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Estimation of the Mice Responses to Flaxseed Oil and Sesame Oil under Methotrexate Effects via Semi-Quantitative RT-PCR and Protein Electrophoresis

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Abstract: The gene expression changes due to the Methotrexate (MTX) effects in many biological systems including mammals are still unsaturated. In this investigation, Semi-Quantitative RT-PCR and protein electrophoresis techniques were applied to evaluate the mice responses to flaxseed oil and sesame oil under Methotrexate effects. The mice individuals (n=30) were divided into six groups including control and five treatments (From T1 to T5). Comparatively with the control sample, the results showed that both the Superoxide dismutase (Sod) and cytochrome oxidase I (COI) relative expression levels were upregulated in all the treated samples except in the T1 (MTX) treatment (downregulated). The T2 treatment (MTX + Sesame oil) exhibited the highest relative COI gene expression values, whereas the lowest values were observed in the T1 treatment (MTX). Similarly, for the relative Sod gene expression, the highest values were found in the T3 treatment (MTX + Flaxseed oil), while the lowest values were observed in the T1 treatment (MTX). The numbers of liver and serum protein subunits separated by SDS-PAGE did not varied compared with the control under the experimental conditions. The results could be useful for exploring the physiological impacts of gene expression investigations under definite experimental conditions. Mitochondrial biogenesis could be affected by oxidative stress and decreasing the antioxidant defense. Additional investigations including molecular, histological, and physiological investigations should be carried out to examine and understand the biological responses triggered by flaxseed oil and sesame oil in the presence of Methotrexate.

Key words: Flaxseed · Sesame · Methotrexate · Protein · Gene Expression

INTRODUCTION

Patients undergoing Methotrexate treatments experienced numerous side effects, such as allergic reactions, skin pain, eye burning, and intestinal mucous. The mammalian hepatotoxicity caused by the Methotrexate treatments was confirmed in some biological studies at histological and physiological levels. On the other hand, at the genetic level, the gene expression changes due to the Methotrexate effects in many biological systems including mammals are still unsaturated [1-6].

Each of the sesame oil and flaxseed oil is usually used as edible oil in many countries. The nutritional benefits of these oils were discussed in some studies. The antioxidant and anti-inflammatory properties of both oils were confirmed.

The usefulness of sesame oil in reducing the risk of cardiovascular disease was also confirmed. The levels of cholesterol ester and free cholesterol might be changed due to sesame oil's effects. Also, flaxseed oil was used as a renal protective element in rats due to its ability to prevent oxidative stress [7-11].

The Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as a discontinuous system and/or native PAGE was commonly used for exploring the variations among the biological samples at the protein subunit level. The sensitivity and accuracy of these techniques in detecting biological tags among treated and control samples were under debate [1].

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The accuracy and/or specificity of the Semi-Quantitative reverse transcription polymerase chain reaction (sqRT-PCR) in detecting the expression levels of many genes were discussed in many investigations [12-16].

The aim of the present investigation is to evaluate the responses of mice to flaxseed oil and sesame oil in the presence of Methotrexate using the Semi-Quantitative RT-PCR and protein electrophoresis techniques.

MATERIALS AND METHODS

Mice male individuals (25±5g) were housed for 7 days under uniform conditions (25°C, and a 12/12 h light/dark cycle). The experiment was carried out under the ethical guidelines of Animal Care (KAU, KSA).

Both sesame oil and Flaxseed oil were purchased from Sigma-Aldrich Corp., St. Louis, MO, USA. The Methotrexate was purchased from Hospira UK Limited (Hurley, SL6 6RJ, UK).

A total of 30 male mice (*Mus musculus*) were approved for participation. The individuals were randomly assigned to six groups, with each group consisting of five mice. Separate cages were labeled as G1 to G6 to house the animals in their respective groups. Based on the experimental design, each group was subjected to a distinct treatment regimen for duration of six weeks (Table 1).

On day 40, blood samples were withdrawn from each mouse. Also, the mice individuals were killed and the liver, kidney and spleen organs were excised, weighed, frozen [4] and kept at -80°C.

Protein Extraction: Protein samples were extracted from the mice's liver tissue using saline solution (0.9%). Additionally, the serum protein samples from the mice were purified and separated. The protein samples extracted using the saline solution were prepared for separation through polyacrylamide gel (14%) electrophoresis, following the methodology described by Mansour *et al*. [4].

Table 1: Experimental groups, description and codes.

Group	Description	Code
1	Control group (IP injected with NaCl solution (0.9%).	C
2	MTX (20 mg/ kg body weight).	T1
3	MTX +sesame oil (600 mg/kg body weight)	T2
4	MTX + Flaxseed oil (2g/kg body weight)	T3
5	Sesame oil (600 mg/kg body weight)	T4
6	Flaxseed oil (2 g/kg body weight)	T5

Protein Separation: The gel preparation, electrophoretic conditions and staining were carried out according to Mansour *et al*. [4], Pasteur *et al*. [17] and Elsebaie and saad [18].

Semi-Quantitative RT-PCR: The RNA samples were extracted from the liver tissue (100 mg) using TRIzol as described by Rio *et al*. [19]. The samples were quantitatively measured as described by Romero *et al.* [20].

The semi-Quantitative RT-PCR method was applied to evaluate COI and Sod gene expressions under experimental conditions. The cDNA synthesis was carried out according to Meadus [21].

As a control, B -actin gene fragments were amplified. The B Actine primer pairs were as follows: B-Actin_F:GGCACCACACCTTCTACAATG and B-ctin_R: GGGGTGTTGAAGGTCTCAAAC. The PCR reaction and condition were carried out according to Wei *et al.* [22].

Regarding the superoxide dismutase (SOD) gene fragments, the primer pairs were as follows: Sod_F: TTTTTGCGCGGTCCTTTCCTG and Sod_R: GGTTCACCGCTTGCCTTCTGCT. The PCR reaction and condition were carried out according to Singh *et al*. [23].

Concerning the *COI* gene fragment, the primer pairs were as follows: F1-TCAACCAACCACAAAGACATT and R1:ACTTCTGGGTGGCCAAAGAAT [24].

Data Analysis: The COI, B-Actin and Sod gen fragments were detected and analyzed via the Gel Analyzer 19.1 software (WWW.gelanalyzer.com).

The protein banding patterns were analyzed as described by Mansour *et al.* [4].

Statistical analysis was conducted using several sample test analysis of variance (ANOVA) for all value comparisons. A significance level of P = 0.05 was considered to indicate statistical significance. The results were statistically analyzed using PAleontological Statistics, (PAST) Version 4.03 (https://past.en.lo4d.com/windows).

RESULTS

Evaluation of Mice's Individual Weight Values: Each mouse individual was weighted and the mean weight values are presented in Fig. 1. These values were 29.77 ± 0.25, 28.6 ± 0.26, 31.07 ± 0.25, 31.73 ± 0.25, 31.63 ± 0.25 and 34.73 ± 0.25 in C, T1, T2, T3, T4 and T5 groups respectively.

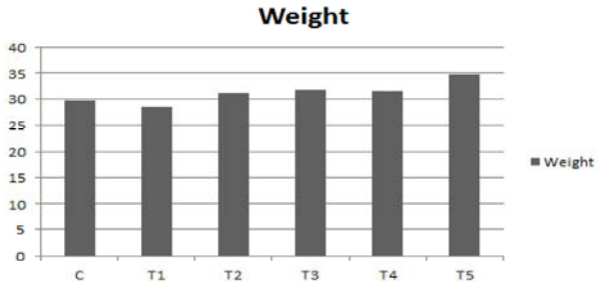

Fig.. 1: The average weights of the mice were calculated after 40 days under the experimental conditions. C= Control group, T1= MTX group, T2= MTX +sesame oil group, T3= MTX + Flaxseed oil group, T4= sesame oil group and T5= Flaxseed oil group.

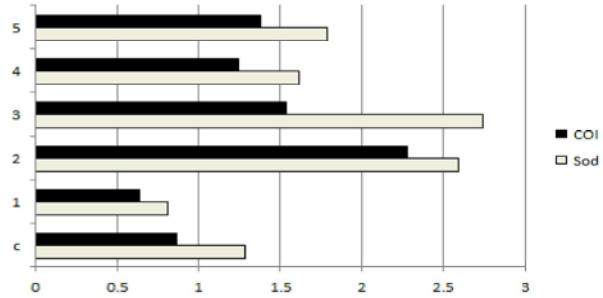

Fig. 2: The relative liver Sod and COI expressions under the experimental conditions. C= Control samples, 1= (MTX), 2= (MTX+ sesame oil) and 3= (MTX+ flaxseed oil), 4 = (Sesame oil) and 5 = (Flaxseed oil). Values are mean ± SD. *p < 0.05, significantly different from control.

Evaluation of the liver COI and Sod gene expressions: Semi-Quantitative RT-PCR technique was applied to verify among the control and experimental mice individuals.

Evaluation of both COI and Sod expressions in the liver tissue showed that the expression levels were affected by the treatment kinds (Fig. 2).

The Relative COI Gene Expression: The highest and lowest relative COI gene expression values were detected in the T2 (MTX+S) and T1 treatment (MTX) respectively.

Comparatively with the control, the results showed that the COI relative expression levels under the treatment conditions were upregulated in all the treated samples except in the T1 (MTX) treatment (downregulated).

The relative expression in the T2 (MTX+S) was higher than the relative expression in the T3 (MTX+ Flaxseed oil). The relative expression of the T5 (Flaxseed oil) was lower than T3 (MTX+ Flaxseed oil).

The relative expression of the T4 (sesame oil) was lower than T2 (MTX+ sesame oil)

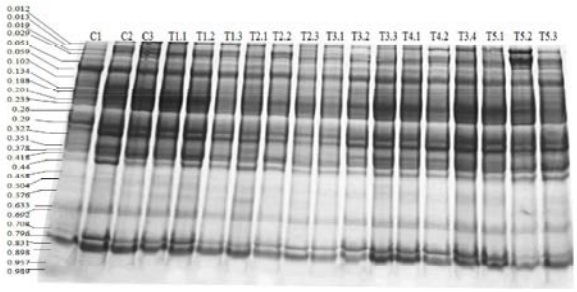

Fig. 3: Separation of the liver protein subunits using native PAGE.

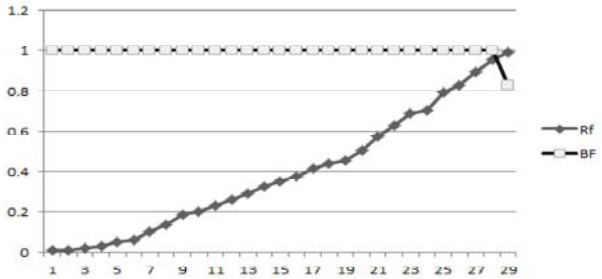

Fig. 4: Distribution of Band frequencies (BF) and Relative fronts (Rf) of the evaluated liver protein subunits.

The Relative Sod Gene Expression: The highest and lowest relative Sod gene expression values were detected in the T3 and the T1 treatment (MTX) treatments respectively.

The results showed that the Sod relative expression levels under the treatment conditions were upregulated in all the treated samples except in the T1 (MTX) treatment (downregulated) compared with the control sample. The relative Sod expression in the T2 (MTX+S) was lower than the relative expression in the T3 (MTX+ Flaxseed oil).

The relative expression of the T5 (Flaxseed oil) was lower than T3 (MTX+ Flaxseed oil).

The relative expression of the T4 (sesame oil) was lower than T2 (MTX+ sesame oil).

Evaluation of Protein Polymorphisms: The electrophoretic patterns of the liver protein separations using native PAGE are presented in Fig. 3. A total of 29 protein bands were detected in the liver protein banding patterns. The distribution of band frequencies (BF) and Relative fronts (Rf) of the evaluated liver protein

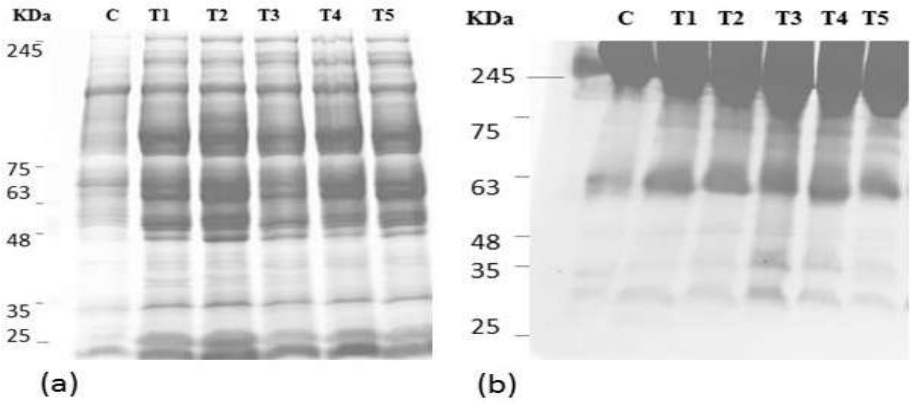

Fig. 5: Separation of the liver (a) and serum (b) protein subunits using SDS-PAGE.

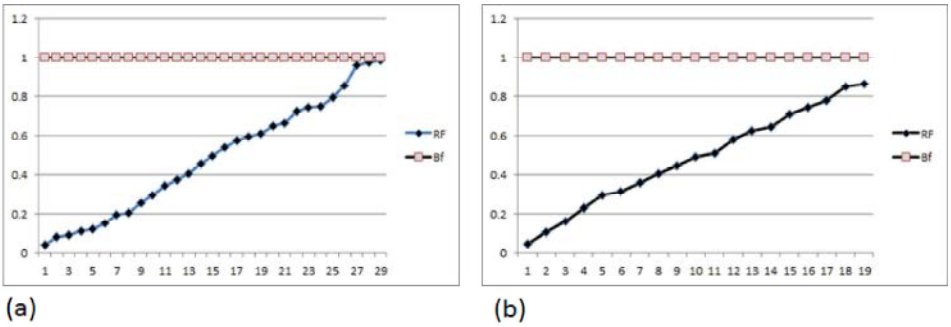

Fig. 6: Distribution of Band frequencies (BF) and Relative fronts (Rf) of the evaluated liver (a)and serum (b) protein subunits.

subunits are presented in Fig. 4. Only one polymorphic protein band was detected (absent from all the T1 samples) at relative front 0.989.

The electrophoretic patterns of the liver and serum protein separations using SDS-PAGE are presented in Fig. 5. A total of 29 and 19 protein bands were detected in the liver and serum protein banding patterns respectively. The Distribution of Band frequencies (BF) and Relative fronts (Rf) of the evaluated liver and serum protein subunits are presented in Fig. 6. No polymorphic protein bands were observed in both the evaluated liver and serum protein banding patterns.

DISCUSSION

In the current study, Semi-Quantitative RT-PCR, SDS-PAGE native PAGE techniques were applied to estimate the mice responses to flaxseed oil and sesame oil under Methotrexate effects.

As recommended by Romero *et al.* [20], all the applied samples were quantitatively estimated for identifying the effectiveness of the RNA extraction technique from the liver cells and the percent value of the mRNA to cDNA.

Both the COI and Sod expressions in the liver tissue showed that the expression levels were affected by the treatment types.

Comparatively with the control, the results showed that both the Sod and COI relative expression levels under the treatment conditions were upregulated in all the treated samples except in the T1 (MTX) treatment (downregulated).

The highest and lowest relative COI gene expression values were detected in the T2 (MTX+S) and T1 treatment (MTX) respectively.

The highest and lowest relative Sod gene expression values were detected in the T3 and the T1 treatment (MTX) treatments respectively.

As known, the MTX (300 µM) treatment caused mitochondrial injury in the Rat liver [25]. Also, Muthuraman and Hwan [26] observed that a lot of isozymes including ALT, LDH and ALP activities and mRNA levels were influenced by the dose-dependent mode under definite experimental conditions. The

alterations of many gene expressions due to various laboratories and/or environmental conditions were discussed in some investigations.

The downregulation in the T1 group for both SOD and COI could be due to cytotoxicity caused by ROS (reactive oxygen species) formation and GSH oxidation leads to oxidative stress. The negative effects of the high concentration of ROS on the mammalian cells were confirmed in some investigations. The mRNA expression of both COI and Sod were reduced suggesting increased mitochondrial oxidative damage after MTX treatment. The decrease in SOD expression suggested decreased antioxidant defense in the mice's liver mitochondria [4, 6, 27].

The relative COI expression in the T2 (MTX+S) was higher than the relative expression in the T3 (MTX+ Flaxseed oil). The relative expression of the T5 (Flaxseed oil) was lower than T3 (MTX+ Flaxseed oil).

The relative SOD expression in the T2 (MTX+S) was lower than the relative expression in the T3 (MTX+ Flaxseed oil).

Both sesame oil and flaxseed oil are usually used as edible oils in many countries. The nutritional benefits of these oils were discussed in some studies. The antioxidant and anti-inflammatory properties of both oils were confirmed. The usefulness of sesame oil in reducing the risk of cardiovascular disease was confirmed. The levels of cholesterol ester and free cholesterol might be changed due to sesame oil effects. Also, flaxseed oil was supposed as a renal protective element in rats due to its ability to prevent oxidative stress [7-11].

The confident influencing of the sesame and/or flaxseed oils was revealed by the upregulation of the liver SOD and COI gene expressions (compared with the control) in both the T2 (MTX+ sesame) and T3 (MTX+ flaxseed).

In the present study, the sensitivity of the Semi-Quantitative RT-PCR technique [12-16] in detecting the expressions of SOD and COI genes under the experimental conditions was established.

Regarding the native PAGE results, only one polymorphic protein band was detected (absent from all the T1 samples) at relative front 0.989. Concerning the SDS-PAGE, the numbers of the separated mice liver and serum protein subunits among the mice individuals are not varied under the experimental conditions. So, application of this technique in such experiments was not recommended [28]. In order to gain a comprehensive understanding of the biological responses exhibited by different animal species to various medical treatments, such as MTX, it is crucial to conduct further studies incorporating molecular, histological, and physiological investigations [4, 6, 29-32].

CONCLUSION

Comparatively with the control, the results showed that both the Sod and COI relative expression levels under the experimental conditions were upregulated in all the treated samples except in the T1 (MTX) treatment (downregulated).

In the native PAGE results, only one polymorphic protein band was detected. However, when using SDS-PAGE, the numbers of liver and serum protein subunits did not show significant variations compared to the control under the experimental conditions.

On the other hand, only one polymorphic protein band was detected (absent from all the T1 samples). The results could be useful for exploring the physiological impacts of gene expression investigations under definite experimental conditions. Mitochondrial biogenesis could be affected by oxidative stress and decreasing the antioxidant defense. Additional investigations including molecular, histological, and physiological investigations should be carried out to examine and understand the biological responses triggered by flaxseed oil and sesame oil in the presence of Methotrexate.

Conflict of Interest: The Authors declared no conflict of interest.

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