Immunomodulatory effect of nigella sativa on bovine peripheral blood mononuclear cells

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Abstract

Nigella sativa (Family Ranunculaceae), commonly known as black seed or black cumin, is an annual plant that has been traditionally used in the Arabian countries. It was found to have a variety of effects including bronchodilator and calcium antagonistic effects, hepatoprotective and immunopotentiating effects.

PBMC are possibly involved in the pathogenesis of various diseases through the release of numerous mediators. In the present study, we investigated the regulation of IL-1β, IL-6, IL-8, IL-10 and TNF- α mRNA expression in bovine peripheral blood mononuclear cells (PBMC) in response to Nigella sativa extract in the absence or presence of lipopolysaccharide (LPS). Blood samples were collected from Holstein cows (aged 2-4 years). PBMC were separated and treated with/or without, LPS 10 ng/ml, Nigella sativa soluble extract 10 µl/ml or combination of both of them for 24 hours. PBMC were then collected and used for the RNA extraction. IL-1β, IL-6, IL-8, IL-10 and TNF-α mRNA were then measured by semi-quantitative RT-PCR. LPS treatment of bovine PBMC stimulated the mRNA expression of tested cytokines. Nigella sativa soluble extract treatment significantly increased the mRNA expression of IL-1β, IL-8. In the presence of LPS, treatment of PBMC with Nigella sativa soluble extract reduced the LPS-induced TNF-α and IL-6 mRNA expression while increased the expression of IL-10 mRNA. These findings suggest an immunomodulatory role for Nigella sativa soluble extract on bovine PBMC through increasing the production of chemokines IL-8, IL-1 β anti-inflammatory effect through down-regulation of IL-6 and TNF-α mRNA expression and a regenerative effect through induction of IL-10 mRNA expression.

Key words: Nigella sativa, bovine PBMC

Introduction

Nigella sativa (family Ranunculaceae), commonly known as black seed or black cumin, is an annual plant that has been traditionally used in the Arabian countries (Sayed, 1980). Historically, it has been recorded that Nigella sativa seeds were prescribed by ancient Egyptian and Greek physician to treat headache, nasal congestion, Toothache, and intestinal worms, as well as a diuretic to promote menstruation and increase milk production El-Dakhakhny et al 1965. Pharmacological study of the plant showed its broad traditional therapeutic value. It was found to have bronchodilator and calcium antagonistic effects (Gilani et al., 2001), analgesic effects (Abdel-Fattah et al., 2000), antiulcer (Akhtar et al., 1996), hepatoprotective (Daba and Abdel-Rehman, 1998) and immunopotentiating

effects (Swamy and Tan, 2000). The seed of Nigella sativa contains more than 30% of a fixed soluble and 0.40-0.45 w/w of a volatile oil. The volatile oil has been shown to contain 18.4-24% thymoquinone and 46% many monoterpenes such as p-cymene, and α-pinene (El Tahir et al 1993).

Cytokines such as TNF-α and IL-1 are produced in large quantities during systemic inflammation and are responsible for the pathophysiological responses that accompany endotoxemia (Luster et al 1994, Harbrecht et al 1994, Aono et al 1997). Interleukin (IL)-10 is a major anti-inflammatory cytokine that potently inhibits production of pro-inflammatory mediators as TNF-α and IL-1 (Cassatella et al 1993). Recombinant IL-10 reduced TNF-α release in response to LPS and protected against LPS induced mortality in mice (Gerard et al 1993). Nigella sativa oil (N.O) was recently subjected to considerable pharmacological investigations that revealed its antioxidant activity in different organs (Houghton et al., 1995; Mansour, 2000). Importantly Nigella sativa oil is traditionally used as anticancer drugs alone or in combination with other anticancer drugs (Salomi et al., 1991; 1992; Worthen et al., 1998). However, the effect of Nigella sativa extract on bovine PBMC remains unknown. In this study we checked the effect of Nigella sativa soluble extract on bovine PBMC function.

Materials and methods

Six Holstein cows (age 2-4 years) without being milked for more than one year and without having any clinical symptoms were used for multiple blood sampling. Blood was collected from jugular vein into heparinized tubes and peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation of blood over Ficoll-Paque PLUS (Amersham Biosciences Co., Piscataway, NJ, USA) as previously described (Yamaji et al., 2004). PBMC were washed twice in phosphate-buffered saline (PBS) containing 1 mM EDTA and resuspended in RPMI1640 medium (Sigma–Aldrich) containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, 2.38 mg/ml HEPES, and 110 μg /ml sodium pyruvate.

Preparation of soluble extract was done as described previously (Afrozul Haq et al 1999). Briefly, 100 g of nigella sativa seeds was washed with 1 L of deionized water, dried and powdered. 20 g of powder was dissolved in 200 ml phosphate buffered saline (pH 6.4) and centrifuged at 10000 rpm for 30 min at 4 Ċ. The clear supernatant was collected as the soluble extract after removal of the oily layer and insoluble pellet. Protein concentration was determined by Lowery. Other chemicals and solvents were of analytical grade.

To examine the effects of Nigella sativa on cytokine mRNA expression in PBMC, the cells (5×10^6 cells) were cultured in 6-well plates either in the absence or presence of Nigella sativa extract (10 μg/ml) and/or lipopolysaccharide (LPS, 10 ng/ml) at 37 Ċ in 5% CO_2 humidified atmosphere for 24 h. Total RNA was extracted from the cells with TRIzol (Invitrogen, Carlsbad, CA, USA), and 2 μg of RNA were reverse-transcribed with oligo-dT primer and M-MLV reverse transcriptase (Invitrogen). The mRNA expression of IL-1β (interleukine-1β), IL-6 (interleukine-6), IL-8 (interleukine-8), IL-10 (interleukine-10), tumor necrosis factor TNF-α, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were detected by a conventional RT-PCR method using 0.5 μM respective primer sets specific for each gene (Table1). The PCR conditions are also summarized in Table 1. The PCR products were analyzed in 1.5 % agarose gel electropheresis

with ethidium bromide staining. Intensities of bands were analyzed densitometrically using NIH Image program (http://rsb.info.nih.gov/nih-image/).

Statistical analysis: Results are expressed as means ± S.E. Statistical analysis was performed using analysis of variance (ANOVA) and Fischer's protected least–significant difference test with $p < 0.05$ as statistically significant.

Results

Effect of LPS and Nigella extract on IL-1ß and IL-6 mRNA expression:

Treatment of the PBMC with LPS upregulated IL-1ß mRNA expression 4 folds that of control. Treatment of the PBMC with Nigella sativa extract also upregulated IL-1ß mRNA expression to the same level induced by LPS while treatment of the PBMC with combination of LPS and Nigella extract showed a tendency to decrease the IL-1ß mRNA than its expression induced by either LPS or Nigella extract treatment alone (Fig .1). Treatment of PBMC with LPS caused induction of IL-6 more than 7 folds that of control while Nigella extract treatment alone did not show any increase. Treatment of PBMC with Nigella extract in combination with LPS caused a reduction of the IL-6 mRNA expression than its expression due to LPS treatment alone (Fig 2). Although the downregulation of IL-6 mRNA by Nigella soluble extract treatment in presence of LPS did not reach the statistically significant level, it shows a clear tendency of downregulation. These results indicate the anti-inflammatory effect of Nigella soluble extract to be at least in part through down regulation of IL-6 mRNA expression.

Effect of LPS and Nigella soluble extract on IL-8 and IL-10 mRNA expression:

Interleukin 8 (IL-8) is a potent chemotactic and activating agent for human neutrophils. Treatment of PBMC with LPS downregulated IL8 than control while Nigella extract treatment upregulated its expression. Moreover Treatment of PBMC with Nigella soluble extract combined with LPS reduced the LPS-inhibitory effect on IL8 expression (Fig 3). IL10 is well known to have an anti-inflammatory effect. Treatment of PBMC with LPS induced the expression of IL10 clearly higher than control. Treatment of PBMC with Nigella extract showed only slight increase in the IL10 mRNA expression however when combined with LPS treatment, Nigella extract produced a further increase in the IL10 mRNA expression (Fig 4). These results indicate the immunomodulatory role of Nigella sativa extract through induction of the chemo-attractant IL8 and the anti-inflammatory IL10 expression.

Effect of LPS and Nigella extract on TNF- α mRNA expression.

Cytokines such as TNF-α and IL-1ß are produced in large quantities during systemic inflammation and are responsible for the pathophysiological responses that accompany endotoxemia (Luster et al 1994, Harbrecht et al 1994). Treatment of PBMC with LPS induced TNF-α mRNA more than seven folds that of control .whereas treatment of PBMC with Nigella sativa soluble extract produced only slight increase of the TNF-α mRNA expression. Importantly, treatment of PBMC with Nigella extract combined with LPS downregulated the LPS-induced TNF-α mRNA expression (fig.5). These results indicate the anti-inflammatory effects of Nigella sativa extract to be through downregulation of TNF-α during inflammatory process.

Discussion

The present study demonstrates that Nigella sativa soluble extract treatment modulated the LPS-induced inflammatory conditions in the PBMC. Nigella sativa is an important medical herb since ancient times as a natural remedy for a wide range of diseases but its mechanism of action is still unclear (Ali and Blunden, 2003). TNF-α and IL-1□ are secreted by peripheral blood mononuclear cells when these cells are exposed to inflammatory agents (Haq et al 1999). In our study, treatment of PBMC with LPS increased IL-1□ mRNA expression that also was increased by Nigella sativa soluble extract treatment. However, Nigella sativa extract downregulated the LPS-induced level of interleukin-6 (IL6). Indeed IL-6 was described as an important mediator of inflammation that is triggered by TNF-α and IL-1□. The anti-inflammatory effect of Nigella sativa soluble extract could be attributed to its ability to downregulate the expression of IL6 rather than IL-1β.

IL-8 is produced by most cells of the body especially macrophages and endothelial cells and is involved in inflammation and cell migration. IL-8 in particular is a powerful inducer of neutrophil chemotaxis (Feldmann, 1998). In our study the induction of IL8 by Nigella soluble extract implies the immmunopotentiation effect through stimulation of the chemotactic IL8 transcription. This result is in line with the reported induction of IL8 in human PBMC by protein fraction of Nigella sativa soluble extract (Haq et al 1999). This was explained, as one of the potential mechanisms that might mediate the modulator effect of N. sativa on inflammatory, immune responses is an alteration of trafficking of the inflammatory cells via modulating expression of chemokines and/or adhesion molecules (Salem 2005). He also stated that the inhibition of the inflammatory cytokines IL-1□, TNF-$\alpha$and enhancement of the chemokine IL-8 by N. sativa might give an indication of this effect (Salem 2005). The anti-inflammatory cytokines down-regulate the inflammatory process, in part by suppressing production of the proinflammatory cytokines, and therefore help to balance the inflammatory response (Gerard et al 1993 and Howard et al 1993).

IL 10 is well known to have an anti-inflammatory effect (Stuart et al 2006). In our study, treatment of PBMC with nigella sativa soluble extract showed induction of IL10 either alone or when combined with LPS.

It was stated that after activation, CD4 T helper cells differentiate into either TH1-type cells, secreting IL-2, IL-12, IFN-□ and TNF-α, or TH2-type cells secreting IL-4, IL-5, IL-10, and IL-13. Indeed, the balance between TH1 and TH2 cytokines is critical for the orientation of the inflammatory response toward cell-mediated or humoral-mediated responses. Thus, any factors that can interfere with TH1/TH2 axis might affect the outcome of the response (Lucey et al 1996). The potentiation of IL10 expression by Nigella sativa soluble extract treatment of PBMC in our study implies its anti-inflammatory role through balancing inflammatory cytokines and favoring the anti-inflammotory ones. Interleukin (IL)-10 was stated to be a major anti-inflammatory cytokine that potentially inhibits production of pro-inflammatory mediators as TNF-α and IL-1β (Cassatella et al 1993). Recombinant IL-10 reduced TNF-α release in response to LPS and protected against LPS induced mortality in mice (Gerard et al 1993). TNF-α is an important cytokine in immune response produced by macrophage (one of PBMC), and is one of major mediators in inflammatory response (Dinarello et al 1989, Kishimoto et al 1989). In the present study, Nigella sativa soluble extract treatment of PBMC increased the TNF-α mRNA expression however it did not reach the statistically significant level. On co-treatment with both Nigella sativa soluble extract and LPS, Nigella sativa soluble extract significantly inhibited the LPS-induced TNF-α mRNA expression that confirms the anti-inflammatory effect of Nigella sativa soluble extract. This result supports the anti-inflammatory effect of Nigella sativa soluble extract and parallel to results of our previous *in vivo* study (Shaban Z. et al, unpublished data) where Nigella sativa soluble extract clearly inhibited TNF-α mRAN expression in rats.

In conclusion, we clearly demonstrated the immmunomodulatory effect of Nigella sativa soluble extract on bovine PBMC. We also demonstrated that Nigella sativa soluble extract has anti-inflammatory effect through inhibiting LPS-induced expression of cytokines including TNF-α, IL-6 mRNA and through induction of IL-10 mRNA expression.

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Table 1. PCR primers

IL-1β (M35589, 726bp) [60 °C, 1 min, 30 cycles]
Forward primer: 5'-ATGGCAACCGTACCTGAACCCA -3'
Reverse primer: 5'-GCTCGAAAATGTCCCAGGAA -3'
IL-6 (EU276071, 624 bp) [58 °C, 1 min, 30 cycles]
Forward primer: 5'- ATGAACTCCCGCTTCACAAG-3'
Reverse primer: 5-CTACTTCATCCGAATAGCTCTCA-3'
IL-8 (EU490318, 308bp) [58 °C, 1 min, 30 cycles]
Forward primer: 5-ATGACTTCCAAACTGGCTGTTGC-3
Reverse primer:5- TCATGGATCTTGCTTCTCAGCTC-3
Bovine IL-10 (EU276074, 416 bp) [61 °C, 1 min, 30 cycles]
Forward, 5'-CTACTCTGTTGCCTGGTCTTCCT-3'
Reverse, 5'-CTTCACCTTCTCCACCGCCTTGC-3;
TNF-α (AF348421, 467bp) [60 °C, 1 min, 30 cycles]
Forward primer: 5'-ACTCAGGTCCTCTTCTCAAGCC-3'
Reverse primer: 5'-ATGATCCCAAAGTAGACCTGCC-3'
G3PDH (U85042, 452bp) [59 °C, 30 sec, 25 cycles]
Forward primer: 5'-ACCACTGTCCACGCCATCAC-3'
Reverse primer: 5'-TCCACCACCCTGTTTGCTGTA-3'

In the parenthesis, GenBank accession number of bovine genes and size of PCR product are shown. In the square brackets, annealing temperature and time, and number of PCR cycle of respective genes are shown, while temperature and time of denaturation and elongation steps of each PCR cycle are 94 °C, 30 sec and 72 °C, 60 sec, respectively.

FIGURE LEGENDS

Fig. 1. Effect of Nigella sativa soluble extract on LPS-induced IL-1ß mRNA expression in PBMC.

PBMC were cultured in 6-well plates at $5x10^6$ cells per well with/or without 10 □g/ml of Nigella sativa soluble extract either in the absence or presence LPS (10ng/ml) for 24 h. Total RNA was isolated and the mRNA expression of IL-1□ , and G3PDH were analyzed by RT-PCR. Shown are representative results of 3 independent experiments and their densitometric analysis.

*p<0.05 vs. control (no treatment).

Fig. 2. Effect of Nigella sativa soluble extract on LPS-induced IL-6 mRNA expression in PBMC.

PBMC were cultured as described in legend of Fig.1. Total RNA was isolated and the mRNA expression of IL-6, and G3PDH were analyzed by RT-PCR. Shown are representative results of 3 independent experiments and their densitometric analysis.

Fig. 3. Effect of Nigella sativa soluble extract on LPS-induced IL-8 mRNA expression in PBMC.

After culturing and treatment of PBMC as described in legend of Fig.1. Total RNA was isolated and the mRNA expression of IL-8, and G3PDH were analyzed by RT-PCR. Shown are representative results of 3 independent experiments and their densitometric analysis.

*p<0.05 vs. control (no treatment). †P<0.05

Fig. 4. Effect of Nigella sativa soluble extract on LPS-induced IL-10 mRNA expression in PBMC.

PBMC were cultured as described in legend of Fig.1. Total RNA was isolated and the mRNA expression of IL-10, and G3PDH were analyzed by RT-PCR. Shown are representative results of 3 independent experiments and their densitometric analysis.

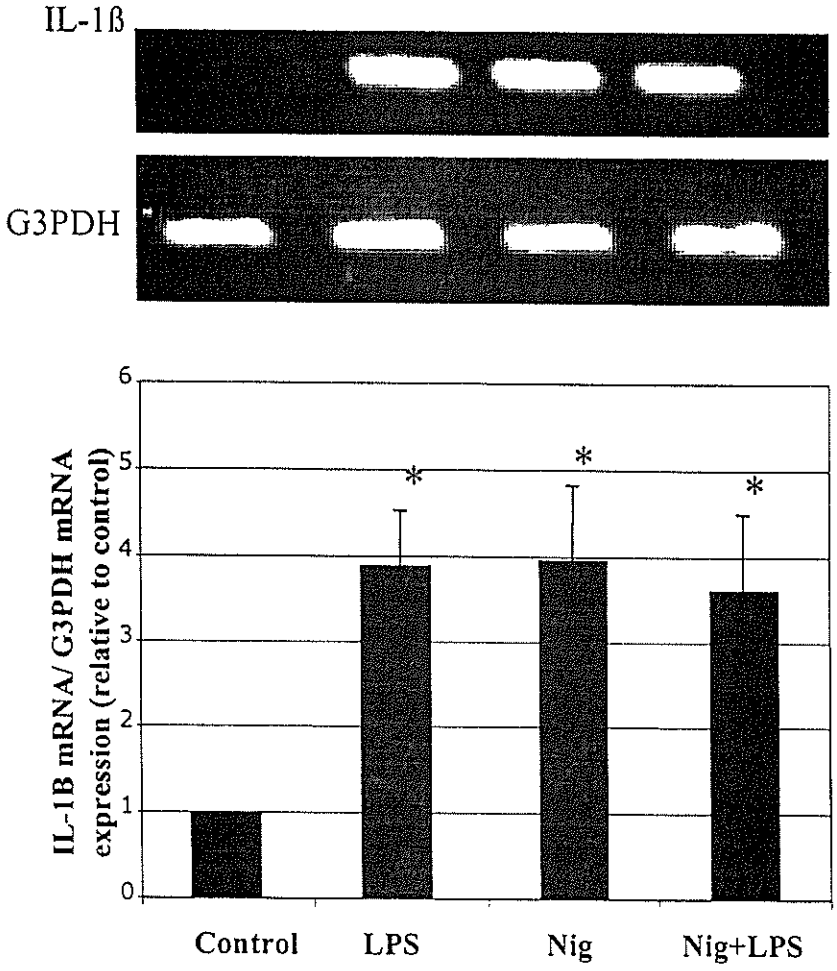
*p<0.05 vs. control (no treatment).

Fig. 5. Effect of Nigella sativa soluble extract on LPS-induced TNF- α mRNA expression in PBMC.

PBMC were cultured as in legend of Fig.1. Total RNA was isolated and the mRNA expression of TNF-α, and G3PDH were analyzed by RT-PCR. Shown are representative results of 3 independent experiments and their densitometric analysis.

*p<0.05 vs. control (no treatment). †P<0.0

Fig1



*P<0.05 vs control

Fig2

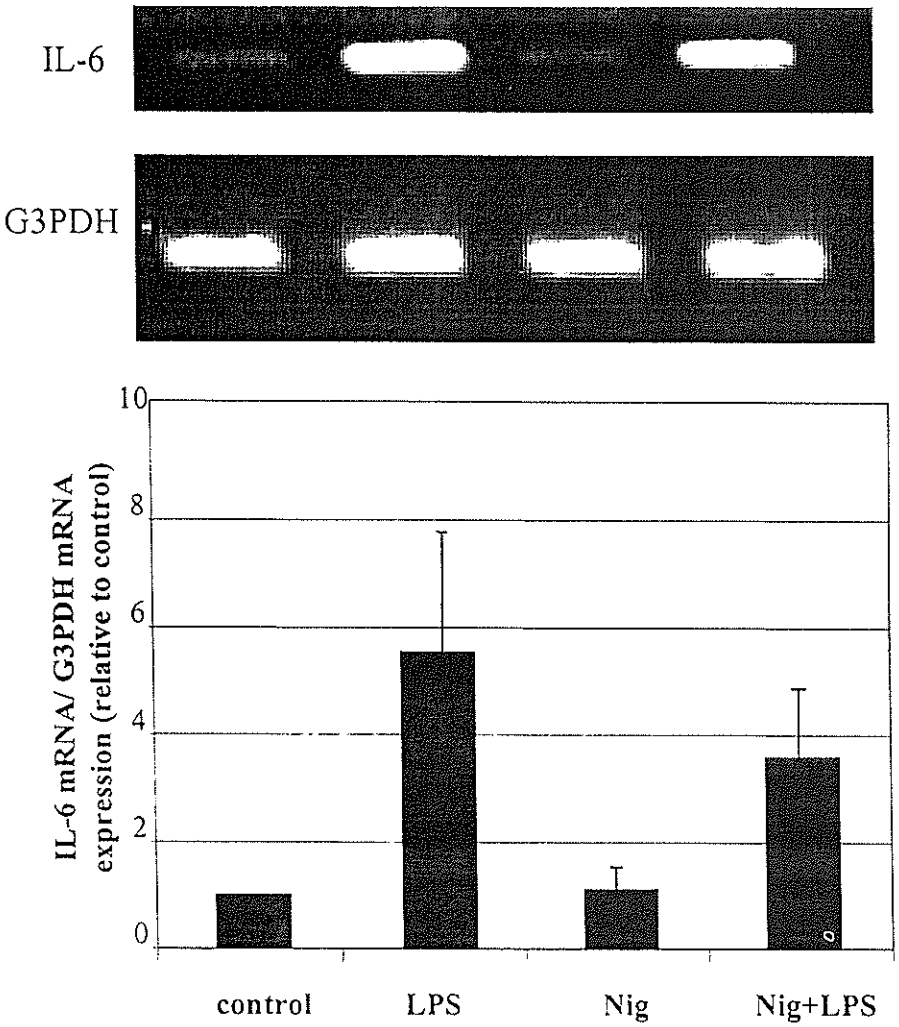
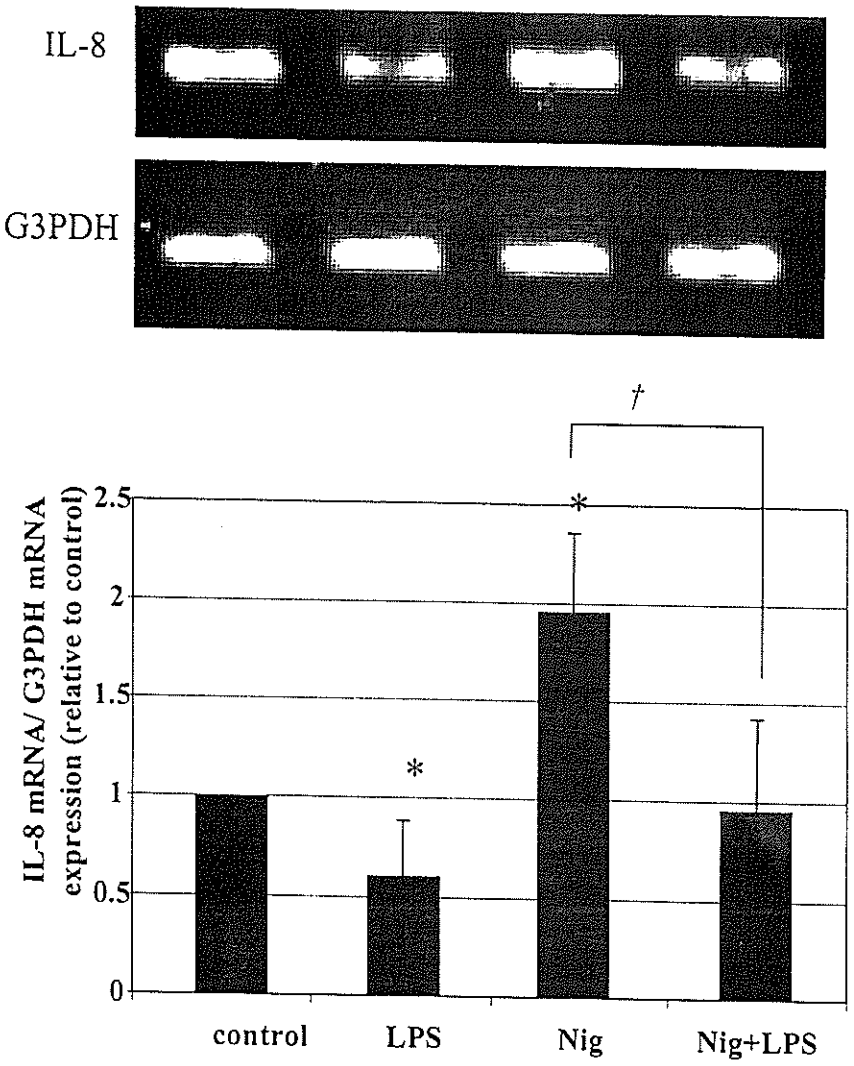

IL-6
G3PDH
IL-6 mRNA/ G3PDH mRNA expression (relative to control)
10
8
6
4
2
0
control
LPS
Nig
Nig+LPS

Fig3



*P<0.05 vs control

†P<0.05

Fig4

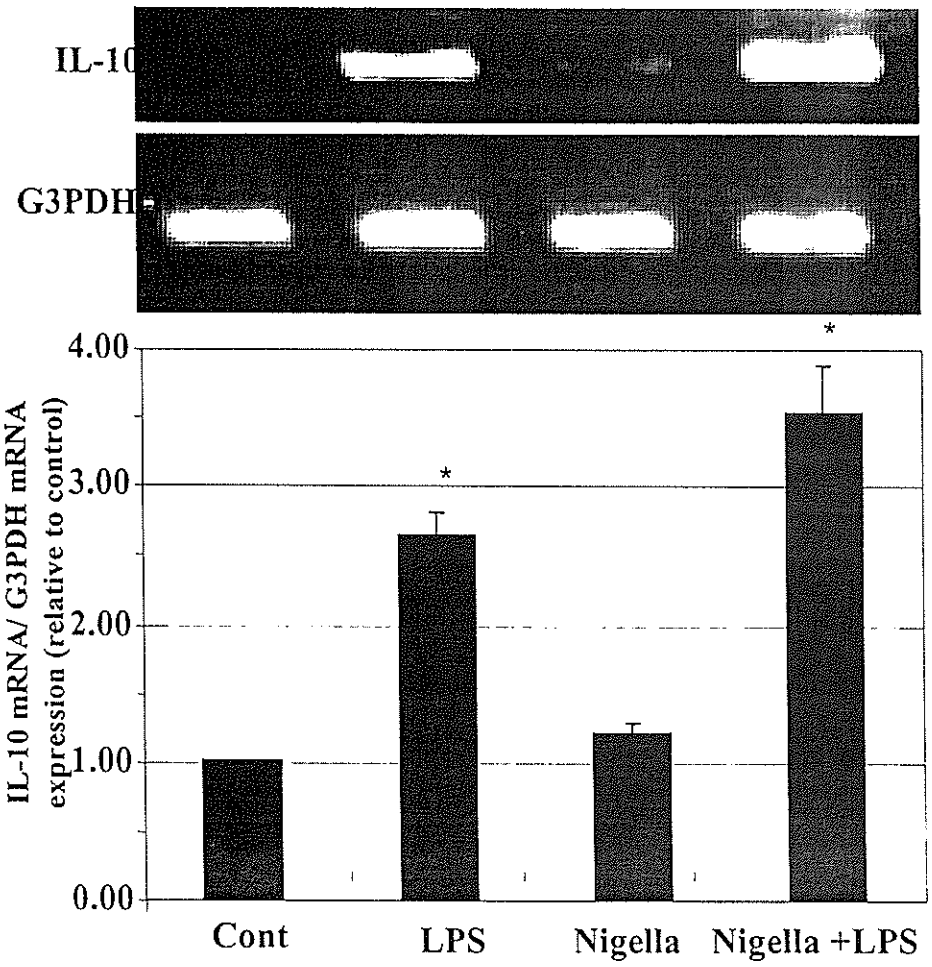
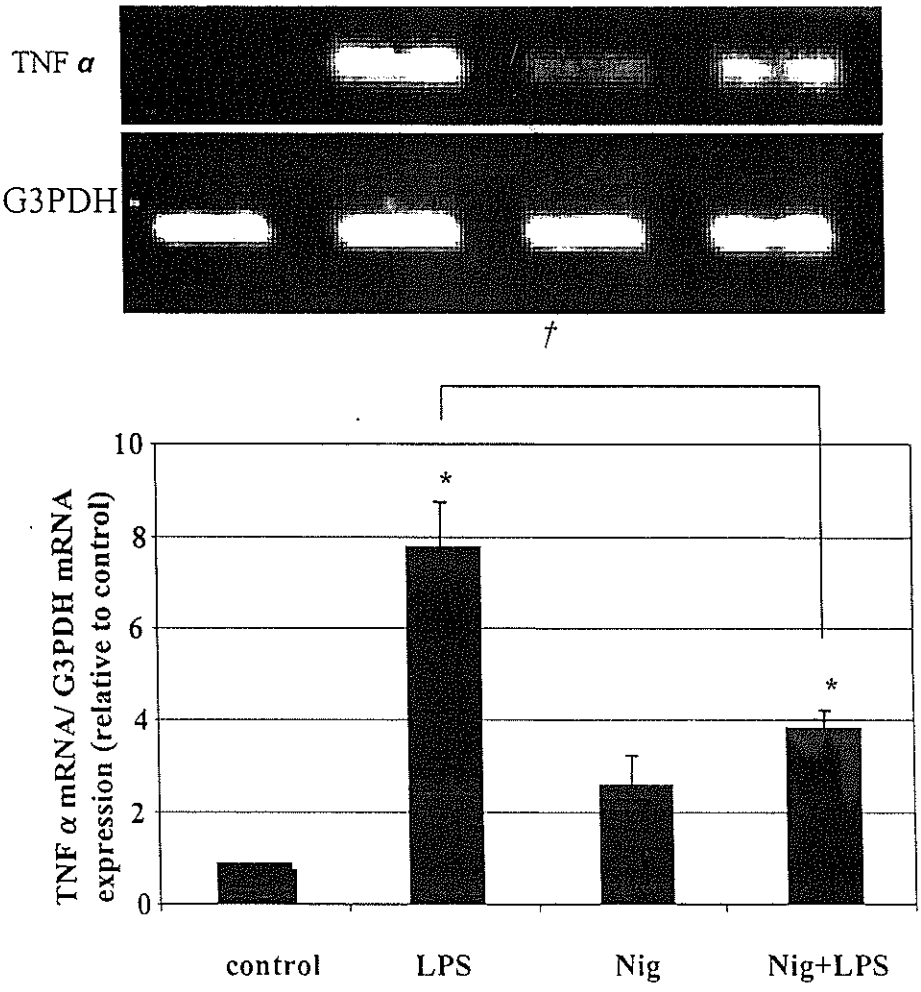

IL-10
G3PDH
IL-10 mRNA/ G3PDH mRNA expression (relative to control)
4.00
3.00
2.00
1.00
0.00
*
*
Cont
LPS
Nigella
Nigella +LPS

Fig5



*P<0.05 vs control

†P<0.05

الملخص العربي

التأثير المناعى لمستخلص حبة البركة على الخلايا أحادية النواة فى الأبقار

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إن نبات حبة البركة المعروف بالحبة السوداء أو الكمون الأسود هو نبات سنوي كثير الاستخدام في الأقطار العربية وقد وجد أن هذا النبات له تأثير موسع للشعب الهوائية كما أن له القدرة علي حماية الكبد وكذلك له تأثير منشط للمناعة.

تعرف خلايا الدم البيضاء الأ حادية النواة بأن لها دور كبير قي مسار معظم الأمراض وذلك بافراز العديد من الوسائط المعروفة بالستوكينات

للوسائط التي تفرزها خلايا الدم البيضاء وقد قمنا في هذه الدراسة بإختبار تنظيم تكوين الرسائل الوراثية) ، الانترلوكين١٠ 8-Il) ،الانترلوكين٨ (6-Il) ، الانترلوكين ٦ (1-IIالأحاديه النواة مثل الانترلوكين ١ (ذلك بعد معالجة هذه الخلايا بمستخلص حبة البركة قي وجود أو عدم وجود المستخلص TNF-α.و)10-Il ( ) وقد جمعت عينات الدم من أبقار متوسط عمرها مابين LPSالبيكتيري المعروق بالليبوبولي ساكارايد (عاميى و أربعة أعوام من نوع الهلوشتاين و قد فصلت الخلايا و جمعت ثم عولجت بالليبوبولي ساكارايد10 ميكروجم لكل مليلتر او بمستخلص حبة البركة ١٠ميكروجرام لكل مليلتر أو عواجت الخلايا بالاثنيين معا لمدة أربع وعشرون ساعة ثم جمت الخلايا واستخلص الحامض النووي ثم استخدم هذا الحامض النووي في تخليق الرسائل الوراثية لكل من الانترلوكين ١ و الانترلوكين ٦ و الانترلوكين٨ و الانترلوكين ١٠وذلك). هذا وقد اظهرت النتائج ان معالجة هذه الخلايا بالمستخلص (PCRبواسطة تفاعل إنزيم سلسلة البلمرة البيكتيري المعروق بالليبوبولي ساكارايد يعمل علي زيادة الستوكينات محل الاختبار

وعندما عولجت هده الخلايا بمستخلص حبة البركة فان مستخلص حبة البركة تسبب في زيادة الانترلوكين ١ و الانترلوكين٨ وعندما عولجت هده الخلايا بمستخلص حبة البركة قي وجود المستخلص البيكتيري المعروق بالليبوبولي ساكارايد فان مستخلص حبة البركةعمل علي تقليل مقدار الزيادة في الانترلوكين ٦ التي تسبب فيها الليبوبولي ساكرايد كما عملت علي زيادة الانترلوكين١٠

ومن هذه الدراسه يمكن استخلاص أن لحبة البركة القدره علي التأثير علي تنشيط المناعه قي الحيوانات من خلال تأثيرها قدرتها علي زيادة انتاج الرسائل الوراثية لكل من زيادة الانترلوكين ١ و الانترلوكين٨ كما أن لها تأثير مضاد للالتهاب من خلال قدرتها علي تقليل انتاج الرسائل الوراثية لكل من الانترلوكين ٦، TNF-α

كما أن لها تأثير منشط لقدرة الخلايا علي التجديد من خلال قدرتها علي زيادة الانترلوكين١٠