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Inhibitory Effects of Dutasteride on TLR4: An *In vitro* Pain Study

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ABSTRACT

Dutasteride was potentially proposed to control chronic pain by Toll-Like Receptor 4 (TLR4) inhibition through its effect on TLR4 expression, Myeloid differentiation primary response 88 (MyD88), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), secretory Interleukin-1β (IL-1β), and nitric oxide (NO) in the Lipopolysaccharides (LPS)-stimulated U-87 MG cell line.

The human astrocytoma U-87 MG cell line was cultured and incubated with 10 μg/mL of LPS for 24 hours to create a neuro-inflammation model, using two different treatment approaches. The first approach included LPS treatment for 24 hours, followed by dutasteride (20 μg/mL) incubation for the next 72 hours. In the second treatment approach, the cells were co-incubated with LPS and dutasteride for 72 hours. Expression of TLR4, MyD88, NF-κBp65, and secretory IL-1 was evaluated by Western blotting while expression of NO was assessed by NO assay.

TLR4, MyD88, NF-κBp65, and secretory IL-1β levels increased in LPS-treated cells after 24 hours. Dutasteride significantly decreased the secretion of NO and also, the levels of TLR4, MyD88, and NF-κBp65 in both treatment approaches. No difference in IL-1β level was seen with the second treatment approach.

Dutasteride has anti-inflammatory properties and probably analgesic effects, by mechanisms different from conventional analgesics.

Keywords: Dutasteride; Neuropathic pain; Toll-like receptor 4

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INTRODUCTION

Neuropathic pain, a prototype of chronic pain, is caused by an injury or a disease in the somatosensory system. Though many patients suffer from neuropathic pain worldwide, the available medications, including antidepressants, opioids, and anticonvulsants, are neither fully efficacious nor free of side effects.[1-3]

The immune system plays a major role in the generation of neuropathic pain; hence, the name 'inflammatory pain' has been used by many researchers and clinicians to define the inflammatory nature of the disease,[4] leading to great attention to the role of Toll-like receptors (TLRs) in neuropathic pain.[5] TLR4 is a known receptor in innate immune cells, and a type 1 membrane glycoprotein is expressed on the surface of some nervous system cells; like primary afferent neurons, cells of the central nervous system including microglia, and astrocytes, and dorsal root ganglia.[4,5] The increased TLR4 expression and activation in the latter cellular locations trigger nociception, potentially leading to the generation, development, and maintenance of neuropathic pain through the production and secretion of pain mediators.[4,6]

TLR4 activation by pathogen-associated molecular patterns, such as Lipopolysaccharides (LPS), as well as damage-associated molecular patterns, triggers two intracellular signaling pathways. These pathways induce the expression of intracellular adaptor proteins such as myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF), resulting in activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Therefore, pain mediators, proinflammatory cytokines such as interleukin (IL)-1, IL-6, tumor necrosis factor-alpha (TNF-α), and nitric oxide (NO) are released that exacerbate neuropathic pain and neutralize the analgesic effect of opioids.[4,7,8]

In some in silico studies, FDA-approved TLR4 inhibitory or antagonistic compounds have been identified as potential analgesics for neuropathic pain, dutasteride being one of the latter.[6,9] Dutasteride, which is a 5 alpha-reductase inhibitor, is usually used to treat the symptoms of benign prostatic hypertrophy (BPH). Therefore, the conventional clinical application of dutasteride is to control BPH. Dutasteride has also been demonstrated to reduce chemically induced prostatic inflammation;[10] however, the potential role of dutasteride with probable analgesic effects on cellular mechanisms leading to neuropathic pain has not been assessed ever. In this in vitro study, we have assessed the potential effects of dutasteride on the expression of TLR4 and its signaling mediators, i.e., MyD88, NF-κB, IL-1β, and NO in LPS-stimulated astrocytoma U-87 MG cell line, as proof of concept.

MATERIALS AND METHODS

Cell Culture

The human astrocytoma cell line of brain origin (glioblastoma; astrocytoma; classified as grade IV since 2007 with cell name U-87 MG) was purchased from the National Center of Genetic and Biological Resources, Iran. Cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, UK) containing 1% L-glutamine, HEPES (10 mM), 1% fungizone, and non-essential amino acids, supplemented with 10% fetal bovine serum (FBS) (Gibco, UK) and 1% penicillin/streptomycin antibiotics (Gibco, UK). U-87 MG cell cultures were incubated at 37°C with 5% CO_2 for 1 week, and during this period, the culture medium was changed twice. The cells reached 70% confluency during this period and were used for subsequent experiments.

Stimulation of U-87 MG Cells by LPS

The U-87 MG cell line was used as a TLR4-positive neuronal cell line in this study. U-87 MG cells (3×10^5 cells/well) were cultured in 6-well plates. Cells were incubated with 10 μg/mL of LPS (Lipopolysaccharides (L2880) from *Escherichia coli* O55:B5 (Sigma-Aldrich, St. Louis, MO)) for 24 hours; LPS was used to create a neuro-inflammation model.[11,12]

Two Approaches for the Treatment of U-87 MG Cells with LPS and Dutasteride

We treated the cells with two different approaches. In the first treatment approach, U-87 MG cells (3×10^5 cells/well) were incubated with 10 μg/mL of LPS for 24 hours. At the end of this incubation period, the culture medium was discarded, and the cells were treated with dutasteride (20 μg/mL)[13] for another 72 hours. The control for this approach included cell culture incubation with LPS for 24 hours and then replacement of culture medium with DMEM (without dutasteride) for 72 hours. In the second treatment approach, the effects of dutasteride (20 μg/mL) on the U-87 MG cells were determined by co-incubation with LPS (10 μg/mL) for 72 hours. The control for this treatment approach involves cell culture incubation with LPS (10 μg/mL) for 72 hours, without dutasteride.

Nitric Oxide Assay (ELISA)

After the treatment of U-87 MG cells with or without non-toxic concentrations of LPS and dutasteride, as described above, cell culture supernatant was collected to quantify the secreted NO. Nitric Oxide Assay Kit (Novin Navand Salamat Pishtaz Co, Iran) was used according to the manufacturer's instructions. The test was repeated three times for each group.

SDS-PAGE and Western Blot

After treatment of U-87 MG cells with LPS and dutasteride, as mentioned before, cellular proteins were extracted using RIPA buffer (CMG, Iran) and Protease inhibitor (Sigma, St. Louis, MO) to evaluate the amount of TLR4, MyD88, and NF-κBp65. Also, cell culture supernatant was collected to measure secretory IL-1β levels by Western blotting. Protein concentrations were measured using the Lowry method.[14] Extracted proteins (50 µg) were denatured and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Thermo Scientific, US). The membranes were blocked with 5% skim milk for 1 hour at room temperature. The membranes were then incubated separately, overnight at 4°C with Rabbit Anti-TLR4 antibody, Rabbit Anti-MyD88 antibody, Rabbit Anti-NF-κBp65 antibody, Rabbit Anti-IL-1 beta antibody (all from Abcam, UK), or Mouse Anti-β-Actin antibody (Santa Cruz Biotechnology, CA) at 1:5000 dilutions. After 3 times washing, the membranes were subsequently incubated with secondary antibodies (Goat Anti-Rabbit IgG H&L (HRP) (Abcam, UK) or Mouse IgGκ BP-HRP (Santa Cruz Biotechnology, CA) at 1:10000 dilutions for 1 hour. Protein bands were then detected on the membrane with ECL Kit (GE Healthcare, UK) and quantified using ImageJ software (version 1.48, NIH, USA).

Statistical Analysis

The data were obtained from at least three independent experiments. Data were presented as mean±SEM. For multiple comparisons, one-way ANOVA with Tukey post-test was used. For comparison of the two groups, Student's t-test was used. $p<0.05$ were considered significant. All calculations were performed using GraphPad Prism 5 software (version 5.04).

RESULTS

Effect of LPS Treatment on the Secretion of NO by U-87 MG Cells

For TLR4 activation, the U-87 MG cell line was incubated with LPS (10 µg/mL) for 24 hours. Treatment with LPS resulted in a significant increase ($p<0.0001$) in the secretion of NO (63.75±0.7484) compared to untreated cells (39.67±1.233) (Figure 1).

Effect of LPS Treatment on the Expression of TLR4, MyD88, and NF-κBp65 and Secretory IL-1β by U-87 MG Cells

On the other hand, the expression of TLR4, MyD88, NF-κBp65, and secretory IL-1β proteins in LPS-stimulated cells was assessed by Western blotting. The U-87 MG cells treated with LPS showed increased TLR4 ($p=0.002$), MyD88 ($p=0.004$), NF-κBp65 ($p=0.006$), and secretory IL-1β ($p=0.02$) levels compared to the untreated group after 24 hours (Figure 2).

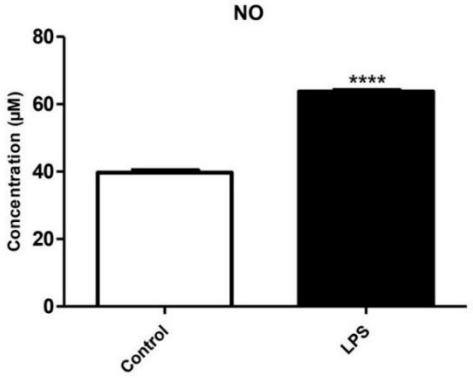

Figure 1. Effect of Lipopolysaccharides (LPS) treatment on the secretion of nitric oxide (NO) by U-87 MG cells. Data are expressed as mean±SEM (n=3). ****$p<0.0001$

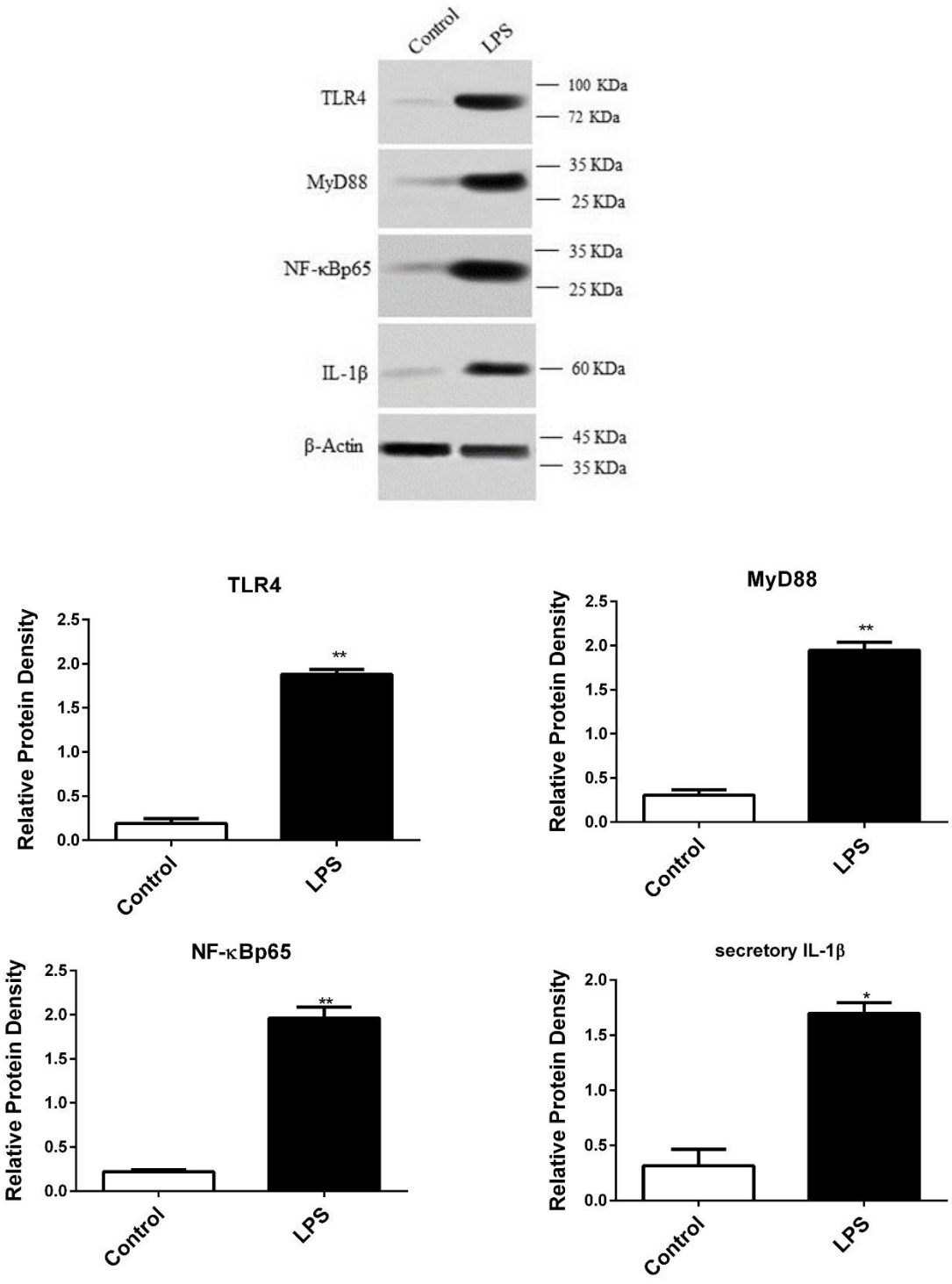

Figure 2. Effect of Lipopolysaccharides (LPS) on the expression of Toll-Like Receptor 4 (TLR4), Myeloid differentiation primary response 88 (MyD88), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κBp65), and secretory IL-1β in the U-87 MG cell line. Data are expressed as mean±SEM. *, ** denote statistical significance at $p<0.05$ and $p<0.01$ compared to the control group.

Effects of Dutasteride on NO Secretion by U-87 MG Cells

Two treatment approaches were used to investigate whether dutasteride can regulate NO production, which was described in the Methods section. The results indicated that treatment with dutasteride in both treatment approaches led to a significant decrease in the secretion of NO by LPS-stimulated U-87 MG cells in the first and second approaches (p=0.007 and p=0.001, respectively) (Figure 3).

Effects of Dutasteride on the Expression of TLR4, MyD88, NF-κBp65, and Secretory IL-1β in U-87 MG Cells

The effects of dutasteride on the expression of TLR4, MyD88, and NF-κBp65 in U-87 MG cells, and secretory IL-1β in the supernatant were evaluated by Western blotting and compared in two treatment approaches. The results demonstrated a significant decrease in TLR4 (p=0.0001), MyD88 (p<0.0001), NF-κBp65 (p=0.0007), and secretory IL-1β levels (p=0.0145) in the first treatment approach compared to untreated controls (Figure 4). Also, in the second treatment approach, the levels of TLR4 (p=0.0016), MyD88 (p=0.0003), and NF-κBp65 (p=0.003) were significantly decreased compared to the untreated control group. Secretory IL-1β expression in the second treatment approach did not show a significant difference (p=0.18) compared to untreated cells (Figure 4).

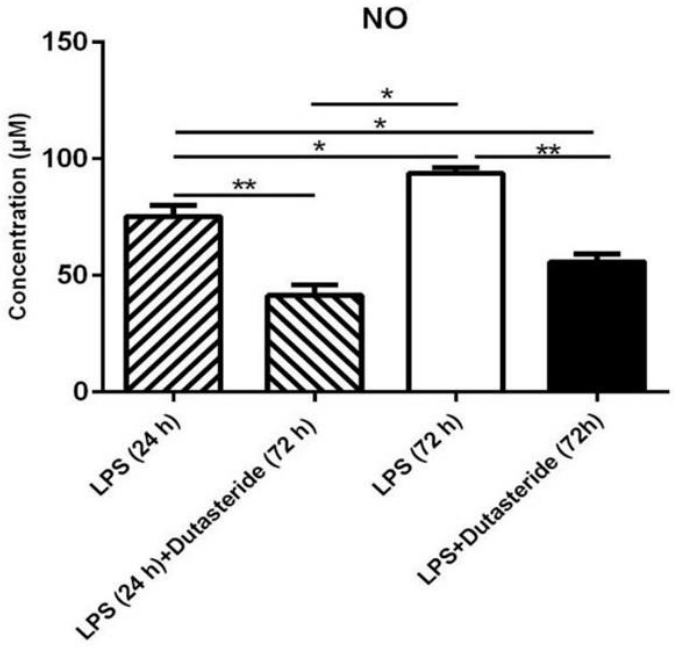

Figure 3. Effect of dutasteride on the secretion of nitric oxide (NO) by LPS-stimulated U-87 MG cell line in two treatment approaches. Data are expressed as mean±SEM (n=3). *p<0.01, **p<0.05

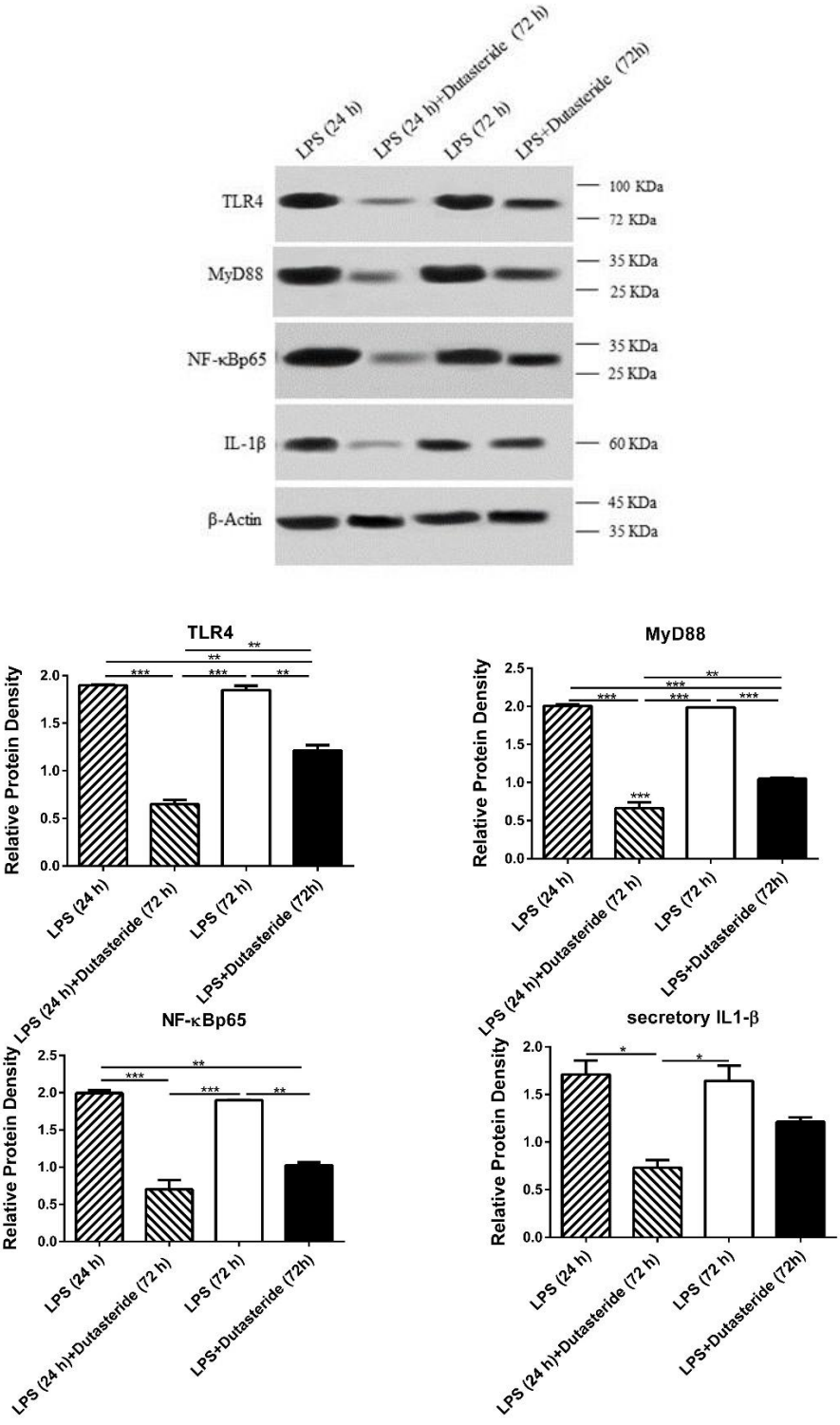

Figure 4. Effects of dutasteride on the expression of Toll-Like Receptor 4 (TLR4), Myeloid differentiation factor 88 (MyD88), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κBp65), and secretory IL-1β. Complete cell lysates were examined by Western blotting for TLR4, MyD88, and NF-κBp65. Cell culture supernatant was used to measure IL-1β. Data are expressed as mean±SEM (n=3). *p<0.05, **p<0.01, ***p<0.001

DISCUSSION

In this study, the effects of dutasteride on the inhibition of TLR4 activity of the LPS-stimulated U-87 MG cell line were investigated. The results of the study demonstrated that dutasteride could significantly suppress inflammatory mediators in LPS-treated cells in the in vitro neuropathic pain cell model. These findings support previous in silico findings indicating the inhibitory effects of dutasteride on TLR4.[6]

The application of docking and in silico methods to find potential dual actions of drugs (like dutasteride) could lead us to novel clinical applications of previously approved pharmaceuticals, including analgesic effects of drugs through mechanisms other than the conventional molecules.

The in vitro model of this study was in concordance with other in vitro pain model studies that describe the inflammatory mechanism of chronic neuropathic pain.[5,15-21] To explain more, the expression of TLR4, and its signaling pathway proteins and proinflammatory cytokines (i.e., MyD88 NF-κB, IL-1β), as well as NO in LPS-stimulated U-87 MG cells, were in favor of the inflammatory mechanisms of the cell line pain model for "chronic neuropathic pain".[5,15-21] The underlying factors leading to chronic pain are not very well known yet; however, persistent noxious signaling in the periphery is one of the proposed mechanisms.[22-24]

In this study, we considered the activation of the TLR4 intracellular signaling pathway by connecting to the MyD88 adaptor. The results of this study may indicate that dutasteride reduces MyD88 expression and nuclear translocation of NF-κB (NF-κBp65) by its antagonistic properties and by inhibiting TLR4 on the surface of astrocytoma cells. As a result, it can reduce the induction of proinflammatory cytokines and algesic mediators such as secretory IL-1β and NO; this inflammatory cascade is in concordance with most inflammation-mediated pain theories.[25,26]

Dutasteride's potential anti-inflammatory effects were proposed in a previous in silico study, which suggested potential FDA-approved drugs as inhibitors of TLR4 to be considered in future studies for the treatment of chronic neuropathic pain, through mechanisms other than the conventional analgesic treatments.[6] In the latter study, one of the potential drugs with a great tendency to bind to TLR4 was dutasteride, a 5 alpha-reductase inhibitor approved by the FDA for the treatment of prostate hyperplasia.[6] The current study was in favor of the latter in silico study regarding the role of dutasteride in a chronic pain cell model.

Finasteride (a drug similar to dutasteride) may also have effects on some processes, such as pain, neuro-steroidogenesis, and behavior.[27-29] For example, finasteride enhances the analgesic effects of morphine, prevents the development of morphine tolerance, and reduces abstinence behavior in rats.[28-31] On the other hand, recent studies have strongly emphasized the role of inflammation in the progress of BPH[10,32] and have considered the role of finasteride as a steroid-like derivative with anti-inflammatory effects; which is a secondary effect in addition to the primary role of finasteride as a competitive inhibitor of 5-alpha reductase. These studies support our findings regarding the potential analgesic effects of dutasteride.[33,34]

High concentrations of NO play an important role in the development of acute and chronic pain phenomena.[35] Hence, regulating NO production is an important goal for the treatment of chronic neuropathic pain,[11,36] supporting our results regarding dutasteride's role in reducing secretory NO by LPS-stimulated U-87 MG cells.

Our results from the 24-hour incubation of U-87 MG cells with LPS (without adding dutasteride) showed no significant difference compared with the 72 hours incubation with LPS (without dutasteride). However, adding dutasteride to the cells incubated with LPS led to significant suppression of the inflammatory pathway, especially if LPS was removed from the cell culture and dutasteride was added (compared with co-incubation of cells with LPS and dutasteride). Penta et al, have demonstrated that the effect of LPS on microglia cells peaked after 24 hours, considering the expression of iNOS protein, and then subsided and increased after 72 hours,[37] which is in contradiction with our results.

Part of the study results may have implications for further acute-versus-chronic pain studies: the significantly increased levels of TLR4, MyD88, and NF-κBp65 after incubation of U-87 MG cells with LPS, and IL-1β and NO levels in cell culture supernatants were reversed considerably by removing LPS and incubating the cells with dutasteride. Furthermore, simultaneous incubation of cells with LPS and dutasteride also reduced the levels of TLR4, MyD88, NF-κBp65, and NO compared to the control group (treatment with LPS for 72 hours). However, the percentage of expression

reduction was lower in the second treatment approach (cells incubated with LPS and dutasteride) than in the first (incubated with dutasteride after LPS removal). These findings may indicate that dutasteride could be more effective in acute pain than in chronic pain. It might also be an indication of the preventive properties of dutasteride from the toxic effects of LPS.

Finally, the current study demonstrated the potential application of dutasteride as an anti-inflammatory agent that could be considered in future chronic neuropathic pain studies, mainly considering its anti-inflammatory properties. This dual effect of dutasteride is much more supported by considering previous findings of anti-inflammatory effects of finasteride, another member of the same 5 alpha-reductase inhibitor family.

Study Limitations

This study was an in vitro pain model performed on a cell line as proof of concept. There is a long distance from these findings to animal proof and clinical studies. Here, we had an in vitro model, and our results should be assessed in future animal and clinical studies.

The present study was conducted to evaluate the in vitro efficacy of dutasteride to inhibit TLR4 and to discover a new application for this drug. The results show that dutasteride has anti-inflammatory effects by preventing and reducing the toxic effects of LPS and thus reducing the production of proinflammatory cytokines and analgesics such as IL-1β and NO. These results may be in favor of dutasteride's ability to inhibit TLR4 as proof of the concept of its potential dual therapeutic effect. However, to further understand the mechanism of action and confirm its potential application in future studies on chronic neuropathic pain, further in vivo and clinical studies are necessary.

STATEMENT OF ETHICS

The protocol of the study was approved by the Research Ethics Committee, Deputy of Research, Shahid Beheshti University of Medical Sciences, Tehran, Iran (IRB code: IR.SBMU.RETECH.REC.1400.017).

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

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