Yogurt: Effect on Leukocytes and Blood Coagulation in an Acute Liver Injury Model

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ABSTRACT   This study determined whether cow or goat yogurt administration has a preventive effect on the hepatic damage undergone during an acute liver injury. Acute liver injury was induced by an intraperitoneal injection of D-galactosamine. Groups of mice were fed with cow or goat yogurt for 2 days or 7 days before the D-galactosamine injection. Blood and liver samples were obtained 12 hours after D-galactosamine inoculation. D-Galactosamine induced an increase in serum aminotransaminases, a reduction in the number of blood leukocytes, an enhancement in neutrophil myeloperoxidase activity, a recruitment of leukocytes toward the liver, an increase in cell death, and an alteration in prothrombin time, activated partial thromboplastin time, and fibrinogen levels. Treatment with cow or goat yogurt was effective at increasing leukocyte number and decrease myeloperoxidase activity. We also observed a decrease in leukocyte accumulation in the liver and a reduction in cell death. Activated partial thromboplastin time and fibrinogen were normalized, but prothrombin time only showed an improvement without reaching normal values. Cow or goat yogurts were effective at protecting against an experimental acute liver injury, especially when administered for 7 days.

KEY WORDS: • *acute liver injury* • *blood coagulation* • *yogurt*

INTRODUCTION

HUMANS ARE FREQUENTLY EXPOSED to many different factors such as viruses, alcohol intake, and various kinds of chemicals that can induce acute liver injury (ALI). Liver injury caused by hepatotoxins is characterized by an inflammatory response with infiltration of neutrophils or mononuclear cells, altered local and systemic expression of inflammatory mediators,[1,2] and hepatocyte degeneration and cell death via either apoptosis or necrosis.[3] In addition, hepatocellular necrosis leads to impaired synthesis of proteins such as coagulation factors.[4] Unfortunately, in the liver, as in other organs, an excessive inflammatory response bears the risk of additional tissue damage.[1]

An experimental ALI model can be induced by D-galactosamine (D-Gal), which provokes depletion of the hepatic uridine triphosphate indispensable for the biosynthesis of macromolecules such as nucleic acids and proteins.[5]

Also, D-Gal increases intestinal permeability and the release of bacterial products. In addition, Kupffer cells are activated to liberation of tumor necrosis factor-α (TNF-α), which leads to cell death in various ways, including the inflammatory process.[6,7]

In recent years research into natural hepatoprotective compounds has become attractive.[8,9]

In the present study we investigated the effect of probiotics, which are live microorganisms that when administered in adequate amounts confer a health benefit on the host.[10] A probiotic product such as yogurt is obtained by the fermentation of milk with cultures of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*.[11] In order to grow in milk, lactic acid bacilli must be capable of hydrolyzing the large proteins contained in milk. It has been proposed that the health-promoting effects ascribed to fermented dairy products arise not only from the bacteria themselves but also from metabolites derived from milk fermentation.[12] Most studies deal with the beneficial effects of yogurt in relation to its immunomodulatory activities, such as protection against enteric and respiratory infections,[13] ability to induce pro- and anti-inflammatory cytokine release, and anticarcinogenic properties.[14–16] Previously, we demonstrated that a pretreatment with oral probiotics prevented the breakdown in intestinal barrier function, reduced bacterial translocation, and attenuated liver injury induced by D-Gal in mice.[17,18]

An alternative probiotic dairy product can be a variety of yogurt obtained by means of the fermentation of goat's milk,

which, compared to cow's milk, has higher bioavailability and digestibility[19] and a greater concentration of selenium, an important antioxidant.[20]

The aim of the present study was to determine whether cow yogurt (CY) or goat yogurt (GY) administration has a preventive effect on the hepatic damage undergone during a D-Gal-induced ALI.

MATERIALS AND METHODS

Animals

Six-week-old BALB/c mice weighing 22–25 g were obtained from the closed colony kept at CERELA (San Miguel de Tucumán, Argentina). Animals were housed in a room at $22 \pm 1°C$ with a 12-hour/12-hour light-dark cycle. Each experimental group consisted of five or six mice, which were housed individually during the experiments. All experiments were conducted following the guidelines of the Ethical Committee for Animal Care at CERELA and the Universidad Nacional de Tucumán, Argentina.

Yogurt preparation

Yogurts were prepared according to the procedure of Villena et al.[13] from a stock culture of L. bulgaricus and S. thermophilus obtained from the CERELA culture collection. These strains were incubated in pasteurized milk from creole goats or cows provided by a farmer from Tucumán, Argentina. Quality control was carried out at the Ecophysiology Laboratory at CERELA. The yogurt was prepared every 48 hours to ensure a constant number of bacteria (2×10^8 cells/mL).

Feeding procedures

Mice were fed a balanced commercial diet supplemented with CY or GY for 2 or 7 consecutive days. Each group received 4 mL of the different yogurts per day per mouse. The control group received only balanced commercial diet ad libitum.

ALI induction

At the end of the feeding period, ALI was induced by intraperitoneal injection of D-Gal (catalogue G-0264, Sigma Chemical Co., St. Louis, MO) at a dose of 800 mg/kg of body weight in sterile saline solution.[21]

The groups were identified as 2dGY-DG, 2dCY-DG, 7dGY-DG, and 7dCY-DG, designating days of supplementation, yogurt type, and D-Gal administration. The ALI control received the D-Gal injection without previous treatment with yogurt (DGC). The normal controls (NC) were animals injected with saline solution only.

Alanine transaminase (ALT) and aspartate transaminase (AST) as liver injury markers

In order to verify the induction of ALI by D-Gal, the serum levels of ALT and AST were determined at 12 and 24 hours post-D-Gal injection. Blood samples were obtained by cardiac puncture from sodium pentobarbital-anesthetized animals. Enzymatic activity was measured in mouse serum by means of a spectrophotometric kinetic assay (Roche Diagnostics, Indianapolis, IN).

Because the highest transaminase levels were found at 12 hours after injury induction, the assays were performed at this time.

Histological examination

In order to determine intrahepatic leukocyte aggregates, the livers were aseptically removed, fixed in 4% formalin, and embedded in Histowax (Leica Microsystems Nussloch GmbH, Nussloch, Germany) according to the technique of Racedo et al.[22] Leukocyte aggregates was determined on 4-μm-thick tissue sections stained with hematoxylin-eosin for light microscopy examination. Four tissue sections from various areas of the liver of each mouse were examined in all animal groups. The results were expressed as the number of leukocyte aggregates per 50 fields (×40).

Total and differential blood leukocyte counts

Blood samples were collected in heparinized tubes. Total number of white blood cells was determined with a hemocytometer. Differential cell counts were performed by counting 200 cells in blood smears stained with May Grünwald-Giemsa stain[22] under a light microscope (×100), and the absolute numbers were calculated.

Phagocytic cell activation

Phagocytic cell activation was assessed by determining the myeloperoxidase (MPO) activity of blood neutrophils using a cytochemical method (Washburn test); cells were graded as negative or weakly, moderately, or strongly positive and used to calculate the score.[22] The scoring was performed by counting 200 neutrophils in blood smears. The score value was calculated by addition of neutrophils with different positive grades.

Cell death quantification

For propidium iodide staining, 2.5×10^5 cells of liver homogenate were suspended in 200 μL of phosphate-buffered saline. Two microliters of staining solution (50 μg/mL propidium iodide; catalogue P-4170; Sigma-Aldrich Co., St. Louis, MO) was added, and the cell suspension was incubated for 5 minutes at room temperature. Then, the cell suspension was diluted with 200 μL of phosphate-buffered saline and analyzed using a Partec Pas flow cytometer (Partec, Münster, Germany).

For each sample, data from 100,000 events were recorded in list mode and recorded on logarithmic scales. Analysis was performed using Partec FloMax® software.

Coagulation tests

Blood samples were obtained by cardiac puncture and collected in a 3.2% solution of trisodium citrate at a 9:1 ratio

according to Agüero et al.[23] Routine coagulation tests such as prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen concentration were performed manually on fresh plasma samples. PT was determined by a one-step method (Thromboplastin-S, Biopool International, Buenos Aires, Argentina). APTT was determined by a one-step technique (STA APTT, Diagnostica Stago, Asnière, France). Fibrinogen was measured using a commercial kit and following the manufacturer's instructions (FIBRI PREST 2, Diagnostica Stago).

Statistical analysis

Experiments were performed in triplicate (five or six animals each time) and results were expressed as mean ± SD values. After verification of a normal distribution of data, two-way analysis of variance was used. Tukey's test (for pairwise comparisons of the means) was used to test for differences between the groups. Differences were considered significant at $P < .05$.

RESULTS

Liver injury markers

A moderate increase in transaminase levels in serum was observed at 12 hours post-D-Gal injection. Administration for 2 days of GY or CY previous to injury induction did not improve enzyme levels, whereas when feeding was prolonged for 7 days, normal values were obtained (Table 1).

Histological examination

The microscopic examination of the liver from DGC mice did not evidence alteration in the liver architecture. Sinusoidal lining cells and endothelium were not affected. However, high numbers of leukocyte aggregates were found in DGC animals, but none could be seen in histological slices from normal mice. Administration of GY or CY for 2 days failed to reduce the number of aggregates, but liver examination of mice treated with GY or CY for 7 days revealed a statistically significant decrease in the number of leukocyte aggregates (Fig. 1).

FIG. 1. Leukocyte aggregates in the liver. The DGC group showed high numbers of leukocyte aggregates in liver slices. The groups fed with yogurt for 2 days showed no improvement, whereas a significant decrease was found in these aggregates after treatment for 7 days: *$P < .05$ relative to the normal control (NC) group; †$P < .05$ relative to the D-Gal control (DGC) group. The 2dGY-DG or 2dCY-DG mice were treated preventively with GY or CY for 2 days before D-Gal injection; the 7dGY-DG or 7dCY-DG mice treated preventively with GY for 7 days before D-Gal injection.

Total and differential blood leukocyte counts

Total leukocyte counts in peripheral blood were diminished in the DGC group ($P < .05$) at 12 hours post-injury. The number of neutrophils and lymphocytes also decreased in these animals ($P < .05$). Mice treated preventively with GY or CY for 2 days before D-Gal injection showed normalization of total leukocyte counts. In the 7dGY-DG group, total leukocyte counts had values higher than in the NC group, whereas 7dCY-DG showed a similar behavior to 2dCY-DG. With respect to neutrophils, this parameter was normalized in 7dCY-DG mice; however, 7dGY-DG induced a significant increase in these cells, reaching higher values than in NC animals (Table 2).

Phagocytic cell activation

A significant increase in MPO activity in polymorphonuclear neutrophils from peripheral blood was observed in

TABLE 1. LEVELS OF AMINOTRANSFERASES IN SERUM

Treatment	ALT (U/L)	AST (U/L)
NC	48.3 ± 3.1	167 ± 9.9
DGC	60.6 ± 8.6*	193.5 ± 13.7*
2d GY-DG	63.1 ± 8.0*	198 ± 8.1*
2d CY-DG	67.0 ± 7.6*	195 ± 7.5*
7d GY-DG	55.1 ± 3.5	175 ± 8.1
7d CY-DG	53.3 ± 3.8	170 ± 7.6

The serum levels of ALT and AST were determined 12 hours post-D-Gal. 2dGY-DG, 2dCY-DG, 7dGY-DG, and 7d CY-DG mice were treated preventively with GY or CY for 2 days or 7 days before D-Gal injection, the DGC group received the D-Gal injection without previous treatment with yogurt, and the NC group was the normal controls.
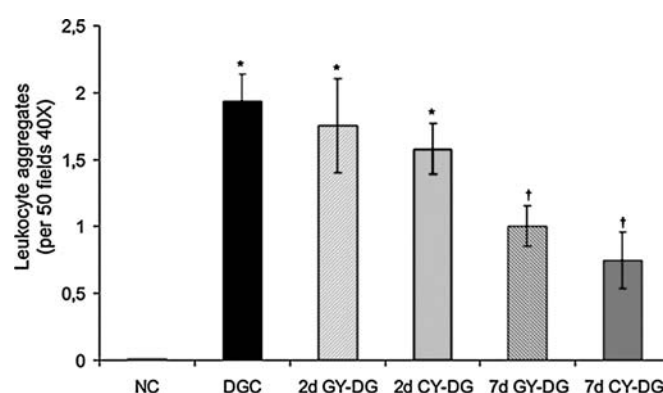*$P < .05$ relative to the NC group.

TABLE 2. TOTAL AND DIFFERENTIAL BLOOD LEUKOCYTE COUNTS

Treatment	WBCs (10^9cells/L)	PMNs (10^9cells/L)	Lymphocytes (10^9cells/L)
2d GY-DG	5.2 ± 0.8	0.6 ± 0.2	4.6 ± 0.9
2d CY-DG	4.9 ± 0.6	0.6 ± 0.1	4.3 ± 0.6
7d GY-DG	7.2 ± 0.8**	1.7 ± 0.1**	5.5 ± 0.7
7d CY-DG	4.9 ± 0.6	0.8 ± 0.1	3.9 ± 0.8
DGC	3.7 ± 0.7*	0.6 ± 0.1*	3.1 ± 0.8*
NC	6.1 ± 0.6	1.0 ± 0.3	4.9 ± 0.7

2dGY-DG, 2dCY-DG, 7dGY-DG, and 7d CY-DG mice were treated preventively with GY or CY for 2 days or 7 days before D-Gal injection, the DGC group received the D-Gal injection without previous treatment with yogurt, and the NC group was normal controls. PMN, polymorphonuclear neutrophil; WBC, white blood cell.
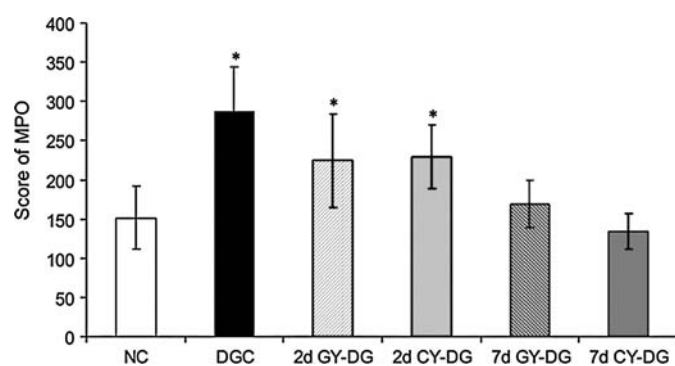*$P < .05$ relative to the DGC group.
**$P < .05$ relative to the NC group.

FIG. 2. MPO activity scores in polymorphonuclear neutrophils from peripheral blood. This activity in the D-Gal control (DGC) group was significantly increased compared to the normal control (NC) group. Yogurt supplementation for 7 days normalized this parameter: *$P < .05$ relative to the NC group. The 2dGY-DG or 2dCY-DG mice were treated preventively with GY or CY for 2 days before D-Gal injection; the 7dGY-DG or 7dCY-DG mice were treated preventively with GY or CY for 7 days before D-Gal injection.

F2 ► DGC animals compared to NC animals (Fig. 2). Administration for 2 days of both yogurts did not significantly improve enzyme activity, whereas when feeding was prolonged for 7 days normal values were obtained.

Percentage of cell death

D-Gal induced a significant increase in the percentage of dead cells in liver. GY or CY administration for 2 days did
F3 ► not modify these values compared to DGC animals (Fig. 3). Feeding for 7 days induced a significant decrease in the percentage of dead cells in liver with respect to DGC but without reaching NC levels.
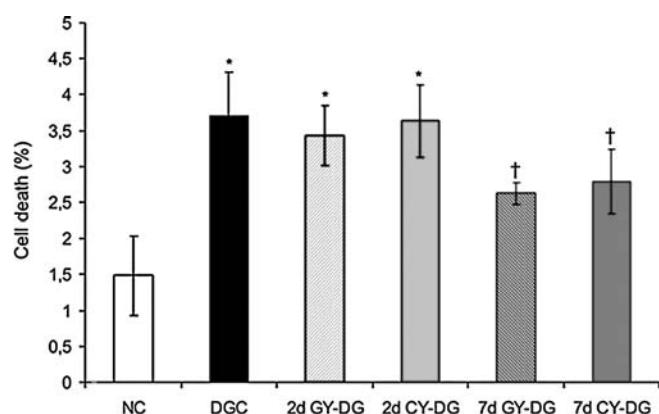

FIG. 3. Cell death quantification of liver homogenate using the cytofluorimetric method. The percentage of dead cells was elevated in the D-Gal control (DGC) group compared to the normal control (NC) group. Only 7-day supplementation enabled a significant decrease in this parameter with respect to the DGC group, without reaching normal values: *$P < .05$ relative to the NC group; †$P < .05$ relative to the DGC group. The 2dGY-DG or 2dCY-DG mice were treated preventively with GY or CY for 2 days before D-Gal injection; the 7dGY-DG or 7dCY-DG mice treated preventively with GY or CY for 7 days before D-Gal injection.

Hemostatic parameters

D-Gal injection induced a significant reduction in the percentage of PT and prolongation of APTT values ($P < .001$). Administration of yogurt at different doses normalized the APTT test. None of the yogurt doses administered caused PT to return to normal values. However, mice treated preventively with GY for 7 days had improved PT values, although they did not reach the activity percentage of the NC group (Fig. 4A and B).   ◄ F4

Fibrinogen concentration was diminished in the DGC group compared to the NC group. Feeding with yogurt for 2 days did not improve this parameter. Only CY supplementation for 7 days enabled normalization of the levels of this protein (Fig. 4C).

DISCUSSION

Hepatotoxicity is one of the most common side effects induced by different therapeutic drugs. Numerous mechanisms are involved in such an effect, with a role for neutrophils and mononuclear cells having been considered in the pathophysiology of liver injury.[24]

In the present work we verified the liver injury induced by D-Gal through the rise in serum transaminase activities, which are markers of hepatic damage. The levels of these enzymes were increased 12 hours after injury induction. Similar results were reported by Izu *et al.*[25] in experimental models with mice.

The increase in the levels of serum transaminases was accompanied by histopathological changes showing leukocyte infiltration. This phenomenon was also observed by Liu *et al.*[26] in rats after lipopolysaccharide/D-Gal injection.

The hepatic infiltration of polymorphonuclear leukocytes is an early response to tissue injury. In addition, neutrophil activation is vital for host defense and removal of cell debris; however, it can also cause additional tissue damage or even liver failure.[27]

In the present work we found a correlation between the presence of leukocyte aggregates in liver and a decrease in total blood leukocytes counts, perhaps due to their recruitment into the liver because of the inflammatory response induced by the hepatotoxic drug.

The recruitment of white blood cells induced by the D-Gal injection would probably be promoted, among other factors, by the release of TNF-α by activated Kupffer cells.[7] The main function of TNF-α is to stimulate the recruitment of neutrophils and monocytes to the site of the injury.[28]

There also was a significant increase in MPO activity in blood neutrophils as a consequence of their activation to defend the organism against aggression. Nevertheless, it has been proposed that HOCl and other oxidizing intermediates generated by MPO also contribute to tissue damage at inflammation sites.[29]

In addition, the animals injected with D-Gal showed an increase in the percentage of dead cells, which could be due to apoptosis or necrosis. At present, apoptosis and necrosis are beginning to be considered as often representing alternate outcomes of the same cellular pathways to cell death.[30]
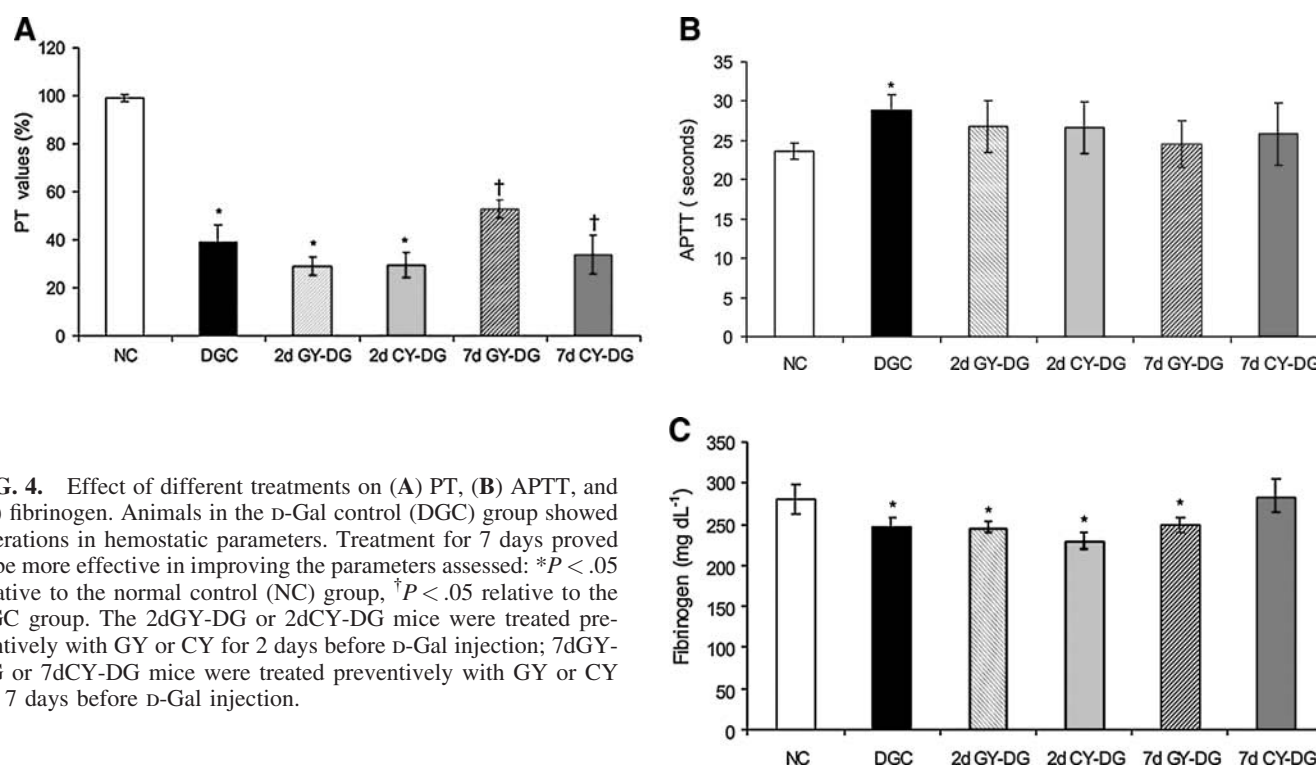
FIG. 4. Effect of different treatments on (**A**) PT, (**B**) APTT, and (**C**) fibrinogen. Animals in the D-Gal control (DGC) group showed alterations in hemostatic parameters. Treatment for 7 days proved to be more effective in improving the parameters assessed: *$P < .05$ relative to the normal control (NC) group, †$P < .05$ relative to the DGC group. The 2dGY-DG or 2dCY-DG mice were treated preventively with GY or CY for 2 days before D-Gal injection; 7dGY-DG or 7dCY-DG mice were treated preventively with GY or CY for 7 days before D-Gal injection.

When GY or CY was preventively administered for 7 days, serum transaminase activities showed normal values. It has been recognized that transaminase levels do not always accurately reflect the extent of liver inflammation and that the correlation between the activity of liver enzymes and the degree of tissue damage can be slight.[31] Histological studies showed that only the 7dGY-DG and 7dCY-DG groups revealed a statistically significant reduction in the number of leukocyte aggregates that could be attributed to the effect exerted by yogurt on leukocyte recruitment, in agreement with the results reported by Roselli et al.[32]

We also observed a decrease in MPO activity in polymorphonuclear leukocytes, probably because yogurt modulates the release of adequate levels of TNF-α and interleukin-10.[14] Aoi et al.[33] also demonstrated that the intake of fermented milk improved the levels of MPO activity, which is increased in muscle after prolonged exercise.

When GY or CY was administered for 7 days before liver injury induction, we noticed an increase the number of total peripheral blood leukocytes, probably as a consequence of the innate immunity regulation induced by the dietary supplement.[34]

GY induced an increase in the number of total peripheral blood leukocytes and neutrophils, with values higher than those of NC animals, while animals fed with CY showed normal leukocyte values. Because the bacterial composition of both yogurts is identical, the values found in animals supplemented with GY compared to those that received CY could be attributed to the different percentages of caseins, vitamins, and fatty acids in both milks.[19]

Lactic acid bacteria utilize milk proteins, mainly caseins, as their prime source of essential and growth-stimulating amino acids.[35]

The peptidic profile of milk proteins is significantly different after microbial fermentation, suggesting that microbial proteolysis can be a potential source of bioactive peptides.[36] Fragments of β-casein have been shown to stimulate phagocytosis of peritoneal macrophages, protect against infections,[37] and enhance the proliferation of murine peripheral blood lymphocytes in vivo.[38]

On the other hand, fatty acids can modulate the function of leukocytes by controlling their proliferation, the production of cytokines, and the expression of adhesion molecules.[39]

The improvement in the number of blood leukocytes, the regulation of the migration of neutrophils, and the decrease in MPO activity caused by yogurt would contribute to regulate the inflammatory response, reducing cell damage and decreasing the percentage of cell death. The anti-inflammatory effect of yogurt has also been reported by Lorea Baroja et al.[40] in bowel disease patients and by Perdigón et al.[14] in a colon cancer model.

Finally, we studied the coagulation process to determine the influence of yogurt in the synthesis of proteins in our experimental model. PT, APTT, and fibrinogen levels showed that coagulation was affected by D-Gal. The PT test evidenced the greatest alteration because the factors involved in it were the ones with the shortest half-life. The modifications induced in the coagulation tests in our experimental model could be due to a decrease in the synthesis of coagulation factors and to their utilization during

coagulation activation. This activation could be triggered by bacterial and endotoxin translocation induced in mice by D-Gal injection.[17]

This translocation phenomenon has been shown to play a key role in the pathophysiology of hemostasis impairment in liver disease patients.[41]

Yogurt administration at different doses enabled the normalization of APTT. However, PT values only improved after 7 days of GY supplementation, whereas fibrinogen concentration was normalized only when CY was preventively administered for 7 days. These differences could also be attributed to the different chemical composition of the milks used.[19,35]

The changes induced by GY and CY would be due to the capacity of these dairy fermented products to control bacterial translocation and the inflammatory response.[17,40]

Taken together, these results suggest that the 7-day treatment was more effective than the 2-day one. In addition, 7dCY was not as effective as 7dGY in the normalization of the number of neutrophils and in the improvement of PT values.

Despite the small differences found in the behavior of the two yogurts, we demonstrated that yogurt can effectively minimize liver damage induced by D-Gal. The effect could be due to the modulation of the innate immune system that prevents the massive secretion of pro-inflammatory cytokines, particularly TNF-α.[42] This effect, associated with a probiotic-induced maintenance of the intestinal barrier and the epithelial function, reduces bacterial translocation and attenuates hepatic injury.[42]

However, much remains to be learned about the cellular and molecular mechanisms implicated in the effect of probiotic products on liver cells involved in the immune response.

ACKNOWLEDGMENTS

AUTHOR DISCLOSURE STATEMENT

The authors declare that they have no competing financial interests.

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