EVALUATION OF THE ANTI-INFLAMMATORY EFFECTS OF MUD THERAPY (PERUÍBE, BRAZIL) ON DIFFERENT EXPERIMENTAL MODELS OF ARTHRITIS

Zélia Maria Nogueira Britschka¹, Walcy Rosolia Teodoro¹, Ana Paula Pereira Velosa¹ and Suzana Beatriz Veríssimo de Mello¹*

¹ Department of Internal Medicine, Rheumatology Division, School of Medicine, University of São Paulo, São Paulo, Brazil.

*Corresponding Author:
S.B.V.Mello, Ph.D.
Associate Professor
Escola de Medicina
Universidade de São Paulo
Av. Dr. Arnaldo 455
São Paulo SP 0124-6903, Brazil.
Telephone: 5511-3066-7200
Fax 5511-3066-7200
e-mail:svmello@usp.br

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**ABSTRACT**

**Objective.** Several studies have demonstrated the beneficial effects of fangotherapy in relieving pain and improving the condition of rheumatic patients. In the present study, we investigated the effectiveness of a Brazilian black mud as a treatment for inflammation in experimental models of arthritis.

**Methods.** The anti-inflammatory effects of mud application during the course of arthritis was compared with warm water and no treatment in rabbits with antigen-induced arthritis or osteoarthritis induced by menisectomy and in Wistar rats with zymosan-induced arthritis. The synovial fluid and membrane were analyzed regarding cellular influx, synovial hyperplasia, vascular proliferation and infiltration, and the proteoglycan and collagen content of the cartilage.

**Results.** Our data showed that mud treatment had no effect on antigen-induced arthritis after a 7-day treatment. However, leukocyte infiltration was significantly impaired in the joints of rats with zymosan-induced arthritis treated with mud compared with both untreated rats or rats treated with hot water. This effect was followed by a partial protective effect on the synovium and cartilage that was also observed in the rabbit osteoarthritis model.

**Conclusion.** The Brazilian mud employed in our study presented an anti-inflammatory effect on animal models of chronic arthritis, reducing cartilage degradation. Even though the mechanism responsible for the observed effects is presently unknown, our data suggest that mud therapy may be useful as a complementary approach to treat chronic articular diseases.
INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease in which inflammation of the synovial lining and subsequent destruction of cartilage produce pain, swelling and progressive erosion of the synovial joints. It is believed that in RA synovial inflammation leads to cartilage destruction. In contrast, osteoarthritis (OA) presents variable degrees of inflammation. Studies using experimental OA models, and clinical observations suggest that synovial inflammation can be preceded by cartilage destruction since mechanical factors have been implicated in causing this pathology\(^1\). Like other chronic diseases, comprehensive management of RA and OA involves procedures that complement regular therapy. Non-pharmacological approaches include education of patients, exercising, rehabilitation procedures and complementary therapies\(^2\).

Fangotherapy, a thermal kind of mud therapy, consists of local application or whole body immersion in the mud and has been recommended for treatment of a great variety of rheumatic diseases\(^3,4,5\) muscular pain disorders\(^6\) as well as for skin treatment\(^7\). Balneotherapy, combined with conventional treatment, has been associated with improvements in the inflammatory signals of arthritis\(^8,9,10\).

Although the exact mechanism by which the mud exerts its actions is unclear, some hypotheses have been suggested to explain their anti-inflammatory effects. One of them is the maturation process of mud, which can induce some alterations on its physical characteristics\(^11,12,13,14,15\). Another one suggests that the high specific heat of thermal mud may generate an
analgesic effect by acting on opioids such as encephalins $^{12}$ and endorphins $^{16}$. It has also been demonstrated that the effects of mud may in part be attributed to their mineral components, which could be absorbed by the skin during application $^5$.

Several studies performed with RA and OA patients have shown decreases in serum levels of TNF-$\alpha$ $^{18}$, prostaglandin E$_2$ (PGE$_2$) and leukotriene B$_4$ (LTB$_4$) $^{19}$, radical-mediated peroxidations, nitric oxide production and myeloperoxidase $^{20,21}$ after twelve days with treatment runs at 42ºC in the Abano and Montegrotto Termes in Italy. In addition, clinical trials using fangotherapy from Dead Sea resulted in an abatement in inflammation in rheumatic patients $^{22,23,24}$. Unfortunately, these clinical studies did not consider the possibility that the environment of the spa center led to the observed improvements in the patients’ physical and mental conditions $^{25}$.

Peruíbe, a city located in southern São Paulo State in Brazil, has a muddy coast known as Black Mud. Its clay is formed by a mixture of sulphurous water and 30 minerals of volcanic origin, such as sulfur, natrium, zinc and calcium $^{26}$ Application of this clay has been widely used by patients and local physicians as a complementary treatment for RA and OA.
This study was designed to test, in vivo, the effectiveness of Brazilian black mud in protecting against the articular destruction verified in arthritis. For this purpose mud was tested on three different models of arthritis: antigen induced arthritis (AIA of 7 days) and partial menisectomy induced osteoarthritis (OA of 4 weeks) in rabbits and zymosan induced arthritis (Zy-IA of 2 days) in rats.

MATERIALS AND METHODS

Animals

Thirty male Wistar rats weighing 200-250g at the beginning of the experiments were employed for zymosan-induced arthritis (Zy-IA) and 36 Male New Zealand White rabbits weighing 2000-2500g were used for antigen induced arthritis (AIA) (n=22) and osteoarthritis (OA) (n=13). The animals were allowed a standard pellet diet and water ad libitum. During experimental procedures the animals were anesthetized to avoid any stressful situation. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Antigen-induced arthritis (AIA)

As previously described male New Zealand White rabbits were sensitized with 5 mg of methylated bovine serum albumin (mBSA, Sigma Chemical Co., St. Louis, MO) in 1 ml Freund's complete adjuvant (FCA, Sigma Chemical Co., St. Louis, MO) and 1 ml of sterile saline through
injections at subcutaneous and muscular sites in the suprascapular and gluteal regions, respectively. Seven days after immunization, animals were boosted with intradermical injections of 1mg/ml of mBSA once a week. Cutaneous Arthus reactions were characterized by central necrosis and observed 24h later. Simultaneously, serum antibody titers against mBSA were quantified by immunodiffusion. The animals used in the experiment were those who appropriately responded to the second cutaneous challenge and had anti-mBSA titers over 1/8. Seven days after the third booster shot, arthritis was induced in the left knee joint by the injection of 0.5 ml of a sterile solution of mBSA (1 mg/ml) into the articular cavity. The contralateral joint was injected with saline. The same procedure was performed a week later and repeated one week after the second intraarticular challenge. The animals were killed with an overdose of ketamine.

Zymosan-induced arthritis (Zy-IA).

Male rats were anesthetized with 0.4 ml of 1.0 mg/kg of xylazine and 0.75 mg/kg of ketamine, and submitted to an intraarticular injection of 1.0mg of zymosan (Sigma Chemical Company, St. Louis, MO) diluted in 50µl of saline on the left knee. The right knee received the same volume of saline. Animals were sacrificed in a CO₂ chamber 21 days after the intraarticular challenge. The joints were washed twice with 0.5ml saline containing 10 mM EDTA, and the synovial washes were collected for determination of total and differential cell counts using a Neubauer chamber and stained smears,
respectively. Synovial membranes were surgically excised, paraffin-embedded and routinely processed.

*Osteoarthritis induction*

Before surgery, rabbits were anesthetized with a mixture of xylazine (5mg/Kg) associated with ketamine (50mg/Kg) by intramuscular route and after tricotomy of the knee, menisectomy was performed as described previously\(^29\). Briefly, under sterile conditions, an anteromedial incision of the right knee was made; subcutaneous tissue and the retinaculum were incised and retracted, along with the articular capsule. The medial compartment was visualized and the peripheral capsular insertion of the medial meniscus was dissected. The anterior half of the meniscus was resected and the capsule, the medial retinaculum the skin were later sutured. All rabbits were housed in regular individual cages and fed *ad libitum*.

*Mud treatment*

Natural Black mud was obtained in Peruíbe, a coastal city in São Paulo state, Brazil and kept for six months in closed buckets with 10–15 cm fresh marine water collected from the same beach. This maturation time in water with high salt content is enough to provide a mud free of contamination\(^26\).

In all protocols, the animals were randomly divided as untreated group (control), water treated group (control) and mud treated group.
In the OA model rabbits were anesthetized and both knees were kept in water or mud packs at 40°C for 30 minutes. Each group received its respective treatment twice a week during fourteen weeks.

The same therapeutic approach was used for AIA, the group was treated daily for one week after induction of arthritis.

Rats submitted to Zy-IA were maintained in cages containing 300 ml of warm water or mud for 30 minutes. This procedure was performed daily during 3 weeks.

*Sampling of the synovial fluid*

Immediately after the sacrifice 2ml and 0.5ml of saline containing EDTA (1mg/ml) was injected into the knee joint of AIA rabbits and Zy-IA rats respectively. The synovial wash was aspirated, the joint was opened and the remainder of the synovial fluid as well as the synovial membrane was recovered. Total and differential leukocyte count was done in a Neubauer chamber under light microscopy. For the differential white cell count smears were prepared from a cell pellet and stained with Giemsa.

*Morphological analysis*

All materials used for histological analysis were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 4µm. The bone of rat whole joints was decalcified using 6.0% EDTA.
The hematoxylin and eosin (H&E)-stained synovial membranes were semiquantitatively evaluated for infiltrating leukocytes, vascular proliferation and lining hyperplasia using a (0 - 3) grade score for each of these parameters. Absence of any of these alterations was given a zero (0) score. These observations were made by 3 experienced pathologists blinded to the treatment protocol. At × 400 of magnification, we used an eyepiece systematic point sampling grid with 100 points and 50 lines to count the fraction of points overlaying infiltrating cells. Ten microscopic fields were counted to obtain a final result expressed as a percentage of cells$^{30}$. Whole rat joints and cartilage slices of rabbits were stained with H&E. Morphometric analyses were done and total cell numbers were expressed as percentages and cells/µm$^2$, respectively. Pannus formation and cartilage erosions were scored by different observers on 0 to 3 grades, which expressed the severity of the alterations. Structure, cells, proteoglycan and collagen amounts were evaluated by Mankin’s grade$^{31}$. For proteoglycan the observation slides were stained with Alcian blue 1.0% pH 2.5 to identify acid polysaccharides and for collagen analysis the observation slides were stained with Masson trichrome and picro-Sirius.

Statistical analysis

Results are expressed as mean ± s.m.e. To compare the differences between means, we used one-way ANOVA followed by student Newman Keuls test. The chosen level of significance was 0.05.
RESULTS

Efficacy of mud treatment on Arthritic experimental models

To investigate the anti-inflammatory effects of Black mud therapy, leukocyte influx into the articular cavity, the synovial cell infiltrate and cartilage alterations were evaluated in experimental models of arthritis, which could represent different human disease stages.

Figure 1 shows total and differential leukocyte counts in the synovial fluid collected from the joints of AIA rabbits, 7 days after intraarticular challenge with 1.0mg of mBSA. Our data demonstrate the inefficacy of mud treatment in impairing the cellular afflux of leukocyte in this model of acute arthritis compared with untreated animals and control rabbits treated with warm water. Migration of both polymorphonuclear (PMN) and mononuclear (MN) leukocytes to the inflamed area remained unaltered.

Figure 2 shows total and differential leukocyte counts in the synovial fluid of rats challenged intraarticularly with 1.0mg of zymosan. This experimental model is characterized by a large infiltration of MN cells into the joint indicating a chronic inflammatory response. The treatment consisted of daily immersion of the animals paws in warmed water or mud during 21 days. Our data demonstrate that mud treatment significantly diminished leukocyte migration into the articular cavity compared with the untreated and warm water-treated groups (PMN, \( p = 0.008 \) and \( p = 0.03 \) and MN, \( p = 0.03 \) and \( p \))
= 0.007, respectively). This observation almost excluded the effect of temperature in the anti-inflammatory efficacy of the mud.

Microscopic analysis of the synovial membrane of Zy-IA rats is depicted in Figure 3 A and B. The membranes from untreated arthritic animals were greatly thickened due to synoviocyte proliferation and infiltration of a large number of lymphomononuclear cells (Figure 3 panel B1). Intense vascular proliferation was also observed. We did not verify any difference between synovium extracted from control animals and those submitted to warm water treatment (Figure 3A). An example of the effect of mud treatment on the synovial membrane from rats is illustrated in Figure 3 - panel B2. The surface of the membrane exhibited a single cell layer of synoviocytes. Although the sub-synovial area had a persistent infiltration with monocytes, the cellular infiltration impairment is documented in Figure 3A by means of cellular counting with eyepiece systematic point sampling grid (p = 0.0001 vs control; p = 0.02 vs water), and observation scores (p = 0.0003 vs untreated; p =0.005 vs water). Additionally, parameters such as hyperplasia (p = 0.006 vs control and p = 0.003 vs water) and vascular proliferation (p = 0.003 vs untreated and p = 0.006 vs water) were significantly reduced by the treatment.

Morphological changes including focal and diffuse erosion as well as necrosis of cartilage were observed in blades stained with H&E (not shown) and Masson trichrome (Figure 4A) in the joints from arthritic animals, untreated and treated with water not shown). Pannus was often seen on the surface of the affected cartilages. In some cases, the inflammatory process
had extended through the articular cartilage and epiphyseal bone into the adjacent bone marrow. Erosion or complete disarrangement of the articular cartilage was seen in most joints. Moreover, untreated control animals exhibited a weak staining by picro-Sirius, demonstrating significant loss of collagen (Figure 4C).

Mud treatment preserved the articular space and cartilage integrity compared with control arthritic rats, as shown in Figure 4A and B. The effect of mud treatment on collagen staining is also evident in Figure 4C and D. Data relative to structure and staining in the cartilage gathered by different observers are summarized in Table I. Warm water treatment did not interfere with morphology or cell counts compared with untreated animals. A moderate increase in the number of chondrocytes \( (p = 0.03) \) and a conserved tissue structure \( (p = 0.04) \) was observed in animals treated with mud compared with control groups, indicating that mud treatment was capable of preventing cartilage damage. Proteoglycan staining was not altered by mud treatment (Figure 4 E and F).

*Effect of mud treatment on the cartilage of osteoarthritic animals*

Data presented in table II show that warm water treatment did not prevent damage in the cartilage of OA rabbits. Morphometric indexes, proteoglycan or collagen staining were not different from those verified in untreated animals.
Changes in the Masson trichrome and Picro-sirius staining in the cartilage of untreated animals were also observed. In contrast, mud treatment appeared to prevent collagen degradation \((p = 0.0003)\). These data implies the occurrence of tissue repair, with chondrocyte proliferation and metabolically active cells. The maintenance of Mankin’s grade indicates that, like in the Zy-IA arthritic group, proteoglycan from the cartilage of OA rabbits did not differ among the control and mud-treated groups (Table II).
DISCUSSION

Although fangotherapy has been considered as a special form of balneotherapy often employed in Europe and Asia as a complementary therapy for RA and OA patients, there are still doubts about its efficacy. This study was designed to evaluate the efficacy of topical application of black mud in reducing the inflammatory parameters of 3 different models of experimental arthritis. It is important to emphasize the importance of employing experimental models to access the efficacy of mud and exclude the influence of medication and psychological factors. For this purpose we chose different experimental models, which partially resemble arthritis in humans. Here we presented the first evidence that treatment with Brazilian Black mud is capable of impairing cartilage destruction and suppressing cellular infiltration in the synovial membrane.

AIA in the seventh day$^{27,32}$ is characterized as an inflammatory response of the sub chronic phase. Our results after topical application of mud or water show that both treatments were ineffective in impairing the cell influx into the articular cavity compared with untreated rabbits. These results suggested that mud was not efficient on this model of inflammation mainly in consequence of the short duration of the treatment.

The Zy-IA previously described by Gegout et al$^{33}$ presents progressive synovitis with articular deformity and has been employed as a model of chronic arthritis. In agreement with these findings, 21 days after challenge we verified that mononuclear cells predominated in the synovial infiltrate. At
this time, mud but not water significantly reduced the influx of both polymorphonuclear and mononuclear cells into the articular cavity, suggesting a time-dependent action. Traditionally, in human treatment, a cycle of twelve applications is used on the basis of patients’ reported relief from pain\textsuperscript{8,18}. In addition, our results showed that mud treatment was not temperature-dependent as proposed by Bellometti & Galzigna\textsuperscript{16}. Our experimental evidence that heat does not interfere with the beneficial effects of mud therapy is consistent with Basili et al\textsuperscript{34} who speculated that mud-pack treatment might counteract the heat-stress-related effects on platelet and endothelial cell function. In their studies, plasma samples from healthy volunteers subjected to a cycle of 12 daily mud-pack applications showed no changes in pro inflammatory cytokines (TNF-\(\alpha\), IL-1\(\beta\)) or adhesion molecules (sP-selectin, sE-selectin and sVCAM). Furthermore, a decrease of IL-6 levels was correlated with the impairment of neutrophil infiltration, suggesting that hot mud-pack does not induce cellular or endothelial activation \textit{in vivo}.

Regarding the mechanism of the anti-inflammatory action, a recent study involving adjuvant-induced arthritis in rats reported a significant reduction of paw volume with a correspondent reduction in the serum levels of TNF-\(\alpha\) and IL-1\(\beta\)\textsuperscript{17}. Similar results were verified in humans subjected to whole body immersion in mud at 42\(^{\circ}\)C/20 min followed by a bath at 37-38\(^{\circ}\) C\textsuperscript{18}. Together these studies suggested that an initial expression of TNF-\(\alpha\) induced the expression of other proinflammatory mediators\textsuperscript{35}. So in patients treated with mud, the decrease of this cytokine could be associated with a reduction of LTB\textsubscript{4} and PGE\textsubscript{2} serum levels that occur simultaneously with pain
amelioration\textsuperscript{19}. IL-1, IL-8 and LTB\textsubscript{4} are potent chemoattractant for leukocytes, and a reduction in their levels at the site of inflammation could be responsible by the observed impairment of cell influx detected here.

We observed the destructive changes typical of RA in the cartilage and synovium of Zy-IA rats. The synovium from the untreated and water-treated groups showed clear signs of synoviocyte hyperplasia such as the predominance of monocytes and neovascularization. The surface of the synovium from arthritic joints treated with mud exhibited a single cell-layer of synoviocytes, despite a slight vascular proliferation and subsynovial cellular infiltration as evidenced by Mankin’s grade. This persistent synovitis can reflect on cartilage degradation, by inhibition of proteoglycan and collagen synthesis, mainly during the acute phase of arthritis\textsuperscript{36}. In this stage, a high neutrophil infiltration with release of litic enzymes and nitric oxide both with potent action on cartilage can be noticed. In control rats, the cartilage damage was characterized by matrix clefts, loss of condhrocytes, and a decrease in the amounts of collagen and glycosaminoglican. Excluding glycosaminoglican content, hot mud application stimulated both hipercellularity and collagen synthesis. It is known that collagenases act mainly on collagens I, II and III while gellatinases degrade collagens and proteoglycans\textsuperscript{37} in response to IL-1 and TNF-\textgreek{z} secreted by synoviocytes and chondrocytes\textsuperscript{38} suggesting that mud therapy can have a possible inhibitory effect on different metalloproteinases.

Similar histological results were observed in the cartilage of rabbits with OA. In the fourteenth week after menisectomy, cartilage from untreated
and water-treated animals presented intense cellular disorganization, significant loss of condhroocytes and less staining for collagen and GAG. In the cartilage from mud-treated joints we observed preservation of the number of condhroocytes, of the structural organization of the tissue and hipercellularity. The increased staining of collagen observed in both arthritic and osteoarthritic cartilage from mud-treated animals may possibly represent a tentative of tissue repair. These histological observations are in agreement with Bellometti et al.\textsuperscript{18} which have reported a decrease in the levels of TNF-\textalpha and a significant increase in IGF-1 after mud treatment, which may act as an extracellular inhibitor of metalloproteinases and a stimulator of proteoglycan synthesis. Additionally, Bellometti et al.\textsuperscript{39} have demonstrated that mud therapy combined with exercising is effective in increasing anabolic parameters of bone metabolism and decreasing bone resorption and pain through regulation the main proinflammatory cytokines.

Taken together, our results demonstrate a beneficial effect of the Brazilian Black mud in the amelioration of arthritis, with possible induction of cartilage tissue repair. Much work remains to be done to demonstrate the mechanism by which mud attains this anti-inflammatory effect. Nevertheless, the effects of Black mud on inflammation have been demonstrated. In the future Black mud may potentially be widely used as a complement to regular therapy to treat pain and inflammation resulting from arthritis.
ACKNOWLEDGMENTS

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Figure 3

A

Score of hyperplasia increased vascular proliferation and infiltrating cells. Cell counts are expressed as percentages (number of points overlying the cells divided by total number of points overlying tissue). Results are expressed as means ± s.e.m. *p < 0.05 vs control; # p<0.05 vs warm water control

<table>
<thead>
<tr>
<th>Histological Parameters</th>
<th>Control (untreated) (n=10)</th>
<th>Water Treated (n=10)</th>
<th>Mud Treated (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperplasia</td>
<td>2.8 ±0.08</td>
<td>2.6 ± 0.18</td>
<td>1.73 ± 0.23**#</td>
</tr>
<tr>
<td>Number of vessels</td>
<td>2.4 ± 0.23</td>
<td>2.3 ± 0.24</td>
<td>1.73 ± 0.22**#</td>
</tr>
<tr>
<td>Cellular infiltration</td>
<td>2.8 ± 0.11</td>
<td>2.55 ± 0.17</td>
<td>1.82 ± 0.18**#</td>
</tr>
<tr>
<td>Cell counts</td>
<td>25.21 x 10^{-2} ± 1.52</td>
<td>21.06 x 10^{-2} ± 2.67</td>
<td>13.8 x 10^{-2} ± 2.13**#</td>
</tr>
</tbody>
</table>
Table I – Histological evaluation of the joint of Zy-IA rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Untreated (n=4)</th>
<th>Water-treated (n=5)</th>
<th>Mud-treated (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Count points</td>
<td>6.65 ± 0.65</td>
<td>5.4 ± 0.65</td>
<td>8.1 ± 0.92*#</td>
</tr>
<tr>
<td>Structure</td>
<td>4.92 ± 0.43</td>
<td>5.27 ± 0.24</td>
<td>3.5 ± 0.73*#</td>
</tr>
<tr>
<td>Cells</td>
<td>1.67 ± 0.30</td>
<td>2.07 ± 0.22</td>
<td>1.42 ± 0.34</td>
</tr>
<tr>
<td>Alcian blue staining</td>
<td>2.75 ± 0.28</td>
<td>2.89 ± 0.16</td>
<td>2.5 ± 0.51</td>
</tr>
<tr>
<td>Masson/Picro-sirius</td>
<td>2.17 ± 0.096</td>
<td>2.67 ± 0.23*</td>
<td>2.08 ± 0.16*#</td>
</tr>
<tr>
<td>Tidimark</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
</tbody>
</table>

Mankin’s score of whole joint. Cell counts are expressed as percentages (number of points overlying the cells divided by total number of points overlying tissue). Results are expressed as means ± s.e.m. *p < 0.05 vs untreated control; # p<0.05 vs water control.
Mankin’s score of OA cartilage. Count point is expressed as cells/ m² of tissue area. Results are expressed as means ± s.e.m. *p < 0.05 vs control; # p < 0.05 vs water control
FIGURE LEGENDS

Fig. 1. Total and differential leukocyte counts assessed in the joint wash from AIA rabbits. Untreated group (n=6), warm water-treated group (n=8) and mud treated group (n=8). PMN = polimorphonuclear, MN = mononuclear. Data are expressed as mean ± s.e.m.

Fig. 2. Total and differential leukocyte counts in the joint wash of rats with ZyIA. Untreated group (n=10), warm water-treated group (n=10) and mud treated group (n=10). PMN = polimorphonuclear, MN = mononuclear. Data are expressed as mean ± s.e.m. * =p < 0.05 vs control; # = p < 0.05 vs warm water.

Fig. 3. Panel B. Synovial samples from rat knee with zymosan stained with H&E. The synovium from the untreated group shows sinovitis (B1) with persistent monotypic infiltration and synovial hyperplasia. Sub-synovial infiltration in samples obtained from mud-treated rats (B2) and maintenance of the synovial lining. Original magnification X100. Arrows → show vessels; ←→ show fibrous and ←→ show cellular infiltration.

Fig. 4. Joint from rat knee with zymosan. Masson trichrome-staining of untreated animals indicates cartilage erosion with development of fibrous cartilage, overgrown pannus and cartilage and subchondral bone damage (A), compared with the mud-treated group (B). Collagen content shown in joint from untreated (C) and mud-treated (D) animals by Picro-sirius staining. Alcian blue-staining shows proteoglycan loss in cartilage from untreated animals (E) and in samples from animals subjected to mud treatment (F). Original magnification X100.
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