Journal of
Babol University of Medical Sciences
JBUMS
e-ISSN: 2251-7170 p-ISSN: 1561-4107

Stereological Study of Mouse Testicular Tissue Treated with Zinc Oxide Nanoparticles and N-Acetyl Cysteine

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Article Type

Research Paper

ABSTRACT

Background and Objective: With the increase in the production of nanoparticles, human beings are exposed to its potential dangers. Research has shown the antioxidant effects of zinc oxide at low doses, but long-term exposure to high doses has pro-oxidant effects and influences the function of the male reproductive system, spermatogenesis and fertility. The present study was conducted to investigate the effect of N-acetyl cysteine on testicular tissue changes and spermatogenesis process in male mice treated with zinc oxide nanoparticles.

Methods: In this experimental study, 24 adult male mice were assigned to groups of control (1 ml/kg saline), zinc oxide nanoparticles (50 mg/kg), N-acetyl cysteine (150 mg/kg) and N-acetyl cysteine + zinc oxide nanoparticles. In all groups, intraperitoneal injections were performed daily for 28 days. At the end of the treatment, mean weight and volume of testicles, length and diameter of seminiferous tubules, height of germinal epithelium and number of germ cells were evaluated.

Findings: In the group of zinc oxide nanoparticles, a significant decrease was observed in volume (38.86±1.48), length (0.89±0.09), diameter (162.69±1.03), and height of germinal epithelium (41.06±1.73) of the seminiferous tubules, number of spermatogonia (4.15±0.11) $\times 10^6$, spermatocytes (21.45±0.83) $\times 10^6$, round spermatid (22.31±0.47) $\times 10^6$, elongated spermatid (21.74±0.76) $\times 10^6$ and Sertoli cell (3.08±0.10) $\times 10^6$ and a significant increase was observed in interstitial tissue volume (18.04±1.84) compared to the control group ($p<0.001$). In the concurrent treatment group, N-acetyl cysteine improved the above parameters compared to the group of zinc oxide nanoparticles ($p<0.01$). These parameters were not significantly different in the N-acetyl cysteine group compared to the control group.

Conclusion: Based on the results of this study, N-acetyl cysteine was able to prevent the damage caused by zinc oxide nanoparticles on testicular tissue and spermatogenesis.

Keywords: *N-Acetyl Cysteine, Stereology, Testis, Zinc Oxide Nanoparticles.*

Received:
Sep 4th 2021
Revised:
Nov 2nd 2021
Accepted:
Dec 4th 2021

Cite this article: Mahmoodi M, Darbandi N, Khosravi Z. Stereological Study of Mouse Testicular Tissue Treated with Zinc Oxide Nanoparticles and N-Acetyl Cysteine. *Journal of Babol University of Medical Sciences*. 2022; 24(1): 109-18.

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Publisher: Babol University of Medical Sciences

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[DOR: 20.1001.1.15614107.1401.24.1.16.2]
[DOI: 10.22088/jbums.24.1.109]

Introduction

Nanotechnology is growing rapidly and with the increasing production of nanoparticles as the main components of this science, people are more exposed to these particles than in the past. When materials change from a mass to nanoscale material, their chemical, biological, and catalytic properties change, and because they are smaller than the cells of the body, they are easily absorbed through the skin, respiration and mucous cells of the membrane and cause dysfunction in various parts of the body (1). Zinc oxide nanoparticles have a diameter of less than 100 nm and considering their size, they have a large surface area and high catalytic activity (2). Zinc oxide nanoparticles are used in many industrial products such as rubber, paints and cosmetics such as sunscreens, and in various scientific, medical and technological fields as anti-cancer, antibacterial, antioxidant, anti-diabetic and anti-inflammatory agent (3, 4). Thus, humans are constantly exposed to these nanoparticles. Although some studies have shown the antioxidant effects of zinc oxide at low doses, long term exposure to high concentration of these nanoparticles has been reported to be harmful to living organisms (5).

Various studies have shown that zinc oxide nanoparticles easily penetrate through epithelium, blood-brain barrier and blood- testis barrier and reach various organs of the body through the circulatory system, including the brain, heart, kidneys and liver. They cause oxidative stress and apoptosis (6-8). Zinc oxide nanoparticles have cytotoxic effects on mice testicular cells, and they are absorbed by Sertoli and Leydig cells and lead to apoptosis in cells by increasing oxygen free radicals and damaging the mitochondrial membrane (9, 10). Zinc oxide nanoparticles reduce the amount of serum testosterone by affecting Leydig cells and thus disrupt the process of spermatogenesis (9). It has been shown that zinc oxide disrupts the integrity of the blood- testis barrier by reducing the expression of proteins and the destruction of cell membranes and mitochondrial outer membranes in Sertoli cells (11). In addition, treatment of male mice with zinc oxide nanoparticles causes structural changes and degeneration of cells in the seminiferous tubules (10, 12).

N-acetyl cysteine is a thiol-derived compound and a derivative of the amino acid L-cysteine (13), which is able to directly and indirectly prevent intracellular damage of free radicals as an antioxidant (14, 15). In clinical studies, oral N-acetyl cysteine supplementation has been able to improve sperm parameters (normal count, motility and morphology) compared to pre-treatment stage (16). N-acetyl cysteine also has anti-inflammatory effects (17). By reducing oxidative stress and lipid peroxidation in testicular tissue and improving its antioxidant status, N-acetyl cysteine shows a protective effect on elliptical injuries and reduces histopathological damage to testes (18) and prevents testicular germ cell apoptosis (19).

Studies show that the use of zinc oxide nanoparticles leads to sperm abnormalities and reduced fertility through hormonal and histological changes, increase in free radicals and induction of apoptosis. On the other hand, the use of zinc oxide nanoparticles in everyday life is undeniable. Therefore, in the present study, we investigated the protective effect of N-acetyl cysteine on the toxicity of zinc oxide nanoparticles on testicular tissue and spermatogenesis indexes.

Methods

To perform this experimental study, after approval by the ethics committee of Arak University with the code IR.ARAKMU.REC.1399.049, 24 adult male mice (NMRI) with an average weight of 35.45±2.11 g were purchased from the Pasteur Institute of Iran and were kept in Arak University's animal house under normal light conditions (12 hours of darkness and 12 hours of light) and normal temperature

[DOR: 20.1001.1.15614107.1401.24.1.16.2]
[DOI: 10.22088/jbums.24.1.109]

(21±2 °C). All animals were kept with the same nutritional conditions and with free access to water. After weighing, the mice were randomly divided into 4 groups (n=6): control (1 ml/kg saline), zinc oxide nanoparticles (Tecnan, Spain, 50 mg/kg) (20, 21), N-acetyl cysteine (Sigma, Germany, 150 mg/kg) (22, 23) and N-acetyl cysteine + zinc oxide nanoparticles. Ethical principles of working with laboratory animals were observed. Treatment was performed daily and intraperitoneally for 28 days in all groups (24). At the end of the treatment period, the mice were first weighed and then anesthetized with diethyl ether. Their left testicles were removed from the body, their wet weight and submerged weight were measured and transferred to Modified Davidson's fluid (mDF) for stereological studies. Total testicular volume, volume of seminiferous tubules and interstitial tissue, length and diameter of seminiferous tubules, height of germinal epithelium as well as germ and Sertoli cell count were measured in all groups. Immersion method was used to calculate the initial volume of the testis (25). Then, sections of testicular tissue were prepared by orientator method and after calculating the amount of shrinkage, the total volume of testis was estimated (26).

Samples were processed by tissue processor (Leica model) and after molding, 5 and 20 µm thick sections were prepared using Leitz 1512 Rotary Microtome and stained by hematoxylin-eosin method. To estimate the volume of seminiferous tubules and interstitial tissue, first the volumetric density of each component was calculated by a four-point probe measurer and then the total volume was estimated by multiplying its volumetric density by the total volume of the testis (27). The density of the length of the seminiferous tubules was calculated by frame count and the absolute length of the seminiferous tubules was obtained through multiplying by the total volume of the testis (28). The diameter of the seminiferous tubules was measured by a Motic Images 3.0 software (2000) (28). To estimate the height of the germinal epithelium, first the sum of the total points hit by the germinal epithelium was divided by the total points hit by the testicular tissue and the volumetric density of the germinal epithelium was obtained. Then, the number of probe surface collisions with testicular tissue images and the lumen surfaces of the germinal epithelium were counted and the surface density of the germinal epithelium was calculated (29). To calculate the number of cells, 20-micron slides with light microscope (100x magnification) and optical dissector method were used and cell types with special frames were selected and counted. After calculating the numerical density and multiplying it by the total volume of the testis, the total number of cells was obtained (29).

For data analysis, SPSS software version 16 and One-Way ANOVA ("analysis of variance") and Tukey statistical test were used and $p<0.05$ was considered significant.

Results

Histopathological changes of testicular tissue: In the control group, regular arrangement of germinal epithelium of seminiferous tubules and normal interconnection of reproductive cells were observed (Figure 1A). In the group of zinc oxide nanoparticles, irregularity and disruption of the germinal epithelium, increase in lumen size of seminiferous tubules, interstitial tissue edema and decrease in the height of the germinal epithelium were observed. Moreover, sperm density in seminiferous tubules was lower than other groups (Figure 1B). In the group of N-acetyl cysteine + zinc oxide nanoparticles, the appearance of the tissue of seminiferous tubules was similar to the control group and tissue damage was largely prevented (Figure 1C). In the N-acetyl cysteine group, normal structure of seminiferous tubules and regular arrangement of germ cells were observed (Figure 1D).

[DOR: 20.1001.1.15614107.1401.24.1.16.2]
[DOI: 10.22088/jbums.24.1.109]

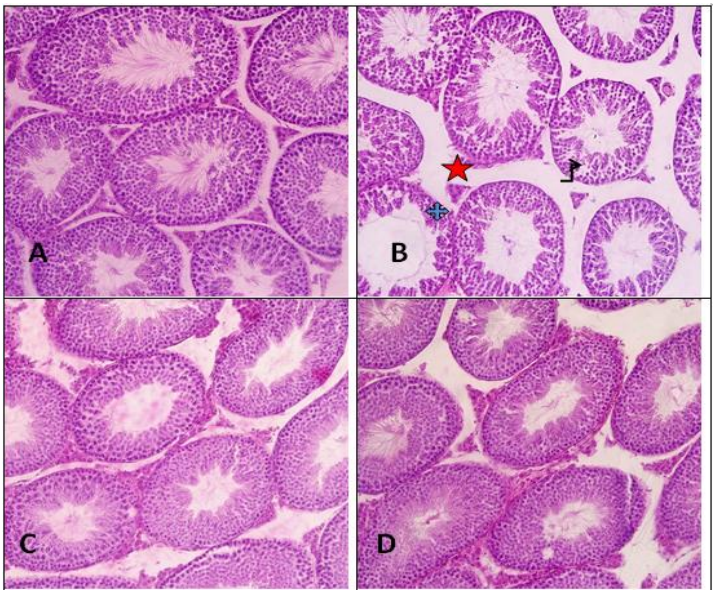


Figure 1. Microscopic images of mice testicular tissue in different groups treated with zinc oxide nanoparticles, N-acetyl cysteine, and N-acetyl cysteine + zinc oxide nanoparticles (5-micron sections, hematoxylin-eosin staining, 200x magnification). A. Normal and regular arrangement of germinal epithelium in control group, B. Disruption, irregular arrangement and decrease in germinal epithelium height (✥) and increase in interstitial tissue area (★) and vacuolation (⚑) in the group treated with zinc oxide nanoparticles, C. Normal structure of germinal epithelium in the group N-acetyl cysteine + zinc oxide nanoparticles, D. Normal and regular arrangement of the germinal epithelium in the group treated with N-acetyl cysteine.

Stereological calculations: Mean mouse weight (g) at the end of the treatment period between control groups (35.73±2.75), zinc oxide nanoparticles (34.48±1.96), concurrent treatment (36.19±1.51) and N-acetyl cysteine alone (37.38±1.05) did not show a significant difference. There was no significant difference in the mean testicular weight in different groups (Table 1).

Comparison of the mean testicular volume of mice (3 mm) between groups of control (59.55±1.38), zinc oxide nanoparticles (56.90±3.08), concurrent treatment (58.06±1.25) and N-acetyl cysteine alone (59.64±0.93) showed no significant difference (Table 2). However, the mean volume of seminiferous tubules in the group of zinc oxide nanoparticles decreased significantly compared to other groups ($p<0.001$) and the volume of interstitial tissue showed a significant increase ($p<0.001$). Concurrent treatment with N-acetyl cysteine + zinc oxide nanoparticles was able to significantly compensate for these values and reach the level of control group ($p<0.001$). The total volume of testis and the volume of seminiferous tubules and interstitial tissue in the N-acetyl cysteine group were not significantly different from the control group (Table 2).

Significant difference was observed in mean length of seminiferous tubules (m) between groups of control (1.33±0.05), zinc oxide nanoparticles (0.89±0.09), concurrent treatment (1.07±0.08) and N-acetyl cysteine alone (1.36±0.13) (Table 3). Furthermore, a significant difference was observed in the diameter of the seminiferous tubules and the height of the germinal epithelium in different groups ($p<0.001$). Mean length and diameter of seminiferous tubules and height of germinal epithelium in the group of concomitant treatment with N-acetyl cysteine + zinc oxide nanoparticles showed a significant increase compared to the group of zinc oxide nanoparticles ($p<0.01$) but did not reach the level of control group. The length and diameter of the seminiferous tubules and the height of the germinal epithelium in the N-acetyl cysteine group did not show a significant difference compared to the control group (Table 3).

[DOR: 20.1001.1.15614107.1401.24.1.16.2]
[DOI: 10.22088/jbums.24.1.109]

Table 1. Comparison of mean mouse weight and testis weight (g) in different groups of mice after treatment with zinc oxide nanoparticles (50 mg/kg/day) and N-acetyl cysteine (150 mg/kg/day) for 28 days.

Groups	Mean initial weight of mice (g)	Mean mice weight at the end of treatment (g)	Mean weight of rat testis (g)
	Mean±SD	Mean±SD	Mean±SD
Control	34.75±2.39[a]	35.73±2.75[a]	0.117±0.008[a]
Zinc oxide nanoparticles	35.85±2.48[a]	34.48±1.96[a]	0.125±0.011[a]
N-acetyl cysteine + zinc oxide nanoparticles	34.98±1.66[a]	36.19±1.51[a]	0.110±0.023[a]
N-acetyl cysteine	36.22±1.99[a]	37.38±1.05[a]	0.135±0.02[a]

The mean values with different letters in a column show significant difference (p<0.05, one way ANOVA, Tukey's test).

Table 2. Comparison of mean testicular volume, seminiferous tubules volume and interstitial tissue volume in different groups of mice after treatment with zinc oxide nanoparticles (50 mg/kg/day) and N-acetyl cysteine (150 mg/kg/day) for 28 days.

Groups	Total testicular volume (3 mm)	Seminiferous tubules volume (3 mm)	Interstitial tissue volume (3 mm)
	Mean±SD	Mean±SD	Mean±SD
Control	59.55±1.38[a]	46.17±0.86[a]	13.38±1.14[a]
Zinc oxide nanoparticles	56.90±3.08[a]	38.86±1.48[b]	18.4±1.84[b]
N-acetyl cysteine + zinc oxide nanoparticles	58.06±1.25[a]	43.86±1.76[a]	14.22±1.06[a]
N-acetyl cysteine	59.64±0.93[a]	46.23±1.70[a]	13.14±1.46[a]

The mean values with different letters in a column show significant difference (p<0.05, one way ANOVA, Tukey's test).

Table 3. Comparison of mean length (m), diameter and height of germinal epithelium (µm) of seminiferous tubules in different groups of mice after treatment with zinc oxide nanoparticles (50 mg/kg/day) and N-acetyl cysteine (150 mg/kg/day) for 28 days.

Groups	Length of seminiferous tubules (m)	Diameter of seminiferous tubules (µm)	Height of germinal epithelium (µm)
	Mean±SD	Mean±SD	Mean±SD
Control	1.33±0.05[a]	177.74±4.79[a]	56.30±0.69[a]
Zinc oxide nanoparticles	0.89±0.09[c]	162.69±1.03[c]	41.06±1.73[c]
N-acetyl cysteine + zinc oxide nanoparticles	1.07±0.08[b]	171.62±1.70[b]	46.64±1.03[b]
N-acetyl cysteine	1.36±0.13[a]	179.55±5.02[a]	55.77±2.73[a]

The mean values with different letters in a column show significant difference (p<0.05, one way ANOVA, Tukey's test).

The mean number of spermatogonia (4.15±0.11) $\times 10^6$, spermatocytes (21.45±0.83) $\times 10^6$, round spermatid (22.31±0.47) $\times 10^6$, elongated spermatid (21.74±0.76) $\times 10^6$ and Sertoli cell (3.08±0.10) $\times 10^6$ showed a significant decrease in the group treated with zinc oxide nanoparticles compared to the control group ($p<0.001$) (Table 4). The mean number of these cells in the group treated with N-acetyl cysteine + zinc oxide nanoparticles showed a significant increase compared to the group of zinc oxide nanoparticles ($p<0.001$) but did not reach the level of control group. In the group treated with N-acetyl cysteine, the number of the above cells did not show a significant difference compared to the control group (Table 4).

[DOR: 20.1001.1.15614107.1401.24.1.16.2]
[DOI: 10.22088/jbums.24.1.109]

Table 4. Comparison of mean number of spermatogonia, spermatocytes, spermatids and Sertoli cells ($\times 10^6$) in different groups of mice after treatment with zinc oxide nanoparticles (50 mg/kg/day) and N-acetyl cysteine (150 mg/kg/day) for 28 days.

Mean number of cells Groups	Spermatogonia ($\times 10^6$)	Spermatocytes ($\times 10^6$)	Round spermatid ($\times 10^6$)	Elongated spermatid ($\times 10^6$)	Sertoli ($\times 10^6$)
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD
Control	6.37±0.23[a]	25.92±0.93[a]	28.33±2.19[a]	29.87±3.19[a]	4.24±0.12[a]
Zinc oxide nanoparticles	4.15±0.11[c]	21.45±0.83[c]	22.31±0.47[c]	21.74±0.76[c]	3.08±0.10[c]
N-acetyl cysteine + zinc oxide nanoparticles	4.83±0.34[b]	23.26±1.21[b]	25.93±0.57[b]	25.01±0.71[b]	3.49±0.14[b]
N-acetyl cysteine	6.19±0.13[a]	25.26±1.17[a]	28.20±1.10[a]	28.25±1.58[a]	4.05±0.21[a]

The mean values with different letters in a column show significant difference ($p<0.05$, one way ANOVA, Tukey's test).

Discussion

In the present study, the mean testicular weight and body weight of mice at the end of the treatment period were not significantly different between different groups. Based on the results of the present study, N-acetyl cysteine is able to prevent tissue damage and the reduction in spermatogenesis caused by zinc oxide nanoparticles, and other studies have reported similar results (20, 30, 31). Therefore, it can be argued that body weight loss and testicular weight loss in mice depend on the time of treatment of mice before and after puberty, breed and age of mice as well as the dose used. In the present study, the dose of zinc oxide nanoparticles could not have a significant effect on appetite, food absorption and metabolic activity of mice due to the maturity of the mice. The findings of this study showed a significant decrease in the volume of seminiferous tubules and a significant increase in the volume of interstitial tissue in the group of zinc oxide nanoparticles compared to other groups. The results of some previous studies, while confirming the results, show that atrophy of seminiferous tubules is due to increased apoptosis and decreased number of reproductive cells (32).

It has also been reported that one of the factors that increase apoptosis is the accumulation of reactive oxygen species in testicular tissue following a decrease in the level of antioxidant enzymes (33). Research has shown that zinc oxide nanoparticles increase the production of reactive oxygen species, mitochondrial damage, and activate apoptotic signaling pathways, including the p53 pathway, and ultimately lead to death of target cells after entering the cell lysosome by producing $^{+}Zn^{2}$ (9, 34). Therefore, according to previous studies, zinc oxide nanoparticles may disrupt the process of spermatogenesis by inducing oxidative stress and autophagy of cells in testicular tissue (9), destroying Sertoli and Leydig cells, degenerating germ cells of seminiferous tubules, and destroying testicular tissue cells, which in turn can reduce the volume of seminiferous tubules and increase the volume of interstitial tissue (12, 35, 36). In the present study, N-acetyl cysteine was able to prevent atrophy and degeneration of reproductive cells and reduce the volume of seminiferous tubules and increase the volume of interstitial tissue caused by zinc oxide nanoparticles and improve it in the control group. It is possible that N-acetyl cysteine, with its antioxidant properties, was able to protect testicular cells from destruction and prevent cell death caused by oxidative damage to the endoplasmic reticulum of testicular cells (19, 29, 37). In this study, a significant decrease was observed in the mean length, diameter and height of germinal epithelium of seminiferous tubules in the group of zinc oxide nanoparticles compared to the control group. Previous studies also confirm these findings (9, 10, 20,

[DOR: 20.1001.1.15614107.1401.24.1.16.2]
[DOI: 10.22088/jbums.24.1.109]

35). Zinc oxide nanoparticles enter the cell through endocytosis or several other mechanisms such as macropinocytosis and cause DNA damage by inducing autophagy. It can also increase toxicity and ultimately cell death by degrading anti-apoptotic and DNA repair proteins (34). Thus, by the increase in the rate of apoptosis and decrease in reproductive cells (32), it leads to a decrease in the length, diameter and height of the germinal epithelium of the seminiferous tubules. On the other hand, zinc oxide nanoparticles can reduce the diameter and height of the germinal epithelium by disrupting the process of spermatogenesis (35) and inducing histopathological changes such as destruction of Sertoli cells and germ cells of the seminiferous tubules (36). In the present study, concurrent treatment with N-acetyl cysteine + zinc oxide nanoparticles was able to increase the mean diameter, length and height of the germinal epithelium of seminiferous tubules. Other studies also confirm this result (18, 29, 37). Due to its antioxidant properties, N-acetyl cysteine seems to prevent tissue damage and apoptosis of spermatogenic and Sertoli cells by increasing the concentration of antioxidant enzymes, reducing lipid peroxidation, increasing testosterone concentration and reducing the free radicals produced (19, 37). Given that the population of spermatogenic and Sertoli cells is closely related to the height and diameter of seminiferous tubules, it can be concluded that N-acetyl cysteine increases the diameter and height of germinal epithelium of seminiferous tubules by preventing degeneration and increasing the population of spermatogenic and Sertoli cells (37).

In this study, the mean number of spermatogonia, spermatocytes, round and elongated spermatozoa and Sertoli cells in the group treated with zinc oxide nanoparticles was significantly reduced compared to the control group. Previous studies also confirm these findings (9, 10, 34). After infiltrating Leydig cells and Sertoli cells by inducing apoptosis (10), nanostructures disrupt the morphology of germinal epithelium (9). Research has shown that by reducing protein expression as well as degrading cell membranes and mitochondrial outer membranes and significantly increasing oxidative stress and inflammatory responses in Sertoli cells, zinc oxide nanoparticles lead to the weakening of the blood- testis barrier and by reducing the expression of genes related to the strong binding in these cells, they lead to the widening of the pores of this barrier and facilitate the penetration of nanostructures into testicular tissues (38). In this study, treatment with N-acetyl cysteine was able to increase the number of reproductive cells and Sertoli significantly. Other studies have suggested that N-acetyl cysteine may save testicular germ cells from death by regulating apoptotic pathways (23) or prevent the destruction and death of testicular cells by preventing oxidative damage in the testicular endoplasmic reticulum (19). Studies have shown that N-acetyl cysteine plays a very important role in neutralizing toxic substances such as xenobiotics (compounds that are foreign to the biological system), peroxides and other free radical-producing molecules (39-41). It can also regulate proinflammatory compounds and anti-apoptotic genes (42, 43).

According to the results of this study, N-acetyl cysteine is able to prevent testicular tissue damage and reduce spermatogenesis caused by zinc oxide nanoparticles in mice testicular tissue. Zinc oxide nanoparticles seem to be the main cause of damage due to oxidative stress and N-acetyl cysteine as a powerful antioxidant was able to prevent the toxicity induced by zinc nanoparticles. However, further studies are necessary to present a precise mechanism.

[DOR: 20.1001.1.15614107.1401.24.1.16.2]
[DOI: 10.22088/jbums.24.1.109]

Acknowledgment

Hereby, we would like to thank Arak University for the financial support as well as the efforts of the experts of the Animal Physiology Research Laboratory of the Faculty of Biology of Arak University.

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