



Article

Response of thymus lymphocytes to streptozotocin-induced diabetes and exogenous vitamin C administration in rats[†]

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Abstract

Diabetes causes oxidative stress, which in turn generates excessive free radicals resulting in cellular damage. Vitamin C is an antioxidant that protects tissues and organs from oxidative stress. The thymus is one of the most important lymphoid organs, which regulates T-lymphocyte proliferation and maturation. The aim of this study is to investigate the protective effects of vitamin C on the thymus of streptozotocin (STZ)-induced diabetic rats. The mitotic activity and cell integrity of thymic lymphocytes were explored. Wistar Albino rats were divided into three groups: control (Group 1), STZ-diabetes (Group 2) and vitamin C-treated STZ-diabetics (Group 3). Rats received a single intraperitoneal injection of 45 mg/kg STZ to induce diabetes. Vitamin C (20 mg/kg) was administered intragastrically. Semithin and ultrathin sections were examined under a light or an electron microscope, respectively. Considerable numbers of mitotic lymphocytes were observed in the thymus of control rats. In the diabetic rats, however, numbers of mitotic lymphocytes decreased to ~57% of controls, and cell division abnormalities were observed. Additionally, diabetic rats showed degeneration in the structure of the thymus including trabecular thickening, accumulation of lipid vacuoles, heterochromatic nuclei and loss of mitochondrial cristae. Degradation of medullar and cortical integrity was also detected. In the vitamin C-treated STZ-diabetic group, the structure of the thymus and mitotic activity of the lymphocytes were similar to the control group. These results suggest that vitamin C protects the thymus against injury caused by diabetes and restores thymocyte mitotic activity.

Key words: thymus, vitamin C, diabetes, electron microscope, mitotic activity

Introduction

Diabetes mellitus is a chronic disease characterized by hyperglycemia, which causes disorders in the metabolism of carbohydrate, lipid and protein [1]. It also affects the thymus, the key organ for the production, maturation and selection of T-cells, which are responsible for the regulation of immune responses as well as cellular immunity [2–6]. In diabetics, susceptibility to inflammation increases due to the reduction in cellular immunity. Type 1 diabetes causes immune dysfunction due to the escape of self-reactive T-cell clones from deletion in the thymus and in the peripheral lymphoid organs; the resulting altered immune balance leads to autoimmunity [7].

Vitamin C is an important antioxidant that protects cells from free radicals [8] and has preventative and beneficial effects on the oxidative stress associated with aging [9,10]. It also considerably reduces the lipid peroxidation in plasma, heart and kidney caused by diabetes [11–15]. There are many studies on the effects vitamin C on the physiology, morphology and pathology of organs in diabetes. However, the diabetic thymus has not yet been comprehensively explored. In this study, the ultrastructure of the thymus was investigated to improve our understanding of the effects of vitamin C on STZ-induced diabetes. Both cell damage and mitotic activity were evaluated.

Methods

Animals and experimental design

This experiment was approved by the Animal Experiments Local Ethics Committee of Gazi University, Turkey. Twenty-nine male adult (2–2.5 months old) Wistar Albino rats weighing 200–220 g were housed in standard laboratory conditions (room temperature $22 \pm 2^\circ\text{C}$, 12:12 h light–dark cycle, and $55 \pm 5\%$ humidity) with free access to food (standard pellet diet) and drinking water.

The duration of the experiment was set at 21 days, and the rats were randomized into three groups, organized as follows:

- Group 1 (G1): control group ($n = 9$)
- Group 2 (G2): diabetic group ($n = 10$)
- Group 3 (G3): vitamin C-treated diabetic group ($n = 10$)

Diabetes induction and vitamin C administration

Diabetes was induced in G2 and G3 rats by a single intraperitoneal injection of streptozotocin (STZ) (45 mg/kg) (Serva 35503, Heidelberg, Germany) dissolved in 0.1 M sodium citrate buffer (pH 4.5). Forty-eight hours after STZ injection, the fasting blood glucose of both diabetic and control rats was measured with a glucose meter (Glucotrend;

Accu-Chek, UK) in blood drawn from tail veins. Animals having a blood glucose level >200 mg/dl were considered to be diabetic. For G3 rats, vitamin C (Sigma Chemical Co., St. Louis, MO) treatment started 1 week after administration of STZ to allow time for diabetes to develop. G3 rats received a dose of 20 mg/kg vitamin C by intragastric gavage for 21 days. Tap water was given to the control group by the same method for the same period. On day 22, the rats were sacrificed under anesthesia (sodium thiopental; 50 mg/kg).

Microscopy

Thymus tissue samples were fixed with 2.5% glutaraldehyde (Sigma Chemical Co.) in 0.1 M sodium phosphate buffer (pH 7.2) and kept cold (4°C) for 2 h for histopathological examination. After rinsing several times in phosphate buffer, the tissues were post-fixed in 1% osmium tetroxide (Sigma Chemical Co.) for 2 h. Fixed tissues were dehydrated in a series of graded ethanol, placed into propylene oxide (Merck, New York) and embedded in araldite (Merck). Semithin sections were obtained with an ultramicrotome, stained with toluidine blue and examined by a light microscope (Leica DM 4000B; Wetzlar, Germany). Ultrathin sections were stained with uranyl acetate and lead citrate and then examined by transmission electron microscopy (Carl Zeiss EM 900; Jena, Germany).

Photomicrographs randomly taken from the histological sections of the thymus were analyzed digitally with ImageJ software (<http://rsb.info.nih.gov/ij/>) for the quantification of mitotic activity.

Statistical analysis

Data are expressed as means $\pm$ SD. Statistical significance was determined by the Kruskal–Wallis test, and *post hoc* multiple comparison tests were performed to check all paired comparisons for possible differences.

Results

Light and transmission electron microscopy

Thymus sections from G2 and G3 rats were compared with those of the control group (G1) for signs of degeneration. Table 1 presents a summary of thymus specimens and associated indicators of degeneration: decrease in mitotic activity, thickened trabecular structure, accumulation of lipid vacuoles and fusion of some lipid vacuoles, loss of some of mitochondrial cristae in lymphocytes and accumulation of material in some giant vacuoles. For each indicator, ‘–’ represents no degeneration, and ‘+’ represents degeneration.

The light micrographs of thymus sections of control group (G1) rats had dark cortical and light medullary areas with mitotically active thymocytes (Fig. 1a and b). In contrast, thymocytes from the diabetic group (G2) showed evidence of both decreased mitotic activity and cell division abnormalities (Fig. 1c). However, in vitamin C-treated diabetics (G3), thymocyte mitotic activity was similar to that of the control group (G1) (Fig. 1d). Again, while the trabecular structure of the control group was normal (Fig. 2a), degeneration of the structure of the thymus was detected in the diabetic group in the form of thickened trabeculae (Fig. 2b) and lipid vacuolization (Fig. 2c). Thymus structure in the vitamin C-treated diabetic group, however, was similar to that of the controls (Fig. 2d).

Table 1. Summary of the structural differences of the lymphocytes between the groups

Electron and light microscopic degenerative criteria	G1	G2	G3
Decreased mitotic activation	–	+	–
Thickened trabeculae structure	–	+	–
Accumulation of lipid vacuoles and fusion in some lipid vacuoles	–	+	–
Loss of some of mitochondrial cristae of lymphocytes	–	+	–
Accumulation of matter in some giant vacuoles	–	+	–

Figures 3 and 4 are electron micrographs of the thymus sections of control group rats and show various phases of mitosis and membrane integrity, respectively, in thymocytes. Electron microscopy of diabetic rat (G2) thymus sections revealed heterochromatic nuclei in some small lymphocytes, accumulation of lipid vacuoles, fusion of some vacuoles (Fig. 5a), accumulation of material in some giant vacuoles and loss of some mitochondrial cristae in lymphocytes (Fig. 5b). No such signs of degeneration could be detected in G3 rats: the thymocytes in the cortical area of the thymus had a normal structure (Fig. 6a) and membrane integrity was preserved in this group (Fig. 6b). Indeed, lymphocytes in mitotic phase were observed in the vitamin C-treated diabetic rats (Fig. 7a–e).

Morphometric results

Using micrographs of thymus sections, both the number of cells and the number of lymphocytes in mitosis were counted with ImageJ. For both parameters, results were converted to the number of cells per 10 000 μm^2 of tissue and are presented in Table 2. The ratio of mitotic cells to the number of cells (in the micrographs) is also given in Table 2 and is expressed as a percentage. For each group (G1, G2 and G3) of rats, mean $\pm$ SD was also calculated. This analysis demonstrated that, compared with the control group,

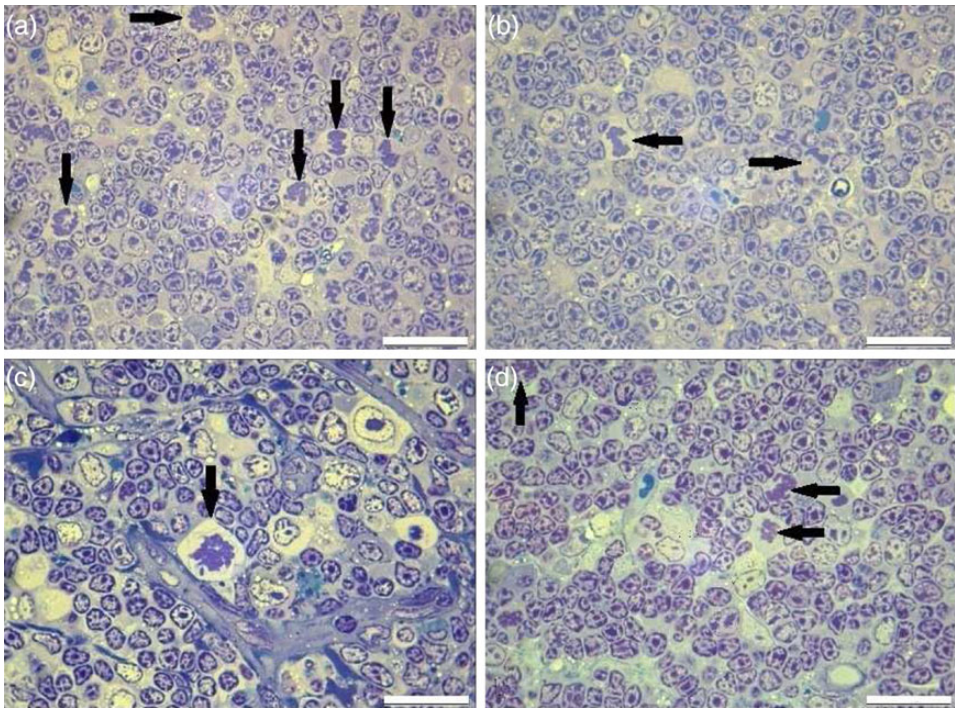


Fig. 1. Micrographs of the semithin sections. (a) Lymphocyte division (arrows) of the control group. (b) The metaphase and the anaphase stages (arrows) were seen clearly in the control group. (c) The division abnormality in prometaphase (arrow) of the STZ-diabetic group. (d) Prophase and metaphase (arrows) seen in the vitamin C-treated diabetic group. Scale bars: 20 μm .

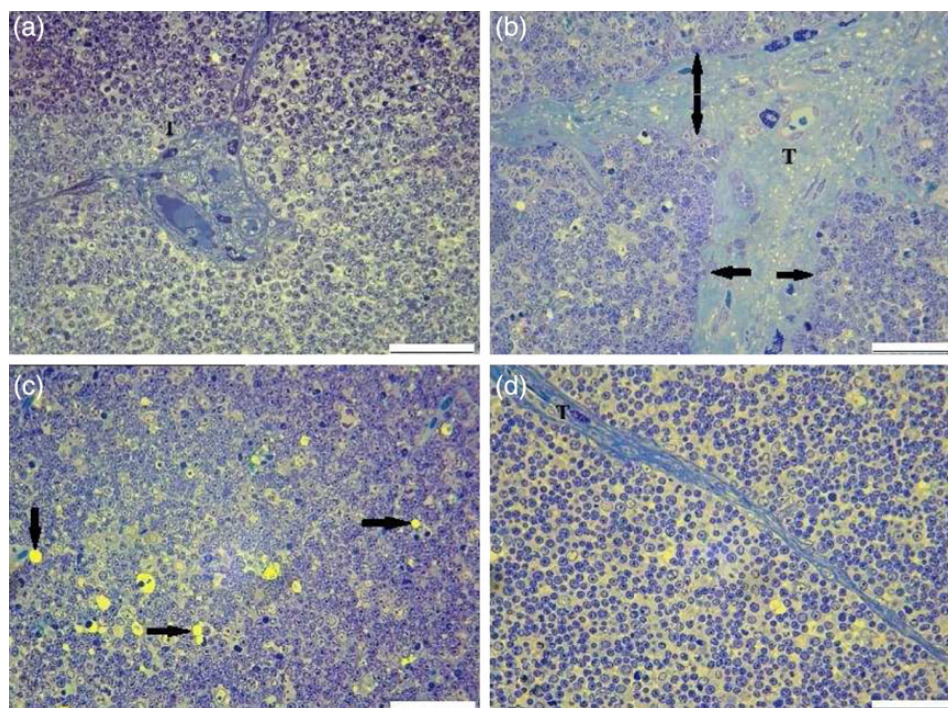


Fig. 2. (a) Trabecule (T) structure of rat thymus in the control group, (b) trabeculae thickening was observed in the STZ-diabetic group, (c) lipid vacuolization (arrows) in the diabetic group and (d) normal structure of the trabeculae (T) in the vitamin C-treated diabetic group. Scale bars: 50 μ m.

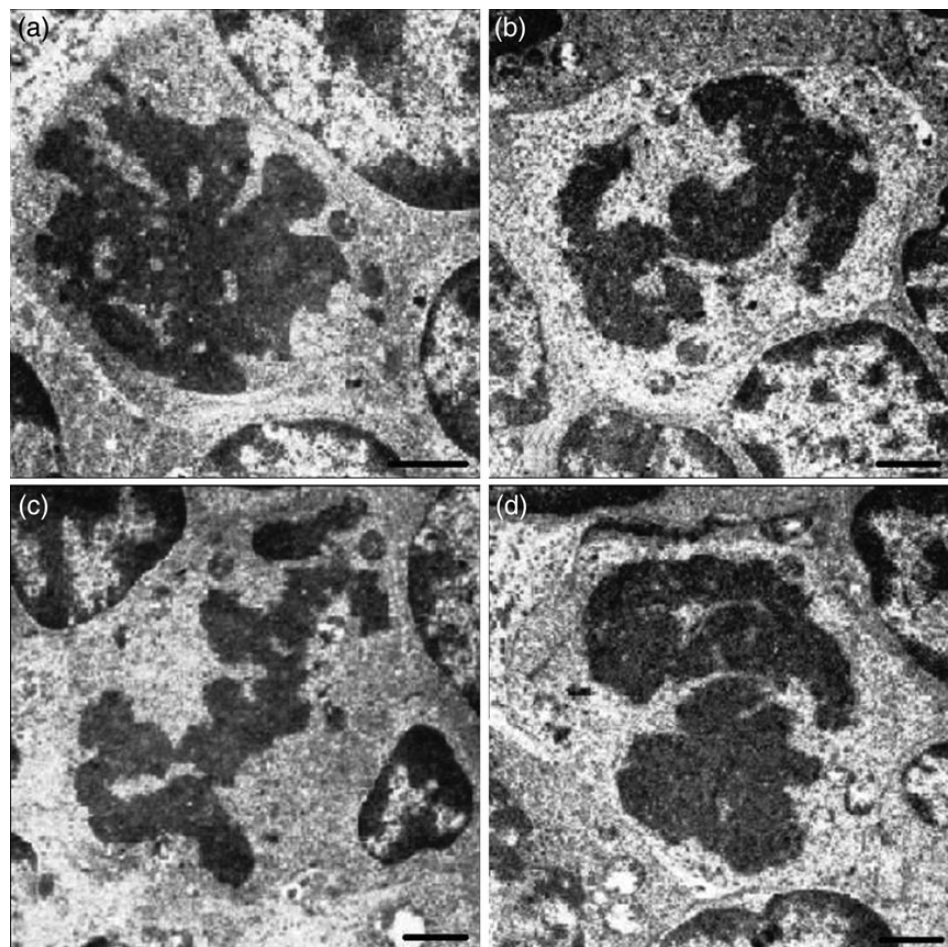


Fig. 3. Electron micrographs of the ultrathin sections showing cells in mitotic phase of the control groups. (a) Late prophase, (b) metaphase, (c) anaphase and (d) early telophase. Scale bars: 2 μ m.

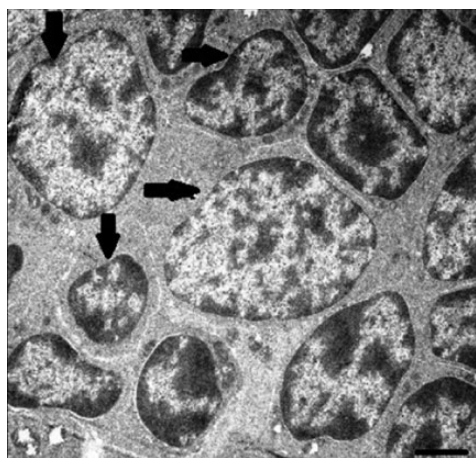


Fig. 4. Small, medium and large lymphocytes (arrows) together in the control group. Scale bar: 2 μm .

both the number of dividing cells and the total number of cells in thymus sections ($10\,000\ \mu\text{m}^2$) of the diabetic (G2) group decreased by 57 and 18%, respectively. This was not the case for the vitamin C-treated diabetic (G3) group, however, which gave similar results to the control group.

The difference in the number of mitotic cells per $10\,000\ \mu\text{m}^2$ ($P = 0.002$) and in the percentage of mitotic cells ($P = 0.026$) between the groups was significant. On the other hand, no statistically significant difference ($P = 0.064$) among the mean number of cells per $10\,000\ \mu\text{m}^2$ of the groups was found. When a *post hoc* multiple comparison test was used to compare all potential combinations, the disparity between groups G1 and G2, with respect to both the number of mitotic cells per $10\,000\ \mu\text{m}^2$ ($P = 0.002$) and the percentage of mitotic cells ($P = 0.024$), was statistically meaningful.

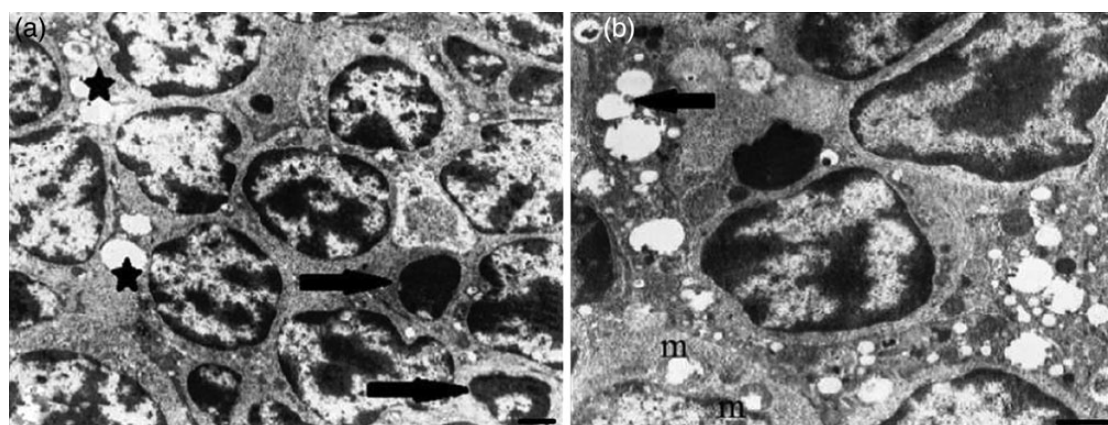


Fig. 5. (a) Vacuolization (stars) and heterochromatic nucleus (arrows) of the lymphocytes in the cortical region of the diabetic group. (b) Accumulation of lipid vacuoles and fusion (arrow) in some lipid vacuoles in the medulla region of the STZ-diabetic rat. Loss of some of mitochondrial (m) cristae of lymphocytes. Scale bars: 2 μm .

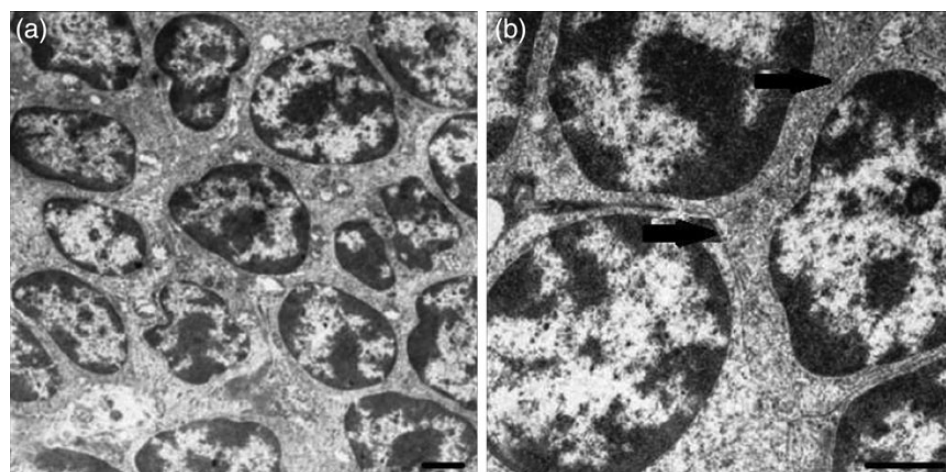


Fig. 6. (a) The cortex region of the thymus in the vitamin C-administered diabetic group. (b) The integrity of the thymocyte cell membranes (arrows) was clearly visible in the vitamin C-treated diabetic group. Scale bars: 2 μm .

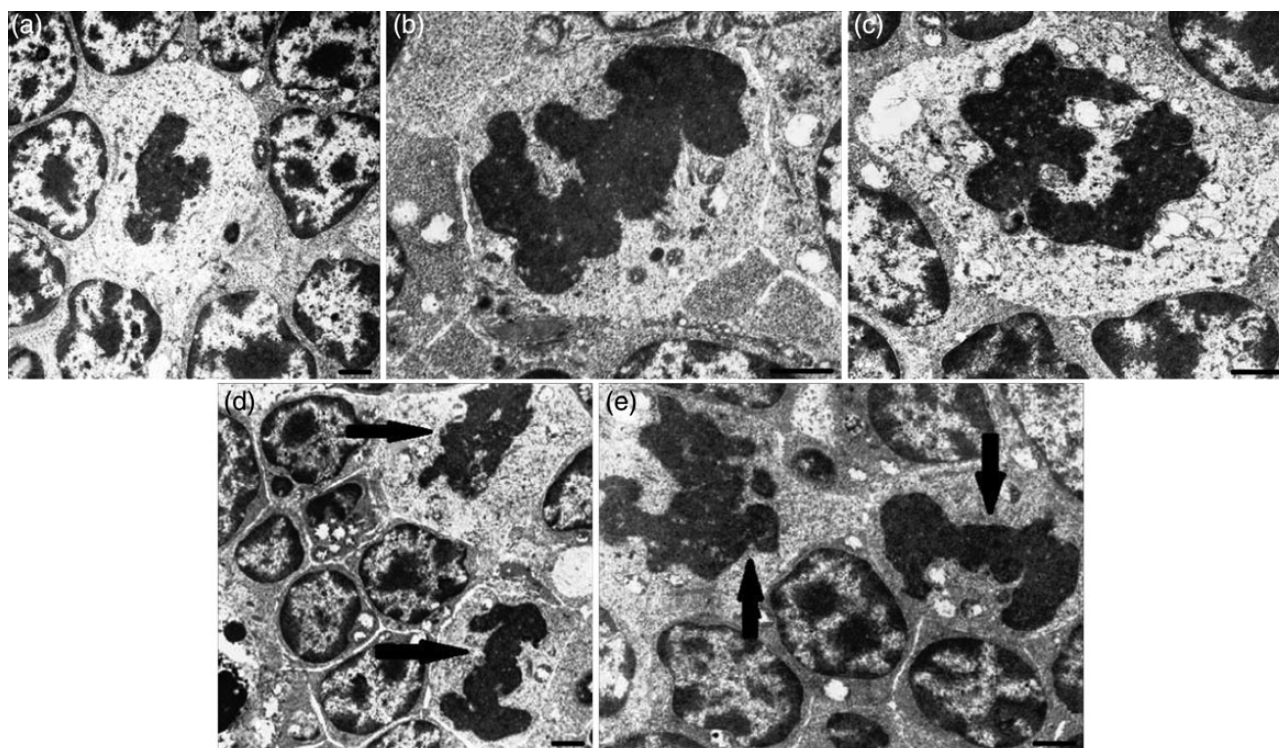


Fig. 7. Thymic lymphocytes show mitotic activity in vitamin C-treated diabetic group of rats. (a) Prometaphase, (b) late metaphase, (c) anaphase, (d) late prophase and metaphase stages (arrows) seen in the same region and increase in the mitotic activation, and (e) thymocyte mitotic activity in the vitamin C-treated diabetic group. Prometaphase and late metaphase phases (arrows) seen together. Scale bars: 2 μ m.

Discussion

The thymus is crucial for the correct functioning of the immune system. It is responsible for the production of self-restricted and self-tolerant T-cells [16], and both facilitate and regulate the interaction of lymphoid and nonlymphoid cells [17]. Various physiological and pathological conditions cause a decrease in the number of thymocytes [18], which leads, in diabetic rats, to thymus atrophy [19–22]. Berzins *et al.* [23] have suggested that abnormal thymocyte differentiation occurs in the non-obese diabetic (NOD) thymus, and the resulting altered cell population is predisposed to autoimmunity. STZ is a nitrosoamide that is frequently used in experimental models to induce diabetes due to its toxic effects on pancreatic β cells [24,25]. Chatamra *et al.* [21] studied thymus atrophy in STZ-diabetic rats, reporting reduced numbers of cortical thymocytes and an increase in fibrous tissues. These findings are corroborated by our own, which show loss of thymocytes and thickening of the trabecular structure in semithin sections of STZ-diabetic rat thymus tissues.

Elmas *et al.* [26] investigated the fine structure of diabetic thymus tissue and found heterochromatic nuclei, crystallization of mitochondria and connective tissue invasion in the thymocytes of the diabetic groups. In our study, similar indicators of degeneration such as loss of

cristae of the lymphocyte mitochondria, hyperchromatic nuclei in some lymphocytes and lipid accumulation in the intra- and intercellular spaces were observed. Kaya-Dağistanlı *et al.* [27] studied neonatal STZ-diabetic rat thymus and found that long-term diabetes causes apoptosis in the thymocytes. Although we did not assess the degree of apoptosis, our analysis revealed cell division anomalies in the diabetic rat thymus, together with a decline in mitotic activity and reduced numbers of thymocytes. Therefore, our observations by both light and electron microscopy, together with those of other workers, are consistent with a pattern of reduced cell proliferation in diabetic rat thymus.

Diabetic rats are known to have decreased levels of endogenous vitamin C [28–30], although the cause of this deficiency has yet to be satisfactorily explained. Nevertheless, researchers have suggested that abnormalities in vitamin C metabolism and a decrease in serum vitamin C levels could be important factors in the pathogenesis of diabetes [30–32]. Vitamin C is a recognized antioxidant and acts to reduce and neutralize reactive oxygen species (ROS) [33,34]; the reduced vitamin C levels in diabetes are likely to result in an increase in ROS activity, which in turn could be the cause of thymus damage. Vitamin C treatment may be expected to fortify the antioxidant

Table 2. Analyses of the mitotic cells in the thymus tissues (area = 10 000 μm^2) of the groups

	Cell counted (n)	Number of mitotic cells (n)	The percentage of mitotic cells (%)
G1			
1	1412	18	1.27
2	1085	32	2.94
3	920	31	3.36
4	1390	33	2.37
5	1326	24	1.81
6	1296	28	2.16
Mean $\pm$ SD	1238 $\pm$ 194	28 $\pm$ 6*	2.32 $\pm$ 0.75**
G2			
7	1096	8	0.73
8	765	8	1.18
9	1114	15	1.35
10	1117	15	1.34
11	1023	14	1.37
12	945	12	1.27
Mean $\pm$ SD	1010 $\pm$ 137	12 $\pm$ 3*	1.20 $\pm$ 0.24**
G3			
13	1387	31	2.24
14	1310	24	1.83
15	1363	17	1.25
16	1211	21	1.73
17	1056	25	2.37
18	1185	16	1.85
Mean $\pm$ SD	1252 $\pm$ 125	22 $\pm$ 5*	1.77 $\pm$ 0.68**

Data are expressed as means $\pm$ SD, Kruskal–Wallis test was used.

* $P < 0.01$.

** $P < 0.05$.

defense system [35–37]; indeed, we observed that the tissue organization of vitamin C-treated diabetic rats was similar to that of the control (non-diabetic) group, indicating that vitamin C can prevent the thymus pathology induced by diabetes. However, the dose and the duration of the vitamin C treatment is crucial, as its transformation products can have pro-oxidant properties, depending on conditions [38].

In humans, exogenous intake of vitamin C is prescribed for diabetics [39], but there is conflicting information in the literature on whether vitamin C acts directly on pancreatic β cells to ameliorate diabetes. Researchers claim either no effect of vitamin C intake on blood glucose concentration [40], or a decline [41–43] in blood glucose concentration proportional to the dosage of vitamin C. Further research is needed to investigate this question. In addition, it has been suggested that ascorbic acid deficiency in the diabetics causes collagen abnormalities [31]. Moreover, it has been reported that vitamin C increases the proliferation of T cells

[44,45], inhibits various forms of T cell death [46] and stimulates cellular immunity [47]. These studies corroborate our findings: T-cell proliferation and mitotic activity returned to normal in the vitamin C-treated group of STZ-diabetic rats examined here.

Concluding remarks

Several physiological and pathological conditions lead to a reduction in the number of cells in various tissues. In this study, we found that diabetes causes reduced mitotic activity, abnormal cell division, lipid accumulation and thickened connective tissue in rat thymus, leading to the destruction of thymus architecture; such tissue damage is a strong indication of thymus atrophy. On the other hand, there was a remarkable increase in mitotic activity in the vitamin C-treated diabetic groups. In conclusion, the vitamin C dosage used was found to be appropriate and effective in decreasing cell degeneration in the thymus tissue of diabetic rats.

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