HISTOPATHOLOGY AND CELL CYCLE ALTERATION IN THE SPLEEN OF MICE FROM LOW AND HIGH DOSES OF SODIUM FLUORIDE

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SUMMARY: Administration of sodium fluoride (NaF) in lower (15 mg/L) and higher (150 mg/L) doses through drinking water for 30 or 90 days in Swiss albino mice resulted in a decrease in the organo-somatic index (OSI) accompanied by a decline in the white pulp content of the spleen. The percentage of dead cells and decrease in white pulp content in the spleen were highest in mice exposed to the lower dose of fluoride (F) for 90 days. An elevated level of G_0/G_1 fraction of cells was noticed in mice exposed to the higher dose of F for 30 days as well as to the lower dose of F for 90 days, suggesting a G_0/G_1 blockage due either to inhibition of DNA synthesis or perturbation of synthesis of cyclin, a regulator of cell cycle. No trace of DNA damage was evident from the DNA fragmentation study in any treatment group.

Keywords**:** DNA synthesis; Fluoride and spleen; Mouse spleen histopathology; Spleen cell cycle; Spleen white pulp.

INTRODUCTION

The spleen is one of the major lymphoid organs of the body and plays a vital role in hemopoiesis especially during fetal life in humans. In mice it is actively involved in blood cell production throughout life.[1,2] Because studies on the toxic effects of fluoride (F) on mammalian spleen are somewhat limited, we decided to examine *in vivo* effects of F on the organo-somatic index (OSI), cell viability, histological architecture, cell cycle pattern, and DNA fragmentation at low (15 mg/L) and high (150 mg/L) concentrations of NaF in drinking water for different time durations in Swiss albino mice. The lower level corresponds to the F-concentration (6.8 mg F ion/L) within the range reported in some potable water supplies in India and other countries.[3] Recently, we reported genotoxic effects of these two drinking water concentrations of NaF on mouse bone marrow cells.[4,5] The aim of the present study was to investigate the effect of the same concentrations of F in spleen of mice to gain a better understanding of the potential immunotoxic insult of F on people in fluoride endemic areas.

MATERIALS AND METHODS

Male Swiss albino mice, aged 2–3 mo, weighing 25–30 g, were maintained in community cages in a controlled-temperature room at 20±2ºC and a 12-hr light/ 12-hr dark cycle. The mice were fed a standard mouse diet procured from NMC Oil Mills Ltd, Pune, India and were provided with water *ad libitum.* All regulations of the Institutional Animal Ethics Committee were strictly followed during the experiment. Mice were randomly divided into four groups with 6 mice in each group (Table 1) and were exposed to 0, 15, and 150 mg NaF/L through

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their drinking water for 30 days and 15 mg NaF/L through their drinking water for 90 days.

Table 1. Experimental design (6 mice per group)

Mice	Treatment
Control	Non-exposed animals (control)
Group I	15 mg NaF/L through their drinking water for 30 days
Group II	150 mg NaF/L through their drinking water for 30 days
Group III	15 mg NaF/L through their drinking water for 90 days

Organo-somatic index (OSI): At the end of the treatment, the body weights of the mice were recorded. The mice were sacrificed by cervical dislocation. The spleen was dissected out carefully, blotted free of blood, and the fresh weight recorded. The OSI was calculated and compared with that of the control mice.

$$\text{Organo-somatic index (OSI)} = \frac{\text{Wt of the organ (g)}}{\text{Wt of the mouse (g)}} \times 100$$

Histological studies: Spleen tissues were treated with Bouin's fixative, dehydrated through graded alcohol, and embedded in paraffin. Tissue sections were stained by the standard hematoxylin-eosin (HE) technique, and the slides were viewed under a light microscope (Olympus, BX 41) with 10 × 10 magnifications and subsequently photographed.

Flow cytometry: Spleen tissues were dissociated using RPMI-1640 medium and passed through a 10-µm diameter mesh to prepare cell suspensions containing 1 x 10^6 cells. Cell viability was assessed by the 0.5% trypan blue exclusion test, and flow cytometric analysis was conducted using propidium iodide as reported earlier.[5]

DNA fragmentation: Agarose gel electrophoresis was carried out for detection of DNA fragmentation. DNA was isolated using "Genei Pure mammalian genomic DNA purification kit (GeNei, Bangalore India)" according to the manufacturer's protocol. The purified DNA was subjected to electrophoresis on 1.2% agarose gel and visualized by ethidium bromide staining.

Results are expressed as mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA). If this analysis indicated significant difference ($p<0.05$) among the group means, then unexposed (control) and exposed groups (Groups I, II, and III) were compared by Fisher's t test using the Sigma plot 8.0 statistical package.

RESULTS

No significant changes were found in body weight of any of the F-treated mice groups compared with the control group (Figure 1).

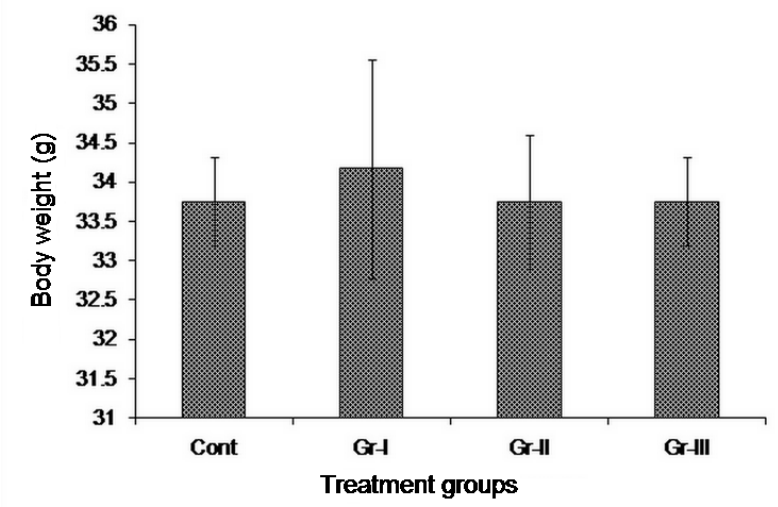

Figure 1. Effect of fluoride on body weight of mouse (n =6) following *in vivo* administration of NaF (mg/L) in the drinking water. Values are mean ± SEM and are not significantly ($p < 0.05$) different from the control group.

Spleen organ weight decreased by an average of 17.44%, 19.39%, and 16.96% (Figure 2) in Groups I, II, and III, respectively, although these changes are not significant.

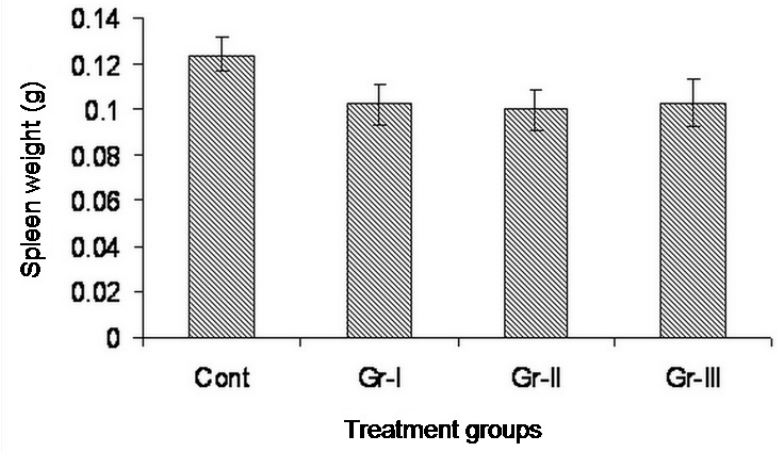

Figure 2. Effect of fluoride on the spleen weight of mouse (n =6) following *in vivo* administration of NaF (mg/L) in the drinking water. Values are mean ± SEM and are not significantly ($p < 0.05$) different from the control group.

As seen in Figure 3, ingestion of F through drinking water resulted in a decrease in the OSI of spleen in all the treatment groups.

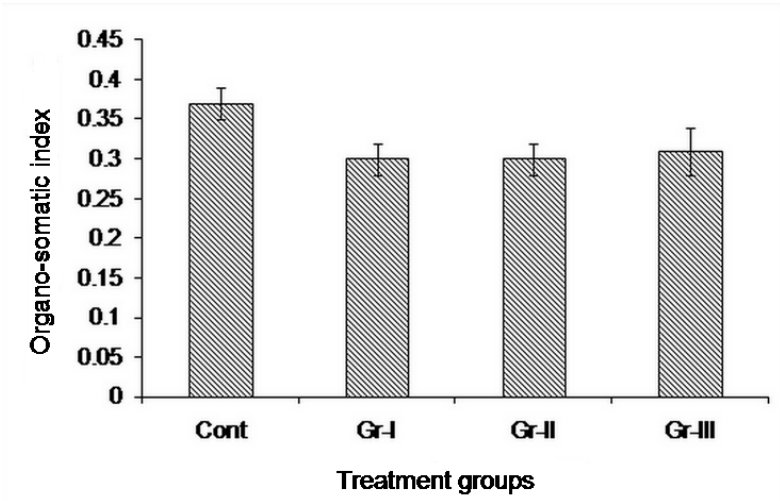

Figure 3. Effect of fluoride on spleen OSI of mouse (n =6) following *in vivo* administration of NaF (mg/L) in the drinking water. Values are mean ± SEM and are not significantly ($p < 0.05$) different from the control group.

The percent of dead cells increased significantly ($df=23$, $F=12.35$, $p=0.0000856$, ANOVA; $p<0.05$, t test) only in the Group III mice exposed to 15 mg NaF/L for 90 days (Figure 4).

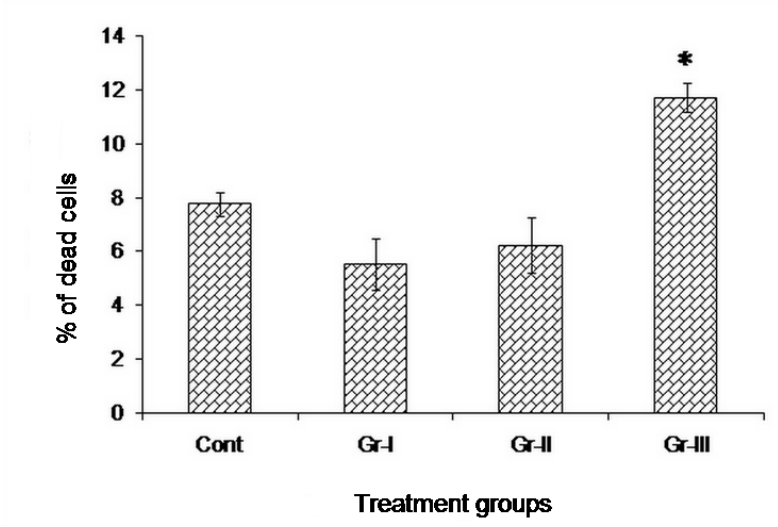

Figure 4. Effect of fluoride on the cell viability of mouse spleen following *in vivo* administration of NaF (mg/L) in the drinking water. Values are mean ± SEM); the value marked * in Group III is significantly ($p < 0.05$) different from the control group.

In the spleen of the control mice, white pulp formed lymphoid nodules surrounded by red pulp (Figure 5).

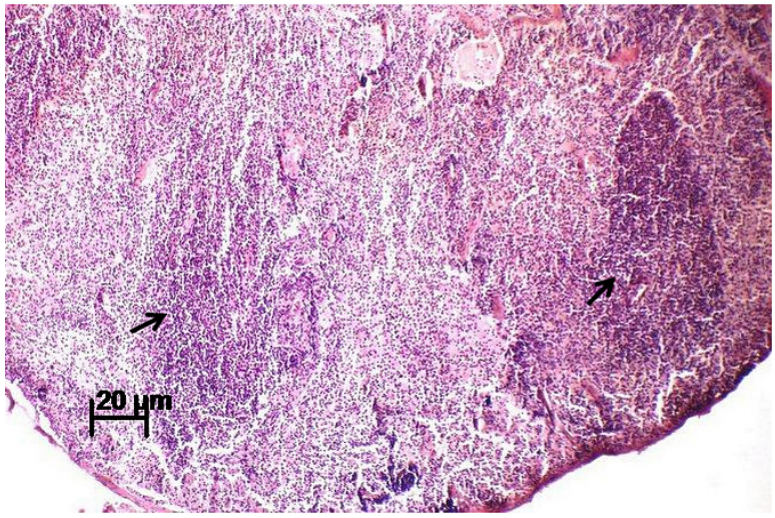

Figure 5. Normal histological appearance of spleen tissue of control mice by HE staining, white pulp (arrows), original magnification x100.

In the spleen of all the treatment groups the amount of lymphocytes in the lymphatic nodules and periarteriolar lymphoid sheaths of white pulps decreased (Figures 6–8) compared with the control group, and red pulps increased and were observed to be infiltrated by lymphocytes. Figures 6 and 7 show the spleen of Group I and II mice exposed to 15 and 150 mg NaF/L for 30 days. The content of the white pulp decreased in these groups with the concomitant increase of red pulps.

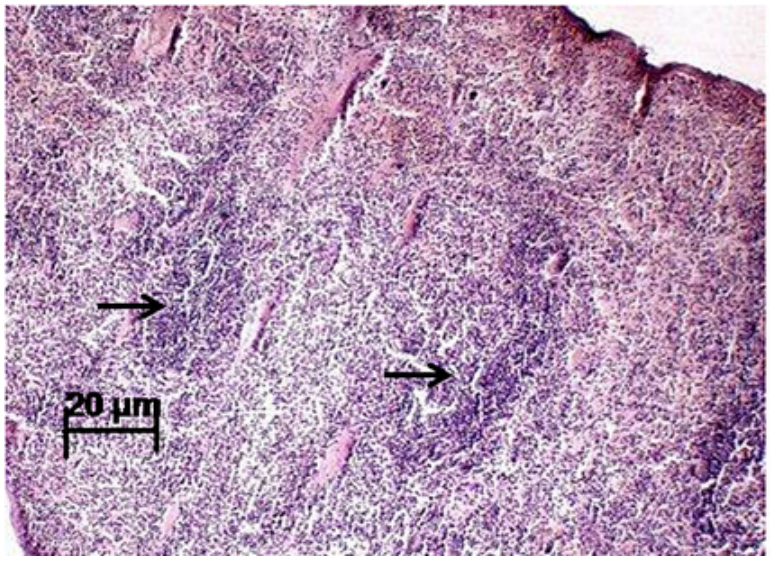

Figure 6. Group I mouse spleen showing decrease of white pulp, white pulp (arrows), original magnification x100.

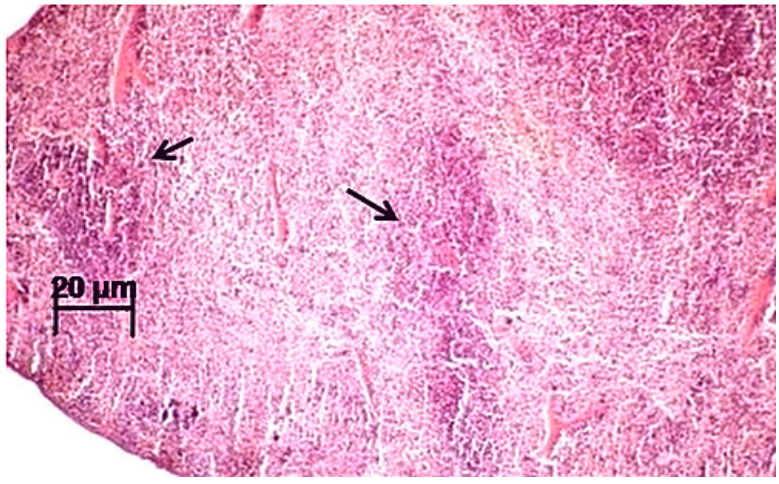

Figure 7. Group II mouse spleen showing decrease of white pulp (arrows), original magnification x100.

The decrease of the amount of the lymphocytes in the white pulps was still visible in the spleen of the Group III mice (Figure 8) exposed to 15 mg NaF/L for 90 days, and wide splenic cords surrounded the white pulps.

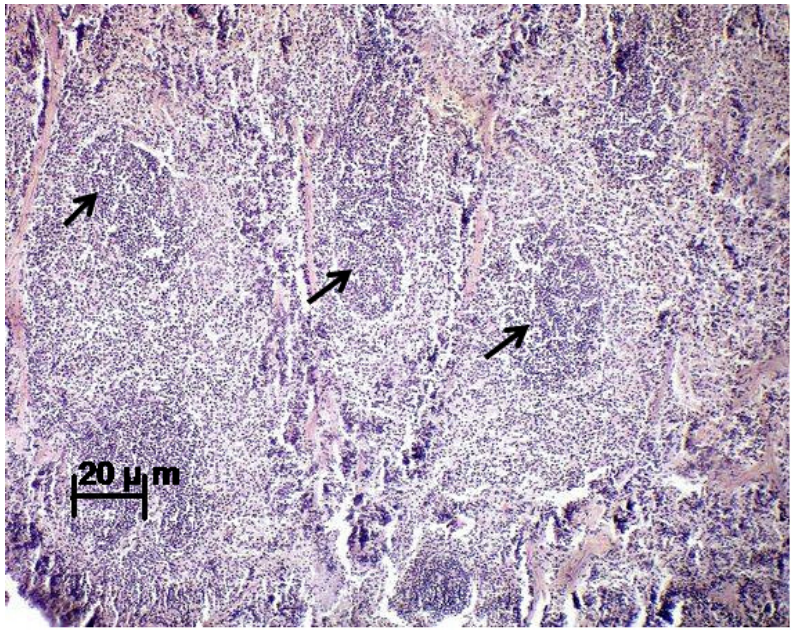

Figure 8. Group III mouse spleen showing decrease of white pulp (arrows), original magnification x100.

Although there was no change in the percentage of splenocytes in the different phases of the cell cycle in the Group I mice (Figure 9), an elevation in G_0/G_1 and G_2/M fraction was noticed in the rest of the treatment groups, which was found to

be significant (df=23, F=5.459911, p=0.006593; and df=23, F= 4.814995, p=0.01106, ANOVA; $p<0.05$, t test) in Group III mice (Figures 9 and 10).

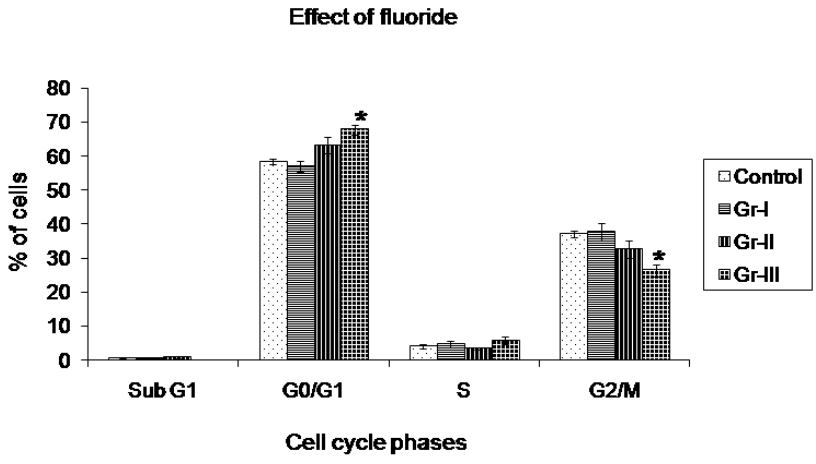

Figure 9. Percentage of splenocytes at different stages of cell cycle in the four groups of mice. Values are mean ± SEM; those marked with * are significantly ($p<0.05$) different from the control group.

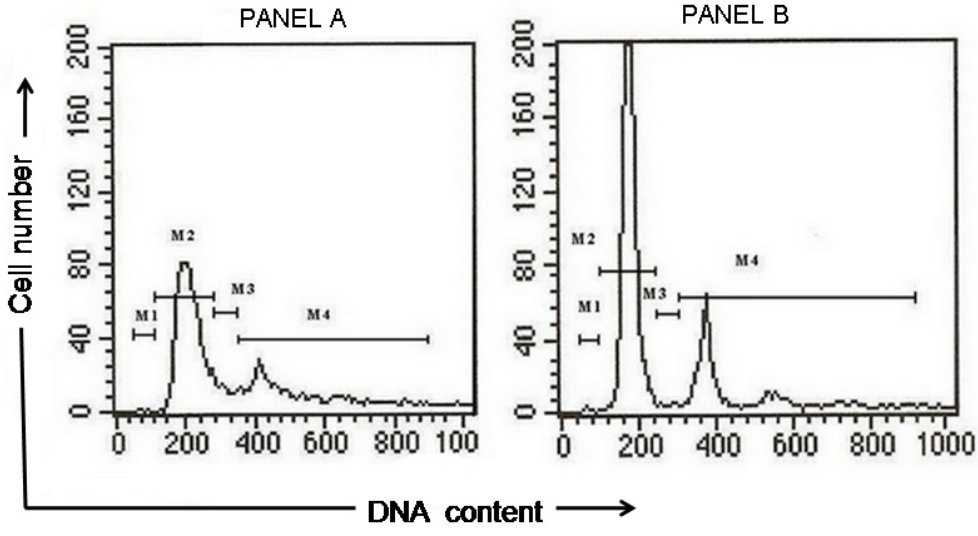

Figure 10. Flow-cytometric analysis of spleen cell cycle in control (panel A) and following the 90-day exposure of the Group III mice (panel B). The DNA content (x axis) and cell numbers (y axis) have been determined. Gates were set to assess the percentage of cells in different cycles. M1 = <2n DNA (Sub G1 cells); M2 = 2n DNA (G0/G1 cells); M3 = 2n - 4n DNA (cells in S phase); M4 = 4n DNA (G2/M cells). Bars denote the boundaries of cell cycle phases.

DNA ladder formation, a characteristic feature of apoptosis, was not discernible in any of the NaF-treated groups of mice (Figure 11).

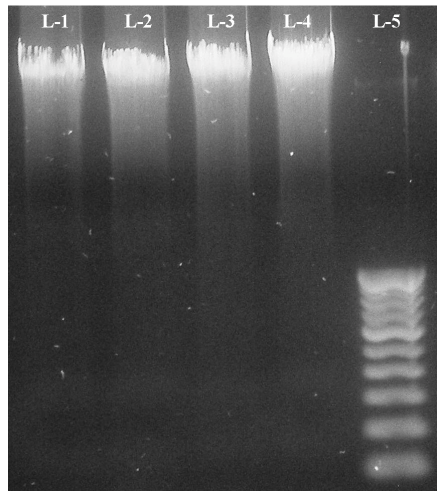

Figure 11. Effect of F exposure on DNA in the spleen of the mice. Lane 1: Control, Lanes 2, 3, 4: Group III mice (three separate samples); Lane 5: marker.

DISCUSSION

The spleen, which is the largest lymphatic organ of the body, produces 20–25% of the total blood cells. Its main functions include destruction of old or damaged red blood cells, and it acts as a reservoir of blood cells and hemopoiesis. There are many reports of F-induced toxicity in organs like liver, kidney and bone marrow[4-11] but fewer on toxicity in the spleen, particularly in mammals. Here, in spite of no significant change in the body weight, a pattern of decreased OSI of the spleen was found in all the treatment groups. The decrease of OSI may be due to the decrease in the spleen weight. White pulp of the spleen contains mainly lymphocytes, and it is the site of blood cell production. Machalinska et al. reported a decrease in white pulp with concomitant increase in red pulp infiltrated by lymphocytes on the 1st, 3rd, and 5th days after injecting 10 and 50 mg of NaF/kg bw into the tail veins of Balb C mice, with termination of the experiment after 7 and 21 days.[6] Recently, another report demonstrated that high F (400, 800, and 1200 mg F/kg) treatment in chickens through their diets decreased the number of splenic nodules, the lymphocyte population with splenic nodules, and periarterial lymphatic sheaths in white pulp.[12] In our study, when mice were continuously exposed to F through their drinking water either for 30 days or 90 days, we noted the decrease in the amount of the white pulp in the spleen of all the treatment groups, which, however, was highest in mice exposed to the lower F level (6.8 mg F ion/L) for 90 days.

F-treatment of the mice through drinking water also caused a significant increase in the G_0/G_1 fraction and a decrease in the G_2/M fraction of the cell cycle, fortifying the interpretation of G_1 blockage of DNA synthesis and inhibition of proliferation of the mouse splenocytes. The decrease in white pulp content likewise signifies perturbation of haemopoiesis and accumulation of G_0/G_1 population, in agreement with the report by Zhou et al. that excessive F intake seriously damaged the specific immune function in rabbits.[13] Moreover, according to Ravel et al., NaF acts as a specific inhibitor of protein synthesis in rabbits.[14] An inhibitory effect of F on DNA synthesis has also been reported by others.[15] One possible pathway of G_1 arrest may occur either through p53 via upregulation of Cdk (cyclin-dependent kinase) inhibitors of Cip/Kip (Cdk-inhibitor protein/kinase inhibitor protein) family proteins like p21, p27 etc., or Ink4 (inhibitor of Cdk4) family proteins if there is any DNA damage. Another pathway is inhibition of cyclin D synthesis in response to a lack of stimulation of proper growth factor involving Ras/ Raf/ ERK signaling pathway.[16]

It should be noted, however, that absence of Sub G_1 fraction in fluorescence activated cell sorter study and DNA ladder formation in DNA gel electrophoresis indicated that DNA damage was not the primary cause for F-induced G_1 blockage. The effect of F on the synthesis of cyclin D and Cdks, required for proper maintenance of cell cycle, could be the probable reason for such delay.[13] Thus the increase in the percent of dead cells in the spleen of the Group III mice might be due to F-induced cytotoxicity and to apoptosis.

From the fact that prolonged exposure to the low concentration of F resulted in a decreased OSI and white pulp content, we conclude there is a significant negative impact on mammalian spleen by F which can potentially impair the process of hemopoiesis.

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