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ROLE OF DEOXYCHOLIC ACID IN MODULATING HUMORAL AND CELL MEDIATED IMMUNE SYSTEM

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ABSTRACT: Deoxycholic acid (DCA) is a secondary bile salt that is a major active constituent of Chinese traditional medicine, "Niuhuang" which is known to have immunoregulatory and anti-inflammatory properties. Although data from the literature indicate possible immunomodulatory effects of Deoxycholic acid on the immune system, little has been reported regarding the mode of activity of DCA in modulating the immune system. The objective in the present study was to determine the effect of Deoxycholic acid (DCA) on immune parameters in mice and rats. The current study employed both *in-vitro* and *in-vivo* protocols in mice and rats. *In-vitro* study results indicate that DCA causes splenocyte proliferation at lower doses but is cytotoxic to splenocytes at higher doses were observed in these supernatants that were sensitized with DCA versus splenocytes derived from control animals. Significant differences between normal and DCA treated animals were observed in the following parameters: white blood cell indices, absolute weights of lymphoid organs, and spleen cell proliferation to DCA stimulation. These results indicate that DCA treatment modulates the immune system by activating both the cell-mediated and humoral immune systems.

INTRODUCTION: The immune system plays a vital and complex role in host defense system. An immune system includes several types of cells, of which some are immunostimulants, and others are immunosupperessors in their functions. Immuno-modulation is the improvement of immune reactions by immunostimulant agents. An immuno-modulatory agent can act *via* its effect on the humoral immune system, cell-mediated immune system, both or through non-specific interaction with various components of immune functions [1].

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Bile acid products, including Niuhuang (Bile stone from Oxen) 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, anti-convulsive, anti-inflammatory, and immuno-regulatory activity.

Deoxycholic acid (DCA) is the major active component of these traditional Chinese drugs. DCA is also known as deoxycholate, cholanoic acid, and 3α, 12α-dihydroxy-5β-cholanate, is a secondary bile acid that is synthesized in higher mammals. Deoxycholic acid 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 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 [2, 3].

In the human body, Deoxycholic acid is used in the emulsification of fats for absorption in the intestine. Outside the body, it is used in the experimental basis of cholagogues and is also in use to prevent and dissolve gallstones. In research, Deoxycholic acid is used as a mild detergent for the isolation of membrane-associated proteins. The critical micelle concentration for Deoxycholic acid (DCA) is approximately 2.4-4 mM. Sodium deoxycholate, the sodium salt of DCA, is often used as biological detergent to lyse cells and solubilize cellular and membrane components. Deoxycholate and bile acid derivatives, in general, are actively being studied as structures for incorporation in nanotechnology [3].

DCA is chemically classified as secondary bile acid, and apart from regular functions of bile acids, it has a completely different function of activation of the immune system. This effect on immune system could be either modulation or stimulation.

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 the gall bladder and found in all the tissues in humans. It is circulated throughout the body in an inactive form. During circulation, if it encounters any tumor or inflammation, it converts into the active form, which induces an 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. Clinical studies conducted in the 1970s and 1980s confirm the expectation that DCA is involved in the natural healing processes of local inflammations, different types of herpes, and possibly cancer [4].

Bile acids involve different physiological processes that include regulation of self-homeostasis, adaptive responses to cholestasis and liver insults, lipid metabolism, and glucose metabolism [5]. FXR knockout mice show an increased incidence of spontaneous hepatic tumors [6]. FXR controls tumor development by inhibiting NF-kB activity and thus antagonizing the inflammation in the liver [7]. The hepato-biliary tract is constantly exposed to enteric bacteria and bacterial products but still maintains a sterile milieu. This is possible through many defense arsenals such as bile salts, bile flow, IgA, and mucous secretion [8]. Bile salts act as direct bacteriolytic owing to their amphipathic nature [9]. Bile salts also increase mucous thickening and bile flow, which reduces the colonization potential of bacteria in epithelium [10]. Bile salt activation of FXR or VDR controls the antibacterial activity in epithelial cells and thus responsible for the innate immunity of epithelium [11-16]. FXR, which regulates the synthesis, absorption, and excretion of bile acids, is expressed on various immune cells. The FXR expressed on immune cells may activate the immunological responses [15, 16].

FXR plays a role in inducing the immune chemokines IL-8 and MIP3α in the Barrett esophagus (BE) patients. FXR is predominantly expressed in the squamous and columnar epithelium of BE, but not expressed in healthy esophagus [17]. Chronic reflux induces FXR expression in healthy squamous epithelium. The patients exposed to DCA (Deoxycholic acid) showed increased expression of FXR and FXR specific chemokines. The increased expression of FXR upregulates the target genes SHP and IBABP, which regulates the bile acid metabolism. These genes have increased expression in BE epithelium. The BE exposed to potentially toxic bile acids has increased the presence of a functional bile acid transport system, an adaptation. 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) [18]. Patients treated with DCA show increased levels of mRNA of IL-8 and MIP3α.

In spite of some literature evidence suggesting the immunomodulatory effects of Deoxycholic acid though, at present, there are no dedicated and extensive studies conducted with DCA to establish

its effects on the complete immune system. The objective of the current work is to evaluate the effects of DCA on the humoral and cell-mediated immune system.

MATERIALS AND METHODS:

Chemicals and Reagents: Deoxycholic acid (Sigma-Aldrich, USA.), DMEM High Glucose Media (GIBCO, CAT# 11995), RPMI 1640 (GIBCO, CAT# 22400), Penicillin-Streptomycin (GIBCO, CAT# 15140), HEPES (GIBCO, CAT# 15630), Fetal Bovine Serum (FBS) (GIBCO, CAT# 10082), Teva-Copaxone (US Sourced), Mannitol (Formulation buffer) (SIGMA,CAT# M4125), 1X PBS (GIBCO, CAT# 14190), RBC Lysis buffer (SIGMA, CAT#R7757), Ammonium Chloride (SIGMA, CAT#A9434), Potassium Bicarbonate (SIGMA, CAT#60339), EDTA (SIGMA, CAT# E6758), Superblock (Thermo Scientific pierce, CAT#37545), CD 3 V500-A (BD, CAT# 260771), CD 45 Per CP-Cy5.5-A (BD, CAT# 550994), CD 8 PE-CY7-A (BD, CAT# 552877), NKp46FITC-A (BD, CAT# 560156), FMO CD4 PE (BD, CAT# 553653) and FMO B220 FITC (BD, CAT# 553087). All other chemicals used in the studies were analytical laboratory grades procured from an approved vendor.

Institutional Animal Ethics Committee: 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 the 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).

Dose Formulation Preparation: The required strengths of dose formulation were prepared in freshly prepared Phosphate Buffered Saline (PBS) or carboxymethylcellulose (0.5%). The dose formulations were suspensions and were mixed thoroughly by a magnetic stirrer with a magnetic bead at the time of dosing.

Repeated Dose Study in SJL 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 the range of 19 to 25°C, relative humidity in range of 30-70%, a light/dark cycle of 12 h each, and at least 15 fresh air changes per hour. The animals had access to a 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. Total 20 mice were segregated into vehicle control and three different treatment groups of five each for DCA. The vehicle control group was dosed with a 10 mL/kg dose of PBS. The three treatment groups were dosed with DCA at 1, 2.5, and 5 mg/kg doses, respectively. The mice were dosed orally using oral gavage attached to a 1 ml syringe. The dose volume to each mouse was 10 mL/kg of body weight. The animals were dosed daily for 14 days. The animals were euthanized on Day 15, and serum and intact spleens were collected [19].

Serum Collection and Spleen Harvest: After the completion of 15 days, approximately 500 µL of blood from each mouse was collected by retro-orbital bleeding. The blood was allowed to coagulate for 1 hr, and serum was separated by centrifugation at 5000 rpm for 20 min. The mice were euthanized on the 15th day; intact spleens were collected aseptically and transferred to ice-cold 10 mL DMEM medium with 10% FBS [20].

Preparation of Single-Cell Suspension of Splenocytes: The spleen was homogenized using GentleMACS Dissociator. Spleen was immersed in a dissociation buffer, and the homogenization was performed using the dissociator. The homogenized material was collected aseptically and passed through 70µm cell strainer. The cell suspension was centrifuged at 1200 rpm for 10 min at RT. The supernatant was discarded, and the pellet was re-suspended in 10mL of gentle MACS buffer and centrifuged at 1200 rpm for 5 min at RT. The supernatant was discarded, and the pellet was re-suspended in 1mL of RBC lysis buffer and incubated for 3 minutes at RT. 10mL of complete DMEM medium was added to the suspension and centrifuged at 800 × g (1200 rpm) for 5 minutes at RT. The supernatant was discarded, and the pellet was re-suspended in 10mL assay medium (RPMI with 10% FBS) [20].

***In-vitro* Stimulation of Splenocytes with DCA and Proliferation Assay:** Deoxycholic acid was diluted in assay medium (complete RPMI with 10% FBS) to get a final concentration of 5µg/mL

and 10 µg/mL. The splenocyte cell count was determined using an automated cell counter, and cells were plated at 5×10^5 cells / 100 µL per well in assay medium into 96 well U-bottom plates in quadruplicates. 125 µL of DCA solution was added to each well and incubated at 37 °C in humidified (70%) CO_2 incubator for 48 h. 0.5 µCi of 3H – thymidine in the supplemented medium was added per well after 74-78 h of incubation and harvested over the G/F plate after 18 hours of incubation [21]. Counts per Minute (CPM) was measured with a Beta counter. Data analysis was performed by using the below-mentioned formula.

Proliferation Assay:

ΔCPM= Average CPM of Test - Average CPM of Cells alone

IL-13 ELISA: Cell culture supernates were evaluated for IL-13 using a commercial kit, Quantikine Mouse IL-13 Immunoassay (R&D Systems, Cat #PM1300CB). The method was a sandwich ELISA and was performed as per the instructions are given in the kit insert [22].

IFN-γ ELISA: IFN-γ evaluation in cell culture supernatants was performed using a commercial kit, Quantikine IFN-γ Immunoassay (R & D Systems, Cat # PMIF00). The method was sand, which ELISA and was performed as per the instructions are given in the kit insert [23].

IL-13 and IFN-γ Assays: The average absorbance of standards, controls, and samples was determined. The mean absorbance of the blank sample was subtracted from the mean absorbance of standards, controls, and samples.

The concentration of standards and corresponding absorbance was converted to log base 10, and a linear curve was plotted. Concentration of the test samples was calculated using the linear equation Y= mX + C where, m = Slope, C = Intercept, Y = Absorbance and X = Concentration of sample. The obtained concentration was converted to antilog and corrected with the dilution factor.

Repeated Oral Dose Study in SD Rats: Sprague Dawley rats were obtained from In-house, Bio needs India Private Limited and housed three animals per cage with paddy husk as bedding. Animals were housed in a controlled environment, the temperature was maintained in the range of 19 to 25 ºC, relative humidity in range of 30-70%, a light/dark cycle of 12 hour each, and at least 15 fresh air changes per hour. The animals had access to a 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. Total 50 rats were segregated in to vehicle control and four different treatment groups of five per sex per group for DCA. The vehicle control group was dosed with 10 mL/kg dose of carboxy methyl cellulose (0.5%). The four treatment groups were dosed with DCA at 30, 100, 300, and 1000 mg/kg doses, respectively. The rats were dosed orally by using ball tipped stainless steel gavage needle (18G for rats). Dose-volume to each rat was 10 mL/kg of body weight. The animals were dosed daily for 28 days. The animals were euthanized on Day 29, and blood, intact spleens, and thymus were collected[19].

Blood Collection and Organs Harvest: Blood samples were collected from all animals on day 29 through retro-orbital plexus puncture under mild Isoflurane anesthesia. Water was provided *ad libitum* during the fasting period. Blood was collected into the tubes containing K_2 EDTA. Hematology parameters was estimated using Advia 2120 Hematology system (Siemens Limited).On the day of necropsy, the spleen and thymus were collected and weighed from all the animals.

Statistical analysis: The experimental results are expressed as mean ± standard error (SE) of triplicate measurements. The data were subjected to One Way Analysis of Variance (ANOVA), and the significance of differences between the sample means was calculated by Dunnett's test. The null hypothesis was rejected when $p<0.05$, and the alternate hypothesis was accepted. Statistical analysis was performed using Graph Pad prism statistical software (version 1.13).

RESULTS:

Repeat Dose Study in SJL Mice: Single-cell suspension of splenocytes was prepared from intact spleen for each animal. These splenocytes were plated at 5×10^5 cells / 100 µL per well and incubated with either 5 or 10 µg/mL concentration of DCA for 48 h. Post 48 h, a proliferation of splenocytes was evaluated by [^{3}H] thymidine assay, and the culture supernatant was analyzed for the levels of IFN-γ and IL-13 by ELISA.

Splenocytes Proliferation: The *in-vitro* proliferation study of mouse splenocytes was evaluated by [^{3}H] thymidine assay. The results are illustrated in **Table 1**. The splenocytes, derived from mice treated with 1 mg/kg, upon stimulation with DCA showed a dose-dependent increase in proliferation (Δ CPM) at 5 µg/mL (~22%) and 10 µg/mL (~88%), respectively. On the contrary, splenocytes derived from mice treated with 2.5 and 5 mg/kg showed a significant decrease (up to 30%) in proliferation. These results suggest that DCA potentiates splenocyte proliferation at lower doses but is cytotoxic at higher doses.

TABLE 1: EFFECT ON SPLENOCYTES PROLIFERATION

Splenocyte proliferation: Δ CPM					
***In-vivo* treatment**		***In-vitro* stimulation**			
		5 µg/ml		**10 µg/ml**	
Treatment	**Dose (mg/kg)**	**Mean**	**SEM**	**Mean**	**SEM**
Vehicle	0	1781	267	1602	533
DCA	1	2183	659	2946	642
DCA	2.5	1873	327	1137	358
DCA	5	1234	473	1243	636

Data expressed in mean ±SEM

TABLE 2: SUMMARY OF IFN-γ LEVELS IN CELL SUPERNATANT

IFN-γ : pg/mL					
***In-vivo* treatment**		***In-vitro* stimulation**			
		5 µg/ml		**10 µg/ml**	
Treatment	**Dose (mg/kg)**	**Mean**	**SEM**	**Mean**	**SEM**
Vehicle	0	BLQ	NA	BLQ	NA
DCA	1	BLQ	NA	9	2
DCA	2.5	BLQ	NA	21	10
DCA	5	BLQ	NA	6	2

Data expressed in mean ±SEM, NA: Not applicable

Effect on cytokines (IFN-γ and IL-13): The splenocyte culture supernates were evaluated for IFN-γ and IL-13 levels using commercial ELISA kits. Quantifiable levels of IFN-γ and IL-13, though non-dependent, were observed in these super-natants that were sensitized with DCA versus splenocytes derived from control animals, suggesting a role of DCA in inducing cytotoxic T-cells. The results are illustrated in **Table 2** and **Table 3**.

TABLE 3: SUMMARY OF IL-13 LEVELS IN CELL SUPERNATANT

IL-13 : pg/mL					
***In-vivo* treatment**		***In-vitro* stimulation**			
		5 µg/ml		**10 µg/ml**	
Treatment	**Dose (mg/kg)**	**Mean**	**SEM**	**Mean**	**SEM**
Vehicle	0	BLQ	NA	BLQ	NA
DCA	1	113	16	80	13
DCA	2.5	32	4	BLQ	NA
DCA	5	BLQ	NA	BLQ	NA

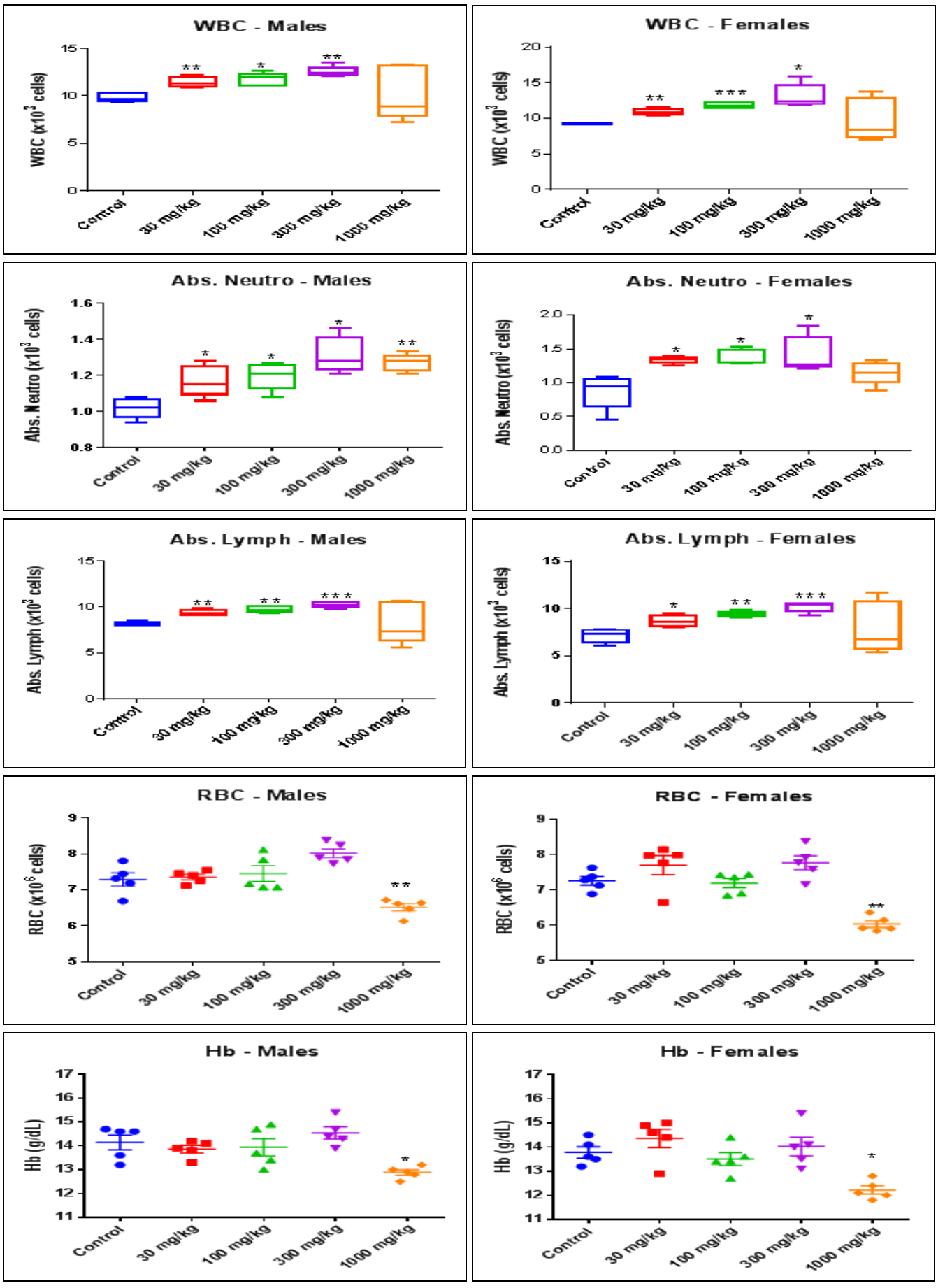
Data expressed in mean ±SEM, NA: Not applicable

Repeated Oral Dose Study in SD Rats: 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. 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 causes an increase in white blood cell indices suggesting an immunostimulatory role on humoral immunity. DCA at 1000 mg/kg caused a 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. The results are illustrated in **Table 4** and **Fig. 1**.

TABLE 4: SUMMARY OF HEMATOLOGICAL PARAMETERS

Dose (mg/kg)	Sex	RBC (×10^6 cells)	HB (g/dL)	HCT (%)	WBC (×10^3 cells)	Abs. Neut (×10^3 cells)	Abs.Lymph (×10^3 cells)
Control	M	7.29±0.41	14.14±0.69	45.02±0.51	9.84±0.44	1.02±0.05	8.22±0.2
	F	7.26±0.28	13.78±0.52	42.66±1.19	9.21±0.07	0.88±0.25	7.16±0.69
30	M	7.36±0.17	13.86±0.35	43.82±1.25	11.40±0.54**	1.17±0.08*	9.38±0.33**
	F	7.71±0.6	14.36±0.85	43.74±2.48	10.91±0.45**	1.34±0.05*	8.71±0.62*
100	M	7.46±0.49	13.94±0.83	43.66±2.82	11.80±0.64*	1.20±0.07*	9.72±0.32**
	F	7.2±0.29	13.5±0.61	41.82±2.01	11.86±0.35***	1.37±0.11*	9.41±0.29**
300	M	8.02±0.28	14.54±0.56	45.74±1.78	12.54±0.55**	1.31±0.1*	10.21±0.3***
	F	7.77±0.45	14.02±0.87	44.22±2.84	13.14±1.65*	1.42±0.26*	10.20±0.54***
1000	M	6.52±0.23**	12.88±0.26*	42.72±0.26***	10.2±2.79	1.27±0.05**	8.25±2.23
	F	6.04±0.22**	12.22±0.39*	40.72±0.77*	9.68±2.93	1.15±0.17	7.94±2.69

M: Male; F: Female; Data expressed as means ± SD, SD: Standard deviation; *: Significantly different from Control group ($p < 0.05$): **$p < 0.01$: RBC: Red Blood Cells; HB: Hemoglobin; HCT: Hematocrit; WBC: White Blood Cells; Abs.Neut: Absolute Neutrophils; Abs.Lymph: Absolute Lymphocytes


WBC - Males
WBC (x10³ cells)
Control
30 mg/kg
100 mg/kg
300 mg/kg
1000 mg/kg
WBC - Females
Abs. Neutro - Males
Abs. Neutro (x10³ cells)
Abs. Neutro - Females
Abs. Lymph - Males
Abs. Lymph (x10³ cells)
Abs. Lymph - Females
RBC - Males
RBC (x10⁶ cells)
RBC - Females
Hb - Males
Hb (g/dL)
Hb - Females

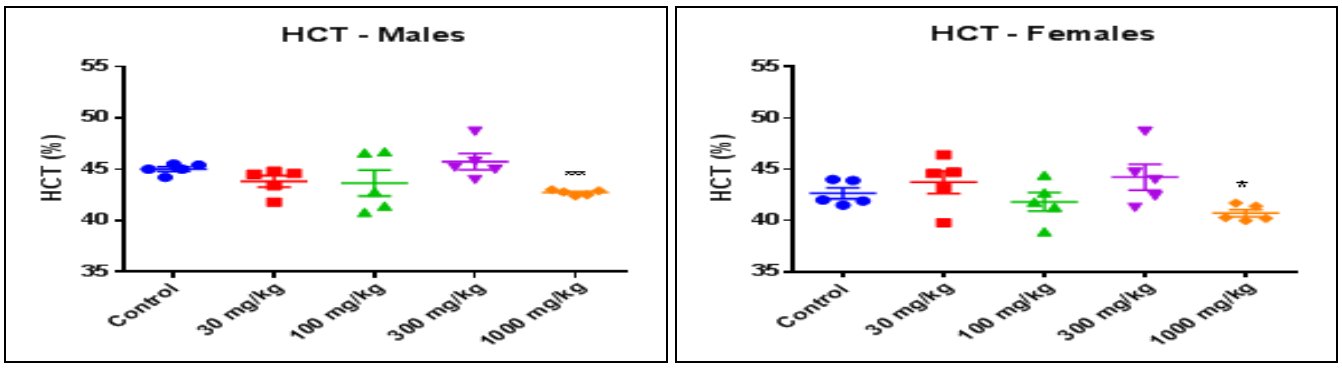


FIG. 1: HEMATOLOGY PARAMETERS OF MALE AND FEMALE

DCA treatment at doses 30, 100, and 300 mg/kg caused a dose-dependent increase ($p<0.05$) in absolute spleen weights and a 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. 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. The results are illustrated in **Table 5** and **Fig. 2**.

TABLE 5: SUMMARY OF ABSOLUTE ORGAN WEIGHTS (g)

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

M: Male; F: Female; Data expressed as means ± SD, SD: Standard deviation; *: Significantly different from Control group ($p < 0.05$): **$p < 0.01$.

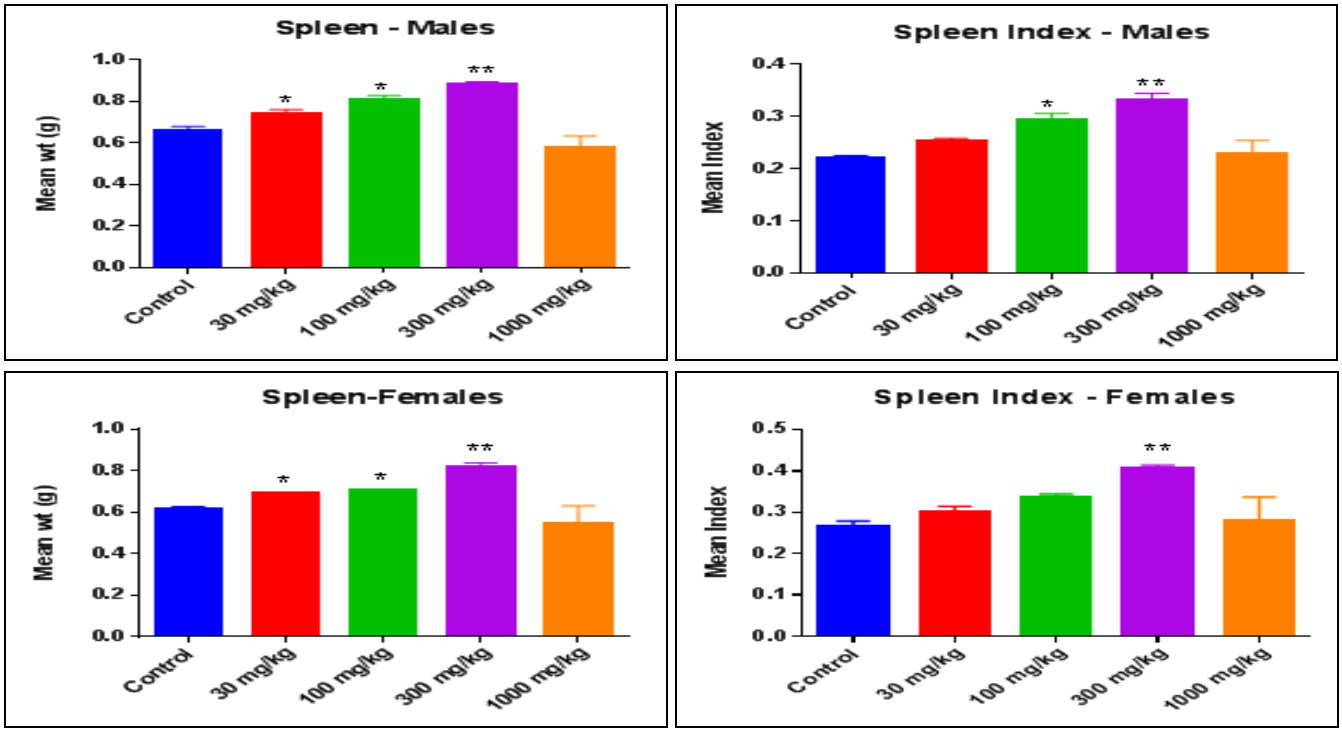


FIG. 2: EFFECT OF DCA ON ABSOLUTE SPLEEN WEIGHTS (g) *p<0.05; **p<0.01

DISCUSSION: Deoxycholic acid (DCA) has been used in traditional therapies and is shown to have immunomodulatory properties. Preliminary evidence have shown that DCA modulates the immune system by way of stimulation and helps in maintaining a disease-free state within an individual. 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 [24]. 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, cardiotonic, hypotensive, anticonvulsive, anti-inflammatory, and immunoregulatory activity [25, 26, 27, 28]. The mechanism of action of DCA as an immunomodulatory agent is not studied in detail and no studies exist in the literature that shows that DCA acts *via* its effect on the humoral immune system or cell-mediated immune system or both or through non-specific interaction with various components of the immune functions.

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.

Effect on Immune System: The primary splenocytes, that were derived from SJL mice treated with 1 mg/kg of DCA for 14 days, when sensitized with DCA, showed a dose-dependent increase in proliferation (Δ CPM) at 5 µg/mL (~22%) and 10 µg/mL (~88%), respectively. On the contrary, splenocytes derived from mice treated with 2.5 and 5 mg/kg showed a significant decrease (up to 30%) in proliferation. These results suggest that DCA causes splenocyte proliferation at lower doses but is cytotoxic to splenocytes at higher doses. The *in vitro* doses of DCA need to be further titrated down (i.e., <5 µg/mL) to establish similar effects at higher *in vivo* doses. Quantifiable levels of IFN-γ and IL-13, though non-dependent, were observed in these supernatants that were sensitized with DCA versus splenocytes derived from control animals, suggesting a role of DCA in inducing cytotoxic T-cells.

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.

Dose (mg/kg)	WBC	Absolute Neutrophil	Absolute Lymphocyte
30	↑18	↑52	↑22
100	↑29	↑56	↑31
300	↑43	↑61	↑42
1000	↑5	↑31	↑11

↑=% Increase (Max among both sexes)

Dose (mg/kg)	RBC	Hemoglobin	HCT
30	NC	NC	NC
100	NC	NC	NC
300	NC	NC	NC
1000	↓17	↓15	↓5

NC=No change (< 5%); ↓=% Decrease (Max among both sexes)

Dose (mg/kg)	Spleen	Spleen Index	Thymus	Thymus Index[&]
30	↑13	↑15	↓35	↓29
100	↑23	↑32	↓33	↓29
300	↑34	↑58	↓39	↓34
1000	↓12	NC[#]	↓40	↓29

NC=No change (< 5%); ↓=% Decrease (Max among both sexes); ↑=% Increase (Max among both sexes); # = Decreased in males, increased in females; & = No statistical significance was observed.

In summary, DCA significantly modulates the cell-mediated immune system as elucidated by; *in-vitro* stimulation of splenocyte proliferation and increased white blood cell indices, the humoral immune system as elucidated by; increased spleen weights. Specifically, DCA-induced secretion of IFN-γ (produced predominantly by Th1 cells) and IL-13 (produced predominantly by Th2 cells[29]) from splenocytes suggests a similar stimulatory effect on Th1 and Th2 cells.

CONCLUSION: The results show that DCA has stimulatory effects on the immune system mainly through activation of cell-mediated immune and humoral immune systems. As the results are

promising the investigator is having a future idea to extend the investigations to further animal models and conduct clinical trials to assess the potential of using DCA as a nutrient supplement in foods to improve or boost the immune system.

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