MARIANA YUKARI HAYASAKI PORSANI

AVALIAÇÃO DA RESPOSTA IMUNOLOGICA E TROFISMO INTESTINAL DE CAMUNDONGOS TRATADOS COM β-GLUCANO E GLUTAMINA APÓS DESAFIO COM ARA-C

LAVRAS - MG

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Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do Programa de Pós-Graduação em Ciências Veterinárias, área de concentração em Ciências Veterinárias, para a obtenção do título de Mestre.

Orientador

Dr. Raimundo Vicente de Sousa

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“Grandes realizações não são
feitas por impulsos, mas pela
soma de pequenas realizações”

Vicent Van Gogh

RESUMO

Mudanças sistêmicas podem predispor o aumento da permeabilidade intestinal, o que torna possível a migração das bactérias e seus produtos a partir do lúmen do intestino para os sítios estéreis, levando a um processo inflamatório local. Em estudos recentes, tem sido demonstrado que a Glutamina e β-glucano desempenham um papel importante na modulação do sistema imunitário e promovem benefícios na saúde intestino. O objetivo deste estudo foi investigado o efeito da respostas inflamatórias e da saúde intestinal de camundongos após tratamento com *Saccharomyces cerevisiae* derivado solúvel β 1,3 / 1,6, β-glucana (80 mg / kg) associados ou não com a glutamina (150 mg / kg). Após serem desafiados com citarabina (Ara-C) (15 mg / kg). As variáveis mensuradas foram: total de leucócitos, IL-1β, IL-10 e INF-γ, morfometria intestinal e imuno-histoquímica de macrófagos, linfócitos T e B no íleo. Não houve melhora na morfometria intestinal no grupo β-glucano, a mensuração das citocinas demonstrou valores mais baixos de IL-10 e IL-1 β, e maiores valores de IFN-γ. Em camundongos tratados com glutamina foram vistos valores intermediário de todas as citocinas mensuradas. Neste grupo a morfometria intestinal apresentou os melhores resultados. O tratamento com β-glucano em associação a glutamina apresentou os valores mais elevados de IL-1β e IL-10 e menores valores do total de leucócitos e INF-γ. A morfometria intestinal deste grupo demonstrou benefícios à saúde intestinal. Com base nestes resultados, a resposta após pré-tratamento com a associação do β-glucano e Glutamina reduziram a inflamação do intestino e demonstraram uma melhorando o trofismos intestinal frente a agressão causada pelo Ara-C.

Palavras-chave: Quimioterápico. Levedura. Saúde Intestinal. Imunomoduladores.

ABSTRACT

Systemic changes can predispose the increase of intestinal permeability, making possible the migration of bacteria and their products from the gut lumen into the sterile sites, leading to a local inflammatory process. Recently, Glutamine and β-glucan, have been demonstrated to play an important role in modulation of immune system and in promotion of intestinal health benefits. The aim of this study was to investigate the effect on inflammatory responses and intestinal health after the mice being pretreated with oral administration of soluble *Saccharomyces cerevisiae,* derived β 1,3/1,6, β-glucan (80 mg / kg) with or without glutamine (150mg/kg) and then challenged with administration of cytarabine (Ara-C) (15 mg / kg). The measured variables were: total of leukocytes, IL- 1β, IL-10 and INF-γ, intestinal morphology and immunohistochemical analysis of macrophage and lymphocytes T and B. Improvement in villi and crypts were not observed in β- glucan group, however, lower values of IL-10 and IL-1 β secretion were observed, whereas they prompt the highest release of IFN-γ. In mice treated with Glutamine was showed the intermediate value of all cytokines measured. The intestinal morphometric showed the best results in this group. The treatment of β-glucan in combination with glutamine presented the highest values of IL-1β and IL-10 and lowest values of the leukocytes total and INF-γ. The intestinal morphometric of this group showed the benefits on intestinal health. Based on these results, the answers of pretreatment with the combination of β-Glucan and Glutamine were reduction of intestinal inflammation and improvement of immune response after challenged with Ara-C.

Keywords: Chemotherapy. Yeast. Intestinal Health. Immunomodulators.

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PRIMEIRA PARTE

1 INTRODUÇÃO

O trato gastrointestinal é atualmente reconhecido não apenas pela sua atuação na digestão, mas por suas funções metabólicas, endócrinas e como um dos principais componentes da imunidade das mucosas (HEIJEDEN, 2007; PEREIRA et al., 2008). O intestino possui uma barreira fisiológica composta por muco, barreira celular, células epiteliais colunares simples, células M, linfócitos e tecido linfoide associado ao intestino, envolvidos em sua defesa (BAUMGART; DIGNASS, 2002).

Algumas alterações sistêmicas como traumas, doenças imunodepressoras, choque, obstrução intestinal, endotoxemia, e pancreatite aguda, podem levar à supressão do sistema imunológico e predispor o intestino ao aumento da permeabilidade (RUYON; SQUIERS; BORZIO, 1994; SANTOS et al., 2010; SARAC et al., 2015). Com o aumento da permeabilidade intestinal, tornando-se possível a migração de bactérias e seus produtos, como as endotoxinas do lúmen intestinal para os sítios estéreis como ductos linfáticos, órgãos abdominais, linfonodos mesentéricos e circulação sistêmica; ocasionando a reposta inflamatória de mucosa no epitélio intestinal (WERSHIL; FURUTA, 2008).

Diversos estudos com substâncias alternativas vem sendo feitos, procurando minimizar complicações no trofismo intestinal e na imunidade de mucosas (HEIJEDEN, 2007; SARAC et al., 2015). Entre eles destaca-se o uso de produtos naturais com origem em plantas, fungos cogumelos e microorganismos vivos (BAUER et al., 2002; VETVICKA, 2011).

A partir da parede de leveduras como o *Saccharomyces cerevisiae*, pode ser extraído o β-glucano, um polímero de glicose com um potente efeito

imunomodulador (VOLMAN; RAMAKERS; PLAT, 2008; XU et al., 2012). Outro produto que vem ganhando destaque é a Glutamina, um aminoácido com grande atuação na imunidade e saúde intestinal (DEMIRKAN; SAVASX; MELLI, 2010; LI et al., 2006; TEIXEIRA, 2009).

A fim de buscar novas alternativas para a diminuição de danos à morfologia intestinal, com aumento da permeabilidade intestinal que possa comprometer a imunidade da mucosa, objetivou-se, neste estudo, avaliar a ação trófica e imunomoduladora do β-glucano de ligação β (1-3), β (1-6) e da Glutamina, isolados e em associação, na mucosa intestinal de camundongos após a indução de lesão por administração do quimioterápico citarabina (Ara-C).

2 REVISÃO DE LITERATURA

2.1 β-glucanos

Os β-glucanos são polímeros de glicose presentes na composição da parede celular de fungos, plantas, bactérias e cereais como aveia e cevada (TSONI; BROWN, 2008; XU et al., 2012). Os estudos de seus efeitos biológicos foram iniciados há mais de 50 anos (VETVICKA; VETVICKOVA, 2014), por meio de linhas euro-americana e japonesa. A vertente euro-americana avaliou as propriedades imunomoduladoras dos β-glucanos oriundos dos *Saccharomy cescerevisiae,* no qual foi possível observar a sua atuação como estimulador da atividade de macrófagos e na liberação de citocinas pelos neutrófilos (SALEH, 2014). Por outro lado, a linha japonesa, direcionou seus nos efeitos biológicos dos β-glucanos provenientes dos cogumelos (VETVICKA, 2011).

Os β-glucanos provenientes da parede de leveduras e fungos possuem resíduos de glicose unidos por ligações β(1→3) - D-glucopiranosil e uma menor parte das cadeias laterais, e ligações do tipo β(1→6) de diferentes tamanhos (SATO et al., 2006; VOLMAN; RAMAKERS; PLAT, 2008; XU et al., 2012). Os β-glucanos derivados da parede celular de uma variedade de fungos e leveduras como o *Saccharomys cescerevisiae*, são capazes de estimular a resposta imune humoral, atuando sob a atividade de leucócitos e liberação de citocinas no sistema imune do hospedeiro (BACON et al., 1969; SATO et al., 2006; VOLMAN; RAMAKERS; PLAT, 2008). Os β-glucanos provenientes das leveduras podem ser reconhecidos pelo sistema imune dos mesmos como padrões moleculares associados a patógenos (PAMPs) (SATO et al., 2006). Nesse sentido, atuam como imunomoduladores, induzindo a resposta imune inata e adaptativa (CHEN; SEVIOUR, 2007; KUSHNER et al., 2014; VETVICKA; VETVICKOVA, 2014).

A ação dos β-glucanos no sistema imune do animal está correlacionada com a sua capacidade de estimular os órgãos linfóides primários e secundários (SALEH, 2014), potencializando a atividade dos macrófagos, monócitos e células *Natural Killers* (NK), promovendo a estimulação indireta de linfócitos T e B através de citocinas (AKRAMIENÉ et al., 2007; OZKAN et al., 2010). Consequentemente, a função antimicrobiana de células mononucleares, e sua habilidade em ativar leucócitos (SANDVIK et al., 2007), ainda, aumentam a quantidade de linfócitos intra-epiteliais, interferon gama (INF-γ) e interleucina 2 (IL-2) modulando a produção de citocinas pró-inflamatórias e anti-inflamatórias (ARAÚJO-FILHO et al., 2006; OZKAN et al., 2010; TSUKADA et al., 2003; VETVICKA; VETVICKOVA, 2014).

Segundo Sonck et al. (2010), além do posicionamento das ramificações, a massa molecular e a conformação possivelmente implicam em diferente capacidade imunológica dos β-glucanos. De fato, os de grande peso molecular conseguem ativar diretamente os leucócitos, estimulando as suas funções citotóxicas e fagocíticas. Em contrapartida, os de peso molecular intermediário possuem poucos efeitos na estimulação da resposta imune celular e os de baixo peso molecular não possuem nenhuma ação na estimulação imunitária (AKRAMIENÉ et al., 2007).

Os receptores de β-glucanos são as Dectinas-1, Dectinas-2, receptor *toll-like*2 (TLR2), receptor de complemento 3 (CR3) e as lactosilceramidas 2 e 6 (GOODRIDGE; WOLF; UNDERHILL, 2009; SONCK et al., 2010; XU et al., 2012). Os receptores CR3 e a dectina-1 são considerados os principais receptores de β-glucanos (SONCK et al., 2010).

O CR3 é um receptor de membrana celulardo sistema complemento, com a função de promover o reconhecimento de polissacarídeos e de patógenos, através de vias não mediadas por anticorpos (VETVICKA, 2011). As Dectinas1 e 2 são proteínas da família das lectinas tipo-C com domínios transmembranae

um domínio único de reconhecimento de carboidratos em sua porção extracelular. Estes receptores são expressos principalmente em células dendríticas e macrófagos. As Dectinas-1 reconhecem os β-glucanos na região extracelular destas células e traduzem os sinais através de imunoreceptores (ITAM). As Dectinas-2, em contrapartida, reconhecem α-mananose e traduzem os sinais através dos ITAM´s e receptores de canais yfc. Desta forma, o baço recruta tirosinas quinases ao ITAM, levando à liberação de citocinas como interleucina 2 (IL-2), interleucina 10 (IL-10) e fator de necrose tumoral (TNF) (SAIJO; IWAKURA, 2011). Os receptores CR3 e o lactosilceramida2 e 6, ativam as proteínas Sky e Raf-1, as quais possuem um importante papel na ativação do sistema imunológico (GOODRIDGE; WOLF; UNDERHILL, 2009).

Os β-glucanos são opsonizados após o reconhecimento de um iC3b (fragmento produto da clivagem proteolítica do complexo 3 dosistema complemento)pelos receptores CR3 das células fagocíticas e NK. Eles levam à fagocitose e de granulação citotóxica, mecanismos importantes para o sistema imunológico (VETVICKA et al., 1997).

A administração dos β-glucanos pode ser feita por via parenteral, endovenosa, subcutânea ou oral. Após a administração oral dos β-glucanos, o mesmo entra em contato com a mucosa do trato gastrointestinal, levando ao estímulo da resposta celular nas placas de Peyer e de linfócitos intra-epiteliais (VOLMAN; RAMAKERS; PLAT, 2008).

Segundo estudos clínicos recentes em pacientes humanos e em animais, a suplementação com β-glucanos mostrou-se benéfica na imunidade, considerado um imunomodulador natural (LI et al., 2006; SANDVIK et al., 2007; VETVICKA; VETVICKOVA, 2014).

O aumento na capacidade de estímulo da hematopoiese foi também observado em estudo feito por Patchen e Macvittie (1983), no qual demonstraram que a ação dos β-glucanos pode reverter a mielossupressão

produzida por fármacos quimioterapêuticos, além de auxiliar na regeneração esplênica após a leucopenia. Desta forma, recomenda-se a imunoterapia com β–glucano em conjunto com tratamento quimioterápico (VETVICKA, 2011).

2.2 Glutamina

A glutamina é um dos aminoácidos mais importantes e abundantes nos organismos animais, representando cerca de 60% do total de aminoácidos livres (DEMIRKAN; SAVASX; MELLI, 2010; TEIXEIRA, 2009; VICARIO et al., 2007). Este aminoácido é necessário para uma série de processos bioquímicos específicos, além de possuir inúmeras funções metabólicas (SANTOS et al., 2013). No entanto, é considerado um aminoácido não essencial, ou seja, pode ser sintetizado pelo animal a partir de outros aminoácidos. Entretanto, esta síntese é influenciada pelo *status* fisiológico do animal, que, em condições metabólicas anormais tais como traumas, sepse, grandes cirurgias, quimioterapia e radioterapia, este aminoácido pode se tornar condicionalmente essencial por aumento de sua demanda (REN et al., 2013).

Este aminoácido torna-se disponível por meio do metabolismo endógeno destes nos músculos. Os aminoácidos de cadeia ramificada sofrem reações de transaminação nos músculos onde seu nitrogênio é retirado (TEIXEIRA, 2009). A glutamina possui a função de carrear o nitrogênio dos órgãos periféricos para os viscerais, doando-os para a síntese de vários compostos, tais como nucleotídeos e mucossacarídeos (LOPES-PAULO, 2005; TEIXEIRA, 2009).

O estoque de glutamina é feito nos músculos esqueléticos (REN et al., 2013;TEIXEIRA, 2009). A sua redução é capaz de prejudicar o organismo diante de infecções, diminuindo a capacidade imune, além de dificultar cicatrizações (TEIXEIRA, 2009), ou prejudicar a função dos linfócitos e neutrófilos (PÉREZ-BÁRCENA et al., 2008;SANTOS et al., 2010). Estudos

recentes tem demonstrado que a glutamina promove a proliferação de células e possui efeitos citoprotetores em reposta a privação de nutrientes, injúrias oxidativas e desafios imunológicos (HAYNES et al., 2009; ZHONG et al., 2011).

A glutamina é também um precursor da síntese protéica, e atua como fonte de energia preferencial para as células de proliferação rápida da mucosa e células do sistema imune (TEIXEIRA, 2009).

Adicionalmente, a glutamina possui um papel importante no controle da barreira intestinal, na produção de nucleotídeos para os enterócitos, hepatócitos, macrófagos, linfócitos e tecido linfóide associado ao intestino (DEMIRKAN; SAVASX; MELLI, 2010), sendo também fundamental para a proliferação dos enterócitos (SANTOS et al., 2013). Assim, a glutamina atua principalmente na homeostasia intestinal como substrato energético para a mucosa (DEMIRKAN; SAVASX; MELLI, 2010).

Nos estados catabólicos, no qual a integridade intestinal é comprometida, a glutamina é liberada dos tecidos musculares afim de nutrir e reparar os enterócitos (LIN et al., 2005; SANTOS et al., 2010); diminuindo a permeabilidade intestinal, prevenido translocação bacteriana e complicações associadas a ela como sepse, síndrome da disfunção múltiplas dos órgãos e óbito (SANTOS et al., 2013).

A glutamina atua em vários componentes da resposta imunológica, sendo importante para a proliferação de linfócitos em resposta à estimulação de células T por mitógenos e ativação da proteína quinase C (WU, 1996). Além disso, este aminoácido estimula o crescimento celular e influencia a modulação e produção de citocinas por monócitos e macrófagos (YAQOOB; CALDER, 1998), fagocitose por macrófagos, produção de imunoglobulina G, apresentação de antígenos e opsonização (NEWSHOLME et al., 2003).

Pacientes com casos críticos de privação alimentar por um longo período, podem desenvolver atrofia em vilosidades intestinais assim como comprometimento imunológico. Estudos demonstraram que a suplementação de glutamina em pacientes com quadros graves como sepse e pneumonia (PÉREZ-BÁRCENA et al., 2008), mantem as funções da mucosa, tais como a secreção de imunoglobulinas, secreção de muco e multiplicação normal dos enterócitos (LOPES-PAULO, 2005; SOUBA et al., 1990). Isso ocorre devido ao fato que a glutamina é fundamental para algumas células do sistema imune como monócitos, macrófagos e linfócitos; sendo um modulador para a imunidade inata (PÉREZ-BÁRCENA et al., 2008).

Em condições *in vitro*, foi demonstrado que o aumento da disponibilidade de glutamina está associado ao aumento da atividade de linfócitos e macrófagos (LIN et al., 2005; TEIXEIRA, 2009).

Foi demonstrado por Ren et al. (2013) que a glutamina auxilia na prevenção de doenças, especialmente as subclínicas e imunodepressoras, podendo ser utilizada em períodos de estresse e desafio imunológico. Além disso, é capaz de preservar a integridade intestinal, reduzindo a permeabilidade intestinal e a translocação bacteriana (SANTOS et al., 2010).

Desta maneira, este aminoácido está intimamente relacionado com a produção de citocinas pro-inflamatórias e macrófagos liberados no organismo (LIN et al., 2005; PÉREZ-BÁRCENA et al., 2008).

2.3 Citarabina (Ara-C)

A citarabina (citosina arbinosideo ou Ara-C) é um análogo do nucleosídeo de arabinosídeo da desoxicitidina, considerada específica para a fase S de replicação do DNA, atuando somente na redução das células em divisão (THAM; VLASVELD; WILLEMENZE, 1990). Sua aplicabilidade terapêutica é

dada no tratamento de pacientes humanos com leucemia e linfoma (ELLI et al., 2009; HIDDEMANN, 1991).

As células epiteliais ao longo do intestino delgado apresentam um alto processo de renovação e reposição celular, tornando-as bastante sensíveis a medicamentos citostáticos utilizados na quimioterapia contra o câncer (ELLI et al., 2009).

Este fármaco possui inúmeros efeitos adversos, expressos principalmente no trato gastrintestinal, tais como náuseas, vômitos, dor abdominal, diarréia, ulceração, estomatite. Estes efeitos podem causar uma grave inflamação da mucosa intestinal (DIAS, 2005; ELLI et al., 2009; LUK et al., 1981; THAM; VLASVELD; WILLEMENZE, 1990), além de alterações funcionais na medula óssea e fígado (THAM; VLASVELD; WILLEMENZE, 1990).

Efeitos adversos intensos foram vistos após a utilização do Ara-C em camundongos, ratos e humanos (LUK et al., 1981; THAM; VLASVELD; WILLEMENZE, 1990). Lesões intestinais como danos à arquitetura jejunal com atrofia de vilosidades, redução do número de enterócitos e infiltração inflamatória foram observadas em camundongos tratados com este fármaco (ELLI et al., 2009).

Em camundongos, dosagens terapêuticas de 15mg/Kg, de Ara-C administradas a cada 3 horas, durante 24 horas via intraperitoneal (IP), provocaram lesões nas células epiteliais da mucosa intestinal. A lesão mais grave ocorre nas primeiras 4 horas após a aplicação, com recuperação completa em 72 horas. A manutenção das doses por mais de 24 horas provoca lesões progressivas da mucosa intestinal e mudanças no tecido hematopoiético culminando com aplasia de medula óssea quando a dose letal de 360 mg/Kg em 72 horas é atingida ou ultrapassada (LEACH et al., 1969).

Ramos et al. (1999), observaram que este quimioterápico induziu graves lesões intestinais como encurtamento de vilosidades, redução do número de enterócitos, infiltração inflamatória e necrose.

Luk et al. (1981), observaram que Ara-C produziu injúrias às células proliferativas das criptas intestinais de ratos e atividade diminuída dos níveis a diamino oxidase –DAO (marcador plasmático de lesões intestinais) à medida que se aumentava as injúrias teciduais causadas pelo Ara-C.

A agressão à mucosa intestinal por quimioterápicos, como a citarabina ou outros fármacos, pode ser avaliada por diversos parâmetros, dentre eles os aspectos histológicos e a morfometria das vilosidades intestinais. Podem ser estudadas a presença de macrófagos na superfície basal do epitélio, a hipercelularidade das criptas, o conteúdo protéico e de DNA das células da mucosa, a ruptura da membrana basal e a diminuição da altura das vilosidades intestinais (RAMOS, 2003).

A utilização do Ara-C em camundongos como indutor de danos intestinais foi referido em outros estudos (GRAZZINELLI, 2014; SILVA, 2012).

3 CONSIDERAÇÕES FINAIS

Diante do exposto, pode-se observar que grande parte dos estudos investigam as ações imunomoduladoras da suplementação com β-Glucanos e Glutamina. Essa suplementação tem como objetivo prevenir danos imunológicos causados por agressões sistêmicas como o uso de fármacos que podem levar à translocação bacteriana. Assim visto, ambas as substâncias têm demonstrado bons resultados quando utilizadas previamente à uma agressão, tornando-se necessário o estudo da associação destas duas substâncias, afim de se obter melhor resposta imunológica e no trofismo intestinal após desafio.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 Evaluate the trophic and immunological action of mice treated with β-glucan and glutamine after challenge with cytarabine (Ara-c).

ARTIGO FORMATADO DE ACORDO COM O PERIÓDICO VETERINARY RESEARCH E ADEQUADO A VERSÃO FINAL DE IMPRESSÃO UFLA.

- **Evaluate the trophic and immunological action of mice treated with β-glucan and Glutamine after challenge with cytarabine (Ara-C).**
- **Trophic effect and immunological action of mice treated with β-glucan and Glutamine after challenge with cytarabine (Ara-C).**
- **Effects of beta-glucanos and Glutamine on the immune system and trophic in micesafter challenge with cytarabine (Ara-C).**
- **Effects of beta-glucanos and Glutamine on the immune system and intestinal tropism in mices previously undergone injury by administration of cytarabine (Ara-C).**
- **Protective effect of β-glucan and Glutamine on intestinal and immunological damage in mice induced by cytarabine (Ara-C)**

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Abstract

Systemic changes can predispose the increase of intestinal permeability, making possible the migration of bacteria and their products from the gut lumen into the sterile sites, leading to a local inflammatory process. Recently, Glutamine and β-glucan, have been demonstrated to play an important role in modulation of immune system and in promotion of intestinal health benefits. The aim of this study was to investigate the effect on inflammatory responses and intestinal health after the mice being pretreated with oral administration of soluble *Saccharomyces cerevisiae,* derived β 1,3/1,6, β-glucan (80 mg / kg) with or without glutamine (150mg/kg) and then challenged with administration of cytarabine (Ara-C) (15 mg / kg). The measured variables were: total of leukocytes, IL- 1β, IL-10 and INF-γ, intestinal morphology andimmunohistochemical analysis of macrophage and lymphocytes T and B. Improvement in villi and crypts were not observed in β- glucan group, however, lower values of IL-10 and IL-1 β secretion were observed, whereas they prompt the highest release of IFN-γ. In mice treated with Glutamine was showed the intermediate value of all cytokines measured. The intestinal morphometric showed the best results in this group. The treatment of β-glucan in combination with glutamine presented the highest values of IL-1β and IL-10 and lowest values of the leukocytes total and INF-γ. The intestinal morphometric of this group showed the benefits on intestinal health. Based on these results, the answers of pretreatment with the combination of β-Glucan and Glutamine

were reduction of intestinal inflammation and improvement of immune response after challenged with Ara-C.

Keywords: Chemotherapy, Yeast, Intestinal Health, Immunomodulators

Introduction

Gastrointestinal tract is recognized not only for its role in digestion, but its metabolic and endocrine functions and as a major component of mucosal immunity [1]. The intestine has a physiological barrier composed of simple barrier of mucus cell, columnar epithelial cells, M cells, lymphocytes and gut associated lymphoid tissue involved in defense [2]. The epithelium consists of a single layer of columnar cells interconnected by tight junctions, it restricts both transcellular and paracellular permeation of molecules [3- 4]

A disturbance of intestinal barrier function such as immunosuppressive illness, intestinal obstruction, trauma and shock, may lead to suppression of the immune system and can predispose the increase of mucosal permeability [5-6-7]. Loss of epithelial contiguity is implicated in increased mucosal permeability, making possible the migration of bacteria and toxins from the gut lumen into the sterile sites. Such fact stimulate the host's immune inflammation [8].

The administration of a range of substances have been studied with aim of minimize the damage to the intestinal tropism and in the immune response [7], highlighting the use of products derived from plants, fungi,

mushrooms and live microorganisms. [9]. β-Glucans are glucose polymers constituents of the cell wall of fungal, plants, bacteria and cereals such as oat and barley [10-11]. They consist of β-1, 3-linked β-D-glucopyranosyl units that forms backbone containing randomly dispersed β-1,6-linked side chains of different sizes [12 Graham 2006). These polysaccharides are not found in the animals, they are considered to be classic pathogen-associated molecular patterns [13]. The recognition of β-Glucans in both systems results in the triggering of innate and adaptive immune responses [14- 15).

The polymers derived of yeast as Saccharomyces cerevisiae, have been shown to modulate effects on the immune system by activation of macrophages, phagocytosis of the pathogen, releasing proinflammatory cytokines [13-16-17-18] Activities associated with β-glucan include the stimulus of both primary and secondary lymphoid organs in the immune system [19], enhancing the activity of macrophages, monocytes and natural killer cells (NK) cells and the promotion of an indirect stimulation of T and B lymphocytes by cytokines [20-21]. The treatment with β-glucan can be an administrator of the route by parenteral, intravenous, subcutaneous or oral administration [16]. After oral administration of β-glucans, it comes in contact with the mucosa of gastrointestinal tract and it stimulates the intestinal immune cells, including Peyer's patches, promoting the increased number of intraepithelial lymphocytes [17-22]. Consequently it enhances mucosal immunity response in the digestive tract, protecting against pathogen infections [17-18].

Another substance studied due to the roles in intestinal tropism and the immunological action is glutamine, an immunonutrient, modulator which can enhance the host's immunity (Li et al., 2006; Teixeira, 2009; Demirkan et al. (2010) [23,24].

Glutamine is an essential amino acid, it is the most abundant free substance in the plasma and tissue fluids [24-25- 26]. This amino acid is required for specific biochemical processes such as metabolic functions in the immune system and in the intestinal functions [27]. Glutamine is a precursor to protein synthesis and a preferred energy source for fast proliferation of mucosal cells and cells of the immune system. [26]

Glutamine is stored in the skeletal muscles [28]. The reduction of stockpile is able to harm the body in front of infections, reducing the immune capacity, hinder scarring or impair the function of lymphocytes and neutrophils [29-30] It plays an important role in the control of gut barrier in the production of nucleotides for the enterocytes, hepatocytes, macrophages, lymphocytes and lymphoid tissue associated with the intestine [24].

It has also been reported an important role of Glutamine in the control of gut barrier, in the production of nucleotides for the enterocytes, hepatocytes, macrophages, lymphocytes and lymphoid tissue associated with the intestine [24-29] and in the proliferation of enterocytes [27]. Thus, glutamine operates mainly in intestinal homeostasis as an energetic substrate for the mucosa [24 – 26].

In catabolic states the increased demand for glutamine happen. [23-26-27]. This amino acid is released from muscle tissue in order to

nourish and repair the enterocytes, if this demand is not enough, it is necessary endogenous supply [23]. Various studies have demonstrated that supplemented glutamin can maintain the morphology and functions of the intestines, in addition to promotingthe enhance of immune system [29- 27-28-30].

Cytarabine (cytosine arbinosideo or Ara-C) is a nucleoside analog to deoxycytidinearabinoside, which is specific for the DNA replication stage S, acting just on the reduction of dividing cells (31THAM et al., 1990). Clinical applicability of Ara-C is given in the treatment of human patients with leukemia and lymphoma [33-34].

This drug has many side effects, expressed in the gastrointestinal tract as well as functional alterations in bone marrow and liver [32]. Intestinal lesions as damage to jejunal villous architecture with atrophy, reduction of enterocytes and inflammatory infiltrate were observed in mice treated with this drug [34].

The aim of this study was to investigate the effects of β-glucan (1®3), β-glucan (1®6), with and without Glutamine, in the trophic action and modulation of the immune system in mice challenged by administration of Ara-C.

Materials and Methods

Animals

This present study was approved by the Ethics Committee on Animal Use (CEU/UFLA) - protocol number 2011/45. All the

proceedings followed the resolutions of the National Council of Animal Experimentation (CONCEA- SBCAL). 30 mice were utilized, with the age of approximately 50 days, lineage Balb/C, males, healthy, originating from the Vivarium of Physiology and Pharmacology Sector of Veterinary Medicine Department, Federal University of Lavras.

The animals were kept in collective cages, ideal temperature conditions (22ºC°± 2°), humidity (45 ± 15° %) and luminosity (light/ dark cycle in 12/12 hours). During all the proceedings standard commercial diet and water *ad libitum* were provided.

Experimental Delineation

The mice were weighed in the beginning of the experiment and distributed in 5 homogeneous experimental groups, each one with 6 animals: Control Group; Ara- C Group and β-glucan Group; Glutamine Group and β-glucan and Glutamin Group.

The β-glucan utilized was the cell wall extract of *Saccharomycescerevisiae*, (Macrogard®/Biorigin, LençóisPaulista-SP), from which daily doses of 80mg/kg of body weight were administered by gavage every 24 hours. The Glutamin (L-Glutamine, SIGMA-ALDRICH®) was also administered by gavage with a daily dose of 150 mg/kg body weight each 24 hours. The Ara-C was administered in all groups, except the Control Group, in the two last days of the experiment. The dose was 15 mg/kg body weight, by intraperitoneal injection, every 12 hours for a total administration of 4 doses.

Euthanasia and Blood and tissue sample collection

After 21 days of experiment the animals were induced in an 8 hours fasting and euthanized by cardiac puncture under anesthesia (Tiopental sódico®40mg/kg, administered by intraperitoneal injection). The blood samples were collected in syringes, wherein the total blood sample was forwarded to leukogram and the plasma was stored for subsequent carrying out ELISA assays. The animals were submitted to abdominal cavity opening with internal organs exposition. For histopathological analysis were collected fragments of: spleen, small intestine (duodenum, jejunum and ileum), liver and mesenteric lymph nodes. The fragments were fixed using formalin 10%.

Performed Analyses

Leukogram

The total count of leukocytes was performed by the hemocytometer method (Neubauer chamber), 1:20 diluted with Türk's solution. The final number of leukocytes/mm^3 was found by the sum of total leukocytes counted across four squares multiplied by 50. The differential leukocyte counting was performed by blood smear fixed and stained with fast Panotic*kit*. 100 cells were counted per glass slide, providing a relative and absolute number of leukocytes.

Cytokines Mensuration: IL-1 β, IL-10, INF-γ, TNF-α

The plasma was stored in an ultrafreezer (-80º C). Subsequently, the plasma was utilized for mensuration of the follow cytokines

concentration: IL-1β, IL-10 e INF-γ by Sandwich ELISA method, according to the manufacturer's suggestion (Uscn Life Science Inc). The results were expressed in pictograms per milliliter based on a standard curve.

Histopathological Analysis

The collected material was fixed in buffered formalin 10% and routinely processed to prepare histological samples, stained by Haematoxylin-Eosin technique. The analysis was executed utilizing light optical microscopy.

Intestinal Morphometry

During the morphometric studies, villi height and width and crypt depth and width were measured in all the segments of the small intestine (duodenum, jejunum and ileum) of all animals. The relation between crypt depth/ villi height was analyzed. The images were obtained by using a binocular microscope, Olympus CX31 (Olympus Optical do Brazil Ltda, São Paulo) with attached camera (SC30 CMOS Color Camera for Light Mirocopy, Olympus Optical do Brazil Ltda, São Paulo). The mensuration was performed using the Image-Pro® Express Software version 6.0 (Media Cybernetics, Rockville, MD, USA), according to the adapted technique described by Carlos (2006), wherein were randomized three histological sections per segment for each animal. Three villi and three crypt per square were measured.

Immunohistochemistry

Histological sections of ileum and mesenteric lymph nodes were submitted to immunohistochemical marking by the streptavidin-biotinaperoxidase method. The antigenic recovery was performed by heating for 20 minutes. Primary antibodies Dako®anti-CD3 (lymphocytes T) in the 1:400 dilution, anti-CD79a (lymphocytes B) 1:200 dilution and anti-CD68 (macrophages) 1:50 dilution were utilized for 14-16 hours at 4ºC temperature. Subsequently, to perform the revelation, DAB (3,3'-diaminobenzidina, Dako) was utilized, and as chromogen, anti- CD3, anti-CD79 and CD68 were used by 4, 4 and 1 minute respectively. The sections were stained with haematoxylin, dehydrated, prepared and analyzed with light microscope. As positive control, histological sections of the mesenteric lymph nodes were used. In these immunohistochemical markings, 5 sections of each slide were selected, wherein were counted the marked cells (lymphocytes T, lymphocytes B and macrophages) and the remaining not marked cells.

The photos were obtained using a system of snapshot and image analysis, consisted of binocular microscope, Olympus CX31 (Olympus Optical Brazil Ltda, São Paulo) with attached camera (SC30 CMOS Color Camera for LightMirocopy, Olympus Optical do Brazil Ltda, São Paulo). The inflammatory cells counting was performed manually using blind evaluation by one experienced observer.

Statistical Analysis

Primarily, all data were submitted to assumption of normality (Shapiro Wilk) and Homoscedasticity (Levene). When there was a

significant effect of a variable, attempts to square root or logarithmic transformation of the data were realized.

Furthermore, the data related to IL- 10 concentration, morphometric evaluations and total leukocytes counting, were submitted to analysis of variance (ANOVA). When there was significance, these means were further compared by Tukey's test.

The data regarding to immunohistochemical analysis and leukocytes differential, were submitted to ANOVA and, when there was significance, the average was compared by Duncan's test. For the data regarding to IL-1b e INF-g concentration (which did not achieve the normality, even after the transformation) was performed the non-parametric analysis by the Kruskal Wallis's test. After the score of the cases, the ranked averages were compared by Tukey's test. The minimum level of significance considered was 5%. All the statistic procedure was performed by the SPSS 20.0 program.

Results

The histopathological analysis(figure 7)did not evidence significant alterations in the spleen, liver and small intestine (duodenum, jejunum and ileum).

It was possible to observe an influence ($p<0,05$) of the treatments on the total leukocytes counting (figure1). The groups treated with β-Glucan in association with Glutamine presented the lowest values and the remaining groups presented intermediary values compared with the control group, which represented the highest mean values.

From the IL- 10 mensuration a difference ($p<0,05$) between the treatments regarding its concentration (Figure 1) was observed, wherein the association between the treatment with β- Glucan and Glutamin indicated the highest values and the treatment with Glutamine indicated intermediary values. On the other hand, the group treated with β-Glucan showed lower values compared with the control group, which showed intermediary values.

From the IL 1- β concentration (Figure 1), was noted a difference ($p<0,05$) between the treatments, wherein the highest cytokine values were found in the group treated with association of β- Glucan and Glutamine. The intermediary values were noticed in the group treated with Glutamine and the lowest values in the Control and β- Glucan groups. In the same way, there was an influence ($p<0,05$) of the treatments on the INF-γ concentration (Figure 1). The treatment with β-Glucan showed the highest values of this cytokine. The treatment with Glutamine presented intermediary values and the remaining treatments presented low values.

In the morphometric evaluation of the duodenum (Figure 2), a difference ($p<0,05$) between the trial groups regarding the width and height of the villi was noticed. Concerning to villi height, the control groups treated with β-Glucan in association with Glutamine presented the highest values for these parameters, the Ara- C and Glutamine groups presented intermediary values and the lowest value was noticed in the β-Glucan group. Regarding to width of the duodenum, the highest value was observed in the group treated with β-Glucan in association with

Glutamine, the intermediary values were observed in the control and in the Glutamine group and the lowest values were noticed in the Ara- C and β-Glucan groups.

There was no difference ($p>0,05$) in the evaluated groups regarding to the mensuration of jejunum.

Concerning to ileum villi there was no difference ($p>0,05$) (Figure 3). There was difference ($p<0,05$) related to depth of the ileum crypts (Figure 3) presenting the highest values in the Glutamine group, intermediary values in the β- Glucan and β- Glucan in association with Glutamine groups and the lowest values in the control and Ara- C groups.

Concerning to villi height and crypts depth there were no differences ($p>0,05$) in any segment (duodenum, jejunum and ileum) of the evaluated groups (Figure 2 and 3)

Immunohistochemical analysis was performed in the ileum of all animals, in which was possible to notice a difference ($p<0,05$) in the marking of B lymphocytes (Figure 4 and 8) and inflammatory cells (Figure 4). The control, Ara- C and Glutamin groups obtained higher values in the total counting of inflammatory cells than the β- Glucan and β- Glucan in association with Glutamin groups. The total counting of B lymphocytes was higher in the control and β- Glucan in association with Glutamine groups than in the remaining groups. The relation between marked B lymphocytes and the total of inflammatory cells was the highest in the β-Glucan and Glutamine groups.

Regarding to marking of T lymphocytes (Figure 5 and 9) and inflammatory cells (Figure 5) there was a difference ($p<0,05$) in the total counting of inflammatory cells, wherein a higher value was noticed in the β- Glucan, Glutamine and β- Glucan in association with Glutamine groups than in the remaining groups. The total counting of T lymphocytes was the highest in the Ara- C group and the relation between marked T lymphocytes and total inflammatory cells was also higher in the Ara- C group than in the remaining groups.

Relating to marking of macrophages (Figure 6 and 10) and inflammatory cells (Figure 6) there was a difference ($p<0,05$), wherein the control, β- Glucan, Glutamin and β- Glucan in association with Glutamine groups obtained the highest values in the total counting of inflammatory cells. The Ara- C group obtained the lowest value in this parameter. The total counting of macrophages was the highest in the groups treated with β- Glucan and β- Glucan in association with Glutamine. Intermediary values were noticed in the Ara- C and Glutamine group. The lowest value was showed in the control group. The values regarding to the relation between the marked macrophages and total of inflammatory cells were the highest in the β- Glucan associated with Glutamine group, the lowest in the control group and intermediary in the remaining groups.

Discussion

Chemotherapy using Ara-C is the usual conduct in the treatment of leukemia and lymphoma in human patients [33-34]. However, there are side effects such as myelosuppression and severe inflammation of the intestinal mucosa with villus atrophy, which may lead to bowel necrosis [35-34].

Considering the above, the Ara-C was used as a challenge in this experiment and the aggression against intestinal tropism and the immunological impairment caused were observed. The total of leukocytes and the intestinal morphometry showed lower values in Ara-C group compared to control group. The results corroborate the studies by Ramos et al. (1999) and Elli et al. (2009) [34-35] who also found impairment of intestinal villi caused by the administration of Ara-C in mice.

According to a study made by Li et al (2005) [23], supplementation with β-glucan in piglet diets worked modulating the immune response, thereby this increased the amount of IL-10, an anti-inflammatory cytokine, and suppressed the secretion of proinflammatory cytokines such as IL-1 β and IFN-γ.

This present work showed different results, we found the lowest values in the IL-10 and IL-1 β and the highest values in the INF- γ concentration. This result demonstrate occur an immune response after previous administration of β-Glucan front of aggression.

In a study performed by Araújo- Filho et al., (2006) [36], the decreased production of IL-1β and IL-10 in mouse treatment with β-Glucan after intestinal ischemia was showed. This experiment suggests β-Glucan working in the modulation of proinflammatory and inflammatory cytokine. Considerations should be given about the IL-10 values, it may be low due to the strong aggression made by Ara-C and the short time to form an immune response.

The values of INF-γ were higher in the β-Glucan group compared to the others groups. These results suggest the INF-γ values inhibited the

liberation of IL-10 [31]. This fact contributed to elucidate the role of β-Glucan as immunomodulator. After their recognition as a classic pathogen-associated molecular patterns, they decrease the secretion of anti-inflammatory cytokine and enhance the immune response by the inflammation.

In this present work, based on the result of intestinal morphometry, improvement in the intestinal morphology in mice treated with β-glucan was not observed and the lowest heights and widths of the duodenal villi were found compared with the remaining groups. This is in accordance with the study made by Reisinger et al., 2012, which reported that the treatment in chickens using yeast extracts containing β-glucans was [38] not beneficial to intestinal morphology.

Despite the group treated with Glutamine had lower values of IFN-γ than β-glucan group, it had the highest values of INF-γ compared to other groups. Our findings also illustrate that this amino acid can modulate the immune response by stimulation of the inflammatory response, in agreement with several studies in mice [28]and rats [27]in challenged animals.

The present study further define the influence of glutamine in the intestinal morphology, the mice treated with glutamine, with or without β-glucan, it also showed statistical significance and biological relevance, illustrated by the greater results in the measurements of the villi and crypts. These findings are in accordance to others studies conducted in humans and animals [6-39- 24-27].

The IL-1β is a multifunctional proinflammatory cytokine and is able to penetrate the site of infection to recruit and activate leukocytes [40]. Bearing in account the evidence, the highest levels of IL-1β were showed in the group of animals with leukopenia. We can suppose there is a recruitment of leukocytes to the site of aggression, decreasing the number of leukocytes in the circulation.

The immunohistochemical analysis showed that all pretreatments, both separated or in combination, decreased the intestinal inflammation, since the recruitment of T and B lymphocytes and macrophages in the intestinal mucosa, thus, demonstrating the benefits of administration of β-glucans and glutamine on intestinal immunity.

Some authors [20,21] suppose that the oral administration of β-glucans induces cell response in Peyer's patches and intraepithelial lymphocytes [17], promoting the indirect stimulation of T and B lymphocytes by cytokines[20,21]. Glutamine also acts in the lymphoid tissue, lymphocytes and intestinal macrophages [24], especially in the proliferation of enterocytes [27].

The β-Glucan group in combination with glutamine represented the highest values of IL-10 and the lowest values of INF-γ when compared to the others groups. Measurements of duodenal villi and depth of ileal crypts showed promising results. This association presumably resulted in a decrease of the lesion by inflammatory response and promoted beneficial effect after aggression of Ara-C.

In summary, the use of the associated β-Glucan and Glutamine provided an improvement of immune response and improved gut health in

the challenged animals, therefore, they are important modulators of immunity in mucous.

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Figure 1: Histologia

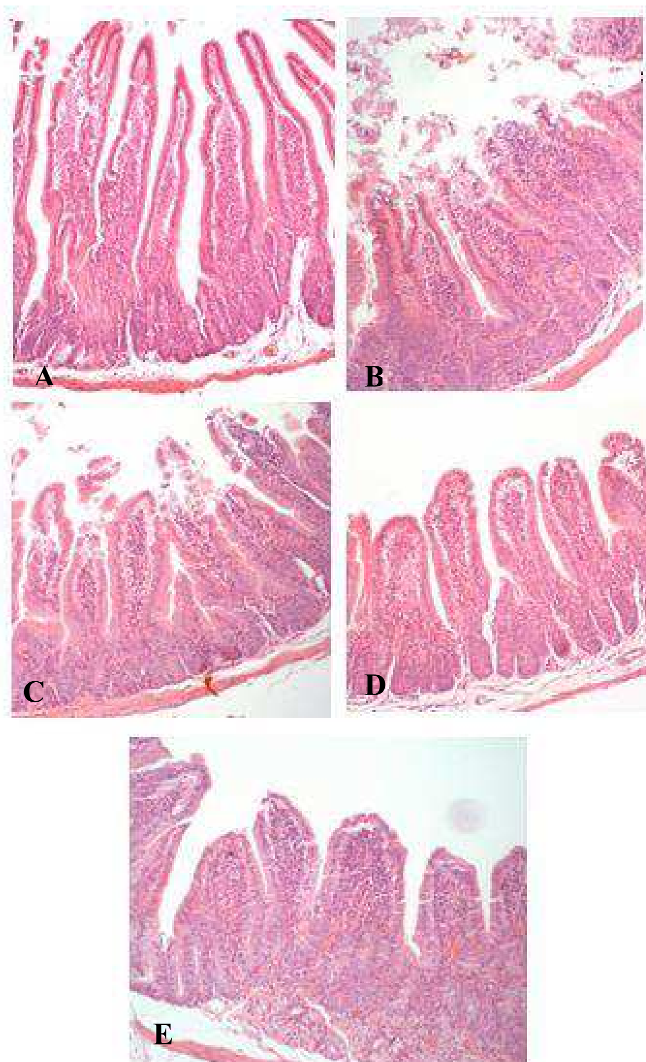

Figure 2: Morphometric

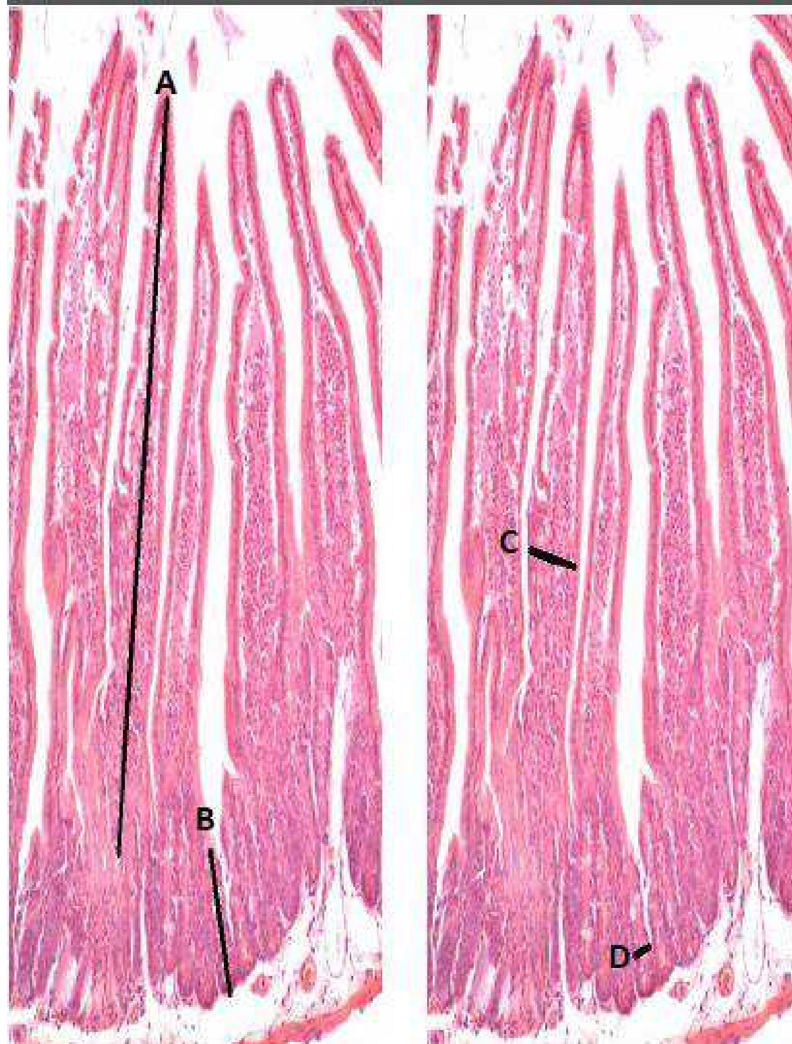

Figura 3: Immunohistochemistry of B-Lymphocyte in ileum of mice of Control group

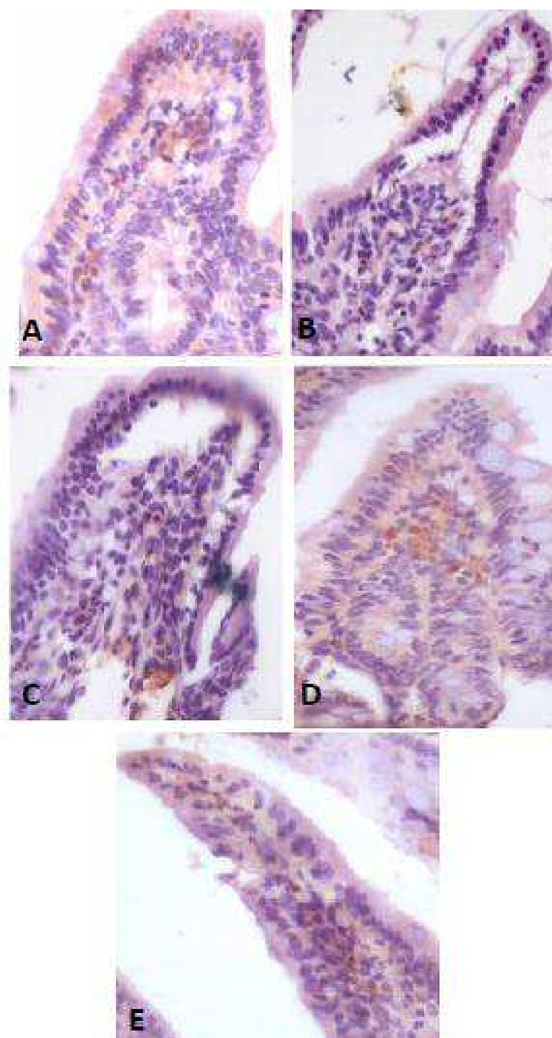

Figura4: Immunohistochemistry of B-Lymphocyte in ileum of mice

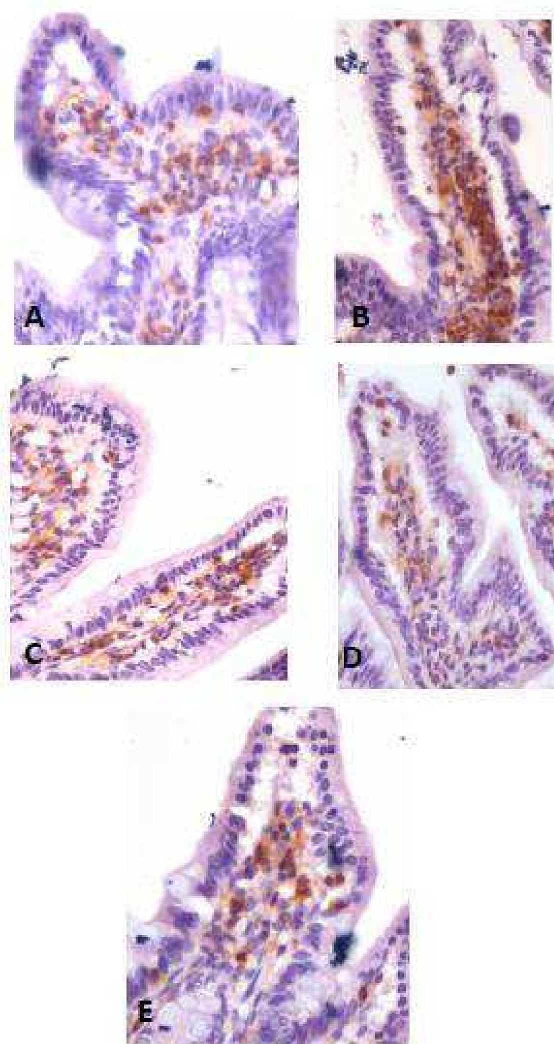

Figura 5: Immunohistochemistryof macrophage in ileum of mice of Control group