Anti-arthritic Effect of Indian Red Scorpion (Mesobuthustamulus) Venom in Freund’s Complete Adjuvant and Collagen Type II Induced Arthritis

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Abstract

Aim of the present study was to observe the effect of Indian red scorpion venom (SV) in the treatment of Freund’s complete adjuvant (FCA) induced arthritis and collagen type II (CII) induced arthritis models in rats. The immunosuppressant action of SV was confirmed by hemagglutination antibody titre. The further results also suggest that SV produce analgesia in both developing and developed phases of arthritis when evaluated on various pain perception models. SV significantly reduced the paw edema volume and ankle joint diameter in arthritic paw of rats. The altered hematological and biochemical parameters were brought to normal by SV in arthritic conditions when tested on experimental animals. Further anti-arthritic activity of SV was confirmed from the histopathological and radiological findings.

Keywords: Indian red scorpion (Mesobuthustamulus) venom; Hemagglutination antibody titer; Freunds complete adjuvant induce arthritis; Collagen type II induce arthritis; Pain parameters

Abbreviations: RA: Rheumatoid Arthritis; SV: Scorpion Venom; ip: intraperitoneal; SGPT: Serum Glutamate Pyruvate Transaminase; SGOT: Serum Glutamate Oxaloacetate Transaminase; FCA: Freunds Complete Adjuvant; CII: Collagen type II; CIA: Collagen type II Induced Arthritis

Introduction

Rheumatoid arthritis (RA) is a chronic, progressive inflammatory polyarticular joint disease predominantly those of the fingers and toes and systemic autoimmune disease [1]. It is characterized by inflammation of synovial cells in multiple joints, which ultimately lead to the cartilage damage, bone erosion and causes further changes in joint integrity [2]. Although non-steroidal anti-inflammatory drugs (NSAIDS) are widely used, there are currently only few disease-modifying antirheumatic drugs (DMARDS) that can modify the progression of the disease [3,4]. These drugs are having severe side effects associated with clinical treatment of RA which limit their chronic use [5-7]. A major problem in the development of rational treatment strategies is that the disease mechanisms and genetic factors remain unknown [8]. Therefore there is need of new and more effective drugs which acts on root cause of RA with minimum side effects.

Traditionally venoms are used from thousands of years to treat pain, inflammation and arthritis. In Ayurveda “SuchikaVoron” and “Shodhona” was practised against pain [9]. Scorpion peptides possess both toxic and therapeutic action; they release the neurotransmitters by acting on sodium and potassium channels [10]. Scorpion extracts both toxic and therapeutic action; they release the neurotransmitters

Materials and Methods

Animals

Animal were purchased from the National Institute of Biosciences, Pune, and Maharashtra, India. Experiments were performed on healthy albino Wistar rats (180-200 gm) and Swiss albino mice (20-25 gm) of either sex used for the present study. The animals were housed in well ventilated cages maintained under standard condition of light, temperature and humidity (Under a 12 hrs light/dark cycle; 25 ± 30°C; 35-60% humidity) and were fed with standard diet and water ad libitum. The care, use and experimental procedures were conducted in accordance with guidelines and procedures for animal experimentation as prescribed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) (Ref: MCP/IAEC/25/2011).

Chemicals

Freund’s complete adjuvant and collagen type II was procured from Sigma-Aldrich. Marketed preparations of Indomethacin and Methotrexate were used. All other chemicals and reagents used were of analytical grade procured from SRL (India), E. Merck (India).

Scorpion venom

Scorpion venom was obtained from Premium serum, Narayangaon, Pune, Maharashtra, India and was preserved at 4°C. The venom was

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weighed and dissolved in phosphate buffer before use. All the venom concentrations were expressed in terms of dry weight.

**Dose selection**

Starting treatment with minimum dose of SV, 1 µg/200 gm, 2 µg/200 gm in rats was found to be effective. No mortality was observed with this treatment.

**Hemagglutination antibody titre**

Animals were divided in 3 groups of 5 mice each.
- **Group I**: Control (saline, i.p.)
- **Group II**: Standard (Methotrexate, 0.5 mg/kg, i.p.)
- **Group III**: Test (SV, 0.6 µg/20g mice, i.p.)

The animals were immunized by injecting 1X108 sheep red blood cells (SRBCs) intraperitoneally (zero day). The SV and methotrexate were administered to all the animals from day 0 to day 7 as shown above. Blood samples were collected from individual animals of all the groups by retro orbital route on day 7 and serum was separated. Antibody levels were determined by the haemagglutination titre technique [15].

**Freund's complete adjuvant induced arthritis**

Freund's complete adjuvant (FCA) was prepared by suspending in liquid paraffin. The arthritis was induced by a single intradermal injection of 0.1 ml of FCA into the left hind metatarsal footpad of rat [16]. FCA produced definite edema within 24 h with progressive arthropathies by day 9 after inoculation. In prophylactic model animals were treated from day 0 to 28 days, while in therapeutic model animals were treated from day 0 to day 28.
- **Group I**: Control (without treatment)
- **Group II**: Arthritic control (Disease control)
- **Group III**: Standard (Indomethacin i.p.)
- **Group IV**: SV (2 µg/ 200 gm rat, i.p.)

After the injection of CII the paw volume, ankle joint diameter, arthritic index, motility test, stair climb ability test, dorsal flexion pain test for the all animal groups was measured at 0, 9, 18, 27 and 36 day. The X-ray of arthritis induced legs of the experimental rats were taken on day 18 and day 36 and examined for the soft tissue swelling, bony erosions and narrowing of the joints spaces. On 37th day, at the end of experiment, all the animals were sacrificed by cervical decapitation and the blood was collected in plain and EDTA containing tubes, respectively for plasma or serum separation. The plasma or serum samples were subjected to examination like RBC count, WBC count and Hb count. Determination SGOT, and SGPT were done. For histopathology, proximal interphalangeal joints were removed, washed with saline and stored in 10% formalin. Mast cell degranulation was also studied.

**Evaluation parameters**

Paw volume and Ankle joint diameter are measured by digital vernier calliper.

**Arthritic index Score**

0 - No sign; 1 - Redness without edema; 2 - Redness with mild edema; 3 - Redness with severe edema; 4 - Redness, severe edema and stiffness in movement [10].

**Motility test score**

The motility pattern of the rats were observed for a period of 5 minutes and scored 0, if the rat walked easily; 1, if the rat walked with little difficulty, with toe touching to the floor; 2, if the rat walked with difficulty and avoided touching the toes of the inflamed paw to the floor [18].

**Stair climb ability test**

Overnight fasted animals were trained for one week to climb a staircase with steps at 5, 10, and 15 cm having water at the second and food at the third step. Climbing ability of the rats in above groups was scored 0 if the rats did not climb; 1, if the rats climbed onto step 1; 2, if the rats climbed onto steps 1 & 2; 3, if the rats climbed all the three steps [18].

**Dorsal flexion pain test**

The rat was held comfortably and the left hind paw was gently flexed 5 times at 5-s intervals, in these conditions a squeak or withdrawal of leg could be elicited; pain was Scored: 0 – when rat showed no squeaking and no withdrawal of leg; 1 - Either squeaking or withdrawal of leg; 2 - Both squeaking and withdrawal of leg [19].
Statistical analysis

All the data are presented as mean ± SEM (n=6). The obtained data was subjected to one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparisons test. A value of p<0.05 was considered statistically significant compared with the respective disease control.

Results

Hemagglutination antibody titer

SV and standard treated groups significantly reduced the no. of wells agglutination (p<0.01) as compared to control group (Table 1).

Paw volume and ankle joint diameter in FCA and CIA induced arthritis

In the FCA and CIA induced arthritis there were a gradual increased in the edema paw volume in the disease control group. In standard and SV treated groups (prophylactic and therapeutic treatment) showed significant (p<0.05) decreased in edema paw volume. FCA and CIA induced arthritis, causes increased in ankle joint diameter in disease control group throughout the experimentation period. There was a significant (p<0.05) decrease in ankle joint diameter of standard and SV treated groups (Table 2 and 3).

Pain perception parameters in FCA and CIA induced arthritis

The arthritic index score, motility test score and dorsal flexion test score were reduced significantly (p<0.05) and stair climb ability 33 was increased significantly (p<0.05) by standard indomethacin treated groups and SV treated groups when compared to disease control group (Tables 4 and 5).

Effect of SV (1 and 2 µg/200 gm) on paw volume and ankle joint diameter in FCA induced arthritis.

Effect of SV (2 µg/200 gm) on paw volume and ankle joint diameter in CIA induced arthritis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean paw volume ± SEM</th>
<th>Mean ankle joint diameter ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>7.89 ± 0.18</td>
<td>7.93 ± 0.30</td>
</tr>
<tr>
<td>STD (P)</td>
<td>6.72 ± 0.21*</td>
<td>6.83 ± 0.25*</td>
</tr>
<tr>
<td>SV1 (P) (1µg/200gm)</td>
<td>7.04 ± 0.26*</td>
<td>6.9 ± 0.21*</td>
</tr>
<tr>
<td>SV2 (P) (2µg/200gm)</td>
<td>7.54 ± 0.11</td>
<td>6.91 ± 0.25*</td>
</tr>
<tr>
<td>STD (T)</td>
<td>7.75 ± 0.08</td>
<td>8.18 ± 0.21</td>
</tr>
<tr>
<td>SV1 (T) (1µg/200gm)</td>
<td>7.55 ± 0.14</td>
<td>8.32 ± 0.31</td>
</tr>
<tr>
<td>SV1 (T) (2µg/200gm)</td>
<td>7.51 ± 0.24</td>
<td>7.05 ± 0.21</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± SEM (n=5); **p<0.01 vs. Control group, Data analysed by One-way ANOVA test followed by Dunnet’s multiple test for comparison. Treatment of various group are as follows, C: Control, STD: Indomethacin, SV: Scorpion Venom.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean paw volume ± SEM</th>
<th>Mean ankle joint diameter ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>9.42 ± 0.5</td>
<td>9.74 ± 0.3</td>
</tr>
<tr>
<td>STD</td>
<td>8.02 ± 0.3</td>
<td>8.54 ± 0.3</td>
</tr>
<tr>
<td>SV (2 µg/ 200gm)</td>
<td>8.45 ± 0.4</td>
<td>8.44 ± 0.5</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± SEM (n=6); *p<0.05, **p<0.01 vs. Disease control group, Data analysed by One-way ANOVA test followed by Dunnet’s multiple test for comparison. Treatment of various group are as follows, DC: Disease Control, STD: Indomethacin, SV: Scorpion Venom, P: Preventive treatment, T: Therapeutic treatment.

Table 2: Effect of SV (1 and 2 µg/200 gm) on paw volume and ankle joint diameter in FCA induced arthritis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Arthritic index score ± SEM</th>
<th>Motility test score ± SEM</th>
<th>Dorsal flexion Score ± SEM</th>
<th>Stair climb ability Score ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC</td>
<td>2.83 ± 0.1</td>
<td>2.03 ± 0.1</td>
<td>2.75 ± 0.2</td>
<td>2.16 ± 0.1</td>
</tr>
<tr>
<td>STD (P)</td>
<td>1.33 ± 0.2</td>
<td>2.0 ± 0.0</td>
<td>1.83 ± 0.1</td>
<td>1.93 ± 0.1</td>
</tr>
<tr>
<td>SV1 (P) (1µg/200gm)</td>
<td>2.0 ± 0.0</td>
<td>1.83 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>SV2 (P) (2µg/200gm)</td>
<td>1.83 ± 0.1</td>
<td>2.0 ± 0.0</td>
<td>1.66 ± 0.2</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>STD (T)</td>
<td>2.33 ± 0.2</td>
<td>2.83 ± 0.3</td>
<td>2.75 ± 0.2</td>
<td>2.16 ± 0.1</td>
</tr>
<tr>
<td>SV1 (T) (1µg/200gm)</td>
<td>2.5 ± 0.2</td>
<td>2.83 ± 0.3</td>
<td>2.0 ± 0.0</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>SV2 (T) (2µg/200gm)</td>
<td>2.6 ± 0.2</td>
<td>2.83 ± 0.3</td>
<td>2.0 ± 0.0</td>
<td>1 ± 0</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± SEM (n=6); *p<0.05, **p<0.01 vs. Disease control group, Data analysed by One-way ANOVA test followed by Dunnet’s multiple test for comparison. Treatment of various group are as follows, DC: Disease Control, STD: Indomethacin, SV: Scorpion Venom, P: Preventive treatment, T: Therapeutic treatment.

Table 3: Effect of SV (2 µg/200 gm) on paw volume and ankle joint diameter in CIA induced arthritis.

Table 4: Effect of SV (1 and 2 µg/200gm) on arthritic index and pain perception parameters in FCA induced arthritis.
compared with the disease control (Figure 1).

absence of edema formation and normal bone marrow. Degeneration of and therapeutic SV treated groups showed less inflammatory signs like scanty cellular infiltrate, mild edema formation. The prophylactic group rat’s joint showed prominent abnormalities from the normal joint like edema formation, degeneration with erosion of the cartilage and extensive infiltration of inflammatory exudates in the articular surface. Standard and SV treated groups showed less inflammatory signs like scanty cellular infiltrate, mild edema formation and normal bone marrow. Degeneration of the ankle joint was less

in the drug treated groups when compared with the disease control (Figure 2).

Haematological parameters

Standard and SV treated groups significantly (p < 0.05) increased Hb count and RBC count with significant (p<0.01) reduction in WBC count when compared to disease control group. SGOT and SGPT levels where significantly (p<0.05) reduced in the standard and SV treated groups when compared to disease control group (Tables 6 and 7).

Histopathological findings

FCA induced arthritis: Slide (A) showed the histopathology of normal ankle joint. Slide (B) FCA disease control arthritic rat joint showed prominent abnormalities from the normal joint like edema formation, degeneration with erosion of the cartilage and extensive infiltration of inflammatory exudates in the articular surface. The prophylactic, therapeutic and standard group rat's joint showed normal bone marrow with less cellular infiltration. The prophylactic and therapeutic SV treated groups showed less inflammatory signs like absence of edema formation and normal bone marrow. Degeneration of the ankle joint was not observed in any of the drug treated groups when compared with the disease control (Figure 1).

CII induced arthritis: Slide (A) showed the histopathology of normal ankle joint. Slide (B) the disease control arthritic rat joint showed prominent abnormalities from the normal joint like edema formation, degeneration with mild erosion of the cartilage, destruction of bone marrow and extensive infiltration of inflammatory exudates in the articular surface. Standard and SV treated groups showed less inflammatory signs like scanty cellular infiltrate, mild edema formation and normal bone marrow. Degeneration of the ankle joint was less
In CII induced arthritis, disease control group showed mast cell degranulation while standard and SV treated group prevents mast cell degranulation (Figure 5).

### Discussion

RA is a chronic inflammatory polyarticular joint disease. It is characterized by synovial proliferation, systemic and local inflammation resulting in cartilage and bone loss [20,21]. Here an attempt was made to investigate its anti-arthritic activity of Indian red scorpion venom.

Haemagglutination titer was performed to check the immunomodulatory activity of SV. Antibody molecules, a product of B-lymphocytes and plasma cells, are central to humoral immune responses; IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonization, neutralization of toxins etc [15]. SV showed a significant inhibition of antibody responses by decrease in haemagglutination titer, this proves that SV possesses immunosuppressant action.

SV significantly reduces the pain, inflammation and also acts as an immunosuppressant and the major manifestation of RA are mainly pain, inflammation, and swelling. Hence further the anti-arthritic activity of SV was evaluated by FCA and CII induced arthritis.

The hind paw volume and bone destruction have been reported to be associated with the accumulation of leukocytes in the arthritic joint fluid and their secreted products. Paw swelling is an index of measuring the antiarthritic activity of various drugs [22] and it is employed here to determine the activity of SV at the dose level 1 & 2 µg/200 gm rat. SV administered in prophylactic and therapeutic groups showed marked reduction in paw volume when compared with the disease control group. SV inhibited the increase in the hind paw volume and bone destruction in the FCA and CII induced arthritic rats. These findings suggest that SV suppresses the migration and accumulation of leucocytes to inflamed area of arthritic rats, due to its immunosuppressive effect.
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Figure 2: Histopathology of rat paw in CII induced arthritis. (A) Normal control, (B) Disease control, (C) STD (Indomethacin), (D) SV (Scorpion Venom – 2 µg/200 gm).

Figure 3: Radiological assessment of rat paw in FCA induced arthritis. (A) Normal control, (B) Disease control-Day 14, (C) Disease control-Day 28, (D) STD(P)-Day 14 (Indomethacin), (E) STD(P)-Day 28 (Indomethacin), (F) STD(T)-Day 14 (Indomethacin), (G) SV(P)-Day 14 (1 µg/200 gm Scorpion venom), (H) SV(P)-Day 28 (1 µg/200 gm Scorpion venom), (I) SV(P)-Day 14 (2 µg/200 gm Scorpion venom), (J) SV(P)-Day 28 (2 µg/200 gm Scorpion venom), (K) SV(P)-Day 28 (2 µg/200 gm Scorpion venom), (L) SV(T)-Day 28 (2 µg/200 gm Scorpion venom).
Figure 4: Radiological assessment of rat paw in CII induced arthritis. (A) Normal control, (B) Disease control-Day 18, (C) Disease control-Day 36, (D) STD-Day 36 (Indomethacin), (E) SV-Day 36 (2 µg/200 gm Scorpion venom).

Figure 5: Effect of SV (2 µg/200 gm) on Mast cell degranulation in CII induced arthritis. (A) Control, (B) STD (Indomethacin), (C) SV (Scorpion Venom – 2 µg/200 gm).
In arthritis IL-10 level decreases [23] IL-10 inhibits macrophage activity as well as IFN γ induced macrophage activation [24]. It has been reported previously that Tityus serrulatus scorpion venom was able to induce an increase in serum levels of IL-10 [25], similar peptides might also be present in Indian red scorpion venom which reduces IL-10 and responsible for its anti-inflammatory activity in FCA and CII induced arthritis.

Several groups have used the arthritic rat as a model for chronic pain and evaluated the hyperalgesia by different means dorsal flexion pain test, stair climbing ability test and mobility test. All 3 of which revealed a rapid onset of hyperalgesia (FCA 14 day and CII 18 day) in disease control groups, followed by a progressive recovery in SV treated groups. Flexion, stair climbing and mobility require movements of joints which were more greatly affected by arthritis, and SV significantly reduced the pain perception over the affected joints.

In this study, the incidence of arthritis increased progressively up to 100% in the disease control group throughout the experiment. In contrast, the mean arthritic index of the therapeutic groups treated with SV increased for day 14 and 18 in FCA and CII respectively, but decreased thereafter till the last day of the experiment. The mean arthritic index of the Prophylactic groups treated with SV in FCA was significantly lower than that of the disease control group from day 7 till day 28.

The observed histopathological changes of proximal interphalangeal joints of the experimental groups reveals that, disease control group of arthritic rats joint showed prominent abnormalities from the normal joint like edema formation, degeneration with partial erosion of the cartilage, destruction of bone marrow and extensive infiltration of inflammatory exudates in the articular surface. The standard (Indomethacin) treated group joint showed normal bone marrow with less cellular infiltrates. Prophylactic SV treated groups had less inflammatory signs like scanty cellular infiltrate, absence of edema formation and normal bone marrow, whereas the therapeutic SV treated groups showed cellular infiltrates on the articular surface with less cartilage destruction in both FCA and CII models. Degeneration of the ankle joint was not observed in any of the SV treated groups when compared with the disease control.

Radiographic changes in RA conditions are useful diagnostic measures which indicate the severity of the disease. Soft tissue swelling is the earlier radiographic sign, whereas prominent radiographic changes like bony erosions and narrowing of joint spaces can be observed only in the developed stages of arthritis [26]. In arthritic rat, soft tissue swellings along with narrowing of the joint spaces were observed which implies the bony destruction in articular condition. The standard (Indomethacin) treated groups have prevented this bony destruction and also there is mild swelling of the joint. Similar to histopathological studies, Prophylactic SV treated had shown significant prevention against bony destruction by showing less soft tissue swelling and narrowing of joint spaces when compared with therapeutic SV treated groups.

In arthritis there is decrease in RBC count and haemoglobin count represents the anemic condition in arthritic rats. The important causes are the abnormal storage of iron in the reticuloendothelial system and synovial tissue and the failure of bone marrow to respond to anemia [27]. The decreased in RBC count and Hb count was restored to normal values in therapeutically treated SV groups in FCA model.

The leukocytes (WBC count) in the peripheral blood increased with the disease development with FCA and CII induced arthritis.

Increased in the leukocyte count in FCA and CII arthritic rats may be due to the stimulation of immune system against the invading antigens. SV significantly decreased peripheral WBC counts when compared to the disease control in both FCA and CII models. Decreased WBC count was mainly due to immunosuppressive effect.

Hence, it can be stated that SV possesses immunosuppressant action. SV able inhibit the release of various inflammatory mediators in RA. SV also inhibits joint bone destruction and deformation in FCA and CII induced arthritis model. Therefore, SV can prove potential therapeutic agent in treating human RA. Isolation and purification of peptides is necessary to develop safer and effective anti-arthritis agent.

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