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Research Article

Combined use of pulse therapy and molecular hydrogen in rats with experimental rheumatoid arthritis: Clinical and histological evaluation of therapeutic effectiveness

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Abstract

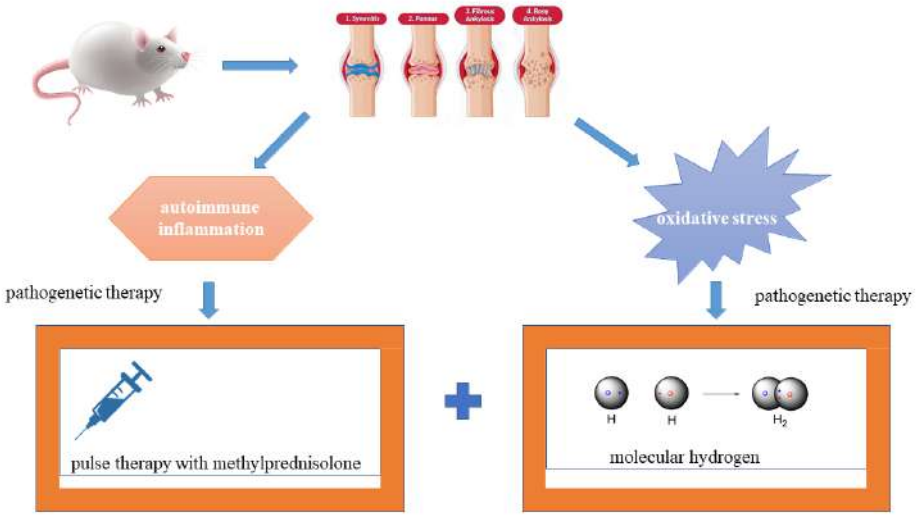
Introduction: Glucocorticosteroid therapy is the basic therapeutic option for rheumatoid arthritis; however, long-term treatment with low and medium doses is associated with the development of negative side effects. As reported, oxidative stress is crucial in the pathogenesis of rheumatoid arthritis. Therefore, pharmacotherapy that combines application of methylprednisolone pulse therapy and an aqueous molecular hydrogen solution seems to be a reasonable treatment option in these cases.

Materials and Methods: The study of the effectiveness of combined pharmacotherapy for rheumatoid arthritis was carried out on mature male rats. Rheumatoid arthritis was simulated by introducing bovine type II collagen into the right knee joint. Animals of the 1st – control – group received placebo; animals of the 2nd group received molecular hydrogen-enriched water intragastrically; animals of the 3rd group received methylprednisolone solution intravenously by catheterization of the tail vein, and animals of the 4th group received water enriched with molecular hydrogen intragastrically and methylprednisolone solution intravenously. After withdrawal of animals from the experiment, microsections of their joint tissues were analysed histologically and biomarkers of the joint inflammation were detected immunohistochemically.

Results and Discussion: Morphological analysis of microsections taken from animals of the 4th group evidenced effectiveness of the combined therapy based on quantitative estimation of the inflammatory biomarker expression. Dynamic polarization of M1/M2 macrophage was manifested with high quality in animals of this group.

Conclusion: The search for new therapeutic options for rheumatoid arthritis is expanded due to major antioxidant substances that can reduce duration of treatment, while ensuring positive dynamics of the course of the disease.

Graphical abstract:



Keywords

immunohistochemical analysis, mast cells, molecular hydrogen, pulse therapy, rheumatoid arthritis, CD68, CD163, TGF-β1

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease that damages joint congruence in the early stages of the disease due to erosive process, pannus formation, hyperproduction of the synovial fluid, inflammation of the ligamentous-muscular apparatus; this results in violation and further loss of joint functions with subsequent disability of a patient as early as in the first two years of the disease (Kearsley-Fleet et al. 2018). Clinical symptoms of RA at early stages with mostly articular manifestations significantly differ from clinical symptoms of RA at later stages occurring in case of delayed health encounters. The early stage of RA is characterized by generalized signs and symptoms of the disease, such as fatigue, flu-like sensations, morning stiffness in the joints, elevated C-reactive protein (CRP) and accelerated erythrocyte sedimentation rate (ESR) (Almutairi et al. 2021). However, undertreated RA is presented by a complex clinical picture with severe systemic manifestations, such as pleural effusion, interstitial lung disease, lymphoma, keratoconjunctivitis, atherosclerosis, hematological disorders (anemia, leukopenia, neutropenia, eosinophilia, thrombocytopenia or thrombocytosis), decreased range of motion, erosive bone changes, cartilage destruction and rheumatic nodes (Ciurtin et al. 2019). Systemic manifestations due to chronic inflammation in RA patients result in a progressively increased mortality rate resulted from RA complications.

Insufficient data about etiological factors of RA do not allow etiotropic therapy application. As pathogenetic mechanisms of the disease become clearer, a need to improve pathogenetic therapy arises. Modern therapeutic strategies have significantly improved prognosis of patients with advanced RA, but they are still associated with safety concerns and are often cost demanding. Glucocorticosteroids were introduced into clinical practice in the 1950s, and low-dose glucocorticosteroid treatment is currently part of the basic RA therapy; however, the balance between beneficial and harmful effects of glucocorticosteroids is still unclear, especially in patients with a long-term medical history of RA (Hua et al. 2020). Although a meta-analysis demonstrates that glucocorticosteroid therapy reduces disease activity and slows progression of joint destruction, nonetheless harmful effects of glucocorticosteroid therapy are being actively discussed nowadays (Roodenrijs et al. 2021; Zhao et al. 2021). Most researchers agree that long-term glucocorticosteroid therapy is dangerous due to developing side effects associated with inappropriate dose titration. That is why current recommendations suggest avoiding hormone therapy or using it only as an "intermediate" therapy; however, such viewpoints are based on observational studies in the absence of valid clinical trials (Takanashietal 2019). This leads to a wide variety of regimens of high prevalent long-term low and medium dose use of glucocorticosteroids in clinical protocols. Methotrexate is currently the gold standard of treatment for RA. Doses applied as therapeutic options for rheumatoid arthritis are typically 10–25 mg per week, much lower than those used in oncology practice (Genovese et al. 2018). However, methotrexate-associated hepatotoxicity is of particular concern for clinicians and patients and may lead to cirrhosis, the fact requiring monitoring liver enzymes every 8–12 weeks (Giachi et al. 2022). Whereas development of liver cirrhosis with methotrexate therapy is thought to be relatively infrequent, gastrointestinal intolerance is considered common even with modern folic acid

supplementation. A recent systematic review has reported that 52 of 174 (30%) patients treated with methotrexate experienced nausea or gastrointestinal disorders (Guo et al. 2021). The prevalence of RA increases with age, reaching a maximum at 70.1 years; therefore, we can expect an increased number of reported RA cases in the elderly and senile population groups (Charles-Schoeman et al. 2022). The elderly patients have the highest risk of side effects considering comorbidity and its treatment (Kalden et al. 2017; Nagy et al. 2022). Thus, application of pulse therapy with glucocorticosteroids seems to be a reasonable option to minimize side effects associated with long-lasting courses of low and medium doses of glucocorticosteroids in patients receiving basic therapy for RA, including the elderly population groups.

In recent years, the global scientific community has focused on studying the role of oxidative stress in the pathogenesis of RA. The synovial fluid and tissue studies in RA have demonstrated occurrence of oxidative damage to hyaluronic acid, lipid peroxidation products, oxidized low-density lipid proteins (LDL), and elevated carbonyl groups reflecting oxidative damage to proteins (Alwazeer et al. 2021; Buch et al. 2021; Rivellese et al. 2022). Molecular hydrogen provides membrane stabilization due to its antioxidant properties: it is able to selectively absorb strong oxidants, such as hydroxyl radicals (Smolen et al. 2020). Water enriched with molecular hydrogen at a therapeutic concentration is the simplest and safest way to introduce hydrogen into the body.

The aim of our research was to study the effectiveness of combined use of pulse therapy with methylprednisolone and molecular hydrogen-enriched water in rats with simulated rheumatoid arthritis.

Materials and Methods

Experimental animals

An experiment to study the effectiveness of combined use of pulse therapy with methylprednisolone and water enriched with molecular hydrogen involved 40 mature male Wistar rats, 10–12 weeks old, weighing 250±20 g. Laboratory animals were kept in the Research Institute of Experimental Biology and Medicine, Voronezh State Medical University named after N.N. Burdenko (RI EBM). All animals were selected using the modified block randomization approach to avoid influence of the researcher's preferences on the formation of experimental groups. Laboratory rats were kept under standard conditions in accordance with Directive 2010/63/EU on Protection of Animals Used for Scientific Purposes, and in accordance with the Sanitary-Epidemiological rules 2.2.1.3218-14 "Sanitary-Epidemiological Requirements to Organization, Fitting and Management of Experimental-Biological Clinics (Vivaria)" (approved by a decree of Chief State Sanitary Doctor of the Russian Federation №51 of 2014 August 29). The experimental protocols were approved by the local Ethical committee of Voronezh State Medical University named after N.N. Burdenko of the Ministry of Health of the Russian Federation (Minutes No. 3 of 20.05.2022). Animals were divided into 4 groups (1 control and 3 experimental), 10 animals in each group. During the adaptation period (2 weeks) and throughout the experiment, rats were kept in open cages, 5 animals in each cage. Each group was provided with 2 cages. All cages were labeled appropriately. Laboratory rats were kept under standard vivarium conditions, air temperature was 20-24 C°, conditions of light were 12 hours of light cycle and 12 hours of dark cycle. Animals received standard granulated feed compound, complete for laboratory animals according to GOST R50258-92 " Complete Feed for Laboratory Animals". Food and water were given ad libitum.

Simulation of rheumatoid arthritis in rats

Simulation of rheumatoid arthritis was performed in the post-adaptation period, daily, for 14 days. Simulation of RA is based on the ability of bovine collagen type II, the major protein in articular cartilages, to stimulate the antibody production against bones and cartilages in mice or rats. The induction of collagen-induced arthritis results from the introduction of native bovine type II collagen with incomplete Freund's adjuvant once intradermally into the area of the right knee at 2 points, 0.4 mg of collagen per rat under anesthesia (Orlovskaya et al. 2015). All injections were performed using local anesthetic lidocaine (topical spray, 4.6 mg/dose).

Treatment of simulated rheumatoid arthritis in rats

Treatment started after the first signs of joint inflammation, 15 days after the onset of the experiment. For a placebo-controlled study, animals of group 1 received 10 ml of boiled water intragastrically via an atraumatic probe for 21 days and 2 ml of 0.9% sodium chloride solution intravenously by the tail vein catheterization during the first three days.

Animals of group 2 received 10 ml of molecular hydrogen-enriched water at a concentration of 1.6 ppm from an Enhel-mini device, intragastrically via an atraumatic probe, daily, for 21 days; simultaneously, during the first three days of treatment, 2 ml of 0.9 % sodium chloride solution were administered intravenously by the tail vein catheterization. The concentration of molecular hydrogen in an aqueous solution was confirmed using a dissolved hydrogen analyzer MARK-501 (Nizhny Novgorod, Russia).

Animals of group 3 received 10 ml of boiled water intragastrically via an atraumatic probe for 21 days; simultaneously, during the first three days of treatment, a solution of methylprednisolone was administered intravenously, dosage 20 mg/kg of the body weight, in 1 ml of a 0.9% chloride sodium solution by the tail vein catheterization.

Animals of group 4 received 10 ml of molecular hydrogen-enriched water intragastrically via an atraumatic probe, daily, for 21 days; simultaneously, during the first three days of treatment, a solution of methylprednisolone was administered intravenously, dosage 20 mg/kg of the body weight, in 1 ml of a 0.9% chloride sodium solution by the tail vein catheterization. There were neither animal deaths recorded nor signs of intoxication noted with intravenous methylprednisolone injections and intragastric administration of 10 ml of hydrogen water.

Investigations of the general condition of experimental animals

During the experiment, a clinical observation was carried out

(within 4 hours after preparation administration) to register signs of intoxication. Physical examination was performed daily; rats were weighed weekly, and the Open Field functional test was conducted to assess locomotor and orientation-exploratory activity. Animals were withdrawn from the experiment 22 days after the treatment onset using a CO_2 chamber.

Morphological analysis

Immediately after withdrawing the animals withdrawn from the experiment, joint fragments isolated from the muscle tissue were fixed in a 10% buffered neutral formalin solution (Samoilenko et al. 2021). Forty-eight hours later, the fixed material was placed in a SoftiDec decalcifying solution (ethylenediaminetetraacetic acid in an acidic buffer solution). Decalcification process was completed 4 days later (with daily replacement of the solution and continuous monitoring with a dissecting needle). Further, the material was washed with running water for an hour, fixed in formalin for 24 hours, histologically processed in the following sequence: a battery of alcohols (6 replacements), mineral oil Blik in increasing concentration (3 replacements), and molten paraffin (2 replacements). The process was completed by embedding the material into paraffin blocks. For observational microscopy, sections 3-4 µm thick were performed from paraffin blocks on a microtome; for immunohistochemical studies, sections 1-2 µm thick were performed from paraffin blocks on a microtome.

Gill's hematoxylin and eosin staining was carried out to visually assess morphological changes in the joint tissues. Finished microsections were analyzed on a ZEISS Axio Imager.A2 research microscope with field lenses 10, 20, and 40 using standard bright-field and polarized light microscopy. When studying sections in polarized light, collagen types I and III were visualized.

Mast cells were selected as polyfunctional agents of inflammatory reactions of various nature to assess the course of the joint inflammation. Metachromatic mast cells were identified by staining with Giemsa solution. Cell populations were analyzed in 25 fields of view using an x40 field lens. The total number of cells, free-lying granules and intercellular contacts, as well as the ratio of degranulated and non-degranulated forms were estimated.

Immunohistochemical detection of TGF-β1 was performed using TGF-β1 rabbit monoclonal antibodies ([EPR21143], ab215715, dilution 1:500) as a factor for cartilage repair. Rabbit monoclonal antibodies CD68 ([EPR20545], ab213363, dilution 1:2000) and rabbit monoclonal antibodies CD163 ([EPR19518], ab182422, dilution 1:500) were used to estimate M2 macrophages and evaluate their role in the inflammatory process. Expressing cells were counted in 20 fields of view using an x40 field lens. Student's t-test was applied to assess the significance of differences between groups.

Results and Discussion

Investigations of the general condition of experimental animals

At the start of the experiment, male rats weighed 250±20 g; within 15 days after RA simulation and 21 days of treatment, positive dynamics of the body weight was noted in both the control group and in groups of animals receiving combined therapy. When being withdrawn from the experiment, rats in groups 1 and 2 weighed 320±27 g, animals in groups 3 and 4 weighed 382±21 g.

Seven days after the treatment onset, animals of groups 1 and 2 demonstrated hypodynamia, lack of activity due to food intake; their hair was dull and rumple. In animals of groups 3 and 4, where methylprednisolone was one of the therapeutic components, there were no changes in behavioral reactions; their appetite was preserved; and their hair did not change. Edema of both hind limbs persisted in animals of all groups after the 15th day of RA simulation.

Fourteen days after the treatment onset, no normal general condition was observed in any animals of groups 1 and 2; there was edema of both hind limbs; in animals of groups 3 and 4, there was a complete normalization of the general condition, edema persisted only on the right hind limb and was absent on the contralateral limb.

Twenty-one days after the treatment onset, there was a complete normalization of the general condition in animals of all groups; however, edema persisted on both hind limbs in rats of group 1 (control). In animals of group 4, there were no signs of edema.

Morphological analysis

Morphological changes in the right and left knee joints were comprehensively analyzed for the signs typical of arthritis with Gill's hematoxylin and eosin staining (Fig. 1).

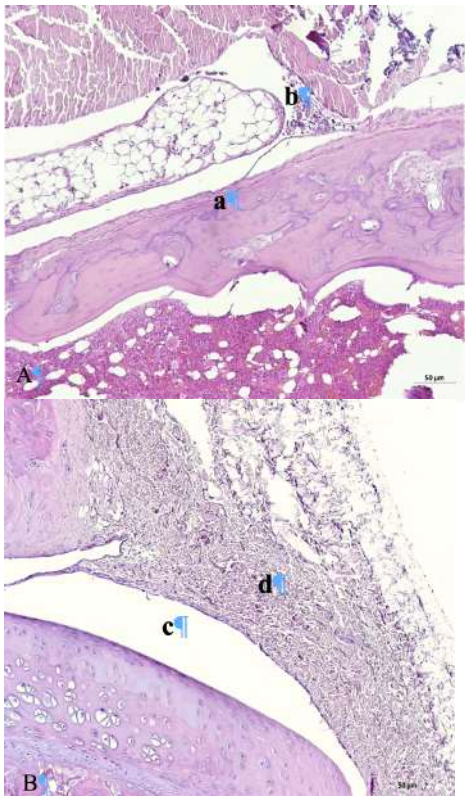


Figure 1. Morphological picture of the right knee joints in rats of group 1 (**A**) and in rats of group 4 (**B**). **Note:** **a** – ridged surface and basophilic resorption boundaries evidence bone erosion; **b** – an inflammatory infiltrate with eosinophilic lumps around the accumulation of adipocytes; **c** – unchanged joint space, **d** – exposure of hypertrophied and hyperplastic synovium in the joint space. Fixation – 10% neutral formalin. Gill's hematoxylin and eosin staining, x20 magnification, light microscopy.

The most pronounced changes typical of the inflammatory process were found in animals of group 1. Isolated cellular elements were observed in the thick fibrin masses, especially in the newly formed growing synovial membrane.

There were revealed pronounced changes in the joint space structure. There were eosinophilic masses and accumulations of inflammatory cellular elements in microsections of the right knee joints in rats of group 1. Visualized fibrin threads, encapsulated oil accumulations detected in animals of group 4 evidenced transition of acute to chronic inflammation.

Analysis of the overgrown synovial membrane of the right knee joints revealed a large number of newly formed vessels. They contributed to formation of the granulation tissue, followed by its replacement with the connective tissue fibers. The degree of angiogenesis was the highest in animals of groups 1 and 2, and the lowest in animals of groups 3 and 4. Notably, the vessels themselves were full-blooded, thrombosed in places, sclerosal, as evidenced by the accumulation of erythrocytes in the lumen. The muscular membrane of the deformed joints was thickened; the intima was divided into layers. Proper synovial membrane of the knee joints of animals was hyperplastic, hypertrophied, and edematous. These changes were detected under morphological analysis of the right and left knee joint tissues in rats of all groups.

In addition to the above changes, in the sections of the tissue joints in rats of group 1, there were found areas of the synovial membrane which resembled the pattern of pannus formation. Tissue exposure to the joint space caused subsequent changes in the bone and cartilage tissue. Importantly, the processes of histogenesis that occurred sequentially as angiogenesis and was especially pronounced in animals of groups 1 and 2 progressed.

The pathological process affected bone structures and cartilage components. In microsections in animals of group 1 (control), we found processes of articular cartilage degeneration, which manifested aschondrocyte loss. The detected areas of chondrocyte loss were compliant with the areas of the synovial membrane growth. Destructive changes in the periosteum (its surface became corrugated, folded, a pronounced layer dissection was observed) evidenced its involvement in the inflammatory process. Bone tissues contained elements of resorption surrounded by osteoclasts; there were signs of matrix vacuolization.

When analyzing microsections of the right knee joints in rats of groups 1 and 4 in polarized light microscopy (Fig. 2), we managed to visualize the following signs: an inhomogeneous pink glow was detected in microsections of rats of group 1 (control), the fact supporting the maturity of the tissue structure and a low regeneration level; on the contrary, in samples obtained from animals receiving combined therapy we managed to visualize a green glow, the fact evidencing regeneration after damage due to inflammation.

Mast cells (MCs) were a key component of the inflammatory response (Fig. 3); they initiated the production of cytokines and recruited neutrophils and eosinophils to the lesion. Perivascular accumulations were found in tissue samples in all study groups (Fig. 4). This location is explained, on the one hand, by MC participation in the process of angiogenesis; on the other hand, it is reasoned by MC decisive role in implementing the body's response to the administered drug. Along with all functional types of mast cells, there was a significantly increased number of degranulating forms, which is likely to support a high activity of the inflammation process. The major mass of degranulating forms was found in animals of group 2 receiving hydrogen-enriched water. In addition, degranulation was provided by macrophage factors of pro-inflammatory agents – M1 macrophages; their number in animals of group 2 was close to that in animals of group 1 (control) (Table 1) (Hasan et al. 2012). In animals of groups 3 and 4, where methylprednisolone was a component of the therapy, the number of compact cells was higher, since glucocorticosteroids provided stabilization of their membranes.

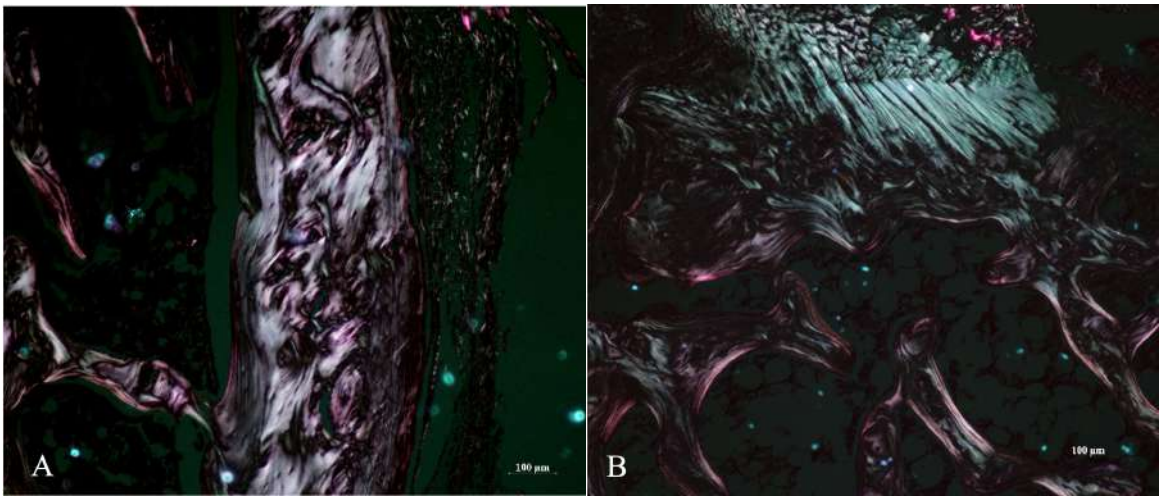


Figure 2. Morphological picture of the right knee joints in rats of group 1 (**A**) and in rats of group 4 (**B**). **Note:** type I collagen – mature (pink glow), type III collagen – immature (green glow). **A** – in the bone tissue, type I collagen prevails, the fact indicating absence of regeneration processes. **B** – green glow evidences active regenerative processes in the bone tissue. Fixation – 10% neutral formalin. Gill's hematoxylin and eosin staining, x10 magnification, polarized light microscopy.

Table 1. Quantitative assessment of biomarkers of the inflammatory process of the knee joints in rats in the experiment (unit of measure – the number of immune-positive cells/mm²)

Group	TGF-β1		CD68		CD163	
	left	right	left	right	left	right
Group 1 (placebo)	1.61±0.4	1.95±0.5	0.32±0.01	9.26±3	-	1.84±0.3
Group 2 (molecular hydrogen-enriched water)	1.01±0.1	2.41±0.1	0.12±0.3	5.28±2	-	4.69±2.5
Group 3 (methylprednisolone)	1.94±0.1	8.22±4*	0.04±0.4	3.33±1.6*	-	13.12±6*
Group 4 (molecular hydrogen-enriched water + methylprednisolone)	2.03±1.2	16.38±8.2*	-	2.24±0.2**	-	13.44±7.2*

Note: for values marked with * – $p<0.1$ compared with control, for values marked with ** – $p<0.05$.

Localization near areas with oil drops is a variant of MC reaction to foreign substances, manifestation of a protective function (Atiakshin et al. 2021). A quantitative analysis of sections of the right knee joints, performed in 25 fields of view with an x40 lens, demonstrated that the average number of mast cells was 14.00±1.7 ($p<0.05$) per 1 mm² in animals of group 1, while in animals receiving combined therapy with hydrogen-enriched water and methylprednisolone the average number of mast cells was 2.39±0.89 ($p<0.05$); this is likely to evidence a decreased inflammatory response in animals of group 4 and correlates with the number of M1 macrophages (Table 1).

Transforming growth factor (TGF-β1) was chosen as a marker to analyze the activity of the inflammatory process. TGF-β1 is produced by M2 macrophages (Fig. 4), T- and B-lymphocytes, erythrocytes and fibroblasts and is crucial in maintaining the constancy of the various tissue structure; it shifts the immune response towards the pattern of tissue repair in the process of inflammation. In RA pathogenesis, it provides a stage of clinical remission, as M2 macrophages release anti-inflammatory cytokines and chemokines (Cutolo et al. 2022). Analyzing the cartilaginous tissue of the articular surface, it is worth noting a critical impact of TGF-β1 on the processes of cell proliferation, differentiation, apoptosis, as well as regulation of the extracellular matrix synthesis and degeneration. In RA pathogenesis, TGF-β1 expression supports participation in the inflammatory response, but its role is ambiguous. On the one hand, the process of cartilage tissue regeneration is stimulated (activation of cell divisions, matrix production); on the other hand, the connective tissue component of the synovium (pannus) grows, which leads to sclerosis, and subsequently to difficulty in moving the joints. In addition to fibrosis, there occurs regeneration and growth of the bone tissue; they were identified in sections in rats of group 4. The predominant number of cells expressing TGF-β1 was found in sections of the right knee joints in animals receiving combined pharmacotherapy (Table 1).

During exacerbation, a higher percentage of pro-inflammatory M1 macrophages demonstrating the specific marker CD68 predominate. Macrophages express leukocyte adhesion molecules on the surface; this, in turn, is significant in T-lymphocyte activation and modulation of a pronounced inflammatory response mediated by chemokines and cytokines (Abdel Jaleel et al. 2021). Among other things, M1 macrophages are involved in the process of bone tissue resorption. Concentrated groups of CD68-positive cells are located in the synovial membrane; the predominant number is in animals of group 1 (Table 1).

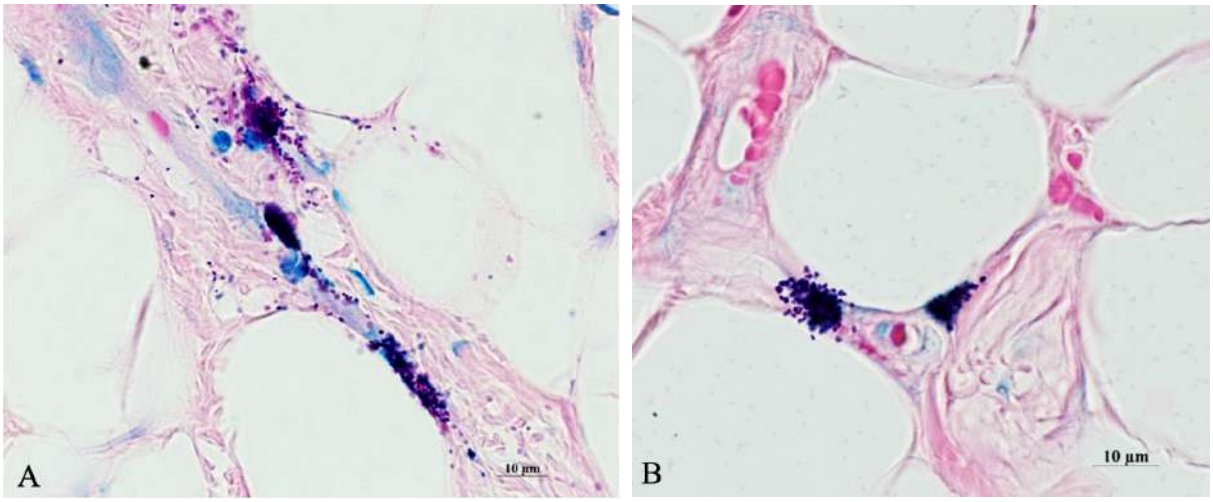


Figure 3. Mast cells in microsections of the right knee joints in rats of group 2 (A) and in rats of group 4 (B). **Note: A** – a group of actively degranulating mast cells and free-lying granules and **B** – a perivascular location of mast cells around clusters of encapsulated oil droplets. Fixation – 10% neutral formalin. Giemsa staining, x20 magnification, light microscopy.

CD163 expression by macrophages supports transition of the process of acute inflammation to chronic inflammation. These receptors are located on the surface of M2 macrophages that provide episodes of remission in the course of rheumatoid arthritis. Isolated CD163-positive cells were found in sections of the knee joints in rats of group 1 (control) (Table 1).

No CD68-positive cells were found in sections of the left knee joints of rats receiving combined pharmacotherapy. No cells expressing CD163 were found in sections of the left knee joints in animals of all four groups.

The results obtained allow concluding on the more rapid course of the major clinical RA signs, absence of the major histological signs of autoimmune inflammation in animals that received combined pharmacotherapy. This supports an aqueous molecular hydrogen solution as the most practical and simple way to introduce hydrogen into the body as a supplement to standard therapeutic options. Elimination of the major clinical signs of simulated RA due to combined pharmacotherapy with molecular hydrogen support results of multiple studies describing therapeutic effect of hydrogen in various acute or chronic inflammatory diseases. Intracellular signaling and metabolic pathways activated in M1 macrophages during the acute phase of RA are associated with a high level of reactive oxygen species. The therapeutic effect of molecular hydrogen, presumably, can be explained by binding free radicals, which are damaging agents of cell membranes (Cutolo et al. 2022).

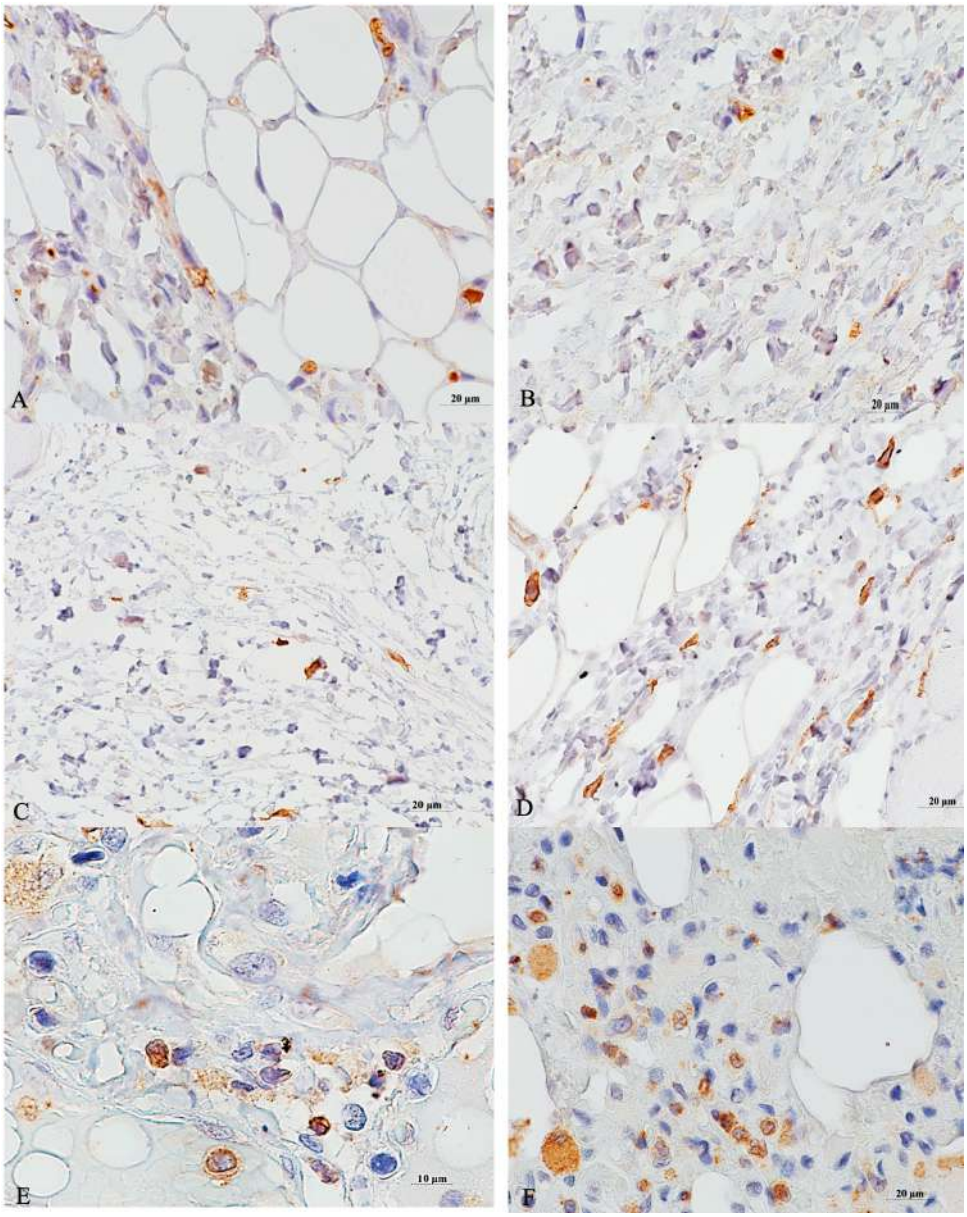


Figure 4. Different levels of CD68 (A, B), CD163 (C, D) and TGF-β1 (E, F) expression in cells of the synovial membrane of the right knee joint in animals of groups 2, 3, 4(experimental). **Note: A** – high level of M1 macrophage CD68 expression in the joint tissues in animals of the placebo-controlled group; **B** – low level of CD68 expression in animals of group 4; **C** – activated M2 macrophages with a low level of CD163 expression in animals of the placebo-controlled group; **D** –activated M2 macrophages CD163 immunoexpression in animals of the group with combined methylprednisolone and an aqueous molecular hydrogen solution treatment; low level of TGF-β1 secretion in animals of group 1 (**E**) and high level of cellular secretion in animals of group 4 (**F**). Fixation – 10% neutral formalin. Immunohistochemical staining with antibodies to CD68 (ab213363), CD163 (ab182422), TGF-β1 (ab215715), nuclei were counterstained with Mayer's hematoxylin. x40 magnification (A-D, F), x100 magnification (E).

Conclusion

A combined approach including methylprednisolone pulse therapy and hydrogen-rich water is stated to be the most effective treatment option. This combination contributes to a more rapid transition of active rheumatoid arthritis into remission. Further studies should be aimed at investigating changes in the structure of adverse reactions resulted from supplementing basic glucocorticosteroid therapy with an aqueous molecular hydrogen solution.

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Conflict of interest

The authors declare no conflict of interests.

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