Modulating effect of the piperine, the main alkaloid from *Piper nigrum* Linn., on murine B lymphocyte function*

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ABSTRACT. Bernardo A.R., da Rocha J.D.B., de Lima M.E.F., Decote-Ricardo D., Pinto-da-Silva L.H., Peçanha L.M.T. & Danelli M. dasG.M. **Modulating effect of the piperine, the main alkaloid from *Piper nigrum* Linn., on murine B lymphocyte function.** [Efeito modulador da piperina, principal alcalóide da *Piper nigrum* Linn., sobre a função de linfócitos B murinos.] *Revista Brasileira de Medicina Veterinária, 37(3):209-216, 2015*. Departamento de Microbiologia e Imunologia Veterinária, Instituto de Veterinária, Universidade Federal Rural de Janeiro, *Campus* Seropédica, BR 465 Km 7, Seropédica, RJ 23890-000, Brazil. E-mail: danelli @ufrrj.br

Piperine is the main alkaloid of black and long peppers and it is conventionally used as immune-enhancers in Indian system of traditional medicine. The main of this study was evaluated for the first time the effect of piperine on B cells functions in vitro and its effects on humoral immune response to T-dependent and T-independent antigens. Different concentrations of piperine (1 µM, 3 µM and 15 µM) were assayed on B cells purified from BALB/c spleen cells and evaluated its effects on proliferation, IgM secretion and expression of CD86 on murine B cells. At 15 µM piperine was able to inhibit the proliferative response induced by LPS and α-IgM antibody and inhibited the secretion IgM antibody in vitro. Also, piperine at 3 µM and 15 µM reduced the CD86 expression on B cells stimulated with LPS and α-IgM antibody in vitro. However, piperine 2.5 and 4.5 mg/Kg did not modulated antibody production for T-independet (TNP-Ficoll) in vivo.

KEY WORDS. Piperine, B lymphocyte, murine.

RESUMO. A piperina é o principal alcalóide das pimentas preta e longa, sendo habitualmente utilizada como imuno-potencializadores na sistema indiano de medicina tradicional. O objetivo desse estudo foi avaliar, pela primeira vez, o efeito da piperina sobre as funções da célula B murina in vitro e sobre a resposta imune humoral para antígenos T-dependentes e T-independentes. Diferentes concentrações de piperina (1uM, 3 uM e 15 uM) foram testadas em células B purificadas a partir do baço de camungongos BALB/ c e, avaliados os seus efeitos sobre a proliferação, secreção de IgM e expressão de CD86 em células B murinas. Quinze micromolar de piperina foi capaz de inibir a resposta proliferativa induzida por LPS e anticorpo α-IgM e inibiu a secreção de anticorpos IgM *in vitro*. Além disso, a piperina, 3 uM e 15 uM reduziu a expressão de CD86 nas células B estimuladas com LPS e α-IgM

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anticorpo in vitro. No entanto, a piperina nas concentrações de 2,5 e 4,5 mg / kg não moduladou a produção de anticorpos T-independet(TNP-Ficoll) in vivo.

PALAVRAS-CHAVE. Piperina, linfócitos B, murino.

INTRODUCTION

B cells are characterized by their ability to differentiate into antibody secreting cells (Mizoguchi & Bham 2006). There are three classes of antigens able to stimuli B cells to product antibodies: T -dependent (TD), T--independent type 2 (TI-2) and T -independent type 1 (TI-1) antigens. The first require the presence of cognate T-helper (T_H) cell interaction to trigger a B-cell response, whereas the TI antigens can induce an antibody response in the absence of T_H (Pereira et al. 2010, Grant et al. 2012). Toll-like receptors (TLRs) are characterized as TI-1 antigens. These molecules comprise a family of conserved pattern recognition receptors (PRRs). Till now, 10 different TLRs have been detected in humans and 11 have been identified in mice. These receptors recognize specific pathogen markers that include lipopolysaccharide, double-stranded RNA, peptidoglycan and CpG oligodeoxinucleotides (Boeglin et al. 2011) and engagement of B cell TLRs induces TI antibody responses (Pone et al. 2012). Polyclonal B-cell activation may plays a pathogenic role in a wide spectrum of inflammatory diseases, including hypersensitivity responses to environmental antigens (allergic disorders), recognition of self-antigen (autoimmune diseases) and immune attack against alloantigens during transplantation (Sharma & Pradeep 2006).

Natural products have been widely used as food additives also exhibit several effects on human health and the side-effects associated with their use are considered to by low (Yoshitomi et al. 2011). Previous studies have shown modulator effect on B cell function by several natural products. Sharma et al. (2006) observed that resveratrol and curcumin suppress proliferation of B cells, inhibit production of both IgG1 and IgG2a in spleen cells and decrease pro-inflammatory cytokines production by BALB/c mice. Decote-Ricardo et al. (2009) demonstrated that the curcumin inhibits the proliferative response and IgM secretion of purified spleen B cells from BALB/c mice. Rocha et al. (2010) suggest that warifterine is a potent inhibitor of B cell response both in vitro and in vivo.

Piperine (Figure 1) is the main natural alkaloid isolated from *Piper longum* Linn. and *Piper nigrum* Linn. These peppers have a long history in traditional medicine in Pacific islands and especially in
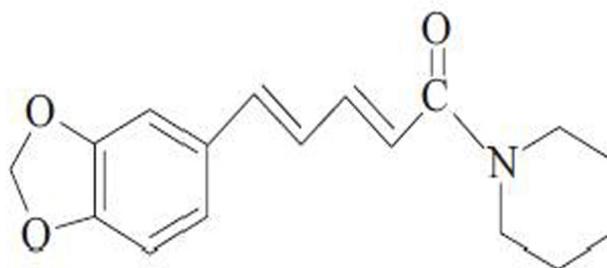

Figure 1. Chemical structures of piperine

Asian medicine (Koleva et al. 2012). Black pepper (*Piper nigrum*) contains 5-9% of piperine and this substance is easily extracted and purified from dried fruits (Ikan 1991). Piperine exhibit a variety of biological activities, among them we highlight its anti-inflammatory as well as its immunomodulatory effects. Previous studies have demonstrated that piperine inhibit the synthesis of both prostaglandins and leukotrienes, inhibit the translocation of the transcription factors NF-kB to the nucleus, decreased expression of the adhesion molecules ICAM-1, VCAM-1 and E-selectin on endothelial cells, inhibit the maturation of dendritic cells and suppresses LPS-induced ERK1/2 and JNK MAPK activation in these cells (Stöhr et al. 2001, Pradeep & Kuttan 2004, Kumar et al. 2005, Kumar et al. 2007, Bae et al. 2012, Chuhawankul et al. 2012). Others studies have shown the important therapeutic effect of piperine in inflammatory diseases such as cardiovascular diseases (Taqvi et al. 2010), arthritis (Bang et al. 2000, Murunikkara et al. 2012), asthma (Kim et al. 2009), sepsis (Bae et al. 2010), neuronal diseases (Fu et al. 2010), gastrointestinal disorders (Mehmood & Gilani 2010) and acute pancreatitis (Bae et al. 2011).

Dogra et al. (2004) demonstrated that spleen B cells isolated from piperine-treated animals (oral administration of 2,25 and 4,5mg/Kg) showed reduced mitogenic response of and a lower number of antibody (IgM) forming cells on cell culture. The serum primary antibody of these mice was also reduced. On the other hand, Sunila & Kuttan (2004) described that intraperitoneal piperine injection (1.14 mg/dose/mouse) raised circulating antibody titers, increased the number of antibody (IgM) forming cells in the spleen, augmented bone marrow cellularity and increased the number of α-esterase positive cells (Sunila & Kuttan 2004). However, this study did not investigate whether the increase in humoral response would be due to a direct effect on B cells.

Taken together, these results suggest that piperine could act directly on B lymphocytes, suppressing its response. To date, there is no study using

purified B cells to verify the effect of this alkaloid on B cell function. In the present study, we investigate the effect of piperine on purified spleen B cell function *in vitro* and evaluated its effect on *in vivo* T-independent B cell response.

MATERIALS AND METHODS

Piperine purification. Piperine was isolated from commercially obtained dried fruits of *Piper nigrum* Linn. according to Ikan (1991). Ethanol was used as solvent with yield of 5-7% and purity high (~98%) determinate by GC-MS, demonstrating melting point of 128-129°C and spectrometric data (^{1}H and ^{13}C NMR, IR e MS) similar to that previously reported in the literature (Araújo-Junior et al. 1997, Siddiqui et al. 1997). The extract was dissolved in DMSO (Sigma-Aldrich) at the moment of use.

Preparation of cell suspensions

A20 cells BALB/c B-cell lymphoma line. The A20 cells (Kim et al. 1979) were kindly provided by John C. Cambier at Department of Immunology, University of Colorado Denver School of Medicine and National Jewish Health, USA. Tumor cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Cripion Biotechnology, Brazil), gentamicin at 40 µg/mL (**Life Technologies**), L-glutamine at 1% (**Life Technologies**) and HEPES at 20mM (Sigma-Aldrich). Cells were grown in suspension culture at 37 °C in 5% CO_2. A20 cells ($2x10^5$) were seeded in 96 well flat-bottom plates.

Spleen cells. Single cell suspension of spleen was prepared under aseptic conditions. The suspensions were filtered through a stainless steel mesh and centrifuged at $200 \times g$ for 10 min at 4°C. Splenocytes were suspended in 5.0 ml hypotonic erythrocyte lysing solution (2.42 g Tris and 7.56 g NH_4Cl in 1.0 l deionised water, pH adjusted to 7.2). After 5 min incubation followed by centrifugation, cells were resuspended in RPMI-1640 complete medium as used with A20 cells. Cell density was adjusted to ca. 1.5×10^5 cells/ml.

Assessment of cell viability. Different concentrations of piperine were added in culture of A20 and spleen cells. These cultures were run in triplicate and were incubated for 24h, 48h or 72h at 37°C in 5% CO_2 to A20 cells culture, and for 24h at 37°C in 5% CO_2 to spleen cells culture. After these periods, 50µL of XTT (1,2mg/mL) was added into each well and incubated for further 2 hours. Plates were read at a microplate reader (Bio-Rad, Model 680) with 450nm filter. Positive control cultures received 1uL of Triton X-100. The percentage of viable cells was determined based on A450 obtained in untreated controls (100%).

Animals. BALB/c mice were obtained from and maintained at the Veterinary Institute from Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil. The animals were bred and housed according to institutional policies for animal care and usage. This work was approved by the Bioethical Committee on Animal Research of UFRRJ, process number 23011803/2011-83.

B cells purification and culture. Total spleen cells were obtained and the depletion of the T cell was performed by treatment with a mixture of anti-T cell Abs (anti-Thy-1, anti-CD4, and anti-CD8) and with Low Tox Rabbit Complement (Cedarlane Inc.) as previously described (Bento et al., 2006). Enriched resting B cells suspension was then applied to a 1.086, 1.081, 1.074 and 1.062 g/mL Percoll (Pharmacia, Sweden) gradient. B cells were cultured in flat bottom 96-well plates in RPMI 1640 medium (**Life Technologies**) supplemented with 10% fetal bovine serum (**Life Technologies**), 2 mM L--glutamine (Sigma-Aldrich) and 40 µg/mL gentamycin (Sigma-Aldrich). Cultures were set in triplicate and were incubated with different doses of piperine and B cell activators at 37°C in 5% CO_2.

B lymphocyte proliferation. B cells ($2x10^5$cells/well) were incubated in absence or presence of LPS 10µg/mL (as TLR4 ligand) or anti-IgM antibody 10µg/mL (as BCR ligand) and treated with different doses of piperine (15µM, 3µM and 1µM). After addition of [methyl-3H]--thymidine, the cultures were incubated for an additional 18h period and plates were frozen until analysis. Liquid scintillation counting was used to measure specific thymidine incorporation. Cultures were set in triplicate and mean cpm values was obtained (Brunswick et al. 1988).

Culture supernatant Ig levels. B cells were cultured in triplicate ($5x10^4$ cells/well) in absence or presence of LPS 10µg/mL and treated with different doses of piperine (15µM, 3µM and 1µM). The cultures were incubated for seven days at 37°C in 5% CO_2 and a pool of culture supernatants was prepared and stored at -20°C until Ig levels were measured by a sandwich ELISA, using a capture ELISA as previously described (Snapper & Paul 1987).

B cell surface markers. B cells were cultured ($1x10^6$ cells/well), in absence or presence of stimuli LPS 10µg/mL or anti-IgM antibody 10µg/mL and treated with different doses of piperine (15µM and 3µM) for 72h. Then the cultures were washed once with PBS containing FCS 1% (Cripion Biotechnology, Brazil) and blocked with PBS containing mouse serum 1% and FCS 2% by 15 minutes at 4°C. B cells were labeled with FITC-conjugated anti B220 and PE-conjugated anti CD86 antibodies (BD Bioscience) by 30 minute at 4°C. The samples were analyzed in a FACScalibur flow cytometer (BD Bioscience) and 10,000 events were collected. Data was analyzed using the CellQuest software. Gated B220$^+$ cells were selected for analysis of CD86 expression.

In vivo assay. The ability of piperine to modulate the humoral immune response induced by thymus-independent antigen was assayed as described: BALB/c female mice with age ranging 6-8 weeks were divided in 6 groups (5 animals per group). Piperine-treated animals received different doses of piperine (2,25mg/Kg or 4,5mg/Kg) intraperitoneally for 5 consecutive days (Dogra et al. 2004). Some groups received an intravenous injection of TNP-FICOLL (50µg/ animal) in the fifth day after the beginning of piperine treatment. The administration of TNP-Ficoll was performed one hour after piperine inoculation (Rocha et al., 2010). Negative con-

trol (untreated) group received injection of DMSO 4% intraperitoneally for 5 consecutive days. Positive control group received TNP-FICOLL intravenously at fifth day besides the treatment with DMSO. Mice's blood was collected 7 and 14 day after treatment by tail vein and the plasma was stored at -20°C until use. Plasma samples were serial diluted and added in polyvinyl chloride round-bottom Falcon Microstest III microplates coated with TNP-FICOLL (50ug/mL). Isotype specific secondary antibody labeled with alkaline phosphatase (Sigma-Aldrich) was used to determine anti TNP-FICOLL antibody binding (Rocha et al. 2010). Plates were read in a 405nm filter in microplate reader (Bio-Rad, Model 680) after one hour of incubation in the dark with phosphatase substrate (Sigma-Aldrich).

Histopathological Examination. At the end of the experiment period, the mice were killed in a CO_2 saturated chamber. The spleen, kidney and liver were removed and fixed with neutral-buffered 10% formalin. The paraffin sections were stained with hematoxylin and eosin and the sections were analyzed to identify the incidence of lesions.

Statistical Analysis. Results were expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) to determine the significant differences between the groups, followed by Student's Newman-Keul's test (Prism Graph Pad 5 software). Level of significance was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Preliminary studies were performed to rule out any toxic effect of piperine on B cells by treating the resting B cell line A20 and spleen cells with 1 - 150 µM piperine (Figure 2). The cytotoxic effect of 150 µM of piperine for A20 cells has been already observed at 24 hours of incubation. The cell viability was inversely proportional to culture exposure time: 44,94% (24 h), 29,82% (48h) and 19,29% (72h). The cell viability was not affected in the doses of 50 µM, 15 µM, 3 µM and 1 µM piperine even after 72 hours of incubation. The IC_{50} obtained for 72 hours was 118 µM ± 9.56.
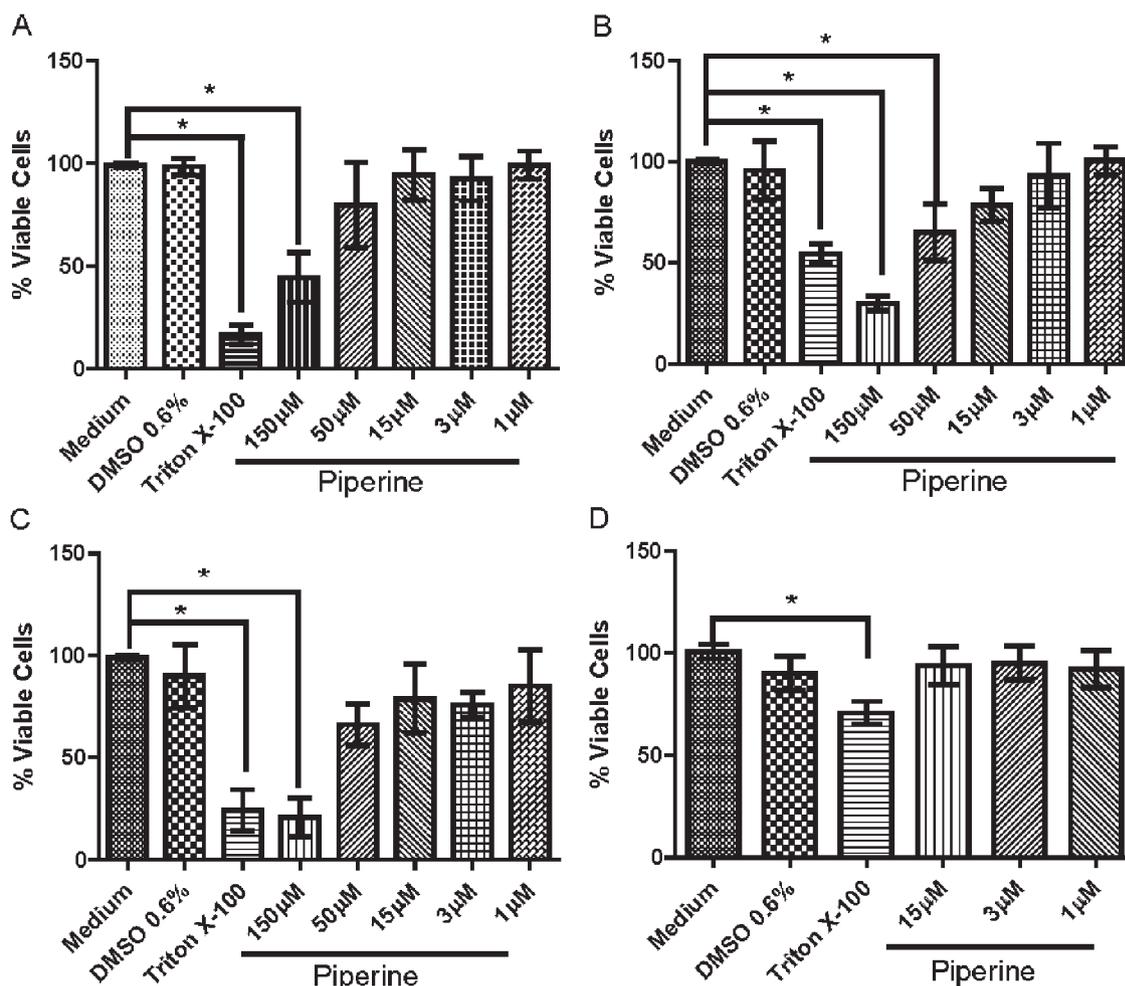

Figure 2. Effect of piperine on the viability of the cell line A20 and murine splenocytes by XTT. A20 cells were incubated with different doses of piperine (150µM, 50µM, 15µM, 3µM and 1µM) by 24h (A), 48h (B) and 72h (C) of incubation and the spleen cells were incubated with the same doses by 24 h (D). It was utilized DMSO 0.6% (negative control of dead) and Triton X-100 (positive control of dead). Bars represent means ± SD from n = 4. Data shown are representative of three independent experiments. *P<0.05%.

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Others studies have also shown that piperine (50 µg/ml or 175 µM) did not have cytotoxic effect in freshly isolated splenocytes and thymocytes obtained from mice after an 18 hours of incubation (Pathak & Khandelwal 2006, 2007). In our study, the spleen cells viability was not affected in the doses of 15 µM, 3 µM and 1 µM piperine after 24 hours of incubation and based on our results, we choose non-cytotoxic piperine concentrations (1, 3 and 15 µM) to evaluate its effect *in vitro* on B cell proliferation and on IgM secretion.

Murine spleen B cells were cultured with either LPS or anti-IgM antibody in the presence of different piperine concentrations. Addition of piperine at 15µM, inhibited the proliferative response induced by both LPS (50.43%) and anti-IgM antibody

Figure 4. Piperine inhibited IgM production induced by polyclonal B cell activator. B cells were incubated by 7 days with different doses of piperine in the presence or not of LPS(10µg/mL). IgM secretion was measured in the pooled supernatants by ELISA. Bars represent means ± SD from n = 3. *P<0.05%.
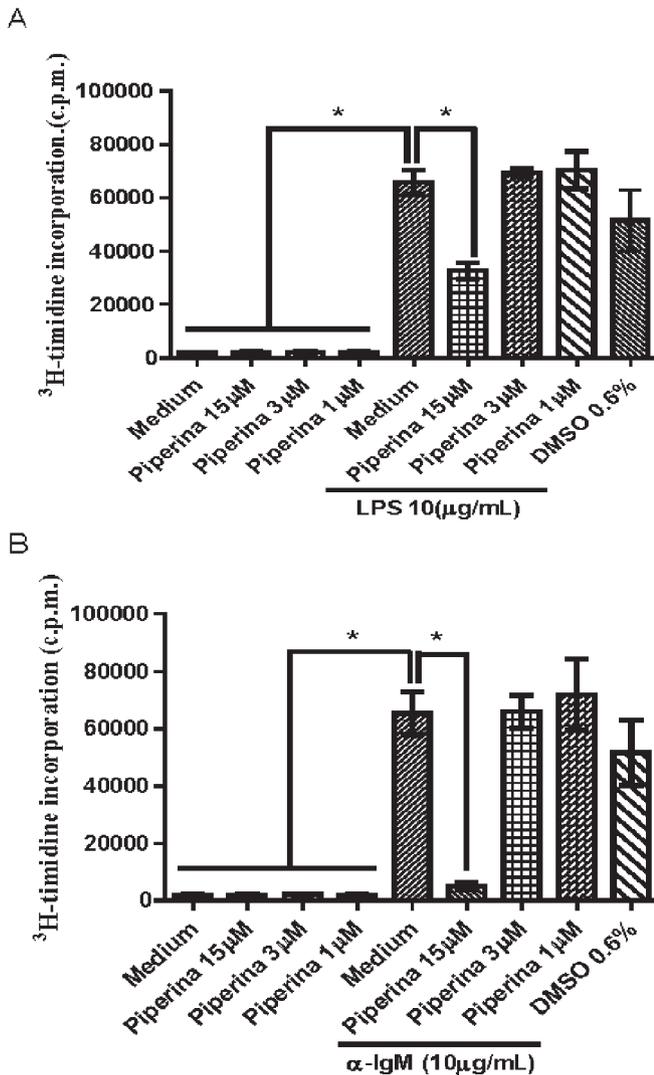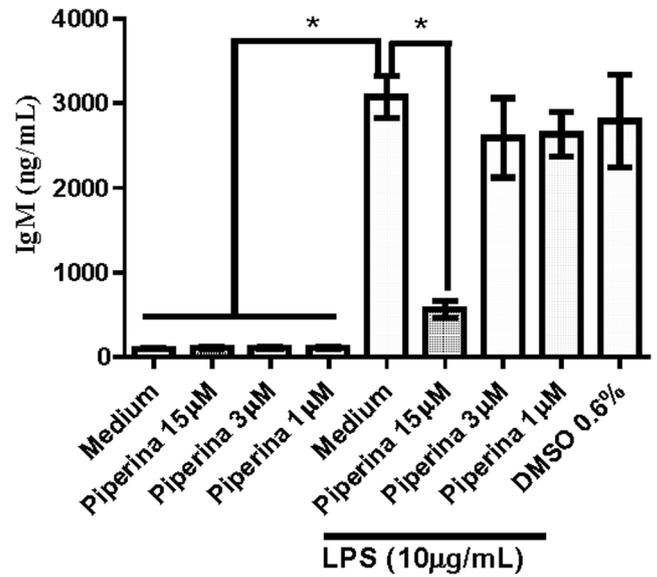

Figure 3. Piperine inhibited B cell proliferation by polyclonal B cell activators. B Cells were incubated for 72h with different doses of piperine in the presence or not of LPS (10µg/mL) (A) or goat anti-mouse IgM Ab (10µg/mL) (B). Data shown are representative of three independent experiments. *P<0.05%.
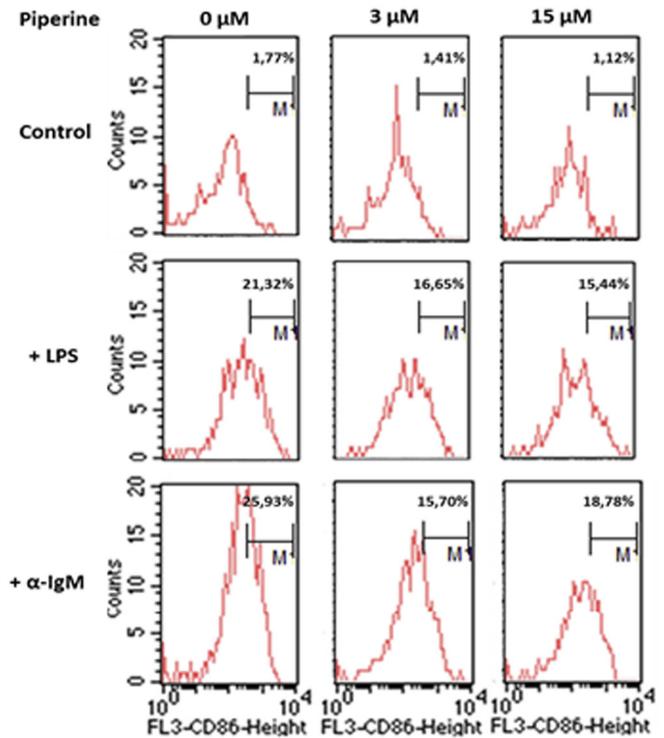

Figure 5. Piperine decreased CD86 expression on B cell subset. B cells were incubated by 48 hours after stimulation with LPS and goat anti-mouse IgM Ab in the presence or not of piperine (15µM and 3µM). B cells were marked with anti-B220 and anti -CD86 antibody and analyzed by FACS. Data shown are representative of the three independent experiments.

(92.47%) as showed in Figure 3. The addition of 3µM or 1µM of piperine was not effective to inhibit the B cell proliferation induced by either LPS (Figure 3A) or anti-IgM antibody (Figure 3B). In addi-

tion, piperine had no significant effect on the pro-liferation of non-stimulated B cells. These results suggest that 15 µM of piperine inhibits the murine B cell proliferation in vitro.

We, then, investigated the piperine effect on IgM antibody production. Murine spleen B cells were cultured in absence or presence of LPS and were in-cubated with different concentrations of piperine. The IgM secretion was reduced for about 81,72% when 15 µM of piperine was added to B cell cultu-res stimulated with LPS. However, this inhibitory effect was not observed the 3µM and 1µM doses of piperine were used (Figure 4). Piperine had no significant effect on the secretion IgM antibody by non-stimulated B cells. These results suggest that the dose of piperine which inhibits the B cells pro-liferation is able to inhibit the secretion IgM antibo-dy by murine B spleen cell in vitro.

To assess whether piperine would modify other B cell activation-induced events, we determined the expression of CD86 for the B220$^+$ cell popula-tion. Activation-induced CD86 (B7-2) expression on a B cell mediates regulatory signaling pathways in a T_H cell, through interaction with CD28 and CTLA-4 (cytotoxic lymphocyte antigen-4). In vitro and in vivo findings suggest that CD86 binding also generates an intracellular signal within a B cell, which positively regulates B-cell activity (Su-vas et al. 2002, Podojil & Sanders 2003). We obser-ved that addition of 15 µM of piperine decreased in 27,58% and 27,57%, respectively, CD86 expression on the B220$^+$ splenic B cell population stimulated with LPS and anti-IgM antibody. This reduction was also observed after the addition of the dose of 3 µM of piperine in B cell cultures stimulated with either LPS or anti-IgM antibody (21,90% and 39,45% inhibition, respectively) as shown in Figu-re 5. The down-modulation of CD86 and other cell surface molecules expression was observed after the treatment of bone marrow derived dendritic cell (BMDCs) cultures with piperine. BAE et al. (2012) cultivated BMDCs for 24 hours in presence of 1, 5 or 10 µM piperine, with and without LPS stimulation. Piperine treatment decreased cultured CD11c$^+$ dendritic cells CD86, CD40 and MHC-II ex-pression in a dose-dependent manner.

The in vivo effect of piperine is controversy and it seems that the administration route and the doses used can influence piperine action on the immune system. Dogra et al. (2004) evaluated the effect of piperine on immunological response and on cell homeostasis of mice in vivo. Oral doses of 1.12, 2.25 or 4.5 mg/Kg (which is equivalent to
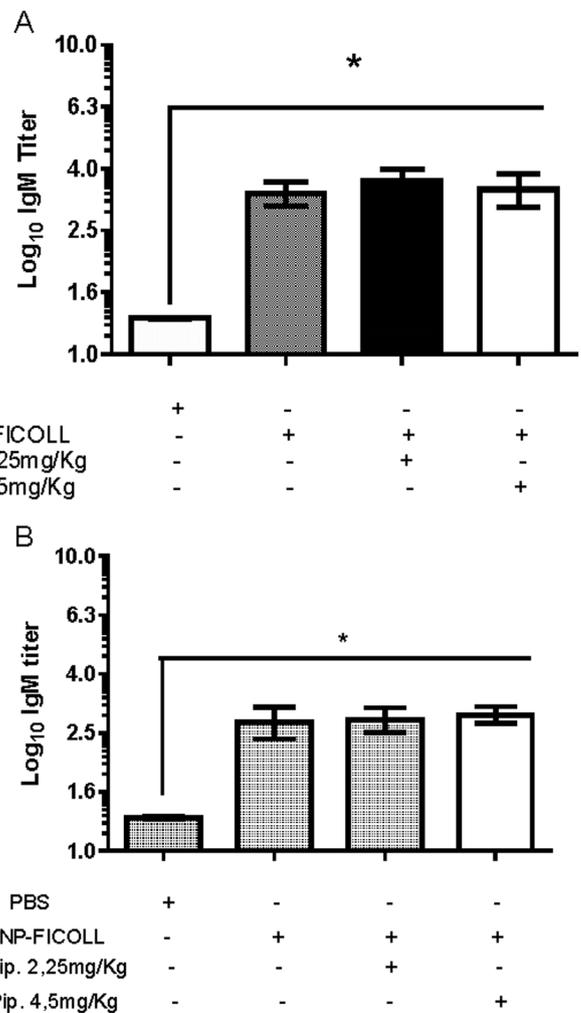

Figure 6. In vivo treatment with piperine does not mod-ulate the IgM production after immunization with polysaccharide antigen. Mice were treated with piper-ine (2,25mg/Kg or 4,5mg/Kg) by 5 consecutive days and at the fifth day, one hour after piperine treatment they were immunized with TNP-FICOLL. The ani-mals' plasma were obtained at 7° (A) and 14° (B) days after inoculation of TNP-FICOLL and their anti-TN-P-FICOLL titers were determined by antigen-specific ELISA. This graph represents analysis of two experi-ments. Bars represent means±SD. * $P<0.05$.

1/8, ¼ and ½ of the generally used dose in human) were used (Piyachaturawat et al., 1983). Dogra and collaborators (2004) observed these dose levels had no overt toxic effect, however, their administration the treatment resulted in significant reduction in total leukocyte counts and in the decrease of cell number in lymphoid organs, except for the spleen, where the doses of 1.12 and 2.25 mg/Kg caused an increase in cellularity and also an increased in the number of antibody (IgM) forming cells. Piperine at 2.25 and 4.5 mg/Kg suppressed the mitogenic response of B-lymphocytes to lipopolysaccharide and decreased both the number of IgM forming cells in the spleen and the level of serum IgM.

An additional study on the immunomodulatory and antitumor activity of *Piper longum* and piperine was performed by Sunila and Kuttan [27]. They described that intraperitoneal administration of either the *Piper longum* extract (10 mg/dose/animal) or piperine (1.14 mg/dose/animal) had antitumor activity and also increased the total leukocyte counts and the number of plaque forming cells.

In the present study, we evaluated in vivo effect of piperine on immunoglobuline production induced by thymus -independent (TNP-Ficoll). The doses 2,25mg/Kg and 4,5mg/Kg of piperine were utilized in this experiment because they did not show any toxic effect on spleen, liver and kidney by histopathological examination (data not show). TNP-Ficoll was able to stimulate the humoral response on 7º days, however piperine did not modulate antibody production in vivo (Figure 6).

CONCLUSION

We demonstrated that piperine could effectively inhibit the B cells proliferation, immunoglobulin production and lower the expression of CD86^{+} *in vitro* though this inhibitory effect was not verified in vivo. Piperine was unable to modify in vivo thymus-independent antigen -induced antibody response.

Conflict of Interest. The authors declare no conflicts of interest.

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