Experimental treatment of autoimmune process with salbutamol, pentoxifylline and their combination

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The therapeutic potential of salbutamol (S), a β-adrenergic agonist, and pentoxifylline (P), a metylxantine derivative and phosphodiesterase inhibitor, was explored in adjuvant-induced arthritis. Female Lewis rats with adjuvant arthritis (AA) were treated with S and P alone and in combination. Treatment was started since day 9–10 of AA and lasted 10 days. At the end of experiment the rats were sacrificed and their joints were evaluated histologically. Daily (except weekends) oral administration of 9 mg/kg of S, 520 mg/kg of P and their combination (8 mg/kg of S and 500 mg/kg of P) had a profound therapeutic effect on the clinical progression of arthritis and protected joints from damage. Administration of P offered a somewhat better protection than S against joint damage as assessed by histology. The best effect was achieved by using a combination of S + P.

Key words: adjuvant arthritis, salbutamol, pentoxifylline, combined therapy

INTRODUCTION

Experimental models of arthritis in rats, which predominantly involves T cell-mediated mechanisms, have been used extensively in studying the role of autoimmunity and inflammation in the pathogenesis of joint disease (1). Freund's adjuvant arthritis (AA) in rats is a well-established experimental model of inflammatory joint disease, which shares many features with human rheumatoid arthritis (RA) (2) and is widely used to evaluate anti-inflammatory and immunosuppressive/immunomodulatory drug properties (3).

Immune system activation during RA and AA leads to increased circulating levels of proinflammatory cytokines (4, 5), where TNFα is a critical mediator of joint inflammation and therefore an important therapeutic target (6, 7).

Previous experimental and clinical reports showed that the phosphodiesterase (PDE) inhibitor pentoxifylline (P) and the β_2-adrenergic agonist salbutamol (S) can inhibit the production of proinflammatory cytokines (8–14). Both drugs promote the accumulation of intracellular cyclic AMP (11, 15–17), which is a potent modulator of the immune system able of either down-regulating the expression of Th1 or upregulating the expression of Th2 cytokines (18). Th1 cytokines promote the recruitment and activation of inflammatory cells and are associated with inflammatory reactions and tissue injuries (19–21). Th1 and not Th2 cells entere the inflamed sites of Th1-based diseases such as arthritic joints (21). There are reports that elevation of intracellular cAMP reduces the production of IL-12 and TNFα, important cytokines in the pathogenesis of RA (6, 22), and augments the production of antiinflammatory cytokine IL-10 (23, 24). IL-12 is critical to Th1 development (20, 25, 26) and plays a central role in both the induction and magnitude of a primary Th1 response (27, 28).

β_2-agonists, such as S, have been shown to inhibit IL-12 production and thus to prevent Th1 development, and this action is mediated by the β_2-adrenoreceptor and correlates with increased intracellular cAMP levels (29). PDE inhibitors such as P also inhibit IL-12 production via cAMP upregulation (17).

So, the therapeutic potential of S and P alone and in combination was explored in adjuvant arthritis induced in rats.

MATERIALS AND METHODS

Two experiments were performed on 56 female Lewis rats weighing 160–240 g at baseline. The animals were purchased from "Bioreglament" (Vilnius,

Lithuania) and kept under standardized conditions in an air-conditioned room and given standard rat chow and water *ad libitum*. After a resting period of one week, the animals were subjected to the experimentals protocols.

The animal studies were carried out in accordance with current guidelines for the care of laboratory animals and were approved by the Institutional Ethical Review Committee.

Induction and evaluation of AA, investigation of blood indices and pathomorphological changes in liver and joints were performed as described in our earlier studies (30).

The ***first experiment*** was performed on 26 rats with AA. On day 10 rats were assigned into 3 groups with the similar mean scores of joint swelling and the treatment with salbutamol (Salbutamol sulfas; Polfa, Poland) in doses 2 mg per rat or 9 mg/kg (1st gr.) and pentoxifylline (Rentyl® 400; Arzneimittel GmbH & Co) in doses 120 mg per rat or 520 mg/kg (2nd gr.) was started.

In the ***second experiment*** (30 rats) animals since day 9 of AA were also assigned into 3 groups and the treatment with salbutamol (S) in doses 1.4 mg per rat or 8 mg/kg (1st gr) and a combination of S (8 mg/kg) and pentoxifylline (85 mg per rat or 500 mg/kg) (2nd gr.) was started.

The drugs were prepared *ex tempore* as a fine homogenized suspension in 1% starch gel and injected orally into the stomach through a metallic sound. The treatment lasted 2 weeks.

General condition, body weight and the severity of joint swelling were monitored 3 times a week. Hematological and pathomorphological changes were evaluated at the end of experiment when the rats were sacrificed. The experiments lasted 23–24 days.

The results were expressed as mean values ± S. E. M. Differences between the control and treated groups were statistically analyzed by Student's t test with $P < 0.05$ considered as significant. The percentage of deviation from the control group was derived by the following formula: (T–C) / C × 100, where T is the data on a test group and C is the data on the control.

RESULTS

1. Effect of salbutamol and pentoxifylline on the development of adjuvant arthritis in rats

Therapeutic treatment of AA with salbutamol (S) and pentoxifylline (P) produced a pronounced anti-inflammatory effect (Fig. 1, A) in the both groups tested. A somewhat better effect was achieved in the rats that received P. S diminished joint swelling by 31–44.5% ($P < 0.05$) and P by 32–48.9% ($P < 0.05$–0.02) during the whole experiment.

Polyarthritis characterizing the development of autoimmune process was absent in the P-treated group. It developed in 10% of rats given S and in 25% of animals in the control group on day 15 of AA and was observed till the end of experiment. ESR was significantly lower in both test groups (Fig. 1, B) and decreased by 48% ($P < 0.001$) and 42% ($P < 0.002$) in animals given S and P, respectively. A significant (by 22%, $P < 0.05$) lowering of leukocyte count (Fig. 1, B) was observed in the P-treated group.

No essential differences in erythrocyte count were observed among the groups, although it was higher in the treated groups than in control.

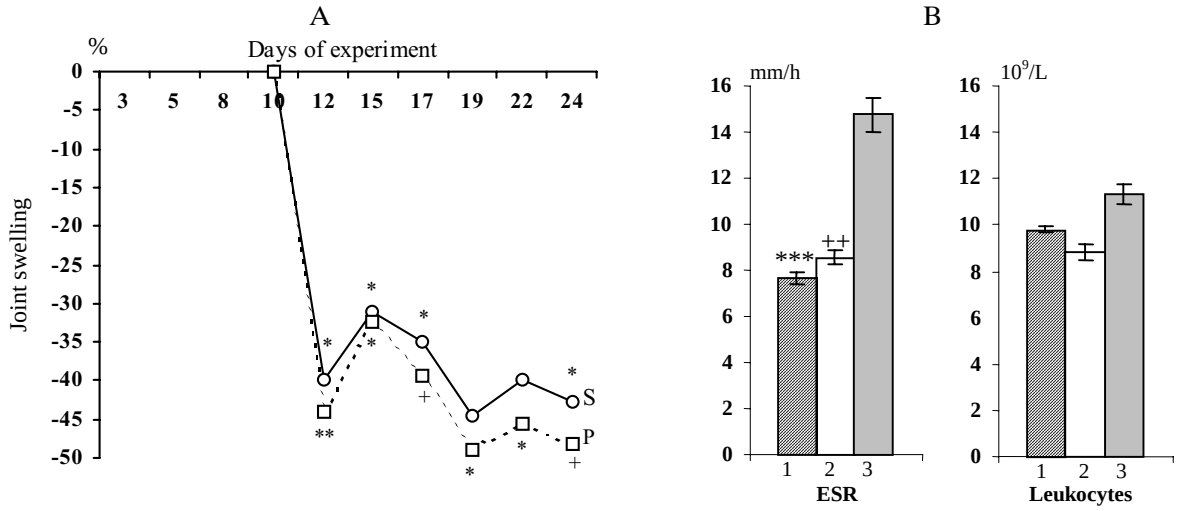

Fig. 1. Joint swelling (A) and blood indices (B) under treatment of established adjuvant arthritis in rats with salbutamol and pentoxifylline (mean indices of joint swelling are expressed as percentages from mean indices in arthritic control). A – salbutamol (S) (2 mg/rat or 9 mg/kg); pentoxifylline (P) (120 mg/rat or 520 mg/kg). B – 1-salbutamol; 2-pentoxifylline; 3 – control. Differences are significant in comparison with control group. *$P < 0.05$; **$P < 0.01$; $^{+}P < 0.02$; $^{++}P < 0.002$; ***$P < 0.001$

Body weight during the experiment increased in both test groups, but no essential differences in this index and in the weight of internal organs were observed among the groups at the end of experiment.

Macroscopic changes in the spleen were pronounced only in 20% of rats in both treated groups *versus* 60% in the control group.

Neither of the preparations exerted a toxic effect on the liver. A pathomorphologic examination showed that P significantly reduced parenchima alterations (0.3 ± 0.15 in the test groups and 1.1 ± ± 0.27 in control; $P < 0.05$) and diminished (insignificantly) hepatic fibrosis (0.3 ± 0.13 in the test group and 0.6 ± 0.17 in control). Both preparations, S and P, reduced general inflammatory reaction in hepatic stroma (0.5 ± 0.09 in both test groups and 0.8 ± 0.10 in control; $P < 0.05$).

A histological examination of joints in control animals with AA showed desorganization of connective tissue collagen fibres, proliferation of new capillars, mucoid edema, foci of fibrinoid necrosis. An expressed inflammatory infiltration with lymphocytes, macrophages, granulocytes, synovitis and pannus formation were observed.

Treatment with S and P diminished desorganization of connective tissue (γ-metachromasia) in soft periarticular tissues and synovium (Table 1). No essential differences in general inflammatory reaction among the treated groups and control were found, although the usage of P diminished it (Table 1) by 25% and significantly decreased inflammatory infiltration with macrophages (1.3 ± 0.13 in test groups and 1.8 ± 0.22 in control; $P < 0.05$). Both preparations suppressed soft tissue and synovium edema, but only the effect of P was significant in synovium ($P < 0.05$). The latter preparation also diminished synovium villi proliferation (by 50%; $P < 0.01$). General inflammatory reaction was decreased in both treated groups, although P action was more significant (Table 1). Both S and P also significantly diminished infiltration of synovium with lymphocytes (1.0 ± 0.09 – 1st gr., 0.6 ± 0.08 – 2nd gr., and 1.6 ± 0.27 – control; $P < 0.05$ and $P < 0.01$, respectively) and P – with macrophages (0.7 ± 0.15 – test group and 1.3 ± 0.21 – control; $P < 0.05$). Fibrotic processes were also pronounced in soft periarticular tissues and synovium under the treatment (Table 1). Pannus formation and cartilage thinning were less in both test groups.

2. Effect of therapeutic administration of S and its combination with P on development of adjuvant arthritis

In this experiment, we evaluated the efficacy of therapeutic administration of S (8 mg/kg) and its combination with P (500 mg/kg) in rats with AA. S significantly ($P < 0.02$) reduced joint swelling (by 33–35%) since day 18 of experiment, and the effect lasted till the end of investigation (Fig. 2, A). Combined treatment with S+P was more effective and markedly ($P < 0.05$–0.01) diminished joint swelling starting from day 16 (by 37%) till the end of experiment (by 41%). Polyarthritis developed in 70% of animals treated with S, 60% with S+P and in 90% of control group. A marked improvement of blood indices was observed in both treated groups (Fig. 2B)

Table 1. **Pathomorphological changes in joints of rats with adjuvant arthritis treated with salbutamol (S) and pentoxifylline (P)**

Morphological changes	Groups		
	1st gr. S (n = 9)	2nd gr. P (n = 9)	Control (n = 8)
Soft periarticular tissues			
Edema	0.4 ± 0.17	0.4 ± 0.19	1.0 ± 0.29
Fibrosis	0.7 ± 0.13**	0.6 ± 0.17*	0.1 ± 0.11
γ-metachromasia	0.3 ± 0.12	0.3 ± 0.19	0.8 ± 0.28
General inflammatory infiltration	2.3 ± 0.23	1.8 ± 0.23	2.4 ± 0.37
Synovium			
Villi proliferation	1.2 ± 0.18	0.8 ± 0.13**	1.6 ± 0.20
Edema	0.2 ± 0.13	0*	0.7 ± 0.32
Fibrosis	1.0 ± 0.02***	1.6 ± 0.15***	0.1 ± 0.08
γ-metachromasia	0.2 ± 0.13	0	0.4 ± 0.25
Inflammatory infiltration	1.2 ± 0.26	0.8 ± 0.13**	1.8 ± 0.24
Cartilage			
Fissure	0	0	0.3 ± 0.21
Pannus	0.25 ± 0.14	0.3 ± 0.16	0.5 ± 0.29
Thinning of cartilage	0	0.2 ± 0.16	0.3 ± 0.16

Note. Differences are significant in comparison with control group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

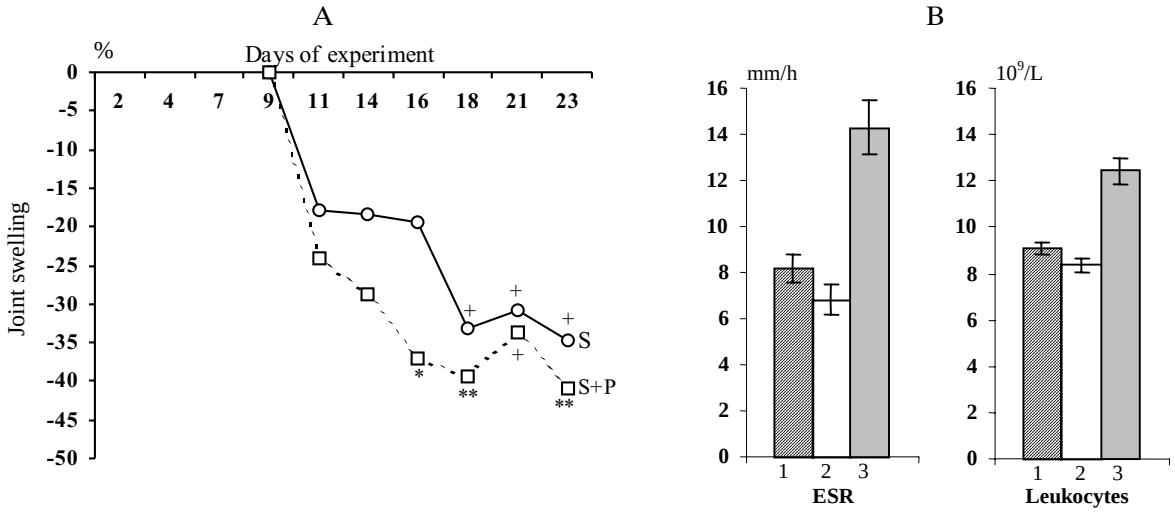

Fig. 2. Joint swelling (A) and blood indices (B) under treatment of established adjuvant arthritis in rats with salbutamol and its combination with pentoxifylline (mean indices of joint swelling are expressed as percentages from mean indices in arthritic control). A – salbutamol (S) (1.4 mg/rat or 8 mg/kg); combination of salbutamol (1.4 mg/rat or 8 mg/kg) and pentoxifylline (P) (85 mg/rat or 500 mg/kg). B – 1-salbutamol; 2-S+P; 3 – control. Differences are significant in comparison with control group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{+}P < 0.02$

as compared to the control, but it was more pronounced under combined therapy. ESR in the group treated with S was lower by 42.67% ($P < 0.05$) and in the group administered S+P by 52.2% ($P < 0.02$). The lowering of the count of leukocytes (by 30.8% and 36.2%, $P < 0.01$) was revealed in both treated groups.

Body weight was higher in treated groups (181.36 ± ± 6.03 g, 185.45 ± 6.52 g; $P < 0.05$ in the 1st and 2nd groups, respectively, and 169.09 ± 3.36 g in control), but no essential differences in the weight of internal organs were observed among the groups at the end of experiment.

The toxic effect of drugs on liver was not obseved. The treatment with S and S+P diminished V. centralis hypervolemia (0.7 ± 0.11, $P < 0.001$ and 1.0 ± 0.19, $P < 0.05$ in test groups and 1.6 ± ± 0.15 – in control), and the usage of combined treatment significantly suppressed inflammatory infiltration with lymphocytes (0.2 ± 0.12 in both test groups and 0.7 ± 0.2 in control; $P < 0.05$) in hepatic stroma.

Table 2. **Pathomorphological changes in joints of rats with adjuvant arthritis treated with salbutamol (S) and its combination with pentoxifylline (P)**

Morphological changes	Groups		
	1st gr. S (n = 10)	2nd gr. S+P (n = 10)	Control (n = 10)
Soft periarticular tissues			
Edema	0.9 ± 0.44	0.7 ± 0.14***	1.8 ± 0.12
Fibrosis	0.8 ± 0.37	1.3 ± 0.10***	0.2 ± 0.14
γ-metachromasia	0.5 ± 0.46^{+}	0.7 ± 0.19***	1.8 ± 0.12
General inflammatory infiltration	1.7 ± 0.40	1.6 ± 0.08***	2.5 ± 0.14
Angiomatosis	0.7 ± 0.41	1.0 ± 0.12	1.4 ± 0.21
Synovium			
Villi proliferation	1.1 ± 0.08***	0.9 ± 0.09***	1.9 ± 0.07
Edema	0.6 ± 0.10^{++}	0.4 ± 0.17^{++}	1.3 ± 0.16
General inflammatory infiltration	1.1 ± 0.08***	1.1 ± 0.17**	1.9 ± 0.17
Angiomatosis	1.1 ± 0.11***	0.9 ± 0.13***	2.1 ± 0.07
Cartilage			
Fissure	0.1 ± 0.13*	0^{+}	0.9 ± 0.34
Pannus	0.5 ± 0.17**	0.3 ± 0.12**	1.6 ± 0.34
Thinning of cartilage	0.1 ± 0.13^{+}	0.1± 0.06**	1.1 ± 0.34

Note. Differences are significant in comparison with control group. $^{*}P < 0.05$; $^{+}P < 0.02$; $^{**}P < 0.01$; $^{++}P < 0.002$; $^{***}P < 0.001$.

Pathomorphological examination of joints showed a marked positive effect of both kinds of treatment on soft periarticular tissues, synovia and cartilage (Table 2). Both treatments diminished all inflammatory reaction (inflammatory infiltration, edema, angiomatosis, γ-metachromasia) in soft periarticular tissues and synovia (Table 2), but combined treatment was more effective (Fig. 3). Inhibition of inflammatory infiltration with lymphocytes (1.5 ± 0.08 – 1st gr.; $P < 0.001$ and 1.6 ± 0.13 – 2nd gr.; $P < 0.01$ and 2.0 ± 0.04 – control) and macrophages (0.9 ± ± 0.23; $P < 0.02$ – 1st gr., 1.1 ± 0.08 – 2nd gr.; $P < 0.001$ and 1.6 ± 0.09 – control) was observed under the both kinds of treatment in soft periarticular tissues, but significant ($P < 0.001$) differences in general inflammatory infiltration were revealed only after combined treatment (Table 2). Combination of S+P inscreased fibrotic processes ($P < 0.001$) in soft tissues.

The same results were observed in the synovium, where inflammatory infiltration with lymphocytes (0.8 ± 0.11 – 1st gr., 0.7 ± 0.16 – 2nd gr., and 1.7 ± 0.21 – control; $P < 0.002$) and macrophages (0.4 ± 0.20 – 1st gr., 0.8 ± 0.13 – 2nd gr., and 1.2 ± 0.12 – control; $P < 0.01$ and $P < 0.05$, respectively) was significantly lower. Inhibition of general inflammatory infiltration was also revealed in both treated groups ($P < 0.001$; $P < 0.01$) (Table 2). Although pathomorphological changes in synovia and cartilage were significantly reduced in groups treated with S and S+P (Table 2), this reduction was more pronounced under the usage of combined treatment (Fig. 3).

Both therapeutic interventions were well tolerated. No toxicity from drug administration was noted during the experimental period. All animals continued to grow and behave normally.

DISCUSSION

The present study was conducted to assess the antiarthritic therapeutic potential of S and P by using them alone or in combination.

Our results clearly demonstrate that all kinds of treatment significantly decrease the symptoms of established arthritis, inflammatory changes in blood and internal organs, prevent the development of polyarthritis and protect joints from destruction. The disease-modifying property was demonstrated not only by supression of joint swelling but also by a significant decrease of pathological changes in periarticular tissues, synovium and cartilage.

In our first experiment where the treatment with S and P was started since day 10 of AA, a relation between the reduction of joint swelling and reduced pathomorphological changes in arthritic joints was observed, and a more pronounced effect was revealed under treatment with P. Although the dose of S in our experiment was rather high (9 mg/kg), no toxic effects of the drug on the general condition of animals during the experiment were observed: all animals continued to grow and behave normally. Treatment with P was also associated with an accelerated weight gain and no toxic effect of the drugs on liver was observed.

A comparison of the treatment of established AA since day 9 with S alone and in com-

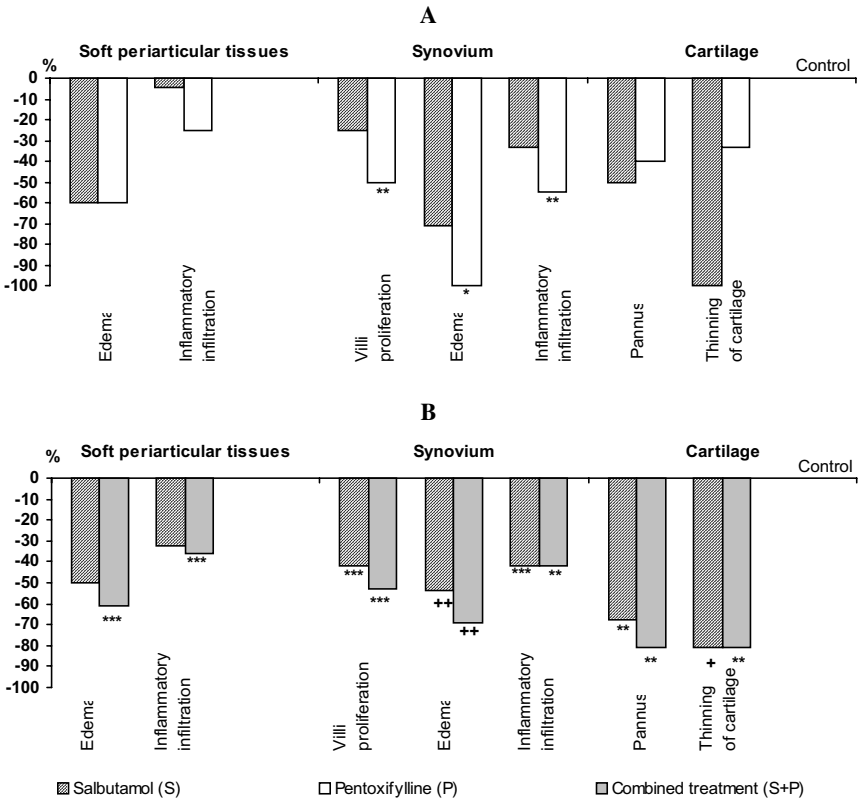

Fig. 3. Comparison of pathomorphological changes in joints under therapeutic treatment of adjuvant arthritis with salbutamol and pentoxifylline (A) and their combinations (S+P) (B) (mean indices of pahomorphological changes are expressed as percentages from mean indices in arthritic control). Differences are significant in comparison with control group; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{+}P < 0.02$; $^{++}P < 0.002$; $^{***}P < 0.001$

bination with P in the second experiment showed that combined treatment induced a more pronounced reduction of joint swelling, development of polyarthritis and joint damage (especially general inflammatory infiltration, angiomatosis, fibrotic processes). No toxicity from drugs administration was noted during experimental period. It should be noted that the doses of drugs in this experiment were somewhat lower (8 mg/kg of S and 500 mg/kg of P) and the treatment was started one day earlier than in the first experiment.

Development of AA is known to be dependent on activation of T lymphocytes and macrophages. Increases in macrophage number in AA synovium closely parallel the progression of clinical disease (31), and macrophage depletion in AA leads to marked disease inhibition (32). NO produced by synovial macrophages has been implicated as an important effector molecule in inflammatory joint disease, and increased production of NO is one of pathogenetic mechanisms of AA (33–35). Inhibition of NO production in AA significantly reduces arthritis severity (36).

In our studies treatment with S, P and S+P inhibited inflammatory infiltration with macrophages in periarticular tissues and synovia.

Our results are in agreement with earlier studies of other authors where it has been postulated that β_2-agonists and PDE inhibitors might be potentially useful in the treatment of Th1-mediated diseases such as RA and their experimental models (13, 37, 38). In animal model of AA, thalidomide-derived PDE4 inhibitors have shown efficacy in suppressing the development of disease as measured by ankle swelling, hind limb radiographic changes and weight gain (6). S treatment ameliorates clinical signs of established collagen-induced arthritis in mice, reduces joint damage, prevents mast cell degranulation in joint tissues and inhibits production of IL-12 and TNF by macrophages (13). Some authors (39, 40) have noted catecholamines and S to exacerbate AA in rats via β_2-adrenoreceptors, but according to Malfait and coauthors (13) this was probably due to the fact that in these studies the compounds were administered in continuous infusion which is known to desensitize β_2-adrenoreceptors (41). There are also data that a combination of S and aminophylline, a weak PDE inhibitor, can prevent AA (37), thus also demonstrating strong antiarthritic properties of the preparations.

P and S are known to elevate the cellular cAMP level (15–18). Elevation of cAMP level in hepatocytes might be one of the possible mechanisms by which these drugs protect from hepatic alterations observed in our study. cAMP also functions as a prominent regulator of the immune system activity and may tentatively be considered to be an upregulator of the production of Th2 cytokines (IL-10, IL-6), but a downregulator of the production of Th1 cytokines (IL-2 and TNFα) (18), which play an important role in joint diseases (4, 5, 42). Although cAMP by itself does not induce NFκβ, it could modulate IL-1α-induced NFκβ activity (43). Elevation of cAMP inhibits NFκβ activity (44). IL-1 together with TNFα are the main proinflammatory and destructive cytokines known to play an essential role in joint damage, acting through stimulation of metalloproteinase production by other cells in inflammed synovium (45).

Inhibition of TNFα and IL-12 secretion and stimulation of IL-6 and IL-10 production can be considered to be a hallmark of the influence of S and P on the joints. This action of drugs through enhanced cAMP on the cytokine network might prove useful in the treatment of various inflammatory processes, including autoimmune diseases (46, 47).

The results presented here allow to conclude that the elevators of cAMP and inhibitors of IL-12 and TNFα, salbutamol (S) and pentoxifylline (P), and their combination have a pronounced antiarthritic effect, show no toxicity and are well tolerated by animals. Joint swelling, development of polyarthritis and pathomorphological changes are reduced by all the kinds of treatment used, but the combined therapy is more effective.

In humans, high doses of S are needed to achieve the same therapeutic effect, and cardiotoxic side effects may occur during such treatment. Combined therapy with S and PDE inhibitor P allows to decrease the doses of both drugs and to get satisfactory treatment results.

We suggest that Th1-driven chronic inflammatory diseases, including RA, might benefit from treatment with S, P and their combination.

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AUTOIMUNINIO PROCESO EKSPERIMENTINIS GYDYMAS SALBUTAMOLIU, PENTOKSIFILINU BEI JŲ KOMBINACIJA

S a n t r a u k a

β-adrenerginio agonisto salbutamolio (S) ir metilksantino derivato bei fosfodiesterazės inhibitoriaus pentoksifilino (P) terapinis poveikis buvo ištirtas sukėlus adjuvantinį artritą (AA) žiurkėms. Lewis veislės žiurkių patelės buvo gydomos S ir P bei jų kombinacija. Gydymas pradėtas 9–10-tą AA dieną, gydymas truko 10 dienų. Bandymo pabaigoje žiurkės dekapituotos ir sąnarių pakenkimas įvertintas histologiškai. Kasdienis (išskyrus savaitgalius) oralinis 9 mg/kg S ir 520 mg/kg P bei jų kombinacijos (8 mg/kg S ir 500 mg/kg P) taikymas buvo efektyvus, slopino artrito progresavimą ir apsaugojo sąnarius nuo pažeidimo. Gydant P gautas šiek tiek geresnis poveikis sąnariams, lyginant su gydymu S (nustatyta histologiškai). Geriausias efektas aptiktas taikant kombinuotą gydymą.

Raktažodžiai: adjuvantinis artritas, salbutamolis, pentoksifilinas, kombinuota terapija