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Topical Bee Venom Nano-emulsion Ameliorates Serum Level of Endothelin-1 in Collagen-Induced Rheumatoid Arthritis Model

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Abstract

Rheumatoid arthritis (RA) patients suffer from the effects of acute and chronic inflammation, and it can be deteriorated by the production of endothelin-1, which is a pro-inflammatory molecule and participates in the pathogenesis of RA. Bee venom (BV) with low doses plays an anti-inflammatory role in the treatment of RA. Besides, nano-emulsions (NEs) increase topical drug delivery and its effectiveness. This project was designed to explore the effects of nano-emulsions containing bee venom (Top-NEs) on the serum level of endothelin-1 in the rats of the collagen-induced RA (CIA) model. After induction of CIA in Wistar rats and grouping, the serum levels of endothelin-1 were measured in four steps. In healthy rats on day 0, in CIA model before, middle, and at the end of treatment on days 7, 14, and 21, respectively. The results demonstrated that serum levels of endothelin-1 had significantly increased in CIA rats before treatment, and they were more in the middle and the end of treatment about blank and negative control groups. However, they had significantly decreased in the Top-NEs groups in the middle and the end of treatment. Topical NEs containing BV can ameliorate the serum level of endothelin-1 in the CIA model; in addition, they can pass BV through the skin. Also, it appears that endothelin-1 can be considered as the main target for the treatment of RA.

Keywords Bee venom · Endothelin-1 · Nano-emulsion · Rheumatoid arthritis

1 Introduction

Rheumatoid arthritis (RA) is a prevalent autoimmune disease that is associated with chronic synovial inflammation and pain in the joints that could be leading to disabling joint destruction [1, 2]. Several chemokines, cytokines, and pro-inflammatory molecules participate in the pathogenesis of RA [3]. Endothelin-1 as one of them is the main pro-inflammatory molecule that plays a potential role in the induction of inflammation during RA [4, 5]. Endothelin-1 is mainly produced by endothelial cells by the activation of the pro-inflammatory transcription factors such as activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in a dependent manner. It induces inflammation by increasing the production of reactive oxygen species (ROS), nitric oxide (NO), and adhesion molecules [6, 7].

Highlights
- Endothelin-1 can be considered as a main target for the treatment of RA.
- Serum level of endothelin-1 increase in collagen-induced arthritis model.
- Using Bee venom as topical nano-emulsions can improve rheumatoid arthritis by prevent from increase serum level of endothelin-1.

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The main aim of the therapeutic strategies is to decrease inflammation and pain [8]. The current therapeutic drugs, such as dexamethasone, are associated with several side effects, including postoperative infection, increased glucose, impaired immune responses, and delayed wound healing [9]. Accordingly, recent investigations are directed to find the effectiveness of local drugs to overcome the side effects.

Bee venom (BV) has several immunosuppressive activities and anti-inflammatory properties in a low dose; hence, it has been introduced as a potential therapeutic strategy for the treatment of inflammatory-related diseases especially RA [10–12]. BV contains a variety of peptides such as melittin, adolapin, apamin, and mast cell degranulating and also non-peptide components such as lipids, carbohydrates, and free amino acids [13, 14]. Melittin, which is nearly half of BV, in high dose causes adverse effects and in low dose produces anti-inflammatory effects [10, 15]. For this purpose, bee venom therapy is performed by live bee stings or injection of BV both of which could have adverse effects, such as pain, itching, or swelling [16].

Nanotechnology is an advanced strategy to increase the effectiveness and topical delivery of the drugs [17]. Nanoemulsions (NEs) are homogeneous systems with mean droplets size less than 100 nm which are prepared by uniform dispersion of at least two immiscible liquids together by surfactants [18]. One of the ways to prepare them is "low-energy methods" that take advantage of the intrinsic physicochemical properties of the components in order to generate droplets, and this process only consists of mixing the liquid phases at room temperature [19]. NEs can be used as drug delivery systems with multiple features including protection of drug content from enzymatic degradation, carrying both hydrophilic and hydrophobic components in a single formulation, increasing the bioavailability and drug loading, and controlling the drug release [20]. Therefore, they can be used as good strategies for topical/transdermal delivery [21].

It has been hypothesized that the topical NE containing BV (Top-NE) may be associated with increased anti-inflammatory properties after passing the BV through the skin as topical/transdermal delivery. Therefore, this project has been designed to evaluate the effects of topical NEs containing BV on serum level of endothelin-1 in the collagen-induced rheumatoid arthritis (CIA) rat models which can be considered the main target for the treatment of RA.

2 Material and Methods

2.1 Chemicals

BV was prepared from healthy and approved hives, *Apis mellifera* strain, by electrical stimulation using protocol suggested by Benton et al. [22]. Sorbitan monooleate (Span-80) and polyoxyethylene 20 sorbitan monooleate (Tween-80) as surfactants were purchased from Merck Chemicals (Germany), olive oil was from Fadak Co. (Iran). Incomplete Freund's adjuvant (IFA) (Sigma-Aldrich, USA), Bovine type II collagen (Xi'an Harmonious Natural Bio-Technology Co., Ltd, China), and Elisa kit of Endothelin-1 (Karmania Pars Gene Co., Iran) were purchased.

2.2 Animals

In this project, 54 Wistar male rats, weighing 200–250 g, were used, which were kept in the Standard Laboratory Animal Guidelines, and the experimental protocol was approved by Rafsanjan University of Medical Sciences Ethical Committee with number IR.RUMS.REC.1398.181.

2.3 Methods

2.3.1 Experimental Groups

This study was performed on 9 groups, including blank (CIAs without any treatment), negative control (topical-treated CIAs with NE without BV), positive control (subcutaneous treated CIAs with dexamethasone 500 μg/kg/day), Top-BV 150 (topical treated CIAs with an aqueous solution containing BV 150 μg/ml), and 5 groups Top-NEs (topical treated CIAs with NEs containing BV150, 75, 37.5, 18.75, and 9.37 μg/ml). Accordingly, treatment began after the maximum inflammatory symptoms started on day 7 for 2 weeks. Topical treatments were done as 5 min massage of rat's paw by 1 ml NEs or BV solution. Also, subcutaneous treatment was injected between two shoulders on the back of rats by 0.1 ml dexamethasone.

The blood samples were collected in 4 steps. On day 0 as an indicator of the healthy rats (1 sample per group, total 9 samples), on day 7 as an indicator of the maximum inflammation CIA and before treatment (1 sample per group, total 9 samples), on day14 as the middle of treatment (6 samples per groups, total 54 samples), and day 21 as the end of treatment (6 samples per groups, total 54 samples). The blood samples were collected in the non-treated anti-coagulant agents and centrifuged (3000 rpm, room temperature, 10 min) to separate the serums, and were kept at − 20 °C for evaluation of the serum levels of endothelin-1.

2.3.2 Preparation of Collagen-Adjuvant Emulsion

To prepare the collagen-adjuvant emulsion, type II collagen was dissolved at 4 mg/ml in acetic acid (0.05 M) by gently stirring overnight at 4 °C and then by using a high-speed homogenizer with a small blade to emulsify the Incomplete Freund's adjuvant with the collagen solution in equal volumes for 30 min in an ice water bath [23].

2.3.3 Induction of CIA

To create CIA, 0.1 ml prepared emulsion was subcutaneously injected into the footpad (as the starter of inflammation) and 0.1 ml was subcutaneously injected into the base of the tail (as amplification of immune system response) [24–26]. Maximum inflammation was seen on day 7 and then treatment started for 2 weeks up to day 21.

2.3.4 Preparation of Top-NEs

A BV stock solution with a concentration of 5000 μg/ml was prepared by solving the BV powder into distilled water with stirring for 1 h at 25 °C and filtered by a 0.22 micron syringe filter. Then, other solutions were diluted (i.e., 2500, 1250, 625, and 312 μg/ml) as an aqueous phase (Aq) for the preparation of NEs.

For the preparation of NEs, 30% surfactant (14% Span-80 and 16% Tween-80) was added to a tube which was on the stirrer 1000 rpm, and RT (MS-300HS, Protraction Intertrade Co., Korea). After 5 min, 3% Aq was added and mixed thoroughly. Finally, 67% olive oil was added to achieve a clear solution approximately after 5 min. This process was repeated for the preparation of all NEs which included different concentrations of BV according to 3% Aq and diluted BV (i.e. 150, 75, 37.5, 18.75, 9.37, and 0 μg/ml).

NEs were assessed for physical stability and thermal stress analyses in three steps included centrifugation (3500 rpm, 30 min, 25 °C), three freeze-thaw cycles (12-h storage at temperature − 20 and then 25 °C), and three heating-cooling cycles (12-h storage at temperature 4 and then 45 °C). Tubes were examined by macroscopic observation for phase separation [27, 28].

2.3.5 Characterization of NEs

The mean size of droplets of NEs and their polydispersity index (PDI) were measured by dynamic light scattering (DLS) at a scattering angle of 90° using Scatteroscope (K-one Ltd. Korea). Viscosity (cP) of NE free BV was measured using a modular compact rheometer (Physica-MCR300, Anton Paar GmbH, Austria) with measurement of shear stress as a function of the shear rate from 0.1 to 100 s^{-1}. Also, its refractive index was measured using an Abbe refractometer (Bausch and Lomb Optical Company, USA). All measurements were at room temperature.

2.3.6 Evaluation of Serum Levels of Endothelin-1

Serum levels of endothelin-1 were measured using the Elisa technique and based on the manufacturing guidelines. Briefly, serums and standards were added to the wells and were incubated for 1 h. Then, the wells were washed and the detection antibody was added and was incubated for 1 h. After re-washing, HRP-Avidin was added with incubation for 30 min. Washing was done again, and substrate was added with incubation without light. Reactions were stopped by acidic solution after 15 min. The optical densities of the wells were read using an ELISA reader (BMG Co. China) at 450 nm in front of the 630-nm reference filter. The concentrations of endothelin-1 were obtained from the diagram of the concentration versus absorption.

2.4 Statistical Analysis

Serum levels of endothelin-1 were analyzed using ANOVA and following the TUKEY test under SPSS software version 16. The differences with p value < 0.05 were considered significant, and finally the analyzed data were reported as mean ± standard error.

3 Results

Viscosity and refractive index of NE without BV were obtained at 172 (cP) and 1.463, respectively. Figure 1 shows the mean droplets size and PDI of NEs containing different concentrations of BV. It shows with the increase of BV concentration in the Aq phase from 0 to 150 μg/ml, size has increased from 11.1 to 29.0 nm and PDI from 0.140 to 0.170. But there was only a significant difference between NE (150) and NE (0) for both size and PDI.

Serum levels of endothelin-1 are shown in Fig. 2 for day14 and Fig. 3 for day 21. The statistical analysis revealed that serum levels of endothelin-1 have significantly increased in the CIA rat models on day 7 as maximum inflammation and before treatment. These figures indicate that the serum levels of endothelin-1 have significantly increased in blank and

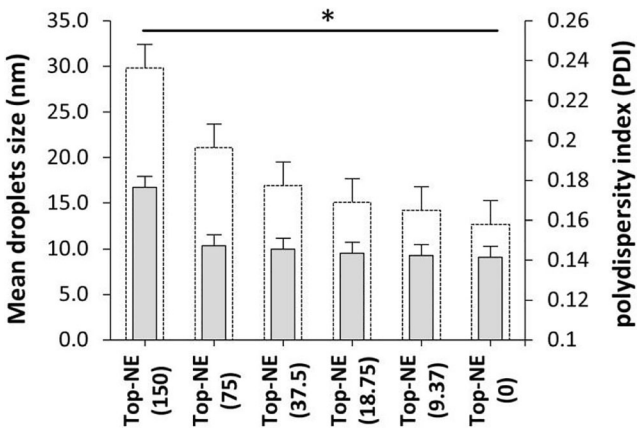

Fig. 1 Mean droplets size according to nanometer (dashed borderline) and polydispersity index (continuous borderline) of NEs containing different BV concentrations (i.e., 150, 62.5, 37.5, 18.75, 9.37, and 0 μg/ml). * shows p value < 0.05

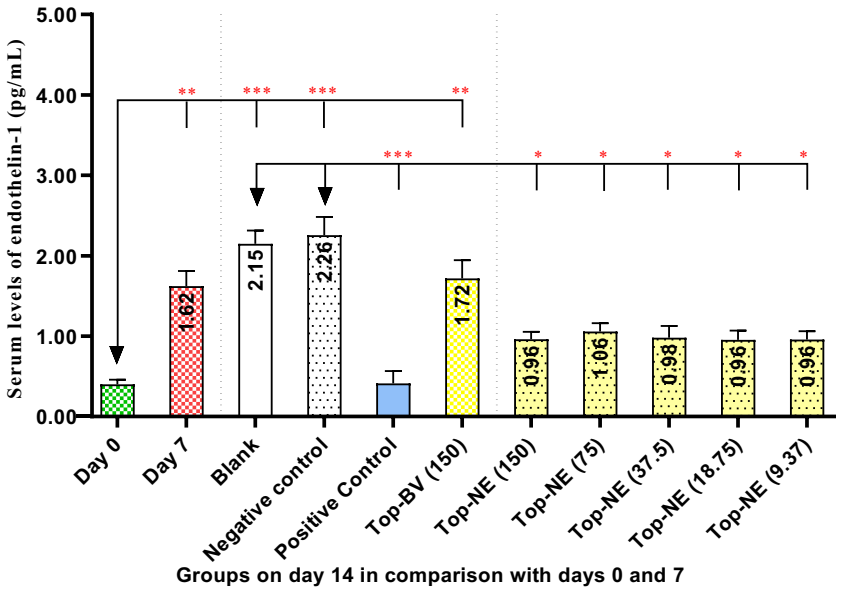

Fig. 2 Mean ± standard error of serum levels of endothelin-1 in the groups on day 14 (in the middle of treatment) in comparison with days 0 and 7. Day 0: Base level of endothelin-1 in healthy rats. Day 7: level of endothelin-1 in collagen-induced arthritis model as maximum inflammation and pre-treatment; other groups are on day 14 including blank (no treatment), negative control (topical treated by nano-emulsion without bee venom (0 μg/ml)), positive control (subcutaneous injection treated by dexamethasone (500 μg/kg/day)), Top-NEs (topical treated by nano-emulsions containing bee venom (150, 75, 37.5, 18.75, and 9.37 μg/ml)), and Top-BV (150 μg/ml) (topical treated by aqueous solution of bee venom (150 μg/ml)). *, **, and *** show p value < 0.05, 0.01, and 0.001, respectively

negative control groups on days 14 (middle of treatment) and more have increased 21 (end of treatment) compared to days 7 (maximum inflammation) and 0 (healthy rats). They show a significant difference between positive control with blank and negative control groups on days 14 and 21. There were significant differences between Top-NE groups with blank and negative control groups on days 14 and 21. Figure 3 presents that there are significant differences between all groups (except positive control) in comparison with the base level of endothelin-1 in healthy rats. The levels of endothelin-1 have increased less in NEs containing BV than in blank and negative control groups, also topical BV (150) on day 21. It follows from both figures that serum levels of endothelin-1 have increased during the CIA model (blank group) from 0.33 to 1.62 (from the base level on day 0 to maximum inflammation level on day 7), from 1.62 to 2.15 (maximum inflammation level on day 7 to middle treatment on day 14), and from 2.15

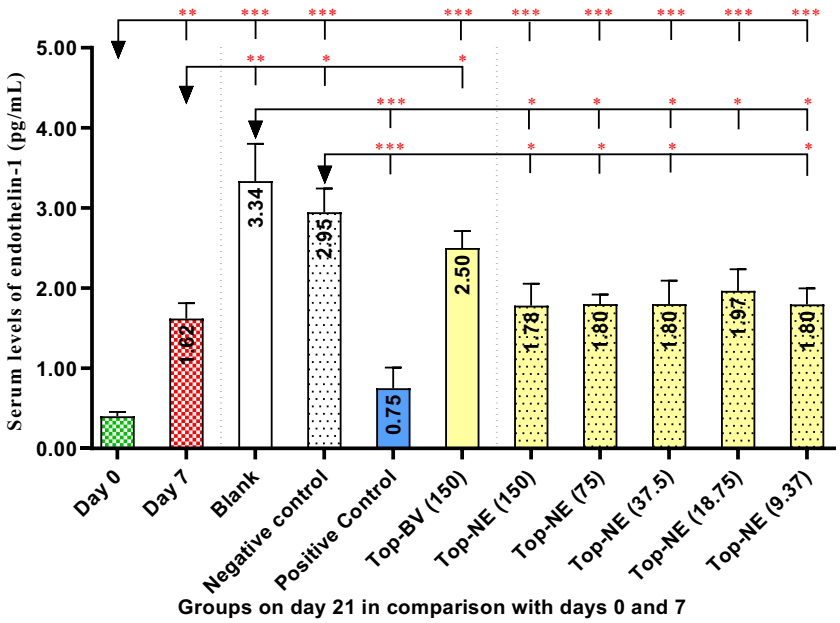

Fig. 3 Mean ± standard error of serum levels of endothelin-1 in the groups on day 21 (at the end of treatment) in comparison with days 0 and 7. Grouping are similar to Fig. 2. *, **, and *** show p value < 0.05, 0.01, and 0.001, respectively

to 3.34 (pg/ml) (from middle treatment on day 14 to at the end of treatment on day 21).

4 Discussion

Based on the results, serum levels of endothelin-1 were significantly increased on day 7 as maximum inflammation in the CIA. During treatment, it stayed high for the blank group on days 14 and 21 and even had increased up to day 21 than days 7 and 14. Thus, it proves that endothelin-1 is the main molecule that participates in the pathogenesis of RA and it is constitutively produced during RA. It has been stated using subcutaneously dexamethasone for treatment that CIA not only can significantly decrease endothelin-1 serum levels but also prove reduction of inflammation during RA. Interestingly, the results demonstrated that CIAs which are topically treated with Top-NE containing BV significantly decreased endothelin-1 serum levels on days 14 and 21. In other words, using topical BV to form nano-emulsion permits that endothelin-1 less increase which can be seen in Figs. 2 and 3 for the topical BV, blank, and negative control groups. Topical solution of BV had no significant effects on the reduction of serum level of endothelin-1 but could reduce it to an extent which may be due to absorption at the site of inflammation and/or maybe because of BV is an anti-inflammatory in very low doses[10, 15]. Also, Based on the results, it may be concluded that BV as nano-emulsion form can regulate production of endothelin-1 in time, while BV as solution form at longer time, which was not seen in the topical treatment. To the best of our knowledge, this is the first experimental study on the effects of BV on the serum level of endothelin-1 in the RA animal models. Furthermore, no investigations were found regarding the effects of BV on the molecule in the human. However, many studies revealed that BV has antioxidant, anti-inflammatory, and immunomodulatory effects on CIAs [29]. BV acupuncture therapy on human RA patients was also associated with a decrease in pain and inflammation [30]. Based on our results, Top-NE can ameliorate RA symptoms via down-regulation of endothelin-1 on days 14 and 21 in a dose-independent manner. Additionally, its aqueous form had anti-inflammatory effects as well as the nano-emulsion, but to a lesser extent. The remarkable results are related to topical using of the BV. As mentioned, the previous investigations have evaluated the injected and acupuncture treatment of BV, which are invasive methods. Using topical nano-emulsion or solution of BV is safe and pleasant with the same results. However, due to the limitations of this project, the authors recommend evaluating other nano-emulsion of BV and also solutions of BV in various doses to treat the RA models.

5 Conclusion

Our study showed that serum levels of endothelin-1 increase after induction RA in collagen-induced arthritis model. BV as topical nano-emulsions or solution can prevent the increase in serum level of endothelin-1 and ameliorate RA.

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Authors' Contributions YY and MA conceived and designed the experiments and wrote the manuscript. YY performed the experiments. MKA analyzed the data. MA, MKA, and AA participated in the design of the study and helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

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Data Availability The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval and Consent to Participate The experimental protocol of animals was approved by Rafsanjan University of Medical Sciences Ethical Committee with number IR.RUMS.REC.1398.181.

Consent for Publication Not applicable.

Competing Interests The authors declare that they have no conflict of interest.

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