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Azithromycin Therapy Changed the Intestinal Microbiota, Caused Oxidative Stress, and Decreased Memory Function in Adult Wistar Rats

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Abstract

Research has shown that the gut microbiome affects memory processes. Different antibiotic treatments lead to changes in the intestine microbiota and the dysbiosis caused by it is associated with changes in brain behavior. This study examined the effect of azithromycin on the intestine microbiome, oxidative stress, and memory in male Wistar rats. Two groups of adult male rats were established (n=16). In both groups, the first collection of the feces samples was done on the first day to identify the gut microbiome, then the animals were gavaged with normal saline or azithromycin (15 mg) in a volume of 1 ml daily for seven days. At the end of treatment, The second phase of collecting feces samples was completed. Then locomotion activity, novel object recognition test, Passive avoidance test, hippocampal neurons count and oxidative stress measurement in blood serum were performed. Azithromycin treatment induced a significant decrease in the number of aerobic and anaerobic colonies and led to the elimination of *Enterococcus faecalis* and *Lactobacillus acidophilus* species in the experimental group intestine. Azithromycin significantly decreased memory function, number of hippocampal healthy neurons, total antioxidant capacity, superoxide dismutase, and catalase enzymes and significantly increased the amount of malondialdehyde in blood serum compared to the control group. In this research, azithromycin by disrupting the intestinal microbiome, reduces diversity and suppresses some bacteria, raises levels of oxidative agents in blood serum, and by reducing the number of hippocampus-healthy neurons decreases cognitive functions.

Keywords: Azithromycin Antibiotic; Gut Microbiome; Memory; Oxidative Stress; Rat.

Introduction

Learning and memory are among the highest functional levels of the central nervous system, occurring as a result of biochemical, morphological, and physiological changes at the synaptic level and neural networks. It is believed that synaptic plasticity is the basis of changes in cognitive functions and provides the conditions for learning and memory (1).

The hippocampus, located in the inner area of the

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temporal lobe, is a region of the limbic system that is engaged in memory and learning. It results in the storage of long-term memories and resistance to forgetting (2). Studies have indicated that processing of spatial information is carried out by the dorsal hippocampus and the ventral hippocampus is involved in emotional learning, fear, and smell (3).

Human intestine microbiota has metabolic, defensive, and nutritional functions(4). Changes in the composition and function of intestine microbiota can alter intestine permeability, digestion, metabolism, and immune responses (5). Environmental factors such as drugs, pesticides, diet, proton pump inhibitors, and antibiotics can cause permanent dysbiosis (6). The interaction between the intestine and the brain takes place through various pathways, including nerve messages, the production of immune mediators, endocrine cells, and the production of microbial metabolites. Disruption in this system causes behavioral changes (7).

Previous studies that have used diet, antibiotics, probiotics, prebiotics, germ-free rats, and analyzed the feces of normal and specific pathogen-free animals have indicated that the structure and function of the hippocampus are affected by the intestine microbiome (8). Studies have shown that Lactobacillus acidophilus, Enterococcus faecalis, and Escherichia coli have probiotic properties (9). Also, the relationship of these species with memory and learning was investigated in previous studies (7, 8).

Because of their antibacterial qualities, antibiotics have been taken into consideration in clinical and laboratory procedures and they're frequently used in the control of infectious diseases. Despite the beneficial effects of antibiotics in the treatment of a large variety of diseases and infections, they lead to damages such as kidney toxicity, asthma in childhood and adolescence, increased risk of abortion, cerebral palsy, and abnormalities of the digestive system, nervous disorders including anxiety, panic attacks, depression, psychosis, and delusions by affecting host cells (10).

Azithromycin is a subset of macrolide antibiotics and is derived from erythromycin. It is used to treat or prevent middle ear infections, streptococcal sore throat, pneumonia, typhoid, bronchitis, and sinusitis (11). It is also used to treat respiratory, genitourinary, skin, and other bacterial infections. It has also immunomodulatory effects in chronic inflammatory disorders (12). This drug was used as a potential therapy in the treatment of SARS-CoV-2 pneumonia in the early months due to its antiviral and immunomodulatory activity (13).

Different antibiotic treatments lead to short-term or long-term changes in the intestine microbiota in humans and animals, and the dysbiosis caused by it is associated with changes in brain behavior and biochemistry. The current study looked into the impact of azithromycin, an antibiotic used extensively in the treatment of secondary infections in COVID-19 patients, on the intestine microbiome and Wistar rat males' memory.

Materials and Methods

1. Animals

In this project, male Wistar rats (220-250 g) were obtained from the Pasteur Institute of Iran. The animals were kept in an animal home with a regulated temperature of 22 ± 2 °C and a 12-hour light/dark cycle and provided freely with water and food. Each experimental group comprised 16 animals subjected to behavioral tests from 9:00 am until 4:00 pm. The local ethical commission approved all procedures (Research and Ethics Committee of the School of Biology, University of Arak; IR.ARAKMU.REC.1401.038).

2. Experimental procedure

The impact of azithromycin on the intestinal

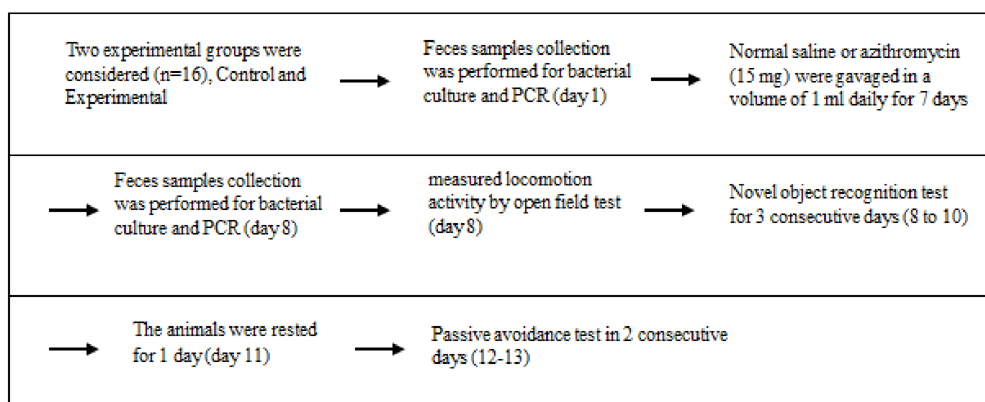

Figure 1. Schematic diagram of the experimental procedures.

microbiome and memory was examined in male Wistar rats of two experimental groups each containing 16 animals (Figure 1). In both groups, samples of feces were first collected on the first day. To collect feces samples, rats were taken out of their cages and given separate cages for a short time until they threw out the samples of their feces (typically 2–3 pellets for sampling). The samples were placed in microtubes and kept on dry ice during the collection period. Part of the collected samples was used for the bacterial culture of feces on the same day, and the other part was kept at -80 °C until the PCR test (14). After collecting feces samples, the animals were gavaged with normal saline or azithromycin (15 mg) in 1 ml daily for 7 days. This dose was selected based on previous studies (15). At the treatment completion, the feces were sampled in the second stage and used to identify the gut microbiome. One day after the end of the treatment, an open field test (OFT) was first used to gauge each animal's general activity, followed by the novel object recognition test (NORT) for 3 days (days 8–10). The animals had a day of rest (day 11), after which the passive avoidance test (PAT) was used for two days (days 12–13).

3. Preparation of bacterial strains

Before starting the experiment, *Escherichia coli* bacteria were observed in the examined feces of the studied animals. However, *Enterococcus faecalis* and *Lactobacillus acidophilus* were absent in the samples. In this study, samples of the *E. faecalis* strain with the ATCC code 19433 and the *Lactobacillus acidophilus* strain with the ATCC code 4356 were obtained from the National Center of Genetic and Biological Resources of Iran in lyophilized ampoules and revived according to the protocol. Then, 1 ml of bacteria with half McFarland turbidity was given to each rat.

4. DNA extraction from feces

To extract fecal DNA using the boiling method, 0.3 g of feces from each animal was transferred to a sterile microtube, and 300 µl of DNase/RNase-free water was added to each microtube, and placed in boiling water for 15 min. Then, the microtubes were removed from the boiling water and placed on ice for 1 min. The suspension was centrifuged at 13000 g for 3 min, and the supernatant of each DNA-containing microtube was transferred to other sterile microtubes and kept at −80 °C to identify bacterial species by the PCR method (16).

5. Bacterial culture of feces

To examine the effect of the antibiotic consumed by the animal on the intestinal microbiome, the feces of each animal were collected and cultured before and after the treatment. The number of aerobic and anaerobic bacteria in 0.1 g of animal feces was counted by preparing a dilution series and using the count's colony method (17).

6. Examining the presence or absence of the studied bacteria in feces by PCR method

The identification of each bacterial strain was confirmed by the PCR method using specific primer sequences fof *E. faecalis* (5'-ATCAAGTACAGTTAGTCTT-3') AKSZ-208-F and (5'- ACGATTCAAAGCTAACTG-3') AKSZ-208-R (18) and *L. acidophilus* (5'-TCATGTTGGGATGCAATGAG-3') NC56 chromosome -F and (5'-TTTCAAAACTTGTCCTGCTG-3') NC56 chromosome -R (19). Based on the previous studies, *E. coli* was identified using the specific primer sequences (5'-GACCTCGGTTTAGTTCACAGA-3') ATCC 8739 chromosome -F and (5'-CACACGCTGACGCTGACCA -3') ATCC 8739 chromosome -R (20).

The reaction mixture for each bacterial strain comprised 1 µl of $MgCl_2$, 2.5 µl of 10x buffer, 0.2 µl of SmarTaq enzyme, 4 µl of the bacterial DNA, 0.4 µl of dNTP, 0.5 µl of each primer (10 µm), and 9.9 15 µl of DNase/RNase-free distilled water. The annealing temperatures were respectively 47, 53, and 58 °C for *E. faecalis*, *L. acidophilus*, and *E. coli* for 60 sec (20). Finally, PCR products were gel electrophoresed with 2% agarose at 70 V for 45 min.

7. Open Field test

The effects of each drug or the combination of drugs on locomotor activity were evaluated using the OFT (21). Learning in rats is related to exploring the environment and objects. At the same time, medical procedures might have an impact on the central nervous system and lead to a decrease or increase in motor activities, drowsiness, anxiety, etc. In this case, it is better to evaluate the motor activity to ensure the results of the behavioral test. Each rat was subjected to a 10-minute OFT (Borj Sanat Co., Iran) on the eighth day. The device, measuring 40 × 40 × 40 cm, has 16 infrared photocells spaced 2.5 cm apart. The number of beam cuts was assessed using the photocell monitoring system. The apparatus was meticulously cleaned following every test. The locomotor activity test was conducted in a reasonably dark, noise-free setting.

8. Novel object recognition test

The long-term nonspatial recognition memory was examined using the novel ORT (21). This apparatus was a white Plexiglas box (approximately 40 × 40 × 50 cm) kept in a separate testing room (a quiet and relatively dark

environment).

This test was completed during 3 continuous days. Animals were free to explore the test box for five min on the first day (the habituation session) without any object. In the training session (the second session), the rats were placed in the task with two identical objects (A1 and A2 - two nuts)with the same texture, color, and size, which were positioned in each cage's two corners, 5 cm from the walls, and had. In this session, animals freely examined the objects and the surrounding area for 15 min. After 24 h (the recognition session), the rats were returned to the testing cage to freely investigate both new and old (A1) objects (T1- one bolt) for 5 min. The chosen nuts and bolts were incapable of rusting and were large and heavy so that the animal could not move them. A camera above the chamber captured movement observations and recordings. Smelling or touching an object with the nose and/or forepaws was considered an act of exploration. The memory performance in this test is indicated by the preference index (PI) for the difference in exploration between two familiar objects and the recognition index (DI) for the difference in exploration between a familiar object and a new object (22).

9. Passive avoidance test

The contextual long-term memory was measured with the step-through PAT to (21). The inhibitory avoidance apparatus is made up of two identically sized compartments: a light compartment and a dark compartment connected through a guillotine door. The light section's walls and floor are composed of white plexiglass, and it is illuminated by a 20 w electric bulb placed 50 cm from the device's floor. The walls of the dark part are made of black Plexiglas, and its floor is composed of metal grid bars positioned 1 cm apart and have a diameter of 0.5 cm. These bars are connected to a shock generator that can produce a shock with an intensity of 1 mA at a frequency of 50 hertz for 3 sec (23).

On the training day, the rats were allowed to become familiar with the apparatus 30 min before the training session. After that, an individual rat was kept in the light compartment for 10 second, after which the door was raised, followed by recording the time taken by the rat to move from the light to the dark compartment. After 2 min, the rat was returned to the light compartment; the door was closed as soon as the rat completely entered the dark compartment, and an electrical foot shock was applied for 3 s. The rat was then removed from the apparatus. The procedure was repeated 2 min later. Animals that did not enter the dark compartment even after the 120 s were removed from the apparatus as successful training. All animals were trained with a maximum of two trials. The testing day was conducted after 24 h of the acquisition trial, where no shock was applied when the rat came into the dark compartment, and retrieval latency was recorded (as step-through latency) by the time taken the rat to re-enter the dark compartment (up to 300 s) (23).

10. Histological staining and tissue preparation

All rats were anesthetized with 3.5% chloral hydrate (35 mg/100 g) and perfused with phosphate buffer (PBS, 0.1 M, pH 7.4), followed by 4% paraformaldehyde in pre-cooled physiological saline through the left ventricle. The brains were removed, immersed in the same solution (24 or 48 h), and then processed and embedded in paraffin. Hematoxylin and eosin were used to stain coronal and serial sections of the dorsal hippocampus with a thickness of 7 µm. Based on the Paxinus Atlas, every slice was cut from the area between -3.8 and -4.30 mm from the bregma, which was marked by the hippocampal structure (Paxinos & Watson, 2006). For counting, four sections with equal intervals were chosen and their average was determined for use in calculating the sample's neuron number (24). Quantitative analysis and counting were performed using a light microscope (BX40, Olympus, New York, USA) connected to a camera (Olympus, DP12) with Image J software.

11. Biochemical analysis

The blood serum samples were centrifuged (13,300 rpm for 10 min) in a refrigerated device (universal device, Germany) at 4 °C, and aliquots of the supernatant were stored at -20 °C for use in biochemical tests. Lipid peroxidation was measured using the Thiobarbituric Acid (TBA) technique, in which aldehydes reacted with thiobarbiturate and generated pink complexes. Using spectrophotometry, this compound can be measured at a wavelength of 535 nm (25). The approach of Benzic et al. was utilized to determine the antioxidant capacity of the whole plasma. Using particular reagents, ferric was reduced to ferrous in an acidic pH to create a blue complex in this approach. A wavelength of 593 nm could be used to measure this compound (26). The level of superoxide dismutase (SOD) enzyme activity in blood serum was measured using pyrogallol, which oxidizes spontaneously in aqueous and alkaline environments. SOD prevents the spontaneous oxidation of pyrogallol. This method is used to measure enzyme activity at 420 nm (27). The catalase (CAT) enzyme activity was matured with the potassium phosphate buffer based on the spectrophotometric measurement of H_2O_2 decomposition at 240 nm (28).

12. Statistical analysis

The data were expressed as mean ± standard error of the mean. A statistical significance of $P < 0.05$ was determined by the independent t-test. The statistical analysis was carried out using SPSS software version 26.0.

Results

1. Comparison of intestinal bacterial colonies in experimental groups

The independent t-test revealed no statistically significant changes in the number of aerobic (Figure 2A) and anaerobic (Figure 2B) bacterial colonies between the groups before treatment with azithromycin ($P > 0.05$). However, the treatment of the experimental group with azithromycin (15 mg/1ml) after one week resulted in a significant decline in the number of aerobic (Figure 2A) and anaerobic (Figure 2B) bacterial colonies as compared with the control group ($P < 0.001$).

2. Comparing the experimental groups' inhibitory avoidance memory

One day following training, the independent t-test revealed significant differences in step-through latency

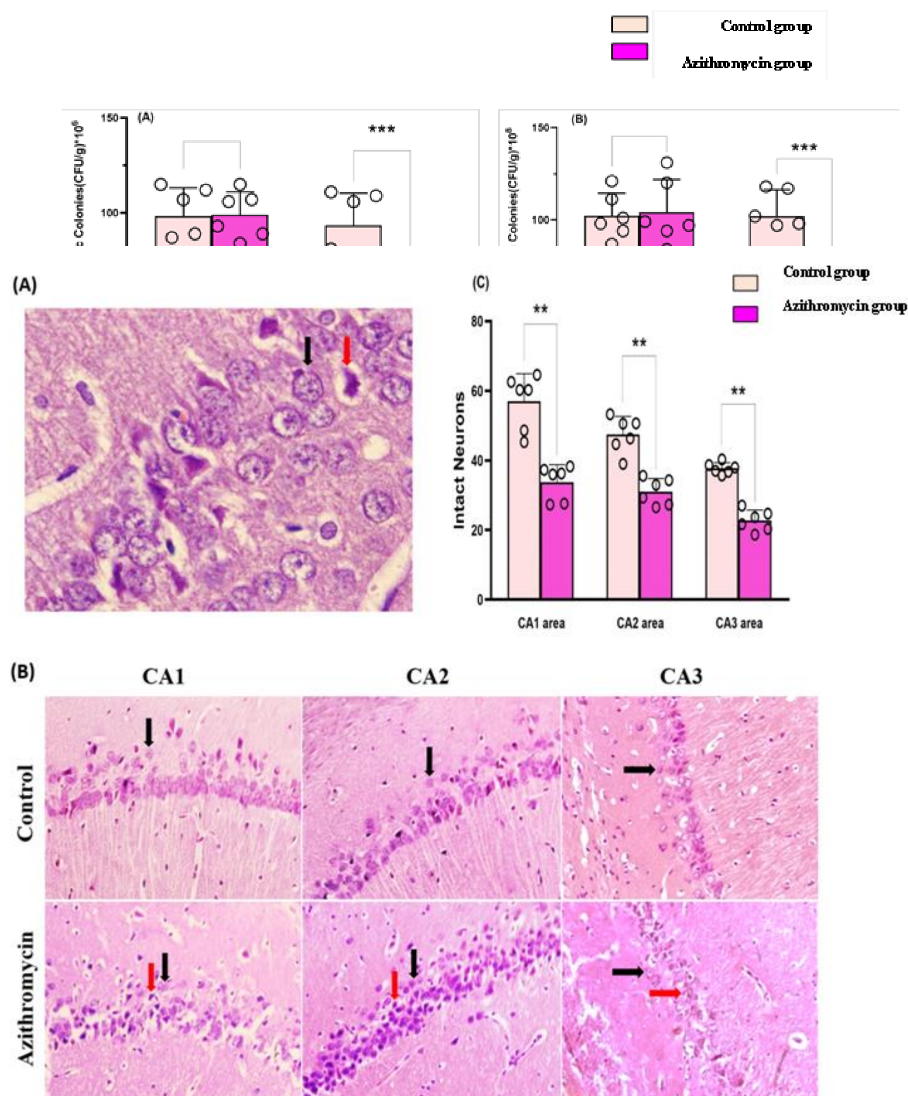

Figure 4. photomicrographs of the hippocampus pyramidal neurons (A) and typical coronal sections across the hippocampal CA1, CA2, and CA3 pyramidal neurons (B) revealing Hematoxylin & Eosin in experimental groups. Red arrows indicate degenerating pyramidal cells, whereas black arrows indicate intact pyramidal cells ×1000 (A) and ×400 (B). Azithromycin's effects on the number of healthy neurons in the hippocampal regions of CA1, CA2, and CA3 (C). Data are presented as mean ± SEM. ***$P < 0.001$ as compared with the control group.

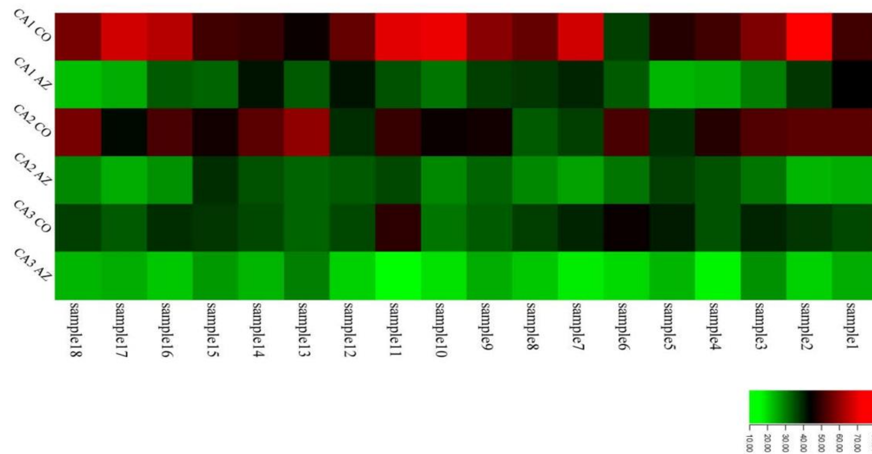

Figure 5. Number of intact neurons in the hippocampal CA1, CA2, and CA3 regions across several experimental groups.

Table 1. Serum malondialdehyde (MDA), the ferric-reducing ability of plasma (FRAP), superoxide dismutase (SOD), and catalase (CAT) enzyme levels were compared between the experimental groups. Mean ± SEM is used to represent all values. Within a single column, the values with distinct superscript letters show a significant difference at P<0.05.

Factor group	MDA (nmol/ml)	FRAP (mmol/l)	SOD (U/ml)	CAT (U/ml)
Control group (saline 1ml)	2/185±0/068 [a]	0/498±0/018 [a]	10/32±0/726 [a]	6/779±0/442[a]
Azithromycin group (15mg/ml)	2/89±0/192 [b]	0/208±0/044 [b]	7/087±0/442 [b]	5/224±0/226 [b]

between the groups (F (1, 12) = 0.15, P < 0.001). According to the analysis, less time was spent by the azithromycin-treated group (15 mg/rat with a volume of 1 ml, 7 days) to re-enter the dark compartment than the control group (P < 0.001), indicating azithromycin antibiotic-induced memory loss (Figure 3A).

3. Comparing the experimental groups' novel ORT

One day following training, the t-test revealed a significant difference in the two groups' results on the novel ORT (F (1, 12) = 3.18, P < 0.05). Treatment with azithromycin (15 mg/rat with a volume of 1 ml) for 7 days significantly reduced the discrimination index in comparison to the control group (P < 0.05) (Figure 3B).

4. Comparing the experimental groups' OFT

The independent t-test showed no significant changes in the OFT between the groups (F (1, 12) = 0.236, P > 0.05). Figure 3C shows that the seven-day azithromycin treatments did not affect locomotor activity on the first day following training.

5. ***Histological investigations in the hippocampal CA1, CA2, and CA3 regions***

In the control group, the pyramidal cells in different hippocampal regions were healthy with a distinct nucleus. A high neuronal density was seen in the CA1, CA2, and CA3 regions, where hippocampus cells were less abundant, and their color was darker in the azithromycin-treated group than in the control group. Additionally, the control group's round cells turned into spindle and triangular shapes with inconspicuous nuclei (Figsure 4A and 4B).

A comparison between the azithromycin and the control groups revealed a significant reduction (P < 0.001) in the number of healthy neurons in the CA1 (P<0.001), CA2 (P<0.001), and CA3 regions of the hippocampus (Figure 4C). The number of healthy neurons in various hippocampal regions in the experimental groups is displayed in the Heat Map graphic (Figure 5).

6. Comparison of MDA, FRAP, SOD, and CAT in experimental groups

According to the results, the levels of serum malondialdehyde (MDA) (F (1, 4) = 4.183, P < 0.05), the total antioxidant capacity of plasma (F (1, 4) = 2.548, P < 0.01), SOD (F (1, 4) = 0.512, P < 0.05), and CAT (F (1, 4) = 0.854, P < 0.05) were significantly different between the experimental groups. In comparison to the control group, the antibiotic-treated group showed a significant increase in serum MDA (P < 0.05) and a significant decrease in the levels of SOD (P < 0.05), CAT (P < 0.05), and total antioxidant capacity of plasma (P < 0.01) (Table 1).

Discussion

In the current investigation, the experimental group's number of aerobic and anaerobic intestinal colonies was significantly lower than the control group's after 7 days of azithromycin gavage (15 mg/1 ml). The results of antibiotic treatment studies indicate changes in the intestine microbiome including a reduction in diversity, disturbance in the composition of the microbiome, the suppression of some bacteria and the strengthening of others, and the increase of antibiotic resistance, which often improves after the end of treatment (29, 30).

In light of the study's findings, the antibiotic gavage of azithromycin (15 mg/1ml) for 7 days led to the elimination of Enterococcus faecalis and Lactobacillus acidophilus species in the animal intestine. However, this treatment did not have a significant impact on the presence of Escherichia coli bacteria in the intestine. Other studies have reported the effect of different antibiotics on the reduction of Enterococcus faecalis and Lactobacillus acidophilus species (31), and the lack of impact on Escherichia coli species (32, 33).

Antibiotics play an anti-metabolic role in bacteria by inhibiting the synthesis of cell walls, inhibiting the synthesis of proteins and nucleic acids, inhibiting metabolic pathways, and interfering with the integrity of the cytoplasmic membrane (34). The mechanism of antibacterial action of azithromycin is similar to other macrolide antibiotics, and it interferes with mRNA translation and inhibits protein synthesis by concentrating on the ribosome's 50S subunit and obstructing the polypeptide exit channel (35). However, the undue use of azithromycin is the most significant cause of antibiotic-resistant bacteria. Improper dosage or duration of treatment leads to the emergence and spread of resistant bacteria (12).

In this study, azithromycin stopped the growth of Enterococcus faecalis, and Lactobacillus acidophilus, and reduced the number of their colonies in the intestine. Additionally, the lack of effect of azithromycin on Escherichia coli can be attributed to the prevalence of antibiotic resistance to macrolides in Escherichia coli strains (33).

The results of this study revealed that azithromycin antibiotic gavage (15 mg/1ml) for 7 days caused a decline in the number of healthy cells in the pyramidal layer of the hippocampus as well as a reduction in cognitive abilities in tests of novel object recognition test and inhibitory avoidance memory when compared to the control group. Other studies also indicate that the use of antibiotics can cause cognitive impairment (21, 36, 37), reduced spatial memory (38), anxiety and depression (39, 40), neurotoxicity in the cerebellum and hippocampus (41), reduced cell proliferation in the hippocampus, functional changes in brain synapses and severe atrophy in hippocampal neurons (21, 42).

The use of antibiotics leads to reduced production of neurotransmitters and a disruption in the vagus nerve signaling by disrupting the intestine microbiome (43). Extended usage of antibiotics can change the bacterial flora in the intestine, which reduces the amount of neurotransmitters and neuropeptides in memory-related regions like the hippocampus, hypothalamus, and prefrontal cortex. This can lead to depressive-like symptoms, anxiety, aggression, and memory loss (44).

Short-chain fatty acids are produced by the large intestine microbiota through the fermentation of complex carbohydrates. These fatty acids are essential for learning and memory because they lower inflammation and control neurotrophic factors in the central nervous system (45). Studies have indicated that antibiotic interventions can reduce short-chain fatty acids in the hypothalamus and hippocampus (45).

The use of antibiotics provides the conditions for dysbiosis, increasing the permeability of the intestinal wall, moning some bacteria to the brain, and the transfer of beta-amyloid plaques from the intestine to the brain by disrupting the bacterial flora in the intestines. The increase of beta amyloids in the brain provides the conditions for cognitive impairment by enhancing immune responses and increasing pro-inflammatory cytokines (43, 46).

Dysbiosis caused by the use of antibiotics activates immune cells and causes neuro-inflammation to induce cell death and memory loss by increasing the permeability of the intestinal epithelium and transferring bacterial extracellular vesicles to the brain (47). Also, increasing the permeability of the intestinal wall and the blood-brain barrier makes neurotoxins reach the brain and damage nerve cells (36).

The intestine microbiome causes the proliferation and activation of glial cells through the intestine-brain axis. Long-term use of antibiotics leads to changes in the activity, number, and morphology of astrocytes by disrupting the microbiome. It also suppresses genes involved in cell activation, pathogen recognition, and microglia defense(43, 44). These cases can provide the conditions for behavioral disorders, and memory defects by disrupting CNS.

In the current examination, treatment with azithromycin led to an increase in the amount of MDA and a notable decline in the FRAP and activity of SOD and CAT enzymes in blood serum in comparison with the control group.

Studies have revealed that mitochondria are targeted by antibiotics due to their bacterial origin. Antibiotics disrupt complexes I and III of the mitochondrial electron

transport chain, which are the primary sources of ROS formation, resulting in a rise in reactive oxygen species generation (48, 49).

Antibiotics also block nucleic acid synthesis processes in mitochondria and thereby lead to increased production of intracellular ROS, damage to mitochondria, and reduced cellular energy levels. In these conditions, waste materials accumulate in the cell with the reduction of autophagy and lysosomal degradation (50).

Additionally, antibiotics increase the rate of mitochondrial fission by disrupting the balance between fission and fusion. Also, it increases the amount of ROS production by changing the morphology and function of mitochondria. Reactive oxygen species not only cause damage to mitochondria but also initiate apoptosis and programmed cell death and induce neurotoxicity (50). Increased production of reactive oxygen species can accumulate in the cell and disrupt the function of nerve cells and induce apoptosis and programmed cell death (48). Increases in ROS are linked to reductions in ATP, glutathione, superoxide dismutase, and catalase enzyme activity, as well as an increase in malondialdehyde levels. These outcomes lead to the suppression of antioxidant defense and the induction of neurotoxicity (44). Azithromycin appears to cause memory impairment via raising ROS, which in turn causes a reduction in the number of healthy cells in several hippocampal regions.

Conclusion

The results revealed that treatment with azithromycin significantly reduced the number of aerobic and anaerobic intestinal bacterial colonies and eliminated Enterococcus faecalis and Lactobacillus acidophilus species in the animal's intestine. However, it did not have a significant impact on Escherichia coli species. This treatment also significantly reduced memory and learning, the number of healthy neurons in various hippocampal regions, and increased oxidative stress factors in blood serum.

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