The effect of *Lactobacillus casei* and *Bacillus coagulans* probiotics on liver damage induced by silver nanoparticles and expression of Bax, Bcl2 and Caspase 3 genes in male rats

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Abstract

In this study, we aimed to evaluate the effect of Bacillus coagulans and Lactobacillus casei probiotics on liver damage induced by silver nanoparticles and expression of Bax, Bcl2 and Caspase 3 genes in rats. 32 adult male Wistar rats were divided into four healthy groups (control), the group receiving silver nanoparticles treated with *L. casei*, the group receiving silver nanoparticles treated with *B. coagulans* and the group receiving only silver nanoparticles. The effect of nanoparticles was induced by intraperitoneal injection of silver nanoparticles prepared from nettle at a dose of 50 mg/kg and entered the liver tissue through the bloodstream. Two days after injection, probiotic treatment with 10^9 CFU was performed by gavage for 30 days. One day after the last gavage, rat liver tissue weight was assessed. Also, the total amount of RNA was extracted from treated, and healthy tissues, as well as induced silver nanoparticles tissues, then evaluated by Real Time PCR. Data were evaluated using one-way Anova, Tukey test. Based on the biochemical results of this study, exposure of rats to different concentrations of silver nanoparticles compared with the control group caused a significant increase in the serum levels of alanine transaminase (ALT) and aspartate transaminase (AST), alkaline phosphatase (ALP), especially at high concentrations. Evaluation of damage and histopathological lesions showed that silver nanoparticles in different concentrations caused different damage to liver tissue, so that, necrosis, inflammatory cell infiltration and vascular degeneration were observed at different concentrations by silver nanoparticles. In the present study, the effects of *L. casei* cell extract on increasing the expression of Bax proapoptotic gene and decreasing Bcl2 gene expression in cancer cells and inducing programmed cell death were shown. In this study, the expression of Bax, Bcl-2 and Caspase-3 genes in the group receiving silver nanoparticles and in the groups treated with probiotics showed significant changes compared to the control group. It can be concluded that the function of silver nanoparticles and the effects of relative improvement of probiotics are from the internal route of apoptosis and factors such as dose, nanoparticle size and nanoparticle coating have an important role in the toxicity of silver nanoparticles, thus the destructive effects on liver tissue could be increased by increasing the concentration of silver nanoparticles.

Key Words: Silver nanoparticles, probiotics, liver enzymes, Bax, Bcl2, Caspase-3, rat.

Eur J Transl Myol 33 (1) 10673, 2023 doi: 10.4081/ejtm.2023.10673

One of the most important and vital organs of the body is the liver, any damage to which can have very wide consequences.[1] Extensive biochemical reactions take place in the liver, including the metabolism of amino acids, carbohydrates, drugs, and detoxification.[2] Silver nanoparticles are one of the various chemical factors that play a role in inducing liver damage.

Nanoparticles (Net Promoter Score) are particles 1 to 100 nanometers in diameter. These particles may be massive or discrete. Silver nanoparticles are used in nanotechnology and are used in disinfection activities, especially in the control of open wounds and burns.[3] Clinical research and animal studies have reported that the adverse effects of silver nanoparticles are systemic

Table 1. *Primers.*

Gene	forward sequence reverse sequence
ß–actin	5'-TCT ATC CTG GCC TCA CTG TC-3' 5'-AAC GCA GCT CAG TAA CAG TCC-3'
Bax	5'-TGC AGA GGA TGA TTG CTG ATG-3' 5'-GAT CAG CTC GGG CAC TTT AG-3'
Bcl2	5'-CCT GGC ATC TTC TCC TTC CAG-3' 5'-GAC GGT AGC GAC GAG AG-3'
Caspase 3	5'GGAGCTTGGAACGGTACGCT-3' 5'-AGTCCACTGACTTGCTCCCA-3'

Table 2. *Thermocycle temperature program.*

Cycle	Time	Temp °c
1	4min	95
35	30 min 30 min 30 min	94 57 72
1	5 min	72

Table 3. *Temperature cycles of the device.*

Cycle	Time	Temp°c
1	15 min	95
40	15-30 sec 30 sec 30 sec	95 55-60 72

and acute exposure to these particles causes toxicity to the nervous system while chronic exposure causes Argyria disease, local inflammation and organ damage.[4] Numerous studies have shown that silver nanoparticles cause toxicity in cells through various mechanisms, including: production of reactive oxygen species (ROS), interaction with intracellular enzymes, disruption of integration, release of silver ions from Nanoparticles and the production of free radicals or oxidative stress markers that have unpaired electrons and cause programmed cell death. Silver nanoparticles cause toxicity to the liver and kidneys, and high doses lead to death.[5] They also cause histopathological changes in the liver, kidneys, and spleen, indicating a tendency for silver ions to bind to thiol groups in the liver. Various studies demonstrated that liver, kidney, and lung tissues in rats exposed to nanoparticles (50 nm) cause damage to liver tissue and kidneys by intravenous injection.[6] Due to the fact that most toxins enter the liver, liver tissue can be vulnerable to nanoparticles because the liver is high in thiol-rich proteins such as glutathione, which can store silver. Silver nanoparticles lead to apoptosis and cell death by altering the morphology and permeability of the membrane and damaging the DNA of the cell cycle. Apoptosis is controlled by both internal and external pathways. Several proteins are involved in the internal pathway, some of which are anti-apoptotic, including bax, bcl2, and Caspase 3 proteins.[7] In recent years, various studies have examined the role of probiotics in preventing tissue and organ damage. The results showed that these beneficial microorganisms are involved in the treatment of some diseases, including *Bacillus coagulans* and *Lactobacillus casei*, which can be used to prevent absorption and possible poisoning by heavy metals.[8] Probiotics are defined as a variety of living microorganisms that have beneficial effects in the prevention and treatment of specific pathological conditions.[9] The effect of probiotics on cytotoxicity caused by high-dose silver nanoparticles on liver tissue

and expression of apoptosis bax, bcl2 and caspase 3 genes has not been studied. Therefore, the aim of this study was to evaluate the effect of *B. coagulans* and *L. casei* probiotics on these factors.

Materials and Methods

Study Design

This experimental study was performed on 30 adult male Wistar rats. Thirty-two adult Wistar rats weighing approximately 200 to 220 g were purchased from the Pasteur Institute Research and Production Complex. Prior to the start of the experiment, the rats were kept for one week under laboratory conditions at 20 °±2 C in 12 hours of darkness and 12 hours of light in order to adapt to environmental conditions. The animals were weighed each time before the start of the experiment. All mice had unrestricted access to water and food.

The 32 adult male Wistar rats were randomly divided into 4 groups of 8 animals.

Group 1 (control group): Rats that did not receive any nanoparticles and were fed only water and food.

Group 2: Rats were injected with a single dose of 50 mg / kg intraperitoneally.

Group 3: Rats receiving silver nanoparticles were gavaged for 30 days with the probiotic *L. casei* at a dose of 10^9 CFU/mL.

Group 4: rates receiving silver nanoparticles were gavaged with *L. coagulans* probiotic at a dose of 10^9 CFU/ml for 30 days.

Probiotic preparations

Two types of probiotics *L. casei* (ATTCC 39392) and *L. coagulans* (ATCC 4356) were prepared from TakGene Company in powder form with log10. To prepare the solution, 1 g of probiotic was dissolved in 9 cc of distilled water for each rat until the probiotic reached log 9.

Production of silver nanoparticles

The purchased nettle plant was approved by a botanist and the extract was prepared from the dried leaves and stems of the nettle plant. To produce nanoparticles from the plant, the extract was exposed to silver metal ions. To do this, first 20 grams of dried parts of this plant

were poured into an Erlenmeyer flask and 97 mL of 1 mM silver nitrate salt was added to a volume of 500 mL of nettle extract. The reaction was performed at room temperature under dark conditions.[10] Silver nanoparticles prepared by nettle plant according to body weight of rat, 50 mg/kg dose of silver nanoparticles were injected intra-peritoneally to the animal as a single dose.

Tissue preparation and RNA extraction

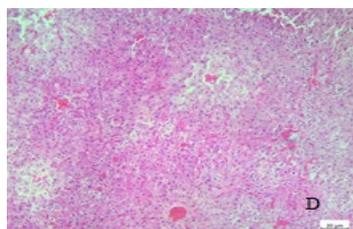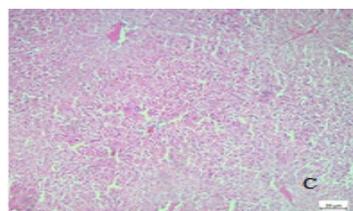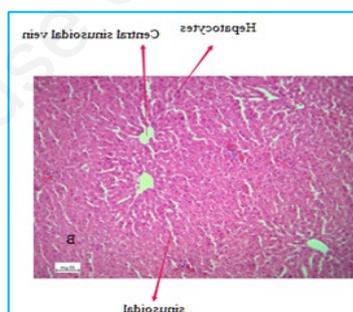To prepare histological sections, the liver was divided into two equal transverse sections. Tissue preparation
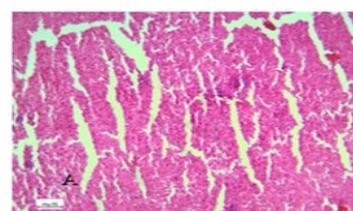

Fig 2. *A) Cross section of rat liver tissue: A) tissue section of the control group that does not show any histological changes. B) Tissue incision from the group receiving the silver nanoparticles clearly shows tissue damage. C) Tissue incision of the group treated with L.casei where tissue repair is visible. D) Tissue incision of the group treated with B.coagulans in which tissue repair is also visible.*

Fig 1. *A) Reaction solution of alcoholic extract of nettle and silver nitrate, B) after production of silver nanoparticles.*

was performed according to the conventional histological method (paraffin method). This was done automatically using a tissue processor, then the liver tissue isolated from rat was homogenized well by a homogenizer.

Total RNA purification kit (Bioneer, German company)

method that is normally used by RNA viruses to convert their genomic RNA to DNA inside a host cell. And then PCR amplification was performed on cDNA. In each sample, 1000 ng of RNA was used as a template for cDNA synthesis. In this study, CycleScript RT PreMix (dN6) kit from (Bioneer Company) was used for cDNA

Table 4. *Effect of silver nanoparticles and probiotics of L. casei and B. coagulans.*

Type of injury	control	**nanoparticles**	*casei*	*coagulans*
Central vein hemorrhage	-	+++	++	++
Cellular order	-	+++	++	+
Cellular rope	-	+++	++	+
Dilation of veins	-	+++	++	+
Vacuolization necrosis	-	+++	++	++

Normal (−), minimal (+), mild (++),and severe (+++).

Table 5. *Results of Real time PCR in different groups.*

Variables	**Ct.ß.action**	**Ct.bcl2**	**Ct.bax**	**Ct.caspase**
Control	20.59±1.65	24.79±1.21	23.54±1.37	33.59±2.12
silver nanoparticles (50mg / kg)	24.59±0.9	23.41±4.28	22.74±0.88	39.34±1.26
silver nanoparticles (50mg / kg)+ *L. casei* (10^{9}CFU/ml)	22.52±1.2	23.85±3.78	22.91±1.2	35.91±4.01
(2mg / kg) silver nanoparticles + *B. coagulans* (10^{9}CFU/ml)	22.66±4.82	24.59±3.8	23.72±1.09	35.7±3.8

Table 6. *ΔCt values of β-actin, Bax, Bcl2 and Caspse-3 genes in different groups.*

Groups	**Δct Bcl2**	**Δct Bax**	**Δct caspse3**
Control	1.98±0.44	2.95±0.28	13±0.47
silver nanoparticles	1.18±0.41	1.85±0.02	14.75±0.36
Silver nanoparticles + *L. casei*	1.33±1.005	1.39±0.002	13.39±0.8
Silver nanoparticles + *B. coagulans*	1.93±1.02	1.06±0.38	13.04±1.02

was used for RNA extraction, according to the manufacturer's instructions, and then the quality of the extracted RNA was checked by agarose gel electrophoresis.

Gene expression

First, RNA was transformed into complementary DNA (cDNA) using a well-known reverse transcription

synthesis. In order to make cDNA, an oligo dt primer that binds to the poly (A) tail of mRNA was used.

Real time PCR

Real time PCR was performed using the Sybr Green method. After finishing the work of the device, Melting and Amplification diagrams were examined and the CT obtained was included in the formula (Table 1-3):

Groups	$-\Delta\Delta ctBcl2$	$-\Delta\Delta ctBax$	$-\Delta\Delta ctcaspase3$	$2^{-\Delta\Delta ctBcl2}$	$2^{-\Delta\Delta ctBax}$	$2^{-\Delta\Delta ctCaspas3}$
Control	0.002±0.20	-0.05±0.20	0.76±0.37	1.68±0.34	2.02±0.81	1.29±0.14
Silver nanoparticles	0.96±0.18	-0.31±0.22	0.55-+23	2.46±0.33	2.06±0.36	1.82±0.13
Silver nanoparticles + *L. casei*	1.05±0.45	1.86±0.37	036±0.19	1.80±0.37	0.68±0.15	2.7±0.5
Silver nanoparticles + *B. coagulans*	0.57±0.41	0.74±0.25	0.45±0.16	0.93±0.14	0.94±0.14	2.02±0.82

Table 7. ΔΔCt and 2-ΔΔCt values of Bcl2, Bax and Caspase-3 genes in different groups.

(B actin gene in healthy sample) Ct - (Gene in healthy sample) Ct = Normalized data of gene in healthy sample

(Normal sample) Δ Ct - (diabetic sample) ΔCt = ΔΔCt of the gene in the diabetic sample compared to the normal sample)

(Normal sample) ΔCt - (normal sample) ΔCt = ΔΔCt of the gene in the normal sample compared to the normal sample

Log2 fold change = -ΔΔCt   Fold change = 2 -ΔΔCt

Gel electrophoresis of PCR products

In order to qualitatively evaluate the expression of the genes studied in this study, PCR products were examined by electrophoresis using 1.5% agarose gel.

Statistical analysis

The data obtained from this experiment were analyzed by One-Way ANOVA ("analysis of variance"). Tukey's test was used to determine groups with differences. Significance level was statistically considered to be P < 0.05 and the obtained values were reported as mean standard error.

Results

Synthesis of nanoparticles

Synthesis of silver nanoparticles was performed using reduction of silver ions by hydroalcoholic extract of nettle. The first sign of the production of silver nanoparticles is a change in the color of the solution. The resulting dark brown color after 24 hours indicates the production of silver nanoparticles in solution. Ultraviolet-visible spectroscopy results of nettle extract before and after the synthesis of silver nanoparticles indicate the production of silver nanoparticles by changing the color of the solution from bright green to brownish green (Figure 1).

Table 4 shows the results of the effects of *L. casei* and *B. coagulans* probiotics on changes in liver tissue cells in the liver of rats exposed to silver nanoparticles. Tissue damage, cellular hypertrophy, hyperemia in the central vein, vacuolization, necrosis, hyperemia in the sinusoids, borderline nuclei were observed (Figure 2).

Rat weight was measured in four groups after injection of silver nanoparticles and after 30-day gavage of probiotics using a digital scale. No significant weight loss was observed in all groups compared to the control group.

The results of Real Time PCR test for expression of apoptosis bax, bcl2 and Caspase-3 genes did not show significant differences in different experimental groups (Table 5). The Ct and ΔCt values of Bax, Bcl2 and Caspase-3 genes in the 4 study groups are shown in Table 6.

Compare the group with the control group p <0.05:

Comparison of group with group of silver nanoparticles p <0.05 #: Comparison of groups with groups

According to the mean comparison test (Kruskal Wallis; Table 7), the mean of the variables (ct. bcl2, ct.bax, ct.caspase and ct.baction) showed statistically significant difference between the treatment groups (p <0.05) (Figure 3)

According to Table 8, Kruskal-Wallis test showed a statistically significant difference between the groups (silver nanoparticles, *L. casei* and *B. coagulans*) with the control group (p <0.05). There is a statistically significant difference between L. casei and *B. coagulans* with silver nanoparticle. Based on the biochemical results of this study, exposure of rats to different concentrations of silver nanoparticles compared with the control group caused a significant increase in the serum levels of ALT, AST and ALP especially at high concentrations (Figure 4)

According to Table 9, we found no statistically significant difference in the study groups.
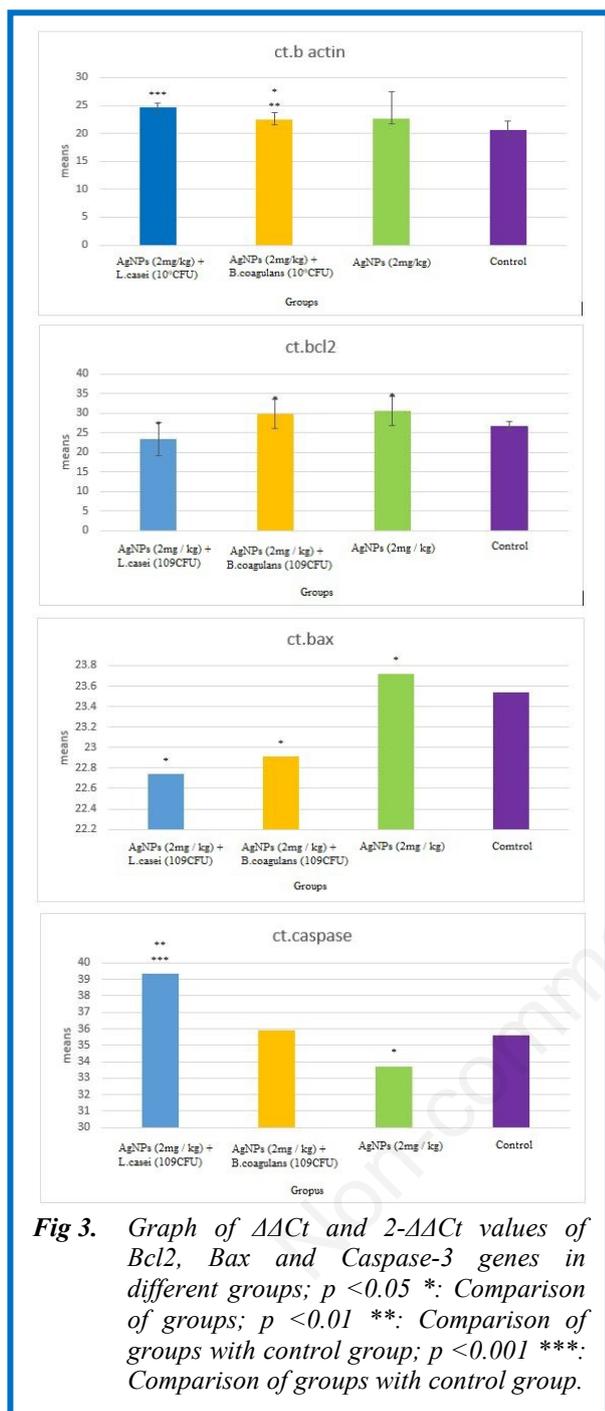
Fig 3. *Graph of ΔΔCt and 2-ΔΔCt values of Bcl2, Bax and Caspase-3 genes in different groups; p <0.05 *: Comparison of groups; p <0.01 **: Comparison of groups with control group; p <0.001 ***: Comparison of groups with control group.*

Fig 4. Graph of average ALT, AST, ALP indices in different groups; ; groups; p <0.05 *: Comparison of groups; p <0.01 **: Comparison of groups with control group; p <0.001 *: Comparison of groups with control group; #: Comparison of groups with AgNps group.**

Discussion

Toxicity of AgNPs has been extensively studied in *in vivo* and *in vitro* systems over the past decade, showing that the main site of AgNP accumulation is the liver,[11] specifically, by silver nanoparticles and the expression of Bax, Bcl2 and Caspase 3 genes in male rats. Therefore, the aim of the present study was to evaluate the effect of *L. casei* and *B. coagulans* probiotics on liver damage induced by silver nanoparticles and to assess expression of Bax, Bcl2 and Caspase 3 genes in male rats.

Based on the biochemical results of this study, exposure of rats to different concentrations of silver nanoparticles, especially at high levels, significantly increases the serum content of ALP, ALT and AST enzymes compared to the control group. ALT and AST are non-functional plasma enzymes that are naturally located in the cells of some organs, including the liver. the increase in serum levels of these enzymes may be due to a change in the permeability of the plasma membrane of liver cells or to cell damage resulting from exposure to silver nanoparticles or both. Naghsh et al. (2012)[12] reported that intraperitoneal injection of nanoparticles in Wistar rats caused the most damage to the liver cell at a dose of 50 ppm.

Evaluation of damage and histopathological lesions revealed that silver nanoparticles in different concentrations caused different damage to liver tissue so that, silver nanoparticles-induced tissue necrosis,

Table 8: *Mean of main variables among the studied groups*

Main variables	ALT(u/L)	AST(u/L)	ALP(u/LL.casei)
Control	62.70±8.12	84.70±9.21	104.90±7.83
silver nanoparticles(50mg / kg)	124±11.83**	325±12.71***	314±8.37*
silver nanoparticles(50mg / kg) + *L. casei* (10^9CFU/ml)	91±4.86*#	156±8.13**#	136±10.21*#
silver nanoparticles(50mg / kg) + *B.coagulans* (10^9CFU/ml)	98.5±5.96*#	184±8.13*#	168±6.94*#

p <0.05 *: Comparison of groups with control group; p <0.01**: Comparison of groups with control group; p <0.001 ***: Comparison of groups with control group, p <0.05 #: Comparison of groups with AgNps group.

Table 9: *Mean and standard deviation of main variables among the studied groups.*

Main variables	Liver weight	Mouse weight
Control	11.63±0.34	200.33±10.01
silver nanoparticles(50mg / kg)	11.44±0.58	192±8.18
silver nanoparticles(50mg / kg) + *L.casei* (10^9CFU/ml)	11.16±0.59	199.62±10.01
silver nanoparticles(50mg / kg) + *B.coagulans* (10^9CFU/ml)	11.39±0.58	202.25±9.62
Total	11.34±0.55	199.63±9.70

p <0.05 *: Comparison of groups with control group; p <0.01 **: Comparison of groups with control group; p <0.0001 ***: Comparison of groups with control group.

infiltration of inflammatory cells and vacuolar degeneration were observed at different concentrations. Zamani et al. (2014)[13] reported that intraperitoneal injection of silver nanoparticles into rat was capable of inducing necrosis, vascular degeneration, and infiltration of inflammatory cells, which is consistent with our histological results. The toxicity of nanoparticles depends on various factors, and smaller particles are more active in creating and inducing toxicity due to their large active surface area.[14] Furthermore, the accumulation and diffusion of nanoparticles in the tissues of the brain, kidneys and liver and its toxicity depends on the way of nanoparticle consumption.[11] Some nanoparticles cause toxicity by producing free radicals and reactive oxygen species. Free radicals from silver nanoparticles damage the liver, causing oxidation of its lipids, resulting in reduced membrane fluidity and loss of structure and function, which in turn leads to an increase in the concentration of liver enzymes in the blood. Therefore, an increase in these enzymes is a sign of liver cell destruction. The increase in liver enzymes after exposure to silver nanoparticles, especially at high concentrations, confirms the damage and histopathological changes observed in the liver.[14,15] According to the obtained results, it can be concluded that factors such as dosage, nanoparticle size and nanoparticle coating have an important role in the toxicity of silver nanoparticles, so that with increasing concentration of silver nanoparticles, the destructive effects on liver tissue increase.

The mechanisms used by probiotic bacteria to reduce the production of carcinogenic mutations are not well understood.[16] Some of them are belived to be: i) binding to mutagenic substances that prevents their absorption by the body; ii) breakdown of some mutagenic compounds; iii) reduction of the activity of harmful enzymes; iv) inhibiting harmful intestinal bacteria; v) producing special metabolites and vi) reducing bile acids. Furthermore, probiotic bacteria are capable of producing some metabolites such as butyrate and folate, which reduce the proliferation of cancer cells by acting on intestinal epithelial cells. Biffi et al. (1997)[17] showed that the production of metabolites by probiotic bacteria is associated with inhibition of proliferation of cancer cells and stimulation of the immune system. In their study, Sekine et al. (1985)[18] demonstrated its positive effects in stimulating the immune system of mice for inhibiting mutagenic substances. All in all, induction of programmed cell death is one of the approaches in cancer treatment. The cell death pathway may involve the activation of proapoptotic events in the cell that begin with the permeability of the mitochondrial organ membrane by Bax and Bak proteins and eventually the release of cytochrome C from it.[19-24]

In the present study, we showed the effects of *L. casei* cell extract on increasing the expression of Bax

proapoptotic gene and decreasing Bcl2 gene expression in cancer cells and inducing programmed cell death.

In addition, Bcl2 and Bax proteins prevent the accumulation of Bax and Bak proteins by being located on the surface of the endoplasmic reticulum, mitochondria and nucleus, thus showing anti-apoptotic activity.[21] Also, the expression of Bax, Bcl-2 and Caspase-3 genes was examined by Real Time method and the expression of Bax, Bcl-2 and Caspase-3 genes in the group receiving silver nanoparticles, and changes were significant in the groups receiving probiotics.

Apoptosis is a natural process in cells that causes the removal of old, damaged and harmful cells. Any disturbance in this pathway will cause disease.[19] Sometimes a decrease in this pathway will lead to growth of abnormal cells such as cancer cells, and sometimes an abnormal increase will lead to the destruction of normal cells. Apoptosis is capable of activating a specific group of aspartate-dependent proteases called caspases, especially caspase-3, both internally and externally. In the external pathway, intracellular caspases are activated by increasing the amount of tumor necrosis factor (TNF-α) secreted by macrophages and T cells and binding to cell surface receptors and trimerization. While in the internal pathway, also known as the mitochondrial pathway, the relative permeability of the mitochondrial membrane to cytochrome C increases with the relative change of proapoptotic (such as Bax) and anti-apoptotic (such as Bcl-2) mediators, and with its release, apoptosis is formed and activated, leading to activation of caspase. [22]

In this study, the expression of Bax, Bcl-2 and Caspase-3 genes in different groups was investigated using real-time method. Significant changes in Bax, Bcl-2 and Caspase-3 gene expression were observed in the group receiving silver nanoparticles and in the probiotic treated groups. It can be said that the function of silver nanoparticles and the effects of relative improvement of probiotics are probably due to the internal pathway of apoptosis. Among all probiotics, bacteria of the Lactobacillus family such as *L. acidophilus*, *L. casei* and *L.delbrueckii* are among the most important components of the normal intestinal flora of humans and animals. In addition, the role of lactobacilli probiotics in facilitating the treatment of colorectal cancer is well known.[23] A number of studies have shown that soluble compounds secreted by *L. casei* and *L. rhamnosus* induce apoptosis in monocytic leukemia cells, so probiotics can be considered as a safe agent for fighting cancer.[16] Another study showed that Heat-killed *Saccharomyces cerevisiae* (HKY) was capable of causing cell apoptosis in three breast cancer cell lines including ZR-75-1, MCF-7, and HCC-70. In addition, they showed that direct injection of HKY into the tumor could cause significant death of cells by inducing apoptosis.[17]

Based on the data presented herein, it can be said that consumption of *L. casei* and *B. coagulans* improves the antioxidant status and reduces oxidative stress in rats receiving silver nanoparticles. Due to the lack of significant changes in the expression of Bax, Bcl-2 and Caspase-3 genes, it seems that these internal pathways of apoptosis induction are not activated in the studied mice. Apoptosis appears to be induced through other pathways. On the other hand, based on results of this study, regular and long-term use of probiotics may be beneficial, as a supplement to the main treatments, to delay the progression of cancer and its complications.

List of acronyms

ALP - alkaline phosphatase
ALT - alanine transaminase
AST - aspartate transaminase
Bax – Bcl-2-like protein 4
Bcl-2 – B-cell lymphoma 2
CFU -Colony-forming unit
HKY - Heat-killed Saccharomyces cerevisiae
ROS - reactive oxygen species

Contributions of Authors

ZK, Ro,RS participated in conception and design of the study, acquisition, analysis and interpretation of data, wrote the manuscript, performed literature review, article drafting and revision, Autyhors reviewed and edited the manuscript critically. All authors have read and approved the final editeded version of the paper.

Conflict of Interest

The authors declare no conflict of interests.

Ethical Publication Statement

We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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