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QUERCETIN ATTENUATES NASO-SINUSAL INFLAMMATION AND INFLAMMATORY RESPONSE IN LUNGS AND BRAIN ON AN EXPERIMENTAL MODEL OF ACUTE RHINOSINUSITIS IN RATS

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This study aimed to investigate the effect of quercetin without intranasal inflammation and oxidative stress in nasal and sinus mucosa, but also in serum, lungs and brain in a rat model of acute nasal and sinus inflammation induced by administration of lipopolysaccharides (LPS) (from *Escherichia coli*). Wistar rats were divided into five groups of 10 animals each. The control group received an intranasal saline solution once/day, for seven consecutive days. Rats in groups 2 and 3, received low-dose (5 μg) and high-dose (10 μg) of LPS, once/day, for seven consecutive days. Rats in groups 4 and 5, received low-dose (5 μg) and high-dose (10 μg) of LPS and after 2 h, 80 mg/kg of quercetin, once/day for seven consecutive days was administered. After the treatment period, the histopathological examination of nasal and sinus mucosa was performed and levels of cytokines (tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6)) and oxidative stress in the blood, nasal mucosa, lungs and brain were also analyzed. High dose of LPS increased TNF-α, IL-6 and IL-1β levels in serum, nasal mucosa, and lungs homogenates while in brain, this effect was only on TNF-α levels. IL-1β enhanced significantly in serum and mucosa, especially after administration of a high dose of LPS ($P < 0.01$ and $P < 0.05$). Histopathological and immunofluorescence analysis revealed acute inflammatory reaction in rats treated with both doses of LPS without significant changes of lipid peroxidation in the studied tissues. Quercetin administration diminished the exudate and degree of inflammation in lamina propria of nasal and sinusal areas, parallel with the decreased secretion of TNF-α (40.2% reduction after the low dose of LPS, and 35.4% reduction after the high dose of LPS) and IL-6 (21.4% reduction after the low dose of LPS and 35.8% reduction after the high dose of LPS). In lungs, quercetin reduced TNF-α (43.3%) and IL-6 levels (24.5%), and in the brain, the protective effect was noticed only on TNF-α (46.5%). The intranasal LPS administration successfully induced acute rhinosinusitis in a rat model and also generated an inflammatory response in the lungs and brain. Intranasal administration of quercetin diminished the nasal inflammation and also exerted protective effect on lungs and partially on brain inflammatory reaction.

Key words: *acute rhinosinusitis, nasal mucosa, inflammation, oxidative stress, quercetin, lipopolysaccharides, pro-inflammatory cytokines, lungs, brain*

INTRODUCTION

Acute rhinitis is one of the most common inflammatory diseases. The major symptoms are nasal obstruction due to the congestion of the nasal mucous membranes and nasal secretions. In its evolution, a frequent complication is acute rhinosinusitis (1, 2), which, if left untreated, can progress to chronic rhinosinusitis, and sometimes to orbital or intracranial complications. Generally, in children, 5 – 3% of upper respiratory tract infections complicate into acute rhinosinusitis (3). Moreover, it is estimated that rhinosinusitis affects about 15% of the population in Western countries, both adult and children (3, 4) and the bacterial etiology is represented mostly by *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus* (5).

From the point of view of symptoms, both acute and chronic rhinosinusitis have clinical similarities, so, the duration of clinical manifestations is one of the selection criteria between the clinical forms of this inflammatory process. In this way, rhinosinusitis is classified as acute (symptoms for up to 3 weeks), sub-chronic (symptoms between 3 to 12 weeks), and chronic (symptoms lasting over 12 weeks) (1, 6).

The pathophysiology of rhinosinusitis is characterized by congestion of the lining membrane of the nose and paranasal sinuses (2), associated with increased local infiltration with inflammatory cells and secretion of pro-inflammatory cytokines such as interleukin (IL)-1α, IL-1β, IL-6 and IL-8 (2, 4, 7), and tumor necrosis factor (TNF-α) (8). These pro-inflammatory cytokines are key factors in initiating and maintaining the inflammation and are considered chemoattractive agents for

neutrophils, which will migrate to the inflammatory area (4). Consequently, the inflammation will be amplified, leading to local edema and swelling of the mucosa and increase nasal and sinus secretions (7, 9). During inflammation, prostaglandin E_2 (PGE_2) levels are also increased, due to induction of cyclooxygenase (COX)-2 and leading to high value of IL-6 (10-12). It was demonstrated that IL-6 plays an important role in the development and progression of the inflammatory responses (13, 14). Inflammation induces oxidative stress, process defined as an imbalance between the production of reactive oxygen species and antioxidant defenses (15, 16). Different studies support an interdependent relationship between inflammation and oxidative stress (16-19). During the inflammatory process, the activated neutrophils and macrophages release large amounts of free reactive radicals (20, 21). Under pathological inflammatory conditions, there is an excessive generation of reactive species, and some of them will diffuse out of the phagocytic cells leading to cell damage and tissue injury (22, 19). These reactive species can attack almost any cell component, but lipids are important targets (20). Lipid peroxidation involves oxidative degradation of polyunsaturated fatty acids to malondialdehyde, a standard marker used for clinical evaluation of oxidative stress in correlation with inflammation (15, 20, 21).

An effective regulatory mechanism is needed to control inflammation in rhinosinusitis due to synthesis of pro-inflammatory cytokines and generation of reactive oxygen species, in order to eliminate the pathogens, with minimal side effects on host (13, 23). Interleukin-10 (IL-10) is an anti-inflammatory cytokine which suppresses the production of pro-inflammatory molecules by neutrophils and macrophages and limits the immune response to pathogens, and by this, protects the host from tissue damage (13, 24, 23). Although a control mechanism occurs, acute bacterial rhinosinusitis requires symptomatic medication and antibiotic. However, rhinosinusitis also needs supportive care treatment in order to reduce local inflammation, edema and mucous hypersecretion (25, 26). Some studies described alternative therapeutics, like medicinal plants used to reduce the inflammatory response (21, 27-30). Polyphenols are natural compounds found in many vegetables, fruits and herbal medicines, which can act as antioxidants and reduce the free reactive radicals generation and also have anti-inflammatory effect (18, 31). Polyphenols includes different sub-classes of compounds such as flavonoids, phenolic acids, lignans and stilbenes; from these, flavonoids are one of the most studied group (31), due to higher antioxidant activity (18, 32). Quercetin is a natural flavonoid found in vegetables and fruits with proven antioxidant capacity (32) and anti-inflammatory, anti-viral, anti-proliferative and anti-atherosclerotic properties (33). The anti-inflammatory effect of quercetin was demonstrated in several studies, by inhibiting the secretion of inflammatory cytokines and chemokines (34, 35). However, the exact role of quercetin on immune cells nasal inflammation is not well understood. It was demonstrated that quercetin inhibited LPS-induced TNF-α production in macrophages (36). In 2006, Nanua *et al.* have hypothesized that quercetin blocked airway epithelial cells chemokine expression *via* phosphatidylinositol (PI) 3 kinase-dependent pathway. They showed that quercetin blocks the airway epithelial cell IL-8 and monocyte chemoattractant protein (MCP)-1 expression by attenuating the signaling through a PI-3 kinase/Akt/NF-κB pathway, and also inhibits chemokine expression *via* transcriptional and posttranscriptional ways (37). There are several studies *in vitro* that investigated the effect of quercetin on allergic rhinitis and it has been reported that beside the anti-inflammatory effect, quercetin inhibited the releasing of chemical mediators (*e.g.*, histamine and leukotriene) from mast cells and eosinophils (38). In addition, quercetin acted as a scavenger of free reactive oxygen species which damage cell membranes and cause cell death, and thus allowed protection against redox misbalance (39-40).

The present study aims to investigate the effect of intranasal quercetin against inflammation and oxidative stress in nasal and sinus mucosa in a rat model of acute nasal and sinus inflammation induced by local administration of LPS (from *Escherichia coli*).

In order to evaluate if the sino-nasal inflammation progresses to the lungs and brain and whether there is a systemic inflammatory response, the cytokines levels and oxidative stress markers in serum, lungs and brain were assessed. The evaluation of inflammation in the brain was done because one of the complications in acute bacterial rhinosinusitis is the spreading of infection from sinus to the brain tissue through the sinus wall. This dissemination is rare involve and affects the frontal sinus (41-43).

Lipopolysaccharides were chosen to induce inflammation of the nasal mucosa due to several qualities: is the primary chemical components of the outer membrane of gram-negative bacteria (44), which can induce acute inflammation by releasing of inflammatory cytokines (45). Intranasal administration of LPS produces an inflammatory response by attracting neutrophils (46). The airway neutrophilia was triggered by two pathways: first, direct activation of the airway epithelial cells by LPS with production of IL-8, a neutrophil attractant chemokines (47), and second, indirect pathway by Th17 cells activation and releasing of IL-17 which in turn activated the airway epithelial cells to generate IL-8 and other pro-inflammatory cytokines (48). In addition, LPS increased COX-2 expression and stimulated production of prostaglandins, including prostaglandin E2, which is considered to be a stimulus for IL-6 production (10, 12). So, bacterial LPS can cause airway inflammation in humans and animals (44, 49). Therefore, some studies used LPS to induce acute (8) or chronic rhinosinusitis (49, 50) in a mouse model.

MATERIALS AND METHODS

Reagents

2-thiobarbituric acid and Bradford reagent were obtained from Merck KGaA (Darmstadt, Germany). Quercetin was obtained from Sigma-Aldrich Chemicals GmbH (Germany) and was prepared on the day of administration in dimethyl sulfoxide (DMSO) 4% in order to prevent its oxidation. This solution was then diluted with saline solution at appropriate concentration for the experiment. ELISA tests for cytokines (TNF-α, IL-1β, IL-6) were purchased from Elabscience (Houston, Texas, USA). All chemicals and reagents were of high-grade purity. Primary antibodies IL-1α, IL-10 and COX-2 used for immunofluorescence analysis were purchased from Santa Cruz Biotechnology, Inc, (Dallas, Texas, USA).

Animals and experimental design

The study was approved by the Local Ethics Committee on Experimental Animal Studies of University of Medicine and Pharmacy "Iuliu Hatieganu", Cluj-Napoca, Romania, according to the Directive 2010/63/EU on the protection of animals used for scientific purposes (no. 68/2019).

The study was conducted with 50 female Wister rats, age 20 days, weighing 100 ± 10 g. The animals were housed for ten days in the Animal Facility of the Physiology Department, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, for acclimatization before treatment. During the experiment, rats were housed under the standard conditions, consisting of a 12/12-h light/dark cycle, temperature of 21ºC and humidity of 60 – 65%. The animals were fed with the standard commercial pellet diet and water *ad libitum*.

Rats were randomly divided into five groups of 10 animals each. The medication used for the study was dropped into the nasal cavities, using a micro-pipette. For the normal control group, 20 µl of sterile normal saline solution with DMSO was administered, once/day, for seven consecutive days. Rats in groups 2 and 3, received low-dose and high-dose of LPS (from *Escherichia coli*), 5 µg, respectively 10 µg of LPS, once/day, for seven consecutive days. Rats in groups 4 and 5, received low-dose and high-dose of LPS (5 µg, respectively 10 µg of LPS), and after 2 h, 80 mg/kg of quercetin was administered in 20 µl solution, once/day, for seven consecutive days. Before and during the administration of the medication, nasal examination was performed in all rats. After four days of repeated intranasal LPS administration, nasal congestion and erythema, was detected in all animals from 2 to 5 groups.

At 24 h after the last treatment, under anesthesia with 10% ketamine and 2% xylasine, the blood samples were collected to assess the parameters of oxidative stress (malondialdehyde) and inflammatory markers (TNF-α, IL-1β, IL-6). Nasal mucosa, lungs, and brain tissue were removed by en bloc dissection. Nasal mucosa was used for histopathological and immunofluorescence analysis, and also for evaluation of inflammation and oxidative stress. From lung and brain tissues homogenates the oxidative stress and inflammation evaluation was also performed.

Oxidative stress markers

In order to assess the biochemical analyses, the harvested tissues were homogenized with a Polytron homogenizer (Brinkman Kinematica, Switzerland) for 3 min on ice in phosphate buffered saline (pH 7.4) at a ratio of 1:4 (w/v), as previously published (51). Then, the suspension was centrifuged for 5 min at 1006 g/min, and 4ºC for preparation of the cytosolic fraction (51). The protein level in homogenates was measured according to the Bradford protocol (52).

In order to quantify the oxidative stress induced by LPS administered into the nasal cavities and the impact of local administration of quercetin, the malondialdehyde levels were assessed in serum, nasal mucosa, brain, and lungs. Malondialdehyde was determined by the fluorimetric method with 2-thiobarbituric acid described by Conti (53). Malondialdehyde values are expressed as nmoles/mg protein.

Pro-inflammatory markers

The concentration of TNF-α, IL-1β, and IL-6 in serum blood, nasal mucosa, brain, and lungs homogenates was evaluated by ELISA assay according to the manufacturer's protocol. Results are expressed either as pg/ml in serum or pg/mg protein in tissue homogenates. The sensitivity of ELISA kits is as follows: TNF-α 46.88 pg/ml; IL-1β 18.75 pg/ml; IL-6 12 pg/ml, and limit of detection for malondialdehyde measurement is 0.38 – 33.33 nmoles/ml.

Histopathological and immunofluorescence analysis

After the complete fixation in 10% neutral-buffered formalin, the heads were cleaned of skin, subcutaneous connective tissue, and muscle and decalcified with Richard-Allan Scientific Decalcifying Solution (Thermo Fisher

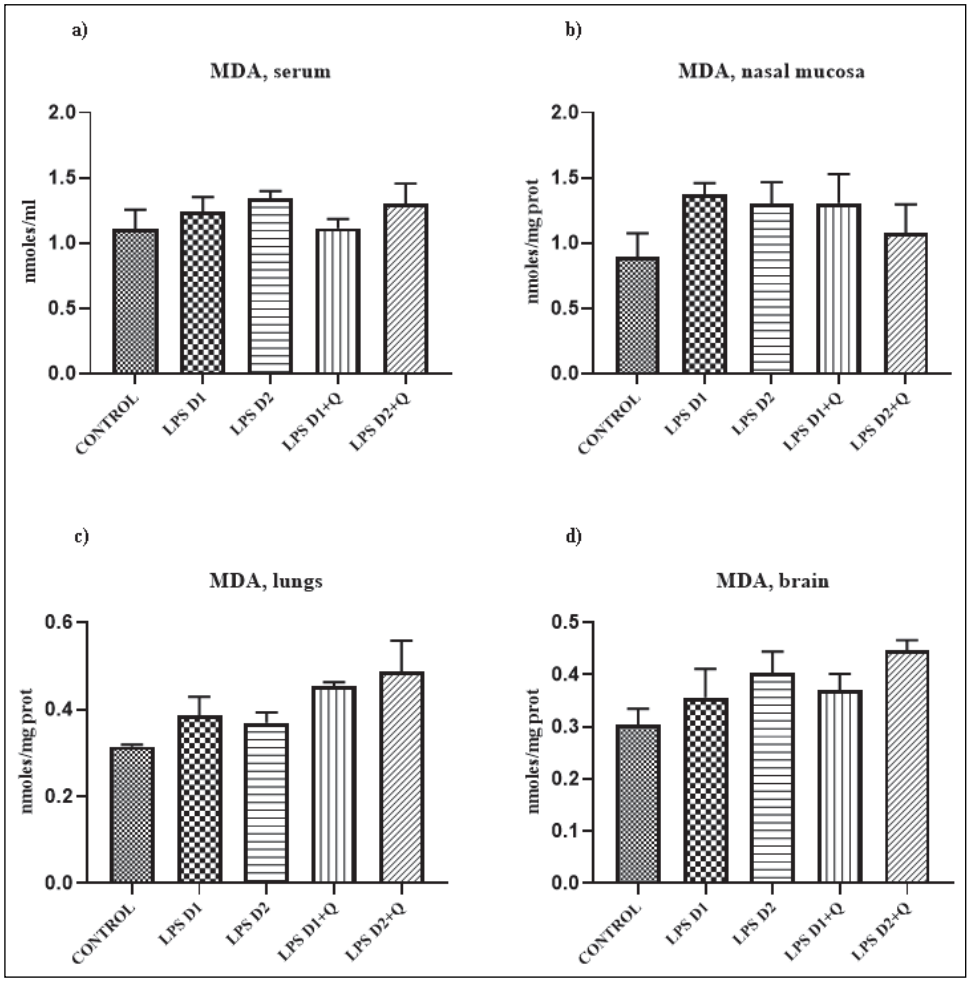

Fig. 1. Malondialdehyde (MDA) in the blood (a), nasal mucosa (b), lungs (c), and brain (d) homogenates in rats treated with lipopolysaccharides (LPS) and quercetin (Q). The rats were intranasal treated for seven days with two different doses of LPS, with or without quercetin. Malondialdehyde levels were quantified in serum, nasal mucosa, lungs, and brain homogenates at 24 h after the last treatment. Data are means ± standard deviation. Statistical analysis was done by a one-way ANOVA, with Tukey's multiple comparisons posttest.

Scientific). The decalcified skulls were transversely trimmed in four planes following the previously described technique by Kittel *et al.* (54) and, following a brief washing step in running tap water, embedded in paraffin-wax with the rostral faces down using routine histology technique. Finally, samples were sectioned to 4-mm thickness with a rotary microtome, stained with hematoxylin-eosin (H & E) and examined using an Olympus BX41 microscope. Histological images were obtained with an Olympus UC30 digital camera and further processed with Stream basic program. For immunofluorescence analysis paraffin-wax embedded tissues were mounted on silane-coated histological slides, deparaffinated in 3 baths of xylene, and rehydrated in descending concentrations of ethanol in water and finally in PBS. Antigen retrieval was carried in 10 mM sodium citrate (pH 6.0) and after several PBS baths and blocking solution (6% bovine serum albumin and 5% normal goat serum in PBS), the slides were incubated overnight with the primary antibodies IL-1 alpha (rabbit polyclonal, dilution 1:50) (SC-7929), IL-10 (mouse monoclonal, dilution 1:50) (SC-365858) and COX-2 (mouse monoclonal, dilution 1:200) (SC-376861) as previously described (55). After washing PBS, the sections were incubated with a secondary antibody: mouse anti-rabbit conjugated with FITC (sc-2359), goat anti-mouse FITC (sc-2010), and goat anti-mouse IgG-PE (sc-3738). Following immunostaining, the tissue samples were examined and collected using a Zeiss LSM 710 Confocal Laser Scanning unit mounted on a biological Axio Observer Z1 Inverted Microscope. Images were recorded using a Plan Apochromat 63 × objective (oil immersion). The image analysis was performed using the ZEN-Zeiss software.

The measurement of the fluorescent intensity of IL-1α, IL-10, and COX- 2 was carried out automatically using the point-by-point fluorescence quantification functions of the ZEN software included in the Confocal Laser Scanning unit (Zeiss LSM 710) as previously described by Clichici *et al.* (56) and Price *et al.* (57). Briefly, ten randomly chosen microscopic fields of vision (obx63, representing an area of 182118 μm2) from the nasal mucosa were scanned, and LSM images acquired under constant acquisition parameters (output power of the lasers, acquisition time, pinhole diameter, master and digital gain, *etc.*) were further quantitatively analyzed for each fluorescent channel (FITC and PE).

Statistical analysis

All data are expressed as mean and standard deviation. The data were analyzed by one-way analysis of variance (ANOVA) follwed by the Tukey's multiple comparasions post test using GraphPad Prism 8 software. A P value < 0.05 was considered statistically significant.

RESULTS

Oxidative stress assessment in the blood, nasal mucosa, lungs and brain

Malondialdehyde (MDA) is a useful marker for clinical evaluation of oxidative stress. After repeated intranasal administration of LPS, an increase of malondialdehyde level was found in groups treated with LPS low and high doses, as

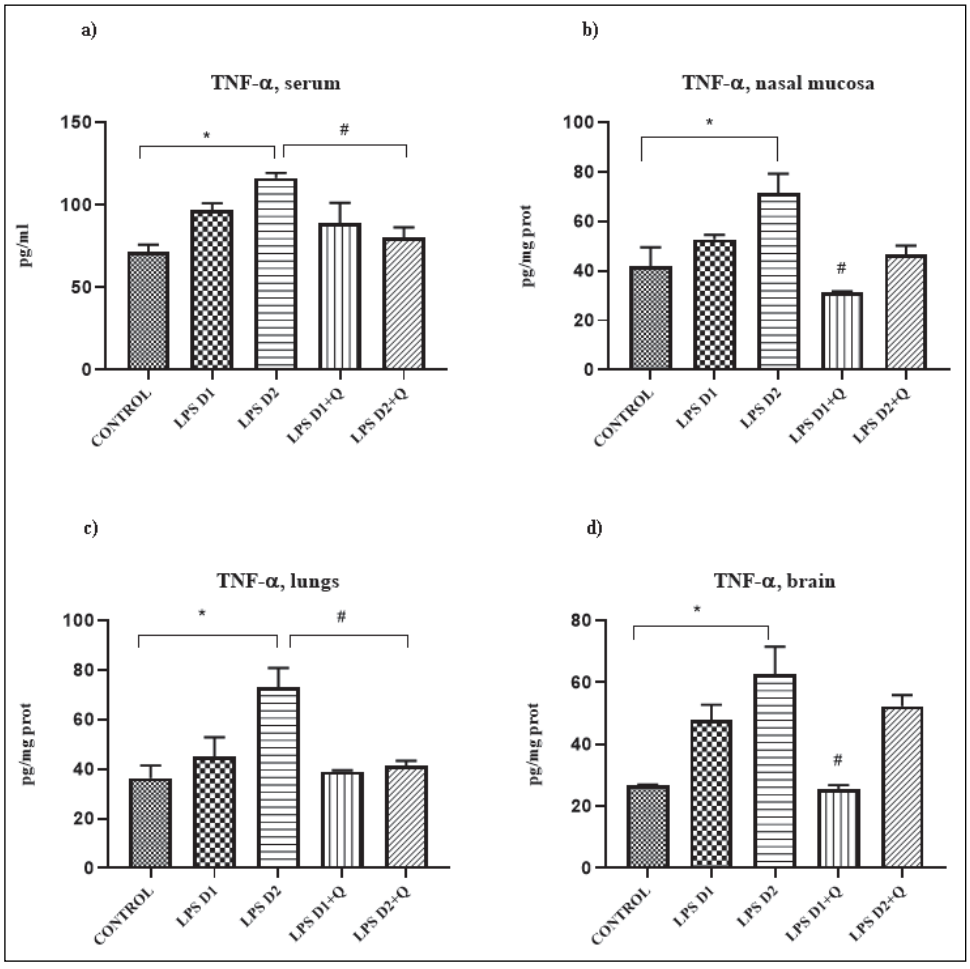

Fig. 2. Tunor necrosis factor-α (TNF-α) levels in the serum blood (a), nasal mucosa (b), lungs (c), and brain (d) in rats treated with lipopolysaccharides (LPS) and quercetin (Q). The rats were intranasal treated for seven days with two different doses of LPS, with or without quercetin. The TNF-α levels were quantified in serum, nasal mucosa, lungs and brain homogenates at 24 h after the last treatment. Data are means ± standard deviation. Statistical analysis was done by a one-way ANOVA, with Tukey's multiple comparisons posttest. * $P < 0.05$ as compared to control group. $^{\#}P < 0.05$ compared to groups treated with LPS.

compared to control group, especially at the nasal mucosa level (an increase of about 30%), but without statistical significance (P > 0.05) (*Fig. 1b*). In blood, lungs, and brain the MDA levels did not change significantly (P > 0.05). Quercetin, administered topically, did not change the malondialdehyde level compared to groups treated only with LPS (P > 0.05).

Inflammation asessment in the blood, nasal mucosa, lungs and brain

TNF-α quantified by ELISA (pg/mg protein) showed that repeated intranasal administration of LPS stimulated TNF-α secretion in blood, nasal mucosa, lungs, and brain homogenates, with a significantly increase of TNF-α level, especially in rats treated with high dose of LPS (P < 0.05), compared with the control group (*Fig. 2a-2d*). In groups treated with quercetin, TNF-α level was lower compared with LPS high dose group, the difference between groups being significant (P < 0.05).

IL-6 quantified by ELISA (pg/mg protein) increased after administration of LPS in all the investigated tissues, especially in serum, nasal mucosa, and lungs. Thus, IL-6 increased significantly in serum in both groups treated with two different doses of LPS (P < 0.05 and P < 0.01) and in nasal mucosa and lungs, in rats group treated with high dose of LPS (P < 0.05), compared with the control group (*Fig. 3a-3c*). Intranasal administration of quercetin resulted in a significantly reduced IL-6 level in nasal mucosa (P < 0.05) (*Fig. 3b*), compared with LPS high dose. In serum and lungs homogenates, the IL-6 secretion was lower compared with groups that received LPS, but the differences were not statistically significant (P > 0.05).

The intranasal administration of LPS for 7 days enhanced IL-1β level in rats treated with LPS high dose, as compared to control group, particularly in serum (P < 0.01) (*Fig. 4a*) and in nasal mucosa (P < 0.05) (*Fig. 4b*). In lungs and brain homogenates, IL-1β did not show significant changes compared to untreated group (P > 0.05). Intranasal administration of quercetin, resulted in a significantly reduced IL-1β level in serum (P < 0.05) (*Fig. 4a*), compared with LPS high dose, without significant changes in the nasal mucosa, lungs, and brain, in groups protected with quercetin.

Histopathological analysis

In order to quantify the inflammation induced by intranasal administration of LPS in two different doses, and the impact of local administration of quercetin, a conventional histopathological examination in hematoxylin-eosin was performed. The histopathological analysis of the nasal and paranasal sinuses cavities evaluated the inflammation of the lamina propria, the aspect of the nasal and sinus epithelium, and the presence of the inflammatory exudate in both cavities.

In the control group, there were no significant findings, with minimal inflammatory infiltrate of lymphocytes in the nasal cavity (*Figs. 5a* and *6a*).

In the group treated with low dose of LPS, an exudate with a large number of neutrophils was present at the nasal mucosa, respectively neutrophils and lymphocytes in the paranasal sinuses. A mild congestion of the lamina propria was observed with inflammatory infiltrate predominantly of lymphocytes at the sinus and nasal epithelium (*Figs. 5b* and *6b*).

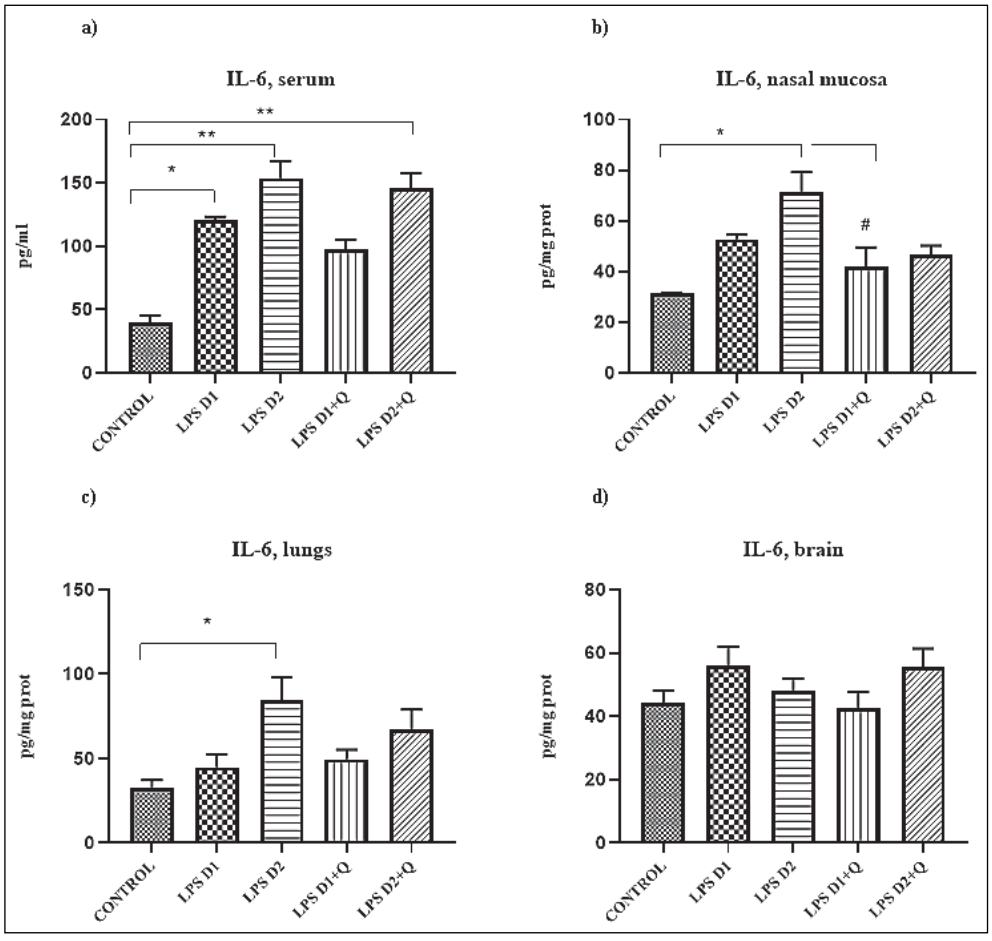

Fig. 3. Interleukin-6 (IL-6) levels in the serum blood (a), nasal mucosa (b), lungs (c), and brain (d) in rats treated with lipopolysaccharides (LPS) and quercetin (Q). The rats were intranasal treated for seven days with two different doses of LPS, with or without quercetin. IL-6 secretion was quantified in serum, nasal mucosa, lungs and brain homogenates at 24 h after the last treatment. Data are means ± standard deviation. Statistical analysis was done by a one-way ANOVA, with Tukey's multiple comparisons posttest. *P < 0.05; **P < 0.01 as compared to control group.

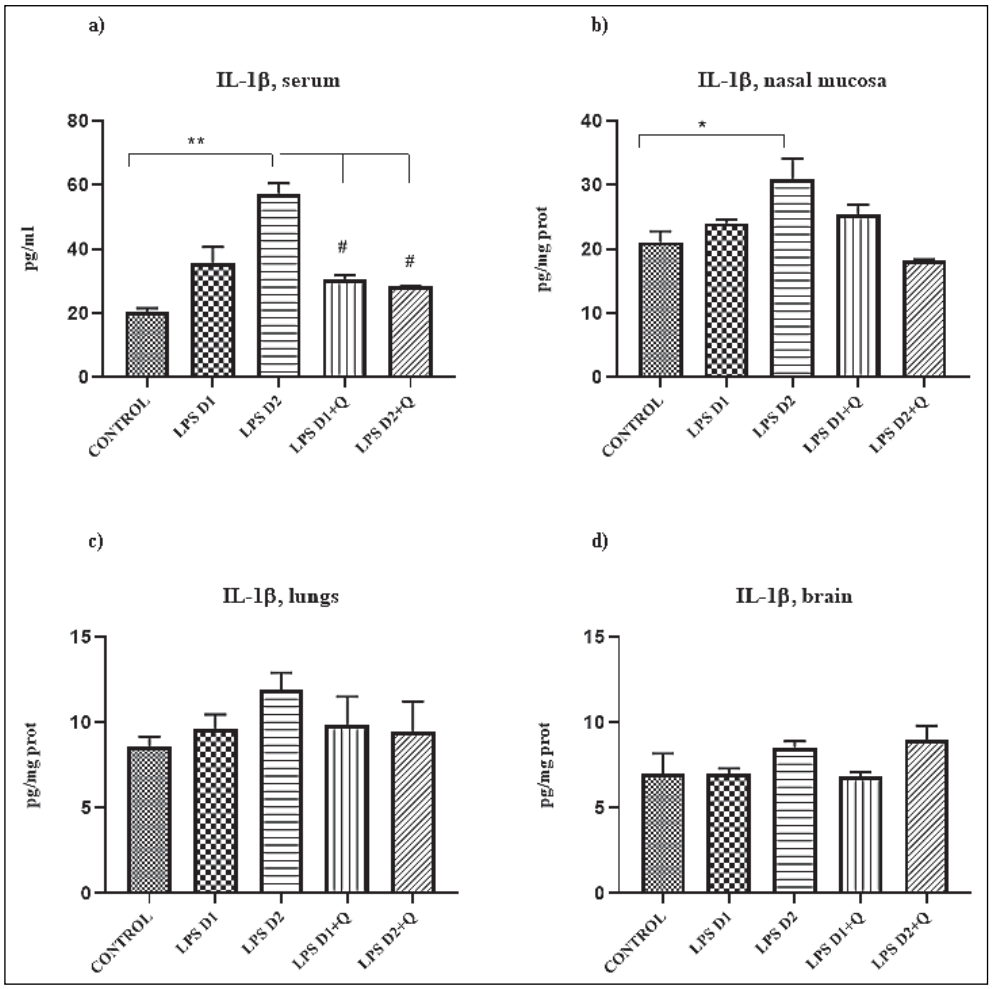

Fig. 4. IL-1β levels in the serum blood (a), nasal mucosa (b), lungs (c), and brain (d) in rats treated with lipopolysaccharides (LPS) and quercetin (Q). The rats were intranasal treated for seven days with two different doses of LPS, with or without quercetin. Interleukin 1β (IL-1β) secretion was quantified in serum, nasal mucosa, lungs and brain homogenates at 24 h after the last treatment. Data are means ± standard deviation. Statistical analysis was done by a one-way ANOVA, with Tukey's multiple comparisons posttest. **P < 0.01 as compared to control group, and $^{\#}$P < 0.05 compared to groups treated with LPS.

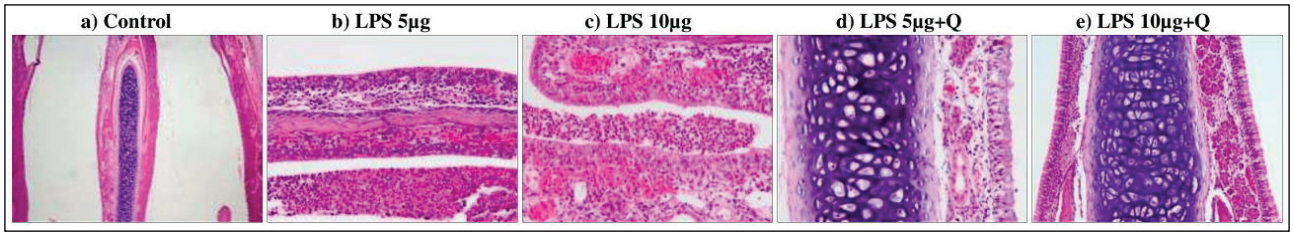

Fig. 5. Histopathological images of nasal mucosa of Wistar rats after seven days of intranasal lipopolysaccharides (LPS) administration and quercetin (Q). All panels represent nasal mucosa sampling areas in H & E-stained sections. In each panel, representative pictures from control (a), LPS 5 µg (b), LPS 10 µg (c), LPS 5 µg + Q (d) and LPS 10 µg + Q (e) are shown. In the control group (a), no significant findings, with minimal inflammatory infiltrate of lymphocytes (H & E, ob ×10), while in LPS groups (b and c) nasal mucosa is infiltrated by lymphocytes and neutrophils, with exudate and congestion of the lamina propria (H & E, ob ×20). Quercetin administration (d and e) reduced the exudate and decreased the degree of inflammation of the lamina propria, a minimal infiltrate with lymphocytes at the surface of the nasal epithelium was present (H & E, ob ×40).

Compared to the group treated with low dose of LPS, in rats treated with high dose of intranasal LPS, substantial congestion with moderate edema and mixed inflammatory infiltrate with a large number of lymphocytes in the lamina propria of the nasal mucosa was observed. An acute inflammatory exudate with an increase of the neutrophils number, was also observed in the nasal mucosa without affecting the paranasal sinuses (*Figs. 5c* and *6c*).

Intranasal administration of quercetin in rats treated with low dose of LPS reduces the exudate and decreases the degree of inflammation of the lamina propria, both in the nasal and sinus areas, with the presence of a minimal infiltrate with lymphocytes that affects only the surface of the nasal and sinus epithelium (*Figs. 5d* and *6d*).

In contrast, in the group treated with intranasal quercetin and high dose of LPS, a minimal infiltrate with lymphocytes in the lamina propria was observed, being present only at the nasal mucosa level, with minimal exudate and without lymphocytes in the thickness of the nasal and sinus epithelium (*Figs. 5e* and *6e*).

Immunofluorescence investigation

In order to detect the distribution of bound antibodies for IL-1α, COX-2 and IL-10 in the nasal mucosa immunofluorescence

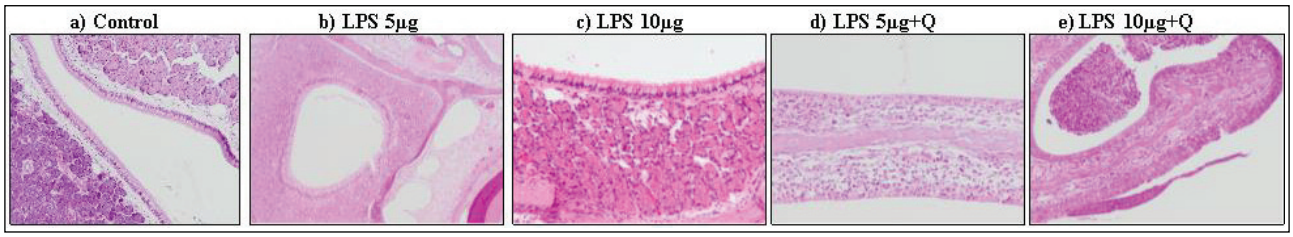

Fig. 6. Histopathological images of sinusal mucosa of Wistar rats after seven days of quercetin (Q) and lipopolysaccharides (LPS) administration. All panels represent sinusal mucosa sampling areas in H & E-stained sections. In each panel, representative pictures from control (a), LPS 5 µg (b), LPS 10 µg (c), LPS 5 µg + Q (d) and LPS 10 µg + Q (e) are shown. In the control group (a), no significant findings, (H & E, ob ×20), while in LPS groups (b and c), mild congestion of the lamina propria was observed with inflammatory infiltrate predominantly of lymphocytes at the sinus epithelium (H & E, ob ×40). Quercetin administration (d and e) reduced the exudate with minimal infiltrate with lymphocytes in the thickness of the sinus epithelium (H & E, ob ×20).

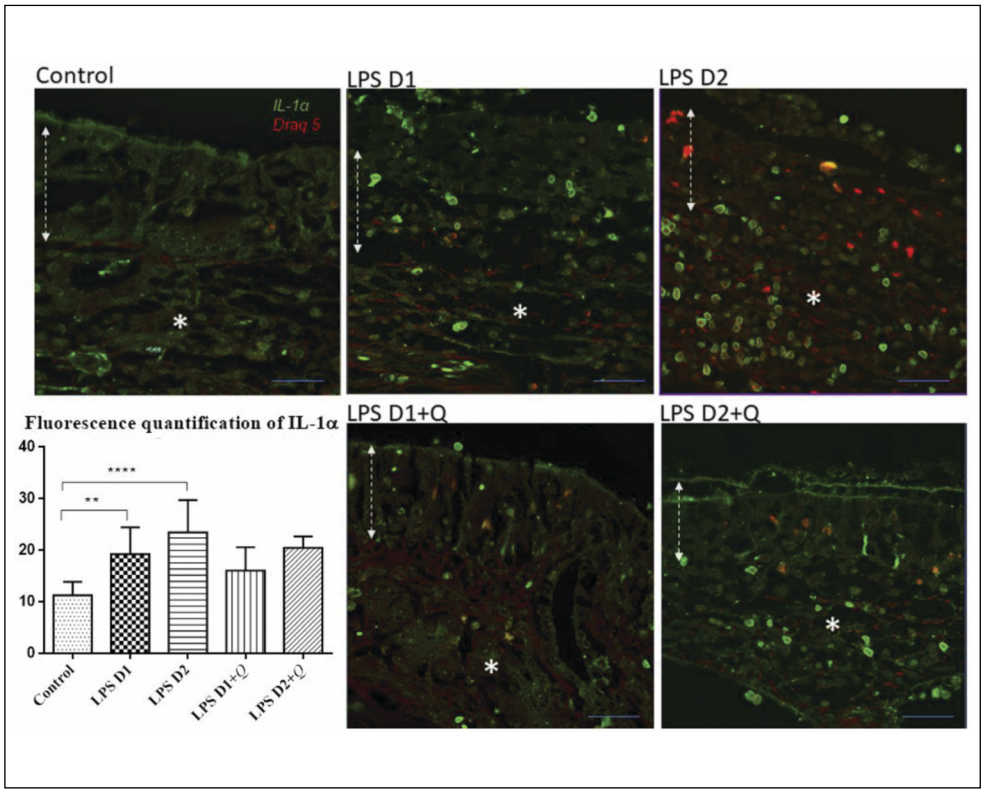

Fig. 7. Immunofluorescence expression (green-channel) and immunofluorescence quantification of interleukin-1α (IL-1α). All panels represent nasal mucosa sampling areas in immunofluorescence-stained sections. In each panel, representative pictures from control, lipopolysaccharides (LPS) D1, LPS D2, LPS D1 + quercetin (Q) and LPS D2 + Q are shown. Draq5 stain of nuclei (red channel). The double arrow indicates the nasal epithelium, and lamina propria is indicated by asterisk. Scale bar = 40 µm. For immunofluorescence quantification data are means ± standard deviation; statistical analysis was done by a one-way ANOVA, with Tukey's multiple comparisons posttest. **P < 0.01 and **** P < 0.0001, as compared to the control group.

and confocal microscopy were used. Scoring of immunofluorescences was based on the extent and intensity of staining in the nasal epithelium and lamina propria of the nasal mucosa. A moderate IL-1α expression (*Fig. 7*) and a minimal COX-2 and IL-10 expressions (*Figs. 8* and *9*) were observed in the nasal epithelium and lamina propria in rats treated with low dose of LPS, as compared to the control group. The number of positive cells (IL-1α, COX-2 and IL-10) increased significantly in rats treated with high dose of LPS (*Figs. 7*, *8* and *9*) while the administration of quercetin, reduced the extent and intensity of staining, especially that of IL-1α (*Fig. 7*).

Immunofluorescence quantification of IL-1α found significant differences between control group and group treated with LPS low dose ($P < 0.001$) and high dose ($P < 0.0001$), (*Fig. 7*). COX-2 fluorescence intensity increased in groups treated with LPS low and high dose (about 40%), as compared to the control group, but the differences were not statistically significant ($P > 0.05$), (*Fig. 8*). Immunofluorescence quantification of IL-10 also showed an increase in IL-10 expression (about 30 – 40%) in both groups treated with different doses of LPS, but without statistical significance ($P > 0.05$), (*Fig. 9*).

Quercetin administration reduced the fluorescence intensity of IL-1α, IL-10, and COX-2, but the differences were not statistically significant ($P > 0.05$), (*Fig. 7*, *8* and *9*).

DISCUSSION

The present study aims to assess the effect of quercetin on LPS-induced acute rhinosinusitis, knowing that quercetin has proven antioxidant capacity (34, 32), and anti-inflammatory activity (33, 32). Several *in vivo* studies demonstrated that quercetin diminished the histological changes of acute inflammation and decreased the inflammatory cytokines (34, 58), and malondialdehyde levels (59, 60, 34, 61). However, the limitations of quercetin are poor oral absorption and rapid metabolism which causes a low serum and tissue concentrations (62, 63). The absorption through nasal mucosa has not been clearly identified, although, nasal administration, could produce

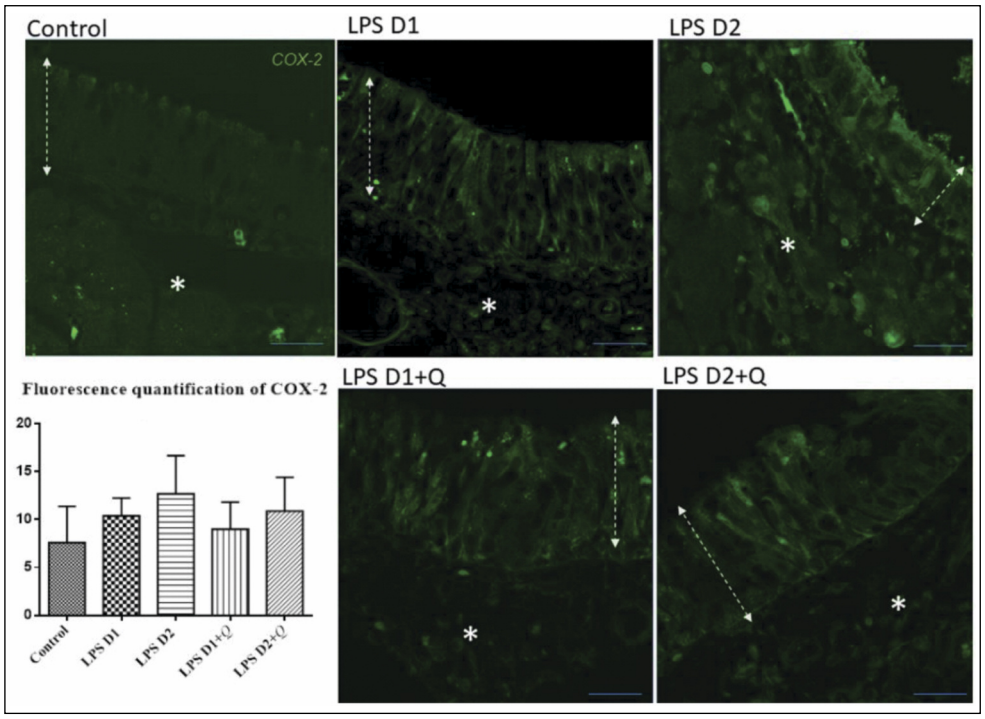

Fig. 8. Immunofluorescence expression (green-channel) and immunofluorescence quantification of cyclooxygenase-2 (COX-2). All panels represent nasal mucosa sampling areas in immunofluorescence-stained sections. In each panel, representative pictures from control, lipopolysaccharides (LPS) D1, LPS D2, LPS D1 + quercetin (Q) and LPS D2 + Q are shown. The double arrow indicates the nasal epithelium, and lamina propria is indicated by the asterisk. Scale bar = 40 μm. For immunofluorescence quantification data are means ± standard deviation, statistical analysis was done by a one-way ANOVA, with Tukey's multiple comparisons posttest.

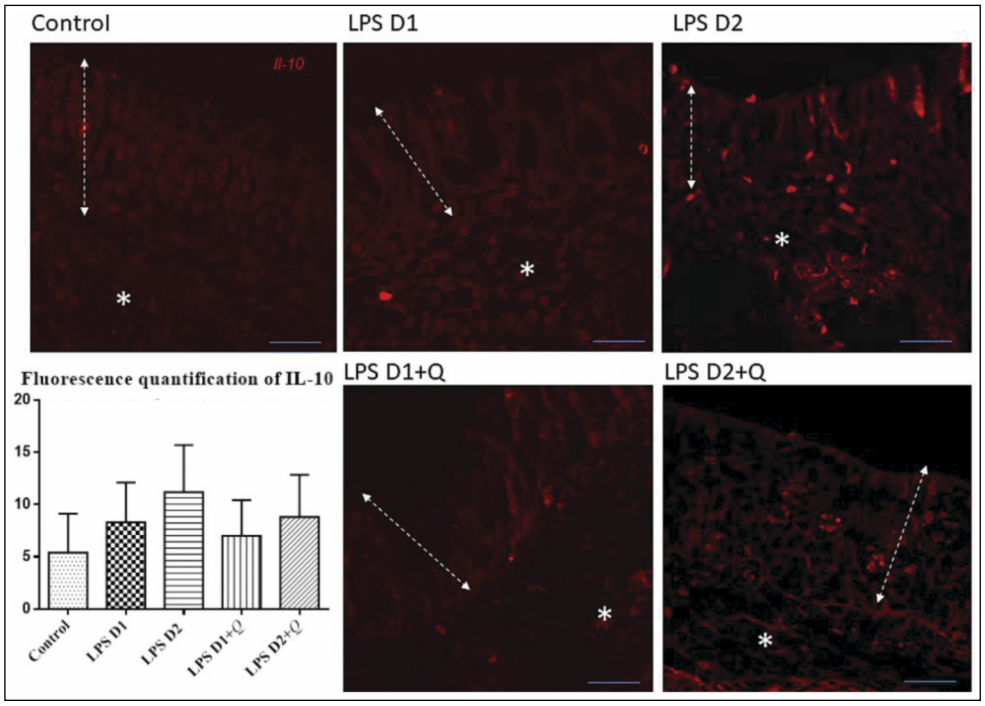

Fig. 9. Immunofluorescence expression (red channel) and immunofluorescence quantification of interleukin-10 (IL-10). All panels represent nasal mucosa sampling areas in immunofluorescence-stained sections. In each panel, representative pictures from control, lipopolysaccharides (LPS) D1, LPS D2, LPS D1 + quercetin (Q) and LPS D2 + Q are shown. The double arrow indicates the nasal epithelium, and lamina propria is indicated by the asterisk. Scale bar = 40 μm. For immunofluorescence quantification data are means ± standard deviation, statistical analysis was done by a one-way ANOVA, with Tukey's multiple comparisons posttest.

a higher bioavailability compared to the oral route due to the nasal cavity which is rich in blood vessels, potentially resulting in rapid substance absorption, and avoiding the hepatic metabolism (62).

So, we chose to perform an experimental rat model of acute rhinosinusitis using intranasal instillation with LPS (from *E. coli*) in two different doses (5 μg, low dose and 10 μg, high dose), for seven consecutive days, in order to investigate the effect of quercetin against local and systemic inflammation. LPS was chose to induce sino-nasal inflammation due to its ability to bind to toll-like receptor 4 and to activate the several intracellular signaling pathway, including nuclear factor (NF)-κB (64), known for triggering the secretion of inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8 (65, 66). In addition, the inflammatory process promotes the oxidative stress with free reactive oxygen species production, contributing to cellular and intracellular disturbances at the sino-nasal mucosa level (22, 20, 21).

During the last years, there are relatively few studies that tried to investigate both, inflammation and oxidative stress in patients or experimental animal models with acute rhinosinusitis (5). Rodents are the most commonly used animals to investigate

the nasal and sinus diseases, and the usual method to induce rhinosinusitis is nasal instillation of inflammatory agents, which triggers an immune response and activate the production of inflammatory cytokines (67). Compared with the number of studies with mice or rats, studies with rabbits are most suitable due to similarity with humans in sino-nasal anatomy and physiology (68). In the present study we chose to use rats because rabbits are more difficult to access, and for the reason that rats are easy to breed and to maintain in the laboratory (67). Although the sinus anatomy of mice is not so similar to that of human beings, the respiratory epithelium is the same (2), so rats are an alternative option to rabbits in performing an experimental model of rhinosinusitis.

In our study, in order to quantify the local inflammation of the sino-nasal mucosa and the redox imbalance, the inflammatory cytokines and malondialdehyde levels were measured and histopathological analysis of the nasal and sinus mucosa was assessed. To determine if the sino-nasal inflammation progresses to the lungs and whether there is a systemic inflammatory response, the cytokines and malondialdehyde levels in the serum, lungs and brain were also measured. Elevated levels of TNF-α, IL-6, and IL-1β in rats treated with both doses of LPS in all the investigated tissues were found. Thus, in serum and nasal mucosa of rats exposed to high dose of LPS, the TNF-α, IL-6, and IL-1β levels increased significantly. Moreover, the low dose of LPS amplified the release of IL-6 in blood and sino-nasal mucosa and increased the TNF-α secretion in the brain. These observations suggested on one hand, that LPS is a useful tool for the induction of the inflammation, and on the other hand, the inflammatory response to local administration of LPS is systemic with the involvement of lungs and brain.

Our findings are in agreement with other literature data (8, 50, 49). Kim *et al.* examined the inflammatory response in rats with LPS-induced rhinosinusitis (8). They used nasal instillation with 0.1 mg LPS (from *Pseudomonas aeruginosa*) once per day for 3 days. Compared to the control group, inflammation and mucus hypersecretion of the sino-nasal tract was observed after LPS instillation (8). Other studies used different bacteria as inductors of inflammation. In 1998, Bomer introduced the first mouse model of acute bacterial rhinosinusitis induced by nasal inoculation of *Streptococcus pneumoniae* (1). Histological analysis showed an increase of neutrophils number within the sinus mucosa and the presence of neutrophils clusters within the nasal sinuses (1). Another mouse model of acute bacterial rhinosinusitis was performed by Jin *et al.*, using methicillin-resistant *Staphylococcus aureus* (MRSA) (69). In this study, the substance was administered with a sponge impregnated with MRSA suspension inserted into the right nasal cavity in a group, and in other group, the substance was dropped directly into the nasal cavity. Histologically, an important local inflammation was observed in all mice treated with a sponge impregnated with MRSA suspension, in contrast with the group treated just with MRSA solution.

To quantify the local inflammation, a conventional histological analysis of the nasal and sinus mucosa was performed. Histological changes suggestive for acute infection were noticed in both groups treated with intranasal LPS. Thus, an exudate with an increase number of neutrophils in the nasal mucosa, respectively neutrophils and lymphocytes in the paranasal sinuses, with mild congestion of the lamina propria, and inflammatory infiltrate predominantly of lymphocytes at the sinus and nasal epithelium were found. Our findings are in agreement with other studies performed before (1, 69). The immunofluorescence analysis confirmed the accumulation of inflammatory cells, within the lamina propria and the epithelium.

As for the oxidative stress, the malondialdehyde levels did not increase significantly in the tissue studied, in rats treated with both doses of LPS. One explanation would be the duration and the intensity of the inflammatory process, which was shorter compared to the studies performed on chronic rhinosinusitis, where the treatment was administered for three weeks or longer (50, 49, 70), thus favoring the production of reactive oxygen species. Another explanation can be the dose and the route of LPS administration. Sah *et al.* measured malondialdehyde, TNF-α, IL-6, and IL-1β levels in brain rats treated with LPS (from *E. coli*; 1 mg/kg body weight) administered intra-peritoneal (59). They found significant increase of malondialdehyde and cytokine secretion in group treated with LPS, compared to the control group. Huang *et al.* evaluated the levels of malondialdehyde and inflammatory cytokines, TNF-α, and IL-6, in lung tissues in rats treated with LPS (from *E. coli*; 100 μg/kg body weight) by intratracheal instillation (60). They found significant values of malondialdehyde and inflammatory cytokines in the group treated with LPS versus the control group. The dose of LPS used was higher compared to our study, and LPS was administered intra-peritoneal respectively, intratracheal through a catheter. These routes of administration and the higher dose probably produced an important inflammatory systemic response, leading to large amounts of reactive oxygen species.

The data from literature are rather contradictory and depended on the model chosen and the dose administered. Thus, Uslu *et al.* (20), and Doner *et al.* (71) used the nasal instillation of *Staphylococcus aureus* for seven days, in rabbits to induce acute sinusitis. Uslu *et al.* used a dose of 0.2 ml of 1×10^9 colony-forming units of *Staphylococcus aureus*, and found no significant difference for malondialdehyde levels of the inflamed sinus mucosa versus the control sinus mucosa (20). In contrast, Doner *et al.* demonstrated that malondialdehyde levels increased in the blood samples and sinus mucosa after nasal instillation of a dose of 0.05 ml of 5×10^6 colony-forming units of *Staphylococcus aureus* (71). The different results were explained by the different number of *S. aureus* colonies used for the experiment.

Previous studies have reported that quercetin inhibited the expression of pro-inflammatory cytokines such as IL-6 and IL-8 induced by various stimuli such as LPS in airway epithelial cells (37, 72, 73), and counteracted the inflammation by inhibiting the TNF-induced NF-κB activation (73, 74, 75). In order to investigate the effect of quercetin against inflammation and oxidative stress, malondialdehyde and inflammatory cytokines, TNF-α, IL-6, and IL-1β, in the nasal mucosa, serum, lungs, and brain, were measured in rats treated with both doses of LPS and quercetin (80 mg/kg/day). Our data revealed that quercetin signifficantly decreased TNF-α levels in all homogenates, compared with the rats treated only with LPS high dose. The secretion of IL-6 in the nasal mucosa and the level of IL-1β in serum were also significantly decreased compared with LPS high dose. The histopathological analysis of the nasal and sinus mucosa showed a lower inflammatory reaction in animals treated with LPS and quercetin, compared to rats that received only LPS. The anti-inflammatory effect of quercetin has also been shown by the immunofluorescence analysis, with a lower extent and intensity of staining in the nasal epithelium and lamina propria of the nasal mucosa for IL-1α, COX-2 and IL-10 expressions. These results suggested that quercetin exerted the anti-inflammatory effects on acute rhinosinusitis and proved the protective effect on different tissues as response to inflammation. In parallel, the quercetin administration did not influence the lipid peroxidation associated with LPS administration. This behavior can be explained either by the low dose of LPS that was used, or by the short duration of the inflammatory reaction.

In conclusions, the current study established a rat model of acute rhinosinusitis using intranasal administration of LPS. The

results of our study suggest that quercetin is effective in reducing inflammatory activity, including secretion of cytokines and histopathological changes in LPS-induced acute rhinosinutistis in rats. The protective effects after intranasal administration were also exerted on the lungs, and partially on the brain, especially in inflammation induced by low dose of LPS.

Authors' contributions: Conceptualization of the research was made by C.N. Tiboc (Schnell) and G.A. Filip. The present study was performed under the supervision of Professor G.A. Filip, who was also responsible for project administration, the analysis of the results, and the writing-review of the manuscript; C.N. Tiboc (Schnell) was involved in methodology of the research, analysis of the results, and writing of the original draft, with support from S.C. Man and V. Sas. N. Decea and R. Moldovan carried out the experiments and contributed to sample preparation. F. Tabaran and R. Opris performed the histopathological analysis.

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