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RESEARCH PAPER

Blood and biochemical changes caused by bee venom-nanoemulsions; a study on animal arthritis model

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ABSTRACT

Objective(s): Traditionally, Bee venom (BV) is used through stinging or injection to treat rheumatoid arthritis (RA). This study aimed to assess the side effects of local bee venom nanoemulsions (BV-NEs) in the collagen-induced arthritis (CIA) model by examining biochemical and hematological parameters.

Materials and Methods: The BV-NEs were prepared, and the CIA model was induced in rats. After the seventh day, the groups were locally treated for two weeks as the following: blank (free treatment), negative control (NE-0), positive control (hydrocortisone acetate ointment 1%, 50 mg/day), BV control (37.5 µg/ml/day), and BV-NEs receiving 75, 37.5, 18.75, and 9.37 µg/ml/day. Three steps of blood sampling were done on days 0, 7, and 21 (healthy rats, before treatment, and at the end of treatment, respectively).

Results: The results revealed that blood levels of Glucose, Cholesterol, Urea, Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), White blood cell (WBC), and %Neutrophil significantly increased before the treatment. Nevertheless, most parameters declined at the end of the treatment compared to the blank and negative control groups about BV-NEs dose-dependently. The drastic changes in biochemical parameters in the CIA model indicated the effect of the immune system function on the metabolic system. Also, NE's impact of BV passed through the skin on these items.

Conclusion: BV-NEs can reduce inflammation caused by arthritis without acute adverse effects on the routine biochemical and hematological parameters.

Keywords: *Bee venom, Biochemical markers, Collagen induced arthritis, Hematological tests, Nanoemulsion*

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INTRODUCTION

Nanoemulsions (NEs), as homogeneous systems in nanotechnology, consist of at least two immiscible liquids thoroughly dispersed by a surfactant(s) and have droplets with a mean size of less than 100 nm. They can be prepared in two forms; water-in-oil (W/O) and oil-in-water (O/W) [1, 2]. Enhancing bioavailability and drug loading, controlling drug release, inhibiting enzymatic degradation, and carrying hydrophilic and hydrophobic components in one form, are advantages of NEs as drug delivery systems. Therefore, they can be used as high-efficiency carriers for local and transdermal purposes [3, 4].

Rheumatoid arthritis is an autoimmune disease that is associated with chronic synovial inflammation and pain in the joints. It can lead

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to joint destruction and the patient's inability to move [5]. Collagen-induced arthritis (CIA) is widely used as a model of human rheumatoid arthritis caused by injecting type 2 collagen that indicates both immunological and pathological features. Among the various models, CIA causes multi-joint inflammation, bone proliferation, and cartilage damage with a more specific and faster onset [6]. Although it is highly used in arthritis studies in rats, there is less evidence available for assessing their biochemical and hematological parameters.

Bee Venom (BV) as a biotoxin is which consists of about 18 ingredients including various enzymes (i.e., phospholipases and hyaluronidase), a variety of peptide components (i.e., melittin, apamin, adolapin, and mast-cell-degranulating), non-peptide components (i.e., carbohydrates, lipids, and free amino acids), as well as active amines (i.e., histamine and epinephrine) [7]. BV has various pharmaceutical properties, whose anti-inflammatory and anti-arthritis effects have been attributed to melittin, adolapin, mast cell degranulating peptides, and apamin [8]. It is used to treat rheumatoid arthritis (RA) through stinging or injection in clinics [9].

In our previous study, BV was encapsulated by W/O NEs and passed BV through the skin by topical delivery [10]. These nanoemulsions (BV-NEs) could reduce the inflammation caused by the CIA model in rats without being stung by bees or the injection route [11]. On the other hand, studies have shown the side effects of BV on the body's organs, such as the liver [12], and kidney [13], as well as its effect on blood glucose and lipids [7] or lytic impact on red blood cells [8]. However, there is no pharmacodynamic information about BV local delivery using NEs. Therefore, this study was aimed for two purposes: first, assessment of side effects of BV encapsulated in NEs that pass through the skin on blood and biochemical parameters. Second, investigation of biochemical and hematological changes in the animal model of CIA to complement studies.

MATERIALS AND METHODS

Drugs and chemicals

BV (Apis mellifera strain) was prepared by Asghapoor Honey and Bee Products Co. (Iran). Sorbitan monooleate (Span 80) and polyoxyethylene 20 sorbitan monooleate (Tween 80) as surfactants were purchased from Merck Chemicals (Germany), and olive oil was from Fadak Co. (Iran). Bovine type II collagen was from Xi'an Harmonious Natural Bio-Technology Co., Ltd (China), and incomplete Freund's adjuvant (IFA) was from Sigma-Aldrich (USA). Biochemistry kits were prepared by Pars Azmoon Co. (Iran). Sampling tubes containing EDTA (Ethylene diamine tetra acetic acid) as an anti-coagulant were prepared from FL Co. (Italy) for collecting whole blood as ready to use.

Animal

88 male Wistar rats weighing ~ 200 g were used in this study, kept according to the Standard Laboratory Animal Guidelines. The Ethical Committee approved the experimental protocols of Kerman University of Medical Sciences (The Ethics approval code is IR.KMU.REC.1399.234).

Preparation and characterization of NEs

NE was prepared as a volumetric percentage formulation according to the previous study [14]. Briefly, a 3% aqueous phase (containing different BV solutions as the following) was added to the container containing 30% surfactant (14% Span 80 and 16% Tween 80) and mixed using a magnetic stirrer (MS-300HS, Protraction Inter-trade Co, Korea) for 10 min with the rotation speed of 1000 rpm at RT. Then, the oil phase (67% olive oil) was added and thoroughly mixed for 10 min.

The average size of droplets and polydispersity index (PDI) were measured by dynamic light scattering at 25 °C using a Scatteroscope (K-one Ltd. Korea). Loading capacity was also calculated in terms of 3% aqueous phase and BV concentrations as 9.37, 18.75, 37.5, and 75 µg/ml.

Induction of CIA and treatment

Bovine type II collagen was dissolved (2 $mg.ml^{-1}$) in 0.05 M acetic acid by gently stirring overnight at 4 °C. Next, an equal volume of IFA was added to the emulsified collagen solution, then mixed by a homogenizer (1000 RPM, 30 min, in an ice-water container) for the collagen-IFA emulsion to be prepared. 0.1 ml of this emulsion was injected subcutaneously into the plantar surface of the left hind paw as the inducer dose and 0.1 ml as a booster dose in an intradermal injection into the root of the tail on the same day to induce CIA in rats. Maximum inflammation was observed on day 7; treatment was followed up from day 7 to day 21 for two weeks [15].

Experimental design

Rats were assigned to 8 groups (n=11). Each group

was treated daily with one of the following treatments; blank (no treatment), negative control (NEs free BV, 0 µg/ml), positive control (hydrocortisone acetate ointment 1%, 50 mg/day), BV control (solution of bulk BV in NS, 37.5 µg/ml/day), and four groups of BV-NE formulations (i.e., 9.37, 18.75, 37.5, and 75 µg/ml/day). The daily treatments were paw circular massage with 1 ml of NE.

Blood sampling

The rats were deeply anesthetized using intraperitoneal injection of 87 mg ketamine/kg and 13 mg xylazine/kg of body weight. 4 ml of blood was taken directly from their hearts, 2.5 ml for biochemical parameters, and 1.5 ml for hematological parameters, and then rats were sacrificed. Three steps of blood sampling were done; including 16 samples in the first step on day 0 as an indicator of healthy rats (2 samples per group), 16 samples in the second step on day 7 as an indicator of maximum inflammatory rats (2 samples per group), and 56 samples in the third step on day 21 at the end of treatment (7 samples per group). After each sampling, rats were sacrificed due to the high volume of blood loss, according to ethical standards. Samples were collected from the EDTA anti-coagulant tubes (1.5 mg/ml whole blood) for hematological parameters and without anti-coagulant tubes for biochemical parameters (The serums were separated after coagulation by centrifuging at 3000 rpm, RT, 10 min).

Measurement of biochemical and hematological parameters

Serums were evaluated for biochemical parameters, including blood glucose (Glu), cholesterol (Ch), triglyceride (Tg), urea (Ur), creatinine (Cr), AST (aspartate aminotransferase or SGOT, serum glutamic-oxaloacetic transaminase), ALT (alanine aminotransferase or SGPT, serum glutamic pyruvic transaminase), ALP (alkaline phosphatase), calcium (Ca) and phosphorus (Ph) using an autoanalyzer (BT 1500, Biotechnica Co., Italy).

Hematological parameters were counted as complete blood cells (CBC) using an automated cell counter (XP-300, Sysmex Co., Japan), including white blood cells (WBCs), and the percentage of neutrophil (%Neu), red blood cells (RBCs), hemoglobin (Hb) concentration, hematocrit (HCT) percentage, and platelets (Plts) count. Also, RBC indices, including mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW), were calculated.

Statistical analysis

One-way analysis of variance and Tukey comparison were performed to assess the statistical significance of differences among groups. Results with a *p*-value≤ 0.05 were considered statistically significant. Statistical analyses were carried out using the SPSS software, v.19 (SPSS, Inc., USA).

RESULTS

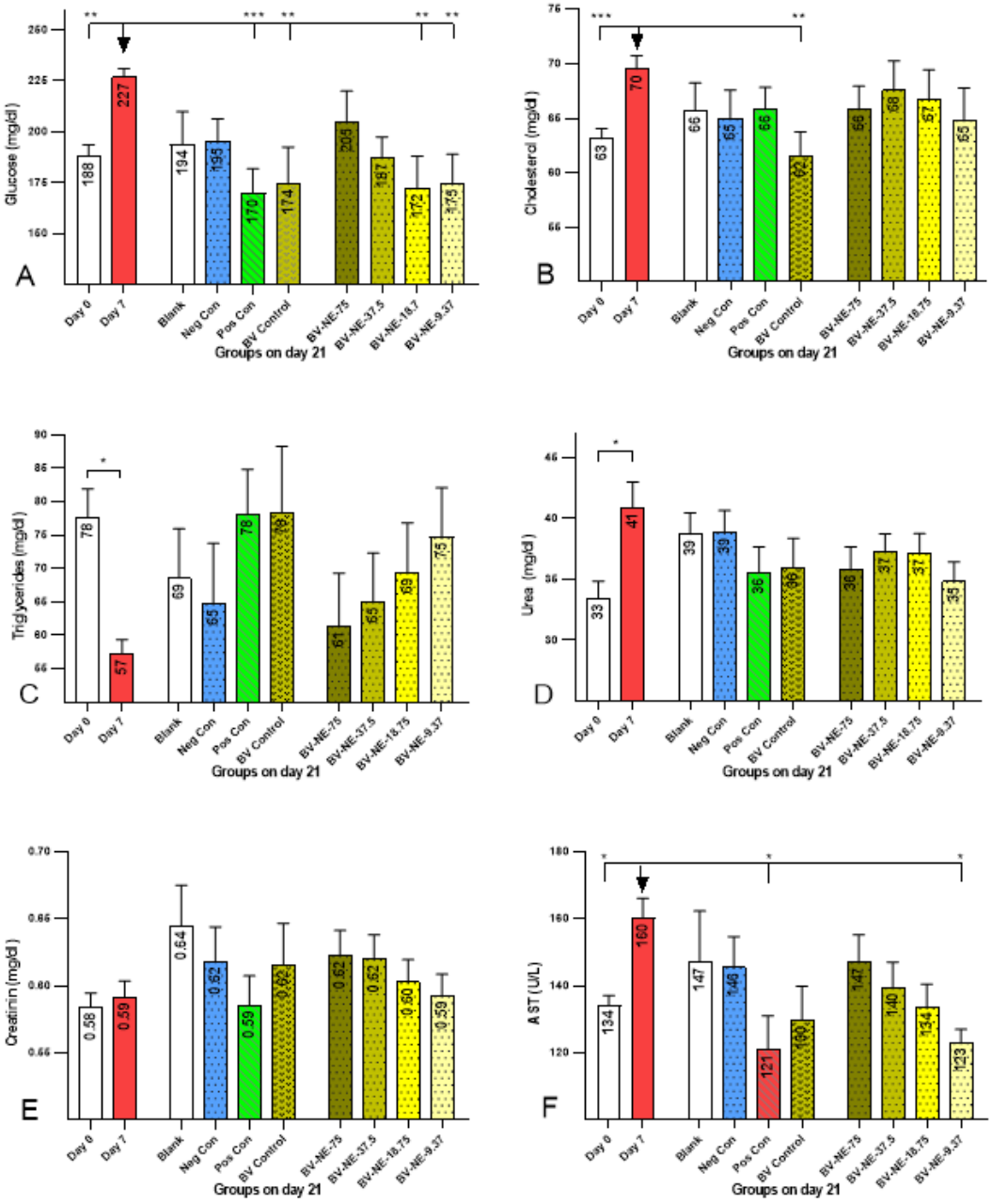
Droplets' mean sizes of NEs were 21.1, 16.9, 15.1, 14.2, and 12.7 nm, as well as PDIs were 0.147, 0.146, 0.143, 0.142, 0.142 for BV-NE-75, BV-NE-37.5, BV-NE-18.75, BV-NE-9.37, and BV-NE-0, respectively. Also, the loading capacity (in terms of 3% aqueous phase) of BV-NEs were 75, 37.5, 18.75, 9.37, and 0, which are named accordingly. It states that in a certain amount of aqueous phase (3%), with increasing the concentration of BV from 0 to 75 µg/ml, the size increased from 12.7 to 21.1 nm, and the PDIs also increased from 0.142 to 0.147. The loading capacities of NEs increased with the increase of BV concentrations from 9.37 to 75 µg/ml.

Fig. 1 (A to J) shows the values of biochemical parameters for healthy rats (day 0), before treatment CIA rats (day 7), and for treated groups (day 21), including blank, negative, positive, and BV controls, as well as BV-NEs including 75, 37.5, 18.75, and 9.37 µg/ml. Fig. 1. A shows the blood Glu level significantly increased in the CIA model from 188 to 227 mg/dl from day 0 to day 7. However, it was significantly returned to near the baseline level only for positive and BV controls, as well as BV-NE 18.75 and 9.37 µg/ml on day 21. In Fig. 1. B, the Chol blood level was significantly increased in the CIA model from 63 to 70 mg/dl from day 0 to day 7. It states that Chol was significantly decreased for BV control on day 21. Fig. 1. C shows which Tg blood level has significantly decreased in the CIA model on day 7. however, it increased on day 21 in all groups. Changes in urea and creatinine in rat blood are shown in Fig. 1. D, and E. These biochemical parameters were not changed on days 0, 7, and 21, except for urea, which significantly increased from 33 to 41 mg/dl in the CIA model. Fig. 1. F, G, and H illustrate blood levels of AST, ALT, and ALP as a function of liver enzymes. Although all of them were increased in the CIA model on day 7, it was only significant for AST from 134 to 160 U/L. The AST and ALP liver

enzymes were decreased in the positive and BV controls, as well as NE 9.37 µg/ml group on day 21 compared to day 7. Ca and Ph blood levels are given in Fig. 1. I and J. for the treated groups, the levels of Ca and Ph decreased on day 21 compared to the CIA model on day 7. Especially about Ca, there were significant differences for BV control and also BV-NEs groups.

Fig. 2 (L to U) illustrates the results of hematological parameters for understudy groups in the pattern of Fig.1. Fig. 2. L shows a significant increase in the counting of WBCs from 4.96 to 9.74 ($\times 10^3/mm^3$) from day 0 to day 7 in the CIA model. However, it was significantly decreased for all treatment groups on day 21, although it was higher for blank and negative control groups.

Hematological parameters of RBC, Hb, and HCT are shown in Fig. 1. M, N, and O, respectively. Fig.



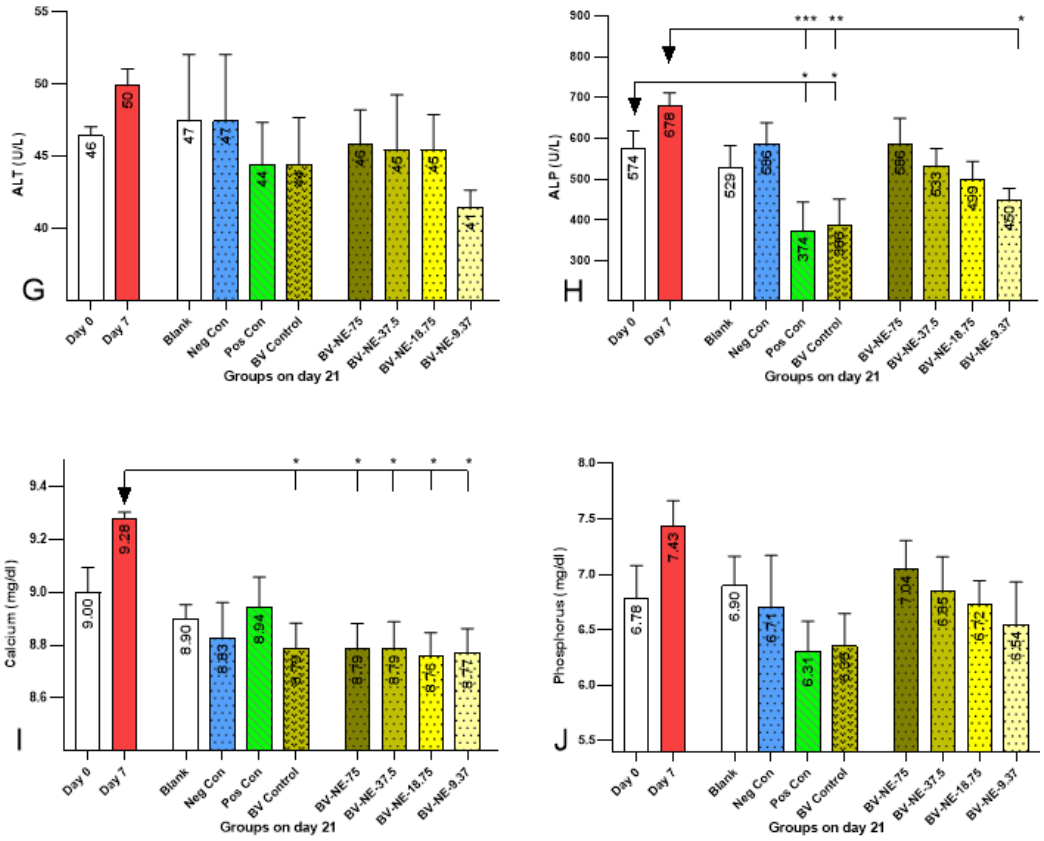


Fig. 1. The values of the rat's blood biochemical parameters were treated with different groups. Day 0: the base levels of biochemical parameters in the rat's blood on day 0. Day 7: biochemical parameters in the rat's blood of collagen-induced arthritis model on day 7 as maximum inflammation and start of treatment. Blank (no treatment); Neg Con: Negative control (nanoemulsion free BV 0 µg/ml); Pos Con: Positive control (hydrocortisone acetate ointment 1%, 50 mg/day); BV Control (BV solution, 37.5 µg/ml); BV-NE-75, BV-NE-37.5, BV-NE-18.75, BV-NE-9.37 (4 groups of nanoemulsions containing bee venom solutions with 75, 37.5, 18.75, and 9.37 µg/ml, respectively). All treatments were followed by 5 min of massage the rat's paw with 1 ml bee venom solution or nanoemulsions. A: Glucose, B: Cholesterol, C: Triglyceride, D: Urea, E: Creatinine, F: AST (aspartate aminotransferase), G: ALT (alanine aminotransferase), H: ALP (alkaline phosphatase), I: Calcium and, J: Phosphorus. *, **, *** show P-value≤ 0.05, 0.01, and 0.001, respectively

2. P, Q, R, and S also indicate indices of RBC (MCV, MCH, MCHC, and RDW). There was no significant difference between treated groups on days 0, 7, and 21. Plts count was increased on days 7 and 21 compared to day 0, but it was not significant (see Fig. 2. T). Fig. 2. U shows %Neu for treated groups. It was significantly increased from 19.9% to 54.4% from day 0 to day 7 in the CIA model and then was significantly decreased for all groups on day 21, although it was more significant for BV-NEs, BV control, and positive control groups compared to blank and negative control groups.

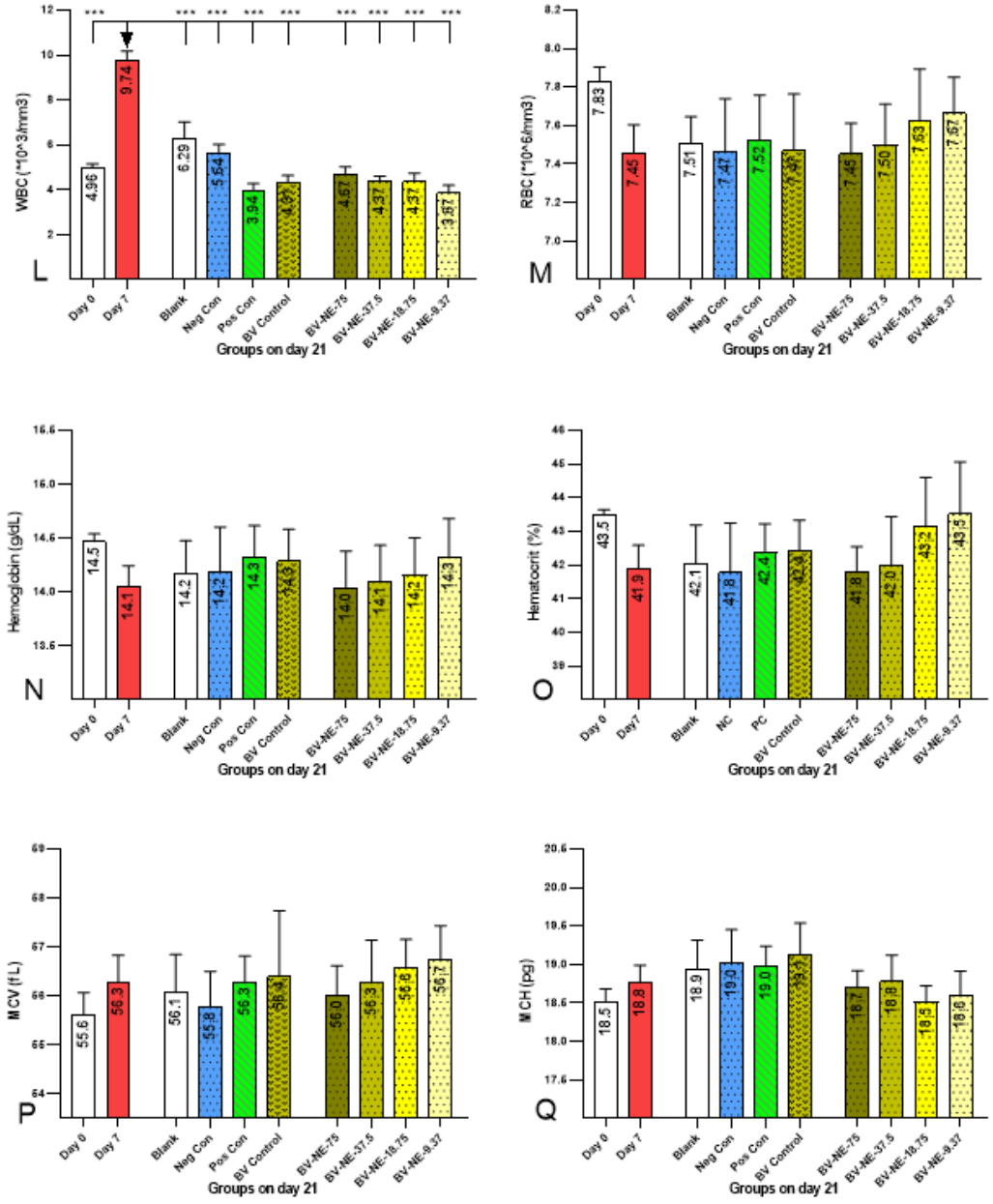
DISCUSSION

Our previous studies found a significant effect of NEs containing BV on improving arthritis in an animal model and its effects on the immune system have been examined. [11, 14]. However, there was no information about its adverse effects after entering the body. Therefore, by measuring these parameters, we discussed its possible effects on blood and metabolic systems. Also, the changes in these parameters have been investigated for CIA model.

The study showed that after 7 days of CIA model induction (maximum inflammation), biochemical parameters, including Glu ($P \leq 0.01$), Ch ($P \leq 0.001$), Ur ($P \leq 0.05$), and AST ($P \leq 0.05$), had significantly increased, and only, TG ($P \leq 0.05$) had significantly decreased. Among the hematological parameters, only WBCs ($P \leq 0.001$) and %Neu ($P \leq 0.001$) had significantly increased. The other biochemical and hematological parameter changes in the CIA were insignificant from day 7 until the end of two weeks of the treatment (day 21, see the blank group). Nevertheless, the %Neu was still high compared to day 0 ($P \leq 0.001$).

Increasing WBCs, especially %Neu in the blood of CIA model is due to calling WBCs to the inflammation site in the rat's paw by some inflammatory cytokines after induction of arthritis [16]. In this regard, inflammatory mediators, immune cells, cytokines, and chemokines play a critical role in dyslipidemia, which cause metabolic effects in arthritis [17]. An increase in Chol and TG has been reported in formaldehyde-induced arthritis, while in the CIA model, only Chol increased and TG decreased [18]. Thus, there was a disorder of metabolism and dyslipidemia in arthritis and animal models of CIA.

BV has several anti-inflammatory and anti-arthritic properties [9]. It has a variety of components with pharmacological and biochemical activities and can destroy location cells or enter the bloodstream and lyse other blood cells, especially RBCs, as it pass through the skin [8]. Studies have shown that BV can affect the body's metabolic systems to change their function, such as reducing blood glucose and affecting the



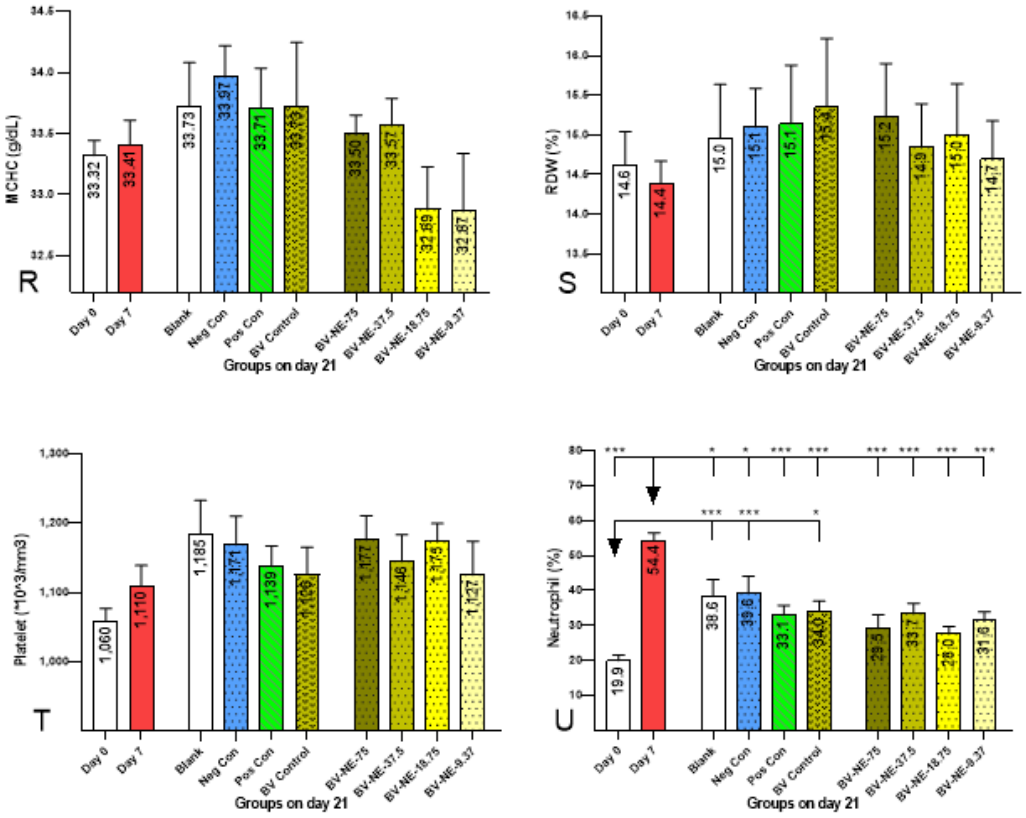


Fig. 2. The values of the rat's hematological parameters were treated with different groups. The details are similar to Fig. 1. L: WBC (white blood cell), M: RBC (red blood cell), N: Hemoglobin, O: Hematocrit, P: MCV (mean cell volume), Q: MCH (mean corpuscular hemoglobin), R: MCHC (mean corpuscular hemoglobin concentration), S: RDW (red cell distribution width), T: Platelet, and, U: Neutrophil (percentage of neutrophil of white blood cell). *, **, *** show P-value≤ 0.05, 0.01, and 0.001, respectively.

blood lipids [19]. Although some unfavorable effects of BV can be neutralized by albumin in the bloodstream [20], it can affect organs such as islands of Langerhans of the pancreas [21], liver [12], kidney [13], adrenal glands, and their hormones [22]. Imani et al. showed that BV injection decreased Glu, total Chol, and Tg levels in treated diabetic rats [23]. It has been shown that serum levels of Chol and TG decreased in rats receiving injection BV, which was similar to our results only about Chol [18]. The study indicated that the blood levels of Glu decreased at the end of treatment and were similar to the base level on day 0 for all groups, except for the BV control, which was lower than the base level. It suggests that local BV can reduce the blood Glu, but this effect was not found for Chol and TG.

It was reported that BV therapy is associated with multiple causes of kidney injury and hepatotoxicity and measuring Ur and Cr blood levels is a simple way to assess kidney injuries [24]. Ur and Cr increased in the CIA model on day 7. At the end of treatment on day 21, Ur and Cr dose-dependently dropped in all groups, compared to day 0 and even day 7. Although these increases were not significant, all groups were especially the blank group. It increased over 21 days, which is more evident for the blank group.

AST, ALT, and ALP are the most common tests used to diagnose liver disorders [25]. Our findings express which blood levels these enzymes have increased on day 7 compared to day 0, although only significant for AST ($p \leq 0.05$). They decreased in a dose-dependent manner for BV-NEs at the end of treatment. These results align with Nicodim et al.'s investigations about rats with RA induced by Freund complete adjuvant treated with BV [26].

Ca and Ph were elevated in CIA rats on day 7, and their reduction was in all treatment groups on day 21. The level of Ca significantly decreased in a dose-dependent manner for all BV-NEs and BV control at the end of treatment. Therefore, BV

could reduce the level of Ca and Ph after entry into the bloodstream, which was recently reported by Kang [27]. It is due to the interaction of melittin as a 26-residue peptide with Ca [28].

BV has an anti-inflammatory effect on pro-inflammatory cytokines and is expected to lower WBC and %Neu [7]. A decrease in the total number of WBCs at the end of treatment in all groups compared to day 7 is related to reduction of inflammation in the animal. There are more reductions in the positive and BV controls, as well as BV-NEs in comparison with blank and negative control groups, due to BV's immunosuppressive effects [18]. After an increase of %Neu on day 7, it was significantly decreased on day 21. The highest %Neu depletion was in the BV-NE-9.37 group. However, this difference was insignificant, and the %Neu did not return to the base level of day 0. In CIA rats treated with BV control and BV-NEs, WBC and the %Neu had diminished, which agrees with the study of formaldehyde-induced arthritis in male rats [18].

On the maximum inflammation day in the CIA rats, anemia could be seen as decreased blood levels of RBCs, Hb, and HCT [7]. After two weeks of treatment, significant changes were not seen by topical BV-NEs, but a dose-dependent increase was observed for BV-NEs (especially BV-NE-9.37 and BV-NE-18.75) about parameters of RBC, Hb, HCT, which could be due to BV improvement of circulation of blood in the micro blood vessels, as well as its role in the stimulation of building erythrocytes [29]. Indices of RBCs, including MCV, MCH, MCHC, and RDW, help elucidate the etiology of anemias. According to the study, there were no crucial changes in these indices on days 7 and 21 which seems that they were less affected by inflammation of CIA and treatment of BV-NEs.

Plts were increased on days 7 and 21; however, these changes were insignificant. An increase of Plts on maximum inflammation day and at the end of treatment in the groups indicates inflammation in RA's early and secondary stages [26]. The critical role of Plts is known in inflammation and immune responses. They actively participate in leukocyte recruitment, especially neutrophils, and host defense regulation in response to exogenous pathogens. This increase in Plts can also be due to the significant effect of cytokines, especially IL-6, which affects the maturation of generated cells of Plts and causes increased production of Plts [30].

CONCLUSION

Based on the findings, induced inflammation in CIA model dramatically changed some routine biochemical and hematological parameters, including increased blood levels of Glu, Chol, Ur, and AST and an increase in the count of WBCs and %Neu. After two weeks of maximum inflammation, Glu, Chol, Tg, Ur, ALT, Ca, and Ph, MCV returned to baseline similar to day 0. Blood levels of Cr, AST, %Neu, MCH, MCHC, RDW, and Plts count increased. Nevertheless, blood levels of ALP, count of WBCs, Hb concentration, and HCT percentage were decreased. Reduction in levels of Glu, liver enzymes, and Ph were higher for the BV control than BV-NE. Overall, the use of BV in NE formulation as a local route has no adverse effects on the routine biochemical and hematological parameters. It can dose-dependently reduce inflammation induced in this model, especially for 18.75 and 9.37 µg/ml.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All methods and experimental protocols were performed following arrive guidelines, also the relevant guidelines and regulations by the Ethical Committee of Kerman University of Medical Sciences (The Ethics approval code is IR.KMU.REC.1399.234).

CONFLICTS OF INTEREST

All authors declare no conflict of interest.

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