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Original article

Morphological changes in spleen after dietary zinc deficiency and supplementation in Wistar rats

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ABSTRACT

Background: Study was conducted to determine the effect of dietary zinc deficiency and supplementation on the spleen morphology.
Methods: Pre-pubertal Wistar rats (40–50 g) were divided into two groups with 6 sub-groups each *viz.* zinc control (ZC, 100 μg/g zinc diet), pair fed (PF, 100 μg/g zinc diet), zinc deficient (ZD, <1 μg/g zinc diet, zinc supplementation control (ZCS), zinc supplementation pair-fed (PFS) and zinc supplementation deficient (ZDS, 100 μg/g zinc control diet). Experiments were set for 2- and 4-weeks followed by 4 weeks of zinc supplementation.
Results: In the present study body weight and BMI decreased significantly along with incidence of splenomegaly as typified by the increased splenic index in deficient groups compared with that of respective control groups. Histopathological changes such as disorganization of red pulp, several infiltered lymphocytes, vacuolization, loss of cellularity, karyolysis, dissolution of matrix, indistinct differentiation between red and white pulp were evident in spleen of 2ZD and 4ZD group animals. Degeneration was more severe after 4 weeks of zinc deficiency as giant cells formation and hypertrophy were also evident.
Conclusion: The findings revealed that zinc deficiency causes growth retardation and splenomegaly. Degenerative and atrophic changes in rat spleen suggest reduced cellular defense potential which will have a direct effect on immunity. Zinc supplementation may prove to be beneficial as there were varying degrees of cellular recovery after cessation of zinc deficiency.

Introduction

Zinc is a vital micronutrient required to sustain life at all age groups [1,2]. Maintenance of total body zinc composition and cellular content in humans is tightly regulated by transporters and metallothionein with approximately 1% of total body zinc content being replenished daily by dietary intake [3–5]. Spleen the largest secondary lymphoid organ has significant immunological and haematological role. Owing to the presence of B and T lymphocytes, the inflammatory effects may be reflected in the histopathological examination of spleen [6,7]. Zinc homeostasis appears crucial for an adequate function of the immune system [8]. In humans and animals zinc deficiency causes thymic atrophy, lymphocytopenia and affects cell- and antibody-mediated responses [9]. Baltaci et al. [10] also reported that zinc deficiency have a negative influence on cellular immunity and showed a significant decline in CD3+, CD4+ and CD8+ lymphocytes sub-groups in rats infected with *Toxoplasma gondii*. Zinc deficiency as well as zinc excess enhanced perturbations in immune cell numbers and their activities, which may be one of the factor leading to the onset of inflammatory diseases [11,12]. Zinc restores the capacity of cell culture of spleen from immunodepressed aged mice to generate an antibody response [13]. Although substantial body of evidence exists describing the impact of zinc deficiency on immune function, less is known regarding the effect of dietary zinc deficiency and supplementation on the spleen histoarchitecture. The present study is thus designed to investigate its effect on spleen morphology.

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Materials and methods

Preparation of basal diet

The basal diet was prepared by ICN Research Diet Protocol (1999). The composition (per kg diet) was as follows: Egg albumin, 180 g; Corn oil, 100 g; Corn starch, 443 gm: Sucrose, 200 g; Cellulose, 30 g; Choline chloride, 2 g; DL-methionine, 7 g; D-biotin, 20 mg; American Institute of Nutrition (AIN)-76 salt mixture, 35 g; AIN-76C vitamin-antibiotic mixture, 10 g. Zinc contents of basal diets from each lot was estimated at 213.9 nm in air–acetylene flame on GBC 902 atomic absorption spectrophotometer (AAS) (GBC Scientific Equipment, Dandenong, Victoria, Australia) and concentration was adjusted to 1.00 μg/g and 100 μg/g by addition of appropriate amounts of zinc sulphate.

Animals

Albino rats of the Wistar strain *Rattus norvegicus* were used. The animals were housed in polypropylene cages in the departmental animal house under hygienic conditions. The animals were maintained on the standard laboratory feed and water *ad libitum* before the period of experimentation. The animal care and handling was carried out as per the guidelines given by the Committee for the Purpose of Control and Supervision on Experiments on Animal, New Delhi, India (1678/GO/Re/S/12 CPCSEA). The Department animal ethical committee (DAEC) approved the experimental protocols for this study.

Experimental design

Pre-pubertal male Wistar rats (30–40 days; 40–50 g) were divided into two groups with 6 sub-groups each:

I Group

(1) **2ZC** (Zinc control): n = 12; Animals were fed ICN zinc control diet, 100 μg/ g
(2) **2PF** (Pair-fed): n = 12; Animals were fed ICN zinc control diet but the amount of feed was equal to the (average) amount consumed by zinc deficient group the previous day. This group was run so as to study starvation effects due to reduced intake of diet and stress effects of the synthetic diet.
(3) **2ZD** (Zinc deficient): n = 12; Animals were fed < 1.00 μg/ g zinc diet.

With the exception of PF group, animals were allowed access to food and water *ad libitum*. Six animals from each of the above group were sacrificed after 2 weeks and remaining six animals were carried forward for 4 week of zinc supplementation.

(4) **2ZCS** (Zinc Supplementation control): n = 6; ICN zinc control diet *ad libitum*.
(5) **2PFS** (Zinc Supplementation Pair-fed): n = 6; ICN zinc control diet *ad libitum*.
(6) **2ZDS** (Zinc Supplementation deficient): n = 6; ICN zinc control diet *ad libitum*.

II Group

(1) **4ZC** (Zinc control): n = 12; Animals were fed ICN zinc control diet, 100 μg/ g
(2) **4PF** (Pair-fed): n = 12; Animals were fed ICN zinc control diet but the amount of feed was equal to the (average) amount consumed by zinc deficient group the previous day. This group was run so as to study starvation effects due to reduced intake of diet and stress effects of the synthetic diet.

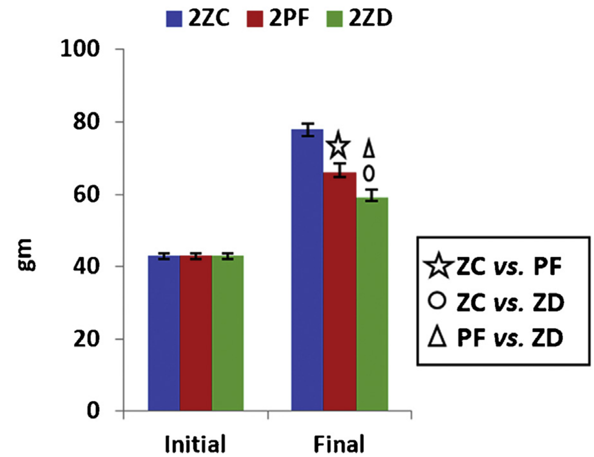

Fig. 1. Body weight of Wistar Rats (n = 12) (2 Wks Expt.).

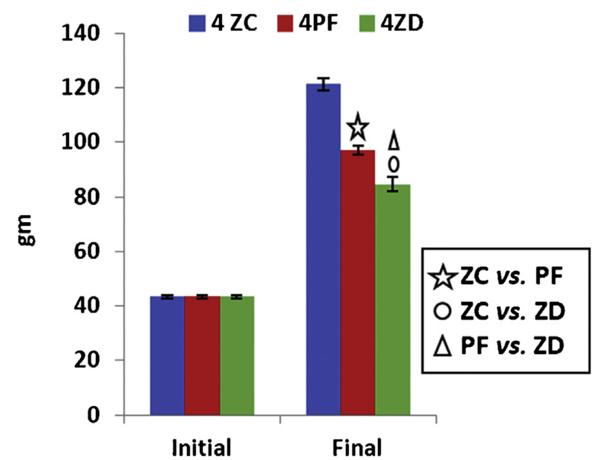

Fig. 2. Body weight of Wistar Rats (n = 12) (4 Wks Expt.).

(3) **4ZD** (Zinc deficient): n = 12; Animals were fed < 1.00 μg/ g zinc diet.

With the exception of PF group, animals were allowed access to food and water *ad libitum.* 6 animals from each of the above group were sacrificed after 4 weeks and remaining 6 animals were carried forward for 4 week of zinc supplementation

(4) **4ZCS** (Zinc supplementation control): n = 6; ICN zinc control diet *ad libitum.*
(5) **4PFS** (Zinc supplementation Pair-fed): n = 6; ICN zinc control diet *ad libitum.*
(6) **4ZDS** (Zinc supplementation deficient): n = 6; ICN zinc control diet *ad libitum.*

Measurement of body weight, BMI, spleen weight and SI

- **Body Weight (BW):** BW of each animal were taken before and after the end of experiment.
- **Body Mass Index (BMI):** BMI was calculated based on ratio of body (BW) and length of animal according to formula:

$$BMI(gm/cm^2) = \frac{Body \quad Weight}{(Length)^2}$$

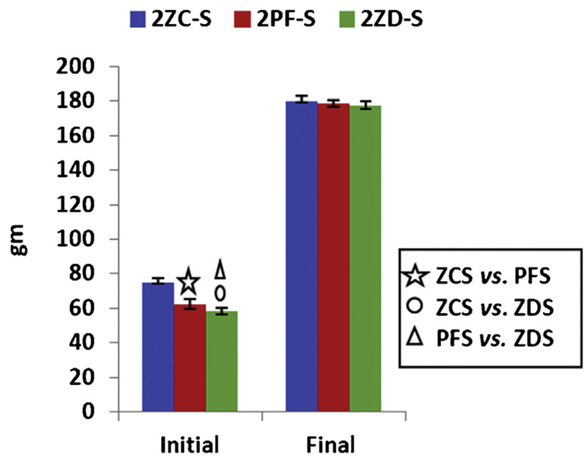

Fig. 3. Body weight of 2 weeks (Wks) zinc deficient Wistar rats after 4 Wks of zinc supplementation (n = 6).

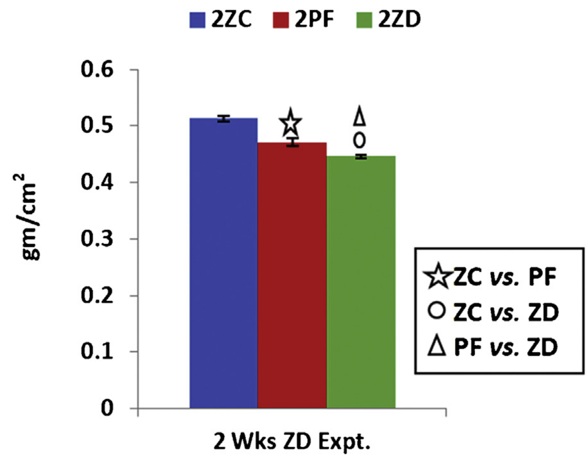

Fig. 5. BMI of zinc deficient Wistar rats (n = 12).

- **Spleen Index (SI):** The excised organ (spleen) of the experimental rats groups were washed with normal saline to remove blood, dried between blotting paper and then weighed. The organ-to-body weight ratios were evaluated according to formula:

$$SI\,(\%) = \frac{Spleen \quad Weight}{Body \quad Weight} \times 100$$

Histopathological examination

Animals were anaesthetized using sodium pentathione (IP) and spleen was weighed and fixed in Bouin's fixative. Microtome cut 5μ sections were stained with hematoxylin and alcoholic eosin (H&E). For histopathological evaluation slides were observed under Leica light microscope (Leica DM 1000) and photographs were taken with Leica Microsystems Ltd. camera (Type-DFC 450 C; CH-9435, Germany). Microscopic examination of spleen sections from each group was carried out separately following guidelines for enhanced histopathology examination of the spleen [6,7].

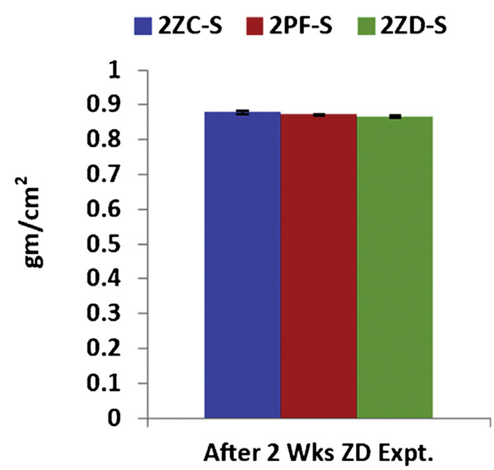

Fig. 6. BMI after 4 Wks of zinc supplementation (n = 6).

Statistical analysis

Data were expressed as mean ± SEM. Further, analysis of 2- and 4-weeks treatment groups was carried out separately using One way Analysis of Variance (ANOVA) and if the difference was found to be significant then *post-hoc* test (Duncan's Multiple Comparison Test) was applied. A $p < 0.05$ was considered to be significant. Statistical analysis was performed by Sigma Stat 3.5 software (Cranes Software International, Banglore, India).

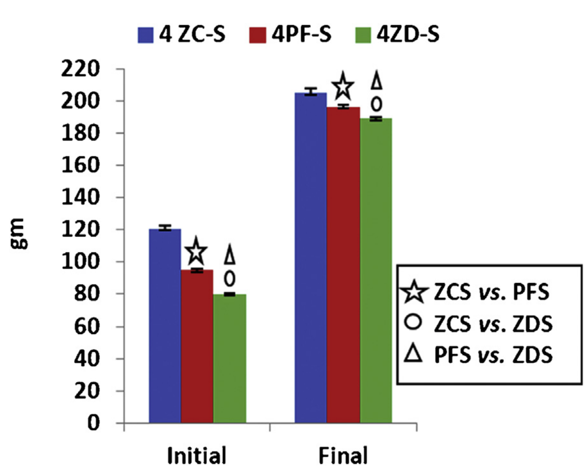

Fig. 4. Body weight of 4 weeks (Wks) zinc deficient Wistar rats after 4 Wks of zinc supplementation (n = 6).

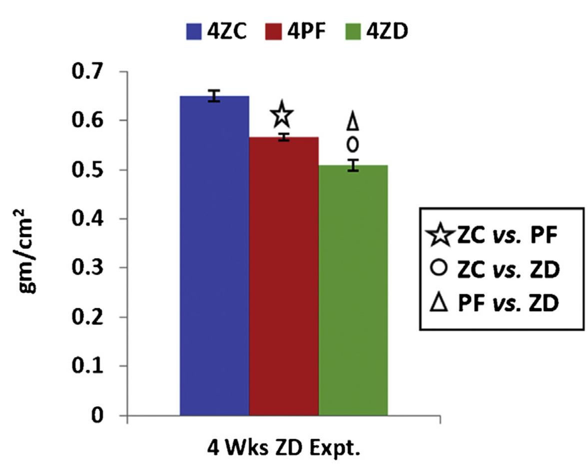

Fig. 7. BMI of zinc deficient Wistar rats (n = 12).

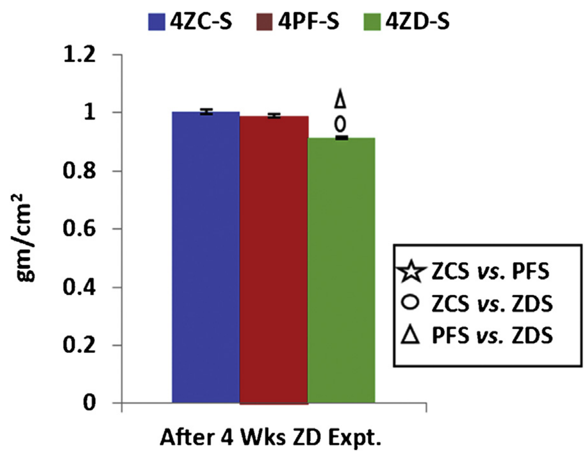

Fig. 8. BMI of Wistar rats after 4 Wks of zinc supplementation (n = 6).

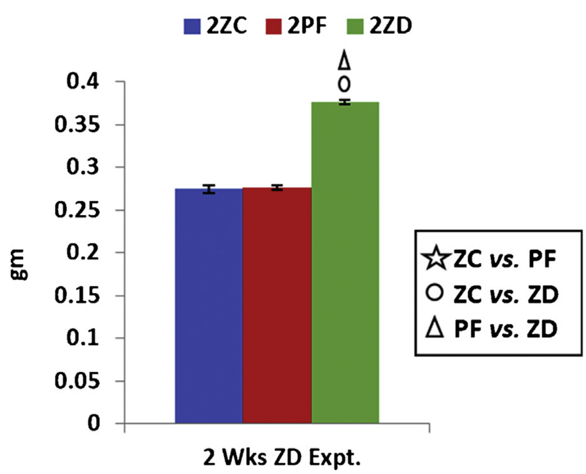

Fig. 9. Spleen weight of zinc deficient Wistar rats (n = 12).

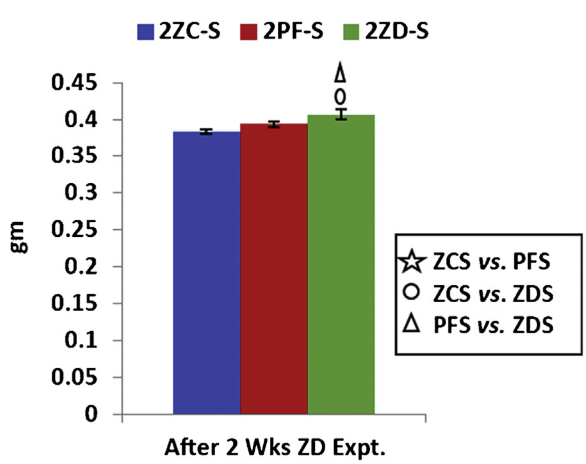

Fig. 10. Spleen weight of Wistar rats after 4 Wks of zinc supplementation (n = 6).

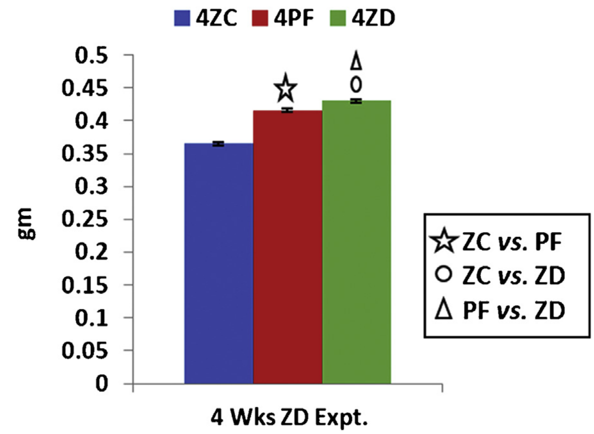

Fig. 11. Spleen weight of zinc deficient Wistar rats (n = 12).

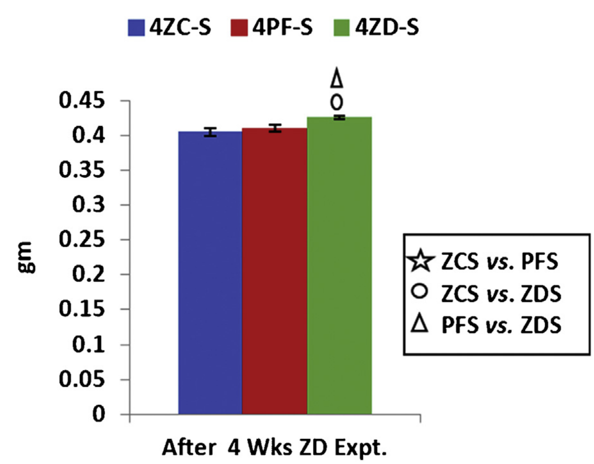

Fig. 12. Spleen weight of Wistar rats after 4 Wks of zinc supplementation (n = 6).

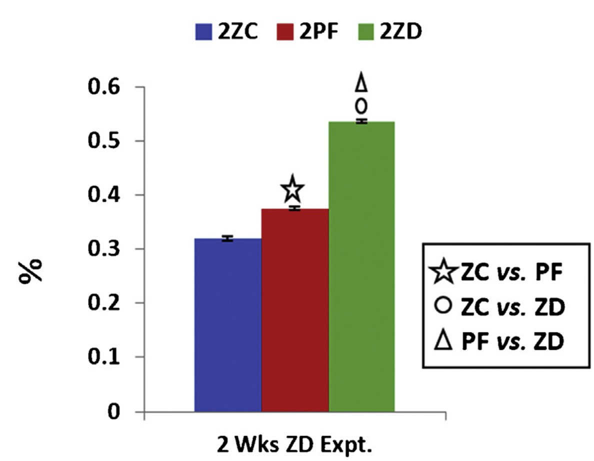

Fig. 13. Spleen index of zinc deficient Wistar rats (n = 12).

Results

Body weight (BW)

Final BW of ZD groups (2-and 4-weeks) decreased significantly ($p < 0.05$) when comparisons were carried out between their respective control and pair-fed groups (Figs. 1 and 2). Zinc supplementation (for 4 weeks duration) improved weight gain in ZDS animals as compared to zinc deficient group (2- and 4-weeks). Two weeks experiment animals after zinc supplementation (2ZDS and 2 PFS) showed a non-significant decline while 4 weeks experimental animals (4ZDS and 4 PFS) revealed significant decline when comparisons were made between ZCS *vs.* ZDS, ZCS *vs.* PFS and ZDS *vs.* PFS groups respectively (Figs. 3 and 4).

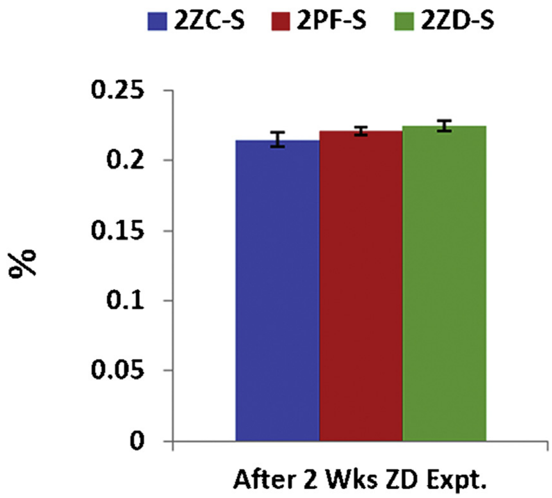

Fig. 14. Spleen index of Wistar rats after 4 Wks of zinc supplementation (n = 6).

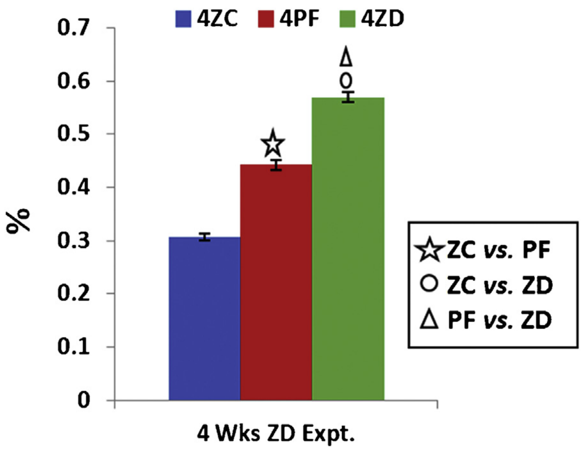

Fig. 15. Spleen index of zinc deficient Wistar rats (n = 12).

Body mass index (BMI)

BMI values were significantly ($p < 0.05$) lower in ZD groups (2- and 4-weeks) as compared to their respective control and pair-fed

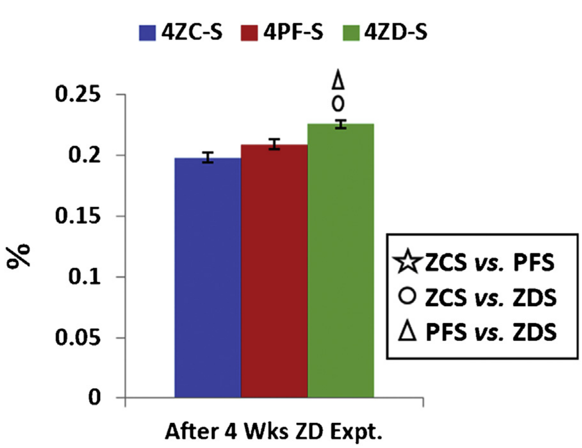

Fig. 16. Spleen index of Wistar rats after 4 Wks of zinc supplementation (n = 6).

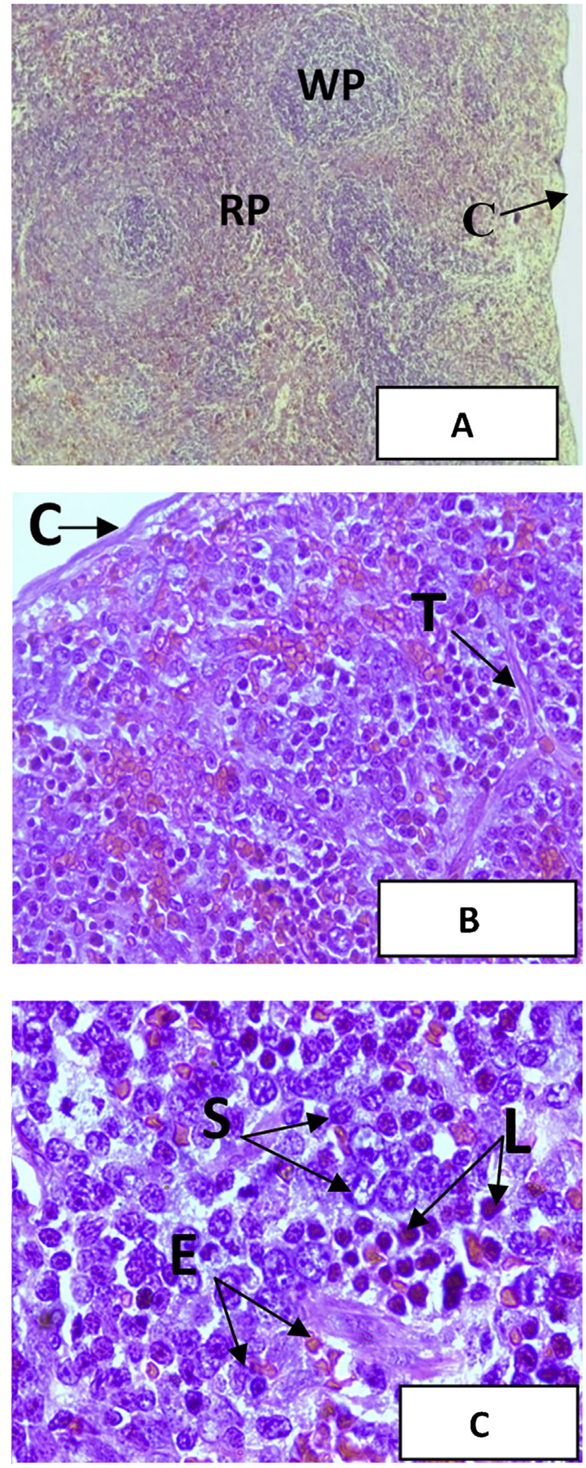

Fig. 17. Photomicrograph of control spleen (2ZC) showing capsule (C), trabeculae (T), visible delineation of red pulp (RP) with splenic cords and white pulp (WP). Erythrocytes (E), splenocytes (S) with few lymphocytes (L) distinct. H&E. (A: ×100; B: ×400 and C: ×1000).

groups (Figs. 5 and 7). Zinc supplemented rats showed slight recovery in BMI and revealed non-significant (2-weeks) and significant (4-weeks) decline in ZDS groups when comparisons were made between ZCS *vs.* ZDS and ZDS *vs.* PFS groups respectively (Figs. 6 and 8).

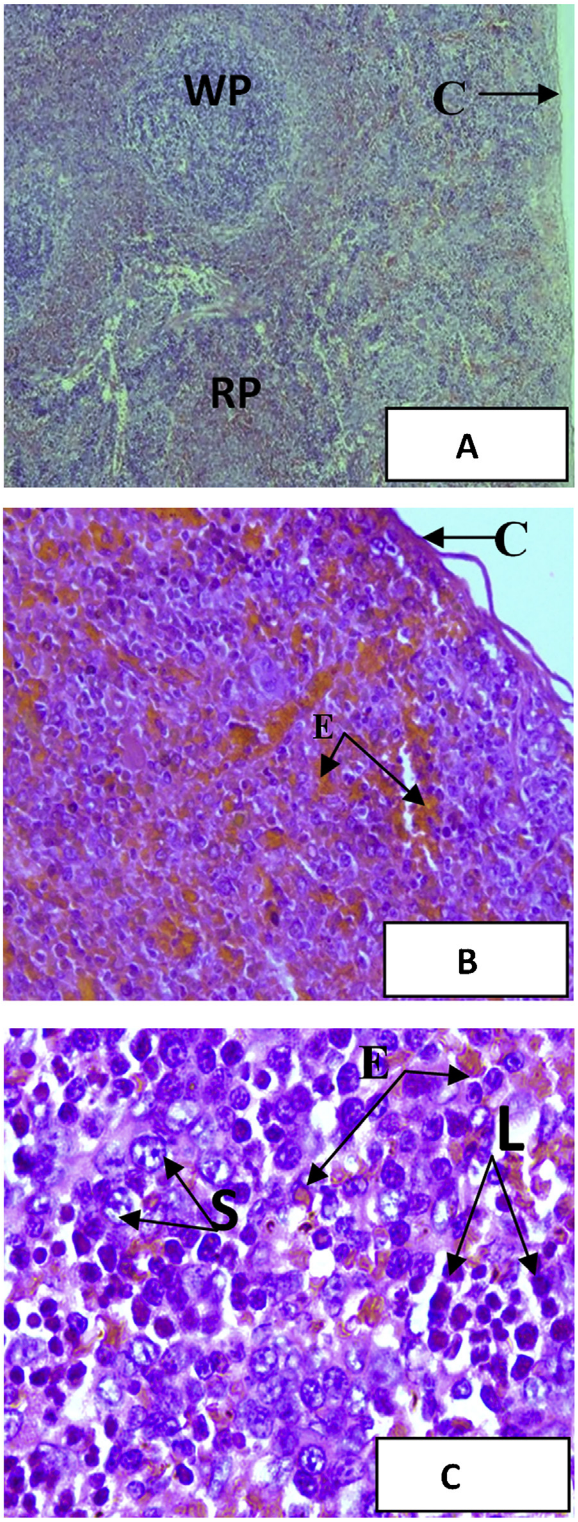

Fig. 18. Photomicrograph of pair-fed group (2 PF) revealing wavy capsule (C), depopulated splenic cells in red pulp (R) and white pulp (W) area. Splenocytes (S), erythrocytes (E), many lymphocytes (L) of different size and darkly stained nuclei visible. H&E. (A: ×100; B: ×400 and C: ×1000).

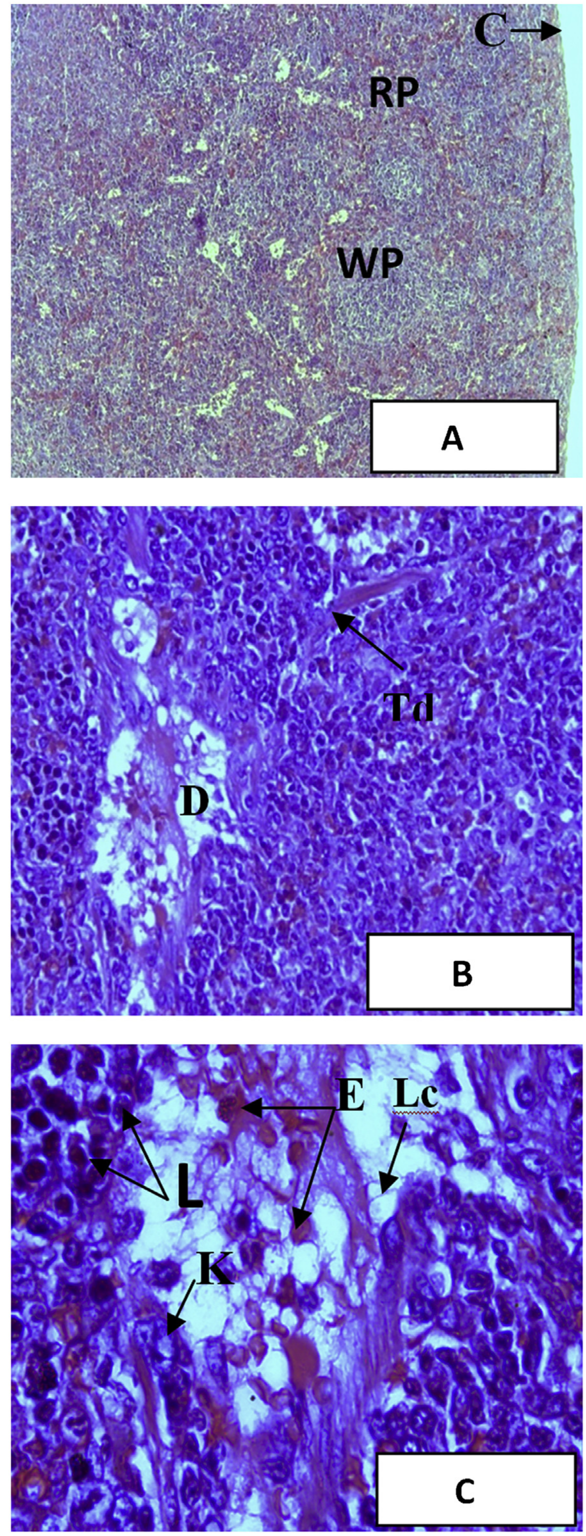

Fig. 19. Photomicrograph of zinc deficient (2ZD) spleen illustrating disorganization of red pulp (RP) and white pulp (WP), degenerated trabeculae (Td), dissolution of matrix (D), loss of cellularity (Lc) and infiltrating lymphocytes (L). Karyolysis (K) in red pulp area evident. H&E. (A: ×100; B: ×400 and C: ×1000).

Spleen weight

Significant increase ($p < 0.05$) was observed in spleen weight (2ZD and 4ZD groups) as compared to respective ZC and PF groups (Figs. 9 and 11). Supplementation studies also revealed increase although the increase was less as compared to 2ZD and 4ZD groups. Non-significant difference was evident between pair-fed and control groups after supplementation (2ZCS *vs.* 2 PFS and 4ZCS *vs.* 4 PFS) (Figs. 10 and 12).

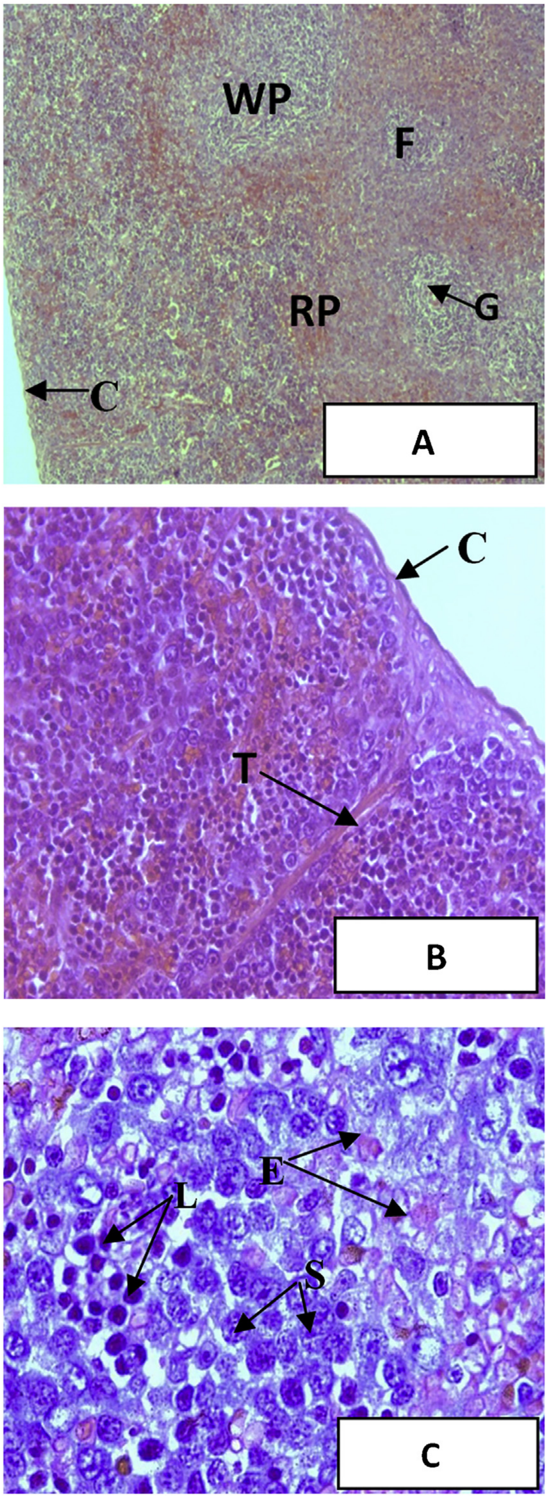

Fig. 20. Photomicrograph of control spleen (4ZC) showing normal histology of rat spleen with red (RP) and white (WP) pulp, lymphatic follicle (F) with pale staining germinal centre (G), trabeculae (T) and capsule (C). Erythrocytes (E), splenocytes (S) and lymphocytes (L) distinct. H&E (A: ×100; B: ×400 and C: ×1000).

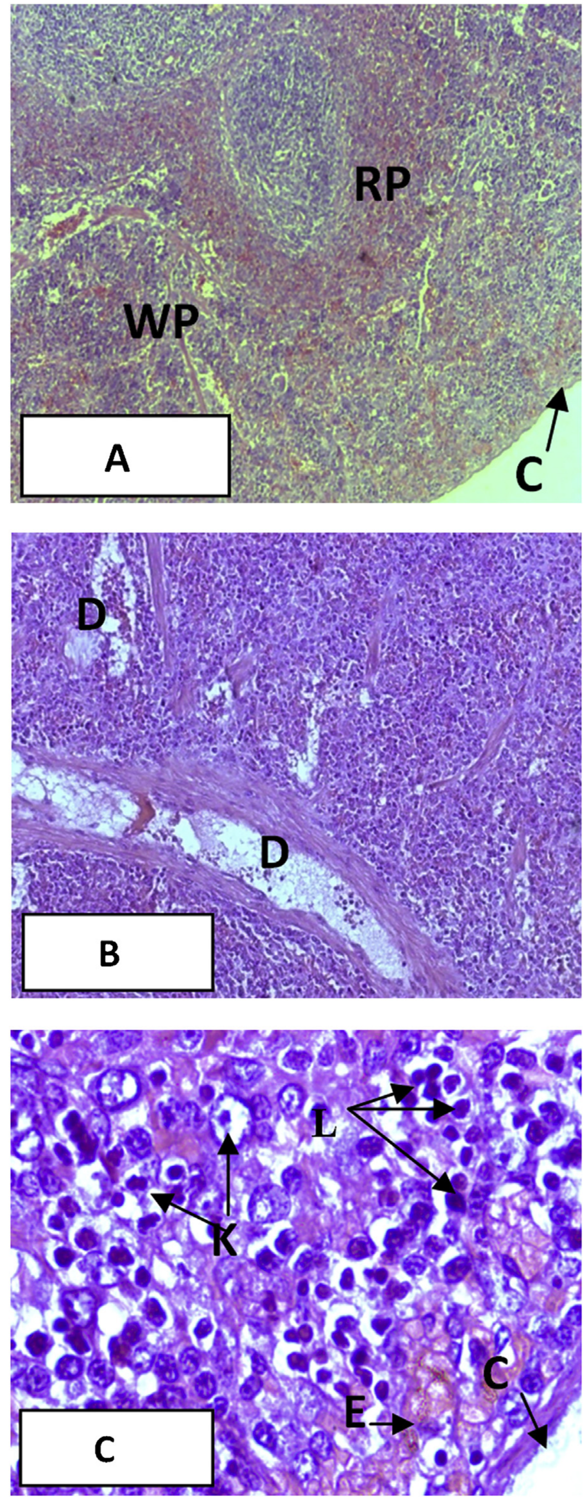

Fig. 21. Photomicrograph of pair-fed group (4 PF) displaying atrophic white pulp (WP) in some areas, distended red pulp (RP) with degeneration (D) evident in red pulp area. Erythrocytes (E), many lymphocytes (L) and karyolysis (K) of splenocytes visible. H&E (A: ×100; B: ×400 and C: ×1000).

Splenic index (SI)

Significant increase ($p < 0.05$) was observed in SI (2ZD and 4ZD groups) compared with their respective ZC and PF groups (Figs. 13 and 15). Supplementation studies revealed decrease in SI on comparison with zinc deficient groups (2- and 4-weeks). However, non-significant (2-weeks) and significant increase (4-weeks) was evident when comparisons were made between ZCS *vs.* ZDS and ZDS *vs.* PFS respectively (Figs. 14 and 16).

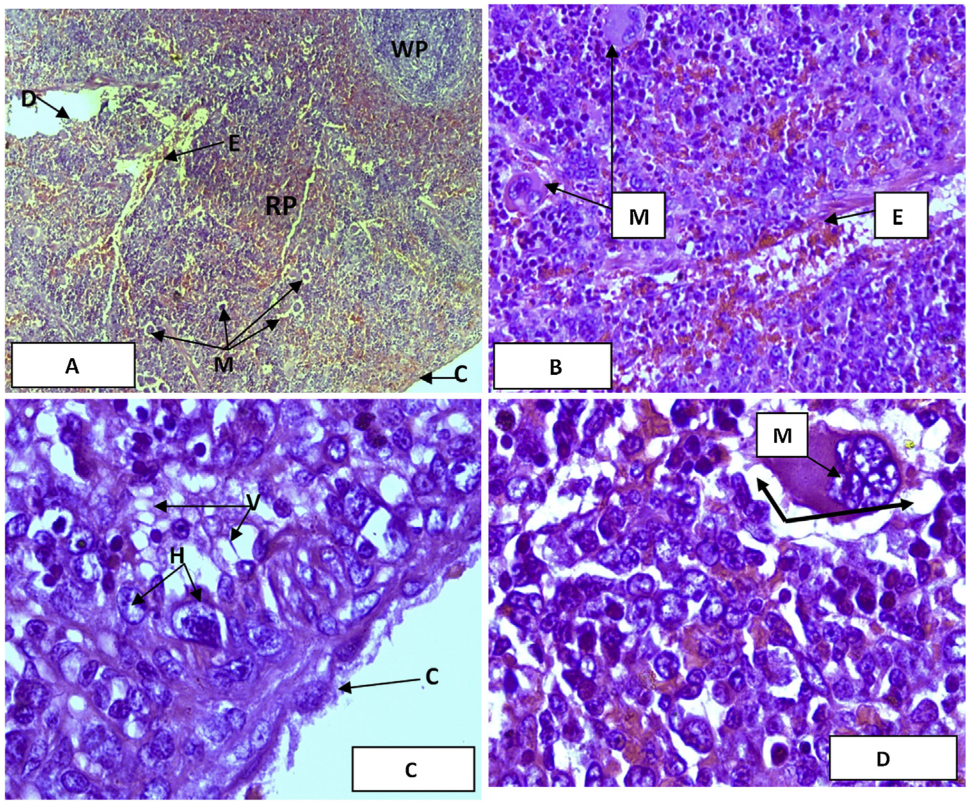

Fig. 22. Photomicrograph of zinc deficient group (4ZD) revealing obliteration of red pulp (RP) and white pulp (WP) and loss of cellularity (Bold arrow), intense proliferation of megakaryocyte (M). Degenerated cells (D), vacuolization (V) and hypertrophy (H) seen at some regions. Lymphatic follicle with absence of germinal centres evident. H&E. (A: ×100; B: ×400; C and D: ×1000).

Spleen morphology

Control groups (both 2- and 4-weeks) revealed normal histoarchitecture of spleen (Figs. 17a–c and 20 a–c). Pair-fed groups (2 PF) showed wavy capsule and depopulated splenic cells in red pulp (RP) and white pulp (WP) area (Fig. 18a–c). 4PF groups display atrophic WP in some areas, distended RP with degeneration. Many lymphocytes (L) and karyolysis (K) of splenocytes were also visible (Fig. 21a–c). Zinc deficient (2ZD) spleen illustrates disorganization of RP and WP, degenerated trabeculae (Td), dissolution of matrix (D), loss of cellularity (Lc), infiltrating lymphocytes (L) and karyolysis (K) in red pulp area (Fig. 19a–c). Degeneration was more severe after 4 weeks as giant cells formation (Extra-medullary haematopoiesis) and hypertrophy were also noticed in 4ZD groups in addition to obliteration of RP and WP, loss of cellularity, intense proliferation of megakaryocyte (M) and vacuolization (V). Lymphatic follicle with absence of germinal centres was also evident (Fig. 22a–c). Zinc-control supplementation (2ZCS and 4ZCS) groups showed normal histoarchitecture. 2 PFS and 4 PFS exhibit slight recovery in the WP and RP (Figs. 23a–c and 25 a–c). 2ZDS and 4ZDS groups also revealed evident recovery with degeneration less severe and no other microscopic lesions in both the RP and the WP areas (Figs. 24a–c and 26 a–c).

Discussion

Zinc is an essential trace element for human nutrition and its deficiency is associated with anorexia, poor food efficiency, protein energy malnutrition, cachexia and growth retardation [14–17]. Zinc deficiency affects the growth hormone (GH) metabolism and may function as a limiting factor in growth regulation [18].Dietary zinc deficiency during pre-pubertal period decreased BMI through mechanism that may depend on disruption of the leptin-signaling pathway that limits food intake by acting on the hypothalamus [19]. This corroborates well for zinc deficiency-induced anorexia accounting for the relationship between zinc and leptin levels [20,21]. These results agree with previous findings that oral zinc supplementation improved appetite and stimulated food intake and enhanced leptin levels in zinc supplemented castrated rats [17,22,23]. In the present study, body weight as well as BMI (measure of body fat based on height and weight) of zinc deficient groups decreased significantly compared with controls. However, there was a statistically significant increase in both weight and BMI after zinc supplementation. Initial body weight from different groups *i.e.* zinc control (ZC), pair fed (PF) and zinc deficient (ZD) revealed non-significant variations but at the end of experiment the zinc deficient groups (both 2 and 4 weeks) exhibited retarded growth or reduction in body weight gain as compared to their respective control and PF groups. Since zinc deficiency leads to altered structure of buccal mucosa, soft palate, dorsal surface of tongue, vallate papillae and taste buds [24,25] that are primarily associated with taste and intake of food, the reduced diet intake is anticipated due to impairment of taste and smell sensitivity [26,27]. The PF groups however exhibited a significant decrease in body weight as compared to control group yet showed significant increase as compared to ZD groups, as they received restricted but zinc sufficient (100 $\mu g/g$) diet. This indicated that multifactorial approaches for growth retardation in ZD groups were not operative for PF groups. The decreased body weight in pair-fed groups as compared to control animals can be explained on the basis of starvation effects due to restricted food supply. Moreover, the restricted food supply for longer duration might have led to low supply of essential nutrients, hence affecting the normal physiology of the animal accounting for decreased BMI and increased spleen index as compared to control animals.

The spleen regulates the immune system on account of its rich and diverse population of immune cells [28,29]. Organ/body weight ratio alterations are characteristic of tissue swellings, atrophy or hypertrophy. The present study revealed incidence of splenomegaly indicative of either haemolytic/ immune compromised infection in the experiment as evident by the increased spleen-to-body weight ratios. Barlak et al. [30] reported that splenic enlargement beyond its normal size had been also found to correlate with increase in the white blood cells. Anatomically, spleen has two functionally distinct areas: (a) red pulp, a hematogenous part which remove damaged cells and acts as a site for iron storage and turns over and (b) white pulp, an organized lymphoid structure [31]. In the present study, control group shows normal histoarchitecure of spleen indicative of normal functional condition of immune organ. The spleen is commonly involved in a wide range of pathologic disorders and the ratio of white to red pulp increases with age due to accumulated antigenic exposure and stimulation [32]. Histopathological changes such as disorganization of red pulp, several infiltered lymphocytes, vacuolization, loss of cellularity, karyolysis, dissolution of matrix, indistinct differentiation between red and white pulp were evident in spleen of zinc deficient animals (2ZD and 4ZD groups). Degeneration was more severe after 4 weeks as evident by giant cells formation (extra-medullary haematopoiesis) and hypertrophy in 4ZD groups. Due to the critical role of spleen during inflammatory response, it can function as sentinel organ for detecting immunomodulatory influences of environmental factors evident by alterations in the size and density of the PALS and/or marginal zone and changes in the number of follicles with germinal centre [33]. Atrophic spleen processes together with numerous lymphocytes suggest reduced cellular defense potential reflecting immunosuppression. The spleen toxicity may either be due to degeneration of the extracellular matrix to which the splenocytes have attached or may be due to the severe erythrocyte depletion triggered due to zinc deficiency. Splenic dysfunction impairs the capacity of the spleen to phagocytose worn-out erythrocytes which results in an increase in abnormal circulating red blood cells.

There is a mobilization of haematopoietic stem cells (HSCs) from the bone marrow to the spleen after haematopoietic stresses which induces extramedullary haematopoiesis (EMH) [34]. In the present study, diffuse expansion of the red pulp by extramedullary hematopoiesis (EMH) characterized by the presence of myeloid, megakaryocytic precursors and morphological predominance of erythroid precursors was observed. EMH refers to the hematopoiesis that occurs in organs other than bone marrow [35,36]. Increased EMH may probably be due to increased demand for these cells as has been reported during hematotoxic insult, stress, systemic anaemia, inflammation *etc.* [37]. Dietary zinc deficiency also revealed the alterations in packed cell volume, erythrocyte indices (MCV, MCH and MCHC) and concentrations of hemoglobin, red blood cells, platelets and total leukocyte count *i.e.* marked deterioration of haematological parameters. Zinc supplementation appears to have beneficial effects by restoring the altered hematological indices [1]. As spleen is the main filter for blood-borne pathogens and antigens, it acts as a key organ for iron metabolism and erythrocyte homeostasis [38]. Enhanced osmotic fragility and altered RBC morphology was reported after zinc deficiency which was reversed to a certain extent after zinc was supplemented for a period of 4 weeks [16]. Growth retardation, iron deficiency anemia and hepatosplenomegaly occur concurrently with zinc deficiency as reported in Prasad's syndrome or hypopituitarism [39]. Dietary zinc supplementation has been shown to enhance immune response in eldery people [40]. Zinc is able to restore T-and B-cells in marginal zone and lymphatic follicles of spleen after cadmium intoxication [41]. Proteinate complex Zn supplementation (PC-ZS) to weaned piglets improved T lymphocyte immune

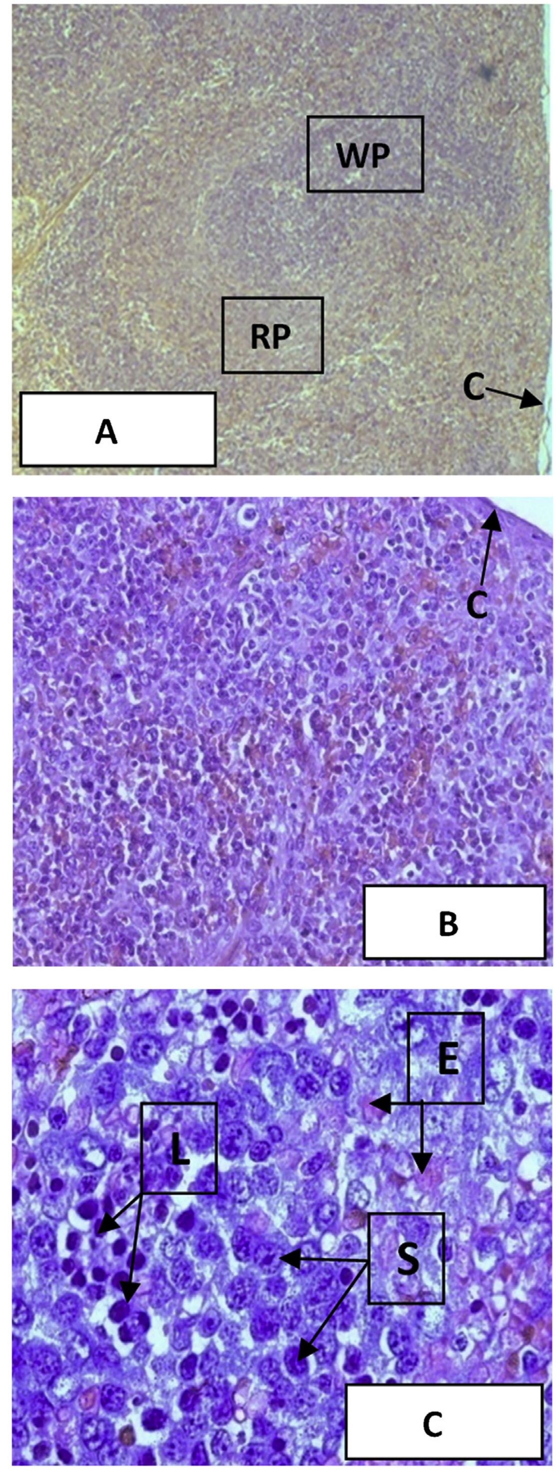

Fig. 23. Photomicrograph of pair-fed supplementation group (2 PFS) exhibiting slight recovery in the white pulp (WP) and red pulp (RP). Lymphocytes (L), erythrocytes (E) and splenocytes (S) visible. H&E (A: ×100; B: ×400 and C: ×1000).

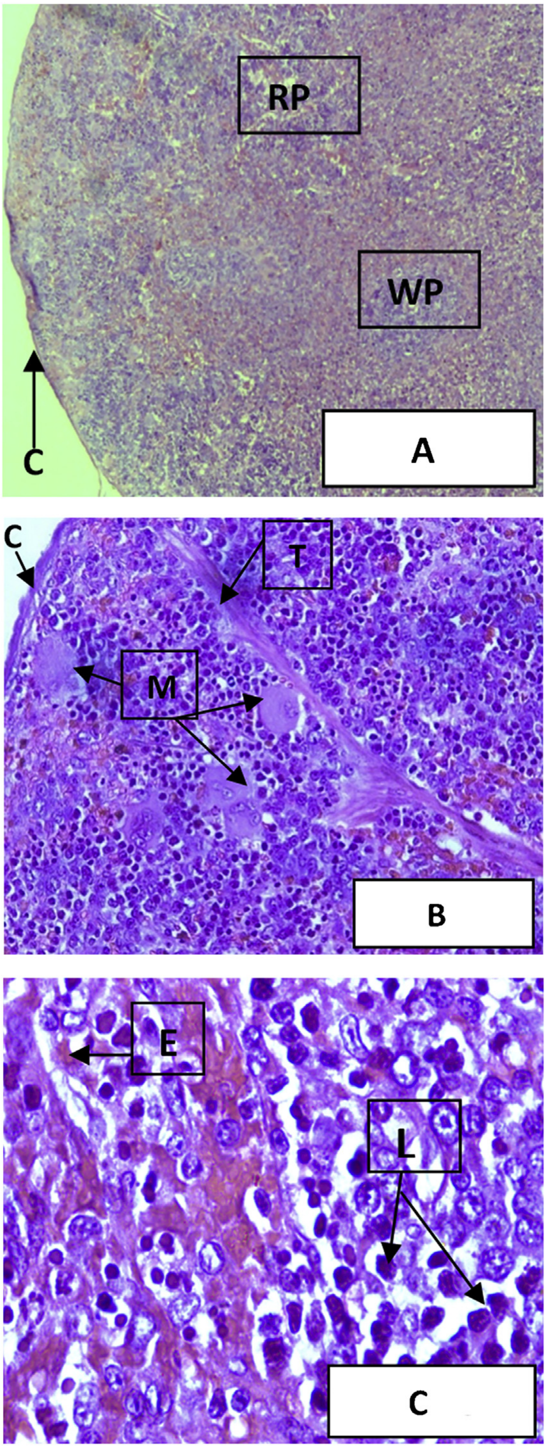

Fig. 24. Photomicrograph of 2ZDS group revealing slight recovery in the morphology with red pulp (RP), white pulp (WP) and trabeculae (T). Many aggregations of darkly stained lymphocytes (L), erythrocytes (E) and abundant megakaryocytes (M) seen. H&E (A: ×100; B: ×400 and C: ×1000).

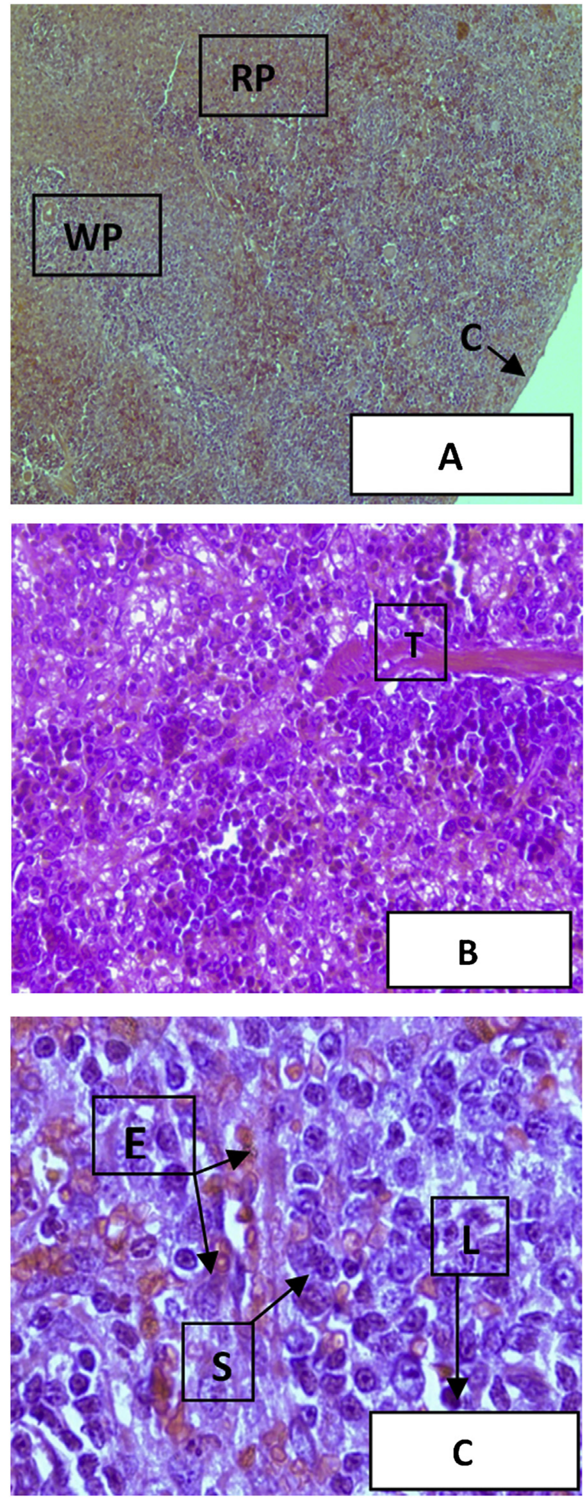

Fig. 25. Photomicrograph of 4 PFS group showing the white pulp (WP) and the red pulp (RP). Splenocytes (S), erythrocytes (E) and few lymphocytes (L) visible. H&E (A: ×100; B: ×400 and C: ×1000).

functions increased the retention of Zn, Cu, Mn, and Fe, enhanced antioxidative function and the proportions of T lymphocyte subsets in the spleen [42]. Dietary zinc supplementation after zinc deficiency reflected the potential to modify the spleen histoachitecture as evident by degeneration being less severe in ZD groups.

Conclusion

The findings revealed that zinc deficiency causes growth retardation and splenomegaly as evident by decreased body weight, BMI and increased splenic index. Degenerative and atrophic changes in rat spleen suggest reduced cellular defense

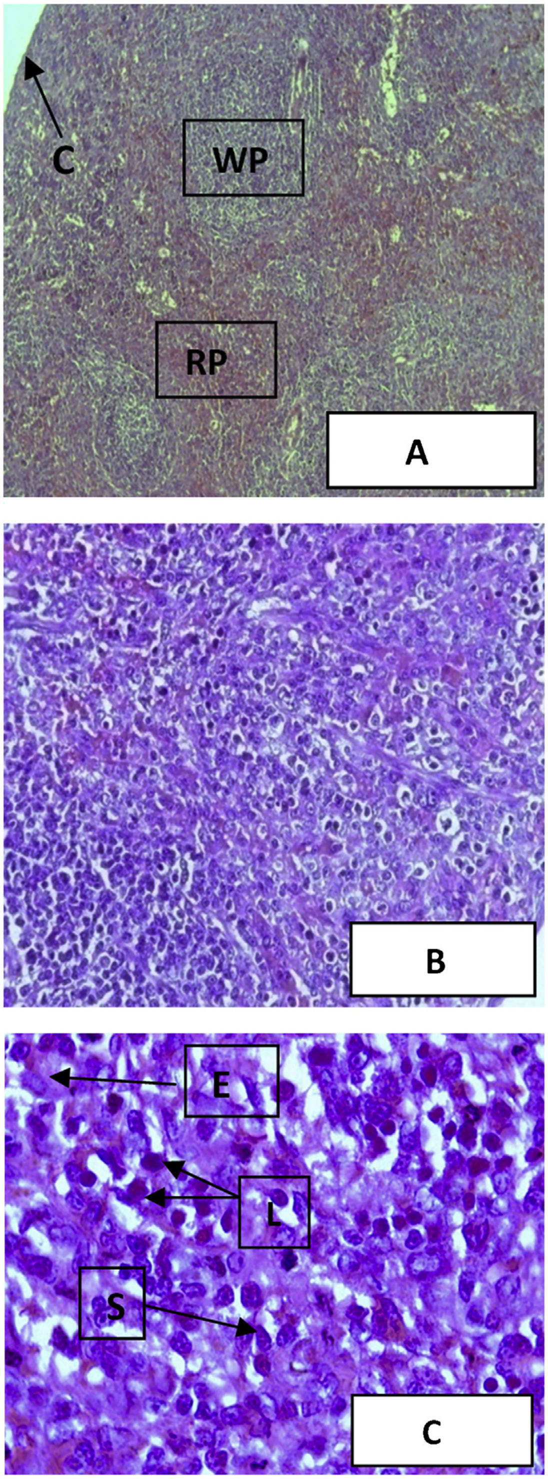

Fig. 26. 4ZDS group with evident recovery. White pulp (WP) is differentiated from the red pulp (RP). Degeneration not severe. Splenocytes (S), lymphocytes (L) and erythrocytes (E) distinct. H&E (A: ×100; B: ×400 and C: ×1000).

potential which will have a direct effect on immunity. Zinc supplementation proves to be beneficial as there were varying degrees of cellular recovery after cessation of zinc deficiency.

Conflict of interest

Authors declare that they have no conflict of interests.

Author contributions

Study Design – D. Kumari, N. Nair; Data Collection – D. Kumari; Statistical Analysis – D. Kumari, N. Nair; Data Interpretation – D. Kumari, N. Nair, R.S. Bedwal; Acceptance of final manuscript version – D. Kumari, N.Nair, R. S. Bedwal; Literature Search – D. Kumari; Fund Collection – D. Kumari.

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