5 - ORIGINAL ARTICLE

Prevention of bacterial translocation using β-(1-3)-D-glucan in small bowel ischemia and reperfusion in rats[1]

Prevenção de translocação bacteriana com β-(1-3)-D-glucana em isquemia e reperfusão intestinal em ratos

Irami Araújo-Filho[2], Amália Cínthia Meneses Rêgo[3], Laíza Araújo Mohana Pinheiro[3], Italo Medeiros Azevedo[3], Vítor Brasil Medeiros[3], José Brandão-Neto[4], Aldo Cunha Medeiros[5].

1. Department of Surgery, School of Medicine, Federal University of Rio Grande Norte (UFRN)- Natal, Brazil. Postgraduate Program in Health Sciences.
2. Fellow PhD degree and Assistant Professor, Department of Surgery-UFRN, Brazil.
3. Graduate student, Scientific Initiation Program, UFRN, Brazil.
4. PhD, Full Professor, Department of Clinical Medicine, UFRN, Brazil.
5. PhD, Full Professor, Department of Surgery, UFRN, Brazil.

ABSTRACT

Purpose: To investigate the role of β-(1-3)-D-glucan on ^{99m}Tc labelled *Escherichia coli* translocation and cytokines secretion in rats submitted to small bowel ischemia/reperfusion injury. **Methods:** Five groups (n=10 each) of Wistar rats were subjected to control(C), sham(S), group IR subjected to 45 min of bowel ischemia/60 min of reperfusion(I/R), and group I/R+glucan subjected to 45 min of bowel ischemia/60 min of reperfusion(I/R) and injected with 2mg/Kg intramuscular. Translocation of labelled bacteria to mesenteric lymph nodes, liver, spleen, lung and serum was determined using radioactivity/count and colony forming units/g(CFU/g). Serum TNFα, IL-1β, IL-6, IL-10 were measured by ELISA. **Results:** CFU/g and radioactivity/count were higher in I/R than in I/R+glucan rats. In C, S and S+glucan groups, bacteria and radioactivity/count were rarely detected. The I/R+glucan rats had enhancement of IL-10 and suppressed production of serum TNFα, IL-1β and, IL-6, compared to I/R untreated animals. **Conclusion:** The β-(1-3)-D-glucan modulated the production of pro-inflammatory and anti-inflammatory cytokines during bowel ischemia/reperfusion, and attenuated translocation of labelled bacteria.

Key words: Bacterial translocation. Glucan. Intestine. Ischemia. Reperfusion. Prophylaxis.

RESUMO

Objetivo: Investigar o papel da β-(1-3)-D-glucana na translocação de *Escherichia coli* marcada com ^{99m}Tc e na secreção de citocinas em ratos submetidos a isquemia e reperfusão intestinal. **Métodos:** Cinco grupos (n=10 cada) de ratos Wistar foram denominados controle (C), *sham* (S), grupo IR submetido a 45 minutos de isquemia do intestino delgado e 60 minutos de reperfusão(I/R), grupo I/R+glucana com 45 minutos de isquemia e 60 minutos de reperfusão(I/R) e tratados com glucana 2mg/Kg intramuscular. Translocação de *Escherichia coli* marcada com ^{99m}Tc, para Linfonodos mesentéricos, fígado, baço, pulmão e soro foi avaliada usando contagem de radioatividade e de unidades formadoras de colônias/g (UFC/g) Dosagem sérica de TNFα, IL-1β, IL-6, IL-10 foi realizada pelo método ELISA. **Resultados:** CFU/g e contagem de radioatividade foi significantemente maior nos ratos do grupo I/R do que no grupo I/R+glucana. Nos grupos C, S e S+glucana bactérias e contagem radioativa foram raramente detectadas. Os ratos do grupo I/R+glucana tiveram aumento de IL-10 sérica e significante redução da expressão de TNFα, IL-1β e IL-6, quando comparados com os animais não tratados do grupo I/R. **Conclusão:** A β-(1-3)-D-glucana modulou a produção de citocinas pró-inflamatórias e anti-inflamatórias durante a isquemia/reperfusão intestinal e contribuiu para reduzir a translocação de bactérias marcadas.

Descritores: Translocação bacteriana**.** Glucana. Intestino. Isquemia. Reperfusão. Profilaxia.

Introduction

Maintenance of bacteria and their products in the intestine is done by both mucin and a layer of epithelial cells, the intestinal barrier that is essential for health and survival. These gut cells are in constant division, metabolizing rapidly and forming an impermeable barrier to harmful intestinal contents. Because they are metabolically active, they are also susceptible to oxygen deprivation with subsequent ischemic damage to enterocytes and their supporting structures[1]. This insult results in epithelial cell damage, decreased absorptive function, and the loss of basement membrane integrity leading to translocation of bacteria[2]. Bacterial translocation (BT) was originally defined and described by Berg and Garlington[3] as the passage of viable bacteria

through the intestinal mucosa into the mesenteric lymph nodes (MLN) and to other tissues and organs. It has been suggested that gut ischemia/reperfusion induces disruption of the intestinal mucosal barrier, allowing translocation of bacteria and endotoxin from within the bowel into the blood, an event that may initiate a systemic inflammatory response and the secretion and activation of inflammatory mediators, including cytokines[4]. Although it has been difficult to show BT in clinical cases, patients suffering from hemorrhagic shock or post-surgical syndrome are quite susceptible to endotoxemia and multiple organ failure[5]. β-(1-3)-glucan purified from fungi have been shown to have broad anti-infective activities[6]. It have been shown to bind to receptors on leukocytes and stimulate some immune responses, such as cytokine release[7], and generation of nitric oxide[8]. Soluble β-glucan has also been shown to enhance the clearance of bacteria from the blood, and reduce mortality in rat sepsis models[9]. The present experiment was designed to analyze the effect of soluble β-(1-3)-glucan in rats submitted to bowel ischemia, with and without reperfusion, on translocation of ^{99m}Tc labelled bacteria from the intestinal mucosa to MLN, liver, spleen, lung and serum. Additionally, the levels of serum cytokines were studied and correlated with BT and β-(1-3)-glucan administration.

Methods

Radiolabelling of bacteria

Escherichia coli were labelled with ^{99m}Tc, as follows. Briefly, a sample (0.1 mL) of *E. coli* ATCC-10536 culture, grown overnight in soybean casein medium, was incubated in 10mL of the same medium, under aeration, for 4 hour at 37ºC. After that, different amounts of stannous chloride were added to 2 mL of the medium to reach final concentrations of 40, 130, 290, 400 and 580 mM, respectively. The samples were then incubated at 37ºC for 10, 20, 40 and 60 min. After incubation, 37.0 MBq of ^{99m}Tc were added to each preparation and kept at 37ºC for 10 min. The tubes were then centrifuged at 3000x g for 25 min, washed and resuspended with normal saline. After three washes with saline, the ^{99m}Tc *E. coli* were incubated at 37ºC for 36h. Aliquots (100 mL) of supernatant and resuspended precipitate in saline were withdrawn for determination of radioactivity. This procedure was repeated three times. In order to evaluate the bacterial viability, aliquots were taken from the last suspension, spread into a solid culture medium and incubated at 37ºC for 24 h. The effect of the procedure on the bacterial viability was assessed by comparing the colony-forming units per mL (CFU/mL) of labelled and unlabelled *E. coli*.

Animals

Male Wistar rats weighing 285±14g were maintained under conditions with controlled temperature, on a 12h light-dark cycle and fed *ad libitum* with commercially available rat chow and water. They were randomly divided into four groups (n=10 each), and named, respectively: C group, for non-operated rats, which were the controls, S group, for sham-operated, I/R for rats submitted to 45 minutes of intestinal ischemia and 60 minutes reperfusion, and I/R+glucan for those ischemia/reperfusion group treated with glucan (2mg/Kg) intramuscular. All the animals were gavaged with ^{99m}Tc *E. coli*, two hours before the operative procedures. After fasting overnight, the animals were anesthetized with intramuscular ketamine (50mg/kg) and xilazine (7mg/kg). In the I/R and I/R+glucan groups, the superior mesenteric artery (SMA) was occluded with a microvascular clamp. The laparotomy incision was then closed, to be opened 45 minutes later for removal of the clamp. Reperfusion was confirmed by the return of pulsation to the mesenteric arcade. The incision was again closed and the animals were killed with overdose of anesthetic 60 minutes later.

Glucan administration

For each experiment, soluble β-(1-3)-D-glucan (Imunoglucan®) was administered intramuscularly to 10 rats of I/R+glucana group, at a dose of 2mg/Kg of body weight.

Measurement of radioactivity, bacterial counting and cytokines

At the end of the procedures, under aseptic conditions, a midline laparotomy was performed and blood was collected from the portal vein for culture, counting and cytokines assays. One mL of serum was aliquoted for radioactivity counting. One gram of MLN complex, spleen, liver and lung were removed for counting and culture, if 1g of tissue was available; otherwise, the entire organ was weighed. Tissues were homogenized and solubilized. Aliquots of 0.2mL were processed and were then counted in a PerkinElmer - Wizard TM Gama Counter. Other portions (0,2mL) were cultured on selective MacConkey's agar and blood agar for detection of gram-negative and gram-positive bacteria, respectively. The plates were examined after 24 and 48 hours of incubation at 37ºC. Portal blood samples were used for measurement of tumor necrosis factor-alfa (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10) assayed using ELISA. Sensitivity of detection was 30 pg/ml for all cytokines. Procedures involving animals and their care were conducted in conformity with the *Guide for the Care and Use of Laboratory Animals,* US National Research Council, 1996. The data analysis were performed using the BioEstat 2.0 program. The results were tabulated and compared by ANOVA using post hoc analysis with Newman-Keuls test. $P<=0.05$ was considered statistically significant.

Results

All animals survived the experimental protocol. The bacterial viability test showed that the number of colony forming units (CFU) of the *E. coli* under radiolabelling

procedure was the same as that grown in absence of ^{99m}Tc (data not shown). When the C and S groups were compared with I/R, and I/R+glucan groups, a significant variation on the labelled bacteria migration to different organs was found. As shown in Table 1, the concentration of radio labelled *E. coli* was the greatest in the MLN, lung, and liver in ischemia/reperfusion (IR) rats. So, the MLN, spleen, liver, lung and serum from I/R rats had significantly higher levels of radioactivity than did the organs from the I/R+glucan ($p<0.01$). The level of positive cultures with CFU was significantly higher in I/R rats than in I/R+glucan group (Table 2). The C group was the only one where the organs and serum were free of any bacterial colony. In the S group the bacteria were rarely detected. As observed with the mean count of radioactivity, bacteria were less detected in the spleen than in the other organs studied (Tables 1,2). The most common bacteria cultured from the organs and serum were *E. coli* and *Enterococcus*. TNF-α, IL-1β, IL-6, and IL-10 were not detected in the serum of the C group, while there concentrations in the serum of S operated rats were 41,7±9,4pg/ml, 34±11pg/mL, 144±17pg/mL and, 94±21pg/mL respectively. Significant increase in serum level of TNF-α (753.7±91pg/ml), IL-1β (588.7±100pg/ml), IL-6 (422.1±56pg/ml) and, IL-10 (311±52pg/mL) was observed in I/R group, when compared with C and S rats ($p<0,01$). The I/R+glucan rats had the serum levels of TNF-α (98±23pg/mL), IL-1β (122±19pg/ml) and, IL-6 (110±31pg/mL) significantly lower than that observed in the I/R rats ($p<0,01$). Nevertheless, an inverse result was observed in the IL-10. There was a significant increase ($p<0,01$) in the level of IL-10 in the I/R+glucan group when compared to the I/R (Table 3).

TABLE 1 - Level of radioactivity (mean counts per minute per gram) from MLN, Spleen, Liver, Lung and Serum after ^{99m}Tc *E. coli* translocation studies.

Groups	n	MLN	Spleen	Liver	Lung	Serum
C	10	0	0	4 ± 1.2	2 ± 1.1	2 ± 0.9
S	10	4 ± 0.9	0	0	3 ± 1.2	0
I/R	10	742 ± 61 *	334 ± 42*	682 ± 83 *	795 ± 132*	420 ± 27*
I/R + Glucan	10	212 ± 29	174 ± 14	322 ± 45	275 ± 95	186 ± 23

C, Control; S, Sham; I/R ischemia/reperfusion; I/R+glucam, ischemia/reperfusion+glucan intramuscular.
* p < 0,01 compared to S, C, I/R+glucan

TABLE 2 - Magnitude (CFU per gram of tissue) of bacterial translocation to several organs and serum, comparing groups with and without β-(1-3)-D-glucan intramuscular.

Groups	n	MLN	Spleen	Liver	Lung	Serum
C	10	0	0	0	0	0
S	10	2 ± 0.2	0	0	4 ± 1.3	0
I/R	10	253 ± 32*	112 ± 12*	178 ± 18*	285 ± 21*	166.5 ± 32*
I/R + Glucan	10	74 ± 18	47 ± 9	82 ± 22	77 ± 12	69 ± 14

C, Control; S, Sham; I/R ischemia/reperfusion; I/R+glucam, ischemia/reperfusion+glucan intramuscular.
*p < 0.01 compared to C, S, I/R+glucan

TABLE 3 - Portal serum levels of cytokines comparing groups with and without β-(1-3)-D-glucan intramuscular.

Groups	n	TNF-α(pg/mL)	IL-1β(pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)
C	10	0	0	0	0
S	10	41,7 ± 9,4*	34 ± 11*	144 ± 17*	94 ± 21*
I/R	10	753.7 ± 91**	588.7 ± 100**	422.1 ± 56**	311 ± 52**
I/R + Glucan	10	98 ± 23	122 ± 19	110 ± 31	430 ± 42

C, Control; S, Sham; I/R ischemia/reperfusion; I/R+glucam, ischemia/reperfusion+glucan intramuscular.
*p<0.01 compared to I/R, and I/R+glucan
** p<0.01 compared to I/R+glucan

Discussion

The gut has been suggested to be a port of entry for bacteria after intestinal mucosal injury and endotoxin challenge[10]. The translocation process involves the initial attachment of the bacteria to the gut wall, which by itself can elicit production of cytokines and initiate the subsequent inflammatory response. Once intact microbes penetrate the mucosa, they may be transported to distant organs of even the systemic circulation[11]. As shown in the present study, bowel ischemia and reperfusion promoted bacteria translocation. In addition, when compared to the control and sham, this phenomenon was significantly higher for MLN, spleen, liver, lungs and, serum in all other groups. Redan et al[12] speculate that the route of BT is through lymphatics into the right side of the heart and then to the lung. The pulmonary vascular bed would then represents the first capillary system in which the translocated bacteria encounter circulating phagocyte cells. In fact, a great amount of colony-forming units of bacteria were found in the lung. The hypoxia, followed by change in intestinal barrier function, generates a vicious cycle of increased permeability, leading to toxic mediators release, and resulting in a further increase in gut permeability, facilitating the BT[13]. However, no significant difference in radioactivity and CFU were found when they were compared the S group, where the intestines were gently manipulated, and in the C group. In this study, increased serum levels of TNF-α, IL-1β, IL-6 and IL-10 reflected the ischemia/reperfusion injury, as demonstrated by other in vivo trials[14,15]. It has been suggested that IL-6 produced by intraepithelial lymphocytes is responsible for the loss of intestinal barrier function following hemorrhage, and the extent of loss can be correlated with plasma levels of this cytokine[16]. In the rats treated with soluble β-(1-3)-D-glucan it was observed a significantly different cytokine response, which was characterized by decreased production of TNF-α, IL-1β, and IL-6, suggesting that immunomodulation with soluble glucan might act to depress the inflammatory cytokine response. The decrease in secretion of these pro-inflammatory cytokines coincided with the increase in IL-10 expression and could, at least in part, be explained by the action of this cytokine known to have anti-inflammatory activity. In fact, IL-10 has been shown to inhibit lipopolissacharide-induced monocyte tissue factor expression in whole blood [17] and to decrease TNF-α production in human monocytes[18]. In a model of murine *E. coli* sepsis, TNF-α and IL-1 levels in soluble glucan-treated mice were significantly lower than in untreated control animals[19]. The levels of radioactivity and colony forming units of bacteria on MLN, spleen, liver, lungs and, serum were lower I/R+glucan rats than the I/R ones, meaning that the use of soluble glucan resulted in an overall decrease in bacterial translocation.

Conclusion

Based on the present data, we conclude that stimulation of the reticuloendothelial system by soluble β-(1-3)-D-glucan modulated the production of pro-inflammatory and anti-inflammatory cytokines during intestinal ischemia/reperfusion, and attenuated the translocation of ^{99m}Tc labelled bacteria.

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Correspondence:
Aldo Cunha Medeiros
Av. Miguel Alcides Araújo, 1889
59078-270 - Natal - RN - Brazil
e-mail: aldo@ufrnet.br

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