Mice (*Mus musculus*) genome responses to methotrexate (MTX) and some plant extracts

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Abstract: Mice genome responses to MTX (anticancer drug) and some plant (Curcuma, Ginger, Green Tee and Pomegranate) extracts were studied. Mice were intraperitoneally injected with 0.75 mg/kg chronic dose of MTX for two weeks. Mortality percentages, bone marrow cell divisions, morphological and biochemical characterization of treated mice groups were estimated. The mortality percentage was 40 % in the positive control while no mortality was observed in both (MTX, Curcumin) and (MTX, Pomegranate) treatments. Some treated mice had ulceration and hair loss on the ears skin. No morphological changes were observed on the negative control group. At cytogenetic level, no bone marrow cell divisions were detected in treated mice. Two Isozyme systems (Esterase and Super oxide dismutase) and Protein electrophoresis were assayed to detect biochemical genetic markers for all mice groups. These analyses reflect mice genome responses under experimental conditions.
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1. Introduction

Methotrexate (MTX, Amethopterin) chemotherapy drug is a cell cycle-specific analog, active in S-phase of the cell cycle, inters cells through specific transport system mediated by the reduced folate carrier and the folate receptor protein. It has a cytotoxic effect with an anti-folate metabolite that inhibit folate-dependent enzymes such as *thymidylate synthase*, which further blocks the de novo purine synthesis by directly inhibiting the activity of *5-aminooimidazole-4-carboxamide ribonucleotide transformylase*, causing an increase in both intracellular and extracellular adenosine (an potent anti-inflammatory mediator) (Cronstein, 2005; Cutolo *et al.*, 2001), and an increase in cAMP (Cutolo *et al.*, 2001), thus blocking the process of DNA synthesis and arrests the cell proliferation. MTX is widely used in treatments of different type of cancers, rheumatoid arthritis (RA) and other chronic inflammatory conditions (Minaur *et al.*, 2002).

MTX like other anticancer drugs has a lot of painful side effects like hair losing, ulcerations, emetic, decreases in attention/concentration, speed of information processing and memory and failure of liver and/or kidney functions (Brezden *et al.*, 2000, Schagen *et al*., 2002, Kreukels *et al.*, 2005, Shilling *et al.*, 2005 and Winocur *et al.* 2006). Up to date, there are no conventional treatments were applied to avoid the MTX side effects on patient. So, unconventional treatments should be applied to avoid side effects of MTX and other drugs (Eisenberg *et al.*, 1993, Murray *et al.*, 1992, and Visser *et al.,* 1992).

Curcuma (*Curcuma longa*), Ginger (*Zingiber officinale*), green tea (*Camellia sinensis*) and pomegranate (*Punica granatum*) plants are rich in different anti-oxidants, anti-inflammatory, and anti-cancer components (Venkatesan *et al.*, 2000, Antunes *et al.*, 2001, Ammon and Wahl 1991, Lin 2007, Srivastava and Mustafa 1992, Sharma *et al.* 1994, Afzal *et al.*, 2001, Rogers *et al.* 1998, Yang *et al.* 1998, and Isemura *et al.* 2000, Mayer *et al.*, 1977; Tanaka *et al.*, 1986a, Du *et al.*, 1975, Lansky *et al.*, 1998, Schubert *et al.*, 1999, Mayer *et al.*, 1977; Tanaka *et al.*, 1986a and Du *et al.*, 1975, Lansky *et al.*, 1998, Schubert *et al.*, 1999). So, the extracts of these plants were chosen as unconventional treatments to test its ability to face the side effects of MTX treatments.

The main aims of this work:

1- Investigation of the mice genome responses to methotrexate (MTX) and some plant extracts (Curcumin, Ginger, Pomegranate fruit and green Tea) as unconventional treatments.
2- Detection the effects of these treatments on the mice morphological characterization.

2. Material and Methods

Mice (*Mus musculus*) animals:

Healthy and genetically pure mice males (20 ± 5g) were housed for one week before experiments for acclimatization to the laboratory conditions.

Treatment with Methotrexate (MTX):

MTX treatments and LD_{50} determination were carried out as described by Pelker *et al.*, (1985) and Wheeler *et al.*, (1995) with some modifications. MTX were intra peritoneal injected (0.75 mg/kg) as chronic doses. The Treatments were divided into six groups (ten male mice in each). Treatment design was presented in Table (1):

Table (1): Mice groups and description of treatments.

Group	Description
Negative control (C-)	Drank water without methotrexate (MTX) injection
Positive control (C+)	Injected with MTX drug once day after day for 2 weeks with a 0.75 mg/kg (Pelker *et al.*, 1985; Wheeler *et al.*, 1995), a dosage similar to clinical therapeutic usage (Friedlaender, *et al.*, 1984).
T1	Intraperitoneally injected with MTX and drank socked Curcumin instead of water.
T2	Intraperitoneally injected with MTX and drank socked Ginger powder instead of water.
T3	Intraperitoneally injected with MTX and drank Pomegranate juice instead of water.
T4	Intraperitoneally injected with MTX and drank socked green Tea instead of water.

Plants material:

Curcumin or Cr (*Curcuma longa*), Ginger or Gn (*Zingiber officinale*), green tea or Te (*Camellia sinensis*) and pomegranate or Pg (*Punica granatum*) plants obtained from Faculty of Agric., Ain Shams Univ., Shobra El-Kheima, Egypt.

Ten grams of each Curcumin, ginger powder and green Tea were soaked separately overnight in 1 litter of hot water (60^{o}C). On the other hand pomegranate fruit (200g) was blinded in 1 litter of fresh water and used directly for mice drinking instead of water for one week before injection with MTX, except negative and positive controls.

Bone marrow preparation and cytogenetical analysis:

Two hours before sacrificing, all mice were intraperitoneally injected with 0.6 mg\kg colchicine (Sharma and Sharma, 1994). The bone marrow cells were collected from mice femurs using a 0.075 M KCl. Centrifuged for 5 min at 1500 rpm, re-suspended in 0.075 M KCl and incubated at 37^{o}C for 15 min. After fixation, Bone marrow cells were dropped onto slides (previously stored at 4^{o}C in cold 70% ethanol) and stained 10% Giemsa (Merk) (Boxio, *et al.*, 2004).

Biochemical genetic analysis:

Protein extraction:

The mice lever (0.5g) samples were obtained from each applied animal and extracted in appropriate volume of 0.85% NaCl solution (1ml). The extraction was homogenized then centrifuged at 12000 rpm/10min. The supernatants were applied for electrophoresis.

Protein separation:

25µl from each protein supernatant was diluted with an equal volume of 2X SDS Leans buffer according to Laemmli (1970) and modified by Abdel-Reheem *et al*., (2007). Samples were applied to 15% polyacrylamid gel. Gel preparation, electrophoresis conditions, staining and destaining gels were done as described by Abdel-Reheem *et al*., (2007) with some modifications.

Isozyme systems:

Esterase (Est.) with different substrates (α-naphthylacetate, α-naphthylvalerate) and Super oxide dismutase (SOD) Isozyme systems were applied to discriminate biochemical variations among treated and control samples. Electrophoretic conditions, gel preparation, staining and distaining were carried out according to Tanksley and Rick (1980), Tanksley and Orton (1983) and Saad (2002).

3. Results

Effects of different Treatments on mice morphological characterization:

The effects of different Treatments with Methotrexate (MTX) alone or doubled with soaked Curcumin (Cr) or Ginger (Gn), green Tea (Te) or Pomegranate juice (Pg) were presented in Table (2).

Table (2): Remarked characters correlated with Methotrexate and plant materials treatments.

Case	C-	C+	Cr	Gn	Pg	Te
Injections (MTX)	0	5	5	5	5	5
Ear Ulceration	-/-	+/+	-/-	+/+	-/+	+/+
Mice Activity	high	low	high	moderate	high	moderate

(+) means ulceration for one ear. (-) means normal ear

The negative control (C-) group exhibit normal (morphology and performance). Ear ulceration was a good remarkable observation of the treated mice. Both ears of treated mice were ulcerated in the positive control (treated only with MTX) as presented in figure (1a). Four mice had ulceration in one/both ears in the group of (MTX, Gn) double treatment (Figure 1b). Five mice of (MTX, Te) group had ulceration in both ears (Figure1c). In the case of MTX with Cr both ears of all mice were normal without any ulceration as shown in figure (1d) comparing with the negative control (figure 1e). While in MTX, Pg treatment, three mice had red spots (beginning of ulceration formation). Only one ear with red spots was noted as shown in figure (1f).

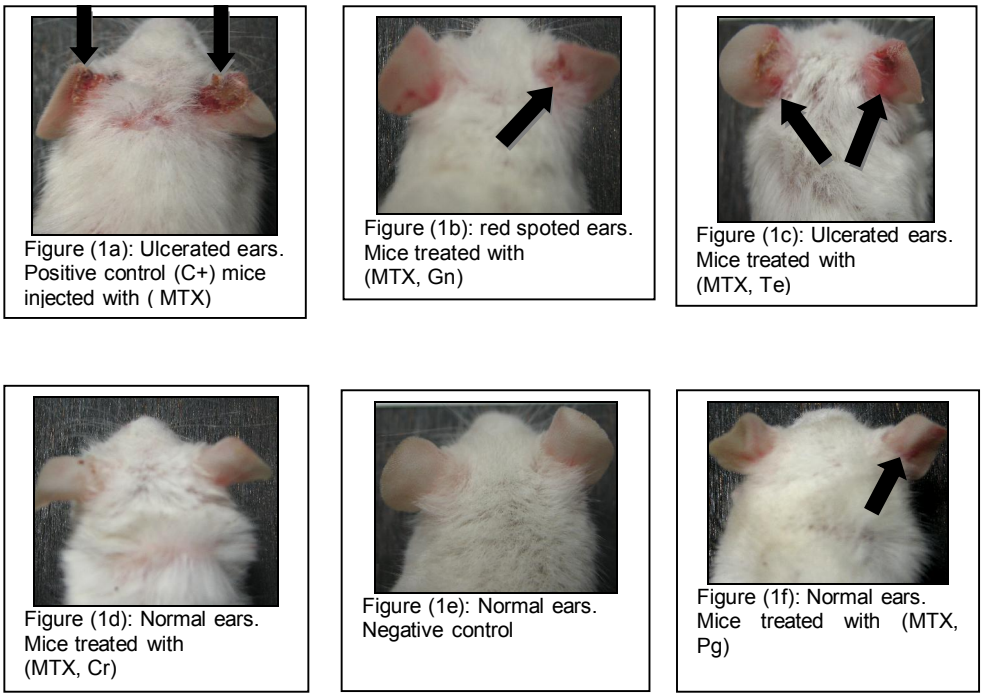

Figure (1a): Ulcerated ears. Positive control (C+) mice injected with (MTX)

Figure (1b): red spoted ears. Mice treated with (MTX, Gn)

Figure (1c): Ulcerated ears. Mice treated with (MTX, Te)

Figure (1d): Normal ears. Mice treated with (MTX, Cr)

Figure (1e): Normal ears. Negative control

Figure (1f): Normal ears. Mice treated with (MTX, Pg)

Figure (1): Morphological characterization of applied mice animals under experiment conditions. (C-) =Negative control, (C+) = Positive Control, (MTX) = methotrexate, (Cr) = Curcumin, (Gn) = Ginger, (Te) =green Tea and (Pg) = Pomegranate

Mortality levels under experiment conditions:

The percentage of mortality was 40% in the positive control (C+) group, (after five MTX injections). The mortality percentage was about (20%) in both (MTX, Gn) and (MTX, Te) treatments. On the other hand, no mortality was noted in both in the (MTX, Cr) and (MTX, Pg) treatments (Figure 2).

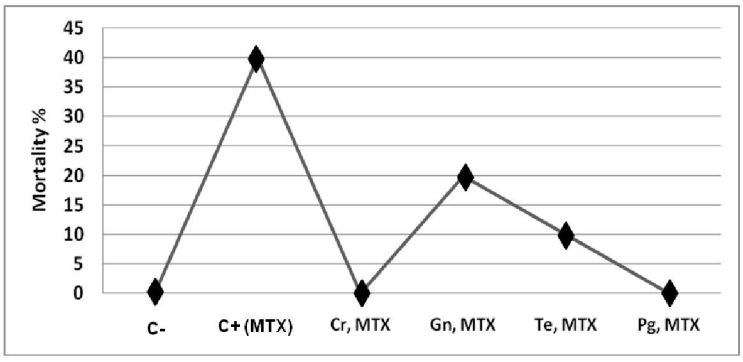

Figure (2): The experiment mortality percentages. (C-) = Negative control, (C+) = Positive Control, (MTX) = methotrexate, (Cr) = Curcumin, (Gn) = Ginger, (Te) = green Tea and (Pg) Pomegranate.

Effects of different Treatments on mice bone marrow cell divisions:

No spread chromosomes were detected in all MTX treatments, only compact nucleuses (undivided) were observed. Negative control showed normal spread chromosomes.

Biochemical genetic analysis:

SDS-PAGE and two isozyme systems (SOD and Est. with two different substrates) were applied to study the effects of different treatments on some liver proteins.

The effects of different treatments on protein electrophoresis:

Protein pattern (figure 3a) showed an extra band in negative control at about 190 kD which disappeared in positive control and other double treatments. It means that, all double treatments (which contain plant extraction) could not prevent MTX effects. In addition, protein banding pattern of the positive control had an extra 2 bands at 40 KD and 35 KD (duo to MTX effects). These 2 bands were absent in negative control and double treatments. Whereas a common band at 34 KD was detected in negative control and double treatments (absent in positive control). One band (200 KD) was detected in double treatment and positive control which is disappeared in the negative control. This band is considered as marker for the effect of MTX and the interaction between MTX and plant extracts on liver gene expressions.

The effects of different treatments on isozyme electrophoresis:

The electrophoresis pattern of SOD isozyme showed that, one band at RF=0.38 was detected in positive control and all other treatments while absent in negative control. This illustrate that this band is created under MTX effect (figure 3b).

The electrophoresis pattern of Est. with α-naphthyl acetate isozyme (Figure 3c) showed that, One band at RF= 0.12 was appeared in T1 (Cr, MTX) and not appeared in the others (C-, C+, T2, T3 and T4).

Esterase (Est.) with α-naphthyl vallerate:

Regarding Esterase (Est.) with α-naphthyl vallerate pattern (figure 3d), only one band with RF about 0.04 was appeared in the treatments (C-, C+, T2, T3 and T4) and absent in (T1).

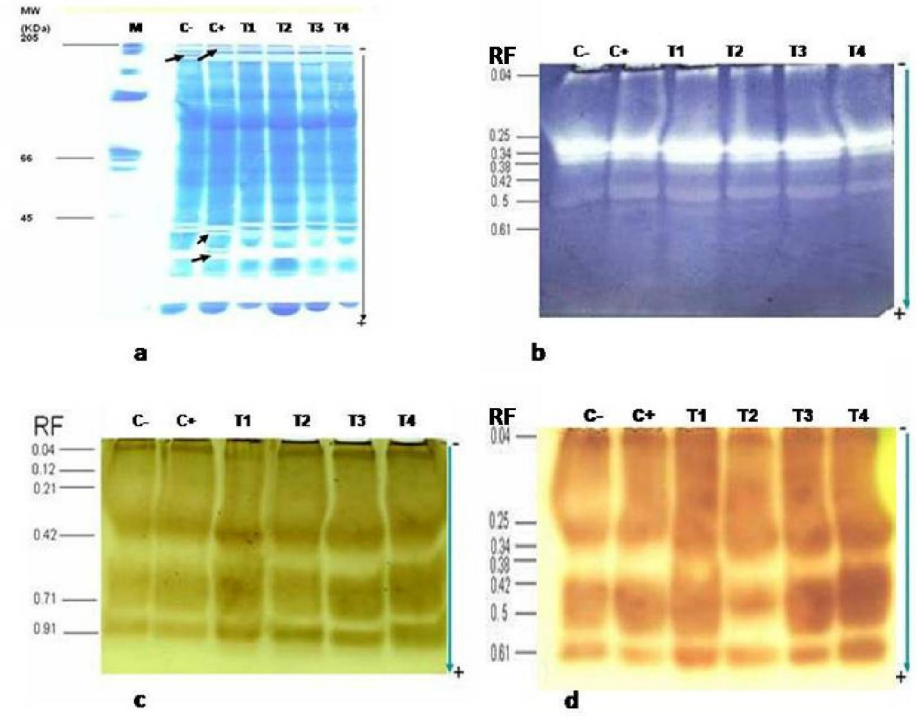

Figure (3): SDS PAGE Protein (a), SOD (b), Est. *α*-naphthyl acetate (c) and Est. *α*-naphthyl vallerate banding patterns. MW = Molecular weight (KD), RF = relative front, C- = Negative control, C+ = Positive Control, and T = Treatment.

4. Discussion

Mice were injected with MTX dosage similar to clinical therapeutic usage (Friedlaender, *et al.*, 1984). The mortality ratio was 40 % in the positive control. Whereas in the double treatment (MTX, Cr) no mortality was observed as well as (MTX, Pg) treatment, these results reflect that Curcumin and pomegranate decreases the toxicity effect of MTX and prevent appearance of ulceration on mice ears (as a secondary symptoms appeared in the positive control). These results agree with the explanation of Venkatesan *et al.*, 2000 and Antunes *et al.*, 2001 that Curcumin prevents or attenuates nephrotoxicity caused by adriamycine or cisplatin chemotherapy treatments respectively. The effects of pomegranate juice may be referred to its enrichment with phenolic compounds and its antioxidant activity (Schubert *et al.*, 1999). In the case of MTX, ginger and MTX, green tea, the viability was near to be same but the ulceration on mice ears was varied, it was only red spots appeared in MTX, ginger treated mice, while it was sever in the case of MTX, Tea treatment. The lower mortality appeared in both cases may be referred to the phenolic compounds of ginger and green Tea (Lee and, Surh, 1998; Wang *et al.*, 2003; Chung *et al.*, 2001) and (Komori *et al.* 1993) respectively.

Methotrexate acts as folate analog. It has an inhibition effect on dihydrofolate reductase (DHFR) resulting in depletion of critical reduced folates, thymidylate synthesis as well as purine synthesis, and incorporates a dUTP bases into DNA resulting in inhibition of DNA synthesis and function, finally arresting cell division in S-phase (Edward and Vincent 2008), for that, there were not any spread chromosomes observed in bone marrow of all treatments compared with untreated negative control, these results can be explained depending on the efficacy of MTX on bone marrow. These also mean that, Curcumin, ginger, green tea, and pomegranate treatments did not affect the main effect of methotrexate (arresting cell division in S-phase). Georgiou *et al.*, 2009 found that acute MTX chemotherapy transiently depletes the bone marrow of its steady-state haematopoietic and stromal progenitors, reducing haematopoietic cellularity and osteogenesis and increasing marrow fat content, which have the capacity to recover by day 14 subsequent to damage.

Severe bone marrow damage is a known adverse effect of cancer chemotherapy. Xian *et al.,* 2007 employed a rat model of an acute Methotrexate (MTX) chemotherapy and demonstrated that daily methotrexate injections at 0.75 mg/kg for five consecutive days caused decreased trabecular bone in tibia as well as an increased adipocyte (AD) density in the bone marrow on day 9 post-treatment.

Several techniques have been used, with different degrees of success, to identify and trace minor differences among animal populations and detect the genetic responses of any treatments. These include morphometries, karyotype analysis, serum protein analysis, immunology and agglutination assay, isozyme polymorphism and protein banding patterns. Among these techniques, however, biochemical studies of genome responses for treatments of populations through electrophoretic analysis are advantageous (Saad *et al*. 2002).

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