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Ameliorative effects of hesperidin on methotrexate toxicity in wistar rats: A hematological study

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Abstract
This present research work was conducted on 40 Wistar rats to study the methotrexate (MTX) toxicity and ameliorative effects of hesperidin. Wistar rats were randomly divided into 4 different groups with 5 male and 5 female in each group. The group were numbered as group I to IV. Group I served as a control and received 1% CMC in distilled water thorough orally for 28 days. Group II received single dose of MTX at the dose rate of 20 mg/kg/b.wt on 21st day of study via i.p. Whereas group III received hesperidin at the dose rate of 100 mg/kg/b.wt and group IV 200 mg/kg/b.wt orally for 28 days and single dose of MTX @ 20 mg/kg on 21st day of study via i.p. The Blood samples were collected from retro orbital venous plexus using rat capillaries at 21st and 28th day of experiment for haematological parameters. group (II) showed clinical signs like decreased feed intake, dullness and diarrhoea after administration of methotrexate. Hesperidin treated both groups (III&IV) showed less severe symptoms than MTX treated group rats. The significant lower mean value of TLC, neutrophils, TEC, Hb, HCT, platelets while significant higher mean value of eosinophils and MCV in MTX treated group (II). In female and male rats, Hesperidin treated both groups 100 mg and 200 mg revealed significant higher mean value of TLC, neutrophils, TEC, Hb, HCT, platelets and significant lower mean value of eosinophils and MCV compared to methotrexate groups on 28th day of study. This study suggests hesperidin have a protective effect against MTX.

Keywords: Methotrexate toxicity hesperidin, chemotherapy, wistar rats, haematological parameters

Introduction
Chemotherapy has been a crucial component of managing cancer in both human and animal patients for the past 40 years. In India, cancer cases or its incidence were abundantly increased in the animals. Cancer is the uncontrolled, abnormal development of cells or tissues in the body that proliferates indefinitely. Chemotherapeutic agents have a cytotoxic effect on cancer cells as well as normal cells of the body. Methotrexate (MTX) is commonly used chemotherapeutic agent which has immunomodulatory, antimetabolite, anti-inflammatory, immunosuppressive and cytotoxic properties, and used in a wide range of clinical practices since 1950 (Peters *et al*., 2000) [9].

Methotrexate (MTX) is a WHO 'essential medicine' that is now widely employed as a first line treatment in auto-immune, inflammatory diseases such as rheumatoid arthritis (RA), psoriasis and Crone's disease. MTX is also utilised in the treatment of lymphoma, osteosarcoma, autoimmune disorders, breast tumours, invasive urinary bladder cancer and in veterinary oncology. Numerous organs and tissues are affected by MTX poisoning, including the liver, kidney, lung, testicles, small intestine, ovary and nerve cells.

Flavonoids are categorized as flavanols (Quercetin, Rutin, Myricetin) flavanones (Hesperidin, Naringin, flavonols), isoflavones (Genistein, Daidzein), flavones (Apigenin, Tangeretin), anthocyanin (Cyanidin, Malvidin) and chalcone (Phlorectin, Arbutin) according to structural variations (Panche *et al*., 2016) [8]. Hesperidin is one of the most commonly used and biologically active compounds in the flavonoid family (Turk *et al.*, 2019) [11]. HSP also has lipid-lowering, anti-inflammatory, antioxidant, antibacterial, antiviral, anti-hypertensive, anti-

carcinogenic, and antioedema effects. Hesperidin has some pharmacological effects like effects on vascular system, action on enzyme, anti-fertility, platelet and cell aggregation inhibition, ultra violet protecting activity and also some miscellaneous effect like analgesic, antipyretic activity, anti-allergic, effect on wound healing and anti-ulcer activity (Garg *et al*., 2001) [3].

Materials and Methods

The study was conducted at small animal house and Department of Veterinary Pathology, College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand. The study was approved by the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary Science and Animal Husbandry, Kamdhenu University, Anand, Gujarat, India. The animal facility of Cadila Pharmaceuticals Company Ltd. in Dholka, Gujarat, India provided a total of 40 Wistar rats (20 male and 20 female), which were used in experiments.

The procedures for animal care and management followed to those given by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. All of the rats were kept in polypropylene cages at a laboratory animal house facility (College of Veterinary Science and Animal husbandry, Anand) in a climate-controlled room with a constant temperature of 22±3 °C and a humidity of 30-70%. There was a 12/12-hour cycle of light and dark. The rats were kept stress-free by using corncob as bedding and following all appropriate management protocols. Before experiments, all rats were acclimated for 15 days.

Rats were given free access to a regular pellet food and palatable water for the duration of the trial. The feed was provided by keval sales corporation, Vadodara, Gujarat. Rats were identified by having picric acid applied to their body coats to mark them. One cage had five rats, each of which had a ring at the base of its tail to represent the number of the animal (rats 1, 2, 3, 4, and 5 consecutively).

Methotrexate and hesperidin were purchased from cadila healthcare Ltd, Ahmedabad and Sigma Aldrich company respectively.

A total of 40 healthy Wistar rats (20 males and 20 females) were divided into four groups at random, each including five female and five male rats. The groups received numbers ranging from I to IV. Group I (control): Rats received vehicle 1% carboxymethylcellulose (CMC) dissolved in distilled water through orally and single dose of normal saline via intraperitoneal route. Group II (MTX): Rats were injected single dose of methotrexate at the dose of 20 mg/kg/bw via intraperitoneally. Group III (MTX and hesperidin 100 mg): Rats received methotrexate at the dose of 20 mg/kg through intraperitoneally at the 21st day and hesperidin @ 100 mg/kg/bw through orally for 28 days. Group IV (MTX and hesperidin 200 mg): Rats were given single dose of methotrexate @ 20 mg/kg i.p at 21st day and hesperidin at the dose of 200 mg/kg/bw for 28 days via oral route.

Every animal in the experiment was examined twice a day for morbidity and mortality. The clinical observations have been recorded once daily during the adaptation period. Clinical and behavioural observations were carried out at least twice on each day of dosing during the experimental period (before and after therapy).

A light isoflurane anaesthesia was given on the 21st and 28th day of the experiment. Using a heparinized capillary tube, blood was collected from the retroorbital plexus of each animal.

One way analysis of variance (ANOVA) was used for analysis of the data from all groups, viz., Control group, Methotrexate group and treatment group, using software SPSS (version 26). The values at <0.05 were taken to indicate statistically difference.

Results

All female and male rats control group (I) did not exhibit any noticeable behavioral and clinical changes throughout the experiment. Female and male rats of group (II) (MTX) showed clinical signs like dullness, decreased feed intake and nasal bleeding was observed after injection of methotrexate at 24th day. Female and male rats of group III (hesperidin 100 mg+MTX) and group IV (hesperidin 200 mg+ MTX) showed anorexia, lethargy and dullness with less severity as compared to methotrexate group (II).

Haematology

The mean ± S.E. values of haematological parameters of different experimental groups on 21st day and 28th day in male have been summarized in table 1 and 2.

The mean ± S.E. values of haematological parameters of different experimental groups on 21st day showed no any statistical changes.

Table 1: Haematological parameter (Mean ± S.E., n=5) in male rats on 21st day of experiment.

Parameter (Unit)	Group I	Group II	Group III	Group IV
Total Leukocyte Count (10^3/µl)	11.19±0.95	11.98±0.58	11.93±1.38	13.75±0.46
Neutrophils (10^3/µl)	2.42±0.39	2.38±0.34	2.30±0.35	2.29±0.31
Lymphocytes(10^3/µl)	8.65±0.91	9.06±0.50	9.56±1.36	10.38±0.69
Monocytes (10^3/µl)	0.39±0.04	0.46±0.13	0.49±0.19	0.46±0.08
Eosinophils (10^3/µl)	0.10±0.02	0.09±0.03	0.11±0.02	0.11±0.01
Basophils (10^3/µl)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Total Erythrocyte Count (10^6/µl)	7.42±0.53	7.82±0.20	7.98±0.12	8.19±0.09
Hemoglobin (g/dl)	14.40±0.11	14.36±0.08	14.92±0.18	15.12±0.18
Hematocrit (%)	43.09±0.36	42.20±1.20	43.34±0.50	43.77±0.47
Mean Corpuscular Volume (fL)	56.00±0.89	54.80±0.97	54.60±0.60	53.80±0.92
Mean platelet volume (fL)	7.68±0.08	7.56±0.05	7.66±0.08	7.48±0.15
Platelets (10^3/µl)	905.40±18.87	864.40±63.34	910.00±33.15	915.80±24.18

I - Control group; II- Methotrexate @ 20 mg/kg; III-Hesperidin @ 100 mg +methotrexate @ 20 mg/kg; IV-Hesperidin @ 200 mg +methotrexate @ 20 mg/kg

The mean ± S.E. values of TLC, neutrophils, TEC, haematocrit, haemoglobin and platelets significantly decreased in group II as compared to Group I, III and IV while mean ± S.E. values of eosinophils, MCV and lymphocyte significantly increased as compared to group I, III and IV.

Table 2: Haematological parameter (Mean ± S.E., n=5) in male rats on 28th day of experiment.

Parameter (Unit)	Group I	Group II	Group III	Group IV
Total Leukocyte Count (10^3/µl)	11.57±0.98^a	10.50±0.36^b	11.20±1.42^c	12.26±0.74^c
Neutrophils (10^3/µl)	1.52±0.29^a	0.82±0.20^b	1.20±0.85^c	1.29±0.37^c
Lymphocytes(10^3/µl)	9.31±0.80^c	10.84±0.56^a	9.81±0.48^a	9.49±0.77^a
Monocytes (10^3/µl)	0.34±0.06	0.69±0.29	0.61±0.23	0.57±0.21
Eosinophils (10^3/µl)	0.10±0.20^a	0.29±0.05^b	0.13±0.02^a	0.12±0.01^a
Basophils (10^3/µl)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Total Erythrocyte Count (10^6/µl)	8.15±0.14^c	6.46±0.32^a	7.13±0.09^b	7.80±0.21^c
Hemoglobin (g/dl)	14.76±0.17^c	12.56±0.27^a	13.36±0.11^b	14.36±0.16^c
Hematocrit (%)	44.71±0.89^b	38.08±0.96^a	39.22±0.42^a	42.75±0.93^b
Mean Corpuscular Volume (fL)	55.00±0.55^a	68.00±2.91^b	57.60±0.93^a	56.00±0.70^a
Mean platelet volume (fL)	7.36±0.09	7.50±0.07	7.48±0.09	7.28±0.07
Platelets (10^3/µl)	809.00±29.43^b	581.80±52.64^a	791.40±20.90^b	804.40±25.93^b

Mean bearing different superscripts in row differ significantly (p<0.05).

I - Control group; II- Methotrexate @ 20 mg/kg; III- Hesperidin @ 100 mg + methotrexate @ 20 mg/kg; IV- Hesperidin @ 200 mg + methotrexate @ 20 mg/kg

The mean ± S.E. values of haematological parameters of different experimental groups on 21st day and 28th day in male have been summarized in table 3 and 4.

The mean ± S.E. values of haematological parameters of different experimental groups on 21st day showed no any statistical changes.

Table 3: Haematological parameter (Mean ± S.E., n=5) in female rats on 21st day of experiment.

Parameter (Unit)	Group I	Group II	Group III	Group IV
Total Leukocyte Count (10^3/µl)	11.89±0.34	12.71±0.24	12.21±0.60	12.57±0.66
Neutrophils (%)	2.58±0.25	2.37±0.37	2.22±0.32	2.36±0.31
Lymphocytes%	9.28±0.36	9.61±0.59	8.92±0.88	8.69±0.97
Monocytes (%)	0.45±0.10	0.53±0.12	0.32±0.15	0.37±0.05
Eosinophils (%)	0.07±0.16	0.05±0.01	0.06±0.02	0.09±0.02
Basophils (%)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Total Erythrocyte Count (10^6/µl)	7.17±0.17	7.43±0.19	7.46±0.093	7.49±0.15
Hemoglobin (g/dl)	14.16±0.14	14.26±0.18	14.00±0.21	14.48±0.13
Hematocrit (%)	43.47±0.80	43.71±0.54	43.26±0.61	45.60±0.61
Mean Corpuscular Volume (fL)	55.60±53.12	56.00±0.55	57.20±0.58	58.00±1.14s
Mean platelet volume	7.66±0.04	7.61±0.08	7.50±0.11	7.66±0.08
Platelets (10^3/µl)	988.60±52.23	930.80±16.96	1010.40±48.26	1006.40±23.18

I - Control group; II- Methotrexate @ 20 mg/kg; III- Hesperidin @ 100 mg +methotrexate @ 20 mg/kg; IV- Hesperidin @ 200 mg + methotrexate @ 20 mg/kg

The mean ± S.E. values of TLC, neutrophils, TEC, haematocrit, haemoglobin and platelets significantly decreased in group II as compared to Group I, III and IV while mean ± S.E. values of eosinophils, MCV and lymphocyte significantly increased as compared to group I, III and IV.

Table 4: Haematological parameter (Mean ± S.E., n=5) in female rats on 28th day of experiment.

Parameter (Unit)	Group I	Group II	Group III	Group IV
Total Leukocyte Count (10^3/µl)	12.28±0.59^c	8.89±0.71^a	10.47±0.30^b	10.76±0.31^{bc}
Neutrophils (%)	1.10±0.25 ^{a}	0.46±0.20 ^{b}	1.09±0.66 ^{a}	1.04±0.46 ^{a}
Lymphocytes%	6.41±0.95^a	11.29±0.78^b	11.19±0.88^b	8.98±1.39^{ab}
Monocytes (%)	0.43±0.13	0.45±0.12	0.61±0.20	0.40±0.20
Eosinophils (%)	0.05±0.01^a	0.14±0.03^b	0.06±0.01^a	0.07±0.01^a
Basophils (%)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Total Erythrocyte Count (10^6/µl)	7.38±0.19^c	5.10±0.40^a	6.15±0.37^b	6.01±0.32^{ab}
Hemoglobin (g/dl)	14.04±0.26^c	9.16±0.78^a	10.88±0.43^b	11.94±0.50^b
Hematocrit (%)	42.98±0.82^a	29.43±2.33^a	35.62±1.55^b	37.36±1.99^b
Mean Corpuscular Volume (fL)	58.20±0.80^a	66.60±2.16^b	59.20±1.20^a	60.00±0.71^a
Mean platelet volume	7.50±0.17	7.42±0.15	7.50±0.11	7.64±0.08
Platelets (10^3/µl)	859.00±48.55^b	643.80±72.60^a	832.20±25.77^b	870.60±35.13^b

Mean bearing different superscripts in row differ significantly (p<0.05).

I - Control group; II- Methotrexate @ 20 mg/kg; III- Hesperidin @ 100 mg + methotrexate @ 20 mg/kg; IV- Hesperidin @ 200 mg + methotrexate @ 20 mg/kg

Discussions

In male and female rats, on 21st day of study, no significant changes were observed in TLC, neutrophils, lymphocytes, monocytes, eosinophils, basophils, TEC, Hb, HCT, MCV, MPV and PLT in group I, II, III and IV. This indicates that administration of hesperidin in group III and IV have no adverse effects on 21st day of experiment compared to control group rats.

In male and female rats, no significant changes were observed in monocyte, basophils and MPV in all experimental group compared to control group on 21st and 28th day of study.

A significantly decreased White blood cells in methotrexate treated rats was observed by Abdul-Wahab (2013) [1] revealed significantly decreased White blood cells in methotrexate treated rats. Ilamkar *et al.* (2020) [4], Aparna *et al.* (2021) [2] and Shwaikh *et al.* (2021) [10] also observed significant decreases in total leucocyte count in methotrexate treated rats. Mahar and Marar (2016) [7] reported significant decreased neutrophils and increased eosinophils in methotrexate treated rats. Ilamkar *et al.* (2020) [4] also reported significant decreased neutrophils in methotrexate treated rats. Aboraya *et al*. (2022) [12] showed significantly increased in mean value of white blood cells in hesperidin treated rats.

These findings of the present study supported by Abdul-Wahab (2013) [1] they reported significant decreased Hb, Red blood cell count and HCT in methotrexate treated rats. Mhatre and Marar (2016) [7], Ilamkar *et al.* (2020) [4], Aparna *et al.* (2021) [2] and Shwaikh *et al.* (2021) [10] also revealed significant decreased TEC, Hb and HCT in methotrexate treated rats. The present study indicated hesperidin has ameliorative effect on methotrexate toxicity with regards to Hb, TEC and HCT value. Similar findings were also observed by Afolabi *et al.* (2019) [13] and Aboraya *et al.* (2022) [12].

These findings were also supported by Abdul-Wahab (2013) [1] and Mhatre. The ameliorative effect of hesperidin in PLT count in other toxicity where also reported by Aborya *et al.* (2022) [14]. The level of RBCs and their related indices was noticeably improved after hesperidin administration. This suggests that the flavonoid can increase erythropoietin's production or release, which in effect promotes bone marrow stem cells to erythrocyte (Afolabi *et al*., 2019) [13]. The finding of present study indicate that hesperidin has a protective effect, against methotrexate-induced toxicity in haematological parameters in male and female rats.

Conclusion

Co-administration of methotrexate and hesperidin in female and male rats showed the protective effect of hesperidin against methotrexate-induced toxicity in clinical signs and haematological parameters.

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