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Complementary effect of vitamin C and Zinc on some blood cells and serum proteins related to immunity in intact and ovariectomized rabbits

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Abstract
The present experiment was investigated to study the complementary effect of vitamin C and Zinc supplementation on some parameters related to the immune system in intact and ovariectomized rabbits. Twenty female rabbits (7-8 weeks) have been used in this study. Ten of them had been subjected to ovariectomy and the other ten were left with intact ovaries. After recovery from the operation and acclimatization, the rabbits were divided into four equal groups as follow: Group one (G1): Intact rabbits received distilled water. Group two (G2): Intact rabbits received complementary vitamin C and Zinc with the dose (10.166 mg/kg/B.W) orally and daily. Group three (G3): Ovariectomized rabbits received distilled water. Group four (G4): Ovariectomized rabbits received complementary vitamin C and Zinc. The daily supplementation of complementary vitamin C and Zinc induces a significant increase in total leukocytes (14.06 and 11.08 × 10^3 /mm^3) in both intact and ovariectomized rabbits respectively. Moreover, the Arneth' s index reveals a significant higher percentage of neutrophils of four and five lobes in both supplemented groups. The total percentage of mature cells was (27.9% and 14.1%) for G2 and G4 respectively in comparison with (9.0% and 8.3%) for G1 and G3 respectively. There is a significant increase in eosinophil's and lymphocytes percentages in Ovariectomized rabbits only. However, the result also revealed a significant increase in total protein and total gamma concentration in both intact and ovariectomized rabbits that supplemented with complementary vitamin C and Zinc. In conclusion, the results from this experiment confirm and for the first time that the complementary vitamin C and Zinc supplementation to rabbits has an important protective role on the immune system in ovariectomized rabbits. This supplementation can overcome the deleterious effect of ovariectomy and ovarian hormones deficiency on bodies' immunity.

Keywords: Vitamin C, Zinc, Ovariectomy, Immunity

1. Introduction
Both vitamin C and zinc give a complementary powerful antioxidant protection against endogenously and exogenously reactive oxygen species (ROS) [1]. There is a true scientific rationale that the combination of vitamin C and zinc can play an important role in the immune functions and also to reduce the risk of diseases [2]. Much evidence pointing towards the significant role of sex hormones in immunity has been reported by human and animal studies on manipulation of hormones. It has been shown in several states that females immune system respond better than males to infections of pathogen and most of programs of vaccination in both mouse models and clinical studies [3].

Generally, steroid hormones exert an opposite role on the immune response, and autoimmune diseases with estrogens as enhancers of humoral immunity and androgens and progesterone as natural immunosuppressant [4, 5].

Aging is playing an important role by declining the immune function affectivity; immune senescence, a phenomenon which believed that it is the cause of greater infectious disease that related to morbidity and mortality in elderly [6]. By expressing the ovarian steroids influences on the immune system functions, their loss could exacerbate the immune senescence during menopause [7].

Some of the immunological alteration occurs in postmenopausal female included decreases in interleukin-2 (IL-2), IL-6, and insulin-like growth factor-1 levels and increase the levels of IL-4and IL-1 [8]. Elevating in production of B-cell, estrogen receptor alpha, CD19+ cells, and the complement, C3 and C4 levels were also documented. Diminished CD8+ checks were always announced as well. In any case, the information on the progressions in different factors like

tumor necrotic factor-alpha (TNFα), interferon-gamma (IFNγ), CD4+, and CD25+ were opposing [8].

From the mentioned literature, it is necessary to find out the protective effect of the complementary vitamin C and zinc on some immunological and biochemical parameters in intact and ovariectomized rabbits.

There for, the objects of this study was to investigated the effect of complementary vitamin C and zinc on some blood cells and serum proteins related to immunity in intact and ovariectomized rabbits.

2. Materials and methods

2.1 Experimental animals and care

Healthy local twenty female rabbits before puberty 7-8 weeks old and there weighing between850-1100 gram (g) were obtained from the drug control center /ministry of health and reared in the animals house of the Veterinary Medicine College /University of Baghdad during the period from December/2016 till February/2017. They were reared in suitable condition of 20-25 ℃ in an air conditioned room and photoperiod of 12 hours daily. The animals were housed at least two weeks for acclimatization before beginning the experiment [9]. Anticoccidiosis (Amprolium) was given via drinking water (1g/litter) for three days during acclimatization period.

2.2 Surgical operation of ovariectomy

Ovariectomy was performed at ten weeks old females for two groups of the experiment. First of all, the site of operation was prepared, then the animal was anesthetized with intramuscular (IM) injection of Ketamine at 35 mg/kg/B.W, and Xylazine at a dose 15 mg/kg/B.W [10].The operation was made according to previous study [11]. The animals were kept after operation individually with post-operative care which lasted for 5 days.

2.3 Preparation of the complementary vitamin C and Zinc dose

The dose of this experiment was calculated according to the recommended dose for human 1-3 tablet/day that provided by MEDCELLPHARMA® CO. (Netherlands). Each one contains three hundred and five mg (vitamin C 300mg + Zinc 5mg). Then each rabbit was received 1ml/1kg/B.W (10.166 mg/kg B.W), the dose was administered daily to each female of two groups for thirty days using gastric intubation.

2.4 Blood samples collection

At the end of period (30 day) of the experiment, Fasting blood was obtained via jugular vein from each rabbit. Samples were divided into two divisions, first part is collected by anticoagulant tube for blood parameters, and the second part for serum collection and was isolated after centrifugation at a speed of 3000 revolution/minute (rpm) for 20 minutes. Serum samples were stored in freezer at -18 ℃ until use [12].

2.5 Experimental design for the experiment

After acclimatization for two weeks the rabbits were divided equally into four groups, two groups was kept intact (G1, G2) and two groups were ovariectomized (G3, G4). Group two and four were administered daily with the complementary vitamin C and Zinc (10.166 mg/kg.Bw). The other two groups (G1, G3) was received distilled water daily.

2.6 Determination of parameters of the experiment

Total WBC count, Red blood cells count and Platelets count was measured manually [13] by using hemocytometer slide (neubauer slide). WBCs types' percentage were measured by using blood smear for each animal and stained by Giemsa stain as described by [13] examined under 100× oil emersion lens light microscope. Neutrophil / Lymphocytes ratio (N/L) and Lymphocytes / Monocytes ratio (L/M) was measured by dividing the percentage of neutrophils on lymphocytes and lymphocyte percentage on monocyte respectively [14]. Arneth's index was estimated by examine 100 (or 200) neutrophils in blood film, noting the number of lobes in each cell and calculates the percentages of each stage [15]. Total serum proteins concentration (g/L) was calculated by using a special kit (LiNear, Spain) [16] and the serum albumin concentration (g/L) was calculated by using a kit of (Bio system, Spain) [17]. Total serum globulin concentration (g/L) was measured as mentioned by [18]. Albumin/Globulin ratio (Alb/Glob) was obtained [19]. Electrophoresis of total gamma globulins was measured by using protein electrophoresis apparatus (Helena Bioscience /Europe) and the results was calculated by control software "analysis-platinum 4V"

2.7 Statistical analysis

The statistical analysis of the data of the experiment was performed by using one-way ANOVA and Least significant differences (LSD) to assess significant differences among means of the groups by using the SAS [20].

3. Results and Discussion

3.1 The complementary effect of vitamin C and Zinc on some blood parameters (total WBCs, RBCs, platelets counts, differential leukocytes numbers, N/L and L/M ratios and Arneth's index) in intact and ovariectomized rabbits

There was a significant ($p \leq 0.05$) decrease in leukocytes and erythrocytes number in the ovariectomized group (G3) in comparison with other groups (Table 1).It also revealed significant ($p \leq 0.05$) increase in platelets count in this group compared with the intact one (G1).On the other hand, administration of the complementary vitamin C and Zinc induces a significant ($p \leq 0.05$) increase in total leukocytes, erythrocytes and platelets numbers in both intact and ovariectomized groups.

Concerning the differential leukocyte count, Table 2 represent the significant ($p \leq 0.05$) decrease in lymphocyte, monocyte, basophile and eosinophil percentages with significant ($p \leq 0.05$) increase in neutrophils percentage in the ovariectomized group (G3). At the meantime, there was an opposite results in groups supplemented with vitamin C and zinc. However, the N/L ratio showed an increase in ovariectomized group (G3) with a decrease in L/M ratio (Table 3).While, there is a decrease in N/L ratio in groups supplemented with the complementary vitamin C and zinc (G2 and G4). The L/M ratio showed higher value in the ovariectomized group that supplemented with the complementary (G4).

Table 4 and Fig.1 represent the complementary effect of vitamin C and zinc on neutrophil maturation stages (Arneth's index). The ovariectomized group (G3) shows a significant ($p \leq 0.05$) large percentage of immature and metamyelocyte stages of neutrophils (40.5%) than the more mature neutrophils that have four, five or more lobes (8.3%) which means shift to left pattern. From the other side, there is a significant ($p \leq 0.05$) higher percentage of the more mature cells in both supplemented intact and ovariectomized groups (G2 and G4) as compared to non-supplemented groups (G1 and G3). The total percentage of cells that exhibited four, five

or more lobes are (27.9%) and (14.1%) for G2 and G4 respectively in comparison with (9 %) and (8.3%) G1 and G3 respectively.

However, the significant decrease in the total leukocyte count in the ovariectomized groups (G3) in the current study is associated with a decrease in lymphocytes and an increase in neutrophils and monocytes percentage. These results in turn induce a higher N/L and lower L/M ratios in this group. It was reported that the amount of mature T- cells were significantly declines [21] by increase glucocorticoid levels. High levels of glucocorticoids had been indicated to produce a serious reduction of the thymus and enhanced apoptosis of pre T-cells [22]. Since the estrogens can regulate antioxidant gene expression and improve antioxidant status via their interaction with estrogen receptors (ER) [23]. And that was proved by the decreases of the activity of the antioxidant enzymes in the intra-abdominal tissue of MS female rats after removal of E2 by ovariectomy [24]. So that will lead to increase the oxidative stress one of the most effective stresses in and trigger the body to generalize physiological responses which will lead to increase the glucocorticoid levels [25, 26].

So, the ovariectomy is consider as a stress physiological condition and could induce such effect by increasing glucocorticoid secretion [25, 26]. and therefor, decreasing lymphocytes number and changes in differential leukocytes [27]. In the present experiment could be explained by the direct action of estrogen on all cellular subsets of the immune system through estrogen receptors-dependent and independent mechanism [5].

There is a clear evidence that estrogen affects the cells of adaptive immune system (T and B cells) and innate immune system which include neutrophils, monocyte, macrophage, dendritic cells and natural killer cells [28, 29]. This in turn, could explain the results of Arneth's index which shows a shift to left which mean increase in immature and metamyelocyte.

Concerning the effect of ovariectomy on red blood cells and the significant decrease of the value in the present experiment could be attributed to the vital role of estrogen on erythrocytes synthesis. The literature review lack such effect about the changes in RBCs number in ovariectomized or postmenopausal females. From our knowledge, the number of erythrocyte is generally more in males than in females.

From the other hand, the mouse had been reported to exhibit sex differences in cycle regulation by estrogen. The hematopoietic stem cells (HSCs) in females divided significantly more frequently than in males and this differences was concluded to be depended on the ovaries but not the testes [30].

The increase of platelets number in the ovariectomized groups of the current study could be associated to decrease nitric oxide function. However, it was reported the loss of ovarian estrogen after ovariectomy induce a decrease in the activity of the NO- synthase enzyme III as well as prostacyclin [31]. This in turn enhance platelets-vessels wall interactive and platelets aggregation which end to produce the arterial occlusive disease after menopause.

Vitamin C has been shown to stimulate both the production [32] and function of leukocytes, especially phagocytes, lymphocytes and neutrophils [33]. This effects of vitamin C could be attributed mainly to the two major functions of it: as antioxidant and as an enzyme cofactor [34]. Vitamin C can protect carbohydrates, lipids, proteins and nucleic acids (DNA and RNA) from damage of free radicals. Moreover, vitamin C also participates in redox recycling of other important antioxidants for example regenerate vitamin E from its oxidized form to its reduced form [35]. Through its antioxidant functions, vitamin C has been shown to protect leukocytes from cells-inflected oxidative damage [36]. The findings of the present experiment reveal a right shift of neutrophils due to an increase in four, five or more mature lobes of neutrophils.

Vitamin C action as a cofactor is by maintaining enzyme-metals in there reduced form [34]. It assist mixed-function oxidases in the synthesis of several critical biomolecules such as collagen, carnitine and catecholamine's [37]. It has been suggested that vitamin C is involved in the metabolism of cholesterol to bile acids, which may have implication for blood cholesterol levels and incidence of gallstones [38]. From the information's discussed above, it's not surprise to fine that the complementary vitamin C and zinc supplementation induces an increase in erythrocytes and platelets counts. Vitamin C was documented to have protection on phospholipids (the main components of all plasma membranes) from the destructive processes in association with glutathione [39].

The significant effect of the essential trace elements zinc for immune functions has been known for several decades. Zinc has been reported to affect both innate and adaptive immune cells. This is highlighted by the effect of zinc deficiency, including thymic atrophy, lymphopenia, impaired cellular and antibody- mediated immune responses and even death [40].

Our explanation for the net results is that zinc induces an absolute increase in total leukocytes counts due to its direct stimulating effect on bone marrow. Zinc is basic and essential for differentiation of T-cells and the components require in this producer are IFN_{γ}, IL-12 receptors β2. Zinc is known to upregulate the mRNAs of every one of these factors [41]. Moreover, zinc supplementation significantly increased NK cells number in whole blood cultures [42] and NK cells activity *in vivo* [43]. NK cells, as all other leukocytes are generated from pluripotent stem cells in the bone marrow that express the clusters of differentiation proteins (CD34) on their surface [44].

3.2 The complementary effect of vitamin C and Zinc on serum proteins (total proteins, albumin, total globulins, AL/GL ratios and total gamma globulins) related to immunity in intact and ovariectomized rabbits.

Table 5 revealed a significant ($p \leq 0.05$) decrease in total serum proteins combined by a decrease in albumin and globulins concentration in the ovariectomized rabbits (G3). Moreover, this has a higher AL/GL ratio and a lower total serum gamma globulins level. The complementary vitamin C and zinc supplementation to the experimental animals induces a significant increase in total proteins of both groups (G2 and G4). On the other hand, Table 5 shows that the supplemented ovariectomized group (G4) has a lower value of serum albumin and a higher significant globulin coincided by a lower AL/GL ratio and higher gamma globulin serum concentration in comparison with all other groups.

From the available reviews, we couldn't find an explanation concerning the alterations in plasma proteins after ovariectomy. However, ovariectomy has been used as an experimental animal model to simulate the physiological condition of menopause and both are associated with changes in body composition, loss of bones rigidity and increase the evidence of autoimmune disease. Estrogens and exactly estradiol 17B(E2) are able to regulate immune responses by cells development, cytokines or/and antibody production, apoptosis and proliferation [45]. Estrogen has also profound effects on B cell differentiation, function, activity [46] and

survival by increasing expression of gens [47]. Estrogen has been shown plasma cells and autoantibody producing cells number [48].

It has been reported that females shows greater antigen presenting activity and mitogenic responses, higher immunoglobulin's levels and more enhanced antibody production than males [49]. Females tend to have more responsive and robust immune system compare to males, it is therefore not surprising that females respond more aggressively to self-antigens and are more susceptible to autoimmune diseases. and the reasons for sex differences in immune responses are not precisely known [5]. Therefore, from the above mentioned discussion, it is not surprising that ovariectomy induced an inhibition of proteins synthesis from liver with increased AL/GL ratio.The present findings show a decrease in total gamma globulins in the ovariectomized group as well. This is coincided with decrease lymphocytes in these animals because only gamma globulins are synthesized from B cells [50] and estrogen has a crucial role in B cell differentiation, activity and function [48].

The increase in total serum proteins off rabbits supplemented with the complementary vitamin C and zinc could be attributed to one or both of the following explanation:

First of the all might be an increase in protein synthesis by the liver (absolute increase) and this is based on the fact that vitamin C could increase the bioavailability of liver which in turn, cause an enhancement of its activity and function for proteins synthesis [51]. Most of plasma proteins produced by the liver [52]. In addition, zinc is an essential element important for the normal growth and reproduction of animal. It's vital for the functions of more than 300 enzymes, for the stabilization of DNA and for gene expression and normal function of cell membranes [53]. Moreover, the increase in total serum globulins in the present experiment is mainly due to the increase in gamma globulins which well known that it's mainly composed from immunoglobulin. This type is produced by the plasma cell that is the mature stage of B lymphocyte cells [50] which exhibit a significant increase by the supplementation [1, 54].

The second explanation for increasing the total proteins and globulins in serum of intact and ovariectomized rabbits supplemented with complementary vitamin C and zinc, could be associated to the antioxidative action of them [1, 55]. They protect the body from the deleterious and damaging effect of free radicles (ROS), that cause oxidation of LDL and the oxidative damage of DNA and proteins [56, 57]. So the role of the complementary is to prevent the damage effect of the free radical by their antioxidant action and that will lead to relative increase total proteins and globulins in the serum.

Table 1: Complementary effect of vitamin C and Zinc on Total Leukocytes counts, RBCs counts and platelets counts in intact and ovariectomized rabbits.

Groups / Parameter	G1 Intact Rabbits Received distilled water	G2 Intact rabbits Received Vit C and Zinc	G3 Ovariectomized rabbits Received distilled water	G4 Ovariectomized rabbits Received Vit C and Zinc	LSD
Total Leukocytes counts ($\times10^3$cells/mm^3).	7.18 ± 0.07 C	14.06 ± 0.02 A	4.75 ± 0.03 D	11.08 ± 0.03 B	0.1354
Red blood cells counts ($\times10^6$cells/mm^3).	5.71 ± 0.005 A	5.72 ± 0.01 A	4.73 ± 0.01 C	4.85 ± 0.008 B	0.0296
Platelets counts ($\times10^3$cells/mm^3).	245.80 ± 0.86 D	330.20 ± 1.93 B	272.60 ± 1.16 C	534.20 ± 2.17 A	4.8758

Values represent mean ± SE (N=5).

Different capital letters denote a significant difference between groups ($p \leq 0.05$).

Table 2: Complementary effect of vitamin C and Zinc on Basophils, Eosinophil's, Neutrophil's, Lymphocytes and monocytes percentage (%) in intact and Ovariectomized rabbits.

Groups / Parameter	G1 Intact Rabbits Received distilled water	G2 Intact rabbits Received Vit C and Zinc	G3 Ovariectomized rabbits Received distilled water	G4 Ovariectomized rabbits Received Vit C and Zinc	LSD
Basophils percentage (%)	1.52 ±0.07 A	1.48 ±0.07 A	0.85 ±0.05 B	1.02 ±0.05 B	0.1882
Eosinophil's percentage (%)	0.68 ±0.06 A	0.71 ±0.04 A	0.36 ±0.03 B	0.60 ±0.01 A	0.1311
Neutrophil's percentage (%)	24.49 ±0.04 B	23.12 ±0.19 C	34.96 ±0.23 A	19.86 ±0.24 D	0.5938
Lymphocytes percentage (%)	70.11 ±0.06 B	70.15 ±0.23 B	60.10 ±0.22 C	75.07 ±0.30 A	0.6758
Monocytes percentage (%)	3.19 ±0.005 D	4.51 ±0.01 A	3.69 ±0.01 B	3.42 ±0.008 C	0.0329

Values represent mean ± SE (N=5).

Different capital letters denote a significant difference between groups ($p \leq 0.05$**).**

Table 3: Complementary effect of vitamin C and Zinc on neutrophil / lymphocyte (N/L) and lymphocyte / monocyte (L/M) ratio in intact and Ovariectomized rabbits.

Groups / Parameter	G1 Intact Rabbits Received distilled water	G2 Intact Rabbits Received Vit C and Zinc	G3 Ovariectomized Rabbits Received distilled water	G4 Ovariectomized Rabbits Received Vit C and Zinc	LSD
Neutrophil / lymphocyte (N/L) ratio	0.34 ± 0.0005 B	0.32 ± 0.003 C	0.58 ± 0.006 A	0.26 ± 0.004 D	0.0127
Lymphocyte / monocyte (L/M) ratio	21.95 ± 0.03 A	15.53 ± 0.09 C	16.26 ± 0.08 B	21.90 ± 0.11 A	0.2602

Values represent mean ± SE (N=5).
Different capital letters denote a significant difference between groups ($p \leq 0.05$).

Table 4: Complementary effect of vitamin C and Zinc on Arneth's index(%) in intact and Ovariectomized rabbits.

Parameter / groups	One lobe	Two lobe	Three lobe	Four lobe	Five or more lobe
G1 Intact rabbits Received distilled water	Ca 12.37 ± 0.008	Bc 25.39 ± 0.007	Aa 53.55 ± 0.01	Dc 6.35 ± 0.04	Eb 2.75 ± 0.006
	37.6%		53.55%	9%	
G2 Intact rabbits Received Vit C and Zinc	Dd 5.77 ±0.007	Cd 19.48 ±0.007	Ad 46.82 ±0.009	Ba 23.27 ±0.06	Ea 4.63 ±0.006
	25.1%		46.82	27.9%	
G3 ovariectomized rabbits Received distilled water	Cb 7.86 ±0.008	Ba 32.72 ±0.007	Ab 50.75 ±0.01	Dd 5.66 ±0.05	Ec 2.65 ±0.01
	40.58%		50.75%	8.31%	
G4 ovariectomized rabbits Received Vit C and Zinc	Dc 6.51 ±0.004	Bb 30.34 ±0.10	Ac 49.21 ±0.08	Cb 9.55 ±0.04	Ea 4.62 ±0.005
	36.85%		49.21%	14.17%	
LSD	0.1078				

Means with different capital letters in the same row significantly different ($P<0.05$).
Means with different small letters in the same Colum significantly different ($P<0.05$).

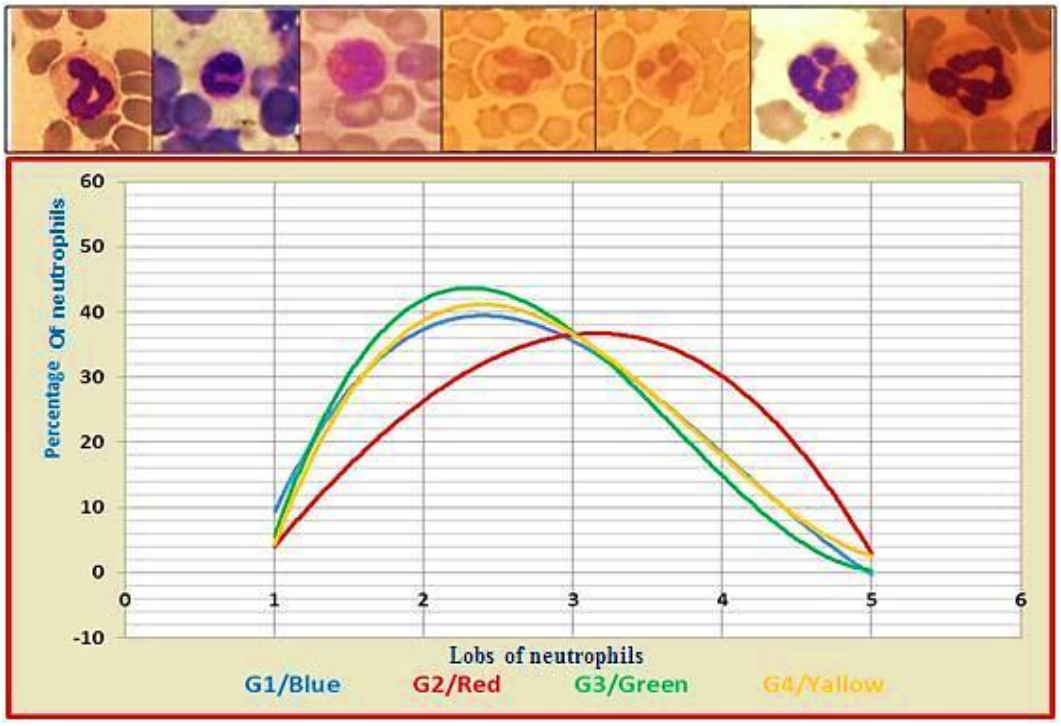

Fig 1: Arneth's index shows different stages of neutrophils maturation

Table 5: Complementary effect of vitamin C and Zinc on Total serum proteins, Serum albumin, total serum gamma globulin concentration (g/L) and Albumin/globulin ratio (AL/GL)in intact and ovariectomized rabbits.

Groups / Parameter	G1 Intact Rabbits Received distilled water	G2 Intact Rabbits Received Vit C and Zinc	G3 Ovariectomized Rabbits Received distilled water	G4 Ovariectomized Rabbits Received Vit C and Zinc	LSD
Total serum proteins concentration (g/L)	72.72 ± 0.30 B	84.12 ± 0.52 A	60.38 ± 0.38 C	84.33 ± 0.61 A	1.4114
Serum albumin concentration (g/L)	41.91 ± 0.22 B	52.28 ± 0.18 A	39.97 ± 0.19 C	34.24 ± 0.50 D	0.9244
Serum globulin concentration (g/L)	30.81 ± 0.41 B	31.84 ± 0.52 B	20.41 ± 0.25 C	50.09 ± 0.56 A	1.3695
Albumin/globulin ratio (AL/GL)	1.36 ± 0.02 C	1.64 ± 0.03 B	1.96 ± 0.02 A	0.68 ± 0.01 D	0.0685
Total serum gamma globulin(g/L)	6.96 ± 0.004 C	9.80 ± 0.003 B	4.37 ± 0.10 D	12.58 ± 0.01 A	0.1627

Values represent mean ± SE (N=5).
Different capital letters denote a significant difference between groups ($p \leq 0.05$).

4. Conclusion

In conclusion, supplementation of the complementary vitamin C and zinc could maintain a normal immune system function. Vitamin C and zinc have a vital role in protection females body against the deleterious effect of estrogen deficiency either by ovariectomy or by normal physiological aged depression.

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