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RESEARCH

INSL3 suppresses LPS-induced inflammation in N9 microglia cells

INSL3, N9 mikroglia hücrelerinde LPS ile indüklenen enflamasyonu baskılar

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Abstract

Purpose: The G-protein coated receptor (GPCR) family, including the Insulin-Like Peptide 3 (INSL3) receptor, is involved in the Nuclear Factor kappa B (NF-ϰB)-mediated pathway in inflammation. In this regard, it can be thought that INSL3 plays a role in inflammation via the NF-ϰB pathway. In this study, we investigated the effect of INSL3 on inflammation and cell viability in the lipopolysaccharide (LPS)-induced N9 microglia cell line.
Materials and Methods: N9 microglial cells were pretreated with INSL3 for 2 hours, and then treated with LPS for 6 hours. Cell viability was identified by WST-8 assay. Immunostaining was performed to evaluate the levels of Interleukin-1β (IL-1β), Tumor necrosis factor (TNF)-α, and NF-ϰB.
Results: The cells in the LPS group showed degenerative changes in morphology and decreased cell viability. In the INSL3+LPS group (1.21±0.06), the general appearance and viability of the cells were more similar to the control group (1.92±0.04) compared to the LPS group (0.61±0.05). It was determined that INSL3 prevented the LPS-induced increase in IL-1β, TNF-α, and NF-ϰB levels and decreased cell death.
Conclusion: INSL3 suppresses inflammation and thus promotes cellular healing and can be considered a therapeutic agent that reduces inflammation.
Keywords:. Inflammation, insulin-like peptide 3, lipopolysaccharide, microglia

Öz

Amaç: İnsülin Benzeri Peptid 3 (INSL3) reseptörünü de içeren G-protein kaplı reseptör (GPCR) ailesi, inflamasyonda Nükleer Faktör kappa B (NF-ϰB) aracılı yolakta yer almaktadır. Bu bakımdan INSL3'ün NF-ϰB yolu üzerinden inflamasyonda rol oynadığı düşünülebilir. Bu çalışmada INSL3'ün, lipopolisakkarit (LPS) ile indüklenen N9 mikroglia hücre hattında inflamasyon ve hücre canlılığı üzerindeki etkisini araştırdık.
Gereç ve Yöntem: N9 mikroglial hücreleri, 2 saat boyunca INSL3 ile ön işleme tabi tutuldu, ardından 6 saat boyunca LPS ile işleme tabi tutuldu. Hücre canlılığı WST-8 analizi ile belirlendi. İnterlökin-1β (IL-1β), Tümör nekroz faktörü (TNF)-α ve NF-ϰB düzeyleri de immünhistokimyasal yöntemlerle değerlendirildi.
Bulgular: LPS grubundaki hücrelerde morfolojide dejeneratif değişiklikler ve hücre canlılığında azalma görüldü. INSL3+LPS (1.21±0.06) grubunda hücrelerin genel görünümü ve canlılığı LPS (0.61±0.05) grubuna göre kontrol grubuna (1.92±0.04) daha benzerdi. INSL3'ün LPS kaynaklı IL-1β, TNF-α ve NF-ϰB seviyelerindeki artışı önlediği ve hücre ölümünü azalttığı belirlendi.
Sonuç: INSL3'ün inflamasyonu baskılayarak hücresel iyileşmeyi desteklediği ve inflamasyonu azaltan terapötik bir ajan olarak değerlendirilebileceği sonucuna varıldı.

Anahtar kelimeler: Inflamasyon, insülin benzeri peptid 3, lipopolisakkarit, mikroglia

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INTRODUCTION

Neurodegenerative and psychiatric disorders are characterized by the crucial involvement of systemic inflammation and neuroinflammation[1]. As the resident macrophages of the Central Nervous System (CNS), microglia are the brain's first line of immune defense[2]. In a resting state under normal physiological conditions, they actively survey the microenvironment, ensuring the clearance of cell debris, protection of neighboring neurons, and provision of neurotrophic support[3]. When exposed to internal or external stimuli, microglia become activated, leading to the production and secretion of cytokines and other inflammatory mediators (2). Microglial activation can lead to different beneficial or harmful outcomes depending on the severity and duration of the stimulus[4]. Transient neuroinflammation serves a beneficial role in combating bacteria and their by-products, and clearing cell debris released from damaged or diseased cells. However, when microglia become excessively overactivated, they release various neurotoxic mediators, such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and proinflammatory cytokines[5]. Activated microglia produces and secretes pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6). These mediators play a role in inducing oxidative stress, neuroinflammation, and mitochondrial dysfunction, leading to the loss of nearby neurons and neuroglial cells. Moreover, they trigger the activation of neighboring glial cells and exert toxic effects on microglia. Long-term activation of microglial cells are also associated with the development of neurodegenerative disorders[6,7].

INSL3 is a member of the relaxin peptide hormone family, including insulin, IGF-1 (insulin-like growth factor-1), IGF-2 (insulin-like growth factor-2), Relaxin-1, Relaxin-2 and Relaxin-3[8-10]. INSL3, together with relaxin, has been included in a new category of hormone family called neurohormones, which have been recently identified. These neurohormones have evolved and are considered novel drug targets and supplements[10]. INSL3 binds to a specific receptor familiar as relaxin family peptide receptor-2 (RXFP2), a member of the GPCR family. Besides Leydig cells, RXFP2 has also been identified in meiotic-post-meiotic germ cells in the testis and ovary, brain, kidney, uterus, thyroid, bone, bone marrow, gubernaculum, epididymis, vas deferens, seminal vesicle, skeletal muscle, and peripheral blood cells. In addition, the same level of RXFP2 is expressed in osteoblasts as in the testes. Although the effects of INSL3 on steroidogenesis and osteoporosis have been reported in the literature, no inflammation-related effects have been found. However, relaxin, another member of the peptide hormone family, which also includes INSL3, is known to have vasodilator, antifibrotic, angiogenic, anti-apoptotic, and anti-inflammatory effects in non-reproductive organs such as the heart, arteries, kidney, lung, liver, blood and brain[9,11]. In the literature, there is also a study showing that the combined treatment of relaxin and estrogen is more effective against inflammation caused by arthritis than the individual administration of each hormone[12]. In another study, it was reported that the relaxin family peptide receptor-1 (RXFP1), a member of the same family as the INSL3 receptor RXFP2, is expressed in decidual macrophages, stimulating cAMP accumulation and proinflammatory cytokine release[13]. It is known that cAMP, which is the key molecule of steroidogenesis, is also important in the regulation of intracellular inflammation. NF-ϰB, which is activated by inflammation in different types of cells such as T cells, monocytes, microglia, and macrophages, can be inhibited by protein kinase-A (PKA)-mediated CREB phosphorylation of cAMP. The GPCR family has also been reported to be associated with the NF-ϰB-mediated pathway in inflammation[14]. In this respect, since the INSL3 receptor RXFP2 is also a member of the GPCR family, it is likely to be involved in inflammation through the cAMP/PKA and NF-ϰB pathways.

On the other hand, the effects of INSL3 on LPS-induced activation of inflammation have not been studied before this study. Lipopolysaccharide is a classical inducer in microglia. LPS, commonly used to stimulate immune responses, is a lipophilic molecule that can cross the healthy or damaged blood-brain barrier into the brain and trigger inflammation. We hypothesize that INSL3 exerts an anti-inflammatory effect on the LPS-induced microglia cell line (N9 microglia cell line) through the NF-ϰB pathway. In this study, we demonstrated that INSL3 treatment increased cell viability and inhibited IL-1β, TNF-α, and NF-ϰB expressions in LPS-activated microglia cells.

MATERIALS AND METHODS

This study was designed as an in vitro study in March

2022 in the Department of Histology and Embryology at Cukurova University. The N9 cell line is well-characterized and widely used. Therefore, N9 microglia cells of rats were kindly supplied by Dr. Paola Ricciardi-Castagnoli (Cellular Pharmacology Center, Italy)[15]. Ethics committee approval is not required as a commercial cell line was used in the study. The cells were multiplied in RPMI 1640 medium supplemented with % 10 Fetal Bovine Serum (FBS), 2 mM L-Glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were cultured in 50 cm^2 flasks at 37 °C and % 5 CO_2 in an incubator. In the treatments with INSL3 and LPS, serum-free RPMI 1640 medium containing 2 mM L-Glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin was used. The cells were removed from the incubator and examined under an inverted microscope (Nikon TS100, Japan) before passaging.

In vitro Assay for WST-8 assay

On the 1st day of the experiment, the cells in confluence were removed from the flasks, and cell counting was performed on a Thoma slide. The cells were seeded into 96-well plates with $1x10^5$ cells per well. As well as the required condition, 3 wells were seeded as many times as the necessary condition. On the 2nd day of the experiment, RPMI 1640 experimental medium containing L-Glutamine, Penicillin, and Streptomycin without FBS was used. For the control groups, only the experimental environment was added. In the INSL3 group, 100 µM INSL3 was added at 100 µl per well[12]. An experimental medium containing LPS at a concentration of 1 µg/ml per well was added to the LPS group and incubated for 6 hours[4]. In the INSL3+LPS group, N9 microglial cells were pretreated with 100 µM INSL3 for 2 hours. Then 100 µl of experimental medium containing 100 µM INSL3 and 1 µg/ml LPS was added and incubated for 6 hours[4,12].

For the assessment of cell viability, the WST-8 (Sigma Aldrich, St. Louis, USA) assay was used. The WST-8 (or CCK-8) assay involves the generation of a water-soluble formazan dye when bioreduction occurs in the presence of 1-Methoxy PMS, an electron carrier specific to WST-8 (2- (2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-3- (4-nitrophenyl)-5- (2,4-disulfophenyl)-5- (2,4-disulfophenyl)-2H-Tetrazolium monosodium salt). After the experiment, 10 µl of WST-8 solution was added to the upper part of the wells and incubated for 4 hours in the incubator. The resulting color change was measured using a multi-plate reader (BioTek ELx800, USA) at wavelengths of 450 nm and 630 nm as a reference. Cell viability was quantified by applying the following equation:

% Cell Viability = (Absorbance found/Maximum absorbance)*100

Immunocytochemistry ethod

After the cells removed with trypsin-EDTA were centrifuged and their supernatants were discarded, the cell count was performed on a Thoma slide. Cell-medium suspension was added to chamber slides (10^4 cells per well in 300 µl of medium for 4 chamber slides). They were kept overnight in the incubator at 37 °C. On the 2nd day of the experiment, the medium in the chamber slides was vacuumed. For the control group, only the experimental environment was added. In the INSL3 group, 100 µM INSL3 was added at 300 µl per well and incubated for two hours. The experimental medium containing LPS at a concentration of 1 µg/ml (300 µl per well) was added to the LPS group and incubated for 6 hours. In the INSL3 + LPS group, 100 µM INSL3 was first added and incubated for two hours, then 300 µl of experimental medium containing 100 µM INSL3 and 1 µg/ml LPS was added and incubated for 6 hours. Following the completion of the treatments, the cells were subjected to fixation using % 4 paraformaldehyde at a temperature of 20 °C for 10 minutes. Subsequently, the cells were rinsed three times with PBS, each rinse lasting for 5 minutes. A blocking solution was then applied, and the cells were allowed to incubate at room temperature for 1 hour, followed by another round of PBS washes. Then, 1/100 diluted anti-IL-1β (IL-1β; anti-rat rabbit polyclonal antibody, STJ118720, St. Johns, United Kingdom), 1/500 diluted anti-TNF-α (TNF-α; anti-rat rabbit polyclonal antibody, ab220210, Abcam, USA) and 2 µg/ml diluted NF-ϰB (NF-ϰB; anti-rat rabbit polyclonal antibody, A00284, Boster, Canada) primary antibodies were added[16].

All the slides with primary antibody added were kept overnight in a humid environment in the refrigerator. Biotin was added to the slides that were passed through PBS and kept at room temperature for 15 minutes. After 15 minutes, Avidin was added to the slides washed in PBS, and kept at room temperature for 20 minutes. Aminoethyl carbazole (AEC) solution was added onto the PBS slides and kept for 5 minutes, washed under running water and the cells

were stained with hematoxylin for counterstaining. They were examined and photographed with an Olympus BX53 (Japan) light microscope.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software Inc., CA, USA). Shapiro–Wilk test was used for the normality test and if distributions were normal, one-way ANOVA with Bonferroni multiple comparison correction was used to compare the experimental groups. Comparisons between the two groups were done by the Mann–Whitney U-test. The significance threshold was taken as 0.05 ($p<0.05$). For descriptive analyses, means ± standard deviations were calculated. In vitro experiments were performed at least 3 times.

RESULTS

The microglial cells were monitored daily, and the growth medium was refreshed and cells were subcultured every 3 days to facilitate cell proliferation. The microglial cells were then treated with INSL3 and LPS and imaged under an inverted microscope. In the control and INSL3 treatment groups, the cells were observed to have normal morphology. The microglial cells had a thin oval nucleus and a narrow cytoplasm. Continuity of the cytoplasm into the branched extensions of the cell was observed. It was found that in the LPS group, the shape of the cells in the normal resting state changed. It was noted that the size of the cells increased and cytoplasmic extensions disappeared. In the INSL3+LPS group, compared to the LPS group, the size of the cells decreased and cytoplasmic extensions in the form of thin threads appeared. It was noteworthy that the general appearance of the cells was more similar to the control group (Figure 1).

96-well plates were seeded with 10^5 cells and those in the treatment group were treated with INSL3 at a dose of 100 μM. The non-toxicity and protective effect of INSL3 was determined by the WST-8 assay. In the viability test of INSL3, a dose of 100 μM was found to have a protective effect. As shown in Figure 2, it was found that the administration of LPS at a dose of 1 μg/ml decreased cell viability ($p<0.0001$), whereas INSL3 significantly increased cell viability compared to the LPS group ($p<0.001$).

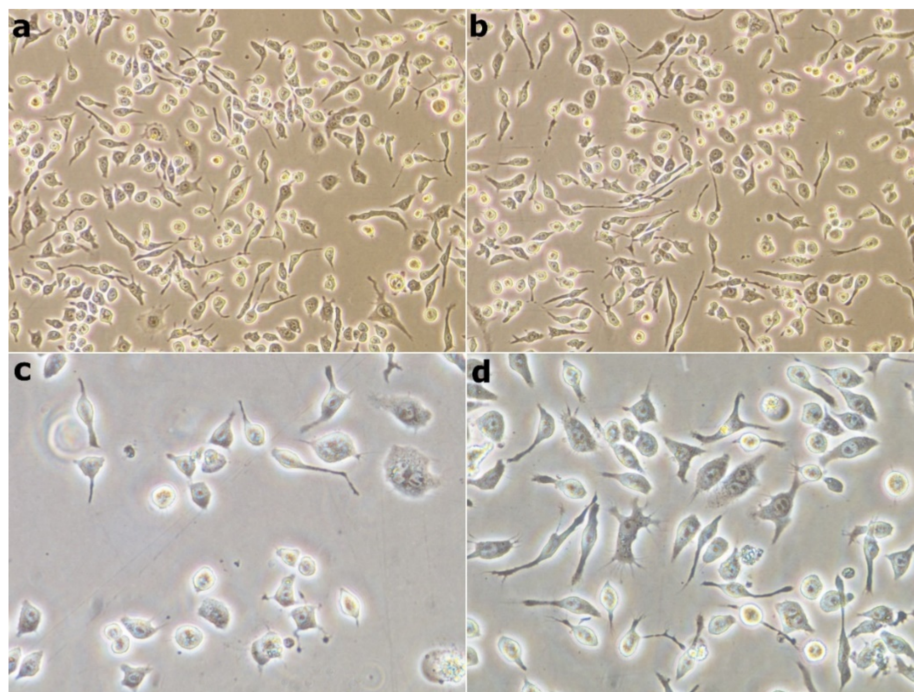

Figure 1. Image of microglial cells under an invert microscope. (a) Control group. (b) INSL3 group. (c) LPS group. (d) INSL3+LPS group.

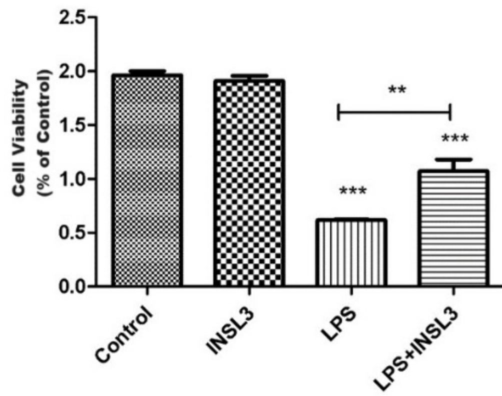

Figure 2. Effect of INSL3 on cell viability in LPS toxicity. Cell viability was evaluated with the WST-8 test (*p<0.0001, **p<0.001).**

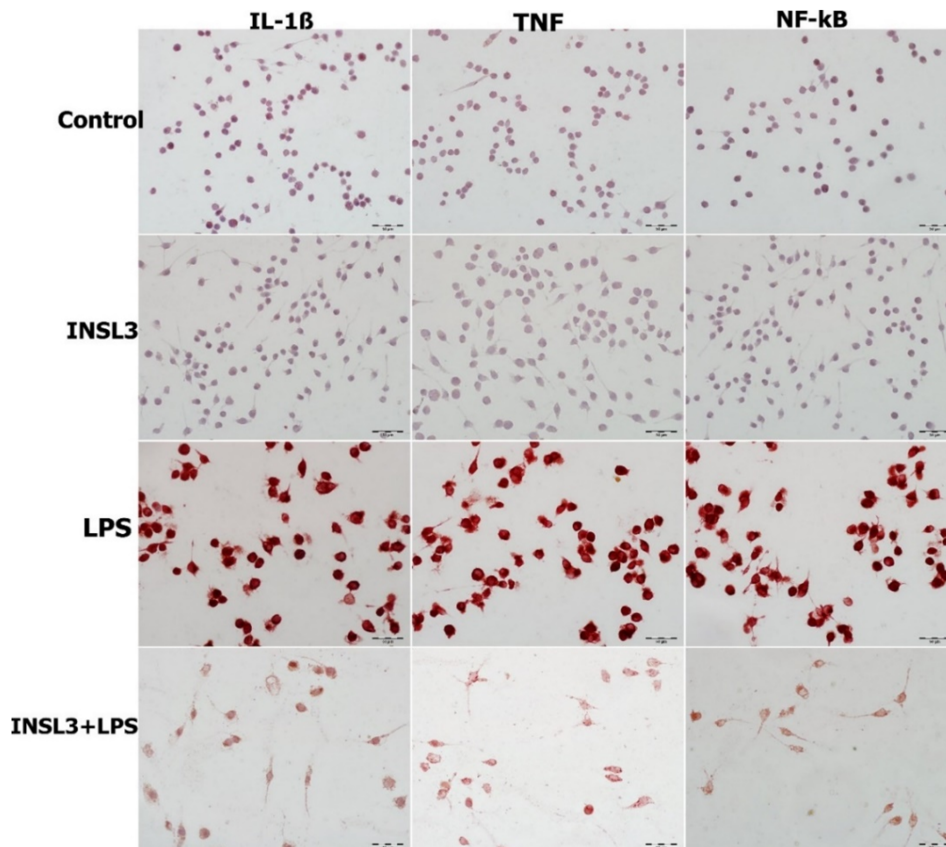

Figure 3. IL-1β, TNF-α, and NF-ϰB immunoreactivities in the control, INSL3, LPS, and INSL3+LPS groups. IL-1β, TNF-α and NF-ϰB immunoreactivities are at a minimum level in control groups. IL-1β, TNF-α, and NF-ϰB immunoreactivities appear to be minimal in INSL3 groups. IL-1β, TNF-α, and NF-ϰB immunoreactivities are high in LPS groups. IL-1β, TNF-α and NF-ϰB immunoreactivities appear to be significantly reduced in INSL3+LPS groups.

After microglial cells were treated with INSL3 and LPS, immunocytochemical methods were applied to investigate the expression of IL-1β, TNF-α, and NF-ϰB. Minimal levels of IL-1β, TNF-α, and NF-ϰB expressions were observed in the microglial cells in the control and INSL3-treated groups. In the LPS group, IL-1β, TNF-α, and NF-ϰB expressions were intense. In the INSL3+LPS group, IL-1β, TNF-α, and NF-ϰB expressions were weakly expressed in the microglial cells (Figure 3).

DISCUSSION

LPS engages toll-like receptor 4 (TLR4) on the cell surface, initiating a cascade of proinflammatory responses via Mitogen-activated protein kinase (MAPK) signaling pathways[17]. The TLR4 pathway activates the NF-ϰB transcription factor, facilitated by the adaptor protein myeloid differentiation primary response gene 88, which in turn promotes the expression of pro-inflammatory mediators[18]. LPS also has the potential to induce cell death by stimulating the MAPK pathway and NF-ϰB transcription factor. Oxidative stress, nitrosative stress, inflammation, and mitochondrial dysfunction interact to lead to cell death[19]. In this study, we employed LPS to establish a model associated with neuroinflammation, as numerous studies have substantiated that LPS induces neuroinflammation in vitro[20]. Upon LPS treatment in N9 microglial cells, both apoptotic and necrotic cell death occurs[4]. In our study, it was found that cell viability decreased in the LPS-treated microglial cells, whereas cell viability increased in the group given INSL3 together with LPS. It was concluded that INSL3 administration decreased LPS-induced cell death. The increase in NF-kB levels in the LPS group decreased with INSL3 administration, suggesting that INSL3 acts via the NF-kB transcription factor.

When microglial cells are activated, morphological changes occur. Microglial cells in a resting state show a round or branched morphology, whereas when stimulated by foreign bodies they acquire an amoeboid morphology with synaptic retraction[21,22]. In our study, the N9 microglial cells activated by LPS tended to be branched amoebic. The INSL3 pretreatment altered the morphology of N9 microglia, indicating the suppressive role of INSL3 in LPS-induced N9 activity from a morphological perspective. Additionally, Zhou et al. also showed LPS-induced microglial activation, as evidenced by a shorter branch length, a lower number of branching points, and reactive phenotype[23].

Inflammatory reactions are an unavoidable pathological process in various CNS injuries. Microglia exhibit two distinct functional polarization states in response to different microenvironmental signals, which play a role in regulating immune and inflammatory responses in the CNS. The M1 phenotype of microglia releases a range of proinflammatory mediators and free radicals that hinder brain repair. Conversely, the M2 phenotype of microglia removes nerve cell debris through phagocytosis and matrix-degrading enzymes, inhibits excessive inflammatory responses, promotes tissue repair and neuronal regeneration, and prevents secondary inflammatory damage[24]. In microglial cells, LPS induces the M1 phenotype and triggers the release of inflammatory cytokines. In our study, IL-1β, TNF-α, and NF-ϰB increased in the microglial cells treated with LPS. It was found that the INSL3 treatment reduced these cytokine levels. INSL3, which is a member of the relaxin peptide hormone family, is a member of the insulin-like peptide family that can bind to the RXFP1 receptor with high affinity[25]. RXFP1 is expressed in neurons, astrocytes, and macrophages/microglia[26]. There are no studies on the anti-inflammatory and antiapoptotic effects of INSL3 in the literature. However, the effects of relaxin, a member of the same hormone family as INSL3, are known.

In a rat model of germinal matrix hemorrhage (GMH), Li et al. demonstrated that the activation of RXFP1 by rh-relaxin-2 can mitigate mast cell degranulation and enhance neurological functions by suppressing NF-ϰB activity. As a result, rh-relaxin-2 holds potential as a promising therapeutic intervention for attenuating neuroinflammation and secondary brain injury in patients with GMH[25]. Kohsaka et al. showed that administration of relaxin in rats with testicular ischemia/reperfusion injury suppressed oxidative stress, testicular dysfunction, inflammation, and germ cell apoptosis. The results of the study unequivocally showcased the protective impact of relaxin in mitigating testicular injury induced by ischemia-reperfusion (IR). This protective effect was observed through the reduction of oxidative stress, apoptosis, and inflammation. These findings suggest that the incorporation of relaxin therapy alongside surgical treatment could potentially serve as an effective strategy for ameliorating

testicular dysfunction after testicular torsion[27]. Additionally, researchers suggested that there exists an association between relaxin-2 and molecules implicated in fibrosis, inflammation, and oxidative stress in atrial fibrillation (AF) patients. This underscores the potential anti-fibrotic protective role of this hormone in normal human atrial cardiac fibroblasts, thereby reinforcing the significance of relaxin-2 in the physiopathology, diagnosis, and treatment of AF[28]. Researchers highlight the significance of the endogenous hormone relaxin-2 as a valuable diagnostic biomarker across various cardiovascular pathologies, including heart failure, atrial fibrillation, myocardial infarction, ischemic heart disease, aortic valve disease, hypertension, and atherosclerosis[29]. In future studies, it is important to understand the roles and mechanism of action of INSL3 on these diseases and could contribute to comprehending the specific role of INSL3 in these diseases.

It should be noted that our study has a limitation in the lack of in vivo experiments. While microglial cell lines demonstrate cellular responses similar to primary microglia when activated by immune stimuli, some variances in gene and protein profiles have been reported[30]. Therefore, it is important to validate our findings using human microglial cells as well. Despite these limitations, cell line cultures offer several advantages, including ease of maintenance, homogeneity, high yield, cost-effectiveness, time-saving, and the ability to avoid animal experiments. The murine N9 microglial cell lines are well characterized and generally used[31]. In the realm of well-designed animal experiments, in vitro studies hold significant value and are considered essential. Additionally, the cell line is well-suited for high-throughput analyses, like studies on cell toxicity and drug discovery.

The effects of INSL3 administration on inflammation were investigated in vitro for the first time. In our study, IL-1β, TNF-α, and NF-κB levels were found to be increased in the LPS-activated microglial cells, whereas the levels of these cytokines were remarkably decreased in the INSL3+LPS-treated group. It was concluded that INSL3 suppressed inflammation in vitro conditions. INSL3 suppresses inflammation and thus promotes cellular healing and can be considered a therapeutic agent for modulating inflammatory responses and cell survival. INSL3 could be a promising candidate based on its therapeutic potential for modulating inflammatory responses and cell survival in CNS. Thus, it is important to validate these findings using human microglial cells and in vivo experiments as well.

Author Contributions: Concept/Design : DŞ, SP; Data acquisition: DŞ, GC; Data analysis and interpretation: DŞ, GC; Drafting manuscript: DŞ, GC, SP; Critical revision of manuscript: DŞ, SP; Final approval and accountability: DŞ, GC, SP; Technical or material support: -; Supervision: -; Securing funding (if available): n/a.
Ethical Approval: Ethics committee approval is not required as a commercial cell line was used.
Peer-review: Externally peer-reviewed.
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