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Immunotoxic potential of sodium fluoride following subacute exposure in Wistar rats

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Fluoride pollution in drinking water is an international problem as the fluoride present is often at levels above acceptable limits. This study was done with an objective to determine the immunotoxic potential of sub acute exposure of sodium fluoride in Wistar rats with special reference to the Dinitroflurobenzene contact skin sensitivity test and pathological alterations in splenic histoarchitecture. The rats were intoxicated orally to sodium fluoride at dose levels of 5, 25 and 50 mg/kg body weight, respectively for 28 days. On day 21 the external surface of right ear pinna of rats were sensitized to 40 μl of 2% Dinitroflurobenzene in vehicle followed by challenge application of 40 μl of 1% Dinitroflurobenzene in vehicle on day 25. The left ear pinna served as control and 40 μl of vehicle (4:1 acetone- olive oil) was applied. The increase in ear thickness (in mm) was measured with Engineers micrometer at 0, 6, 12, 24 and 48 h post challenge. The results revealed a dose dependent decrease ($p < 0.001$) in mean ear thickness (in mm) of sodium fluoride intoxicated rats following challenge application of 1% Dinitroflurobenzene in vehicle. The blood picture revealed dose dependent leucocytosis associated with neutrophilia and lymphocytopaenia. There was also dose dependent decrease in organosomatic index of spleen and severe depletion of lymphocytes in white pulp of spleen. This report highlights the proposition that prolonged exposure to fluoride contaminated drinking water is likely to result in immunotoxicity.

Key words: Fluoride, immunotoxicity, rats, dinitroflurobenzene, spleen.

INTRODUCTION

Environmental pollution is a dangerous problem globally and one of the most complex health hazards results from fluorides. Fluorine being most electronegative element holds its ubiquitous presence. Fluorides are naturally occurring harmful contaminant in the environment (Raghuvansi et al., 2010). It is a cumulative poison and thus leads to fluorosis; a serious public health problem. Endemic fluorosis is prevalent in many parts of the world where drinking water contains more than 1 to 1.5 ppm of fluoride and causes damage not only to hard tissues of teeth and skeleton, but also to soft tissues, such as brain, liver, kidney, spleen and endocrine glands (WHO, 1984;

Santhakumari and Subramanian, 2007; Sharma et al., 2007; Ozsvath, 2009).

Fluoride can be an adjuvant for mucosal and systemic immunity and is reported to affect oral immunity in chickens. Studies with sodium fluoride (NaF), however, indicate that fluoride can damage human lymphocyte chromosomes, induce adverse effects in the spleen (Podder et al., 2010) inhibit growth and general health in rabbits and increase their nonspecific immune-related acid phosphatase and lysozyme activities (Liu et al., 2012).

Spleen is the largest secondary lymphoid organ

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containing about one-fourth of the body's lymphocytes and initiates immune responses to blood-borne antigens (Nolte et al., 2002; Balogh et al., 2004). This function is charged to the white pulp which surrounds the central arterioles. The white pulp is composed of lymphocytes (B cells, CD4+ T cells and CD8+T cells), macrophages, dendritic cells, plasma cells, arterioles, and capillaries in a reticular framework (Cesta, 2006). Fluoride is reported to alter the histoarchitecture of spleen by causing reduction in the content of white pulp with concomitant increase in red pulp infiltrated by lymphocytes (Machalinska et al., 2002). Das et al. (2006) also observed disorganization in the histoarchitecture of the spleen and thymus of male albino rats after NaF treatment. Fluoride causes depletion of energy reserves due to its adverse effects on several enzymes and thus impairs the immune functions of white blood cells leading to reduction in the immune functions (Sharma et al., 2004).

The relationship between fluoride and immunity in animals is an ongoing topic of discussion and debate. The present communication is intended to put forth the immunotoxic potential of sodium fluoride on cell mediated immune response in Wistar rats being assessed by Dinitroflurobenzene contact skin sensitivity test together with the pathological alterations in blood picture pertaining to the leucocytes and histoarchitecture of spleen.

MATERIALS AND METHODS

Experimental design and test chemical

The experimental investigation was planned to adjudge the toxicopathological effects of sodium fluoride on cell mediated immune response in Wistar rats after obtaining approval from Institutional Animal Ethics Committee. The rats were maintained under regular supervision in controlled environment with 12 h light dark cycle and provided with standard feed and water *ad libitum* throughout the experimental period. The rats were acclimatized for 10 days before commencement of the experimental study. Twenty four healthy Wistar rats weighing 195 to 215 g (females) and 220 to 235 g (males) were randomly divided into four different groups having 6 (3 males + 3 Females) rats each. Rats of Group I served as control and were given only distilled water, orally. Rats of Groups II, III and IV were administered NaF orally, dissolved in distilled water, at the dose rate of 5.0, 25.0 and 50.0 mg/kg body weight for 28 days. These doses were selected based on the results of acute toxic dose of NaF determined by approximate lethal dose method. The test chemical Sodium fluoride (NaF) of 99% purity was obtained from Merck Limited, Mumbai- 400018.

Assessment of cell mediated immune response by dinitroflurobenzene (dnfb) contact skin sensitivity test

Cell mediated immune response; based on delayed type hypersensitivity reaction was measured by Dinitroflurobenzene (Sigma Chemical Co. Ltd., U.S.A.) contact skin sensitivity test as described by Phanuphak et al. (1974) and later slightly modified by Tamang et al. (1988). DNFB contact skin sensitivity test for

monitoring cell mediated immune response was studied on day 25 following primary sensitization at day 21.

Briefly, on day 21 of the experiment, 4 rats were randomly selected from each group and marked individually for this test. Dorsal aspects of ears (external pinna) of the selected rats were cleaned. Left ear was kept as control and only 40 µl of vehicle (4 acetone: 1 olive oil) was applied. The dorsal aspect of right ear of each rat was used to measure the contact sensitivity to DNFB. All the selected rats of each group were sensitized with 40 µl of 2% DNFB in 4: 1 acetone olive oil solution to the right ear. Four days following primary sensitization; on day 25, the sensitized rats were challenged with 40 µl of 1% DNFB in 4: 1 acetone olive oil solution to the right ear and the left ear was treated as respective control. Ear thickness (in mm) was measured with Engineer's micrometer at 0, 6, 12, 24 and 48 h post challenge. The clinical observations were recorded.

Haematology

At the end of the experiment on day 28 under diethyl ether anaesthesia blood samples were collected in heparinised vials from retro-orbital venous plexus of rats as described by Moore (2000). Thin blood smears were prepared for differential leukocyte count during blood collection. The total leucocytic count (TLC) was done as per Jain (1986), by using W.B.C. diluting fluid (Merck Limited, Mumbai - 400018) and Haemocytometer (Neubauer's chamber and WBC diluting pipette). Total leucocytes counts are expressed in thousands/cu.mm of blood. The differential leucocytic count (DLC) was done as per Coles (1986), using Leishman's stain (Merck Limited, Mumbai- 400018). The percentages of different leucocytes were determined by examining the stained blood smear under oil immersion objective lens of light microscope.

Organo-somatic index of spleen

Following humane sacrifice and after recording the gross lesions, the spleen were carefully removed, blotted free of blood and weighed (in grams) over electronic digital balance. The organo-somatic index (OSI) was calculated by using the following formula as per Chattopadhyay et al. (2011).

$$\text{Organo-somatic index (OSI)} = \frac{\text{Organ weight (g)}}{\text{Live body weight (g)}} \times 100$$

Histopathology of spleen

The tissue samples of spleen were collected in 10% neutral buffered formalin for histopathological studies. The tissues were thoroughly washed in running water; dehydrated in ascending grades of alcohol; cleared in benzene and embedded in paraffin at 58°C. The paraffin embedded tissue sections of 4 to 5 µm were obtained as described by Luna (1972) and stained with haematoxylin and eosin (H and E) as per the method described by Bancroft and Stevens (1990) with slight modifications. The stained sections were examined under light microscope and the lesions were recorded, if any.

Statistical analysis

Statistical analysis was done using complete randomized design (CRD) -single factor analysis of variance by Snedecor and Cochran (1968). The mean values between treatment and control groups

Table 1. Contact skin sensitivity response (Mean increase in ear thickness in mm) after challenge application of 1% Dinitroflurobenzene of different experimental groups during sub acute sodium fluoride toxicity (Left side served as vehicle control and right side treated with DNFB) n = 4.

Experimental groups	Ear side	Ear thickness before sensitization	Ear thickness after challenge with DNFB at different time interval (hours)			
			6	12	24	48
Group I	Left	0.80 ± 0.90	0.80 ± 0.29	0.80 ± 0.41	0.80 ± 0.22	0.80 ± 0.30
	Right	0.80 ± 0.86[a]	1.14 ± 0.39[ab]	1.15 ± 0.54[b]	2.17 ± 0.19[a]	1.97 ± 0.48[a]
Group II	Left	0.80 ± 0.82	0.84 ± 0.36	0.87 ± 0.24	0.89 ± 0.31	0.88 ± 0.59
	Right	0.81 ± 0.65[a]	1.06 ± 0.44[b]	1.31 ± 0.51[a**]	1.86 ± 0.43[b**]	1.74 ± 0.65[b**]
Group III	Left	0.81 ± 0.75	0.83 ± 0.43	0.87 ± 0.81	0.91 ± 0.55	0.89 ± 0.73
	Right	0.81 ± 0.73[a]	1.16 ± 0.50[a*]	1.27 ± 0.92[a**]	1.62 ± 0.16[c**]	1.50 ± 0.86[c**]
Group IV	Left	0.80 ± 0.81	0.84 ± 0.22	0.89 ± 0.80	0.92 ± 0.31	0.88 ± 0.70
	Right	0.81 ± 0.65[a]	1.13 ± 0.31[ab]	1.25 ± 0.71[a**]	1.48 ± 0.50[d**]	1.38 ± 0.84[d**]

Values indicate Mean ± S.E. Superscripts may read column wise for comparison of means. Mean values with similar superscripts do not differ significantly from each other. *significant at $P \leq 0.05$ from control group (Group I) and **significant at $P \leq 0.01$ from control group (Group I).
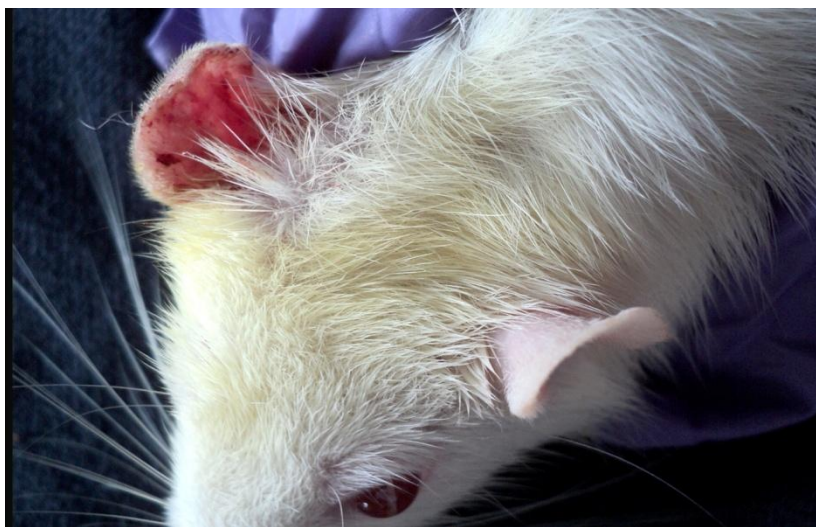

Figure 1. Ear of a control rat showing erythema 6 h post challenge application of 1% DNFB.

were tested for critical difference (CD), if any. The results are expressed as Mean ± S.E.

RESULTS

Cell mediated immune response to DNFB contact skin sensitization test

The observations of mean increase in ear thickness (in mm) of Wistar rats post challenge at different time interval is summarised and presented in Table 1.

Challenge application of 1% DNFB to the right ear caused erythema (Figure 1) followed by vesiculation and scabbing. Vesiculation was observed at 12 h which persisted up to 18 h in control rats. The ruptured vesicles were also noticed (Figure 2). The ear lesion turned into a scab between 20 and 24 h post challenge application (Figure 3) accounting for very intense increase in ear thickness. All these changes (erythema, vesicle and scabbing) were most prominent in rats of control group. However, there was moderate and mild erythema and vesiculation in rats of Groups II and III exposed to NaF at 5 and 25 mg/kg body weight, respectively which gradually

Figure 2. Ruptured vesicle on dorsal surface of right external pinna in a control rat 12 h post exposure to 1% DNFB challenge.

Figure 3. Profound increase in thickness of right external pinna in a control rat 24 h post exposure to 1% DNFB challenge due to scab formation.

became normal by 24 h. Furthermore, the intensity of ear lesion was very meagre in the rats of Group IV being intoxicated by the highest dose of NaF at 50 mg/kg body weight (Figure 4). A dose dependent decrease (P ≤ 0.01) in ear thickness of NaF intoxicated rats was observed at 12 to 48 h post challenge in comparison to the control rats. Moreover, the maximum immunological response was observed 24 h post challenge in the rats of control group (Group I). The scabs were found to be persisting even at 48 h in control group (Figure 5).

Haematology

The administration of NaF orally for 28 days to Wistar rats caused leucocytosis in rats of Group III (P ≤ 0.05) and Group IV (P ≤ 0.01) as compared to rats of Groups I and II. There were remarkable lymphocytopaenia (P ≤ 0.01) and neutrophilia (P ≤ 0.01) of all the rats belonging to Groups II, III and IV as compared to rats of control group (Group I). Non significant alterations were recorded for the monocyte percent and eosinophil percent in NaF

Figure 4. Very mild change in right ear thickness in a rat of Group IV (exposed to sodium fluoride at 50 mg/kg body weight, orally) 24 h post exposure to 1% DNFB challenge.
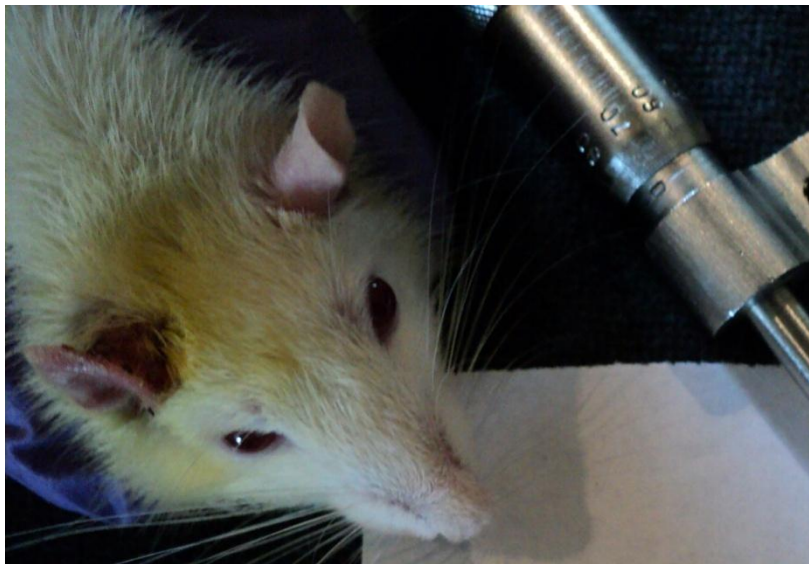

Figure 5. Persisting scab and increased thickness of right external pinna in a control rat 48 h post exposure to 1% DNFB challenge.

intoxicated groups with respect to the control rats. The results have been summarised and presented in Table 2.

Organosomatic index

The organosomatic index of spleen was found to be decreased (P ≤ 0.01) in comparison to the control rats following intoxication of rats with NaF. Further, the decrease was in a dose dependent manner. The results

have been represented in Figure 6.

Pathmorphology of spleen

At necropsy, spleen of control (Group I) rats appeared as normal. On the contrary, mild to moderate decrease in the size of spleen was observed in the rats of Group III and IV. The histopathological examination of spleen of Group IV represented several necrotising lymphocytes

Table 2. Effect of daily oral administration of sodium fluoride for 28 days on the leucocytic parameters of Wistar rats (n = 6).

Parameter	Groups			
	Group I	Group II	Group III	Group IV
TLC (Thousands/ cu.mm)	7.01 ± 0.01^c	7.02 ± 0.05^c	$7.20 \pm 0.04^{b*}$	$7.42 \pm 0.09^{a*}$
Lymphocyte (%)	66.75 ± 0.25^a	$58.00 \pm 0.82^{b**}$	$48.00 \pm 0.41^{c**}$	$41.25 \pm 0.25^{d**}$
Monocyte (%)	4.75 ± 0.25^a	4.25 ± 0.28^a	4.25 ± 0.24^a	4.25 ± 0.25^a
Neutrophil (%)	24.75 ± 0.48^d	$33.75 \pm 0.75^{c**}$	$44.25 \pm 0.48^{b**}$	$51.25 \pm 0.48^{a**}$
Eosinophil (%)	3.75 ± 0.25^a	4.00 ± 0.41^a	3.50 ± 0.29^a	3.25 ± 0.25^a
Basophil (%)	00.00	00.00	00.00	00.00

Values indicate Mean ± S.E. Superscripts may read row wise for comparison of means. Mean values with similar superscripts do not differ significantly from each other. *significant at $P \leq 0.05$ and **significant at $P \leq 0.01$ from control group (Group I).
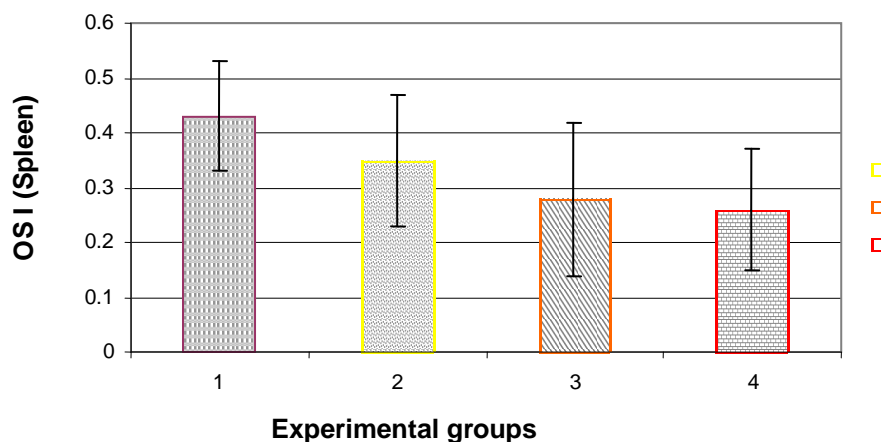

Figure 6. Organosomatic index of spleen in various experimental groups intoxicated orally with sub acute sodium fluoride toxicity.

and proliferation of reticuloendothelial cells. In addition to these changes, there was marked depletion of lymphocytes in the white pulp of spleen particularly in the periarteriolar lymphoid sheath (Figure 7). The splenic histoarchitecture in rats of Group III revealed a moderate depletion of lymphocytes in the white pulp (Figure 8) as compared to those from Group IV rats. On the other hand very mild depletion was noticed in splenic sections of Group II rats. No abnormalities could be identified in the splenic histological sections from control rats (Figure 9).

DISCUSSION

Contact hypersensitivity is a T- cell mediated cutaneous immune response to reactive haptens (Elmets and Bowen, 1986). After exposure of the skin to contact allergens, haptens covalently bind to discrete amino acid residues on carrier proteins. The epidermal Langerhans cell, a member of the dendritic-cell family, takes up haptenated proteins and processes them into antigenic peptides which are transported to the cell surface in association with major histocompatibility complex molecules (Griem et al., 1998; Wang et al., 2001). Matos et al. (2005) reported that DNFB induces the activation of the extra cellular signal-regulated kinases ERK1/2 and p38, and also up regulates CD40 expression.

The results of this study are in close agreement with Das et al. (2006) who reported that NaF treatment lowered cellular immunity in the rats. They observed a significant diminution in peripheral blood lymphocyte, monocyte and neutrophil counts in conjunction with a reduction in splenocyte counts. They had also reported the effects of NaF treatment on humoral immunity with lowered levels of plasma IgG specific to bovine serum albumin. On the other hand, De Vos et al. (2004) reported that mercurous chloride and NaF significantly suppressed concanavalin- A induced gamma-IFN
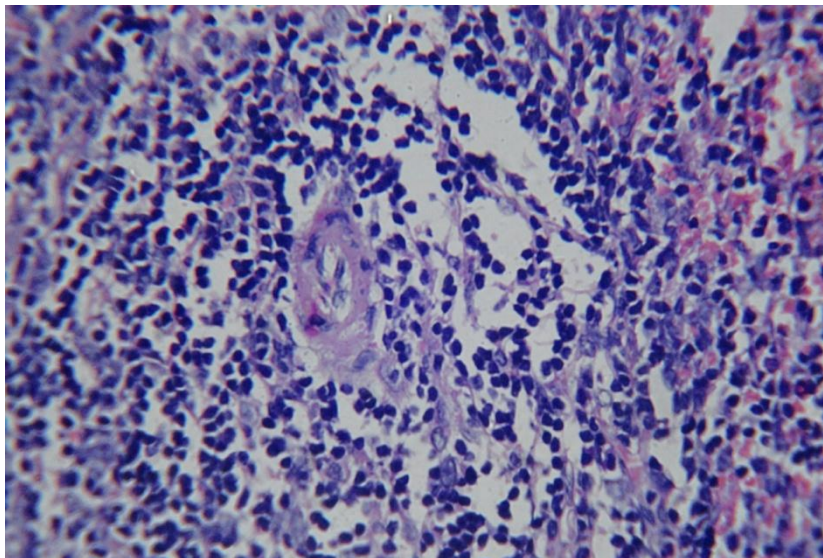
Figure 7. Microphotograph of spleen from Group IV rat (given NaF at 50 mg/kg body weight, orally) representing severe depletion of lymphocytes from white pulp, and proliferation of reticuloendothelial cells (H and E X 400).
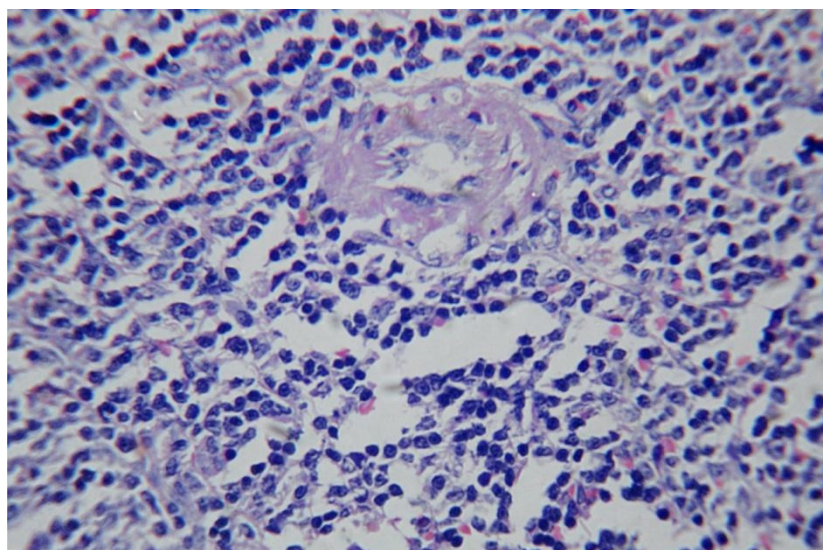

Figure 8. Microphotograph of spleen of a rat from Group III (given NaF at 25 mg/kg body weight, orally) showing moderate depletion of lymphocytes from white pulp (H and E X 400).

(interferon) production. They also reported suppressed Th_1 activity and stimulated Th_2 cytokine production in vitro on human peripheral blood mononuclear cells.

Our findings of significant increase in TLC is in accordance with the previous reports by Pillai et al. (1988) in Swiss albino mice exposed to 5.2 mg fluoride/kg body weight for 35 days and in rats treated with fluoride water containing1.5, 3, 4.5 and 6 ppm for 60 days (Sharma et al., 2004). Contrary to this Sharma et al.

(2007) reported no change in TLC in female rats exposed to water containing 6 ppm NaF for 15 and 30 days. Rao and Vidyunmala (2010) also documented dose and duration independent normal values in case of mice intoxicated with 5 and 10 mg fluoride/day for 30 and 60 days. Moreover, Santhakumari and Subramanian (2007) observed dose and duration dependent leucocytopaenia in Wistar rats exposed to 25 mg fluoride /l/rat/ day for 8 and 16 weeks. The decreased immune response in our
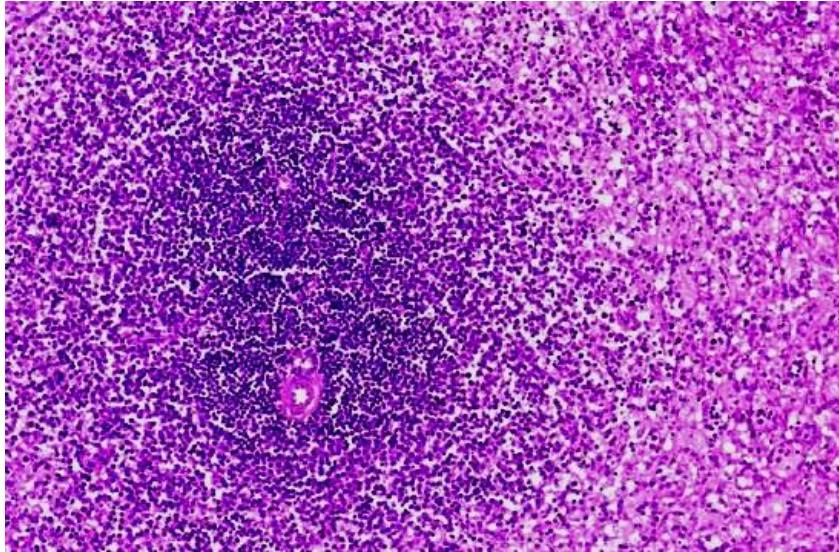
Figure 9. Normal histoarchitecture of spleen of a control rat (Group I) depicting the cell population in white pulp and adjoining red pulp.

study might be due to fluoride induced stress which further adds to immunosuppression. As fluoride alters cell membrane stability and integrity, it is also possible that membranous targets within immunocompetent cells which are essential for the induction of the contact hypersensitivity response are particularly vulnerable by fluoride. Immunosuppression could also have occurred due to complement-mediated damage to immune cells as opined by Elmets and Bowen (1986).

The reduction in organosomatic index of spleen are also in same line with those recorded by Podder et al. (2010) who observed that NaF when given at doses of 15 and 150 ppm through drinking water for 30 or 90 days in Swiss albino mice results in a decreased organo- somatic index of spleen without any change in body weight. However, in the present study a significant reduction in body weight of the rats was also recorded in NaF intoxicated groups. Fluoride causes a significant increase in the G0/G1 fraction and a decrease in G2/M fractions of cell cycle, fortifying the interpretation of G1 blockage of DNA synthesis and inhibition of proliferation of the mouse splenocytes (Podder et al., 2010). The decrease in white pulp content likewise signifies perturbation of haemopoiesis and accumulation of G0/G1 population; in agreement with the report by Zhou et al. (2009) that excessive fluoride intake seriously damages the specific immune function in rabbits. The nature of altered histoarchitecture of splenic pulps observed herein is in concordance with the findings of earlier workers. High fluoride (400, 800 and 1200 mg/kg) treatment in chickens through their diets decreases the number of splenic nodules, the lymphocyte population within splenic nodules and periarterial lymphatic sheaths in white pulp (Chen et al., 2009).

Podder et al. (2010) attributed the histopathological changes in spleen to fluoride induced cytotoxicity and to apoptosis. Alike our findings they too reported, decreased white pulp, increased red pulp and mononuclear infiltration in red pulp. They also recorded an increase in the percent of dead cells in the spleen. The mononuclear cells in white pulp of spleen of Group IV appear very dark which might be due to fluoride induced cytotoxicity as fluoride has been reported to generate toxic free radicals inducing oxidative stress (Xi et al., 2012). The dose dependent, mild to severe depletion in lymphocytes from white pulp of spleen of rats in Groups II, III and IV also explains the decrease in immune response with respect to the rats of control group and justifies the observations of contact skin sensitivity to rat ear against challenge dose of DNFB in the present study. From the observations and the results of this study, it is concluded that fluoride impairs the cell mediated immunity owing to a marked decrease in the number of leucocytes and the associated stress. Moreover, the immunotoxic potential of fluoride is marked and very significant in sufficiently higher doses. There is an urgent need to get rid off the dreadful and spreading tentacles of fluoride problem globally by exploring the possible alternatives against fluoride induced immunotoxicity.

Abbreviations: NaF, Sodium fluoride; **DNFB,** Dinitroflurobenzene.

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