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Research Article

ASSESSMENT OF ACUTE AND SUB CHRONIC TOXICITY OF DEOXYCHOLIC ACID ON ORAL ADMINISTRATION IN RODENTS

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ABSTRACT

Deoxycholic acid (DCA) is one of the secondary bile acids, which are metabolic byproducts of intestinal bacteria and used traditionally to treat immune related disorders. The objective of the present study was to evaluate acute and sub-acute toxicity of DCA in mice and rats after oral administration following OECD test guidelines. Toxicological profile of DCA were evaluated by single-oral toxicity (5, 10, 100 and 200 mg/kg), 14-day repeated oral dose toxicity (1, 2.5 and 5 mg/kg) in mice and 28-days repeated-oral dose toxicity (30, 100, 300 and 1000 mg/kg) in healthy SD rats. Oral single dose of DCA caused decreased body weight and the mice were found lethargic. DCA was lethal at doses ≥ 10 mg/kg and caused mortality/morbidity by 48 hours post dosing. In 14-days repeated oral toxicity study with DCA as oral gavage at 1, 2.5 and 5 mg/kg, no effects on body weight and no treatment related clinical signs were observed. 28-day repeated oral toxicity demonstrates dose-dependent significant increase in WBC (up to 43%), absolute neutrophil (up to 61%) and absolute lymphocyte (up to 42%) counts suggesting an immunostimulatory role on humoral immunity. DCA at doses >1000 mg/kg was toxic and caused anemia. Minimum lethal dose (MLD) and MTD of DCA in mice was estimated to be equal or greater than 10 mg/kg and 5 mg/kg/day, respectively. Oral NOAEL of DCA was30 mg/kg/day in SD rats. DCA did not cause any delayed effects. DCA appears to be safe for human consumption.

Keywords: Deoxycholic acid (DCA), MTD, NOAEL, LD_{50}, RBC, HB, HCT.

1. INTRODUCTION

Deoxycholic acid (DCA) is one of the secondary bile acids, which are metabolic byproducts of intestinal bacteria. The two primary bile acids secreted by the liver are cholic acid and chenodeoxycholic acid. Intestinal microflora metabolizes chenodeoxycholic acid into the secondary bile acid *i.e.* lithocholic acid, and they metabolize cholic acid into deoxycholic acid. There are additional secondary bile acids, such as ursodeoxycholic and tauroursodeoxycholic acid. Deoxycholic acid is soluble in alcohol and acetic acid [1, 2]. Deoxycholic acid (DCA) is a secondary bile salt that is a major active constituent of a Chinese traditional medicine "Niuhuang" that is known to have immunoregulatory and anti-inflammatory properties. Other references in Chinese medicine mentioned the use of Niuhuang in coma and delirium due to febrile diseases, epileptic convulsion by high fever, convulsions in infants, ulcerative gingivitis, retropharyngeal abscess, aphthous stomatitis, large caruncle and furuncle [2]. DCA is also believed to have a secondary function as a hormone that counteracts the effects of some stress hormones, thus indirectly possessing the antioxidant activity. DCA is synthesized in gall bladder and found in all the tissues in human. It is circulated throughout the body in an inactive form. During circulation if it encounters any tumor or inflammation, it converts into active form which induces immune reaction that is beneficial. The immune response induced by DCA is specific and local. Some European publications point towards the effect of DCA as an immunostimulant of the unspecific immune system, activating its main actors, the macrophages. According to these publications, a sufficient amount of DCA on the human body would correspond with a good immune reaction of the unspecific immune system [3]. Bile acids, in either free or conjugated form, bind to the ligand-binding domain of Farnesoid X Receptor (FXR), which forms a heterodimer with retinoid X receptor (RXR). FXR regulates bile acid synthesis, transport, and excretion [4, 5] and lipid and glucose metabolism [4-9].

Additionally, bile acids bind and activate Vitamin D receptor (VDR) [10], and Pregnane X receptor (PXR) which are important in regulating detoxification of bile acids, and xenobiotics [11-13]. Bile acids also modulate cellular signaling pathways that include cyclic AMP synthesis, calcium mobilization, and protein kinase C activation [14] and activate the protein kinase C/Janus N-termina kinase pathway [15]. Bile acids stimulate Kupffer cells to secrete the pro-inflammatory cytokines (TNFα, and IL-1β) that activate the TNF receptor signaling and the MAPK/JNK pathway [16, 17]. Conjugated bile acids activate the EGFR (Epidermal growth factor receptor) and Raf-1/MEK/ERK signaling pathway [18, 19] by inducing mitochondrial reactive oxygen species. Conjugated bile acids activate the ERK and PI3K/AKT pathways via a pertussis toxin-sensitive mechanism through Gαi protein-coupled receptor [20, 21]. FXR knockout mouse show increased incidence of spontaneous hepatic tumors [22]. FXR controls tumor development by inhibiting NF-kB activity and thus antagonizing the inflammation in liver [23]. Bile salt activation of FXR or VDR controls the antibacterial activity in epithelial cells and thus responsible for the innate immunity of epithelium [24-28]. FXR is predominantly expressed in the squamous and columnar epithelium of Barrett esophagus (BE), but not expressed in healthy esophagus [29]. The induced FXR expression enhances the release of chemokines that promote the influx of immune cells, such as neutrophils (IL-8) and B-cells (MIP3α) [30]. Patients treated with DCA show increased levels of mRNA of IL-8 and MIP3α.

Although Deoxycholic Acid was used traditionally as a natural therapeutic product with a variety of beneficial properties, data on systemic oral toxicity are lacking. Therefore, the objective of this study was to determine the maximum tolerated dose (MTD) and no-observed-adverse-effect-level (NOAEL) of DCA after oral acute and sub-acute toxicity of DCA in rodents (mice and rats) after oral administration following Good Laboratory Practice (GLP) regulations and Organization for Economic Cooperation and Development (OECD) test guideline [31].

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

Deoxycholic acid (Sigma-Aldrich, USA.), 1X PBS (GIBCO, CAT# 14190) were used in the study. All the other chemicals used in the studies were analytical laboratory grades procured from approved vendor(s).

2.2. Institutional animal ethics committee clearance

All experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and approved by Institutional animal ethical committee (IAEC) and confirmed to national guidelines on the care and use of laboratory animals (Mice: BIO/IAEC/3263 and Rats: BIO/IAEC/3249).

2.3. Test organisms

2.3.1. Mice

Female SJL mice weighing (18-25 g) were obtained from Jackson laboratory and housed three animals per cage with paddy husk as bedding. Animals were housed in a controlled environment, the temperature was maintained in range of 19 to 25°C, relative humidity in range of 30 - 70 %, a light/dark cycle of 12 hours each and at least 15 fresh air changes per hour. The animals had access to commercial diet and autoclaved potable water *ad libitum*. The animals were identified by marking at the tip of tail using a black indelible marker pen.

2.3.2. Rats

Specific pathogen-free Sprague-Dawley (SD) rats were obtained from In-house, Bioneeds India Private Limited, Devarahosahally, Sompura Hobli, Nelamangala Taluk, Bangalore Rural District, Karnataka, India. The animals were housed under controlled conditions (temperature, 22±2°C; humidity, 40-60%) in the experimental animal facility at Bioneeds accreditedby AAALAC International in accordance with Guide for the Care and Use of Laboratory Animals. These animals were allowed free access to their diet (manufactured by Altromin Spezialfutter GmbH & Co. KG) and tap water with a 12 h light: dark cycle. The rats were adapted to this environment for 1 week prior to study initiation. The animals were identified by marking at the tip of tail using a black indelible marker pen.

2.4. Experimental design

2.4.1. Experimental design for single oral dose toxicity study

The acute toxicity of DCA was carried out according to the up and down procedure (OECD guidelines no. 425). For single oral dose toxicity study, healthy adult female SJL mice weighing between 18 to 25 g were used for the study. Limit test for 200 mg/ kg was carried out in which one animal was dosed with the test dose to evaluate its survival. Additionally, 12 animals were

divided in to 4 groups of 3 each and dosed with vehicle, 5, 10 or 100 mg/kg of DCA administered to mice by oral gavage at dose volume of 10 ml/kg of body weight. The animals were fasted for approximately 6 hours before dosing. Body weights were recorded on day 1 and day 3.The mice were observed for mortality and clinical signs every 0.5, 1.0, 4.0 24 and 48 hours. At study termination, all mice were euthanized by CO_2 asphyxiation.

2.4.2. Experimental design for 14-days repeated oral dose toxicity study

For 14-day repeat-dose toxicity study, healthy female SJL mice were randomly assigned to four groups (5/F/ group). Vehicle (PBS buffer) or graded doses of DCA (1, 2.5 and 5 mg/kg of body weight) were administered to mice by oral gavage once daily for 14 days at dose volume of 10 ml/kg of body weight. The mice were observed daily for mortality and clinical signs for 14 days. Body weights were recorded on days 1, 4, 7, 10 and 14 after the treatment. At study termination, all mice were euthanized by CO_2 asphyxiation.

2.4.3. Experimental design for 28-days repeated oral dose toxicity study

For 28-day repeat-dose toxicity study, healthy SD rats were randomly assigned to five groups (5/sex/group). The test item formulations were administered by oral route at the dose level of 30, 100, 300 and 1000 mg/ kg/day of DCA for 28 days. Similarly, vehicle (0.5% carboxy methyl cellulose) was administered to vehicle control for 28 consecutive days. The dose volume administered was calculated for individual animals on the first day of the treatment and was adjusted according to the most recent body weights. Dose volume was 10 mL/kg body weight. Individual animal body weights were recorded on day of treatment (Day 1) and twice weekly thereafter. Fasting body weight of all animals was recorded at their scheduled terminal sacrifice. Feed consumption was measured at twice weekly intervals. Average feed intake per rat (g/rat/day) was calculated using the amount of feed given and left over in each cage and the number of rats in each cage. Blood samples were collected from all animals on day 29 through retro-orbital plexus puncture under mild Isoflurane anaesthesia. Water *ad libitum* was provided during fasting period. Blood was collected into the tubes containing K_2 EDTA for hematology, and lithium/sodium heparin for clinical chemistry. On day 29, all the animals were sacrificed using carbon dioxide followed by exsanguination and subjected to necropsy and detailed gross pathological examination. Fasting body weight was recorded before the scarification. On the day of necropsy, the spleen and thymus organs were collected and weighed from all the animals.

2.5. Statistical analysis

The experimental results are expressed as mean ± standard deviation (SD) of measurements. The data was subjected to One Way Analysis of Variance (ANOVA) and the significance of differences between the sample means was calculated by Dunnett's test. Null hypothesis was rejected when $p<0.05$ and alternate hypothesis was accepted. Statistical analysis was performed using Graph Pad prism statistical software (version 1.13). Differences were considered to be statistically significant for a *p*-value lower than 0.05 ($p < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Single oral dose toxicity study

As shown in table 1, mild reduction (< 10%) in mean body weight was noticed in all DCA treated animals on day 3. Animal dosed with 200 mg/kg showed lethargy and was found moribund 48 hours post dosing. Similarly, one animal each at 10 and 100 mg/kg showed lethargy and were found moribund 48 hours post dosing. Animal's dosed with 5 mg/kg did not show any signs of toxicity. The maximum tolerated dose (MTD) of DCA after a single dose under fasting condition in female mice was 5 mg/kg.

DCA when administered as a single oral dose in fasted (4-6 hours) female SJL mice at doses 10, 100 and 200 mg/kg was severely toxic. Single dose of DCA caused loss in body weight and the mice were found lethargic. DCA was lethal at doses ≥ 10 mg/kg and caused mortality/morbidity by 48 hours post dosing. The single dose MTD of DCA in SJL mice was 5 mg/kg. Based on this study a high dose 5 mg/kg and subsequent lower doses were selected for repeat dose studies in mice.

3.2. Fourteen-day repeated oral dose toxicity study

No significant effects on body weights were noticed (table 2). No treatment related mortality and clinical signs were observed. In the subsequent study SJL mice that were treated daily for 14 days with DCA as oral gavage at 1, 2.5 and 5 mg/kg, showed no effects on body weight and showed no treatment related clinical signs. This study suggests that the DCA when treated under fasting condition is severely toxic, whereas well tolerated under fed conditions.

3.3. Twenty eight-days repeated oral dose toxicity study

No clinical signs or mortality/morbidity were noted in any of the treated group animals in either sex until terminal sacrifice (table 3).

SD rats were treated for 28 days with DCA as oral gavage at 30, 100, 300 and 1000 mg/kg. A vehicle treated group served as a concurrent control. No clinical signs or mortality/morbidity were noted in any of the treated group animals in either sex.

Table 1: Summary of single dose clinical signs and body weight for mice

Treatment	Dose (mg/kg)	Animal Number	Body weight (g)		Clinical signs				
			Day 1	Day 3	0.5 hr	1 hr	4 hr	24 hr	48 hr
Vehicle control	0	1	22.3	18.07	NAD	NAD	NAD	NAD	NAD
		2	23.6	24.08	NAD	NAD	NAD	NAD	NAD
		3	22.1	22.12	NAD	NAD	NAD	NAD	NAD
		Mean	**22.7**	**21.4**					
		SD	**0.8**	**3.1**					
Treatment	**Dose (mg/kg)**	**Animal Number**	**Body weight (g)**		**Clinical signs**				
			Day 1	**Day 3**	**0.5 hr**	**1 hr**	**4 hr**	**24 hr**	**48 hr**
Deoxycholic acid	5	4	20	19.8	NAD	NAD	NAD	NAD	NAD
		5	21.7	16.93	NAD	NAD	NAD	NAD	NAD
		6	24.9	23.25	NAD	NAD	NAD	NAD	NAD
		Mean	**22.2**	**20**					
		SD	**2.5**	**3.2**					
	10	7	20.9	20.85	NAD	NAD	NAD	NAD	NAD
		8	19.7	17.02	NAD	Lethargy	Lethargy, hunched	Lethargy, hunched	Moribund
		9	22.3	21.23	NAD	NAD	NAD	NAD	NAD
		Mean	**21**	**19.7**					
		SD	**1.3**	**2.3**					
	100	10	20.6	20.55	NAD	NAD	NAD	NAD	NAD
		11	21.9	18.43	Lethargy	Lethargy, hunched	Lethargy, hunched	Lethargy, hunched	Moribund
		12	20.3	19.84	NAD	NAD	NAD	NAD	NAD
		Mean	**20.9**	**19.6**					
		SD	**0.9**	**1.1**					
	200	13	22.2	20.72	Lethargy	Lethargy, hunched	Lethargy, hunched	Lethargy, hunched	Moribund

NAD: No abnormality detected; Data expressed in mean ± SD

Table 2: Summary of 14-day repeated dose of body weights for mice

Dose (mg/kg)	Body Weight (g) mean ± SD				
	Day 1	Day 4	Day 7	Day 10	Day 14
Control	18.6±1.1	19.6±1.0	19.5±1.3	19.7±1.2	19.7±1.1
1	19±0.5	19±0.2	20±0.3	19.2±0.6	21.7±1
2.5	18.6±1.7	18.7±1.2	19.6±1.0	19.5±0.5	19.9±0.4
5	17.8±0.7	17.5±0.2	18.7±0.6	19.4±1.0	18.6±0.5

Table 3: Summary of clinical signs in rats

Dose	Day																										
(mg/kg/day)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	26	27	28
0	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
30	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
100	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
300	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
1000	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

N= Normal

DCA at doses 300 and 1000 mg/kg caused a dose dependent reduction in body weight in males and females from Day 8 till end of the study and was statistically significant on Day 8 (Males), Day 11 (Females) and Day 28 (Males and Females). No treatment related effects on body weights were observed at 30 and 100 mg/kg (table 4).

DCA at doses 100, 300 and 1000 mg/kg caused a dose dependent moderate reduction (15%) in body weight from Day 4 till end of the study (Day 28). These changes in body weight were not associated with any changes in food consumption. No treatment related effects were observed in feed consumption (table 5).

Table 4: Summary of body weights in rats

Dose (mg/kg)	Sex	Day 1	Day 4	Day 8	Day 11	Day 15	Day 18	Day 22	Day 25	Day 28	Necropsy Day
Control	M	172.79±9.89	191.91±9.41	217.8±12.37	234.62±9.44	259.81±12.67	278.27±11.71	272.32±15.29	311.89±15.98	310.47±12.75	300.36±6.12
	F	145.16±7.87	159.29±8.02	174.47±11.38	192.4±6.75	199.33±12.33	208.99±8.07	211.27±11.61	236.73±13.33	240.96±14.73	234.2±16.69
30	M	173.67±10.04	192.83±9.38	212.65±17.5	229.94±17.63	254.62±23.97	277.79±24.11	282.36±22.2	309.39±29.22	313.4±28.47	296.08±26.04
	F	145.37±8.88	159.11±8.84	175.66±12.7	187.58±15.19	203.53±16.38	211.62±14.97	215.09±15.96	235.5±24.68	239.82±25.06	233.24±24.24
100	M	175.47±10.4	194.73±10.96	208.67±11.01	229.03±13.4	260.44±19.48	279.87±23.06	284.37±23.85	280.2±39.31	282.68±31.41	276.44±33.01
	F	145.35±9.05	158.07±9.79	167.87±3.28	180.21±13.26	200.89±16.4	209.78±13.63	213.33±12.47	226.68±26.55	217.38±12.28	210.64±11.32
300	M	174.66±9.77	193.03±9.59	206.81±9.96*	224.85±9.3	250.64±11.58	265.5±15.18	269.64±14.13	294.61±17.45	273.11±14.38**	267.07±18.86
	F	144.41±8.71	157.92±7.85	166.4±8.68	177.82±8.17*	197.56±10.99	205.43±12.15	208.64±12.05	231.05±14.05	211.70±11.1*	202.21±9.31
1000	M	174.72±7.33	193.14±6.57	197.34±10.35	214.64±16.5	241.9±12.07	256.51±10.67	260.58±11.52	290.73±15.64	263.34±11.2*	253.24±9.31*
	F	144.97±7.37	158.53±7.87	167.87±8.03	163.62±15.61*	191.08±13.36	201.77±14.65	205.59±15.34	214±22.73	209.20±15.74*	199.59±19.16

*M: Male; F: Female; n: number of animals; SD: Standard deviation; *: Statistically significant than the control group ($p<0.05$)*

Table 5: Summary of food consumptions in rats

Dose (mg/kg)	Sex	Day (1-4)	Day (4-8)	Day (8-11)	Day (11-15)	Day (15-18)	Day (18-22)	Day (22-25)	Day (25-28)
Control	M	16.75±2.13	20.3 ±0.22	22.41±3.24	16.38±1.31	22.89±1.55	18 ±1.67	23.68±1.78	22.91±0.13
	F	12.11±3	17.61±1.94	18.84±2.42	19.04±0.19	18.89±0.84	13.86±0.52	20.25±2.8	18.28±2.47
30	M	15.44±1.28	25.68±9.82	18.06±1.68	22.92±1.99	19.07±4.05	14.6±2.56	25.68±9.83	25.64±8.76
	F	12.39±1.84	17.18±0.72	18.6±1.74	18.01±1.71	18.79±2.53	14.13±1.74	17.88±1.73	17.06±2.11
100	M	13.98±0.44	20.78±0.1	22.05±1.47	22.06±1.09	21.93±0.73	16.83±0.48	22.07±0.57	23.09±0.24
	F	10.99±0.09	16.57±1.67	16.99±2.51	19.34±2.02	19.74±1.33	14.42±0.93	19.05±0.21	18.06±0.43
300	M	13.18±0.73	20.41±1.12	21.45±0.24	22.06±0.16	22.66±0.2	17.33±0.71	22.08±1.07	23.22±2.87
	F	12.6±1.06	17.01±2.41	19.54±2.66	19.29±3.16	20.33±2.2	16.75±3.38	22.58±6.47	18.16±2.49
1000	M	10.2±1.4	13.05±2.45	16.17±2.76	12.82±2.39	15.47±0.4	12.44±0.62	16.85±0.68	18.78±4.63
	F	13.01±1.59	16.67±2.53	18.43±0.88	16.88±1.66	17.51±1.71	13.9±0.1	19.01±0.08	18.09±0.39

M= Male, F=Female, Mean± SD: Mean ±Standard deviation;

The hematological parameters analyzed in DCA treated animals of both sexes were compared with concurrent vehicle control. DCA at doses 30, 100 and 300 mg/kg in a dose-dependent manner significantly increased WBC, absolute neutrophil and absolute lymphocyte counts in both males and females (Table 6, Figure 1). Significant increase in absolute neutrophil counts was also observed at 1000 mg/kg in males. All the other significant findings were considered not related to treatment as they were incidental and not dose dependent. This data shows that repeat doses of DCA cause an increase in white blood cell indices suggesting an immunostimulatory role on humoral immunity. DCA at 1000 mg/kg caused significant decrease in red blood cell indices (RBC, HB and HCT). This data suggests that DCA at doses >1000 mg/kg is toxic and causes anemia in rats (table 6, fig.2).

DCA at doses 30, 100 and 300 mg/kg in a dose-dependent manner significantly increased WBC (up to 43%), absolute neutrophil (up to 61%) and absolute lymphocyte (up to 42%) counts in both males and females. This data shows that repeat doses of DCA cause an increase in white blood cell indices suggesting an immunostimulatory role on humoral immunity. The effects were lower at 1000 mg/kg, as at this dose body weight loss was higher and it caused anemia.

DCA at 1000 mg/kg caused significant decrease in red blood cell indices (RBC, HB and HCT). This data suggests that DCA at doses >1000 mg/kg is toxic and causes anemia in rats.

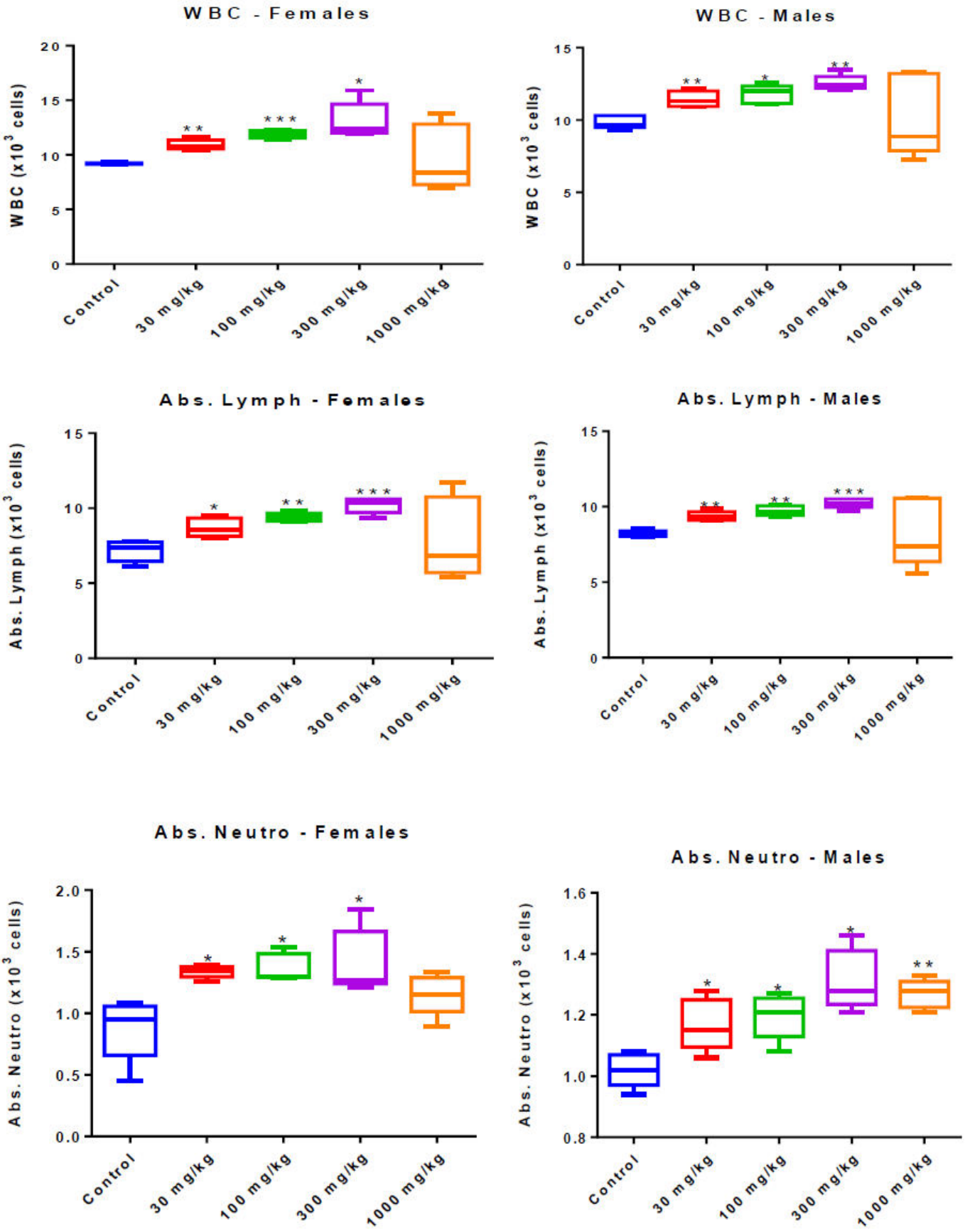

Fig. 1: White blood cell parameters in male and female rats

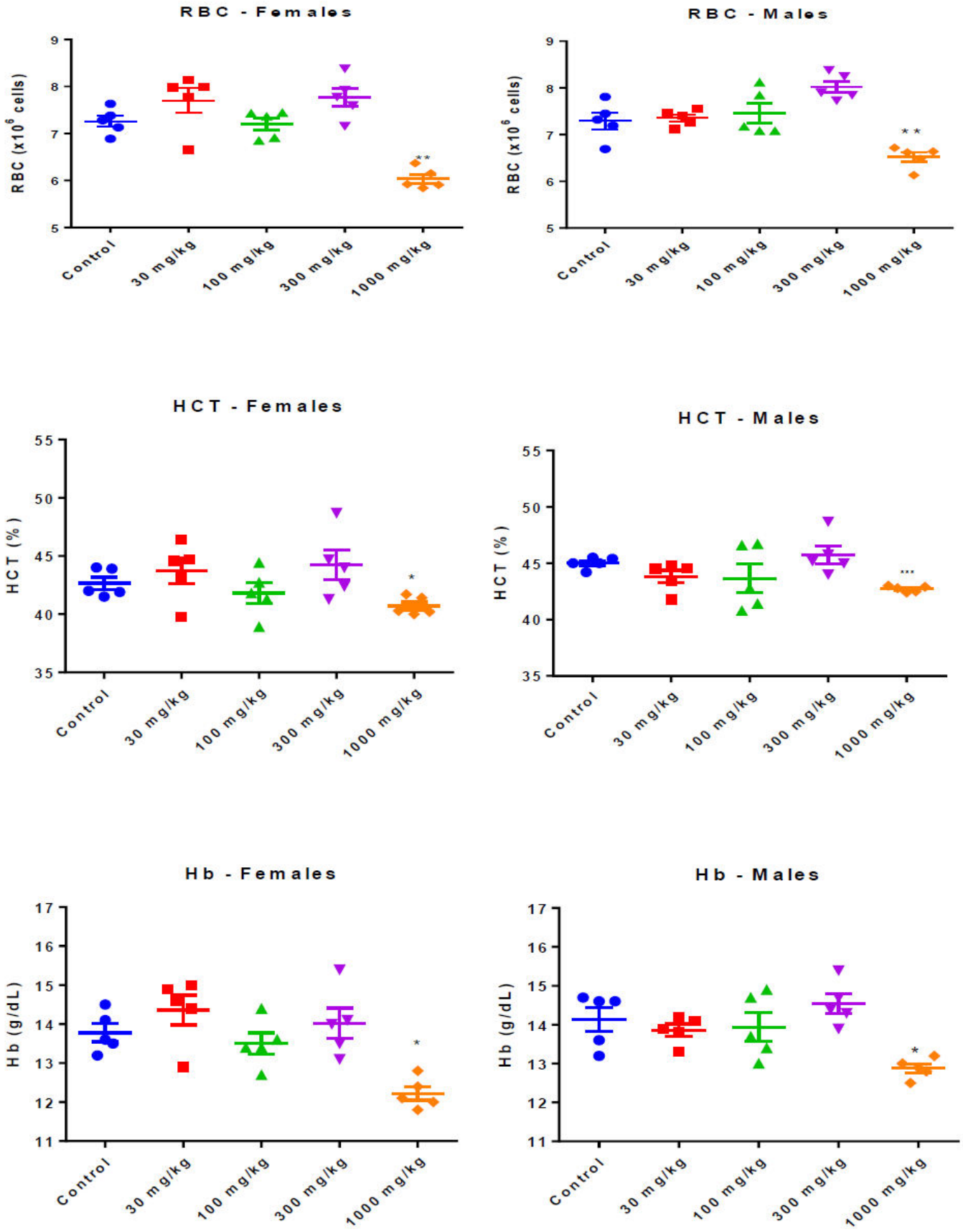

Fig. 2: Effect of high doses of DCA on red blood cell indices in male and female

No treatment related effects on clinical chemistry parameters were observed (table 7).

No treatment related gross pathological findings were observed in any of the tissues/organexamined. DCA treatment at doses 30, 100 and 300 mg/kg caused a dose dependent increase ($p<0.05$) in absolute spleen weights and corresponding increase ($p<0.05$) in spleen index in both sexes. Reduced absolute spleen weights were noticed at 1000 mg/kg, although not statistically significant (table 8 and fig. 3). DCA at 30, 100, 300 and 1000 mg/kg showed a trend of reduction in the absolute thymus weights in both sexes but significantly in females at 100 and 1000 mg/kg (table 8).

Table 6: Summary of haematology parameters in rats

Haematology parameters	Control		30 mg/kg		100 mg/kg		300 mg/kg		1000 mg/kg	
	M	F	M	F	M	F	M	F	M	F
WBC (x103 cells)	9.84±0.44	9.21±0.07	11.40±0.54**	10.91±0.45**	11.80±064*	11.86±0.35***	12.54±0.55**	13.14±1.65*	10.2±2.79	9.68±2.93
RBC(x10^{6}cells)	7.29±0.41	7.26±0.28	7.36±0.17	7.71±0.6	7.46±0.49	7.2±0.29	8.02±0.28	7.77±0.45	6.52±0.23**	6.04±0.22**
HB (g/dL)	14.14±0.69	13.78±0.52	13.86±0.35	14.36±0.85	13.94±0.83	13.5±0.61	14.54±0.56	14.02±0.87	12.88±0.26*	12.22±0.39*
HCT (%)	45.02±0.51	42.66±1.19	43.82±1.25	43.74±2.48	43.66±2.82	41.82±2.01	45.74±1.78	44.22±2.84	42.72±0.26***	40.72±0.77*
MCV (fL)	61.88±3.16	58.78±1.51	59.55±1.49	56.86±1.76	58.56±1.37	58.11±1.91	57.03±1.46*	56.91±1.19	65.61±2.26	67.52±3.08*
MCH(pg)	19.43±1.16	18.98±0.61	18.83±0.29	18.66±0.43	18.7±0.41	18.76±0.59	18.13±0.44	18.04±0.32	19.77±0.42	20.27±1.21
MCHC(g/dL)	31.4±1.25	32.3±0.46	31.63±0.38	32.83±0.51	31.94±0.32	32.29±0.32	31.79±0.24	31.71±0.13	30.15±0.51	30.00±0.45**
PLT(x10^{3}cells)	876.8±171.31	1026.4±82.13	1018.6±121.13	1032.6±121.46	965.6±90.67	1074.8±128.94	1015.6±69.06	1009.4±54.26	1100±150.33	1108.6±148.61
MPV(fL)	6.4±0.12	6.6±0.35	6.26±0.11	6.52±0.35	6.46±0.35	6.76±0.36	6.86±0.46	6.82±0.23	6.62±0044	6.98±0.37
Abs.Retc (x10^{9}cells)	175.76±32.26	110.66±33.61	148.94±28.04	102.46±24.41	177.86±104.64	125.4±26.55	156.34±18.34	122.62±21.15	157.1±46.72	91.98±19.45
Abs.Neut (x10^{3}cells)	1.02±0.05	0.88±0.25	1.17±0.08*	1.34±0.05*	1.20±0.07*	1.37±0.11*	1.31±0.1*	1.42±0.26*	1.27±0.05**	1.15±0.17
Haematology parameters	**Control**		**30 mg/kg**		**100 mg/kg**		**300 mg/kg**		**1000 mg/kg**	
	M	F	M	F	M	F	M	F	M	F
Abs.Lymph (x10^{3}cells)	8.22±0.2	7.16±0.69	9.38±0.33**	8.71±0.62*	9.72±0.32**	9.41±0.29**	10.21±0.3***	10.20±0.54***	8.25±2.23	7.94±2.69
Abs.Mono (x10^{3}cells)	0.28±0.08	0.33±0.1	0.45±0.2	0.39±0.09	0.35±0.08	0.41±0.13	0.45±0.12	0.33±0.09	0.4±0.14	0.28±0.06
Abs.Eosino (x10^{3}cells)	0.15±0.1	0.11±0.03	0.14±0.04	0.07±0.02	0.1±0.04	0.11±0.03	0.1±0.02	0.1±0.02	0.07±0.03	0.13±0.08
Abs.Baso (x10^{3}cells)	0.05±0.01	0.05±0.03	0.05±0.01	0.05±0.01	0.05±0.01	0.04±0.01	0.05±0.02	0.04±0.01	0.05±0.02	0.05±0.02
%Neutro	10.4±0.93	9.51±2.7	10.24±0.56	12.25±0.54	10.15±0.72	11.59±1.18	10.5±0.94	10.72±0.6	13.25±3.73	12.43±2.7
% Lymph	83.66±4.03	77.77±7.14	82.32±3.29	79.84±5.55	82.42±2.75	79.44±3.94	81.52±2.77	78.27±6.93	80.89±3.32	81.29±3.03
% Mono	2.78±0.67	3.6±1.04	3.9±1.64	3.6±0.77	2.95±0.59	3.45±0.99	3.63±0.96	2.48±0.67	3.97±1.3	3.07±0.87
%Eosino	1.52 ±0.93	1.22±0.34	1.22±.035	0.68±0.13	0.82±0.33	0.89±0.25	0.82±0.18	0.79±0.15	0.71±0.2	1.4±1.09
% Baso	0.49±0.11	0.54±0.33	0.42±0.11	0.44±0.13	0.39±0.08	0.34±0.08	0.36±0.13	0.33±0.07	0.48±0.1	0.47±0.12

*M= Male, F=Female, Mean± SD: Mean ±Standard deviation; *: Statistically significant than the control group ($p<0.05$); Abs.Retc=Absolute Reticulocyte; Abs.Neut=Absolute Neutrophil; Abd.Lymph=Absolute Lymphocye; Abs.Eosino=Absolute Eosinophil; Abs.Baso=Absolute Basophil; Neutro=Neutrophil; Lymph=Lymphocyte; Mono=Monocyte; Eosino=Eosinophil; Baso=Basophil.*

Table 7: Summary of Clinical chemistry parameters in Rats

parameters	Control		30 mg/kg		100 mg/kg		300 mg/kg		1000 mg/kg	
	M	F	M	F	M	F	M	F	M	F
Glucose	74.4±8.62	85.6±11.93	82.6±15.32	83.4±8.02	90±7.65	87.8±10.73	90±7.65	87.8±10.73	85.6±11.84	97.6±18.46
Urea	29.44±2.07	34.16±3.57	33.48±3.45	35.02±4.33	32.04±2.01	38.58±5.98	32.04±2.01	38.58±5.98	38.48±4.61*	38.42±6.88
CRE	0.48±0.04	0.56±0.01	0.52±0.05	0.56±0.04	0.5±0.06	0.56±0.02	0.5±0.06	0.56±0.02	0.53±0.04	0.57±0.03
CHO	52.2±5.17	67.4±7.57	57.8±5.81	64±9.92	54.8±5.02	65.2±14.06	54.8±5.02	65.2±14.06	58.2±7.89	77±6.71
Trig	62.8±17.47	67.2±18.46	45.2±9.52	40.6±9.15	52.2±16.07	55.8±27.24	52.2±16.07	55.8±27.24	58±13.69	52.2±12.13
T. Bil	0	0	0.01±0.02	0.05±0.06	0	0	0	0	0.01±0.01	0.01±0.02
TP	6.32±0.48	7.28±0.28	6.82±0.37	7.34±0.38	7.12±0.55	7.32±0.39	7.12±0.55	7.32±0.39	6.94±0.17	7.56±0.51
parameters	**Control**		**30 mg/kg**		**100 mg/kg**		**300 mg/kg**		**1000 mg/kg**	
	M	F	M	F	M	F	M	F	M	F
Alb	2.78±0.09	3.18±0.19	2.89±0.2	3.29±0.36	3.07±0.32	3.09±0.14	3.07±0.32	3.09±0.14	3.07±0.15*	3.25±0.14
ALT	37.2±8.56	32±11.51	41±4.95	36.8±14.04	43.4±11.37	42.6±12.18	43.4±11.37	42.6±12.18	71.6±51.62	43.4±13.99
AST	82±25.86	75.4±18.04	79.8±10.43	86.80±15.9*	79±16.51	90±12.75	79±16.51	90±12.75	109.4±68.41	78±4.74
ALP	181.4±26.01	109.4±7.09	210±54.29	140.00±39.88**	234.8±29.32	156.8±37.99	234.8±29.32	156.8±37.99	207.2±27.4	120.4±37.82
CA	9.1±0.59	10.02±0.51	9.64±0.35	10.18±0.4	9.82±0.73	10.04±0.4	9.82±0.73	10.04±0.4	9.7±0.22	10.42±0.65
Phos	6.28±0.78	6.3±0.58	6.56±0.58	6.4±0.44	7.04±0.96	6.16±0.23	7.04±0.96	6.16±0.23	6.72±0.35	6.66±0.39
Glb	3.54±0.42	4.1±0.23	3.57±0.42	4.29±0.49	3.93±0.27	4.05±0.11	4.05±0.25	4.23±0.29	3.87±0.13	4.31±0.42

M= Male, F=Female, Mean± SD: Mean ±Standard deviation; Cre=Creatinine; CHO=Cholesterol; Trig=Triglycerides; T. Bil=Total Bilirubin; TP=Total Protein; Alb=Albumin; CA=Calcium; Phos=Phosphorus; Glb=Globulin.

Table 8: Summary of Absolute organ weights (g) in rats

Absolute Organ Weights (g)	Control		30 mg/kg		100 mg/kg		300 mg/kg		1000 mg/kg	
	M	F	M	F	M	F	M	F	M	F
Thymus	0.83±0.22	0.67±0.06	0.54±0.18	0.54±0.16	0.56±0.12	0.51±0.06*	0.51±0.07	0.54±0.1	0.5±0.07	0.54±0.03*
Spleen	0.66±0.04	0.61±0.03	0.74±0.04*	0.69±0.01*	0.81±0.05*	0.70±0.02*	0.88±0.04**	0.82±0.04**	0.58±0.12	0.54±0.19
Spleen Index	0.22±0.01	0.26±0.03	0.25±0.01	0.3±0.03	0.29±0.02*	0.33±0.02	0.33±0.03**	0.41±0.02**	0.23±0.05	0.28±0.13
Thymus Index	0.28±0.07	0.29±0.02	0.2±0.03	0.24±0.08	0.2±0.05	0.25±0.04	0.19±0.02	0.27±0.06	0.2±0.03	0.27±0.02

*M= Male, F=Female, Mean± SD: Mean ±Standard deviation; *: Statistically significant than the control group (p<0.05)*

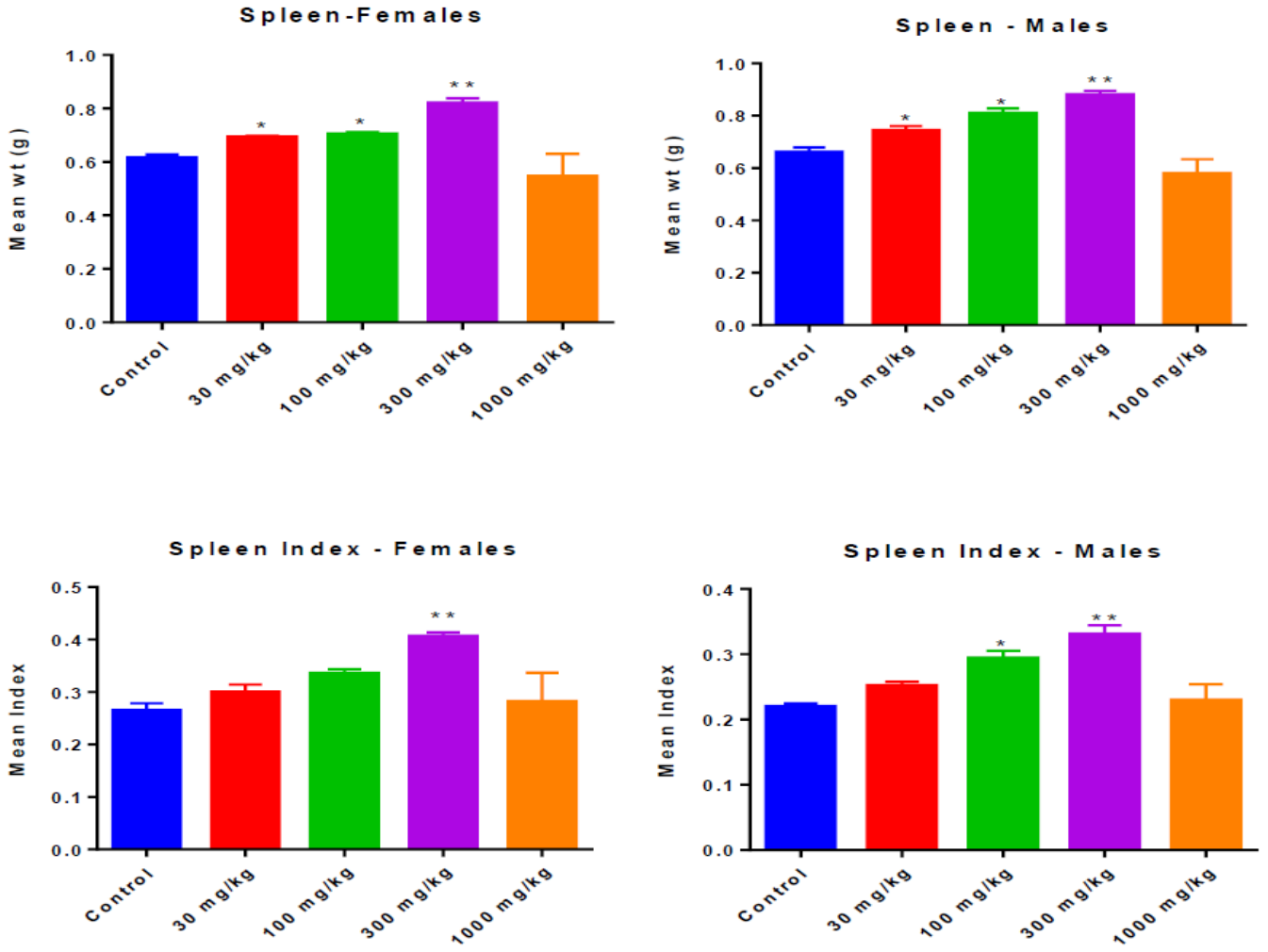

Fig. 3: Effect of DCA on absolute Spleen weights (g)

DCA treatment at doses 30, 100 and 300 mg/kg caused a dose dependent increase (up to 34%) in absolute spleen weights and corresponding increase (up to 58%) in spleen index in both sexes. Reduced absolute spleen weights were noticed at 1000 mg/kg, although not statistically significant. DCA at 30, 100, 300 and 1000 mg/kg showed a trend of reduction in the absolute thymus weights in both sexes but significantly in females at 100 and 1000 mg/kg. No histopathological evaluations of lymphoid organs were performed.

Niuhuang (Bile stone from Oxen) is indicated treatment of smallpox, madness and delirium. Other references in Chinese medicine mentioned the use of Niuhuang in coma and delirium due to febrile diseases, epileptic convulsion by high fever, convulsions in infants, ulcerative gingivitis, retropharyngeal abscess, aphthous

stomatitis, large caruncle and furuncle [32]. Bile acid products, including Niuhuang and bear bile, have been used in China and other Asian countries as therapeutic for thousands of years and possess anti-spasmodic, anti-pyretic, cardio-tonic, hypotensive, anticonvulsive, anti-inflammatory, and immunoregulatory activity [33-36]. As DCA was well tolerated under fed conditions, higher doses were selected for assessment of its toxicity profile in future studies. The current study is the first to provide important background on safety concerns of DCA by analyzing the traditional sub-acute toxicity profile of DCA comprehensively under OECD guideline.

4. CONCLUSION

Results of single and 14-days repeated oral dose toxicity studies clearly supported that administration of DCA did not exert adverse effect in female mice for most toxicological factors. In particular, Single dose of DCA caused loss in body weight and the mice were found lethargic. DCA was lethal at doses ≥ 10 mg/kg and caused mortality/morbidity by 48 hours post dosing. The single dose MTD of DCA in SJL mice was 5 mg/kg. Sub-acute exposure of mice to DCA showed no effects body weight and treatment related clinical signs. Taken together, sub-acute exposure of SD rats to DCA exhibited no observed adverse effect levels (NOAEL) at dose up to 30 mg/kg. This corresponds to about 4.86 mg/kg in human by using the conversion help according to dosing adjustment guidelines of the US Food and Drug Administration.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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