Cytokines profile in knee cartilage of rats during monoiodoacetate-induced osteoarthritis and administration of probiotic

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Aim. To identify the effect of probiotic (PB) on cytokines profile in knee cartilage of rats with experimental osteoarthritis (OA). **Methods.** The animals of 1st (Control) and 3rd (PB) groups got 50 µL of saline to both hind knees. The animals of 2nd (MIA-OA) and 4th groups (MIA-OA-PB) got single injection of 1 mg monoiodoacetate (MIA) dissolved in 50 µL of saline to knee, after which MIA-OA developed for 28 days. PB feeding [was] provided daily for 14 days during the MIA-OA progress. The levels of cytokines (IL-1β, TNF-α, IL-6, IL-8, IFN-γ, IL-4, IL-10, TGF-β, IGF-1) were measured in cartilage homogenates by [the] enzyme-linked immunosorbent assay (Biotrak ELISA System, GE Healthcare, USA). **Results.** MIA-OA caused [an] increase in the levels of IL-1β, TNF-α, IL-6, IL-8, IFN-γ, IL-10, TGF-β, decreased the level of IGF-1 and did not change the level of IL-4, compared to Control. PB during MIA-OA increased the level of IGF-1, decreased the levels of IL-1β, TNF-α, IL-6, IL-8, IFN-γ, TGF-β, compared to MIA-OA, but did not reach the Control values (unlike IL-4 and IL-10 that equaled to Control). **Conclusions.** MIA-OA caused significant changes in the levels of studied cytokines in knee cartilage. An application of PB has positive local anti-inflammatory effect in cartilage tissue of rats with MIA-OA.

K e y w o r d s: osteoarthritis, cytokines, growth factors, probiotic, cartilage.

Introduction

Osteoarthritis (OA) is a widespread pathology of the musculoskeletal system with complex etiology. This disorder strikes the movable limb joints, leading to the irreversible degeneration of cartilage, damage of joints with impaired movement, acute pain [1]. According to different sources, from 14 % to 25 % of adults, 55-60 years old, have a risk of OA [2, 3]. The development of OA is associated with the local synthesis of inflammatory mediators

that transfer from the joint tissue into the bloodstream and can lead to systemic inflammatory responses [4]. Signaling pathways of cytokines play a key role in the OA development through the control of pro- /anti-inflammatory balance and the response to the synthesis of matrix metalloproteinases and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) through [the] degradation of extracellular matrix collagen II and aggrecan [5]. The OA-related mediators include interleukins (ILs) -1α, -1β, -1ra, -6, -7, -8, -15, -33, tumor necrosis factor α (TNFα), matrix metalloproteinases -1, -2, -3, -10, -13, -14, C-reactive protein, chemokine ligands -5, -8, tissue inhibitors of metalloproteinases -1, -2, leptin, adiponectin and some others [6]. The investigation on cytokines as potential biomarkers for OA opens new strategies of the disease treatment [7]. Modern strategies of nonsurgical treatment of knee OA are based on efficiency of pain-reducing and functional outcomes restoring [8]. They include walking cane, biomechanical interventions, corticosteroids, nonsteroidal anti-inflammatory drugs (NSAIDs), capsaicin, paracetamol [9]. However, long-term administration of NSAIDs induces a number of side effects via nephrotoxicity, cardiovascular adverse effects, gastrointestinal toxicity [10]. Except for dyspepsia, gastroduodenal ulcers, gastrointestinal bleeding and perforation, NSAIDs alter the species diversity and stability of microbiome [11].

In clinical practice, joint diseases are mainly associated with obesity and non-alcoholic fatty liver disease. The dysbiosis of intestinal microbiota leads to obesity, insulin resistance and systemic inflammation, so it is feasible that gut microbiota is ascribed to pathogenesis of OA. The relation between OA and obesity was appointed to exceeding joint loading as a consequence of increased body weight; and yet disturbed lipid metabolism, low-grade inflammation, and adipokines on joint tissues are the former etiology for obesity-stimulated OA. Additionally, clinical and experimental studies have shown that administration of probiotics and nutraceuticals exhibited notable health benefits in obesity and corresponding diseases [12-16].

Recent studies show tight link between alteration of intestinal microbiota and pathogenesis of various disorders, such as inflammatory bowel disease, cancer, OA [17]. The risk factors of OA like gender, age, obesity, and diet are profoundly associated with intestinal microbiota via regulation of immune response, cognitive function, hormonal factors, nutrient-induced changes [18]. The possible strategies that can alter, modify and reinstate gastrointestinal microbiota are forcedly discussed in the past few decades. Most of them described beneficial effects of some probiotic (PB) strains *(Bifidobacterium, Lactobacilli, S. boulardii, B. coagulans.),* prebiotics and synbiotics supplements [7]. Our previous works showed promising results of PB administration to rats with experimental OA on cartilage remodeling [19, 20], oxidative/antioxidant balance [21] and cytokines profile of blood [22]. The PB complex included live symbiotic biomass that contains 14 strains of microorganisms belonging to 10 species: *Bifidobacterium bifidum, B. longum, Lactobacillus acidophilus, L. delbrueckii, L. helveticus, Propionibacterium freudenreichii, P. acidipropionici, Lactococcus lactis, Acetobacter aceti, Streptococcus sali-*

varius. In this study, we concentrated on the effect of PB mix on the levels of some important cytokines (IL-1β, TNF-α, IL-6, IL-8, IFN-γ, IL-4, IL-10, TGF-β, IGF-1) in cartilage homogenates of rats during MIA-OA.

Materials and Methods

Animals and ethical statements. This study involved male non-linear white rats (weighing 180-240g, n=20). All manipulations were conducted according to the general ethical principles of experiments on animals adopted by the Sixth National Congress of Ukraine on Bioethics [23], Guide for the Care and Use of Laboratory Animals [24] and approved by bioethics commission of Educational and Scientific Center "Institute of Biology and Medicine", Taras Shevchenko National University of Kyiv.

Design of experiment. The rats were randomly divided into four groups with five animals in each group and placed under standard living conditions of the vivarium. Each animal was weighed once a week to correct doses of PB. Single intra-articular injection of monoiodoacetate (MIA, Sigma, USA) to ligament medial side of hind knee was used to provide MIA-induced OA (MIA-OA) [25, 26]. The animals of 2nd and 4th groups got 1 mg of MIA dissolved in 50 µL of saline to right hind knee. Left hind knee got 50 µL of saline only (Fig. 1). The animals of 1st and 3rd groups got 50 µL of saline to both hind knees. The rats of 3rd and 4th groups got intragastric administration of PB composition (O.D. "Prolisok", Ukraine) at a dose of 140 mg/kg diluted in freshwater daily for 14 days of the experiment model. The animals of 1st and 2nd groups received intragastric irritation with freshwater for 14 days.

Sampling. The cartilage sampling was provided on the 30th day of the experiment after sacrificed of the animals, according to the protocol of the ethics committee. Knee joints were carefully removed and stored in plastic tubes at -80°C for no more than 3 months
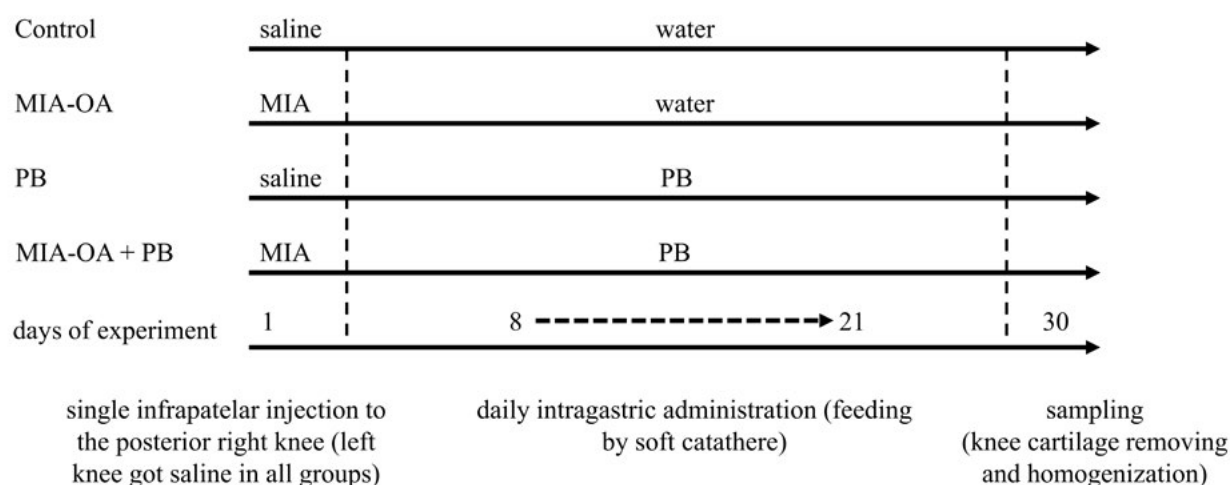

Fig. 1. Design of experiment.

before homogenization. The joints sliced in 2mm pieces and chopped in a porcelain mortar with liquid nitrogen. Then added 1X PBS (Sigma, USA) in 1:10 ratio to a weight of cartilage and homogenized in automated homogenizer Ultra Sonic T10 Basic (IKA, Germany) at 3000 rpm for 3 min. Unhomogenized parts were removed by centrifugation on 1-15K Centrifuge (Sigma, USA) at 3000 rpm for 15 min. Supernatants was stored in plastic tubes for further biological tests no more than 1 week at -20°C.

ELISA tests. The samples were measured for total protein by the Bradford method [27, 28]. Aliquots of PBS were added to equalize total protein in each sample. The levels of the studied parameters (IL-1β, TNF-α, IL-6, IL-8, IFN-γ, IL-4, IL-10, TGF-β, IGF-1), were measured in prepared samples by enzyme-linked immunosorbent assay (Biotrak ELISA System, Healthcare, USA), using manufacturer's recommendations.

Statistical analysis. SPSS 23 (IBM, USA) was used to conduct statistical calculations. Normal distribution was checked via the Shapiro-Wilk test for normality and conducted to one-way ANOVA with Tukey`s post hoc test for multiple comparisons. Two-sided $P \leq 0.05$ was considered statistically significant. The results were presented as average arithmetic ± standard error meaning (SEM). GraphPad Prism 8 (GraphPad Software Inc., USA) was used to build figures.

Results and Discussion

Many studies described IL-1 as a central factor of immune disorders. It can be produced in many types of the immune cells (monocytes, macrophages, lymphocytes, synovial lining cells) and activated intracellular T-cells interactions by binding with (IL-1R) on the T-cells surface [29]. The expression of IL-1β is linked with the response to microbial patterns and is able to form trimolecular signaling complex with IL-1R and IL-1AcP and further to activate the pro-inflammatory cytokine expression [30]. Medical trials showed elevating of IL-1β in human with OA in synovial fluid, cartilage and subchondral bone layer [31]. In present study, MIA-OA caused an increase of IL-1β in homogenates of cartilage 2.4 times compared to Control group, (Figure 2 A). Administration of PB to rats without OA caused no significant changes in the level of IL-1β, compared to Control group. Administration of PB to animals with MIA-OA decreased the values of IL-1β 1.3 times compared to MIA-OA group though they remained 1.9 times higher than Control. The similar effect had the administration of mono-strain PB (*L. casei*) that decreased the level of IL-1β in rats with collagen-induced arthritis whereas summarized effect occurs during mixture administration in rats with collagen II type [32].

IL-1β has a synergistic relationship in local and general inflammatory processes, mainly with TNF-α, IL-6 and TGF-β that creates the key links of inflammatory response [33]. TNF-α has two isoforms (soluble and membrane-bound), which initiated various signal pathways and response for apoptosis, pro-inflammatory cytokine expression, inflammation and cell survival [30]. The study of platelet lysate towards MIA-OA focused on an ability to regulate TNF-α signal-related mechanisms considering this approach as a promising strategy of an alternative option for OA therapy [34]. In this work, MIA-OA caused an increase
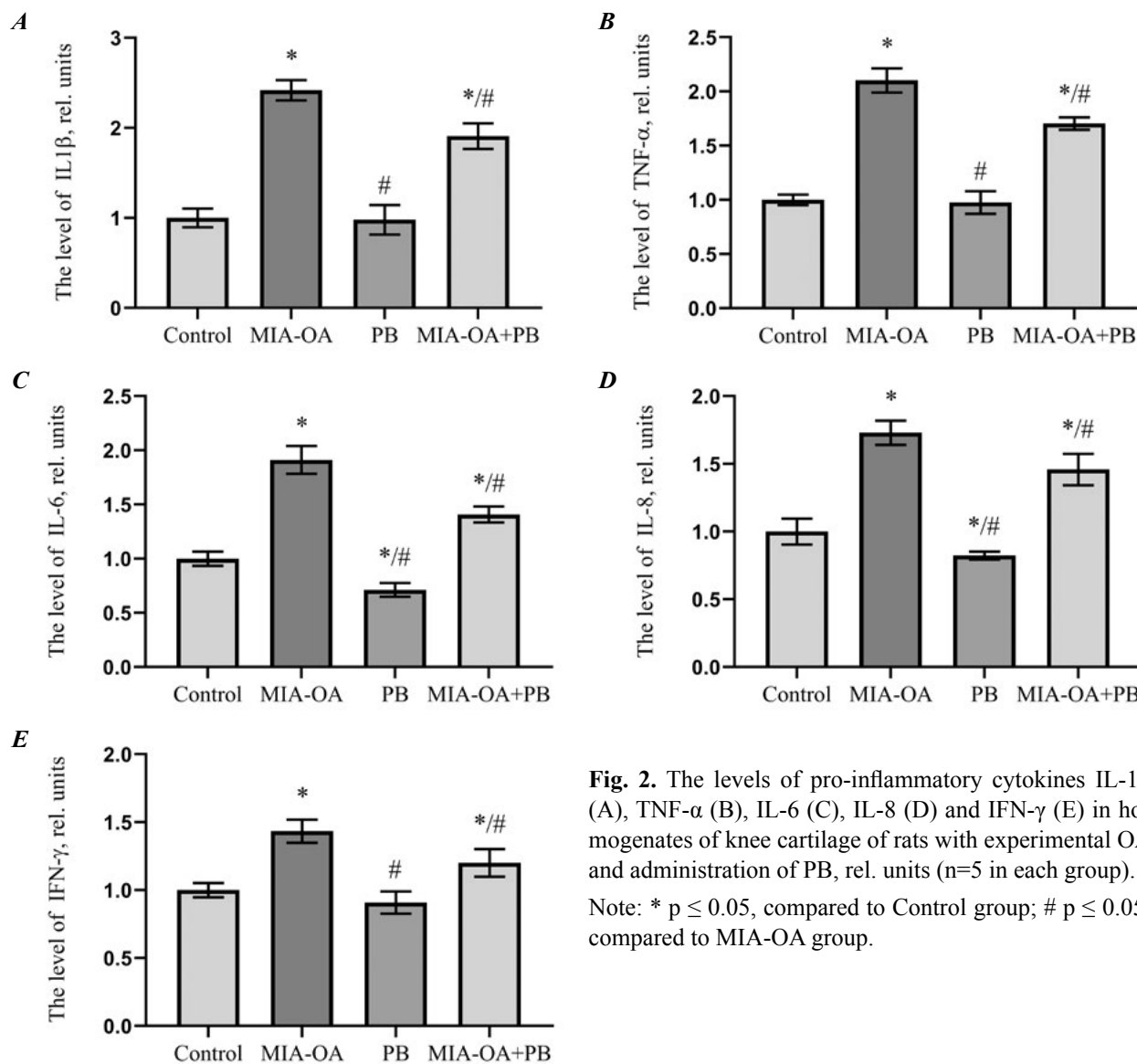
Fig. 2. The levels of pro-inflammatory cytokines IL-1β (A), TNF-α (B), IL-6 (C), IL-8 (D) and IFN-γ (E) in homogenates of knee cartilage of rats with experimental OA and administration of PB, rel. units (n=5 in each group).

Note: * $p \leq 0.05$, compared to Control group; # $p \leq 0.05$, compared to MIA-OA group.

of TNF-α 2.1 times, compared to Control group, (Fig. 2 B). The review of recent data showed that increasing soluble TNFα in blood serum and intracellular matrix of cartilage affected the prevalence of joint space narrowing and predict knee cartilage loss [35]. Administration of PB to animals without MIA-OA (PB group) did not cause difference compared with Control. Administration of PB to animals with MIA-OA decreased the values of TNF-α by 1.2 times compared to MIA-OA group but they remained 1.7 times higher than Control. Showed the effect of PB during OA in the experimental rats, which is associated with the

triggering of production of TNFα- in CD4 T cell, synovial fibroblasts, and chondrocytes [36].

Among the immunomodulatory cytokines present in the OA joint, IL-6 and IL-8, are also considered to be pro-inflammatory, and the synthesis of both can be stimulated by IL-1 and TNF-a [37]. However, neither IL-6 nor IL-8 alone was capable of stimulating cartilage degradation directly [38]. In this study, we showed [that] MIA-OA caused an increase in the levels of IL-6 and IL-8 in homogenates of knee cartilage by 1.9 and 1.7 times, respectively, compared to the Control group (Figure 2 C, D). Administration of PB to animals without MIA-OA decreased the levels of IL-6 and IL-8 by 1.4 and 1.2 times, respectively, compared to Control group. Administration of PB to animals with MIA-OA decreased the levels of IL-6 and IL-8 by 1.4 and 1.2 times, compared to MIA-OA group, but it was 1.4 and 1.5 times higher than the values of Control group, respectively. We suggest that the modulating effect of PB may be associated with [an] indirect systemic influence due to change in the number of microbial and food metabolites, e.g. acetate, butyrate, propionate. Li and Abdollahi-Roodsaz showed that these metabolites play an important role in disorders of the musculoskeletal system [18, 39]. The investigation of acute gouty arthritis in human showed that butyrate also decreased the production of IL-1β, IL-6, IL-8 in peripheral blood mononuclear cells from healthy donors [40]. Our results show similar down-regulating of IL-6, IL-8 in rats with no MIA-OA, whereas IL-1β and TNF-α were on the same level. It may be associated with different sources of synthesis of these cytokines [41].

It is generally recognized that IL-1β and TNFα are the main cytokines in the early and late stages of the development of OA, whereas IL-6, IL-8 can be regulatory and IL-4, IL-10, IFNγ are classified as inhibitory cytokines, because they may block the actions of catabolic cytokines during the inflammatory process of OA in the joint [37]. However, IFN-γ is independent from IL-1β and TNF-α signaling, it is a part of STAT1 pathway in T-cells, increases neutrophil and monocyte functions [42], inhibits CII synthesis in articular chondrocytes [43]. The action of IFN-γ is not restricted by syntactical intermediations; cytotoxic lysis is confined to antigenic targets. IFN-γ is stable and active at low concentrations in extracellular media, whereas isolated lytic granules have a very weak lytic capacity [44]. We have previously shown that the administration of PB to rats on the background of hypoacidity causes increased production of IFN by thymocytes and splenocytes [45, 46]. IFN-γ plays important role in the development of RA, whereas the data on the rodent models of OA are controversial [47]. In this study, we showed that the level of IFN-γ in cartilage homogenates increased in MIA-OA group 1.4 times, compared to Control (Figure 2 E). Our previous result showed an increase of IFN-γ in blood serum during MIA-OA [22]. Administration of PB did not cause differences in the level of IFN-γ in animals without MIA-OA (PB group) and Control. PB decreased the level of IFN-γ in MIA-OA+PB group 1.2 times, compared to MIA-OA group, however, the value did not reach that ofControl (the latter was 1.2 times higher). These results correlated with the data of the regulation of IFN-γ in CD4+ T cells

during [the] MIA-OA and PB supplementation [36].

Unlike the cytokines described above, IL-4 run[s the] anti-inflammatory signal response, responsible for proliferation of B and cytotoxic T cells, enhances MHC II expression, stimulates IgG and IgE production [48]. That is why, IL-4 has been considered [as] therapeutic targets in many studies [49, 50]. MIA-OA did not change significantly the level of IL-4, compared to Control group, (Figure 3 A). The administration of PB decreased the level of IL-4 in both group, with MIA-OA and without it, compared to Control group.

Along with IL-4, IL-10 has a strong anti-inflammatory effect; its receptors are expressed on the cells of synovial tissue and chondrocytes. IL-4 and IL-10 not only reduce the synovial inflammation but also reverse the TNF-alpha-induced production of prostaglandin E2 by synovial fibroblasts and inhibit the apoptosis of chondrocytes and cartilage breakdown [51]. Moreover, IL-4 and IL-10 have partially overlapping and complementary activities. The experimental models of synovial tissue cultures showed the effect of IL-10 itself on inhibition of the TNFα and IL-1β productions and a decrease of the levels of MMPs produced
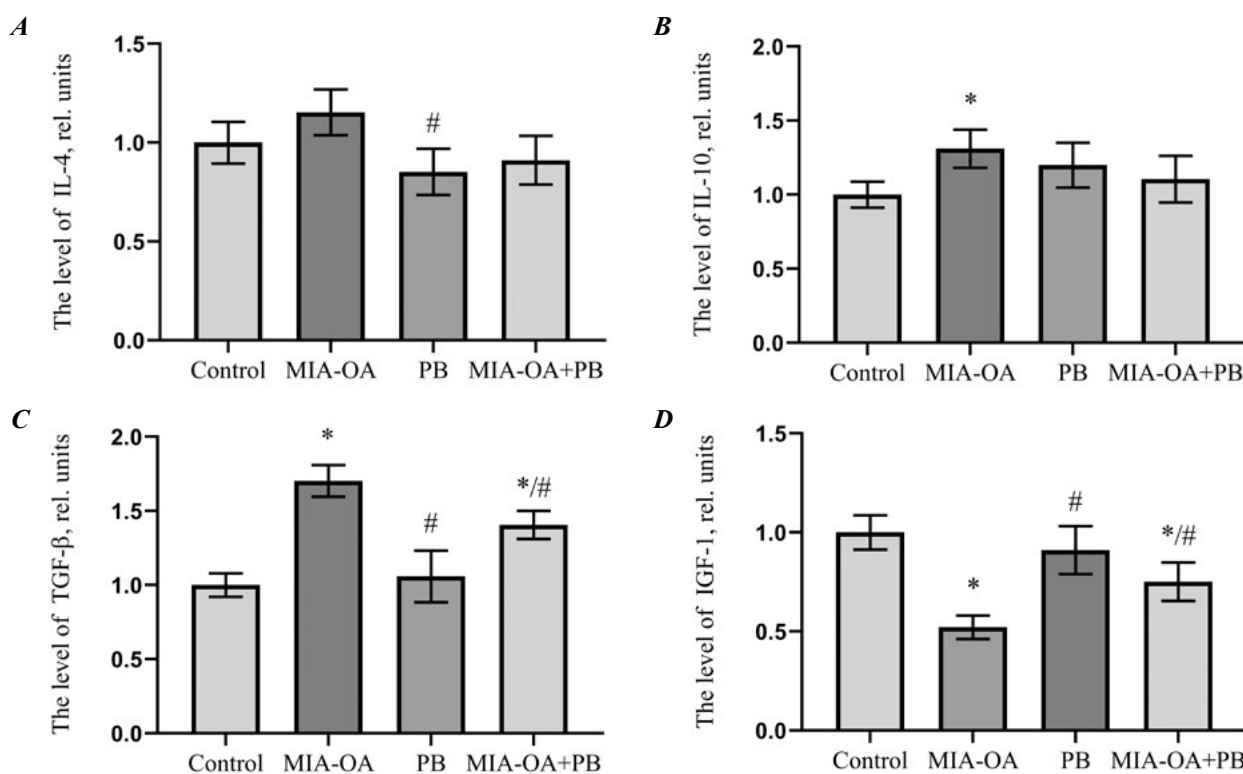

Fig. 3. The levels of anti-inflammatory cytokines IL-4 (A), IL-10 (B), TGF-β (C), and IGF-1 (D) in homogenates of knee cartilage of rats with experimental OA, rel. units (n=5 in each group).

Note: * p ≤ 0.05, compared to Control group; # p ≤ 0.05, compared to MIA-OA group.

by macrophages and fibroblast-like synoviocytes [52]. Our results showed the increasing of IL-10 1.3 times in MIA-OA group, compared to Control, (Figure 3 B). PB did not cause significant changes in healthy animals (PB group). Administration of PB to rats with MIA-OA decreased the level of IL-10 to Control values. The observed levels of IL-4 and IL-10 in MIA-OA correlate with the growth of pro-inflammatory cytokines.

Additionally to the anti-inflammatory effects of IL-4 and IL-10, the controversial role of TGF-β is actively discussed in the literature. On the one hand, the TGF-β supplementation can enhance cartilage repair and is therefore a potential therapeutic tool [53]. On the other, TGF-β induces synovial cells to produce inflammatory factors, such as IL-1 and TNF-a, which can further stimulate articular chondrocytes terminal hypertrophy, depositing type X collagen instead of type II collagen and aggrecan [54]. The TGF-β supplementation provides problems in other tissues of the joint and results in the fibrosis and osteophyte formation [55]. The TGF-β signaling depends on modulation of the receptor-Smads signaling and plays an important role in both the regulation of chondrocyte differentiation and osteoarthritis development and progression [56]. In this study, the experimental model of OA increased the level of TGF-β 1.7 times, compared to Control group (Figure 3 C). The administration of PB to rats with MIA-OA reduced TGF-β 1.2 times but it did not get the level of Control group. Despite an increase in the level of TGF-beta in MIA-OA that correlates with an increased expression of the TGF-β gene in cartilage [57], our other study showed negative histological changes [19]. This may indicate insufficient compensatory effort against a significant increase of pro-inflammatory cytokines in the cartilage and blood [58], and may also be associated with fibrosis and cartilage degeneration [59].

IGF-1 plays an important protective and anabolic role during OA. IGF-1 can enhance the cartilage-osteochondral graft for the fibrin regeneration and type II collagen formation [60]. We showed that IGF-1 reduces the loss of chondrocytes and matrix integrity in guinea pig [61]. In this study, MIA-OA caused a decrease in the level of IGF-1 twice, comparing to Control (Figure 3 D). Administration of PB to animals without MIA-OA did not cause significant changes, compared to Control. PB upregulated the level of IGF-1 in animals with MIA-OA 1.5 times, compared to MIA-OA, however, did not reach the level of Control group. IGF-1 itself cannot induce substantial production of cartilage matrix but it could function in a paracrine and autocrine manner to stimulate matrix synthesis and to inhibit matrix degradation by down-regulating matrix metalloproteinases and inflammatory cytokines [62].

We do not try to "humanize" the intestinal microbiome of rats and the effect of PB on it. There is a huge difference between rodent and human microbiome diversity; host and microbe have tight relationships [63]. Only clinical trials may show the therapeutic effect of composition during the development of the pathology. The systematic clinical data show controversial effect of PB on human in wide range of hospital cases, including OA and pain-related conditions [64].

Summarizing the data, administration of different PBs has a modulating effect on the

cytokines profile in experimental models of OA. Recent studies showed that the administration of L. acidophilus restored the balance between anabolic and catabolic factors in chondrocytes of rats and can alleviate OA-associated pain [65]. Also, reducing serum level of C-reactive protein in rats with OA was shown during the administration of *L. casei* Shirota [66]. The Lyophilized inactivated culture of *B. longum* decreased the cartilage structure lesions and type ii collagen degradation suggesting a potential effect of PB on the OA development [67]. The combination of PB complex, rosavin, and zinc reduced the expression of IL-6 and TNF-α in the joints of MIA-OA rats [68]. Our previous works showed promising results of combination of PB and chondroitin sulfate administration on rats model of OA [19].

Conclusions

We showed the local positive effect of PB in rats with experimental MIA-OA. It was revealed by decreasing levels of pro-inflammatory cytokines in the cartilage homogenate (IL-1β, TNF-α, IL-6, IL-8, IFN-γ) and normalization of anti-inflammatory cytokines (IL-4, IL-10, TGF-β, IGF-1). The use of PB in the pathology of the musculoskeletal system is promising to expand existing approaches of the correction and prevention such disorders, including OA. The mechanisms of influence of the microbiome on the development of OA are of great interest and undoubtedly require further study.

Acknowledgements

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Цитокіновий профіль у колінному хрящі щурів при моноіодоацетат-індукованому остеоартрозі та введенні пробіотику

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Мета. Виявити вплив пробіотику (ПБ) на цитокіновий профіль у колінному хрящі щурів з експериментальним остеоартрозом (ОА). **Методи.** Тварини 1-ї (Контроль) та 3-ї (ПБ) груп отримували 50 мкл фізіологічного розчину в обидва задніх коліна. Тваринам 2-ї (МІА-ОА) і 4-ї груп (МІА-ОА-ПБ) вводили одноразову ін›єкцію 1 мг моноіодоацетату (МІА), розчиненого в 50 мкл сольового розчину, в коліно щурів, після чого МІА-ОА розвивався протягом 28 днів. Введення ПБ здійснювалося щодня протягом 14 днів під час прогресування МІА-ОА. Рівні цитокінів (IL-1β, TNF-α, IL-6, IL-8, IFN-γ, IL-4, IL-10, TGF-β, IGF-1) в гомогенатах хряща вимірювали за допомогою імуноферментного аналізу (Biotrak ELISA System, GE Healthcare, USA). **Результати.** МІА-ОА викликав підвищення рівнів IL-1β, TNF-α, IL-6, IL-8, IFN-γ, IL-10, TGF-β, знижував рівень IGF-1 і не змінював рівень IL-4 порівняно з контролем. ПБ під час МІА-ОА підвищував рівень IGF-1, зменшував рівні IL-1β, TNF-α, IL-6, IL-8, IFN-γ, TGF-β, порівняно з МІА-ОА, але дані показники не досягали контрольних значень (на відміну від IL-4 і IL-10, які були рівні контрольним). **Висновки.** МІА-ОА викликав значні зміни рівнів досліджуваних цитокінів у колінному хрящі. Застосування ПБ справляло місцеву протизапальну дію на хрящову тканину щурів з МІА-ОА.

К л ю ч о в і с л о в а: остеоартроз, цитокіни, фактори росту, пробіотик, хрящ

Цитокиновый профиль в коленном хряще крыс при моноиодоацетат-индуцированном остеоартрозе и введении пробиотика

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Цель. Выявить влияние пробиотика (ПБ) на цитокиновый профиль в коленном хряще крыс с экспериментальным остеоартрозом (ОА). **Методы.** Животные 1-й (Контроль) и 3-й (ПБ) групп получали 50 мкл физиологического раствора в оба задних колена. Животным 2-й (МИА-ОА) и 4-й групп (МИА-ОА-ПБ) вводили однократную инъекцию 1 мг моноиодоацетата (МИА), растворенного в 50 мкл солевого раствора, в колено крыс, после чего МИА-ОА развивался в течение 28 дней. Введение ПБ осуществлялось ежедневно в течение 14 дней во время прогрессирования МИА-ОА. Уровни цитокинов (IL-1β, TNF-α, IL-6, IL-8, IFN-γ, IL-4, IL-10, TGF-β, IGF-1) в гомогенатах хряща измеряли с помощью иммуноферментного анализа (Biotrak ELISA System, GE Healthcare, USA). **Результаты.** МИА-ОА вызывал повышение уровней IL-1β, TNF-α, IL-6, IL-8, IFN-γ, IL-10, TGF-β, снижал уровень IGF-1 и не изменял уровень IL-4 по сравнению с контролем. ПБ вовремя МИА-ОА повышал уровень IGF-1, уменьшал уровни IL-1β, TNF-α, IL-6, IL-8, IFN-γ, TGF-β, по сравнению с МИА-ОА, но не достигал контрольных значений (в отличие от IL-4 и IL-10, которые были равны контрольным). **Выводы.** МИА-ОА вызывал значительные изменения уровней изучаемых цитокинов в коленном хряще. Применение ПБ оказывало положительное местное противовоспалительное действие на хрящевую ткань крыс с МИА-ОА.

К л ю ч е в ы е с л о в а: остеоартроз, цитокины, факторы роста, пробиотик, хрящ