DOI: https://doi.org/10.2298/SARH221212039B
UDC: 613.287.1:577.112.85

ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Oral intake of bovine lactoferrin alleviates intestinal injury induced by perinatal hypoxia and hypothermia in newborn rats

Nenad Barišić[1,2], Vesna Stojanović[1,2], Slobodan Spasojević[1,2], Milica Milojković[1,2], Tanja Radovanović[1,2]

[1]University of Novi Sad, Faculty of Medicine, Novi Sad, Serbia;
[2]Institute for Child and Youth Healthcare of Vojvodina, Novi Sad, Serbia

SUMMARY

Introduction/Objectives To investigate whether oral administration of lactoferrin attenuates intestinal injury induced by perinatal hypoxia and hypothermia.

Methods Newborn Wistar rat pups were exposed to perinatal asphyxia, followed by global hypothermia. The pups were distributed into two groups: lactoferrin group (LG) – pups that received lactoferrin orally for seven days (20 mg/day), control group (CG) – pups that received normal saline orally during first seven days of life. After seven days macroscopic examination of the bowels and pathohistological analyses of tissue samples have been performed.

Results The incidence of macroscopic injuries was significantly lower in LG group compared to CG. The incidence of pathological findings, as well as the values of injury scores used to assess the intensity and extent of intestinal injury at microscopic level were significantly lower in LG group.

Conclusions Lactoferrin attenuates perinatal hypoxia/hypothermia-induced intestinal injury in newborn rats.

Keywords: lactoferrin; intestinal injury; perinatal hypoxia; hypothermia; neonatology

INTRODUCTION

Close to 80% of all neonatal deaths are due to three leading causes: prematurity and low birth weight, perinatal complications and asphyxia, and infection with variations according to the region and neonatal period [1]. Perinatal asphyxia, especially when combined with uncontrolled accidental hypothermia, may result in decreased perfusion of the gastrointestinal tract, manifesting with vomiting, diarrhea, gastrointestinal hemorrhage and even necrotizing enterocolitis (NEC). The incidence of these events among the neonates born in perinatal asphyxia is around 29% [2].

Intestinal injury caused by hypoxia/reperfusion has been studied in various animal and human models [3–6]. The first histological manifestation of intestinal ischemia is the appearance of small gaps in subepithelial space at the tips of the villi followed by the loss of mature enterocytes. If ischemia lasts long enough, process extends to the base of the villi and causes complete destruction of the villi. Enterocytes located at the top of the villi are most susceptible to injury. Death of enterocytes during hypoxia/reperfusion is a consequence of apoptosis or anoikis (i.e., apoptosis induced by separation of the cells from its natural niches and loss of interplay with the extracellular matrix) [5]. The last mechanism is responsible for the death of enterocytes during hypoxia and apoptosis causes cell death during the reperfusion phase.

Upon termination of hypoxia, in reperfusion phase, the process may go in two directions: migration and proliferation of enterocytes and reestablishment of the continuity of the intestinal epithelium, or amplification of inflammatory response, with neutrophil infiltration and activation of the complement system, what favors apoptosis and leads to further deterioration of the initial epithelial injury and culminates into destruction of all layers of intestinal wall [7].

Hypothermia impairs digestive function and prolongs gastric emptying [8]. At the cellular level, the effects of hypothermia on the gastrointestinal tract are the consequence of redirection of the blood, hemoconcentration, reduction of blood flow and oxygen supply. This initiates a cascade of cellular injury and leads to cell death.

Lactoferrin (LF) is one of the most represented and important bioactive proteins in human and mammal milk. In humans, it is responsible for several actions targeting anti-infective, immunological, and gastrointestinal domains in neonates, infants, and young children. Evidence-based data vouch for the ability of supplemented LF to prevent sepsis and NEC in preterm infants and to reduce the burden of morbidity related to gastrointestinal and respiratory pathogens in young children [9]. LF exists in two forms, as iron (Fe) saturated holo-lactoferrin and as apolactoferrin that do not contain Fe. Two forms of LF differ in their tertiary configurations. LFs of different species differ in secondary and

Received • Примљено: December 12, 2022
Revised • Ревизија: May 7, 2024
Accepted • Прихваћено: May 20, 2024
Online first: May 23, 2024

Correspondence to:
Nenad BARIŠIĆ
University of Novi Sad
Faculty of Medicine
Hajduk Veljkova 3
21000 Novi Sad
Serbia
nenad.barisic@mf.uns.ac.rs

tertiary configurations, mostly in the degree glicolization. For potential medical and research purposes in humans, most commonly are used bovine LF, human milk LF and recombinant human LF. LF has diverse biological actions: non-specific defense against bacteria, fungi, and viruses, immunomodulation, modulation of cell proliferation and activation of gene transcription [10]. Action of LF is mediated by several types of LF receptors (LFRs). These receptors modulate inflammation by affecting cell signaling systems, gene expression and transcription of the DNA and post-transcriptional protein processing [11]. The main LFRs are sulfonated proteoglycans that bind up to 80% of the available LF [12]. Especially important LFR, which is present in the enterocytes, is low-density lipoprotein receptor related protein (LDR) whose activation promotes cell mitosis [13]. It is important to note that enterocytes have specific LFR, which is a protein of 34 kDa and which activates the Ras-dependent extracellular signal regulated mitogen activated protein kinase (Ras-ERK) signaling cascade and stimulates proliferation and synthesis of antiapoptotic proteins. Human LF shares ~70% sequence homology with bovine LF [14]. Bovine LF binds to human LFRs and produces all biological effects as LF from human milk [15].

The aim of this experiment was to examine if oral application of LF during the first week of life will reduce incidence and extent of intestinal injury caused by hypoxia in combination with hypothermia.

METHODS

We used adult female Wistar rats and their pups. Females that were in the proestrus phase were mated with sexually mature males (coupled in the same cage for 24 hours and then separated). On the 22nd day of gestation, pregnant females were induced in general anesthesia with ketamine (90 mg/kg) and laparotomy was performed. Blood vessels of the uterus were ligated and the wombs were immediately submersed in a bath tub with 0.9% NaCl heated at 38°C and kept in those conditions for 15 minutes. Pups were delivered by Cesarean section. Immediately after the birth, the pups were reanimated (sweep, aspiration, tactile stimulation). Upon initial reanimation, rapid cooling was conducted in a Styrofoam padded box with adjustable cooling cartridge. Core body temperature of the pups was continuously controlled and measured with rectal probes (RET-3, rectal probe for mice; Physitemp Instruments LLC., Clifton, NJ, USA) and was kept at 32°C. The total duration of hypothermia was one hour, following which the pups were gradually warmed (0.5–1°C/h) in the thermostat, to a normal rectal temperature of 38°C.

The pups were randomly designated in two groups:

1. LF group (LG): 10 pups who survived perinatal hypoxia, exposed to hypothermia, and fed via orogastric tube with 0.2 ml of 10%-suspension (20 mg) of bovine LF (apolactoferrin form of LF; Jarrow Formulas®, Los Angeles, CA, USA) in 0.9% NaCl, once daily. The first dose of LF was given one hour after the birth. Subsequent doses of LF were administered at regular intervals of 24 hours.

2. Control group (CG): 10 pups who survived perinatal hypoxia, exposed to hypothermia and fed via orogastric tube with 0.2 ml of 0.9% NaCl, once daily. The first dose of normal saline was given one hour after the birth and subsequent doses were administered at regular intervals every 24 hours.

After they were adequately labeled, the pups were handed to surrogate mothers. The pups from both groups were breastfed by surrogate mothers, *ad libitum*. The pups were sacrificed on the seventh day of life when laparotomy was made.

Intestines were examined for the presence of any macroscopic changes (discoloration, bleeding, distension or stenosis).

Three tissue samples from the distal part of the ileum (the last 2 cm proximal to the ileocecal valve) were taken from each animal (total of 30 samples in each group). Histological sections were stained with hematoxylin-eosin.

Post-hoc sample size and study power calculation showed that minimum sample size for ideal study power of 80% is 36 histological samples per study group. In our case, 30 specimens were obtained per study group, what sets the study power at the level of 73%. For analysis of macroscopic changes, inclusion of 10 animals per study group, sets study power at 81.7%.

Mucosal injury was assessed in a blinded manner by pathologists. Mucosal injury was quantified using the score previously described by Chiu et al. [3] (0 – indicates absence of mucosal injury (normal finding), values 1–5 indicate different degrees of mucosal injury, 5 being the most devastating).

To access the areal (superficial) distribution of the intestinal injury and compare it between animals, we derived an additional variable – cumulative injury score, defined as the number of positive findings among three histological samples taken from the same animal.

Data are presented as absolute numbers, frequencies, percentages or means ± 2SD. Fisher's exact test, Z-test and Wilcoxon signed-rank test were used for statistical analysis, as appropriate.

The experiment was approved by the Ethical Committee for Animal Care and Use of the University of Novi Sad (approval No 01-237/6).

RESULTS

Macroscopic changes

Discoloration of the intestine was observed in 20% of animals from LG and in 80% of animals from CG. This difference was statistically highly significant (Fisher's exact test; $p = 0.0007$). Other macroscopic changes of the intestines (bleeding, intestinal distension, and stenosis) were not observed in any animal.

Mucosal injury score

Normal histopathological findings were described in 26.67% (8/30) of histological samples in the LG group,

while in the CG were observed in 3.33% (1/30) samples. This difference was statistically significant (Z-test; Z-score is 2.5309; p = 0.0114).

The mean value of mucosal injury score in the LG group was 1.033 ± 1.300. The average value of mucosal injury score in the CG was 2.533 ± 1.363.

The values of mucosal injury scores were significantly lower in group LG (Wilcoxon signed-rank test; p < 0.001), indicating less severe injury in animals from the LG. Distributions of values of mucosal injury scores, in both groups are shown in Figure 1.

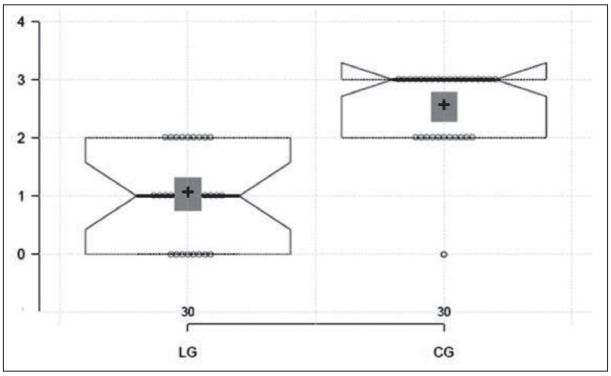

Figure 1. Minimal and maximal values, medians and inter-quartile ranges of mucosal injury scores in lactoferrin group (LG) and control group (CG); center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; crosses represent sample means; bars indicate 95% confidence intervals of the means; data points are plotted as open circles

Cumulative injury score

Normal histopathological findings in all three tissue samples taken from the same animal, were not observed in neither group (there were no animals with cumulative injury score 0).

The mean value of cumulative injury score in the LG group was 1.500 ± 1.054. The average value of cumulative injury score in the CG was 2.900 ± 0.632.

The values of cumulative injury scores were significantly lower in group LG (Wilcoxon signed-rank test; p = 0.005). Distributions of values of cumulative injury scores in both groups are shown in Figure 2.

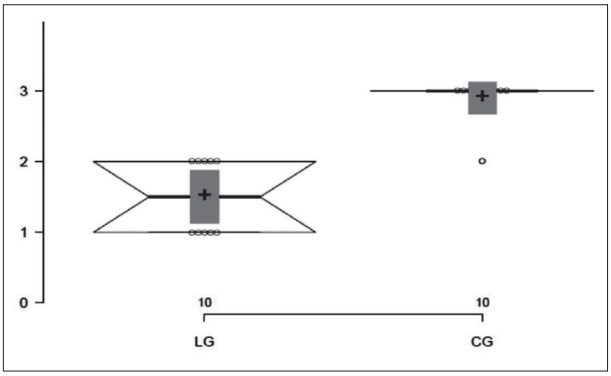

Figure 2. Minimal and maximal values, medians and inter-quartile ranges of cumulative injury scores in lactoferrin group (LG) and control group (CG); center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots; crosses represent sample means; bars indicate 95% confidence intervals of the means; data points are plotted as open circles

DISCUSSION

The objective of this study was to investigate whether oral administration of LF attenuate intestinal injury induced by perinatal hypoxia and hypothermia. In this experiment, application of LF was initiated one hour after hypoxia and after process of reperfusion has already begun. LF was administered via orogastric tube, once daily, during a one-week period. It was observed that macroscopic changes of the intestines were more often present in animals that were not fed with LF. The indicators, used to assess the intensity and extent of intestinal injury, showed that intestines were significantly less damaged in animals treated with LF. The animals received 20 mg of LF daily (cca 2000 mg/kg/day). As estimated by the Clark's formula, this dose of LF is equivalent to human dose of 280 mg/kg/day.

In similarly designed experiments conducted by other authors, similar or analogous doses of LF have been applied to experimental animals. For example, Sarkar et al. [16] showed that supplementation with bovine LF in early-life (first seven days, 100 mg/day) with or without added probiotic, reduced the mortality in the suckling piglets by promoting the systemic immunity and enhancing the intestinal integrity.

Cerven et al. [17] studied possible adverse effects of oral administration of LF (1000 mg/kg/daily), during a four-week period. Their results showed no toxic or adverse effects on nutritional, hematological or biochemical status of experimental animals.

In a recently published review article by Ashraf et al. [18] the authors stated that the European Food Safety Authority approved bovine LF as a safe novel food additive for infant formula at the level of 1000 mg/l, and recommend that the highest intake of bovine LF for infants should be 1.1 g/day. Referring to different literal sources, the same group of authors suggested that LF produce desired positive effects when it is added to infant formula at the level of 850 mg/l or 600 mg/kg/day (what is twofold higher dose than estimated human dose used in our study) [18].

In serval in vitro experiments, it was found that, in dose-dependent manner, LF stimulates proliferation and differentiation of intestinal epithelium by interfering with the processes of DNA transcription, promotes production of transformational growth factor-β1 and secretion of interleukin-18. The 2017 Cochrane review included six randomized controlled trials, and meta-analysis suggested that substantial reductions in late-onset infection and NEC was associated with LF supplementation in very preterm infants [19]. Other experiments showed that LF reduces production of free radicals and lipid peroxidation products and thereby minimizes the effects of inflammation on intestinal mucosa [20]. Experiments on adult rats, conducted

by Zhang et al. [20], demonstrated that orally administered LF had protective effect on intestinal ischemia-reperfusion injury if LF was applied prior induction of hypoxia (as premedication, during preparation for elective surgery of the intestine). This protective effect was dose-dependent and was attributed to inhibition of pro-inflammatory cytokines, inhibition of apoptosis and increased local production of tissue antioxidants.

The exact mechanism through which LF exerts protective effect on enterocytes has not been fully elucidated. In the intestine LF is partially degraded to polypeptide fragments that bind to receptors on the enterocytes, lymphocytes and dendritic cells, and trigger intracellular signaling cascades, stimulate proliferation and suppress proapoptotic processes, thus maintaining the integrity of the intestinal barrier. LF can be transported into the cytoplasm or the nucleus of cells, where it can bind to DNA and alter expression of genes, including those that control apoptosis and cell death [21]. LF modulate expression of more than twenty genes associated with immune response, suppresses the production of tumor necrosis factor alpha (TNF-α) and Interleukins IL-1β, IL-6 and IL-8 in human mononuclear cells and improves production of IL-10 and IL-4 [22]. LF stimulates increased intestinal stem cell marker Lgr5 + expression and increased nuclear β-catenin - indicating upregulated Wnt pathway, as well as increased Ki67 positivity, suggesting enhanced proliferation [23]. By binding to micro-organisms or their toxic products, LF directly regulates composition of intestinal microbiome [12].

In our experiment, we used iron-free form of bovine LF (apolactoferrin). The question arises whether exogenous LF may interact with the LFRs on human enterocytes and cause any biological effect? Jiang and al. demonstrated that both forms of human LFs bind to receptors on human enterocytes and subsequently may be transported within enterocytes, allowing involvement in intracellular processes and signaling [24]. However, these authors showed that effects of apo- and holo-lactoferrin were different and that only apolactoferrin stimulated proliferation of enterocytes. This difference is explained by the different tertiary configuration of these two types of LF (holo-lactoferrin is less reactive) and the higher affinity of cellular receptors for apolactoferrin.

The results of our experiment indicate that repeated, oral administration of LF attenuates intestinal injury induced by hypoxia. On the other hand, data reported by the ELFIN trial investigators group, showed that enteral LF supplementation (150 mg/kg per day until 34 weeks' postmenstrual age) does not reduce the risk of late-onset infection, other morbidity, or mortality in very preterm infants [25].

CONCLUSION

Our experiment showed that repeated, oral administration of bovine LF attenuates intestinal injury induced by perinatal hypoxia/hypothermia in newborn rats. Similar data, from other animal and human-based studies have been reported in literature. Most experiments and studies demonstrated positive effects of bovine LF with no toxic or adverse effects, so we may assume that bovine LF is a potent agent that may be safely used for prevention of post-hypoxic intestinal injury in neonates. The precisely assessed dosage, methods of application and other details of LF application are still controversial and additional studies on this topic are needed.

Conflict of interest: None declared.

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Орална примена говеђег лактоферина делује протективно на оштећење црева индуковано перипарталном хипоксијом и хипотермијом код новорођених пацова

Ненад Баришић[1,2], Весна Стојановић[1,2], Слободан Спасојевић[1,2], Милица Милојковић[1,2], Тања Радовановић[1,2]

[1]Универзитет у Новом Саду, Медицински факултет, Нови Сад, Србија;
[2]Институт за здравствену заштиту деце и омладине Војводине, Нови Сад, Србија

САЖЕТАК

Увод/Циљ Циљ рада је био истражити да ли орална примена лактоферина ублажава интестинално оштећење изазвано перипарталном хипоксијом и хипотермијом.

Методе Младунци пацова соја вистар су одмах по рођењу изложени хипоксији/асфиксији и глобалној хипотермији. Распоређени су у две групе: лактоферин група – младунци који су примали лактоферин орално током седам дана (20 *mg*/дан) и контролна група – младунци који су примали физиолошки раствор орално током првих седам дана живота. После седам дана урађени су макроскопски преглед црева и патохистолошка анализа узорака ткива.

Резултати Инциденција макроскопских оштећења била је значајно нижа у групи младунаца који су примали лактоферин у односу на контролну групу. Инциденција патолошких налаза, као и вредности хистолошких скорова који су коришћени за процену интензитета и обима оштећења црева биле су статистички значајно ниже у групи која је третирана лактоферином.

Закључак Лактоферин ублажава интестиналну повреду изазвану перипарталном хипоксијом/хипотермијом код новорођених пацова.

Кључне речи: лактоферин; оштећење црева; перипартална хипоксија; хипотермија; неонатологија