] as compared to the red blood cells and white blood cells. In current study, 3 different doses of APPE were used to find out right therapeutic dose. The study results demonstrated that all 3 doses of APPE caused an overall numerical increase in peripheral blood cell counts and megakaryocyte percentage of all experimental groups, but statistics revealed that the APPE in a dose of 50 mg/kg is more significant as compared to the rest of 2 doses. The significantly better results of 50 mg/kg dose as compared to 25 mg/kg dose demonstrates dose dependent preventive effect, while inferior effect of 75 mg/kg dose may be explained by the fact that antioxidants in a high dose act as pro-oxidants.[20]

Conclusion

Apple Peel Extract is effective in preventing the fall in platelet count & megakaryocyte percentage induced by the carboplatin driven oxidative stress. Apple peel extract produced better significant results in a dose of 50 mg/kg.

Department of Pharmacology
D. G. Khan Medical College, Dera Ghazi Khan.

www.esculapio.pk

References

1. Sulya, M., R. Malik, and K. Patel, Evaluation of various causes of throm bocytopenia in patients attending Ham idia Hospital, Bhopal. Annals of Applied Bio-Sciences, 2016. 3(1): p. A72-74.
2. Bhatia, D., Platelet Count Using Image Processing Techniques. University of Delhi.
3. Wang, Y., V. Probin, and D. Zhou, Cancer therapy-induced residual bone marrow injury: mechanisms of induction and implication for therapy. Current cancer therapy reviews, 2006. 2(3): p. 271-279.
4. Deutschman, C.S. and P.J. Neligan, Evidence-based practice of critical care. 2015: Elsevier Health Sciences.
5. Kuter, D.J. and C.G. Begley, Recombinant human thrombopoietin: basic biology and evaluation of clinical studies. Blood, 2002. 100(10): p. 3457-3469.
6. Kuter, D.J., Managing thrombocytopenia associated with cancer chemotherapy. Oncology, 2015. 29(4): p. 282-282.
7. Tahir N, Zaheer Z, Kausar S, Chiragh S. Prevention of fall in platelet count by carica papaya leaf juice in carboplatin induced thrombocytopaenia in mice.

Biomed 2014; 30(1):21-25.
8. Abd-Allah AR, Al-Majed AA, Al-Yahya AA, Fouda SI, Al-Shabana OA. L-Carnitine halts apoptosis and myelosuppression induced by carboplatin in rat bone marrow cell cultures (BMC). Arch Toxicol 2005;79(7):406-413.
9. Weijl NI, Elsendoorn TJ, Lentjes EG, Hopman GD, Wipkink-Bakker A, Zwinderman AH et al. Supplementation with antioxidant micronutrients and chemotherapy-induced toxicity in cancer patients treated with cisplatinbased chemotherapy: a randomised, double-blind, placebo-controlled study. Eur J Cancer 2004; 40(11):1713-1723.
10. Pratibha R, Sameer R, Rataboli PV, Bhiwgade DA, Dhume CY. Enzymatic studies of cisplatin induced oxidative stress in hepatic tissue of rats. Eur J Pharmacol 2006; 532(3):290-293.
11. Das B, Yeger H, Baruchel H, Freedman MH, Koren G, Baruchel S. In vitro cytoprotective activity of squalene on a bone marrow versus neuroblastoma model of cisplatin-induced toxicity: implications in cancer chemotherapy. Eur J Cancer 2003; 39(17):2556-2565.
12. Karaman S, Tütem E, Başkan KS, Apak R. Comparison of antioxidant capacity and phenolic composition of peel and flesh of some apple varieties. J Sci Food Agric 2013; 93(4):867-875.
13. Manzoor M, Anwar F, Saari N, Ashraf M. Variations of antioxidant characteristics and mineral contents in pulp and peel of different apple (Malus domestica Borkh.) cultivars from Pakistan. Molecules 2012; 17(1):390-407.
14. Xu Q, Ming Z, Dart AM, Du XJ. Optimizing dosage of ketamine and xylazine in murine echocardiography. Clin Exp Pharmacol Physiol 2007; 34(56):499-507.
15. Gibson FM, Michael AC, Diamanti P, Rizzo S, Macharia G, GordonSmith EC et al. A new model of busulphan-induced chronic bone marrow aplasia in the female BALB/c mouse. Int J Exp Pathol 2003; 84(1):31-48.
16. Huitema, A., et al., Relationship between exposure and toxicity in high-dose chemotherapy with cyclophosphamide, thiotepa and carboplatin. Annals of oncology, 2002. 13(3): p. 374-384.
17. Bennett, C.L., et al., Colony-stimulating factors for febrile neutropenia during cancer therapy. New England Journal of Medicine, 2013. 368(12): p. 1131-1139.
18. Smith, T.J., et al., Recommendations for the use of WBC growth factors: American Society of Clinical Oncology clinical practice guideline update. Journal of Clinical Oncology, 2015. 33(28): p. 3199-3212.
20. HE, F., et al., The Effect of Eclipta Prostrata on Mice of Thrombocytopenia by Carboplatin. Acta Chinese Medicine and Pharmacology, 2012. 1.
21. Nasri, H. and M. Rafieian-Kopaei, Medicinal plants and antioxidants: why they are not always beneficial? Iranian journal of public health, 2014. 43(2): p. 255.