SUMMARY

The process of wound healing consists of the following phases: inflammation, proliferation, remodeling. Non-steroidal anti-inflammatorratory drugs may be important in this process, especially in a stage called angiogenesis. For this reason, it was decided to investigate the effect of selective COX-2 (cyclooxygenase 2) inhibitor (NS-398) on the proliferation of endothelial cells and their ability to secrete bFGF (fibroblast growth factor) for vascular endothelial cells (HMEC-1). For determination of the secretion of bFGF in a cell line HMEC-1 immunosorbent ELISA assays were used. In turn, the cell proliferation assay was performed using the MTT method. Using MTT method, it was found that NS-398 at 10 μM did not affect cell viability. Whereas selective COX-2 inhibitor at 100 μM decreased cell viability in a statistically significant manner and inhibited the proliferative effect of 100 μg/mL LPS at concentrations of 10 and 100 μM. In the further step, application of NS-398 (10 and 100 μM) with LPS (100 μg/mL; inflammatory environment) reduced the secretion of bFGF in a statistically significant manner. The investigations showed that NS-398 has an antiangiogenic effect which is based on reducing the proliferation of vascular endothelial cells and inhibiting the secretion of bFGF- factor responsible for angiogenesis during wound healing.

Keywords: angiogenesis, selective COX-2 inhibitor, fibroblast growth factor, vascular endothelial cell
INTRODUCTION

The process of angiogenesis is an important step in the process of wound healing, e.g. after a surgery. The process of wound healing consists of several phases: inflammation, proliferation and remodeling. The first stage occurs in the first days after the injury or after the surgery, then there is the process of blood coagulation and the release of growth factors such as platelet derived growth factor (PDGF – platelet-derived growth factor), transforming growth factor type α (TGF-α – transforming growth factor α), epidermal growth factor (EGF – epidermal growth factor), and vascular endothelial growth factor (VEGF – vascular endothelial growth factor) [4, 9, 13]. Growth factors induce the proliferative phase, which consists of keratinocyte proliferation and angiogenesis, and also stimulate inflammatory cell influx into the wound site. One of the key processes of wound healing is angiogenesis; its disorders occur in difficult to heal wounds. In the initial stage of angiogenesis there is the increased vascular permeability to form a matrix required for migration and proliferation of endothelial cells. Then, as a result of growth factors VEGF and FGF (fibroblast growth factor), there is the proliferation of endothelial cells, formation of blood vessels, the ripening and in the end, the recruitment of smooth muscle cells and pericytes to newly formed vascular network [4, 9, 13].

Prostaglandins are mediators of cell proliferation, differentiation, and angiogenesis, all of which are important for tissue repair. They regulate the induction and resolution of inflammation that accompany the tissue response to injury. However, non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, diclofenac, celecoxib, that are widely used for a treatment of pain after surgery and inflammation that can be associated with surgical procedure, inhibit synthesis and effects of prostaglandins. NSAIDs may be classified according to their mechanism of action. Nonselective NSAIDs (ibuprofen, diclofenac, naproxen) inhibit cyclooxygenase-1 (COX-1) and -2 (COX-2), and selective COX-2 inhibitors like celecoxib and NS-398. COX-2 is inducible enzyme that is upregulated in response to injury, resulting in the production of prostaglandins E₂ (PGE₂) [8, 12, 14].

The use of drugs, particularly non-steroidal anti-inflammatory drugs after the surgery may be important due to slowing the processes (proliferation and angiogenesis) that occur during wound healing [4, 9]. For this reason, it was decided to investigate the effect of NS-398 on the proliferation of endothelial cells and their ability to secrete the fibroblast growth factor (bFGF is responsible for angiogenesis) for vascular endothelial cells (HMEC-1 – human microvascular endothelial cells, cells derived from the blood of the skin) under normal and inflammatory conditions.

MATERIALS AND METHODS

CELL CULTURE

HMEC-1 (human microvascular endothelial cells) were purchased from ATCC (Rockville, MD, USA), catalog number ATCC-CRL-10636 (depositor Centers for Disease Control, Dr. Ed- win W. Ades, Atlanta, GA, USA). For experimentation, cells between 10–31 passages were used. HMEC-1 cells were cultured in 25 cm² flasks in MCDB 131 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 10 ng/mL epidermal growth factor, 1 μg/mL hydrocortisone and penicillin-streptomycin solution (Sigma-Aldrich Chemical Co. Ltd., St. Louis, MO, USA) in a humidified atmosphere of 95% O₂ and 5% CO₂ at 37°C. Cells were harvested every third day in a trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA). HMEC-1 cells were cultured according to the method described in the literature [1, 15] and the author’s own modification.
MTT CONVERSION
The viability of the HMEC-1 (human microvascular endothelial cells) cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemical Co. Ltd., St. Louis, MO, USA) conversion method. Cells were seeded (50,000 cells/well) into 96-well plates. The treated cells were incubated for 24 h with LPS 100 µg/mL, NS-398 10 or 100 µM, LPS and NS-398 or without tested chemicals (control group). All the substances were added at the same time. After incubation, 50 µl MTT (1 mg/mL, Sigma-Aldrich Chemical Co. Ltd., St. Louis, MO, USA) was added and the plates were incubated at 37°C for 4 h. At the end of the experiment, the cells were exposed to 100 µl dimethyl sulphoxide, which enabled the release of the blue reaction product: formazan. The absorbance at 570 nm was read on a microplate reader and results were expressed as a percentage of the absorbance measured in control cells.

ELISA ASSAYS
bFGF concentrations in cell culture media were determined by commercially-available ELISA kits according to the vendor’s protocols (R&D System, Abingdon, UK).

STATISTICAL ANALYSIS
Statistical comparisons between the groups were performed using ANOVA, and post-hoc comparisons were performed using the Student-Newman-Keuls test. The normal distribution of parameters was checked by means of the Shapiro-Wilks test. If the data was not normally distributed or the values of the variance (test F) were different, ANOVA with Kruscal-Wallis and Mann-Whitney’s U test were used. All parameters were considered significantly different if \( p < 0.05 \). The statistical data analysis was performed using Statgraphics 5.0 plus software (STSC Inc., Rockville, MD, USA).

RESULTS AND DISCUSSION
The effect of bacterial LPS and NS-398 on cell proliferation. Using MTT method, it was found that LPS at 100 µg/mL stimulated proliferation of HMEC-1 cells by 33.5%. This result was statistically significant (Fig. 1).

The next set of experiments addressed the influence of NS-398 at concentrations of 10 and 100 µM on human microvascular cell viability. NS-398 at 10 µM did not affect cell viability. Selective COX-2 inhibitor at 100 µM decreased the viability of endothelial cells by 20% and down regulated their viability in LPS stimulated culture at 10 and 100 µM by 30 and 38%, respectively. The observed effects were statistically significant.

The effect of bacterial LPS and NS-398 on bFGF secretion in HMEC-1 cells. LPS induced secretion of bFGF in HMEC-1 cells by 147% in a statistically significant manner. NS-398 at concentration of 10 and 100 µM had no effect on bFGF formation (Fig. 2). Application of NS-398 (10 and 100 µM) with LPS (100 µg/mL) reduced the level of bFGF in a statistically significant manner. The addition of 10 and 100 µM NS-398 decreased the secretion of LPS-induced bFGF by 8% and 34%, respectively. The observed effects were statistically significant. The effect of 100 µM NS-398 on LPS-induced bFGF was also statistically signifi-
cant in comparison with the effect evoked by NS-398 at 10 μM. Endothelial cells produce prostaglandins from arachidonic acid in physiological processes and in pathophysiological such as wound healing. The first phase of wound healing is inflammation, therefore in this study there was used bacterial lipopolysaccharide from \textit{Salmonella enteritidis} cell wall (LPS), mimicking an inflammatory process, in experiments. LPS is responsible for releasing of inflammatory cytokines and prostaglandins [5, 10]. LPS is a less potent stimulator of VEGF secretion than hypoxia and, in contrast to hypoxia, stimulates the secretion of bFGF [17]. In the present study, LPS used at concentration 100 µg/mL stimulated proliferation in MTT test and secretion of bFGF.

The aim of this work was to determine whether selective COX-2 inhibitor can reduce the cell proliferation and inhibit angiogenesis and thus hinder wound healing. In the present study, NS-398 decreased proliferation in a concentration-dependent manner (Fig. 1). This effect was also found when cells were treated with NS-398 in the presence of LPS. Previous reports indicated that LPS was responsible for the induction of cyclooxygenase-2 in bovine aortic endothelial cells (BAEC) and intensified cell proliferation. Thus, COX inhibitors, inhibiting prostaglandin synthesis, show antiproliferative activity. The described effect may also explain the proliferative effect of LPS on HMEC-1 in the present study, because LPS can activate COX-2 [2] while NS-398, inhibiting COX-2, reduces

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**Fig. 1.** Effects of NS-398 (10 and 100 µM) on cell viability of cultured hMEC-1 cells in the presence of LPS (100 µg/mL) measured by MTT conversion assay. The results are presented as a percentage in relation to the control value. Bars represent the means (±SEM of 4–15 experiments). *p < 0.05 vs. control; a – p < 0.05 vs. LPS (100 µg/mL). Abbreviations used in this figure denote: LPS 100 – 100 µg/mL of LPS, NS 10 – 10 µM of NS-398, NS 100 – 100 µM of NS-398.
proliferation of HMEC-1 (3). These results are in a close agreement with those of Flis et al. [7] who demonstrate that sulindac sulfide (non-selective COX-2 inhibitor) and celecoxib (selective COX-2 inhibitor) inhibit the survival of HMEC-1 cells and induce their apoptosis. A similar effect is shown by Niederburger et al. (11) in human umbilical vein endothelial cells (macrovessels-derived endothelial cells) and in our previous studies (15, 16, 18). It was also investigated whether NS-398 could modulate bFGF secretion. NS-398 used at both concentrations (10 and 100 μM) did not influence the generation of bFGF (Fig. 2). The simultaneous application of 10 or 100 μM NS-398 and LPS (100 μg/mL) inhibited secretion of LPS-induced bFGF in a dose-dependent manner. Also, 100 μM NS-398 decreased LPS-stimulated bFGF to a greater degree than 10 μM NS-398. Selective COX-2 inhibitor can inhibit angiogenesis by an antiproliferative effect and by decreasing secretion of bFGF, which in turn may be an important factor in the wound healing (19).

![Graph](image)

**Fig. 2.** Effects of NS-398 (10 and 100 μM) on bFGF levels in HMEC-1 cells in the presence of LPS (100 μg/mL). Bars represent the means (±SEM of 3–5 experiments). *p < 0.05 vs. control; a – p < 0.05 vs. LPS (100 μg/mL); b – p < 0.05 vs. LPS (100 μg/mL) & NS-398 (10 μM). Abbreviations used in this figure denote: LPS 100 – 100 μg/mL of LPS, NS 10 – 10 μM of NS-398, NS 100 – 100 μM of NS-398

**CONCLUSIONS**

These researches show that selective COX-2 inhibitors exhibit an anti-proliferative effect while LPS augments HMEC-1 cell proliferation. Moreover, selective COX-2 inhibitor weakened the proliferative effect of LPS, which may indicate the participation of COX-2 and their products in the proliferation of HMEC-1 stimulated by LPS. The release of bFGF by the endothelium under the influence of
inflammation may take place in important cellular processes like wound healing. LPS, mimicking an inflammatory process, can have a pro-angiogenic influence on other cells such as vascular smooth muscle cells, and it can stimulate their proliferation. Stimulation of proliferation of HMEC-1 and release of proangiogenic factors like bFGF by LPS can play an important role in wound healing. By contrast, selective COX-2 inhibitors may delay the wound healing due to the inhibition of endothelial proliferation and angiogenesis (inhibiting bFGF). These observations can be explained according to Fairweather et al. (6), who report that reepithelialization and wound closure are delayed by celecoxib (selective COX-2 inhibitor) treatment. The observations presented in this paper and others (7, 11, 16, 18) may indicate that very popular OTC medicines, for instance common use of pain killers (diclofenac, ibuprofen) in wounds or in cancers, prolong wound healing.

In summary, a selective COX-2 inhibitor NS-398 has an antiangiogenic effect which is based on reducing the proliferation of vascular endothelial cells and inhibiting the secretion of bFGF - factor responsible for angiogenesis during wound healing. In summary, non-steroidal anti-inflammatory drugs are often used after surgery for pain control, they can inhibit the healing process due to the anti-angiogenic effect. These findings have potential clinical implications in postoperative wound management.

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