

# Trivalent Chromium Promotes Healing of Experimental Colitis in Mice by Suppression of Inflammation and Oxidative Stress

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## Abstract

Ulcerative colitis (UC) has reactive oxygen species (ROS) and immunologic pathways implicated in its pathogenesis. The search for new therapeutic protocols in managing UC is tailored in suppressing or preventing these pathways. The influence of trivalent chromium ( $\text{Cr}^{3+}$ ), an essential mineral on experimental colitis was investigated. Mice were grouped into 3; group 1 (control) received clean drinking water while groups 2 and 3 received 10 and 100 ppm  $\text{Cr}^{3+}$  respectively for 12 weeks through drinking water. After  $\text{Cr}^{3+}$  administration, 5 animals per group were sacrificed on day 0. Thereafter, experimental colitis was induced intra-rectally using acetic acid (4%, 0.3mL) and 5 mice per group were subsequently sacrificed on days 3, 7 and 14. Blood and colonic tissues were obtained and processed appropriately. Blood  $\text{Cr}^{3+}$  level, haematological variables, gross and microscopic colitis scores, colonic myeloperoxidase (MPO), Superoxide Dismutase (SOD) and malondialdehyde (MDA) levels were determined using standard methods. Colon cytokine mRNA genes were quantified using real-time PCR. There was a significant decrease in colon gross and histology scores on days 3 and 7 in chromium treated compared with control. The MPO and MDA in chromium groups reduced significantly compared with control while SOD activities increased significantly in  $\text{Cr}^{3+}$  groups compared with control. Total RNA increased in chromium groups compared with control on day 3 post-colitis. There was up-regulation of IL-10, down-regulation of TNF- $\alpha$  and IFN- $\lambda$  in chromium administered groups compared with control. Chromium enhanced healing of colitis by suppression of ROS, inflammation and promotion of antioxidant activities.

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## Keywords

Colitis, Oxidative Stress, Trivalent Chromium, Inflammation, Mice

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## 1. Introduction

Inflammatory bowel disease (IBD) comprises of both ulcerative colitis (UC) and Crohn's disease (CD) which is often characterized by extensive and severe intestinal inflammation resulting from deranged immune system in response to increasing loads of commensal micro-biota [1]. Although, the pathophysiology is still vague, reactive oxygen species (ROS) and immunologic processes are key theories that have been proposed as the possible mechanism of its pathologic nature [2] [3] [4] [5]. This has driven the search for therapeutic strategies that can be focused or targeted to the needs of individual patients. Conditions such as the patient's genetic components, disease states, and environmental influences have all played vital roles in the treatment and prognosis of the disease [5] [6]. Nevertheless, increase in antioxidant activities are also known to provide relieve for these conditions [7] [8].

Chromium, specifically trivalent ( $\text{Cr}^{3+}$ ) is an essential mineral that human requires in trace amounts, although its mechanism of actions and the quantity that is beneficial to health have no clear-cut definitions [9]. It exists mainly in two forms, trivalent which are the more stable bio-available forms and present in food substances; hexavalent, the toxic form available from industrial pollutions. Chromium in this study is limited to trivalent form and has been reported to promote actions of insulin [10] [11], aid metabolism of fats, carbohydrates and protein [12] [13], among other beneficial effects.

The non-specificity of available treatment in the clinical management of ulcerative colitis and the increasing use of trivalent chromium as dietary supplement have increased our curiosity towards evaluating the probable activities of oral chromium on normal colon and experimental colitis. This study aim was to investigate the effect of chromium exposure basically on the reactive oxygen species and inflammatory pathways in both normal and experimental colitis in mice.

## 2. Materials and Methods

### 2.1. Ethical Considerations

This study was carried out in strict adherence to the recommendations in the Guide for the Care and Use of Laboratory Animals released by US National Institute of Health [14]. The protocol was approved by the Committee of Animal Care and Use of Maebashi Institute of Technology, Japan (No. 15-009).

### 2.2. Animals and Treatment Protocols

Sixty male, slc:ddY mice (  $25.3 \pm 2.1$  g, 5 weeks old) were purchased from SLC

Incorporation, Japan, kept in the animal room at the Department of Biotechnology, Maebashi Institute of Technology and were used for the experiments. The animals were acclimatized for 2 weeks with access to standard feeds and water *ad libitum*. They were grouped into 3: Control (n = 20) were allowed free access to clean drinking water and groups 2 and 3, were administered oral 10 ppm, (n = 20) and 100 ppm (n = 20) trivalent chromium respectively through their drinking water for 12 weeks.

### 2.3. Chemicals and Drugs

Chromium was obtained from Koshin Chemicals, Japan. Hexadecyltrimethylammonium Bromide, o-Dianisidine Dihydrochloride, and Sodium nitrite were procured from Tokyo Chemical Industry, Co., Limited, Japan. All Other chemicals were of highest purity and analytical grade. RNA retraction and reverse transcription kits were obtained from Qiagen, Japan. Real-time PCR kits were obtained from Brilliant Agilent<sup>®</sup>, UK; Primers for standards and RT-PCR assays all from qStandard<sup>®</sup>, London.

### 2.4. Acute Colitis Induction, Stool Consistency and Gross Assessment of Colonic Damage

After Cr<sup>3+</sup> exposure, mice (n = 5 per group) were sacrificed (day 0) for gross, histology, haematological variables, biochemical parameters and gene expression studies. Thereafter, experimental colitis was induced using the modified method by Choudhary *et al.* (2001) [15]. Briefly, 0.1 mL of 4% acetic acid was introduced using a 3 mm soft paediatric catheter which was advanced 3 cm from anal opening under light ketamine anaesthesia (0.01 mL/g) for 60 seconds in 24 h fasted mice. Mice were maintained in a head-down position for 30 seconds following introduction of acetic acid to prevent immediate extrusion of the solution (Control had normal saline of the same proportion instilled). Stool consistency was scored starting from 24 h post induction and daily for 7 days after colitis induction using the method described by Fukuda *et al.* [16] where 0 = normal stool, 1 = loose stool without visible blood, 2 = loose stool with visible blood, 3 = bloody diarrhea. The mice were anaesthetized with xylaxine (0.0005 mL/g) and ketamine (0.015 mL/g) cocktails. Thereafter, laparotomy was conducted and the distal 8 cm of the colon was resected for gross colitis scores, histology, biochemical assessment and cytokine mRNA gene expression on day 0 (before colitis induction) and days 3, 7 and 14 after induction of colitis in the mice. The gross scoring method described by Morris *et al.* [17] where, no damage = 0, localized hyperemia with no ulcers = 1, linear ulcers with no significant inflammation = 2, linear ulcer with inflammation at one site = 3, more site of ulcers and inflammation, the size of ulcer <1 cm =4 and multiple inflammations and ulcers, the size of ulcer >1 cm =5 was adopted.

### 2.5. Histology and Histomorphometry Assessment of Colon Tissues

All sections for light microscopy were fixed in 10% buffered formalin, embedded

in paraffin wax, sectioned, and stained with haematoxylin and eosin (H&E). Accuscope TS view, China was used to capture images and to evaluate morphological changes and measurement of histomorphometry variables.

## 2.6. Determination of Blood Parameters

Blood samples (1 mL) were analyzed using an automated machine following blood sample collection from cardiac puncture into a 5 mL heparinized bottle. Each of the samples collected were ran sequentially using KX-21 haematological analyzer by Symex Kobe® Japan.

## 2.7. Lipid Peroxidation Determination

This was assessed by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method described by Varshney and Kale [18]. The method described the value of malondialdehyde (MDA) in the tissue homogenates during lipid peroxidation with thiobarbituric acid (TBA) forming a pinkish MDA-TBA complex that was read spectrophotometrically at 532 nm.

## 2.8. Determination of Antioxidant Status in Colonic Tissues

Catalase activity was determined according to the method described by Sinha [19]. The procedure described the reduction of dichromate in the presence of acetic acid to chromic acetate when heated in the presence of H<sub>2</sub>O<sub>2</sub>, with the formation of perchromic acid as an unstable intermediate. The resultant chromic acetate was determined spectrophotometrically at 530 nm. Superoxide Dismutase (SOD) method described by Misra and Fridovich [20] was adopted using 0.5 mL of tissue homogenates which was added to 2.5 ml of 0.05 M carbonate buffer at pH 10.2 to equilibrate in glass cuvette. The reaction commenced with the addition of 0.3 mL freshly prepared 0.3 mM epinephrine. Increase in absorbance at 480 nm was observed every 30 s for 1 min and 1 unit of SOD activity was given as amount of SOD expected to cause 50% inhibition of the oxidation of adrenaline.

## 2.9. Determination of Total Nitrite

Total tissue nitrite determination was done using the method described by Ignarro *et al.* [21]. The assay relies on a diazotization reaction that was originally described by Griess [22], which uses sulfanilamide and *N*-1-naphthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions.

## 2.10. Determination of Myeloperoxidase Activity

The method described by Kim *et al.* [23] was adopted using tissue homogenates.

## 2.11. RNA Extraction and Quantification of Absolute Real-Time PCR

Total RNA was obtained with RNeasy Mini Kit from Qiagen, Japan and the

protocol was followed strictly. Briefly, 30 mg of sample were stored in RNeasy lysis buffer (Qiagen<sup>®</sup>) pending homogenization. Tissue was thawed on ice and subsequently homogenized after lysing with appropriate lysis buffer at 4°C. The lysate was centrifuged for 3 minutes at maximum speed and supernatant was carefully pipetted. Ethanol (70%) was added to the pipetted supernatant, volume for volume and mixed well. The mixture (700 µL) was added to 2 mL RNeasy spin column and centrifuged for 15 seconds at >8000 x g. The flow-through was discarded and another 700 µL wipeout buffer (RW1) added to the spin column, this was centrifuged at >8000 x g for 15 sec.

The flow-through was also discarded and 500 µL RPE buffer added to wash out membrane-bound RNA and also centrifuged for 15 seconds at >8000 x g. This was repeated for 2 minutes. The spin-column is now placed in a new collection tube and spin for 1 minute to dry the membrane. Then 50 µL of RNase-free water was added to the column directly and centrifuged for 1 minute at >8000 x g to elute the RNA. The eluted fraction was quantified using 1 µL of total RNA sample on Nanodrop<sup>®</sup> 2000 spectrophotometer at 260/280nm. Total RNA was further expressed on 2% Agarose gel electrophoresis. The eluted RNA was then stored at -80°C pending reverse transcription, thereafter 150 ng/µL RNA was reverse transcribed into complementary DNA.

QuantiTect Reverse Transcription Kit from Qiagen<sup>®</sup> was used and all reactions set-up was on ice. Briefly, genomic DNA elimination reaction was done using gDNA wipeout buffer, template RNA and RNase-free water mixture incubated for 2 minutes at 42°C, then returned immediately on ice. The reverse-transcriptase master mix was constituted with 1 µL Quantiscript reverse transcriptase, 4 µL Quantiscript RT Buffer and 1 µL RT Primer mix all added to the entire purified genomic DNA reaction described above and quickly returns on ice. This was incubated for 15 minutes at 42°C. Incubation was repeated for 3 minutes at 95°C to inactivate Quantiscript Reverse Transcriptase. The reverse-transcription reaction was then placed on ice and immediately proceeds for real time PCR. Prior to use in PCR, the cDNA was diluted 10-folds (1:10) by tRNA (10 µg/mL), to reduce contamination if any.

Thereafter, Brilliant III Ultra-Fast SYBR<sup>®</sup> Green QPCR Master Mix obtained from Agilent Technologies, UK. Standard assays, primers and cytokine assays were all gotten from qStandards<sup>®</sup>, UK with the following document number for standards, mmu\_Actb\_001; mmu\_Gapdh\_001; mmu\_Rpl13\_002; mmu\_Iifng\_001, mmu\_Il1a\_001; mmu\_Il6\_001; mmu\_Il10-001 and mmu\_Tnf\_001 were used. Applied Biosystems<sup>®</sup> 7500 Fast Real-Time PCR System was used to amplify the eight mRNA genes, three were reference genes (B-Actin, Gapdh and Rpl13) and five cytokines of interest (IL-1 $\alpha$ , IL-6, IL-10, TNF- $\alpha$  and IFN- $\lambda$ ) (**Table 1**).

The protocols were strictly followed. Briefly, a total volume of 20µL was used for the PCRs. 4 µL diluted cDNA, 10 µL Brilliant III SYBR green mix, 1 µL forward primer, 1 µL reverse primer and 4 µL Samples were first denatured at 95°C for 3 min, this was followed by amplification for 40 cycles; denaturation, 95°C

**Table 1.** Oligonucleotide primers used for real time quantitative PCR.

Genes	Primer Sequence	
	Forward	Reverse
$\beta$ -actin	5'-caccatgaagatcaagatcattgct-3'	5'-taaaacgcagctcagtaacagt-3'
GAPDH	5'-catttctggtatgacaatgaatac-3'	5'-ggatagggcctctctgctc-3'
RPL13	5'-tgattggcgttgagattggc-3'	5'-gcttcagtatcatgccattccg-3'
IFN- $\lambda$	5'-cggcacagtcattgaaagcc-3'	5'-tgtcaccatcctttgcccagt-3'
IL-1 $\alpha$	5'-cgcttgagtcggcaagaaat-3'	5'-tggcagaactgtagtctctc-3'
IL-6	5'-gccttctgggactgatgct-3'	5'-gccattgcacaactctttctca-3'
IL-10	5'-gccgggaagacaataactgc-3'	5'-ttggcaaccaagtaacct-3'
TNF- $\alpha$	5'-gtctactgaactcggggtgat-3'	5'-tgagaagatgatctgagtgtgag-3'

for 5 sec, annealing and extension was at 60°C for 27 seconds and ramping was done between 65°C and 95°C rising by 1°C per step. Polymerase chain reaction amplification was performed in triplicate. The standard curves for each gene were generated using the specific standard fragments from qStandards<sup>®</sup>. The absolute copy numbers were generated by the PCR machine and the amounts determined by normalization against the three reference genes were subsequently analyzed.

The RNA and PCR products were subjected to electrophoresis on 1.5 % Agarose gels with 1 × running buffer Tris-Acetic acid-EDTA (TAE × 1), at 50 V for 90 min and visualized by means of ethidium bromide stain under ultra violet light before they were transferred for digital gel photographs with Quantum LAS 4000.

## 2.12. Statistical Analysis

Data were recorded as mean ± S.E.M and analyzed using descriptive statistics one way ANOVA followed by a post-hoc test (Newman-Keul's comparison test) with GraphPad Prism version 5 (GraphPad software, San Diego, CA). Differences were considered significant at  $P < 0.05$ .

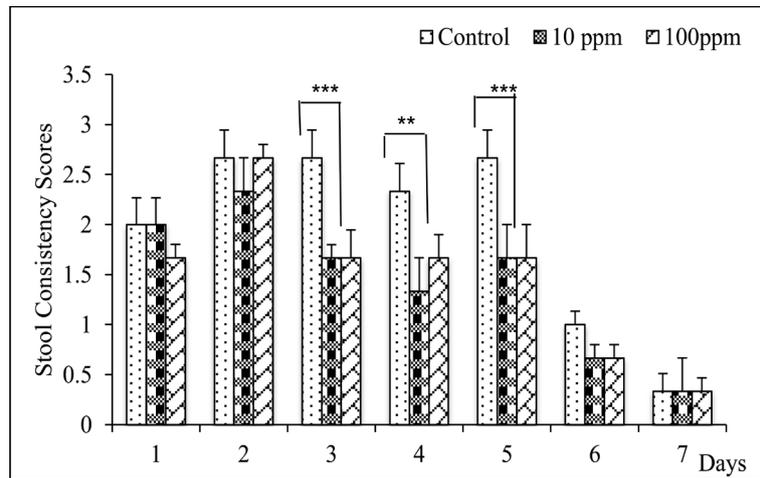
## 3. Results

### 3.1. Effect of Oral Chromium on Stool Consistency in Acetic Acid-Induced Colitis

The stool consistency scores were significantly low in the chromium groups by days 3, 4 and 5 following colitis induction compared with control group (Figure 1).

### 3.2. Effects of Oral Chromium on Colon Macro and Micro Architecture (MAG X100), Pre- and Post-Induction of Colitis

The colon histoarchitecture varied with significant increase in crypt heights in the chromium groups, 10 ppm ( $163.1 \pm 10.59 \mu\text{m}$ ) and 100 ppm ( $165.71 \pm 12.26 \mu\text{m}$ ) compared to control ( $132.13 \pm 8.24 \mu\text{m}$ ). Mucosa width increased signifi-



\*\*Significant at  $p < 0.01$ , \*\*\*significant at  $p < 0.001$  compared with control.

**Figure 1.** Stool consistency post colitis induction in chromium administered groups and control.

**Table 2.** Effect of oral chromium exposure on colon architecture before colitis induction.

Day 0	Control	10 ppm	100 ppm
<b>Macroscopic</b>			
<b>Microscopic</b>			
<b>Gross Colitis Scores</b>	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
<b>Histology scores</b>	0.12 ± 0.06	0.08 ± 0.06	0.11 ± 0.09
<b>Mucosa width (µm)</b>	260.81 ± 9.3	273.01 ± 6.44	298.35 ± 2.78 <sup>+</sup>
<b>Crypt height (µm)</b>	132.13 ± 8.24	163.10 ± 10.59 <sup>*</sup>	165.71 ± 12.26 <sup>*</sup>
<b>Mucous Glandular Unit/61,634.95 µm<sup>2</sup></b>	48.25 ± 3.57	47.10 ± 2.90	50.25 ± 4.56

The gross and histology of all tissues appears normal. <sup>\*</sup>significant difference at  $p < 0.05$  compared with control (MAG. X100), H & E stain.

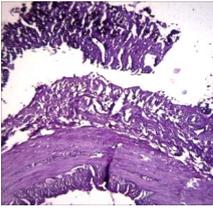
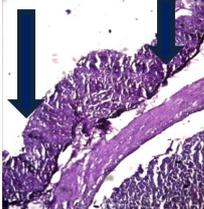
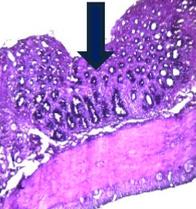
cantly and not significantly only in the 100 ppm ( $298.35 \pm 2.78 \mu\text{m}$ ) compared with control ( $260.81 \pm 9.3 \mu\text{m}$ ) after exposure (**Table 2**). The control group on day 3 (**Table 3**) shows gangrenous and severe ulceration in both the gross and histology compared with the chromium treated groups with milder form of injuries. Day 7 (**Table 4**) shows an improved healing in all the groups studied.

### 3.3. Effect of Oral Chromium Administration on Haematological Variables before and after Colitis Induction

**Table 5** shows significant increase in platelet counts on days 0 and 3 in 10 ppm

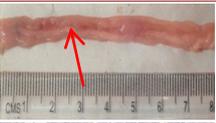
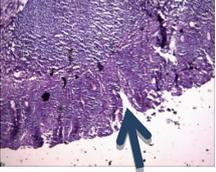
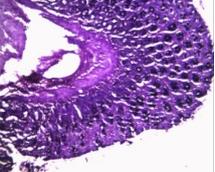
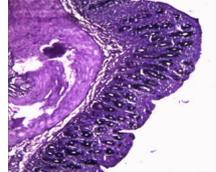
( $117.7 \pm 9.5$ ;  $187.7 \pm 30.3$ ) and 100 ppm ( $105.3 \pm 7.4$ ;  $181.0 \pm 28.8$ ) chromium groups compared to the control ( $78.9 \pm 4.1$ ;  $143.7 \pm 17.6$ ), respectively. There

**Table 3.** Effect of oral chromium exposure on colon architecture 3 days after colitis induction.

COLON DAY 3	Control	10 ppm	100 ppm
Macroscopic			
Microscopic			
Gross Colitis scores	$4.52 \pm 0.88$	$2.62 \pm 0.67^*$	$2.38 \pm 0.60^{**}$
Histology scores	$4.32 \pm 0.81$	$2.33 \pm 0.33^*$	$2.07 \pm 0.33^*$
Mucosa width ( $\mu\text{m}$ )	$217.12 \pm 9.3$	$296.30 \pm 4.91^{**}$	$339.70 \pm 7.26^{**}$
Crypt length ( $\mu\text{m}$ )	$115.73 \pm 2.69$	$119.60 \pm 5.08$	$141.20 \pm 6.22^{*#}$
Mucous Glandular Unit/ $61,634.95 \mu\text{m}^2$	$38.33 \pm 1.46$	$39.33 \pm 4.41$	$40.67 \pm 1.77$

The thick red arrow indicates dead area of the mucosa; thin red arrow shows points of inflammation on the colon tissues of 10 and 100 ppm. Blue arrows show degree of mucosa defects following colitis induction. \*significant at  $p < 0.05$ , \*\*significant at  $p < 0.01$ , compared with control, #significant at  $p < 0.05$  compared with 10 ppm.

**Table 4.** Effect of oral chromium exposure on colon architecture after day 7 of colitis induction.

COLON DAY 7	Control	10 ppm	100 ppm
Macroscopic			
Microscopic			
Gross Colitis scores	$1.63 \pm 0.33$	$0.67 \pm 0.33^*$	$0.00 \pm 0.00^*$
Histology scores	$3.45 \pm 0.51$	$2.17 \pm 0.67^*$	$1.40 \pm 0.37^*$
Mucosa width( $\mu\text{m}$ )	$282.90 \pm 10.34$	$322.40 \pm 6.79$	$318.2 \pm 14.71$
Crypt height( $\mu\text{m}$ )	$130.32 \pm 5.98$	$155.41 \pm 8.97$	$149.2 \pm 13.57$
Mucous Glandular Unit/ $61,634.95/\mu\text{m}^2$	$40.67 \pm 1.2$	$38.00 \pm 2.33$	$40.67 \pm 2.08$

Red arrow indicates ongoing inflammation in the gross colon tissue of control while the blue arrow shows persistent defects on the wall of the mucosa. \*Significant at  $p < 0.05$  compared with control.

**Table 5.** Effect of oral chromium administration on haematological variables before and after colitis induction.

Hematological Parameters	DAY 0			DAY 3			DAY 7		
	Control	10 ppm	100 ppm	Control	10 ppm	100 ppm	Control	10 ppm	100 ppm
PCV (%)	44.2 ± 1.6	41.3 ± 1.3	41.3 ± 0.7	44.3 ± 1.2	42.3 ± 3.8	43.4 ± 1.8	41.0 ± 1.5	42.3 ± 2.6	47.5 ± 2.6
Haemoglobin (g/dL)	15.1 ± 1.1	13.3 ± 0.9	13.7 ± 0.7	14.7 ± 0.4	13.4 ± 1.2	14.1 ± 0.8	13.7 ± 0.5	14.0 ± 1.0	16.1 ± 1.0
RBC count (×10 <sup>6</sup> /μL)	7.8 ± 0.3	7.4 ± 0.7	7.0 ± 0.1	7.8 ± 1.0	6.9 ± 0.7	7.7 ± 0.4	6.8 ± 0.2	7.0 ± 0.4	8.0 ± 0.4
WBC count (×10 <sup>3</sup> /μL)	2.5 ± 0.3	2.5 ± 0.4	2.6 ± 0.3	8.1 ± 0.7	9.6 ± 2.1	9.2 ± 1.8	6.2 ± 0.5	9.0 ± 2.2	6.4 ± 1.4
Platelets Count (×10 <sup>3</sup> /μL)	78.9 ± 4.1	117.7 ± 9.5 <sup>++</sup>	105.3 ± 7.4 <sup>++</sup>	143.7 ± 17.6	187.7 ± 30.3 <sup>+</sup>	181.0 ± 28.8 <sup>+</sup>	129.0 ± 16.3	135.3 ± 36.4	113.8 ± 3.1
Lymphocyte Count (%)	65.8 ± 1.3	66.3 ± 9.7	64.0 ± 3.2	32.3 ± 0.8	46.0 ± 2.5 <sup>+</sup>	47.0 ± 4.3 <sup>+</sup>	56.7 ± 0.3	61.7 ± 5.2	62.8 ± 1.8
Neutrophil Count (%)	29.8 ± 2.1	30.3 ± 5.3	33.3 ± 2.7	62.0 ± 1.7	50.0 ± 2.5 <sup>+</sup>	51.3 ± 3.3 <sup>+</sup>	40.3 ± 0.9	35.0 ± 4.5	34.0 ± 2.3
Monocyte (%)	0.4 ± 0.2	2.3 ± 1.2	1.0 ± 0.3	2.0 ± 1.0	3.0 ± 1.3	1.3 ± 0.9	1.0 ± 0.3	1.0 ± 0.6	1.5 ± 0.7
Eosinophils (%)	2.0 ± 0.7	3.0 ± 1.5	2.2 ± 0.7	2.3 ± 0.3	1.0 ± 0.3	4.3 ± 1.8	2.0 ± 0.3	2.3 ± 0.3	2.0 ± 0.6
Neutrophil-Lymphocyte Ratio	0.67 ± 0.06	2.31 ± 0.21	1.37 ± 0.19	0.59 ± 0.09	3.66 ± 0.28 <sup>++</sup>	3.44 ± 0.19 <sup>++</sup>	0.62 ± 0.06	3.72 ± 0.13 <sup>+</sup>	2.37 ± 0.22 <sup>+</sup>

\*significant at  $p < 0.05$ , \*\*significant at  $p < 0.01$  compared with the control.

**Table 6.** Effect of oral chromium administration on biochemical parameters before and after colitis induction.

Biochemical Parameters	DAY 0			DAY 3			DAY 7		
	Control	10 ppm	100 ppm	Control	10 ppm	100 ppm	Control	10 ppm	100 ppm
MDA (nmol/mg protein)	3.07 ± 0.22	2.88 ± 0.29	2.75 ± 0.39	5.36 ± 0.30	4.18 ± 0.17 <sup>*</sup>	4.17 ± 0.22 <sup>*</sup>	3.59 ± 0.29	2.30 ± 0.28 <sup>*</sup>	2.20 ± 0.25 <sup>*</sup>
Superoxide Dismutase (μmol/mg protein)	6.93 ± 0.67	6.15 ± 0.29	6.11 ± 0.50	7.52 ± 0.39	9.39 ± 0.22 <sup>*</sup>	9.42 ± 0.16 <sup>*</sup>	7.24 ± 0.34	8.78 ± 0.38 <sup>*</sup>	8.70 ± 0.29 <sup>*</sup>
Catalase (μmol/min/mg protein)	923.9 ± 67.2	1044.2 ± 82.1	1010.2 ± 77.7	1128.3 ± 112.0	1173.2 ± 52.1	1185.1 ± 99.3	993.0 ± 88.4	1160.1 ± 73.3	1140.0 ± 97.3
Tissue Total Nitrite	0.11 ± 0.01	0.12 ± 0.01	0.13 ± 0.01	0.22 ± 0.01	0.19 ± 0.01	0.21 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.18 ± 0.01
Myeloperoxidase (U/mg tissue)	0.46 ± 0.17	0.34 ± 0.17	0.34 ± 0.15	3.08 ± 0.24	2.29 ± 0.12 <sup>*</sup>	1.82 ± 0.26 <sup>**</sup>	2.42 ± 0.05	1.77 ± 0.19 <sup>*</sup>	1.76 ± 0.12 <sup>*</sup>

\*significant at  $p < 0.05$ , \*\*significant at  $p < 0.01$  compared with control.

was also a significant increase in lymphocyte counts on day 3 in the 10 ppm (46.0 ± 2.5) and 100 ppm (47.0 ± 4.3) compared with control (32.3 ± 0.8). Neutrophil reduced significantly in the chromium groups, 10 ppm (50.0 ± 2.5) and 100 ppm (51.3 ± 3.3) on day 3 compared with the control (62.0 ± 1.7) (**Table 5**).

### 3.4. Effect of Oral Chromium Exposure on Biochemical Parameters Pre- and Post-Colitis Induction

There was no significant change after period of exposure (on day 0) compared to days 3 and 7 after colitis induction (**Table 6**). The MDA values reduced signifi-

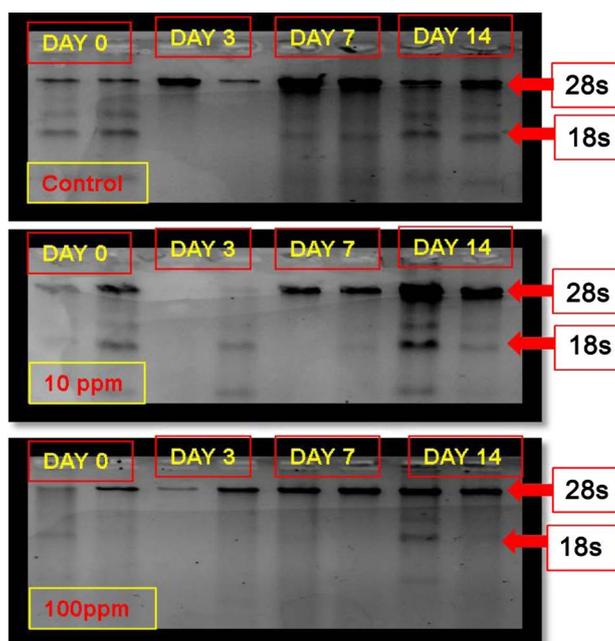
cantly on days 3 and 7 in the chromium groups, 10 ppm ( $4.18 \pm 0.17$ ;  $2.30 \pm 0.28$ ) and 100 ppm ( $4.17 \pm 0.22$ ;  $2.20 \pm 0.25$ ) compared with control ( $5.36 \pm 0.30$ ;  $3.59 \pm 0.29$ ) respectively, while SOD increased significantly on days 3 and 7 in the 10 ppm ( $9.39 \pm 0.22$ ;  $8.78 \pm 0.38$ ) and 100 ppm ( $9.42 \pm 0.16$ ;  $8.70 \pm 0.29$ ) compared with control ( $7.52 \pm 0.39$ ;  $7.24 \pm 0.34$ ) (**Table 6**), respectively. **Table 6** further shows significantly reduced myeloperoxidase activities on days 3 and 7 in the 10 ppm ( $2.29 \pm 0.12$ ;  $1.77 \pm 0.19$ ) and 100 ppm ( $1.82 \pm 0.26$ ;  $1.76 \pm 0.12$ ) compared with control ( $3.08 \pm 0.24$ ;  $2.42 \pm 0.05$ ), respectively.

### 3.5. Effect of Oral Chromium Exposure on Colon Cytokines mRNA and DNA Pre- and Post-Colitis Induction

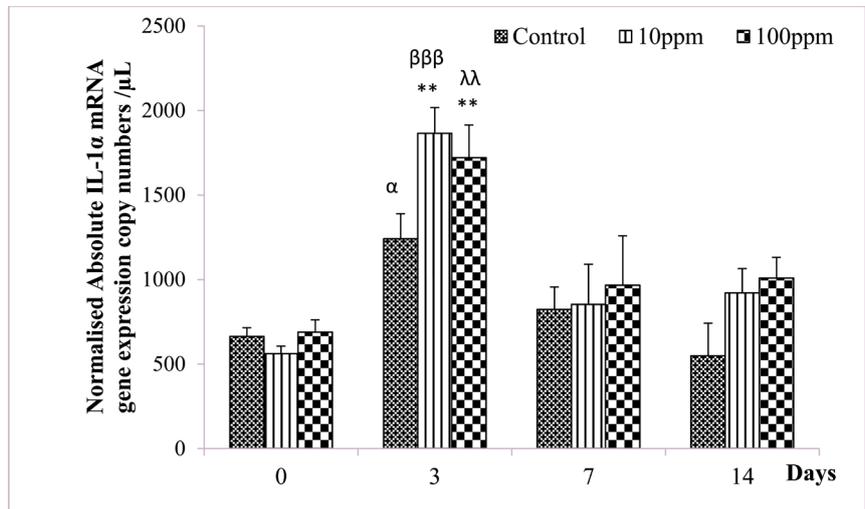
**Plate 1** revealed depletion of 18 subunit of ribosomal RNA in all the groups on day 3 (after colitis induction) and a gradual return to normal days 7 and 14. **Figure 2** and **Plate 2** shows a significant increase in the copy numbers of mRNA IL-1 $\alpha$  in the chromium groups compared with the control on day 3. Expression of IL-6 mRNA increased significantly in all the groups on day 3 compared to their counterparts on day 0 (**Figure 3** and **Plate 3**). The expression of Interleukin-10 mRNA significantly increased in the chromium groups on days 3, 7 and 14 compared with control (**Figure 4** and **Plate 4**). **Figure 5**, **Plate 5** and **Figure 6**, **Plate 6** shows significant decrease in expressions of TNF- $\alpha$  and IFN- $\lambda$  mRNA in the chromium groups on days 7 and 14 compared with the control.

## 4. Discussion

This study emphasizes the effect of oral chromium administration on intact co-

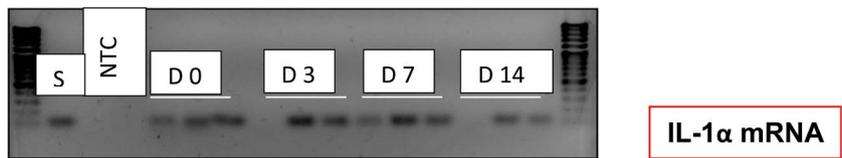


**Plate 1.** Colon total RNA on Agarose-Gel electrophoresis after oral chromium administration and colitis induction. It shows 28 s and 18 s ribosomal RNA bands, an important observation is the depletion of the 18 s bands especially on days 3 in almost all the groups.



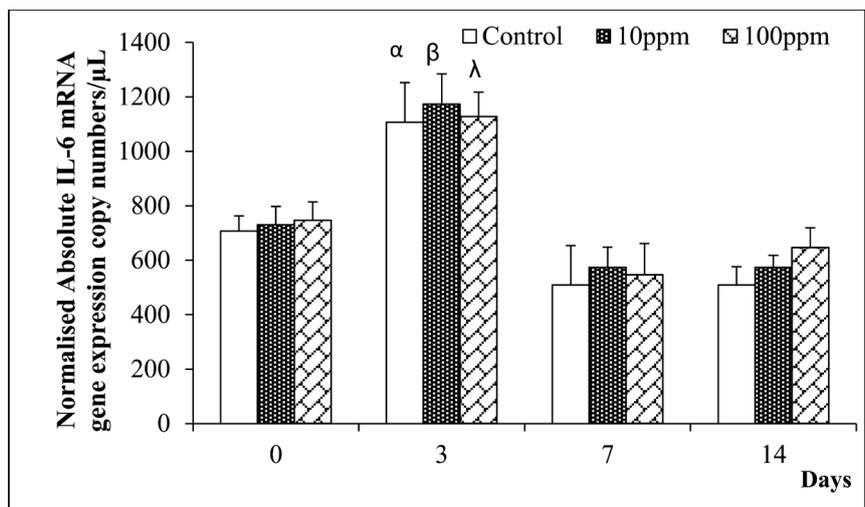
$\alpha$ - Significant at  $p < 0.05$  compared with control on day 0, \*significant at  $p < 0.05$  compared with control,  $\beta\beta\beta$ - significant at  $p < 0.001$  compared with 10 ppm on day 0;  $\beta\beta, \lambda\lambda$ -significant at  $p < 0.01$  compared with 100 ppm on day 0.

**Figure 2.** Real-Time PCR Absolute copy numbers of colon IL-1 $\alpha$  mRNA gene expression following oral chromium administration before and after colitis induction. Values were normalised with the three reference genes, B-actin, GAPDH and RPL13.



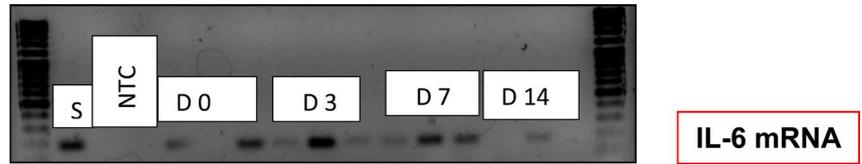
D0 = Day 0, D3 = Day 3, D7 = Day 7 and D14 = Day 14, S = Standard, NTC = No template control.

**Plate 2.** The plate represents the corresponding Agarose-Gel electrophoresis following real-time PCR amplification.



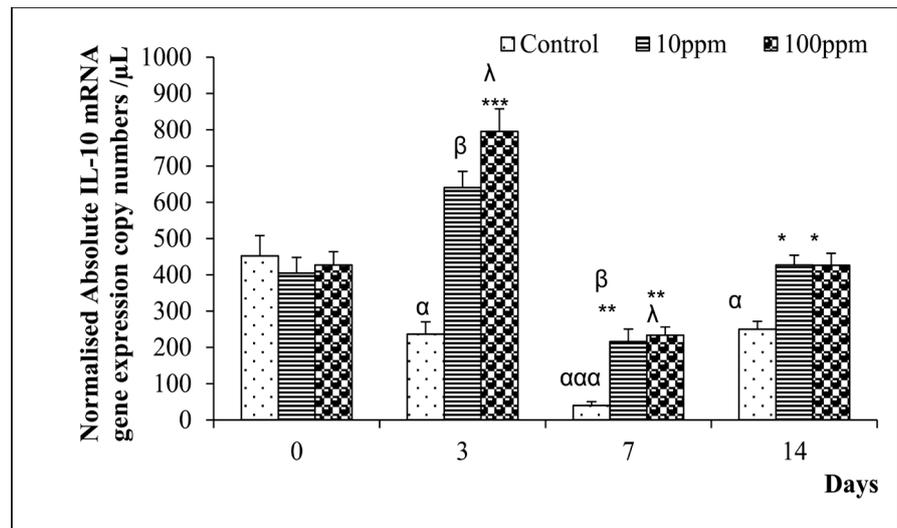
$\alpha$ - Significant at  $p < 0.05$  compared with control on day 0,  $\beta$ -significant at  $p < 0.05$  compared with 10 ppm on day 0,  $\lambda$ -significant at  $p < 0.05$  compared with 100 ppm on day 0.

**Figure 3.** Real-Time PCR Absolute copy numbers of colon IL-6 mRNA gene expression following oral chromium administration before and after colitis induction. Values were normalised with the three reference genes, B-actin, GAPDH and RPL13.



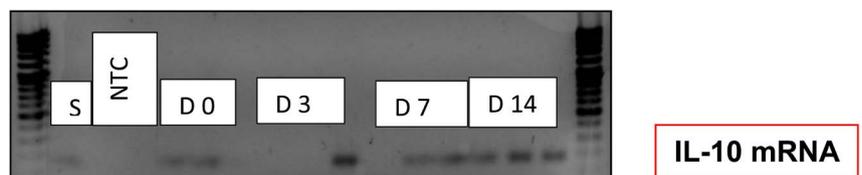
D0 = Day 0, D3 = Day 3, D7 = Day 7 and D14 = Day 14, S = Standard, NTC = No template control.

**Plate 3.** The plate represents the corresponding Agarose-Gel electrophoresis post real-time PCR amplification.



$\alpha$ -significant at  $p < 0.05$  compared with control on day 0,  $\alpha\alpha$ -significant at  $p < 0.05$  compared with control on day 0;  $\alpha\alpha\alpha$ -significant at  $p < 0.001$  compared with control on day 0; \*significant at  $p < 0.05$  compared with control on day 14, \*\*-significant at  $p < 0.01$  compared with control, \*\*\*-significant at  $p < 0.001$  compared with control,  $\beta$ -significant at  $p < 0.05$  compared with 10 ppm on day 0,  $\lambda$ -significant at  $p < 0.05$  compared with 100 ppm on day 0.

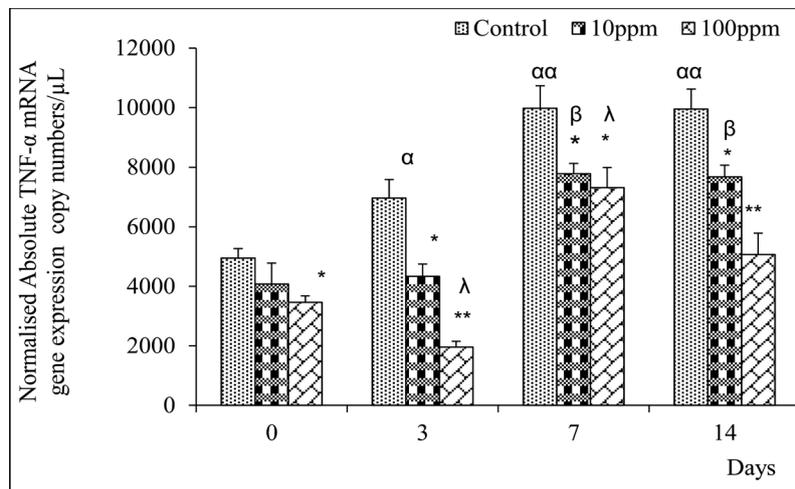
**Figure 4.** Real-Time PCR Absolute copy numbers of colon IL-10 mRNA gene expression following oral chromium administration before and after colitis induction. Values were normalised with the three reference genes, B-actin, GAPDH and RPL13.



D0 = Day 0, D3 = Day 3, D7 = Day 7 and D14 = Day 14, S = Standard, NTC = No template control.

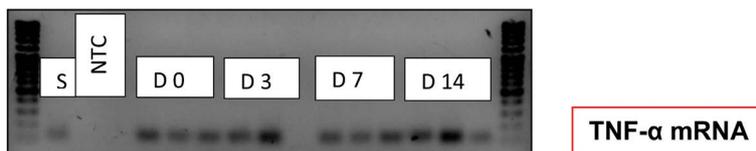
**Plate 4.** The plate represents the corresponding Agarose-Gel electrophoresis post real-time PCR amplification.

lon tissue and acetic acid-induced colitis in mice following the exposure period to chromium. The low scores for stool consistency reported in the test groups compared with control on days 3, 4 and 5 post inductions, is an indication of early recovery which was evident by gross and micro architecture rapid re-epithelization [24]. The increase in mucosa architecture is suggestive of colon mucosa growth enhancement. These findings underscore possible tissue toxicities from trivalent chromium administration.



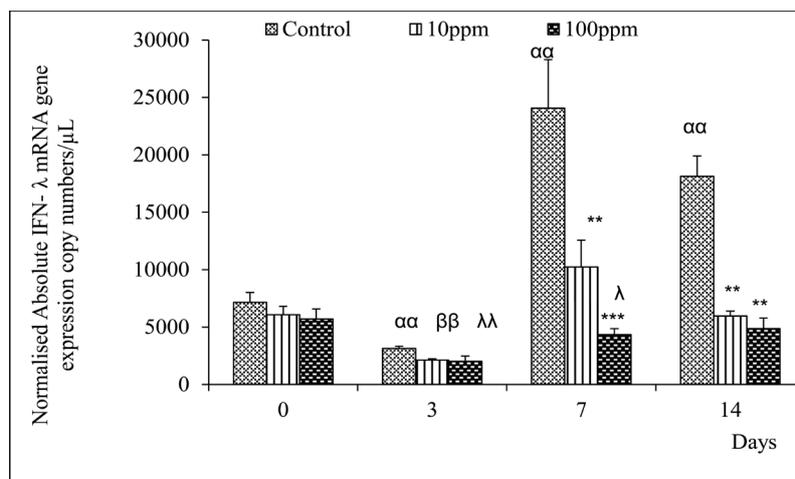
$\alpha$ - significant at  $p < 0.05$  compared with control on day 0,  $\alpha\alpha$ -significant at  $p < 0.05$  compared with control on day 0, \*significant at  $p < 0.05$  compared with control on day 14, \*\*-significant at  $p < 0.01$  compared with control,  $\beta$ -significant at  $p < 0.05$  compared with 10 ppm on day 0,  $\lambda$ -significant at  $p < 0.05$  compared with 100 ppm on day 0.

**Figure 5.** Real-Time PCR Absolute copy numbers of colon TNF- $\alpha$  mRNA gene expression following oral chromium administration before and after colitis induction. Values were normalised with the three reference genes, B-actin, GAPDH and RPL13.



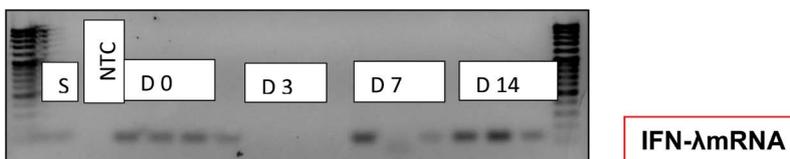
D0 = Day 0, D3 = Day 3, D7 = Day 7 and D14 = Day 14, S = Standard, NTC = No template control.

**Plate 5.** The plate represents the corresponding Agarose-Gel electrophoresis post real-time PCR amplification.



$\alpha\alpha$ -significant at  $p < 0.01$  compared with control on day 0,  $\beta\beta$  significant at  $p < 0.01$  compared with day 0, \*\*-significant at  $p < 0.01$  compared with control,  $\beta$ -significant at  $p < 0.05$  compared with 10 ppm on day 0,  $\lambda$ -significant at  $p < 0.05$  compared with 100 ppm on day 0.

**Figure 6.** Real-Time PCR Absolute copy numbers of colon IFN- $\lambda$  mRNA gene expression following oral chromium administration before and after colitis induction. Values were normalised with the three reference genes, B-actin, GAPDH and RPL13.



D0 = Day 0, D3 = Day 3, D7 = Day 7 and D14 = Day 14, S = Standard, NTC = No template control.

**Plate 6.** The plate represents the corresponding. Agarose-Gel electrophoresis post real-time PCR amplification.

The platelet counts increased in relation to control in the chromium treated groups after the administration period. There is a growing appreciation of roles of platelets in immune system regulation and inflammation [25]. The roles played by platelets during immune responses are observed at sites of its activation and deposition or systemically at locations that are far from platelet activation [25]. The increase in platelet count and reduction in neutrophil-lymphocyte ratio noted in the injured states of chromium treated groups compared to control colon suggest an anti-inflammatory effect. This further suggests that, platelet interactions with inflammatory cells might produce pro-inflammatory responses, which could be beneficial in limiting the progression of infection. In this present study, the platelets pro-inflammatory activities were observed thus accelerating the inflammatory phase of colitis healing. Acetic acid-induced colitis in mice largely mimics clinical form of human colitis especially with the acute inflammatory responses [26].

Certain studies have indicated that an increase in ROS production decreases antioxidant defenses in IBD patients [27] [28], apart from protection by the epithelial layer. Food particles and disease causing organisms may cause inflammation by activating certain unregulated factors within the colon epithelium. The infiltration of polymorphonuclear neutrophils (PMNs) and macrophages can synthesize inflammatory cytokines (and other mediators) that contribute further to oxidative stress [29]. In physiological conditions, exogenous (dietary) and endogenous enzymatic antioxidants protect tissues from dangers posed by ROS. The malondialdehyde, myeloperoxidase, catalase, Superoxide Dismutase and colon total nitrites were essentially within normal range compared with control. The raised SOD, platelet counts, lymphocyte counts and the reduced MPO, NLR and MDA are all suggestive of an anti-inflammatory activity of chromium, partly by its suppression of neutrophil infiltration and consequently reducing its over activation. The suppression of MDA values is an important indicator of reduced ROS activity of which the gastrointestinal tract itself is a major producer [29].

Chemokine and cytokines are released following injuries and inflammatory cells release inflammatory mediators which ultimately cause cell death or programmed cell death [30]. Leukocyte's over-activation is a very important step in this process. The level of MPO, an enzyme mainly existing in neutrophil, reflects the level of neutrophil infiltration. The amount of MPO clearly decreased in chromium treated group which suggests that chromium reduced neutrophil in-

filtration *in vivo*.

Trivalent chromium is involved in the structure and expression of genetic information in animals [31] and it also protects RNA from heat denaturation. Chromium is concentrated in cell nuclei and has increased *in vitro* RNA synthesis in mice [32] thus buttressing the hypothesis of its effect on gene functions. Depletion of rRNAs, especially the 18 s rRNA noted on day 3 in all groups and both tissues might imply that it was used up in protein synthesis during epithelial restitution. As the functional organelle for protein synthesis, ribosomes bound to the endoplasmic reticulum (ER) perform complex surveillance of various pathologic stresses [33] [34]. Ribosomal alteration by endogenous and external insults can be a trigger of a variety of pathogenic processes, including inflammatory responses [35] [36] [37].

Furthermore, Interleukin-1 mRNA expression was down-regulated in the colon and being an early response pro-inflammatory cytokines acts with TNF- $\alpha$  (another early response pro-inflammatory cytokine). These two cytokines act through similar pathways [38], by enhancing permeability and coagulation property of the endothelium, induction of arrays of both inflammatory and immune response genes [38]. Interleukin-6 mRNA expression was insignificant except by day 14 in the colon. Interleukin-6 being a complex regulatory cytokine is initially a pro-inflammatory cytokine which then stimulates anti-inflammatory responses through its regulatory influence on TNF- $\alpha$  and IL-1 receptors [39]. This present result is in keeping with the above stated role as continuously elevated levels of IL-6 was observed during the post injury phases in the colon tissues at all points examined. This suggests that IL-6 is acting as an anti-inflammatory regulator throughout the latter stages of injury and healing in this particular study.

Interleukin-10 is a recognized and established anti-inflammatory cytokine [40] and was up-regulated at all point examined after injury compared with control. It acts by down regulating inflammatory immune responses via suppression of macrophages activities and dendritic cells thus inhibiting TH1 responses [40]. Interleukin-10 is also a potent inhibitor of IFN- $\gamma$  [41]. There was a decrease in IFN- $\lambda$  expression on days 7 and 14 after colitis induction in the chromium groups. Certain researchers early reported that pro-inflammatory cytokines, IFN- $\lambda$  and tumor necrosis factor (TNF) blocked intestinal epithelial cell proliferation (IEC) and delayed healing by promoting IEC programmed cell death [42] [43].

However, TNF- $\alpha$  was down regulated on day 3 post colonic injury compared with the control. TNF- $\alpha$  is usually one of the first set of cytokines released after tissue injury and has a spectrum of activities such as, increasing vascular permeability, stimulation of acute phase protein secretion and induction of many pro- and anti-inflammatory cytokines such as IL-6, IL-8, INF- $\gamma$ , and IL-10. Studies on experimental traumatic brain injury models have described strong rise in TNF- $\alpha$  level at injured sites within a few hours following injury, and this cyto-

kine had returned to baseline within the first 24 hour [41] [44] [45]. TNF- $\alpha$  had most probably been up-regulated at an earlier time and had induced an array of pro and anti-inflammatory cytokines, and by now itself down regulated under the influence of elevated levels of anti-inflammatory agents such as IL-10 and IL-6.

## 5. Conclusion

In conclusion, trivalent chromium promotes healing of acetic acid induced injuries by suppressing reactive oxygen species, inhibiting inflammatory tissue markers and down-regulating pro-inflammatory cytokine genes, while promoting expression of anti-inflammatory cytokine mRNA. This study did not report any adverse effect on the normal tissue exposed to chromium in terms of tissue integrity, generation of oxidative radicals and gene expression. This is very important in allaying fears of toxicity to the gastrointestinal cells and tissues. Finally, consumption of Cr<sup>3+</sup>-rich natural products may be beneficial for reversal of some gastrointestinal dysfunctions.

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## Conflict of Interest

Authors declared no conflict of interest.

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