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Study on the anti–inflammatory effect of 3–(4–hydroxyphenyl) propionic acid in an *in vitro* LPS–stimulated acute kidney inflammation model

Estudio sobre el efecto antiinflamatorio del ácido 3–(4–hidroxifenil) propiónico en un modelo de inflamación renal aguda estimulado por LPS *in vitro*

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ABSTRACT

Acute kidney injury (AKI) is a syndrome defined by a rapid decrease in glomerular filtration that can be caused by sepsis, ischemia/reperfusion injury (IRI), or nephrotoxic drugs. Human microbiota makes significant contributions to human health by enzymatic transformation of such active substances and the release of molecules such as 3–4 hydroxyphenyl propionic acid (4–HPPA). Biological effects of 4–HPPA such as anti–inflammatory and antioxidant have been reported in many studies. The aim of the research is to reveal the anti–inflammatory activity of 4–HPPA, one of the microbiota products of flavonoids (especially naringin) found in many fruits, in an *in vitro* LPS (lipopolysaccharide) stimulated kidney inflammation model. HEK 293 kidney cells of human origin were used as material in the research. The trial consisted of 4 groups: control group, LPS group, 4–HPPA group and 4–HPPA+LPS group. LPS and 4–HPPA were applied to the cells at different concentrations for 24 hours. Effective concentrations of LPS and 4–HPPA were investigated by MTT viability test. Finally, IL–1β, TNF–α and NFkβ gene expression analyzes responsible for inflammatory responses were investigated by qRT–PCR method. According to the findings, after 24 hours of incubation, LPS at 2.5 ng·mL^{-1} and 4–HPPA at 6.25 µg·mL^{-1} were determined to be effective concentrations for the experiment. Again, it was observed that 4–HPPA downregulated LPS–induced IL–1β, TNF–α and NFkβ gene expressions by 7, 42 and 40%, respectively. According to the data obtained from the research, it was revealed that 4–HPPA had effective anti–inflammatory properties in the *in vitro* LPS–stimulated kidney inflammation model. However, it was concluded that *in vivo* and more advanced molecular methods are needed to fully elucidate the issue.

Key words: Acute kidney injury; 4–HHPA; inflammation

RESUMEN

La lesión renal aguda (IRA) es un síndrome definido por una rápida disminución de la filtración glomerular que puede ser causada por sepsis, lesión por isquemia/reperfusión (IRI) o fármacos nefrotóxicos. La microbiota humana contribuye significativamente a la salud humana mediante la transformación enzimática de dichas sustancias activas y la liberación de moléculas como el ácido 3–4 hidroxifenilpropiónico (4–HPPA). En muchos estudios se han informado efectos biológicos del 4–HPPA, como antiinflamatorios y antioxidantes. El objetivo de la investigación es revelar la actividad antiinflamatoria del 4–HPPA, uno de los productos de la microbiota de los flavonoides (especialmente la naringina) que se encuentran en muchas frutas, en un modelo de inflamación renal estimulada con LPS (lipopolisacárido) *in vitro*. Como material de investigación se utilizaron células renales HEK 293 de origen humano. El ensayo constaba de 4 grupos: grupo control, grupo LPS, grupo 4–HPPA y grupo 4–HPPA+LPS. Se aplicaron LPS y 4–HPPA a las células en diferentes concentraciones durante 24 horas. Se investigaron las concentraciones efectivas de LPS y 4–HPPA mediante la prueba de viabilidad de MTT. Finalmente, los análisis de expresión de los genes IL–1β, TNF–α y NFkβ responsables de las respuestas inflamatorias se investigaron mediante el método qRT–PCR. Según los hallazgos, después de 24 horas de incubación, se determinó que LPS a 2,5 ng·mL^{-1} y 4–HPPA a 6,25 µg·mL^{-1} eran concentraciones efectivas para el experimento. Nuevamente, se observó que 4–HPPA regulaba negativamente las expresiones de los genes IL–1β, TNF–α y NFkβ inducidas por LPS en un 7; 42 y 40%, respectivamente. Según los datos obtenidos de la investigación, se reveló que el 4–HPPA presento propiedades antiinflamatorias efectivas en el modelo de inflamación renal estimulada por LPS *in vitro*. Sin embargo, se concluyó que se necesitan métodos moleculares *in vivo* y más avanzados para dilucidar completamente el problema.

Palabras clave: Daño renal agudo; 4–HHPA; inflamación

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INTRODUCTION

Sepsis is the most common cause of acute kidney injury and plays a role in 40–50% of cases [1]. Importantly, the development of acute kidney injury (AKI) in the setting of sepsis increases the risk of in-hospital death by 6–8 times [1, 2] and survivors are at predominant risk of progression to chronic kidney disease [3]. Despite this, the mechanisms by which sepsis causes AKI are not fully understood and therefore current treatment is reactive and nonspecific. A growing body of evidence suggests that, at least in some patients, AKI may occur in the absence of overt signs of hypoperfusion, and thus other mechanisms may be at play. Langenberg *et al.* [4] showed that AKI developed in septic animals despite normal or increased renal blood flow (RBF).

Citrus fruits are important for human health due to their protective effects against many diseases such as inflammation and cancer. In particular, naringin, which is predominantly found in its structure, is a flavonoid with a strong antioxidant effect [5 ,6]. In addition, the most abundant polyphenols in the diet are procyanidins, which have health benefits as antioxidants [7], anti-inflammatory [8, 9], anti-aging [10] and preventing cardiovascular diseases. Although potential health beneficial effects have been attributed, procyanidins are poorly absorbed in the gastrointestinal tract. However, the colonic microbiota breaks down procyanidins and converts them into largely absorbable metabolites, which are responsible for the biological effects in the body [11]. Procyanidin A2 (PCA2) is abundantly found in cranberry (*Vaccinium macrocarpon*)[12], avocado (*Persea americana*) [13], red peanut shell *(Arachys hypogaea)* [14] and litchi fruit pericarp (*Litchi chinensis*). The main microbial product of procyanidin A2 is 3-(4-hydroxyphenyl) propionic acid (HPPA) [15]. In particular, the major microbial biotransformation product of both naringin and Procyanidin A2 is 4-HPPA. In particular, the major microbial biotransformation product of both naringin and Procyanidin A2 is 4-HPPA, and biological effects are attributed to this component [15, 16]. In the light of this information, the aim of the study is to reveal the anti-inflammatory activity of 4-HPPA, one of the microbiota products of flavonoids (especially naringin) found in many fruits, in an *in vitro* LPS-stimulated kidney inflammation model.

MATERIALS AND METHODS

Experimental Trial

Kidney cells of human origin (HEK293 ATCC CRL-1573) from continuous cell lines were used in the study. Cells were grown in media Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% Penicillin/streptomycin. LPS and 4-HPPA were commercially available and applied to the cells at different concentrations (LPS 0.125, 0.25, 1.25, 2.5 ng·mL^{-1}; 4-HPPA 2.5, 6.25, 12.5, 25 µg·mL^{-1}, respectively) for 24 hours. Effective concentrations of these substances were determined by Thiazolyl Blue Tetrazolium Bromide (MTT) viability tests. At the end of the period, the expression levels of genes encoding IL-1β (Interleukin-1 Beta), TNF-α (tumor negrosis factor-alpha) and Nuclear Factor kappa B (NFkβ), which are important markers of inflammation, were investigated by full-time quantitative polymerase chain reaction (qRT-PCR) from the RNA samples taken from the experimental groups.

Cell Viability Test

The cell viability rate can be determined quantitatively by using Thiazolyl Blue Tetrazolium Bromide (MTT), on the principle that the mitochondrial reductases of living cells in a cell population can cleave the tetrazolium ring in the compound. With the disintegration of the tetrazolium ring, the yellow MTT molecule turns into a blue-violet formazan molecule. In this method, briefly, after 24 hours of exposure of LPS and 4-HPPA to the cells, the medium in the wells was removed and 200 µL of complete medium containing 20 µL of MTT reagent (obtained by dissolving it in 5 mg·mL^{-1} PBS) was added. The cells were incubated for 4 h at 37°C, and the MTT products were dissolved with 100 µL of 0.04 M hydrochloric acid/isopropanol for 15 min at 37°C. At the end of the period, the samples taken were centrifuged (Heal force, Neofuge 13-JAP) at 12000 G and +4°C and their absorbance was taken at 570 nm light wavelength in a microplate (µQuant ELISA reader). With this method, the effects of LPS and 4-HPPA on cell viability were analyzed (n=6).

RNA isolation and qRT-PCR analysis

After 24 hours, the kidney cells were harvested with trypsinization, and total RNA was isolated using a RNA ekstraction kit (Thermo Fisher, EU) from four groups, and its amount and purity (OD260/OD280 nm ratio) were determined on a spectrophotometer (UV mini 1240, Shimadzu, JAP).

For the PCR reaction, a one-step qualified, ready-made commercial kit with syber green probe marking (5x Hot FIREPol EvaGreen qPCR Mix plus(Rox)-EU) was used. cDNA conversion was carried out in a thermal cycler (BioRAD CFX96) by adding appropriate amounts of kit components (Hot Firepol DNA polymerase, reaction buffer, $MgCl_2$, dNTPs) to the RNA procedure taken from the samples, and a pair of primers specific to the genes under investigation (oligonucleotide – TABLE I), and PCR cycles were performed. PCR cycles were carried out as 40 cycles, with initial activation at 95°C for 12 min, followed by 15 s denaturation at 95°C, 20 s "Annealing" at 60°C and 20 s "Elongation" steps at 72°C. With this method, the expression levels of genes important in the inflammatory process, such as Interleukin 1 Beta (IL-1β), tumor necrosis factor alpha (TNF-α) and nuclear factor kappa B (NFkβ), were investigated in the RNA samples taken at the end of the period by optimizing them with the levels of the control gene GAPD

TABLE I
Specific gene sequences used in the research

Genes	Forward	Reverse
GAPDH	5′-CGTGGCCATCTCTTGCTCGAAG-3′	5′-CATCGTCACCAACTGGGACGAC-3′
IL-1β	5′-GGTCATTCTCCTGGAAGGAGGTCTGTGGGC-3′	5′-GCAAGGGCTTCAGGCAGGCCGCG-3′
TNF-α	5′-CAGAGGGAAGAGTTCCCCAG-3′	5′-CCTTGGTCTGGTAGGAGACG-3′
NFkβ	5′-AGGCAAGGAATAATGCTGTCCTG-3′,	5′-ATCATTCTCTAGTGTCTGGTTGG3′

Statistical analysis

Viability data were revealed using the "One-way ANOVA" analysis method of the SPSS 22.0 (Statistical Package for Social Sciences) package program. Statistical differences were determined by the "Duncan test". A *P*-value less than 0.05 was considered statistically significant. The findings of the study were presented as mean ± standard error (SE). PCR findings are given with percentage standard errors.

RESULTS AND DISCUSION

Viability results

Human kidney cells (HEK 293) were treated with different concentrations of LPS and 4-HPPA for 24 hours. After the incubation period, the viability rates in the cells were revealed by MTT analysis In the viability findings, when compared to the control group (0.324 ± 0.014), the cell count at 2.5 and 6.25 µg·mL^{-1} concentrations of 4-HPPA increased by 5.24% (0.341 ± 0.07) and 5.86% (0.343 ± 0.011), respectively (FIG. 1; *P*<0.05). However, concentrations of 12.5 and 25 µg·mL^{-1} of 4-HPPA reduced by 17.9% (0.266 ± 0.09) and 31.79% (0.221 ± 0.04), respectively (*P*<0.05). Based on the data obtained, since 4-HPPA was effective at a concentration of 6.25 µg·mL^{-1}, this concentration was applied to the cells in gene expression analysis. Consistent with our data, Zhang *et al* (15), reported the effects of HPPA at the concentrations of 6.25, 12.5, 25, 50 and 100 µg·mL^{-1} for 24 h on the cell viability of RAW 264.7 macrophage and the concentration of 6.25 mg·mL^{-1} was shown to significantly increase cell number.

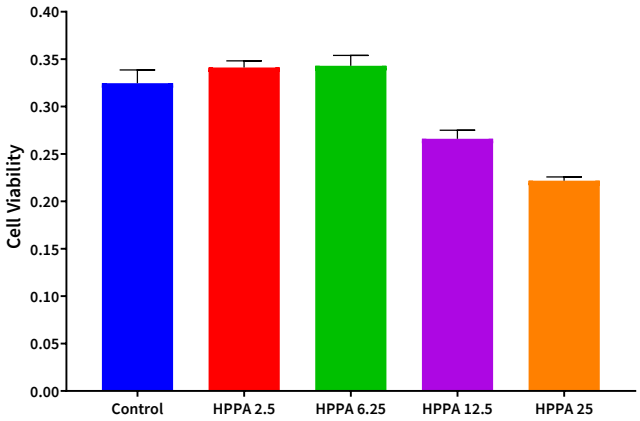


FIGURE 1. Effects of 4-HPPA on human kidney cell (HEK 293) viabilities. The effective concentration was determined with the use of different concentrations (2.5, 6.25, 12.5 and 25 µg·mL^{-1}) of 4-HPPA and MTT analyse was used for all of tests. All data are mean±SE and are representative of at least three independent experiments

The results of LPS, indicated that this increased by 0.02% (0.332 ± 0.010) at 0.125 ng·mL^{-1} concentration compared to control cells (0.3246 ± 0.014) (*P*<0,05). However, increasing concentrations of LPS decreased the viability by 3.7% (0.312 ± 0.015), 11.7% (0.286 ± 0.08) and 17.5% (0.267 ± 0.03) compared to the control, respectively (FIG. 2) (*P*<0,05). Based on the data obtained, LPS was found to be effective at a concentration of 2.5 ng·mL^{-1} and was chosen for gene expressions.

Lipopolysaccharides (LPS) is a microbial-derived stimulant as it is a component of gram negative bacteria cell wall. Utilising LPS

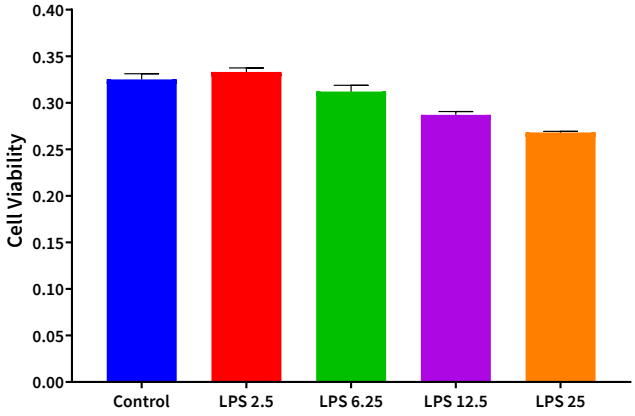


FIGURE 2. Effect of LPS at different concentrations on the cell viabilities.The effective concentration was determined with the use of different concentrations (0.125, 0.25, 1.25, 2.5 ng·ml^{-1}) of LPS and MTT analyse was used for all of tests. All data are mean±SE and are representative of at least three independent experiments

rather than inflammatory stimuli such as TNF-α or IFN-γ simulates bacteriainduced inflammation. Therefore, LPS have been used as a stimuli in *in vitro* experiments [17-19]. It was shown in a previous research that LPS caused significant death in HEK cells [20]. Chen *et al*. [21] reported that, in their *in vitro* microglia LPS-stimulated dopamine neurotoxicity, LPS application significantly reduced cell density, It is shown that these studies are compatible with our data.

When the effects of 4-HPPA and LPS at effective concentrations on cell viability were examined, it was determined that the cells reduced by 17.5% (0.267± 0.03) in the trial group in which LPS 2.5 ng·mL^{-1} was added, compared to the control group (0.324 ± 0.015) (*P*<0.05). Again, it was revealed that the cell viability was preserved by 11.23% in the experimental group in which 4-HPPA and LPS were used together (0.300 ± 0.04) at effective concentrations, compared to the group in which only LPS was applied (*P*<0.05) (FIG. 3).

In literature reviews on the subject, no reports showing the effects of HPPA on cell viability in LPS-stimulated studies were

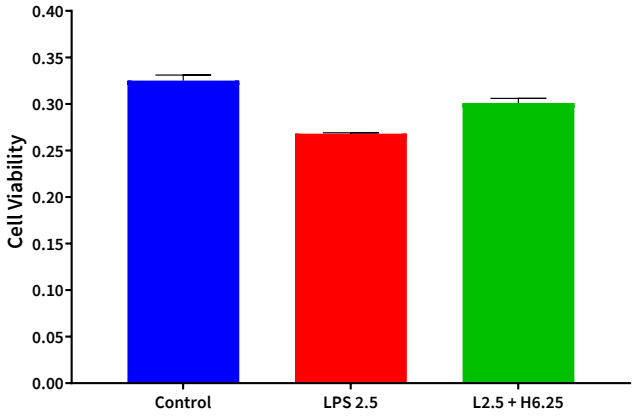


FIGURE 3. Effects of HPPA and LPS on the viability. The effective concentrations of LPS and LPS+4-HPPA on cell viability was determined by MTT analyse. All data are mean±SE and are representative of at least three independent experiments

identified. Naringin which belong to a subclass of flavonoid is found predominantly in citrus fruits. Due to their beneficial effects on human health, they have been discussed in many studies [5, 6, 22, 23]. With its low bioavailability, naringin is poorly absorbed in the blood circulatory system, suggesting that orally administered naringin accordingly remains in the gastrointestinal tract for a long time and is inevitably influenced by the intestinal microbiota [24]. However, since HPPA is the metabolite product of naringin, biological activity can be correlated both naringenin and HPPA [25]. In this context, in LPS-stimulated dopamine neurotoxicity, it has been that LPS application naringin administered together with LPS protected it significantly compared to LPS [25].

Gene expression results

According to this results, it was found that LPS at the selected concentration upregulated the NFkβ gene expression level with 16% relative fold changes compared to the control group. In the group in which LPS was applied together with 4-HPPA, it was found to be downregulated by 40% compared to the group in which only LPS was applied (FIG. 4). Inflammation is an important aspect of the pathogenesis of several types of acute and chronic diseases such as acute kidney injury (AKI) [1]. Macrophages are considered to play an essential role in inflammation. When activated by endotoxin (such as LPS), macrophages produce inflammatory cytokines, which in turn activate other macrophages and other nearby cells to promote inflammatory gene expression [26, 27]. Binding of exogenous LPS or endogenous ligands like members of the heat shock protein family and proteoglycans to TLR4 (e.g. by stimulating the NFkβ signaling pathway) activates the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, iNOS and IL 6 [17, 28, 29, 30]. Many studies have reported that LPS causes inflammasome formation by stimulating the expression of inflammatory mediators through the NFkβ signaling pathway. However, naringin or its metabolite HPPA have been shown to use various mechanisms to interfere with cancer development, promotion and progression, modulating several unregulated signaling pathways associated with inflammation, proliferation, apoptosis, autophagy, angiogenesis, invasion and metastasis [15].

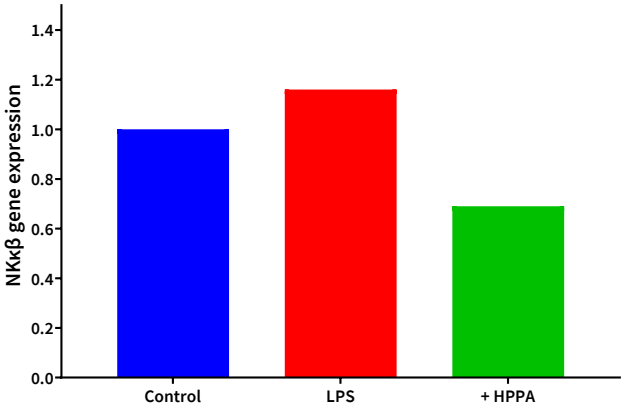


FIGURE 4. NFkβ expression levels (relative fold change). NFkβ gene expression levels were determined with the use of LPS alone and in combination with 4-HPPA. qRT-PCR method was applied for all tests. All data are representative of at least three independent experiments. Test time was 24 h. All specific gene expression levels were optimized for the house keeping gene, Standard errors in the experiment are shown as percentages (%)

In previous studies, Feng *et al.* [24] and Fu *et al.* [19] showed that in AKI, endotoxins such as lipopolysaccharide (LPS) bind to TLR4, leading to the activation of the NFkβ pathway and the production of TNF-α and IL-1β cytokines, leading to infiltration and activation of immune cells. Zhang *et al.* [15] revealed that PCA2 and its major microbial metabolite (HPPA) significantly suppressed the activation of NF-κB pathways induced by ox-LDL, confirming the fact that inhibition of NF-κB can reduce foam cell formation. It was observed that the above-mentioned studies coincide with our findings.

Moreover, LPS up-regulated IL-1β expression by 184% compared to the control group, while mixed application down-regulated IL-1β gene expression levels by 7% compared to the LPS-only group (FIG. 5). In a study parallel to our data, it has been reported procyanidin A2 (PCA2) and its major microbial metabolite (HPPA) significantly that decreased the ox-LDL-increased levels of IL-6 and IL-1β [15].

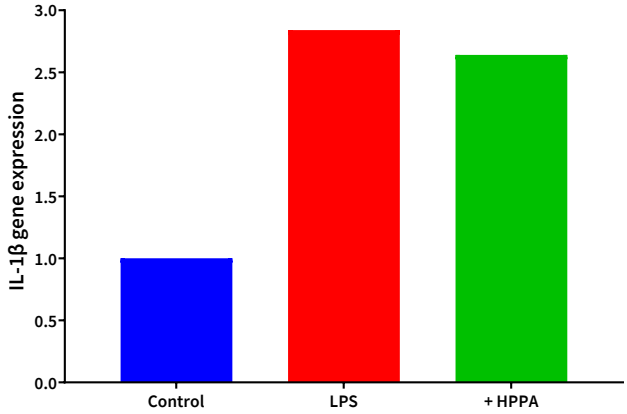


FIGURE 5. IL-1β gene expression. (relative fold change). IL-1β gene expression levels were determined with the use of LPS alone and in combination with 4-HPPA. qRT-PCR method was applied for all tests. All data are representative of at least three independent experiments. Test time was 24 h. All specific gene expression levels were optimized for the house keeping gene, Standard errors in the experiment are shown as percentages (%)

According to TNF-α gene expression results, it was found that LPS at effective concentration upregulated this gene by 12% compared to the control group. Again, it was found that in the group in which 4-HPPA was applied together with LPS, TNF-α expression was downregulated by 42% compared to the group in which LPS was applied (FIG. 6). Peng *et al.* [30] revealed that IL-6, IL-1β and TNF-α gene expressions increased significantly as a result of the interaction of LPS with TLR-2 in an *in vivo* septic acute kidney injury model.

Cell Images

In the research, after applying effective concentrations of 4-HPPA and LPS to kidney cells for 24 hours, cell images (FIG. 7) were taken from one of the cell wells with a 20x objective of an inverted microscope (Olympus CKX41-JAP).

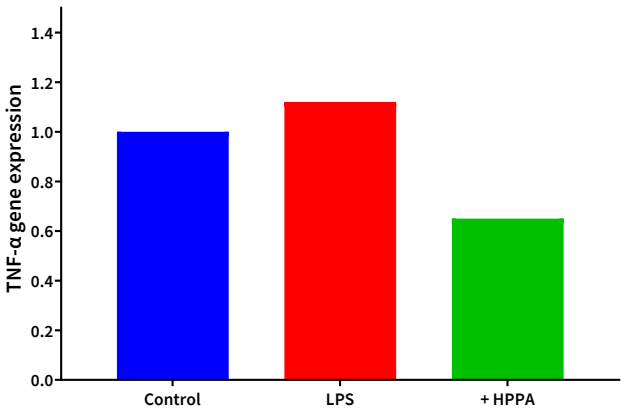
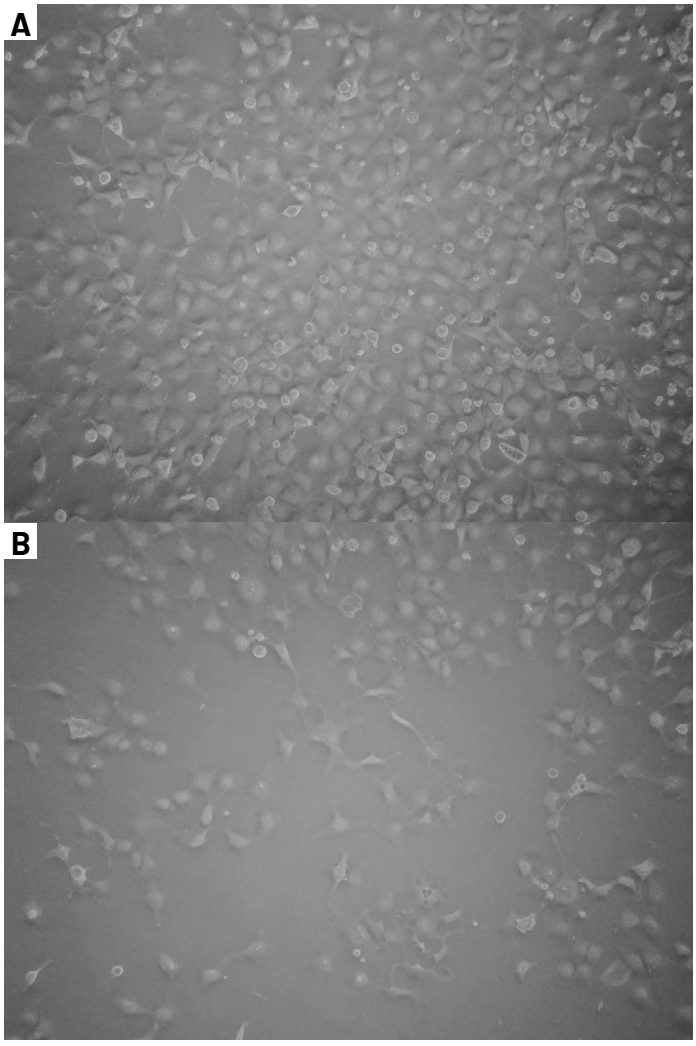


FIGURE 6. TNF-α gene expression levels. TNF-α gene expression levels were determined with the use of LPS alone and in combination with 4-HPPA. qRT-PCR method was applied for all tests. All data are representative of at least three independent experiments. Test time was 24 h. All specific gene expression levels were optimized for the house keeping gene, Standard errors in the experiment are shown as percentages (%)



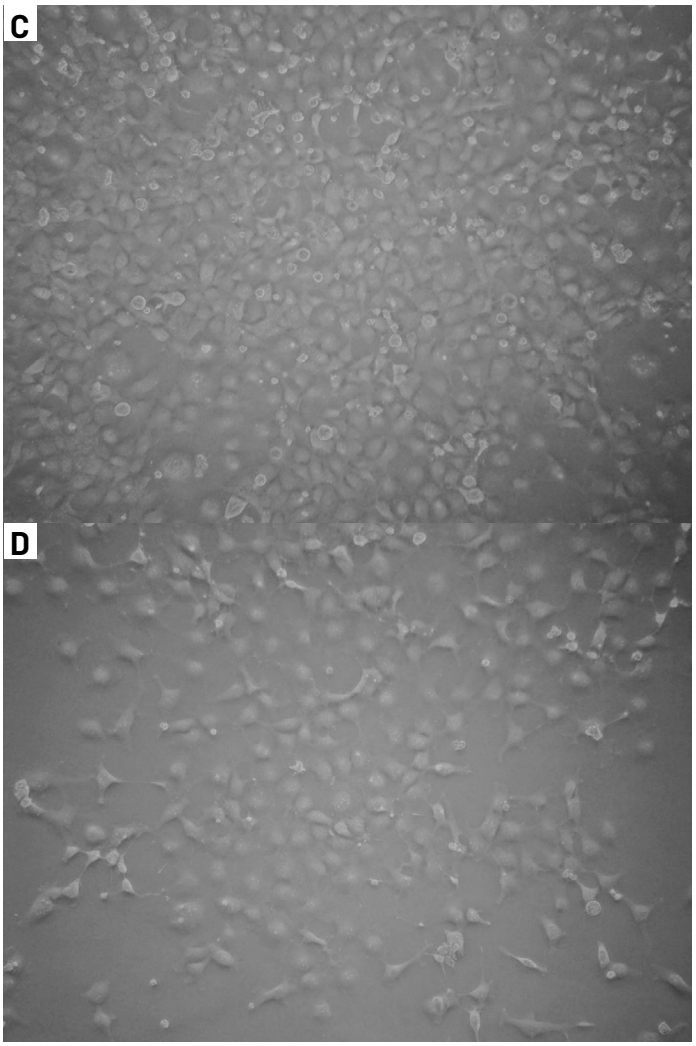


FIGURE 7. Cell images in the control (A), LPS (B), 4-HPPA (C) and LPS+4-HPPA (D) applied groups (24 h)

CONCLUSION

In this research, it was observed that LPS applied to cells in an *in vitro* kidney inflammation model effectively stimulated inflammation and caused cell death. This situation is supported by NFkβ transcriptor factor, IL1β and TNF-α expression level measurements and MTT cell viability tests. In addition, we found that 4-HPPA, significantly increased cell numbers and reduced the expression levels of inflammatory mediators stimulated by LPS. As a result of our findings, the positive effects of HPPA on cell number may be associated with the suppression of NFkβ, IL-1β and TNF-α gene expression which have an active role in inflammation. In addition, 4-HPPA may be alternative in the treatment of LPS-induced acute renal injury. However, *in vivo* experiments and advanced analyzes are needed to fully elucidate the issue.

Ethical approval

This study was approved by Hatay Mustafa Kemal University Non-invasive Clinical Trials Local Ethics Committee (Decision No: 2021/09-15).

Conflict of interests

The authors of this study declare that there is no conflict of interest with the publication of this manuscript.

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