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Reduction of NO production in LPS-stimulated primary rat microglial cells by Bromelain

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Abstract

Microglia, the sentries of the brain, is highly implicated in neurodegeneration as in neuroprotection. Chronic microglial activation endangers neuronal survival through the release of various potentially neurotoxic mediators including Nitric Oxide (NO). Thus, negative regulators of microglial activation have been considered as potential therapeutic candidates to target neurodegeneration, such as those in Alzheimer's and Parkinson's diseases and even in chronic epileptic syndromes. Bromelain, a mixture of cysteine proteases, derived from pineapple stem, has been reported to have anti-inflammatory and immunomodulatory effects. Neonatal rat primary microglial cells were isolated from the brain according to the Floden's method. The purity of the cultures was determined by immunostaining with an OX-42 antibody which showed a purity greater than 95%.The activation profile of microglia was investigated by determining the effects of Bromelain (1, 10, 20, 30, 40 and 50 µg/ml) on the level of neurotoxin, NO, mitochondrial activity and morphological changes in treated microglia with lipopolysaccharide (LPS) (1µg/ml), as an endotoxin. Our results showed that pretreatment of primary rat microglia with bomelain (30 µg/ml), decreased the production of NO induced by LPS (1µg/ml) treatment in a dose-dependent manner, which prevented the deramification of microglia and its phagocytic morphology. Moreover, bromelain does not show cytotoxicity at any of the applied doses, suggesting that the anti-inflammatory effects of bromelain are not due to the cell death. In conclusion, Bromelain reduces the NO synthesis *in vitro* by potentially exerting its anti-inflammatory effects. Bromelain naturally found in pineapple stem, can be considered as a useful agent for neuroprotection and alleviation of symptoms in neurodegenerative diseases.

***Keywords*:** Microglia, Bromelain, CNS inflammation, NO, Neurodegeneration

Introduction

Microglia are the primary immune cells or resident macrophages which regulate inflammatory responses in the central nervous system (CNS) and often referred to as the tissue macrophage of the brain. In the unflustered brain, these cells invariably survey their environment (Hanisch and Kettenmann, 2007; Schwab and McGeer, 2008) and disturbances during neuropathological conditions, initiate reactive responses, known as 'activation', which are modelled through specific changes in their immunological and morphological phenotype to ultimately maintain brain homeostasis (Garden and Moller, 2006).

Activation of microglial cells involves proliferation, migration to the injured site, increased expression of immunomodulators, and transformation into phagocytes to access capability of scavenging effects (Dheen et al., 2007; Glezer et al., 2007). Secretion of some soluble proinflammatory molecules such as cytokines, chemokines, oxidative radicals, and nitric oxide is linked to activation of microglia (Minagar et al., 2002). The beneficial or detrimental effects of these molecules are related to their concentrations. Proinflammatory and cytotoxic factors produced by microglia have been shown to induce neurodegeneration. However, it is increasingly more appreciated that these factors may work synergistically to damage neurons (Chao et al., 1995; Jeohn et al., 1998; Skaper et al., 1996). Inflammation is now widely accepted to underlie the pathology of various neurodegenerative diseases, with evidence that the overactivation of microglia is a main participant and thus may be a target for therapeutic benefit (Block et al., 2007).

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Moreover, therapeutic intercession might be achieved by targeting primary microglia. In the healthy brain, microglia have a typical ramified morphology consisting of a small cell body and long processes with secondary branching. Changes in the microglial morphology are observed in a wide range of the CNS pathologies such as brain injury, ischemia, infection, autoimmunity, and neurodegenerative diseases (Suzumura et al., 1991). Under these pathological conditions, ramified microglia transform into amoeboid microglia, with their processes retracted and their cell body enlarged. Such a morphological transformation is associated with microglial activation and is induced *in vitro* by a variety of stimuli including LPS (Bohatschek et al., 2001; Giulian et al., 1995; Kloss et al, 2001).

LPS is an endotoxin from Gram-negative bacteria, which arouses inflammatory and immunological responses. Through binding to LPS-binding protein (LBP) in plasma, LPS is delivered to the cell surface receptor CD14. Then, LPS is transferred to the transmembrane signaling receptor toll-like receptor 4 (TLR4) and its accessory protein MD2. LPS-stimulated murine microglia, macrophages, and Kupffer cells activate the inflammatory responses and subsequently, production of inflammatory molecules (Geppert et al., 1994; Jeng et al., 2005).

Since chronic microglial activation has been implicated in the neuronal destruction associated with various neurodegenerative diseases, the activation of counter-regulatory mechanisms is essential to avoid the exacerbation of CNS inflammatory processes (McCarthy, 2006). This may be possible via the identification of agents that target overactivated microglial cells and the determination of their anti-inflammatory mechanisms.

Bromelain is an extract from pineapple stem (*Ananas comosus*) which is known for its anti-inflammatory effect (Seligman, 1962). Many Pharmacological activities of bromelain have been reported, such as regulation of immune functions, anti-inflammation, anti-edema, anti-hypertension, reduction of thrombogenesis and inhibition of cancer cell growth (Chandler and Mynott, 1998; Maurer, 2001; Taussing and Batkin, 1988). The pleiotropic therapeutic effects of bromelain are considered to be due to the complex natural mixture of closely related cysteine proteinases, proteinase inhibitors, phosphatases, glucosidases, peroxidases, and other undefined compounds (Harrach et al., 1998; Harracj et al., 1995). In addition, bromelain has demonstrated to have both antiproliferative and antimetastatic effects in tumor models *in vitro* and *in vivo* (Grabowska et al., 1997; Taussig et al., 1985). Bromelain can simultaneously enhance and inhibit immune cell responses *in vitro* and *in vivo* through a stimulatory action on accessory immune cells and a direct inhibitory action (Engwerda et al., 2001; Hou et al., 2006).

In human macrophages/monocytes and mixed lymphocyte culture, bromelain induced a significant increase in interleukin (IL)-6, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFNγ) (Barth et al., 2005; Rose et al., 2005). Bromelain also enhanced IFNγ mediated TNF-α and NO production by murine macrophages. Bromelain's effect is independent of endotoxin receptor activation and is not caused by direct modulation of IFNγ receptors. Instead, bromelain either enhances or acts synergistically with IFNγ receptor-mediated signals (Engwerda et al., 2001).

On the other hand, bromelain blocks activation of extracellular signal-regulated kinase (ERK) in Th0 Cells stimulated via the T cell receptor, or stimulated with combined PMA and calcium ionophore. However, this inhibitory activity of bromelain is dependent on its proteolytic activity (Mynott et al., 1999). Bromelain also reduced LPS-induced cyclooxygenase 2 (COX2) mRNA, in bv2 microglial cells (Jeng et al., 2005). However, the efficacy of bromelain in reducing inflammation of primary microglial cell has not been reported.

Thus, we examined whether bromelain, as a natural plant extract, repress microglia activation and thereby confer neuroprotection against inflammation-related neuronal injury. So, in this study, highly enriched microglial primary cultures obtained from newborn rat cortical regions were cultured in the presence of LPS and bromelain. Activation, cytotoxicity and release of the oxidative and inflammatory factor, NO, were investigated.

Materials and Methods

Materials and reagents

Fetal bovine serum (FBS), Dulbecco's Modified Eagle Medium (DMEM) and Griess reagent were purchased from GibcoBRL (Grand Island, NY, USA). LPS (E5:055), 2', 7'-dichlorodihydrofluororescein diacetate, bromelain, E64 and Thiazolyl blue (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). FITC conjugated anti-OX42 antibody to rat CD11b/c was from CALTAG (Sketty, Swansea).

Inhibition of proteolytic activity of bromelain

Bromelain (10 mg/ml) diluted in 3 μM dithiothreitol (DTT) was incubated with 100 μM E-

64 and 60 mM sodium acetate (pH 5) for 10 min at 37°C. The inactivated bromelain was then dialyzed overnight in phosphate-buffered saline (PBS) at 4°C. The total inactivation of bromelain was achieved as assayed with the casein (Huang et al., 2008).

Cell culture

Primary microglial cells were prepared from cerebral cortices of one-day-old rat pups as described previously (Jung et al., 2003; McCarthy and De Vellis, 1980). Briefly, Cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 100 UI/ml penicillin G, 100 µg/ml streptomycin, 2 mmol/L L-glutamine, 0.011 g/L pyruvate, and 10% fetal calf serum, seeded on polystyrene culture dishes (Nunc), and incubated in a humidified atmosphere with 5% CO2 at 37°C. To obtain primary microglia rich mixed cultures, after 2 days, all media and tissues were removed and fresh media was replaced.

Isolation of microglia

After the cells became confluent at 12–14 days (figure 1), the flasks were shaken to remove the microglia (Floden and Combs, 2007). Microglia was plated onto 96 well tissue culture plates (20,000 cells/well). The detached microglial cells were incubated for 1 h, and the non-adherent cells were removed. The adherent microglial cells were cultured for 24 h, and the purity of the cultures was routinely greater than 95% as judged by immunostaining with an anti-OX-42 antibody (figure 2).

Drug treatment

Primary microglia were pretreated with bromelain (1, 5, 10, 20, 30, 40 and 50 µg/ml) in fresh medium containing 1% fetal bovine serum (FBS) for 1 h before LPS (1 µg/ml) was added and then were incubated for 24 and 48 h.

Cell viability assay

For the cell viability assay, After various treatments, the medium was removed and the cells were incubated with MTT (3-4-5-dimethylthiazole-2-yl-2, 5-diphenyl-tetrazolium bromide) solution (1 mg/ml) in two volumes of culture medium for 4 h at 37˚C. The MTT solution was then removed, the formazan crystals in cells were dissolved in DMSO (dimethyl sulfoxide), and the level of MTT formazan was determined by measuring its absorbance at 580 nm using a microplate reader.

Nitrite assay

The production of NO was assessed as nitrite (NO_2) accumulation in the culture medium of three independent experiments 24 and 48 h after treatment, using a colorimetric test based on Griess reagent (Wilms et al., 2007) and a standard $NaNo_2$ solution was used for the standard curve.

Statistical analysis

One way ANOVA followed by the LSD test was used to determine the statistical differences among groups. Student's t-test was used to compare two groups. Values of $p<0.01$ were considered as significant, compared with the LPS-treated group without bromelain.

Results

Morphological assessment of primary rat microglia exposed to bromelain

Different morphological phenotypes of primary rat microglial cells observed with phase contrast microscope such as ramified morphology in the control isolating microglia, ameboid activated Microglial cells and intermediate phenotypes in the effective concentrations (10-30 µg/ml) of bromelain, which prevented the deramification of microglia in a dose-dependent manner (figure 3).

Bromelain decreases production of NO in LPS-stimulated primary microglia

We initially determined the effect of bromelain on NO expression in LPS-stimulated primary microglia. To analyze NO production, primary microglia were pretreated with bromelain (1, 5, 10, 20, 30, 40 and 50 µg/ml) for 1 h prior to stimulation with LPS (1 µg/ml) for 24 and 48h, the levels of NO in the culture media were determined using the Griess assay. As shown in figure 4A, LPS alone was able to markedly induce NO production from the cells as compared to that in the control. However, bromelain at 10-30 µg/ml effectively diminished the levels of NO production in LPS-stimulated primary microglia in a dose-dependent manner and a significant decrease of NO was observed only in LPS-stimulated microglial cultures exposed to 30 µg/ml of bromelain after 48 h.

Evaluation of toxicity of bromelain concentrations

To exclude the possibility that the cytotoxic action of bromelain inhibited LPS-stimulated NO production, we investigated the effect of bromelain on cell viability. Under the experimental conditions described above, there was no significant reduction

in cell viability using the MTT assay (figure 4B). Therefore, the inhibitory effect of bromelain on LPS-stimulated NO production was not due to bromelain's cytotoxic action on primary microglia.

Effects of post-treatment of bromelain on LPS-stimulated inflammatory responses

To determine if post-treatment of bromelain has neuroprotective action as well, microglia were stimulated with LPS first and then exposed to bromelain. Thus, we performed the experiment by adding bromelain 1 h after the LPS exposure. Bromelain didn't confer protective effects against LPS-induced nitrite release when exposed after the LPS stimulation. These results demonstrated that only pre- and not the post- treatment with the bromelain, suppressed the LPS-induced NO production in primary rat microglial cells.

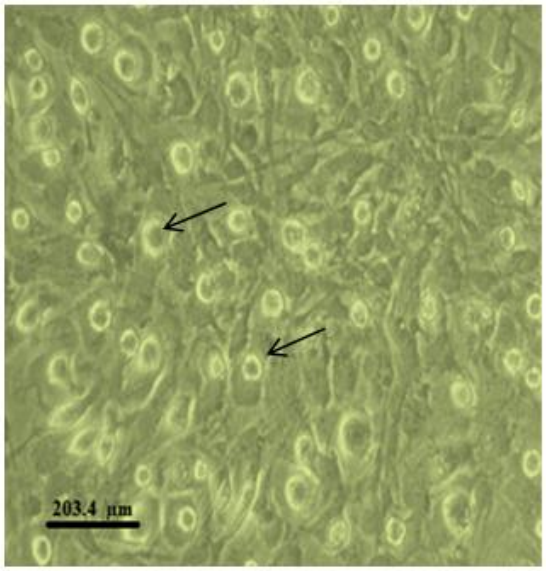


Figure 1. Confluent primary microglia-rich mixed culture at 12–14 days. Loosely adherent microglial cells (arrows) can be removed easily by shaking such a culture.

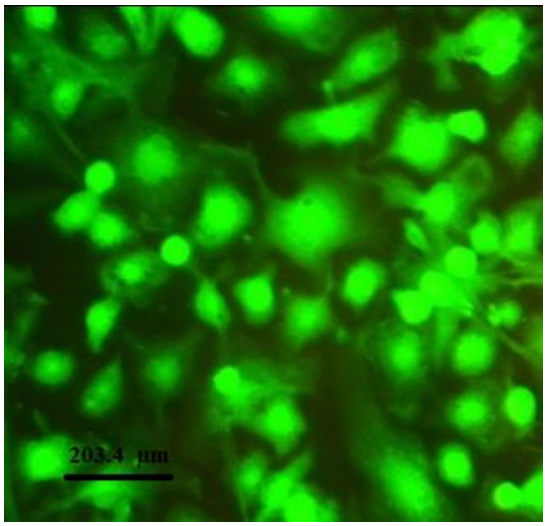


Figure 2. Isolated microglial cells immunostained with an anti-OX-42 antibody, showed more than 95% purity.

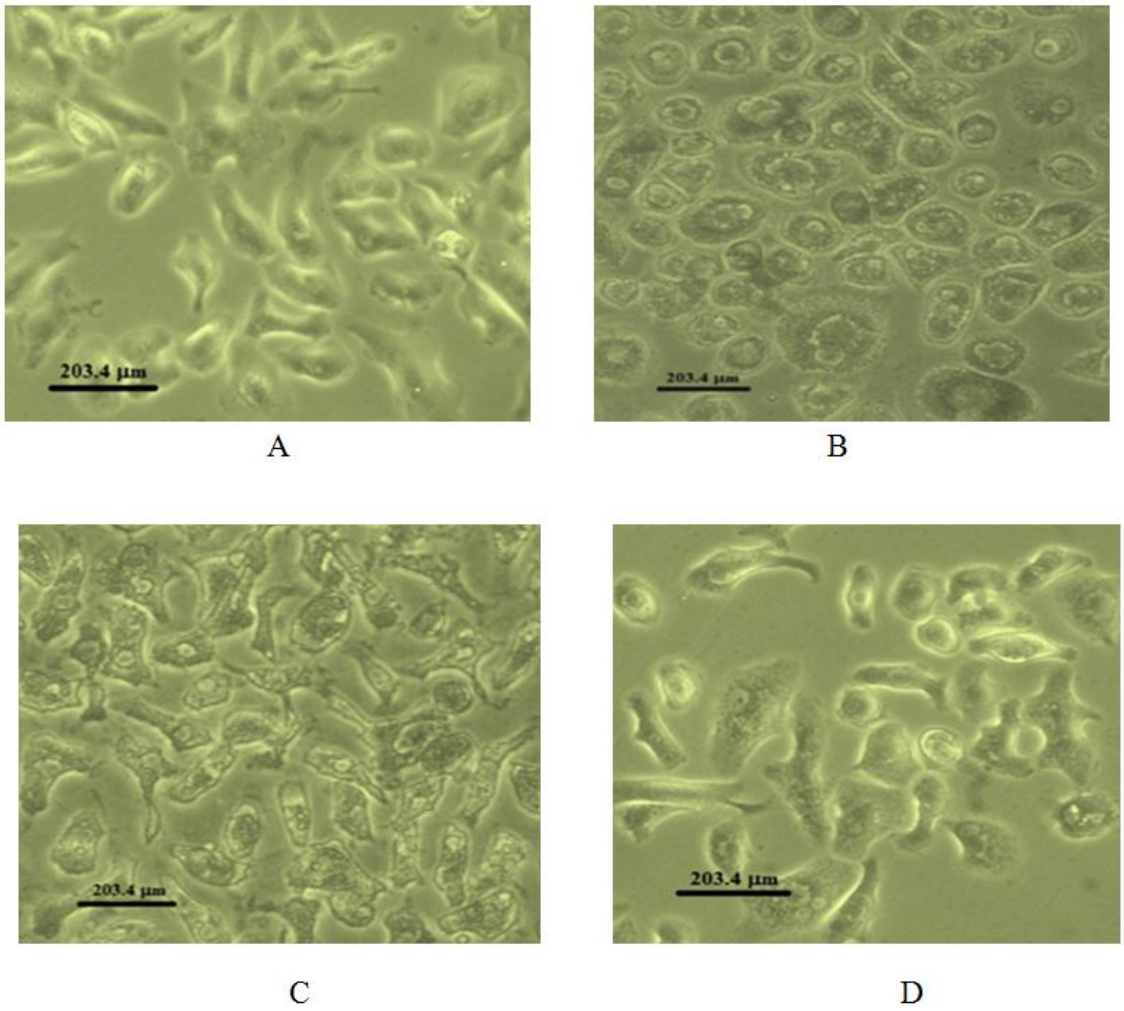
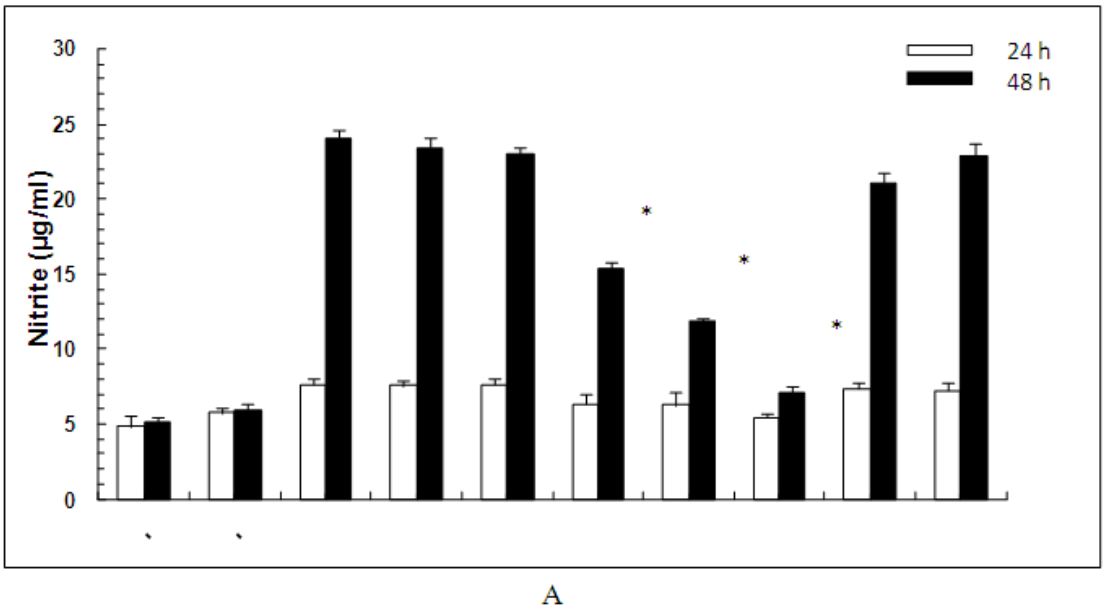


Figure 3. The effects of bromelain on microglial morphology. Isolated microglial cells were either untreated (A) or treated for 48 h with LPS 1 µg/ml (B), bomelain 10 µg/ml (C) and bromelain 30 µg/ml (D) for 1 h before LPS, and then different cellular morphology was examined under phase contrast microscope such as typical ramified morphology in control cells (A), amoeboid morphology in LPS-treatment microglia (D) and intermediate phenotypes in the effective concentrations of bromelain (B, C) which prevented the deramification of microglia in a dose-dependent manner. It should be mentioned that figures are from different fields and in fact treatments didn't have any effect on the number of the cells.



A

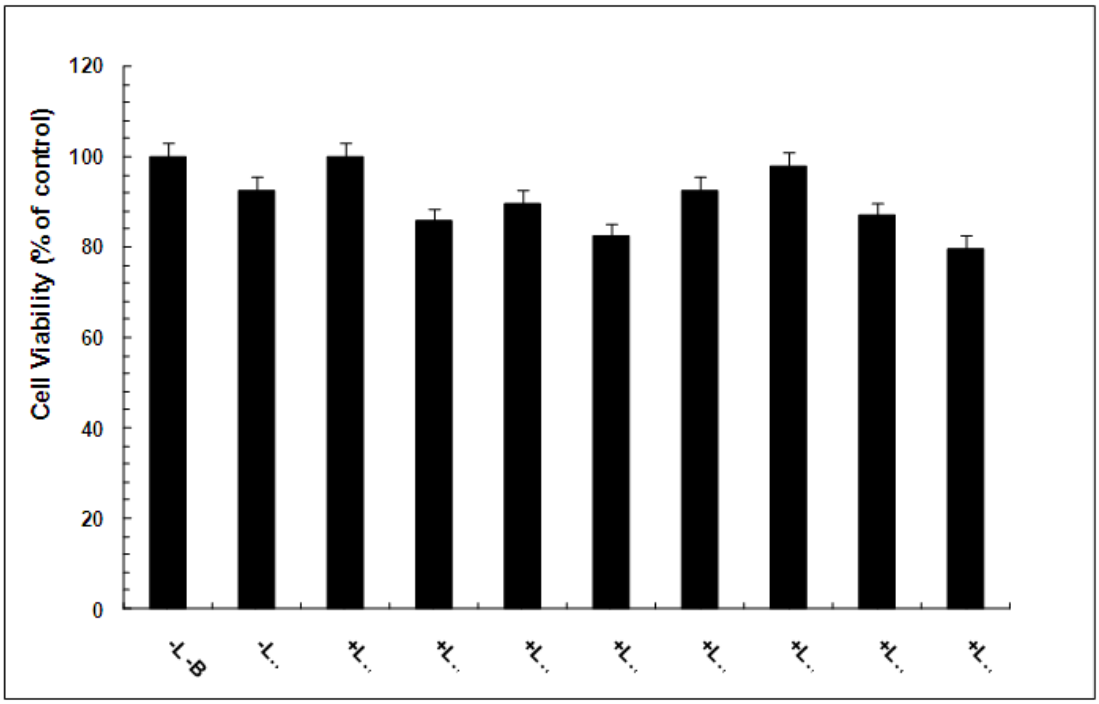


Figure 4. Effects of bromelain on NO production in LPS-stimulated microglial cells. Primary microglial cells were incubated in the absence or presence of LPS (1 µg/ml). The cells were pretreated with various amounts of bomelain (1, 5, 10, 20, 30, 40 and 50 µg/ml) for 1 h before LPS was added. The cultures were subjected to a nitrite assay after 24 and 48 h (A) and a cell viability assay after 48 h (B). *P < 0.01 as compared with the LPS-treated group without bromelain.

Discussion

Activated microglia produce various proinflammatory cytokines and free radicals such as NO, that have an important role in the process of neuroinflammatory diseases. Several lines of evidence have indicated that the NO production is upregulated in the activated microglia (Mcgeer et al., 1993). In this study, we demonstrated that bromelain, a mixture of cysteine proteases of the pineapple stem, decreased inflammatory activation of microglia in culture.

So far, contradictions exist for bromelain effects on inflammation. Bromelain has been shown to simultaneously enhance and inhibit immune cell responses *in vitro* and *in vivo* (Engwerda et al.,2001; Hou et al., 2006). Although bromelain 50 µg/ml increased the production of IFN-γ stimulated nitrite in murine macrophage cell line (Engwerda et al., 2001), elsewhere, in concentration of 100 µg/ml, it significantly inhibited the enhanced production of LPS-induced nitrite in same cell line (Wen et al., 2006). Bromelain also have anti-inflammatory effects on LPS activated microglial cell line (Hou et al., 2006).

Our results showed that bromelain (30 µg/ml) reduced the LPS-stimulated NO production in primary rat microglia cells in a dose- and time-dependent manner. Based on the previous reports and the present results, it is clear that bromelain effects differ due to the cell type and the used doses.

It is also demonstrated that only pre and not the post- treatment with the bromelain, suppressed the LPS-induced NO production in primary rat microglial cells. So, this result makes bromelain more useful for prevention than for treatment of microglia- mediated inflammation.

Microglia has been related to disease progression and pathology in several neuroinflammatory diseases such as Alzheimer's diseases, Parkinson's diseases and HIV dementia (Block et al., 2007; McCarthy, 2006). Microglia activation has both beneficial and harmful effects on neuronal injury in neurodegenerative diseases. Overactivation of microglia contribute to neurodegenerative processes through the production of various neurotoxic factors including NO (Klegeris et al., 2007).

In this study, the level of NO as a proinflammatory cytokine was evaluated in activated primary microglia treated with bromelain. NO, as well as other proinflammatory cytokines have been implicated as important mediators in the

process of inflammation (Possel et al., 2000). Microglia activation induced by CNS injury or infection is associated with neurodegeneration and the release of NO (Gonzalez-Scarano and Baltuch, 1999). Excessive production and accumulation of nitric oxide is deleterious to neurons in the inflammation-mediated neurodegenerative processes (Schmidt and Walter, 1994). Thus, it is suggested that the search for the efficient anti-inflammatory compounds that attenuate microglial activation may lead to an effective therapeutic approach against many neurodegenerative conditions.

Our results as well as previous reports showed that LPS stimulation of microglia induced a morphological change of the cells into the round shape with a loss of processes, and activates the production of NO as a proinflammatory cytokine (Nakajima et al., 2003; Suzumura et al., 1991).

We found that pretreatment of primary microglia cultures with bromelain 30 µg/ml, largely prevented the deramification of microglia.

We also showed that the potent anti-inflammatory effects of bromelain due to a decreased production of NO is dose-dependent (10-30 µg/ml). Moreover, bromelain does not show cytotoxicity at any of the applied doses, suggesting that the anti-inflammatory effects of bromelain are not due to the cell death. The effect of bromelain on reducing proinflammatory mediators such as NO suggests that bromelain is a useful therapeutic agent.

Further studies are, however, required to evaluate a neuroprotective property of bromelain in the animal models of neurodegenerative diseases, and to understand the precise molecular mechanisms of anti-inflammatory actions of the bromelain *in vitro* as well as *in vivo*.

Nevertheless, this is the first study that has demonstrated anti-inflammatory effects of bromelain in primary microglia, suggesting the neuroprotective effects of bomelain against inflammation-mediated neurodegeneration. Future works along with this line will give rise to a novel therapeutic use of the bromelain for the treatment of neurodegenerative diseases and other inflammatory disorders.

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