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Protection against Methotrexate Induced Hepato-Renal Toxicity in Rats by Zinc and its Combination with Vitamin C and Vitamin E

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Abstract

Methotrexate, a well-known cytotoxic chemotherapy, anti-folate, immunosuppressant and effectively treats all major disorders like cancer, psoriasis, refractory rheumatoid arthritis, etc. It is widely and mostly used drug having multitherapy properties and well tolerated, however, its cytotoxic nature leads to many serious side effects, including hepato-renal toxicity, so it is necessary to reduce its side effect and get its benefit. Among all hypothesis, oxidative stress and toxic metabolite are the noticeable cause of toxicity. The present study investigated the protective effects of Zinc alone and its combination with vitamin C and vitamin E against the methotrexate induced hepato-renal toxicity in male albino rats. Zinc is a well-known potent anti-oxidant that scavenges the free radical in tissues. Vitamin C and vitamin E are water soluble and insoluble antioxidant, respectively. In conclusion, the rats treated with zinc alone and its combination with vitamin C and vitamin E had a significant decrease in the level of creatinine, urea, total bilirubin, SGOT, SGPT in blood serum when compared with methotrexate treated rats. Similarly the activity of catalase and reduced glutathione were enhanced and lipid peroxidase was decreased. Histopathology report confirmed the hepato-renal protectivity of Zinc and its combination with vitamin C and vitamin E by restoring the normal architecture of liver and kidney.

Keywords: Methotrexate; Vitamin C; Vitamin E; Zinc; Antioxidants; Histopathology

Introduction

Methotrexate (MTX) also known as amethopterin, is an antimetabolite (capable of blocking the metabolism of cells) and antifolate drug used in treating a variety of cancers like skin, breast, neck, lung cancer etc., [1]. It also treats autoimmune diseases like rheumatoid arthritis and psoriasis. It acts by inhibiting the metabolism of folic acid by inhibiting an enzyme dihydrofolate reductase, which is essential for cancer cells to divide and multiply. It also act by inhibiting the activity of the immune system by blocking several enzymes involved in the immune system and affects the growth of cancerous cells [2,3]. It is reported that liver and kidney damage occur following high dose or chronic administration of MTX [4-6]. MTX, a part of its multi-use, it leads to severe cells and organ damage, including Fallopian tubes [7], causes liver and kidney toxicity [7-10], hence are essential to prevent the MTX side effect as it is mostly used in many complications.

Zinc, an essential trace element and is relatively non-toxic and is essentially used in many important functions of metabolism [11]. Zinc has shown to have an antioxidant effect and stabilizes cell membranes [12,13].

Studies suggested that zinc protects liver from paracetamol toxicity [14], chlorpyrifos [15], chloroform [16] etc. Moreover, zinc alone or its combination with vitamin E also protects the kidney from lithium toxicity [17], indomethacin [18]. It also protects against peroxidative damage and promotes membrane integrity [19] and intestinal damage [20-22].

Materials and Methods

Animals

30 male albino rats weighing 180-230 g were used in this study. Rats were purchased from "*In vivo* Biosciences, #23 katha no.3169, assessment no. 154, kodigehalli village, off Magadi road, yeswantapur Hobli, Bangaluru north tank, Bangaluru [560091].

The animals were stabilized for 1 week in Central animal house of the department of pharmacology, RIPER. The animals were maintained in controlled temperature and humidity and photocycle of 12:12 h of light and dark. The animals were housed in sanitized polypropylene cages containing sterile paddy husk as bedding with free access to standard pellets as diet and water ad libitum. The study was conducted under the approval of the Institutional Animal Ethical Committee (IEAC) of the Raghavendra Institute of Pharmaceutical Education and Research (RIPER), Ananthapuramu. Approval No. 878/ac/05/CPCSEA/001/2016, according to prescribed guidelines of

Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

Testing kits

All the testing kits (bilirubin testing kit, Liquixx creatinine kit, SGOT, SGPT and urea testing kit) were purchased from "Sri Sai Agencies" #10-517, Adimurthy Nagar, and Anantapuramu-515001. All the reagents used in the experiment were of analytical grade. All these kits were manufactured by Transasia Bio-Medicals Ltd., in technical collaboration with ERBA diagnostics Mannheim GmbH, Germany.

Medicines

Zinc sulphate tablets 200 mg of brand "ZINFATE" manufactured by Yash Pharma Laboratories Pvt. Ltd. Haridwar, Uttarakhand, India.

Method: One tablet of 200 mg was crushed and dissolved in 50 mL of distilled water and the dose was given orally on the basis of rat weight.

Vitamin C tablets 500 mg of brand "Celin chewable (500 mg)" manufactured by GlaxoSmithkline Pharmaceuticals Ltd.

Method: One tablet of 500 mg was dissolved in 5mL of distilled water and the dose was calculated by weight and given orally.

Vitamin E 400 mg soft gelatin capsules of brand "Evion 400" manufactured by Merck Biopharma India.

Method: One soft gelatin capsule was squeezed and the content was dispersed in 1% acacia gum. The rat was given orally on the basis of rat weight.

MTX 5 mg tablets of brand "Folitrax 5 mg tab" manufactured by IPCA laboratories Ltd.

Method: One tablet of 5 mg was dissolved in 20 mL and was given orally on the basis of their weight.

Treatment Schedule

Rats were divided into five groups of six each, Control group was treated with distilled water regularly from the first day, MTX-treated group were treated with MTX from the third day of protocol started and followed at intervals of two days, Test 1, 2, and 3 groups were first treated with zinc and vitamins regularly as per the table below and MTX was started from third day and continued at intervals of two days till the end of the protocol.

Groups were treated as follows:

Group	Treatments	Serum parameter testing days	Oxidative parameter in liver and kidney
Control group	Untreated	Creatinine, Urea, Total bilirubin, SGOT and SGPT were tested on Day 1, 13, 25 and 37	Catalase, Reduce glutathione, and LIPID PEROXIDASE were tested on the last day of treatment
Methotrexate-treated group	Methotrexate of 0.675 mg/kg PO [23] at the interval of two days. Note: the oral dosing is given at two days interval by considering the prescription of human and the sum of regular doses of rat in reference almost match the dose of humans. So there slight changes in the protocol from the reference.		
Test 1	Zinc (18 mg/kg PO) [24] with methotrexate (0.675 mg/kg PO)		
Test 2	Zinc (18 mg/kg PO) +vitamin C (250 mg/kg PO) [25] with methotrexate (0.675 mg/kg PO)		
Test 3	Zinc (18 mg/kg, PO) +vitamin C (250 mg/kg, PO)+vitamin E (250 mg/kg PO) [25] with methotrexate (0.675 mg/kg PO)		

Table 1: Treatment Schedule followed at intervals of two days, Test 1, 2, and 3 groups.

Serum Parameter

Blood collection and serum separation: Blood was collected at intervals of 1, 13, 25 and 37 day by means of retro-orbital sinus puncture under inhaled diethyl ether anesthesia. The blood obtained was subjected to centrifugation at 2500-3000 rpm for 25 min and supernatant serum was separated. Following parameter was tested from the supernatent serum by using all the kits manufactured by Transasia Bio-Medicals Ltd., which is in collaboration with ERBA diagnostic Mannheim GmbH, Germany.

Creatinine

Creatinine was estimated by Jaffe's method using Liquixx Creatinine, commercially available.

Method:

Pipette	Standard	Test
Working Reagent	1000 µL	1000 µL
Standard	100 µL	-
Test (serum)	-	100 µL

Mix well and read initial absorbance (A1) 20 sec after mixing and final absorbance (A2) 80 sec after mixing.

Calculation: Calculate the result as follows:

$\Delta A = A2 - A1$

Creatinine (mg/dL)=(ΔA of Test)/ (ΔA of Standard) x Concentration of standard (mg/dL)

SGOT: SGOT was estimated by IFCC Method using SGOT kit, commercially available.

Method:

Reagent reconstitution: The reagent bottle and Aqua-4 (supplied in the kit) to attend room temperature (15°C to 30°C). Add the amount of Aqua-4 indicated on the label to contents of each vial. Swirl to dissolve, do not shake vigorously.

Pipette	Volumes
Working reagent	1000 µL
Test	100 µL

Calculation: Determine the mean absorbance change/min (ΔA/min) for every reading. Find the mean value.

Activity of AST at 37°C (IU/L)=(ΔA/min) x Factor (1768)

SGPT: SGPT was estimated by IFCC Method using SGPT kit, commercially available.

Method:

Reagent reconstitution: The reagent bottle and Aqua-4 (supplied in the kit) to attend room temperature (15°C to 30°C). Add the amount of Aqua-4 indicated on the label to contents of each vial. Swirl to dissolve, do not shake vigorously.

Pipette	Volumes
Working reagent	1000 µL
Test	100 µL

Calculation: Determine the mean absorbance change/min (ΔA/min) for every reading. Find the mean value.

Activity of ALT at 37°C (IU/L)=(ΔA/min) x Factor (1768)

Total Bilirubin: It was estimated by Diazo Method, end point Using Liquixx Bilirubin, commercially available.

Method:

Pipette into test tubes marked	Blank	Test
Working reagent	500 µL	500 µL
Distilled water	25 µL	-
Test		25 µL

If the standard is not supplied, we can multiply with factor [factor 546/630 is 23].

Mix well; incubate for 5 min at 37°C. Read the absorbance at 546/630 nm against reagent blank.

Calculation with factors

Total bilirubin (mg/dL)=absorbance of test x factor [factor for 546/630 is 23].

Urea: It was estimated by GLDH-urease method using urea kit, commercially available.

Method:

Pipette into test tubes marked	Standard	Test
Working reagent	1000 µL	1000 µL
Standard	20 µL	-
Test		20 µL

Mix well, and aspirate standard followed by samples.

Calculations: Determine absorbance change (ΔA) for the standard and unknown samples by using formula:

ΔA=A1-A2

$$\text{Urea}\left(\frac{\text{mg}}{\text{dL}}\right)=\frac{\Delta\text{A of Test}}{\Delta\text{A of Standard}}\times\text{Concentration of standard}\left(\frac{\text{mg}}{\text{dL}}\right)$$

Oxidative parameters in liver and kidney tissue homogenate

Catalase (CAT): 2 mL of tissue homogenate was diluted with 1 mL of H_2O_2 and took the absorbance at 240 nm for 3 min with interval of 30 sec. Add H_2O_2 just before taking O.D [26].

Reduced glutathione (GSH): The supernatant homogenate was precipitated with 20% trichloroacetic acid (TCA) and centrifuged. 0.25 mL of supernatant was taken for GSH estimation using freshly prepared DTNB solution (2 mL) and volume was made up to 3 mL with phosphate buffer pH 8. The intensity of yellow colour formed was read at 412 nm against blank for each sample without reagent was run. The GSH content was calculated by using ε=13.6 x 103 M-1 cm^{-1} expressed as nmol/g of wet tissue [27].

Lipid peroxidation (LPO): LPO was evaluated as an index of oxidative damage and was assessed by measuring thiobarbituric acid reactive substances (TBARS) i.e., Malandialdehyde (MDA) in liver and kidney. MDA, an end product of the LPO process. Its level is well known as a marker of oxidative stress.

Procedure: 500 µL supernatant of tissue homogenate, 500 µL of reagent TBA and 1.5 mL of 15% trichloroacetic acid were combined with a 10 mL screw cap Pyred centrifuge tube, mixed well and heated for 45 min in boiling water. Cooled in an ice bath, 3 mL of n-butanol was added, mixed and centrifuged. The chromogen was extracted. The absorbance was determined Spectro-photometrically at 512 nm against a blank. The amount of LPO was determined by using the formula ε=1.56 x 105 M^{-1} cm^{-1} and expressed as nmol of MDA per mg of wet tissue [28,29].

Histopathological Analysis: The specimens were fixed in 10% formalin and embedded in paraffin. Sections were prepared, stained with hematoxylin and eosin and examined by a pathologist Dr. Varalaxmi in the Anantapur diagnostic centre, Ramnagar, Anantapur, India, under a light microscope fitted with camera and changes were observed under 10x and 40x magnification.

Statistical Analysis

Data were expressed as Mean±SED. The differences among various groups were tested for statistical significance using one-way analysis of variance (ANOVA) test followed by Bonferroni's compare all pairs of column by using Graphpad prism 5.0 software. A p value of less than 0.05 denotes the presence of a statistically significant difference.

Results

Effect of Zinc and its combination with vitamin C and vitamin E on ALT (SGPT) (U/L)

ALT level was significantly and gradually increased in MTX-treated group in comparison to Control group. Test 1, 2, and 3 were found to have less ALT in comparison to MTX-treated group. Test 3 is having slightly less ALT level than Test 2 and Test 2 is having less ALT than Test 1 as shown in Table 1.

Group.	Treatment	Day 1	Day 13	Day 25	Day 37
Control	Untreated	29.74±2.27	32.09±1.02	28.62±0.937	26.02±1.113
Methotrexate-treated group	Methotrexate (0.675 mg/kg PO)	25.54±2.349^{ns}	87.31±5.68$^{\#}$	174.3±5.546$^{\#}$	296.3±7.001$^{\#}$
Test 1	Zinc (18 mg/kg PO) +Methotrexate (0.675 mg/kg PO)	19.59±0.894^{ns}	67.34±1.766*	65.67±3.283***	52.67±2.404***
Test 2	Zinc (18 mg/kg PO) +Methotrexate (0.675 mg/kg PO)+vitamin C (250 mg/kg PO)	30.06±4.00^{ns}	61.59±4.175**	49.33±4.564***	39.01±3.356***
Test 3	Zinc (18 mg/kg PO) +Methotrexate (0.675mg/kg PO)+vitaminC(250 mg/kgPO) + vitamin E (250 mg/kg PO)	34.98±2.268^{ns}	61.93±1.889**	46.60±5.208***	29.6±0.6099***

Values are expressed as Mean ± SEM (n=6). One-way ANOVA by Bonferroni's test, $^{\#}$P<0.001 when compared to normal, *P<0.05 when compared to the negative control, **P<0.01 when compared to the negative control, ***P<0.001 when compared to the negative control ns represents not significant, SEM represents Standard Error of Mean

Table 1: Effect of Zinc and its combination with vitamin C and vitamin E on ALT (SGPT) (U/L).

Effect of Zinc and its combination with vitamin C and vitamin E on AST (SGOT) (U/L)

AST level was significantly and gradually increased in MTX-treated group in comparison to Control group. There was a significant decrease in AST level in Test 1, 2 and 3 in comparison to MTX-treated group. Test 3 was found to have less AST than Test 2 and Test 1 as shown in Table 2.

Group.	Treatment	Day 1	Day 13	Day 25	Day 37
Control	Untreated	58.63±10.77	72.29±5.227	67.87±6.334	70.92±3.833
Methotrexate-treated group	Methotrexate (0.675 mg/kg PO)	44.26±6.999^{ns}	133.6±4.709$^{\#}$	181.6±10.86$^{\#}$	311.1±17.70
Test 1	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kgPO)	45.53±6.94^{ns}	107.6±4.58*	97.25±6.433***	81.09±4.963***
Test 2	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kg PO) + vitamin C (250 mg/kg PO)	41.91±2.39^{ns}	97.62±5.7**	92.00±2.8***	78.47±1.3***
Test 3	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kg PO) + vitamin C (250 mg/kg PO) + vitamin E (250 mg/kg PO)	36.41±2.59^{ns}	88.31±1.580***	77.06±2.61***	71.41±1.94***

Values are expressed as Mean±SEM (n=6). One-way ANOVA by Bonferroni's test, $^{\#}$P<0.001 when compared to normal, *P<0.05 when compared to the negative control, **P<0.01 when compared to the negative control, ***P<0.001 when compared to the negative control, ns represents not significant, SEM represents Mean of Standard Error

Table 2: Effect of Zinc and its combination with vitamin C and vitamin E on AST (SGOT) (U/L).

Effect of Zinc and its combination with vitamin C and vitamin E on Total Bilirubin (BIT) (mg/dL)

BIT level was significantly and gradually increased in MTX-treated group in comparison to Control group. Test 1, 2 and 3 were found to have decrease BIT level in comparison to MTX-treated group. Test 3 was found to have fewer BITS than Test 2 and Test 1 as shown in Table 3.

Group.	Treatment	Day 1	Day 13	Day 25	Day 37
Control	Untreated	0.530±0.036	0.5067±0.0409	0.480±0.017	0.440±0.043
Methotrexate-treated group	Methotrexate(0.675 mg/kg PO)	0.470±0.015^{ns}	0.910±0.055 $^{\#}$	1.507±0.17$^{\#}$	1.817±0.115$^{\#}$
Test 1	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kg PO)	0.423±0.020^{ns}	0.690±0.028*	0.636±0.0328***	0.570±0.025***
Test 2	Zinc (18 mg/kg PO)+ Methotrexate (0.675 mg/kg PO) +vitamin C (250 mg/kg PO)	0.366±0.023^{ns}	0.666±0.039*	0.576±0.020***	0.520±0.017***
Test 3	Zinc (18 mg/kg PO)+ Methotrexate (0.675 mg/kg PO)+vitamin C (250 mg/kg PO)+vitamin E (250 mg/kg PO)	0.363±0.038^{ns}	0.596±0.0088**	0.523±0.01***	0.413±0.014***

Values are expressed as Mean±SEM (n=6). One-way ANOVA by Bonferroni's test, $^{\#}$P<0.001 when compared to normal, *P<0.05 when compared to the negative control, **P<0.01 when compared to the negative control, ***P<0.001 when compared to the negative control, ns represents not significant, SEM represents Mean of Standard Error

Table 3: Effect of Zinc and its combination with vitamin C and vitamin E on Total Bilirubin (BIT) (mg/dL).

Effect of Zinc and its combination with vitamin C and vitamin E on Urea (mg/dL)

Urea level in blood serum was significantly increased in MTX-treated group in comparison to Control group. Test 1, 2 and 3 were found to have decrease urea level in comparison to MTX-treated group. Test 3 was found to have less urea than Test 2 and Test 1 as shown in Table 4.

Group.	Treatment	Day 1	Day 13	Day 25	Day 37
Control	Untreated	41.12±2.636	37.15±2.511	39.74±0.943	39.72±0.516
Methotrexate-treated group	Methotrexate (0.675 mg/kgPO)	42.36±3.506^{ns}	69.17±1.061$^{\#}$	137.9±3.341$^{\#}$	156.3±6.770$^{\#}$
Test 1	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kg PO)	39.45±1.901^{ns}	62.24±3.055^{ns}	56.47±3.867*	52.74±3.080*
Test 2	Zinc (18 mg/kg PO)+ Methotrexate (0.675 mg/kg PO) + vitamin C (250 mg/kg PO)	38.52±3.710^{ns}	59.86±2.951^{ns}	58.10±1.970*	49.28±2.880*
Test 3	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kgPO) + vitamin C (250 mg/kg PO) + vitamin E (250mg/kg PO)	39.68±0.497^{ns}	58.89±1.989^{ns}	46.33±3.635*	36.19±2.128*

Values are expressed as Mean±SEM (n=6). One-way ANOVA by Bonferroni's test, $^{\#}$P<0.001 when compared to normal, P<0.001 when compared to the negative control, ns represents not significant, SEM represents Mean of Standard Error

Table 4: Effect of Zinc and its combination with vitamin C and vitamin E on Urea (mg/dL).

Effect of Zinc and its combination with vitamin C and vitamin E on Creatinine (mg/dL)

Creatinine level was significantly and gradually increased in MTX-treated group in comparison to Control group. Test 1, 2 and 3 were found to have decrease Creatinine level in comparison to MTX-treated group. Test 3 was found to have less creatinine than Test 2 and Test 1 as shown in Table 5.

Group.	Treatment	Day 1	Day 13	Day 25	Day 37
Control	Untreated	0.616±0.042	0.593±0.021	0.563±0.021	0.560±0.020
Methotrexate-treated group	Methotrexate (0.675 mg/kgPO)	0.516±0.068^{ns}	2.533±0.284$^{\#}$	4.273±0.289$^{\#}$	4.333±0.260$^{\#}$
Test 1	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kg PO)	0.473±0.023^{ns}	1.600±0.200*	0.876±0.035***	0.643±0.084***
Test 2	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kg PO) + vitamin C (250mg/kg PO)	0.503±0.061^{ns}	1.283±0.152**	0.766±0.077***	0.640±0.047***
Test 3	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kg PO) + vitamin C (250 mg/kg PO) + vitamin E (250 mg/kg PO)	0.653±0.033^{ns}	1.067±0.072**	0.640±0.055***	0.530±0.064***
Values are expressed as Mean±SEM (n=6). One-way ANOVA by Bonferroni's test, $^{\#}$P<0.001 when compared to normal, *P<0.05 when compared to the negative control, **P<0.01 when compared to the negative control, ***P<0.001 when compared to the negative control, ns represents not significant, SEM represents Mean of Standard Error					

Table 5: Effect of Zinc and its combination with vitamin C and vitamin E on Creatinine (mg/dL).

Effect of Zinc and its combination with vitamin C and vitamin E on oxidative parameter of Liver tissue

MTX-treated group was found to have significantly decreased catalase and GSH level and significantly increased lipid peroxidase in comparison to Control group.

Test 1, 2 and 3 were found to have significantly increased catalase and GSH level and significantly decreased lipid peroxidase in comparison to MTX-treated group as shown in Table 6 and graphically in Figure 1.

Group.	Treatment	Catalase (µmol/mg of tissue)	GSH (nmol/mg of tissue)	LPO (nmol MDA/mg of tissue
Control	Untreated	282±12.90	20.42±0.719	0.31±0.053
Methotrexate-treated group	Methotrexate (0.675 mg/kg PO)	79.70±1.721$^{\#}$	8.563±0.843$^{\#}$	0.98±0.017$^{\#}$
Test 1	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kg PO)	164.7±13.87*	15.38±0.617**	0.68±0.017**
Test 2	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kgPO) + vitamin C (250 mg/kg PO)	181.8±9.218**	16.82±0.722**	0.59±0.038**
Test 3	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kgPO) + vitamin C (250 mg/kg PO) + vitamin E (250 mg/kg PO)	222.7±7.513**	21.2±0.895**	0.51±0.020**
Values are expressed as Mean±SEM (n=6). One-way ANOVA by Bonferroni's test, $^{\#}$P<0.001 when compared to normal, *P<0.01 when compared to the negative control, **P<0.001 when compared to the negative control, SEM represents Mean of Standard Error				

Table 6: Effect of Zinc and its combination with vitamin C and vitamin E on oxidative parameter of Liver tissue [Oxidative parameter in liver homogenate].

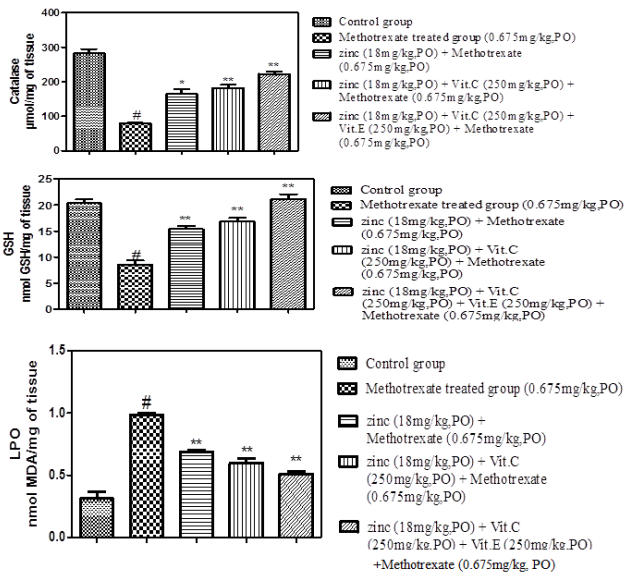

Figure 1: Graphical representation of oxidative parameter in liver homogenate.

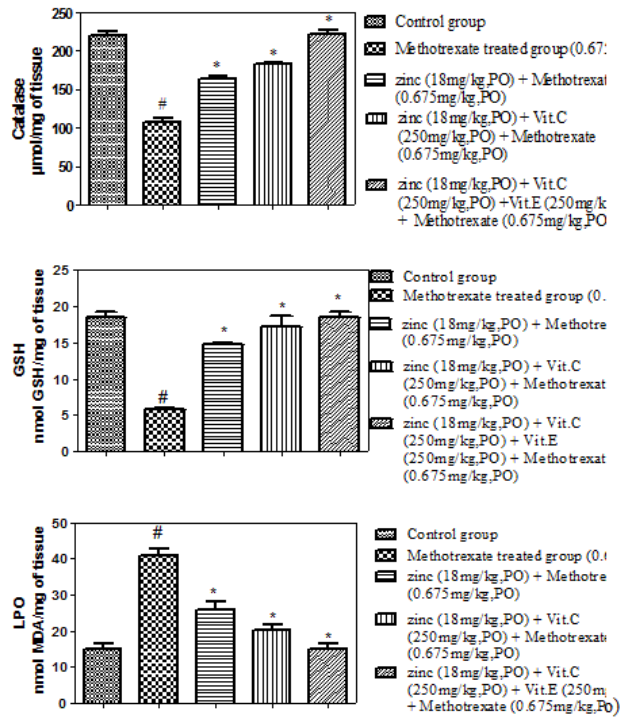

Figure 2: Graphical representation of oxidative parameter in kidney tissue homogenate.

Effect of Zinc and its combination with vitamin C and vitamin E on oxidative parameter of kidney tissue

MTX group was found to have significantly decreased catalase and GSH level and significantly increased lipid peroxidase in comparison to Control group.

Test 1, 2 and 3 were found to have significantly increased catalase and GSH level and significantly decreased lipid peroxidase in comparison to MTX group as shown in Table 7 and graphically in Figure 2.

Effect of Zinc and its combination with vitamin C and vitamin E on Histopathological Analysis

Histopathology of liver Figure 3 (A-F) and kidney Figure 4 (G-K) where 'A' indicates Control group showing normal hepatic architecture, 'B' and 'C' indicates MTX-treated group showing fatty changes or steatosis and periportal inflammation, respectively, 'D' indicates Zinc+MTX-treated group showing degeneration of fatty changes indicating the sign of recovery, 'E' and 'F' indicates Zinc +vitamin C+MTX-treated group and Zinc+vitamin C+vitamin E +MTX-treated group, respectively, showing normal hepatic architecture. Similarly 'G' indicates Control group kidney showing normal glomeruli and tubules, 'H' and 'I' indicates MTX-treated group showing renal injury, shrinking of glomeruli with periglomerular space and tubules with cystic dilation, 'J' indicates Zinc+MTX-treated group showing tubules with very mild dilation or almost looking normal, 'K' and 'L' indicates Zinc+vitamin C+MTX-treated group and Zinc +vitamin C+vitamin E+MTX-treated group, respectively, showing normal kidney architecture with normal looking glomeruli and tubules.

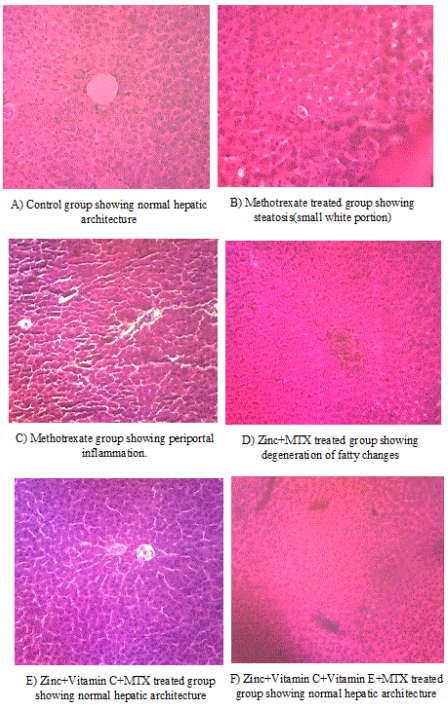

Figure 3: Histopathology of liver sample.

Group	Treatment	Catalase (µmol/mg of tissue)	GSH (nmol/mg of tissue)	LPO (nmol MDA/mg of tissue
Control	Untreated	220.7±4.7	18.40±0.75	15.07±1.41
Methotrexate-treated group	Methotrexate (0.675 mg/kg PO)	107.3±4.8#	5.76±0.29#	40.97±1.63 #
Test 1	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kg PO)	164.0±2.8*	14.70±0.25**	25.7±2.33**
Test 2	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kgPO) + vitamin C (250 mg/kg PO)	183.3±2.3**	17.27±1.36**	20.27±1.31**
Test 3	Zinc (18 mg/kg PO) + Methotrexate (0.675 mg/kgPO) + vitamin C (250 mg/kg PO) + vitamin E (250 mg/kg PO)	222.7±4.2**	18.50±0.72**	14.87±1.40**
Values are expressed as Mean±SEM (n=6). One-way ANOVA by Bonferroni's test, # P<0.001 when compared to Control, * P<0.01 when compared to the Methotrexate treated group				

Table 7: Oxidative parameter in kidney tissue homogenate.

Discussion

The present study demonstrated that Zinc and its combination with vitamin C and vitamin E is having a protective activity against hepato-renal toxicity produced by MTX. This finding is supposed to be very beneficial and novel for the patient who consumes MTX as therapy.

Doctors usually prescribe MTX along with folic acid. This folic acid is to maintain folate in dividing cells and recover the side effect on GI tract, mouth, hair, follicles and liver produced by folate deficiency. However, to protect the toxicity of the active metabolite of MTX, this study suggests that Zinc alone or its combination with vitamin C and vitamin E is having protective action.

The study reported that MTX damages tissue by (1) oxidative stress [4] mediated by oxygen free radical. (2) It accumulates inside the cell in a polyglutamated form causing folate levels decreased and hence toxicity occurs. It binds to dihydrofolate reductase with greater affinity than folic acid limiting the conversion of folic acid to tetrahydrofolate, a molecule necessary for the synthesis of DNA [30]. It inhibits the synthesis of purine and pyrimide thymidilate results in the improper DNA synthesis and subsequent apoptosis [31]. MTX dose is usually less for rheumatoid arthritis and high for antitumour. Its antitumour activity is due to inhibition of folic acid reductase and prescribing folic acid at such situation may boost cancer cell. At such stage, Zinc and vitamin C and vitamin E can be taken into consideration.

Zinc has shown to have an antioxidant effect and stabilizes cell membranes [12,13]. A study reported that Zinc protects the liver from Carbontetrachloride (CCl4) toxicity (Dhawan, [16]. Similarly, it is reported that zinc and vitamin E protect against peroxidative damage and promote membrane integrity (Bettger [19]). A study suggested that zinc protects against acetaminophen induced hepato-toxicity in mice (Chengelis, [14]). Vitamin C is hydrophilic and is an important free radical scavenger in extracellular fluids, trapping radicals and protecting bio-membranes from peroxide damage. It scavenges the radicals and hypochlorous acid [21]. Vitamin E, a lipid soluble antioxidant that has an important role in scavenging free oxygen radicals and stabilizing the cell membranes, maintaining the permeability [22]. Another study on rat kidney suggested that zinc and vitamin E protect against lithium induced kidney toxicity (Omar, [17]).

Thus, it is likely that Zinc and its combination with vitamin C and vitamin E have protective activity on hepato-renal toxicity induced by MTX.

In this study, MTX caused a significant increase in serum parameter like creatinine, urea, total bilirubin, SGOT and SGPT. In addition, MTX also causes oxidative tissue damage, as assessed by decreased catalase and GSH and increased LPO in the hepatic and renal tissue. While Zinc and its combination with vitamin C and vitamin E protected against the MTX-induced hepato-renal toxicity.

Conclusion

From the Serum parameter, oxidative parameter of tissue (liver and kidney) and Histopathological report, the present investigation suggest that Zinc alone and also in combination with vitamin C and vitamin E can protect liver and kidney from MTX induced hepato-renal toxicity in rats. This data suggest that Zinc and its combination with vitamin C and vitamin E prevents hepato-renal toxicity and may enhance the selectivity of drug of anticancer, psoriasis, refractory rheumatoid arthritis in patients who required MTX as a treatment.

Conflict of Interest

We declare that we have no conflict of Interest.

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