



Efficacy of parenteral administration of bee venom in experimental arthritis in the rat: A comparison with methotrexate



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ABSTRACT

The use of bee venom (BV) to treat inflammation and pain in arthritis has become increasingly common. This study aimed to compare the effects of BV and methotrexate (MTX), the most used disease-modifying anti-rheumatic drug, in arthritic rats. Edema, erythema, cyanosis, hyperalgesia, reduction of the body mass gain, high circulating tumor necrosis factor alpha (TNF- α) and anti-type II collagen antibodies (AACII), and altered activity of basic (APB) and neutral (APN) aminopeptidases and dipeptidyl peptidase IV (DPPIV) are present in arthritic rats. MTX and/or BV do not affect AACII in healthy or arthritic individuals, but restores TNF- α to normal levels in arthritic rats. BV restores body mass gain to normal levels and MTX ameliorates body mass gain. BV contains DPPIV. BV restores APN in synovial fluid (SF) and in soluble fraction (S) from synovial tissue (ST), and DPPIV in solubilized membrane-bound fraction (M) from peripheral blood mononuclear cells (PBMCs). MTX restores APN of SF, as well as ameliorates APB of S-PBMCs, APN of S-ST and DPPIV of M-PBMCs. The combination therapy does not overcome the effects of BV or MTX alone on the peptidase activities. Edema is ameliorated by MTX or BV alone. MTX, but not BV, is effective in reducing hyperalgesia. Data show that anti-arthritic effects of BV at non-acupoints are not negligible when compared with MTX.

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1. Introduction

The use of pharmacopuncture with bee venom (BV) to treat various diseases has become increasingly common, especially in many Asian countries and some underdeveloped ones. It is well-known that the annual global trade of animal-based medicinal products accounts for billions of dollars. The pharmacopuncture preconizes that the injection of herbal extracts, or sublethal doses of venoms from animal and plant origins, or subclinical doses of

traditional drugs into acupoints are pharmacologically efficient and, in the case of traditional drugs, with fewer undesired side effects than the traditional routes of administration. In fact, nowadays acupuncture in general is much more than a cultural practice, being used in many parts of the world by traditional health-care professionals. However, the majority of studies does not support its effectiveness and acupuncture remains under controversy. This is precisely the case for using BV to treat inflammation and pain in rheumatoid arthritis (RA).

RA is an inflammatory, chronic, systemic and autoimmune disease that is difficult to treat and with unknown ethiology (Upchurch and Kay, 2012). Methotrexate (MTX) is the conventional standard and most used disease-modifying anti-rheumatic drug (DMARD). However, the mechanisms of its action in RA have not yet been completely understood (Abolmaali et al., 2013). In turn, BV administered by acupuncture has become increasingly popular as an alternative for pain relief in many diseases (Lee et al., 2005). Possible anti-arthritic actions of BV administered subcutaneously (Kang et al., 2002) or by acupuncture (Kwon et al., 2001; Baek et al., 2006; Saad et al., 2010) in various kinds of animal arthritic models

Abbreviations: AACII, anti-type II collagen antibodies; ADA, adenosine deaminase; Ang, angiotensin; APB, basic aminopeptidase; APN, neutral aminopeptidase; BM, body mass; BV, bee venom; CII, collagen type II; CIA, collagen-induced arthritis; DMARD, disease-modifying anti-rheumatic drug; DPPIV, dipeptidyl peptidase IV; LTA4H, leukotriene A4hydrolase; M, solubilized membrane-bound fraction; MTX, methotrexate; NF κ B, nuclear factor κ -light chain-enhancer of activated B cells; PBMCs, peripheral blood mononuclear cells; RA, rheumatoid arthritis; S, Soluble fraction; SF, synovial fluid; TNF- α , tumor necrosis factor alpha.

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(Kwon et al., 2001; Kang et al., 2002; Baek et al., 2006; Saad et al., 2010), or administered by acupuncture in clinical trials (Kwon et al., 2001; Liu et al., 2008), have been reported, but the results are still insufficient for demonstrating the efficacy of BV in arthritis.

Collagen type II (CII) is probably a primary antigen in RA, since it is the major constituent of cartilage, and also because anti-CII antibodies (AACII) exist in RA patients, and CII-induced arthritis (CIA) and RA have similar characteristics (Hietala et al., 2004). Furthermore, the transference of AACII can generate arthritis, due to the activation of complement followed by binding of these antibodies to normal cartilage (Hietala et al., 2004). The onset, development and persistence of synovitis in RA are related to several cell types, including peripheral blood mononuclear cells (PBMCs) (Hashimoto et al., 2011; Li et al., 2011; Mendes et al., 2011; Meugnier et al., 2011; Yamasaki et al., 2012; Mendes and Silveira, 2013). The tumor necrosis factor (TNF)- α is well-recognized as one major pro-inflammatory cytokines in RA (Doan and Massarotti, 2005).

Given that any recommendation for use in pharmacopuncture should be preceded by exhaustive evaluation about the effects of employed extracts or drugs through well-established pharmacological methods, the present study aimed to know more about the mechanisms and targets of BV in RA, performing the comparison of BV and MTX effects on key aspects of arthritis in CIA model, such as edema, erythema, cyanosis, algesia, altered body mass (BM) gain, TNF- α and AACII levels. The mechanism of anti-arthritic action of MTX is not yet fully understood, and the activities of basic aminopeptidase (APB) (Mendes and Silveira, 2013), dipeptidyl peptidase IV (DPPIV) (Yamasaki et al., 2012) and neutral aminopeptidase (APN) (Mendes et al., 2011) emerged recently as potentially new valuable targets that may lead to a better understanding of arthritic processes and the development of novel treatments. In this way, the effects of MTX and BV on these enzyme activities were also checked in plasma, soluble (S) and solubilized membrane-bound (M) fractions from PBMCs and from synovial tissue (ST), and in synovial fluid (SF) of the femoro-tibial-patellar joint (knee).

2. Materials and methods

2.1. Ethics statement

The conducts and procedures involving animal experiments were approved by the Butantan Institute Committee for Ethics in Animal Experiments (License number CEUAIB 432/2007) in compliance with the recommendations of the National Council for the Control of Animal Experimentation of Brazil (CONCEA). All invasive procedures were performed under anesthesia, and all efforts were made to minimize suffering.

2.2. Chemicals, reagents and equipment

Enzyme-linked immunosorbent assay (ELISA) kit for anti-type II collagen antibodies was from Chondrex Inc. (USA). BV (*Apis mellifera* venom, code V3375; lot 092K 0693), the β -naphthylamine, collagen type II from chicken, DL-dithiothreitol, Freund's incomplete adjuvant, L-alanine- β -naphthylamide, L-arginine- β -naphthylamide, methoxy- β -naphthylamine, and NaCl were from Sigma-Aldrich (USA). TNF- α ELISA kit was from Biosource (USA). Acetic acid was from Labsynth Produtos para Laboratório Ltd. (Brazil). Bio-Rad Protein Assay reagent was made by BioRad Laboratories, Inc. (USA). H-Gly-Pro-4-methoxy- β -naphthylamide was from Peninsula Laboratories, Inc. (USA). Ketamine 5% (Vetanarcol[®]) was from König do Brasil (Brazil). MTX (Miantrex[®] 25 mg/mL) was from Laboratórios Pfizer Ltd. (Brazil). Percoll ($\rho = 1.077$ mg/mL) was manufactured by GE-Healthcare (USA). Sodium heparin 25,000 UI/5 mL (Liquemine[®]) was from Roche (Brazil). Xylazine 2.3%

(Anasedan[®]) was from Sespo Ind. Co., Ltd., Vetbrands Division (Brazil). All other chemicals and reagents were of analytical grade and purchased from Merck KGaA (Germany). 96-well flat bottom microplates was from Corning Inc. (USA). Centrifuge model CR31 was from Joaun, Inc. (USA). Container for rodents was from Alesco Ind. Co., Ltd. (Brazil). The digital analgesimeter (Von Frey) was from Insight Equipamentos Científicos Ltd. (Brazil). The homogenizer model Polytron-Aggregate was from Kinematica (Switzerland). The micrometer was from Mitutoyo do Brasil (Brazil). Microplate Fluorescence/Absorbance Reader model FL600FA was from Bio-Tek (USA). The ultracentrifuge model CP60E was from Hitachi one (Japan).

2.3. Animals and induction of arthritis

Adult male Wistar rats, weighing 150–160 g, were kept in polyethylene cages (interior length \times width \times height = 56 \times 35 \times 19 cm) with food and tap water *ad libitum*, in a container with controlled temperature (25 °C), relative humidity (65.3 \pm 0.9%) and 12 h: 12 h photoperiod light:dark (lights on at 6:00 AM). The animals were anesthetized with a solution of ketamine (3.75%) and xylazine (0.5%) at a dose of 0.2 mL/100 g, via intraperitoneal (i.p.). Baseline values were then evaluated for BM and dorsal-plantar thickness in the region of the metatarsus (both paws were measured by paquimeter and calculated the average thickness of each animal). Subsequently, two animal groups with the same average BM and dorsal-plantar thickness were formed, and each group was subjected to induction of arthritis or sham induction as previously described (Mendes et al., 2011; Yamasaki et al., 2012). Briefly, CII from chicken dissolved in 0.01 M acetic acid and emulsified in an equal volume of Freund's incomplete adjuvant (prepared at 4 °C just before use) was administered via a single intradermal dose of 0.4 mg/0.2 mL/animal, into the proximal one-third of the tail (induced animals), or with 0.9% saline at the same scheme of administration (sham induced).

2.4. Treatments and evaluation of BM, edema and hyperalgesia

On day 21 after induction/sham induction rats were classified by macroscopic evaluation of the hind paws according to the following score: 0- undetectable swelling without erythema and cyanosis; with erythema and cyanosis and 1- detectable swelling in a joint, 2- in two joints, 3- in three joints, 4- swelling throughout the paw. The maximum possible score for each animal is 8 points. Animals submitted to sham induction with score 0 were grouped as healthy (100%), CII-treated animals with score 6–8 were grouped as arthritic (60%) and both were selected to be used in this study; those with score 1–5 (10%) were not included in the present study and thus they were euthanized; and those with score 0 (30%, called resistant) were included in another study on resistance to CII-induced arthritis (Mendes and Silveira, 2013; Mendes and Silveira, 2014). Dorsal-plantar thickness was then evaluated as described in 2.3. Subsequently, the therapy with vehicle (0.9% saline) or MTX and/or BV was started in healthy and arthritic rats, three times per week, on alternate days during 3 weeks, between the fourth and sixth hour of the light period of the photoperiod as specified. Sick animals formed the following groups: Arthritic, administered with 0.9% saline, 50 μ L subcutaneously (s.c.) dorsal route and 300 μ L, i.p.; Arthritic + BV, administered with 0.25 mg BV/kg (Roh et al., 2004; Baek et al., 2006) in a maximum volume of 50 μ L s.c. dorsal route; Arthritic + MTX, administered with 0.3 mg MTX/kg (Mirshafiey et al., 2006) in a maximum volume of 300 μ L, i.p.; Arthritic + BV + MTX, administered with 0.25 mg BV/kg in a maximum volume of 50 μ L s.c. dorsal route and with 0.3 mg MTX/

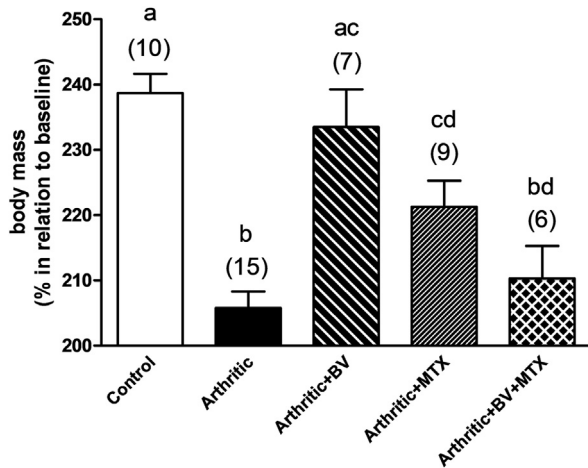


Fig. 1. Percentage change in body mass 41 days after induction/sham induction in relation to baseline (immediately before the induction and considered as 100%). BV = bee venom, MTX = methotrexate. Values are means \pm S.E.M. Number of animals in parenthesis over the bars. ANOVA Control vs Arthritic \times Arthritic + BV vs Arthritic + MTX vs Arthritic + BV + MTX ($P < 0.003$); post hoc Tukey's multiple comparison test, different letters indicate significant differences ($P < 0.05$).

kg in a maximum volume of 300 μ L, i.p. Healthy animals formed the following groups: Control, Control + BV, Control + MTX and Control + BV + MTX, respectively treated as Arthritic, Arthritic + BV, Arthritic + MTX and Arthritic + BV + MTX. BV was dissolved in 0.9% saline at 4 $^{\circ}$ C. MTX was diluted in 0.9% saline at 23 $^{\circ}$ C.

On day 41 after induction/sham induction, the mechanical hyperalgesia threshold was evaluated with a digital analgesimeter as previously described (Guerrero et al., 2006). Briefly, the animals were kept in individual acrylic boxes with a metal grid floor for 15–30 min in a room without noise, for adaptation to the environment. Then, a perpendicular and growing force was applied by the analgesimeter sensor in the center of the plantar surface of one hind paw, inducing dorsal flexion of the tibio-tarsal joint, until the withdrawal of the paw. The test was repeated to obtain three measures with a maximum variation lower than 2 g. Subsequently, at the same day the animals were anesthetized, using the same scheme specified above, and submitted again to the quantification of BM and dorsal-plantar thickness as described above and then for obtainment of samples.

2.5. Sample collection, fractionation of ST and PBMCs and measurements of AACII and TNF- α

The blood was withdrawal from the left ventricle with heparinized and non-heparinized syringes. Heparinized blood was centrifuged (at 200 g for 10 min at 25 $^{\circ}$ C) to separate plasma from the pellet containing PBMCs and other blood cells. The non-heparinized blood was left at 25 $^{\circ}$ C for 10 min and then centrifuged under the same conditions to obtain serum. SF and ST were subsequently removed from both knees of each animal as previously described (Mendes et al., 2011; Yamasaki et al., 2012). The procedures for separation and counting of PBMCs, fractionation of ST and PBMCs into S and M, and measurements of plasma AACII and serum TNF- α were performed as previously described (Mendes et al., 2011; Yamasaki et al., 2012).

2.6. Amino-peptidase activities and protein

Total protein was measured photometrically at 630 nm, in

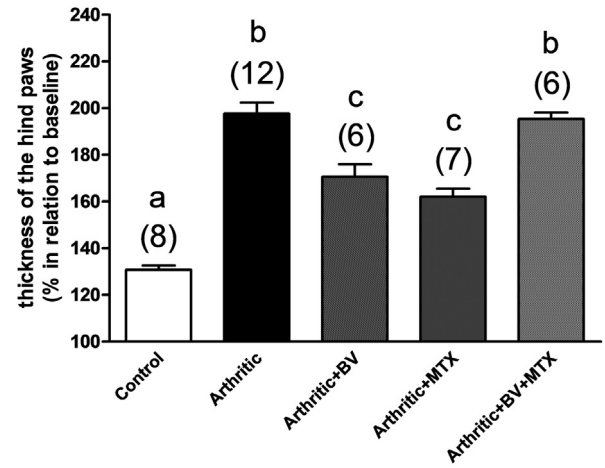


Fig. 2. Percentage change in hind paw thickness 41 days after induction/sham induction in relation to baseline (immediately before the induction and considered as 100%). BV = bee venom, MTX = methotrexate. Values are means \pm S.E.M. Number of animals in parenthesis over the bars. ANOVA Control vs Arthritic \times Arthritic + BV vs Arthritic + MTX vs Arthritic + BV + MTX ($P < 0.0002$); post hoc Tukey's multiple comparison test, different letters indicate significant differences ($P < 0.05$).

triplicates of 40 μ L of MTX (0.3 mg/mL), BV (1.25 mg/mL), SF and S (diluted 10-fold), and M (diluted 2-fold) from ST and PBMCs making use of the Bradford method (1976). Protein contents were interpolated by comparison with standard curves of bovine serum albumin (BSA) in the same diluent. Peptidase activities were measured as previously described by Gasparello-Clemente et al. (2003), using 25 μ L of MTX (without protein) and BV (0.05–0.199 mg of protein/mL), both at concentrations of 50 μ g, 500 μ g and 5000 μ g per mL, and 10–25 μ L of SF (5–49 μ g protein), 10–25 μ L of S (1.3–20 μ g) and 25 μ L of M (2.6–15 μ g protein) from ST, 50 μ L of S (22–72 μ g protein) and 100 μ L of M (13–44 μ g protein) from PBMCs, and 25 μ L of plasma (0.2–112 mg protein) according to the amount of β -naphthylamine released as a result of the enzyme activities of samples incubated at 37 $^{\circ}$ C for 30 min in 96-well flat bottom microplates with prewarmed substrate solution of: 0.5 mM L-arginine β -naphthylamide in 0.05 M phosphate

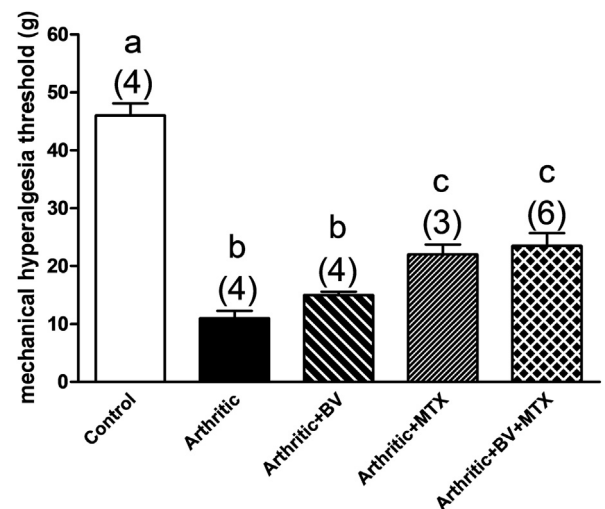


Fig. 3. Hyperalgesia in hind paws. BV = bee venom, MTX = methotrexate. Values are means \pm S.E.M. Number of animals in parenthesis over the bars. ANOVA Control vs Arthritic \times Arthritic + BV vs Arthritic + MTX vs Arthritic + BV + MTX ($P < 0.0002$); post hoc Tukey's multiple comparison test, different letters indicate significant differences ($P < 0.05$).

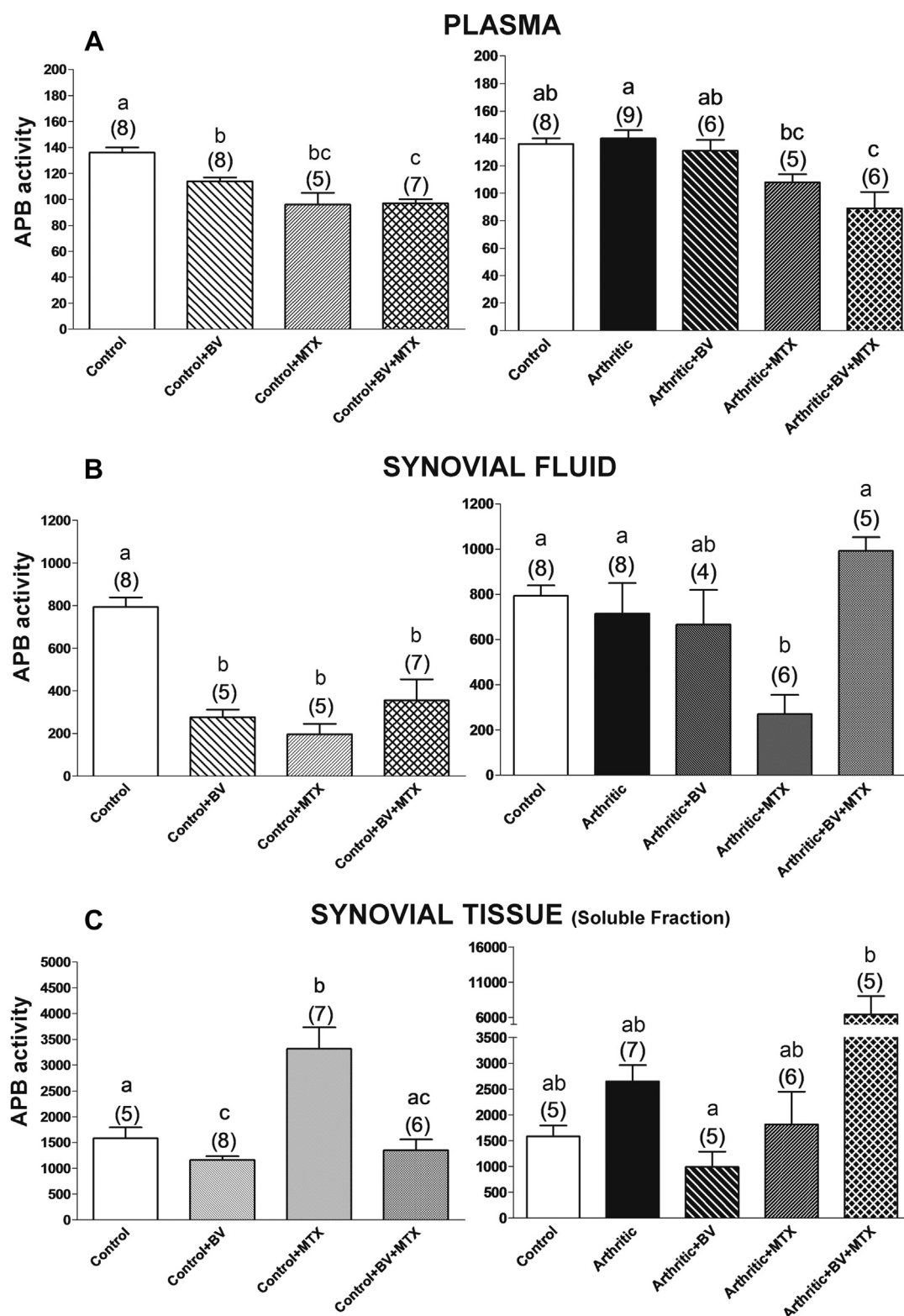


Fig. 4. Basic aminopeptidase (APB) activity (pmoles hydrolyzed substrate/min/mg protein). Animals are treated with saline (control and arthritic), or bee venom (BV) and/or methotrexate (MTX). Values are means \pm S.E.M. Number of animals in parenthesis over the bars. ANOVA Control vs Control + BV vs Control + MTX vs Control + BV + MTX (left) and Control vs Arthritic vs Arthritic + BV vs Arthritic + MTX vs Arthritic + BV + MTX (right): 4(A): left and right $P < 0.0001$; 4(B): left $P < 0.0001$, right $P < 0.001$; 4(C): left $P < 0.0001$, right $P < 0.02$; 4(D): left $P = 0.07$, right $P < 0.01$; 4(E): left and right $P < 0.0001$; 4(F): left $P = 0.93$, right $P < 0.0002$; post hoc Tukey's multiple comparison test, different letters indicate significant differences among the bars in each graph ($P < 0.05$).

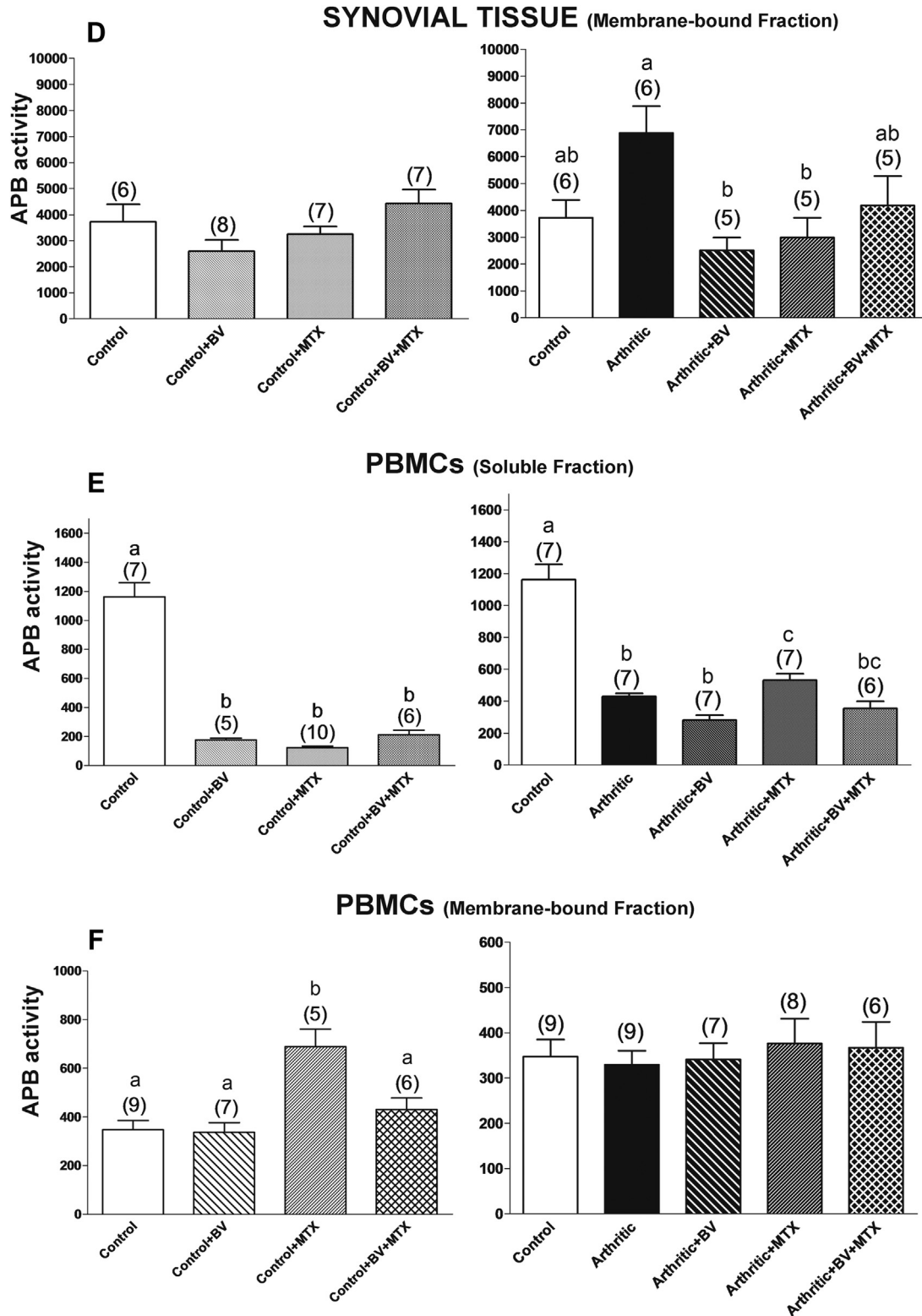


Fig. 4. (continued).

buffer, pH 6.5, with 150 mM NaCl, 0.02 mM puromycin and 0.1 mg BSA, for APB; 0.125 mM L-alanine β -naphthylamide in 0.05 M phosphate buffer, pH 7.4, with 1 mM DL-dithiothreitol and 0.1 mg BSA, for APN; and 0.2 mM H-Gly-Pro-4-metoxi β -naphthylamide in 0.05 M Tris-HCl buffer, pH 8.3, with 0.1 mg BSA, for DPPiV. β -naphthylamine was estimated fluorimetrically using the Bio-Tek

FL600FA Microplate Fluorescence/Absorbance Reader, at 460/40 nm emission wavelength and 360/40 nm excitation wavelength. The values of incubates at zero time (blank) was subtracted and the relative fluorescence was converted to picomoles of β -naphthylamine by comparison with a correspondent standard curve. Peptidases activities were expressed as picomoles of hydrolyzed

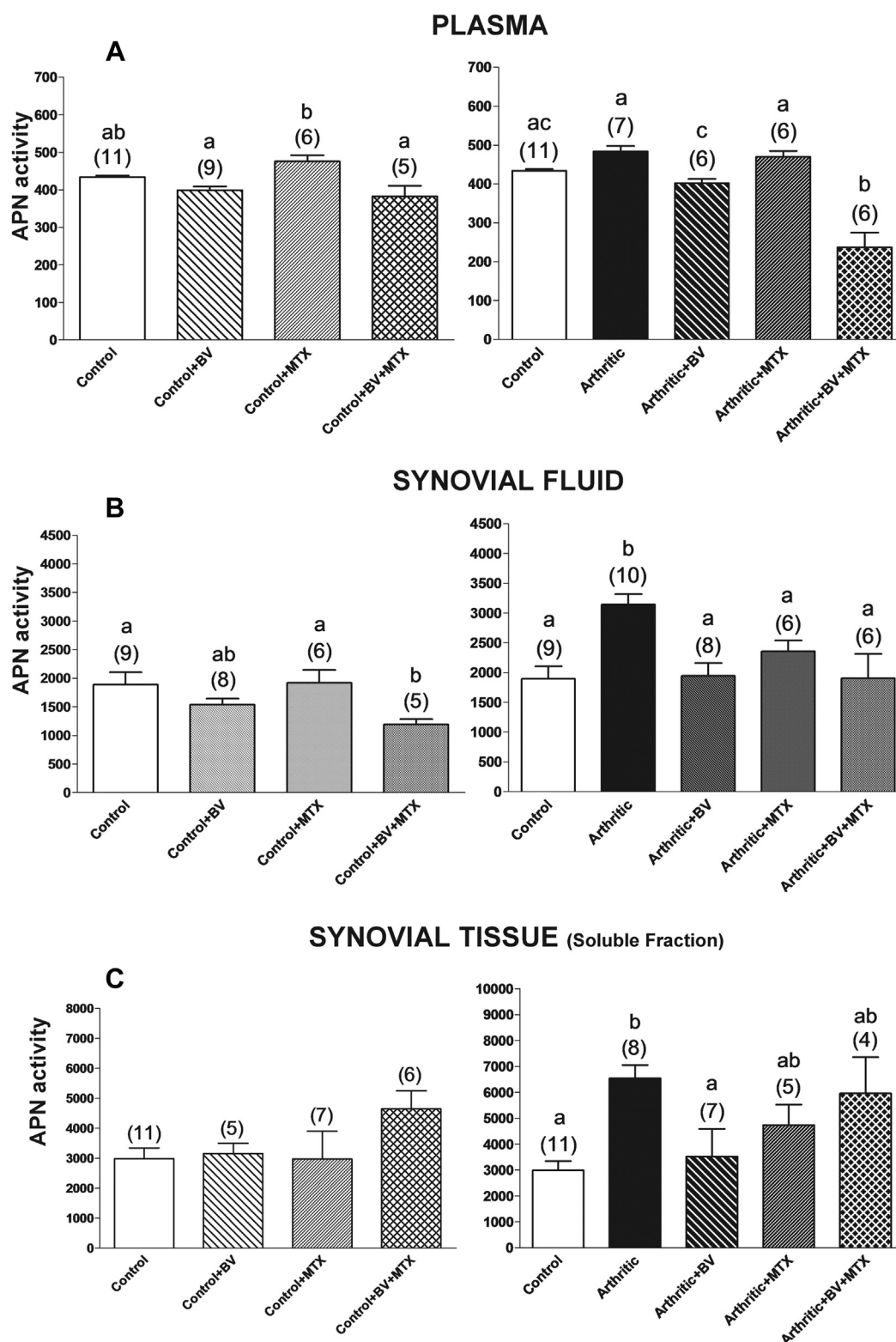


Fig. 5. Neutral aminopeptidase (APN) activity (pmoles hydrolyzed substrate/min/mg protein). Animals are treated with saline (control and arthritic), or bee venom (BV) and/or methotrexate (MTX). Values are means \pm S.E.M. Number of animals in parenthesis over the bars. ANOVA Control vs Control + BV vs Control + MTX vs Control + BV + MTX (left) and Control vs Arthritic vs Arthritic + BV vs Arthritic + MTX vs Arthritic + BV + MTX (right): 5(A): left $P < 0.0003$, right $P < 0.0001$; 5(B): left $P < 0.0003$, right $P < 0.0001$; 5(C): left $P < 0.1999$, right $P < 0.004$; 5(D): left $P = 0.003$, right $P < 0.002$; 4(E): left $P < 0.0001$, right $P < 0.008$; 4(F): left $P < 0.008$, right $P < 0.0002$. Post hoc Tukey's multiple comparison test, different letters indicate significant differences among the bars in each graph ($P < 0.05$).

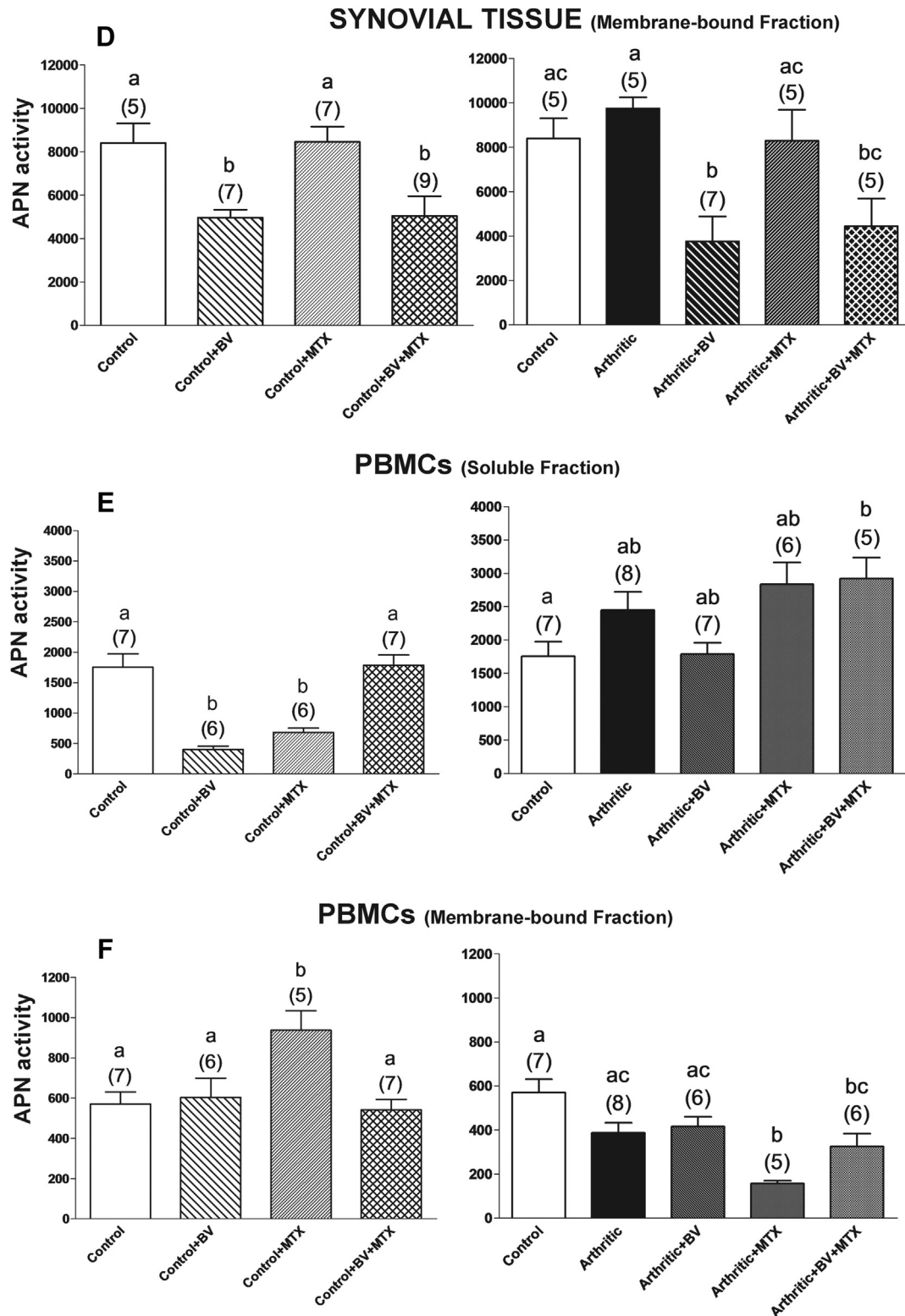


Fig. 5. (continued).

substrate $\text{min}^{-1} \text{mg protein}^{-1}$.

2.7. Statistics

Data are presented as mean values \pm standard error of the means (S.E.M.) for n individual experiments or n animals, and were

analyzed statistically using the GraphPadPrism™ software package. Regression analyses were performed to obtain standard curves. One-way analysis of variance (ANOVA) followed, when differences were detected, by the Tukey multiple comparison test to compare values among three or more variables. In all the calculations, a minimum critical level of $P < 0.05$ was set.

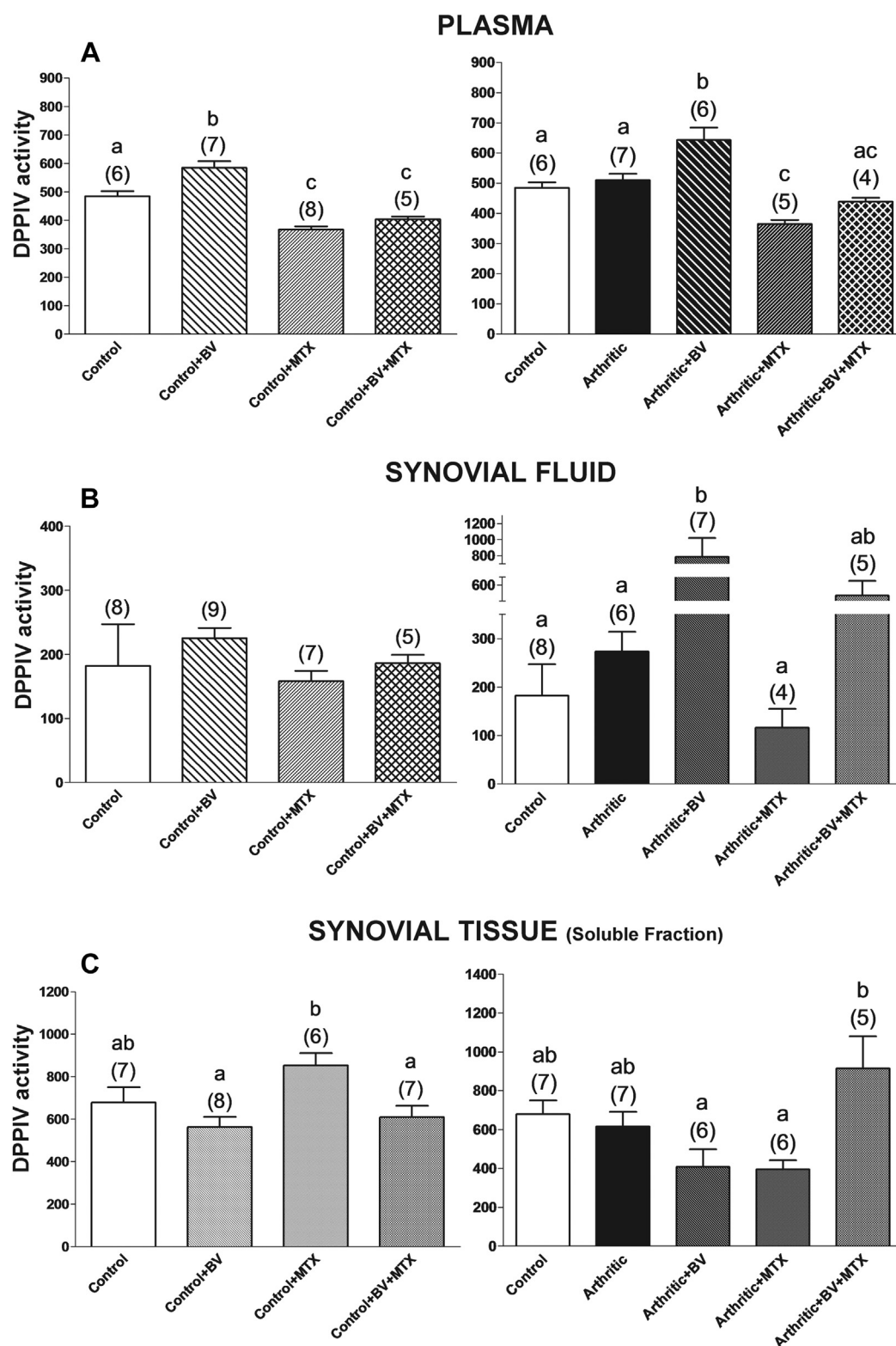


Fig. 6. Dipeptidyl peptidase IV (DPPiV) activity (pmoles hydrolyzed substrate/min/mg protein). Animals are treated with saline (control and arthritic), or bee venom (BV) and/or methotrexate (MTX). Values are means \pm S.E.M. Number of animals in parenthesis over the bars. ANOVA Control vs Control + BV vs Control + MTX vs Control + BV + MTX (left) and Control vs Arthritic vs Arthritic + BV vs Arthritic + MTX vs Arthritic + BV + MTX (right): 6(A): left and right $P < 0.0001$; 6(B): left $P < 0.6326$, right $P < 0.009$; 6(C): left $P < 0.02$, right $P < 0.004$; 6(D): left $P < 0.0001$, right $P < 0.003$; 6(E): left $P < 0.001$, right $P = 0.431$; 6(F): left $P < 0.0007$, right $P < 0.0001$. Post hoc Tukey's multiple comparison test, different letters indicate significant differences among the bars in each graph ($P < 0.05$).

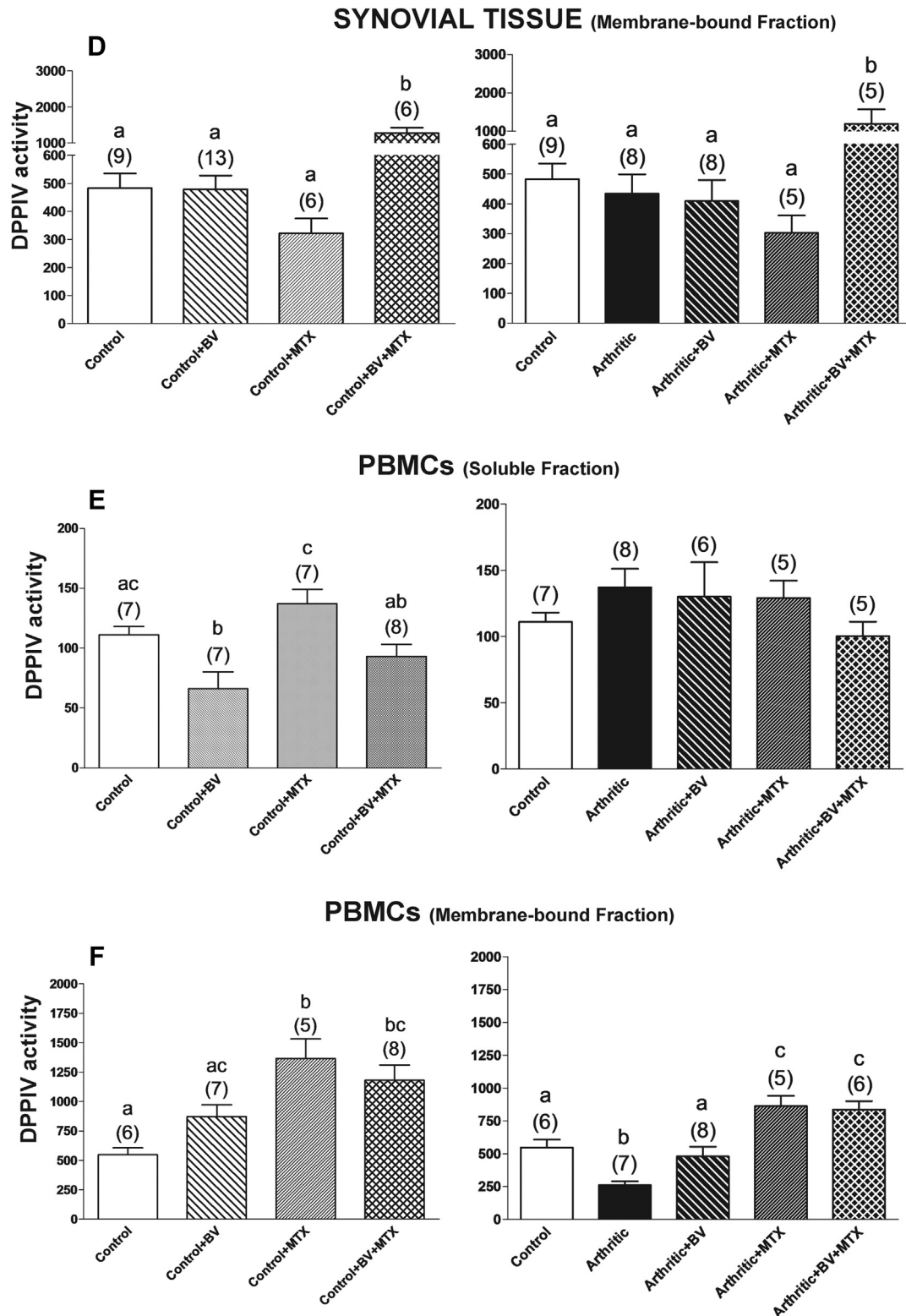


Fig. 6. (continued).

3. Results

3.1. BM gain

On day 41 after induction/sham induction, there were no significant differences of BM values among healthy groups (ANOVA,

$P = 0.4403$). The values are: 370 ± 11 ($n = 10$) (control); 376 ± 10 ($n = 9$) (control + BV); 399 ± 8 ($n = 9$) (control + MTX); and 387 ± 18 ($n = 11$) (control + BV + MTX). As shown in Fig. 1, on day 41 after induction/sham induction percent BM gain was about 33% lower in arthritic individuals than in control ones. MTX ameliorates and BV fully preserves BM gain at control levels in arthritic rats. The

Table 1

Overall summary of alterations detected in all treatments compared with control treated with saline.

Parameter	Sample	Control			Arthritic			
		BV	MTX	BV + MTX	Vehicle	BV	MTX	BV + MTX
Body mass		=	=	=	↓	=	=	=
Edema		=	=	=	↑	↑	↑	↑
AACII		=	=	=	↑	↑	↑	↑
TNF- α		=	=	=	↑	=	=	=
Hyperalgesia		=	=	=	↓	=	=	↓
APB	Plasma	↓	↓	↓	=	=	=	↓
	Synovial fluid	↓	↓	↓	=	=	↓	=
	S-synovial tissue	↓	↑	=	=	=	=	=
	M-synovial tissue	=	=	=	=	=	=	=
	S-PBMCs	↓	↓	↓	↓	↓	↓	↓
	M-PBMCs	=	↑	=	=	=	=	=
APN	Plasma	=	=	=	=	=	=	↓
	Synovial fluid	=	=	↓	↑	=	=	=
	S-synovial tissue	=	=	=	↑	=	=	=
	M-synovial tissue	↓	=	↓	=	↓	=	=
	S-PBMCs	↓	↓	=	=	=	=	↑
	M-PBMCs	=	↑	=	=	=	↓	↓
DPPIV	Plasma	↑	↓	↓	=	↑	↓	=
	Synovial fluid	=	=	=	=	↑	=	=
	S-synovial tissue	=	=	=	=	=	=	=
	M-synovial tissue	=	=	↑	=	=	=	↑
	S-PBMCs	↓	=	=	=	=	=	=
	M-PBMCs	=	↑	↑	↓	=	↑	↑

(=) equal; (↓) decrease; (↑) increase.

combined treatment is ineffective against reduction of the BM gain.

3.2. Hind paw thickness

Basal dorsal-plantar thickness (in mm) of hind paws was 4.26 ± 0.08 ($n = 39$). On day 21 after sham induction healthy animals had thickness of 5.56 ± 0.1 ($n = 37$) (control), while the animals submitted to CIA induction had thickness of 8.40 ± 0.40 ($n = 31$). On day 41 after sham induction healthy animals untreated or treated with MTX and/or BV had similar dorsal-plantar thickness than untreated healthy animals on day 21 (ANOVA, $P = 0.4945$). The values were: 5.50 ± 0.04 ($n = 8$) (untreated control); 5.59 ± 0.09 ($n = 9$) (control + BV); 5.69 ± 0.06 ($n = 9$) (control + MTX); and 5.54 ± 0.05 ($n = 11$) (control + BV + MTX). As shown in Fig. 2, on day 41 the presence of edema persisted in all sick groups, but its level was ameliorated with MTX or BV treatment. The combined treatment is ineffective against the edema.

3.3. AACII and TNF- α

AACII titer varies from 0.015 to 0.039 $\mu\text{g/mL}$ among control ($n = 8$), control + BV ($n = 8$), control + MTX ($n = 5$) and control + BV + MTX ($n = 7$), and from 186 to 193 $\mu\text{g/mL}$ among arthritic ($n = 9$), arthritic + BV ($n = 6$), arthritic + MTX ($n = 5$), arthritic + BV + MTX ($n = 6$). Grouping these animals to form only two groups, control and arthritic ones, the intra-group changes based on the applied treatments are not statistically significant (ANOVA, $P > 0.05$), and the comparison between control (mean \pm S.E.M., $n = 28$, 0.04 ± 0.01) and arthritic (mean \pm S.E.M., $n = 26$, 186 ± 6) groups reveals a very significant difference (unpaired two-side Student's t test, $P < 0.0001$). The presence of TNF- α is found only in untreated arthritic (21.1 ± 1.2 pg/mL , $n = 4$), being below the threshold of detection (<4 pg/mL , $n = 17$) in the other groups under study (control, control + BV, control + MTX, control + BV + MTX, arthritic + BV, arthritic + MTX and arthritic + BV + MTX) (ANOVA, $P < 0.0001$).

3.4. Hyperalgesia

The values of the mechanical threshold of hyperalgesia among healthy animals show no significant differences (ANOVA, $P = 0.7535$). The values of this parameter (g) are: 46 ± 2 ($n = 4$) (control); 45 ± 3 ($n = 5$) (control + BV); 45 ± 1 ($n = 3$) (control + MTX); and 45 ± 1 ($n = 3$) (control + BV + MTX). Reduced mechanical threshold of hyperalgesia persists in all arthritic groups, but with amelioration in relation to untreated arthritic individuals (11 ± 1.30 , $n = 4$), in Arthritic + MTX (22 ± 1.70 , $n = 3$) and Arthritic + BV + MTX (23.5 ± 2.20 , $n = 6$) (Fig. 3).

3.5. Peptidase activities

3.5.1. Screening of APB, APN and DPPIV activities in BV and MTX

MTX at used concentrations has no APN, APB and DPPIV activities, while BV has only DPPIV activity ranging between 1.1 and 2.8 pmoles of hydrolyzed substrate per min per μg of BV (containing 0.05–0.199 mg of protein/mL).

3.5.2. Changes in plasma, SF and S and M from ST and PBMCs

In healthy rats BV and MTX decrease APB in plasma (Fig. 4-A), synovial fluid (Fig. 4-B) and soluble fraction of PBMCs (Fig. 4-E). BV decreases APN in solubilized membrane-bound fraction of synovial tissue (Fig. 5-D). BV decreases and MTX increases APB in soluble fraction of synovial tissue (Fig. 4-C). BV and MTX decrease APN in soluble fraction of PBMCs (Fig. 5-E) and MTX increases APN in solubilized membrane-bound fraction of PBMCs (Fig. 5-F). DPPIV is increased in plasma (Fig. 6-A) and decreased in soluble fraction of PBMCs (Fig. 6-E) by BV, while DPPIV is decreased in plasma (Fig. 6-A) and increased in solubilized membrane-bound fraction of PBMCs (Fig. 6-F) by MTX. Arthritic rats are characterized by decreased APB in soluble fraction of PBMCs (Fig. 4-E), increased APN in synovial fluid (Fig. 4-B) and soluble fraction of synovial tissue (Fig. 4-C), and decreased DPPIV in solubilized membrane-bound fraction of PBMCs (Fig. 4-F). In arthritic rats BV restores APN in synovial fluid

Table 2
Overall summary of effects of BV and MTX on altered parameters in arthritic.

Parameter	Sample	Arthritic treated with		
		BV	MTX	BV + MTX
body mass		R	A	U
edema		A	A	U
AACII		U	U	U
TNF- α		R	R	R
hyperalgesia		U	A	A
APB	S-PBMCs	U	A	U
APN	synovial fluid	R	R	R
	S-synovial tissue	R	A	A
DPPIV	M-PBMCs	R	A	A

R = restore to values of control treated with saline.

A = ameliorate in relation to control treated with saline.

U = unaltered in relation to arthritic treated with saline.

(Fig. 5-B) and soluble fraction of synovial tissue (Fig. 5-C), and DPPIV in solubilized membrane-bound fraction of PBMCs (Fig. 6-F). MTX restores APN in synovial fluid (Fig. 5-B), and ameliorates APB in soluble fraction of PBMCs (Fig. 4-E), and DPPIV in solubilized membrane-bound fraction of PBMCs (Fig. 6-F). Other changes or the absence of those changes occur when certain groups are compared, but besides the above mentioned, none of these changes occurred in relation to control or arthritic groups (Figs. 4-D, 4-F, 5-A, 5-C, 6-B, 6-C, 6-D).

4. Discussion

The comparison between BV and MTX effects on key aspects of experimental arthritis, as well as the possibility that these effects may be related with APB, APN and DPPIV have not been addressed until now. We demonstrated that BV and MTX act similarly ameliorating or restoring compartmentalized peptidase activities (with exception of APB) that are altered in arthritis. Mainly, MTX normalized TNF- α and ameliorated BM gain and attenuated hyperalgesia and swelling. In addition, we provide evidence that BV also attenuated swelling, restored BM gain, inhibited TNF- α , besides the fact that it possesses DPPIV activity. Acting on activity levels of APN and DPPIV, MTX and BV downregulate some pro-inflammatory factors, thus inducing the anti-edematogenic and anti reduction of BM gain responses. Furthermore, the antinociceptive effect and amelioration of activity levels of APB were concomitantly induced by MTX. Therefore, the contribution of APB to antinociceptive effect of MTX can be hypothesized. Since edema and BM gain with combined treatment with MTX and BV did not improve, their concomitant use cannot be recommended. These findings contribute to novel insights to explain mechanisms and targets of MTX and BV, administered by non-acupoint, in arthritis. Data also show that MTX and BV, alone or associated, were not innocuous on peptidases under study in healthy animals. The deleterious or beneficial impact of altered peptidase by BV and MTX in healthy subjects is difficult to deduce, since these peptidases play multiple roles, such as regulation of hormones, cytokines and peptides relevant to cell proliferation, adhesion, cell signaling, cell activation, differentiation and cell communication. The results are summarized in Tables 1 and 2.

4.1. Dosage and route of administration for BV and MTX

0.25 mg BV/kg by acupoint is known to promote a decrease in hyperalgesia (Roh et al., 2004; Baek et al., 2006). It was administered here by s.c. dorsal route, since systemic effects can be evaluated through this route without interference of mapped acupoint. MTX, at same dose and i.p. route ameliorated histopathological

injuries in CIA rats (Mirshafiey et al., 2006). A dose of 0.33 mg MTX/kg, with a two-day interval along four weeks, was also beneficial (Xinqiang et al., 2010), while 0.75 mg MTX/kg, administered daily during one week, promoted defects on bone formation (Xian et al., 2007). The rationality for using BV combined with MTX in this study was to evaluate the possible synergism, potentiation or antagonisms given that a significant number of patients treated with BV are concomitantly treated with MTX.

4.2. MTX and BV on altered parameters of arthritic

4.2.1. BM gain

The cachexia is a common occurrence in RA and could be symptomatic of an inability to eat due to pain (Hartog et al., 2009; Roubenoff, 2009). In the arthritis model in rodents a loss of BM is frequent (Simjee et al., 2007; Hartog et al., 2009), and we now demonstrate the amelioration of BM gain by MTX and its restoration by BV. MTX diminishes TNF- α via cAMP (Wessels et al., 2008), and the whole crude BV, or its isolated component, mellitin, inhibits nuclear factor κ -light chain-enhancer of activated B cells (NF κ B) transcription (Park et al., 2004), which in turn is responsible for TNF- α transcription. BV and MTX action on recovery of BM gain is probably due to TNF- α and another unknown factor(s), since only anti-TNF- α therapy was reported as insufficient to promote this effect (Roubenoff, 2009). Aminopeptidases related with energy balance (Zambotti-Villela et al., 2008; Alpointi and Silveira, 2010), that are altered in arthritic individuals and ameliorated by MTX and BV, could help to promote this benefit. However, this effect against reduction of BM gain disappears when using BV combined with MTX. A tentative explanation of this finding is outlined below.

4.2.2. Edema, AACII, TNF- α and hyperalgesia

Anti-edematogenic effect of BV and MTX confirmed literature data (Cutolo et al., 2001; Saad et al., 2010). On the other hand,

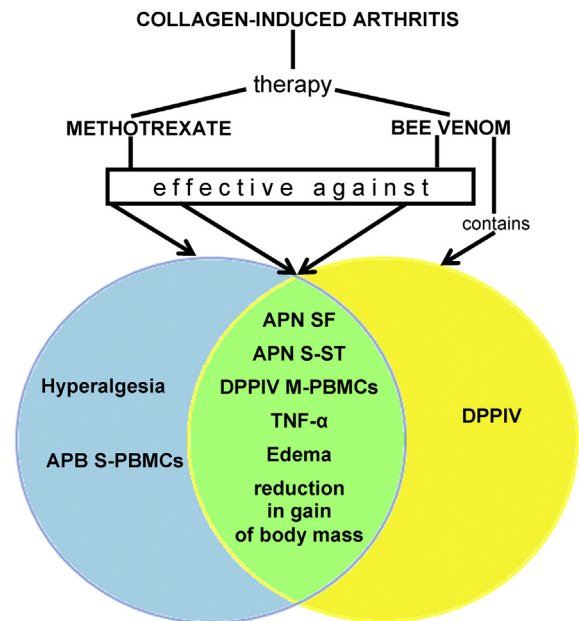


Fig. 7. Schematic depicting proper and shared effects of BV and MTX on key aspects of CIA model. MTX, associated or not with BV, normalizes TNF- α , and attenuates body mass (BM) and hyperalgesia. MTX and BV alone attenuate the edema and normalize APN of synovial fluid (SF). MTX ameliorates APB of soluble fraction (S) from PBMCs. MTX ameliorates and BV restores APN in S from synovial tissue (ST) and DPPIV in solubilized membrane-bound fraction (M) from PBMCs. BV attenuates BM and normalizes TNF- α independently of MTX. BV is a source for obtaining DPPIV enzyme.

regarding only BM gain, this effect disappears when BV combined with MTX was used. To our knowledge there is no previous study that supports the existence of anti-inflammatory or stabilizer of BM gain effects caused by different agents that cease to exist by combined use of these same agents. However, desensitization and interaction are relatively common pharmacological phenomena that should be investigated in this case as possible causes. Painful stress exists at the time of BV's administration (Stojanovich, 2010), but it is difficult to infer its involvement in this disruption cause. The demonstration that the anti-edematogenic effect of MTX (and BV) is unrelated to AACII titer in arthritic (Tables 1–2) was remarkable. It reinforces the multifactorial ethiology of RA, as well as the hypothesis that B cell response in CIA occurs against different epitopes of the triple helix of CII (Nandakumar, 2010), and that CII is immunoreactive to varying degrees of pathogenic and non-pathogenic antibodies (Mendes et al., 2011). The treatments of arthritic with BV, MTX or a combination of the two were efficient to reduce TNF- α . Despite the well-known relationship of TNF- α with the improvement of hyperalgesia and/or edema (Simjee et al., 2007; Wood, 2009), an action upon TNF- α is not the sole responsible for the antinociceptive effect of MTX, since it was absent in BV. Patients with RA often have a higher perception of pain (Wood, 2009). Arthritic CIA mice have higher level of nociception than healthy mice, and treatment with MTX was previously reported to cause slight yet significant improvement (Al-Abd et al., 2010), findings that were confirmed here in CIA rats. While MTX alone or in combination with BV significantly reduced the arthritis-associated hyperalgesia, BV administered on its own did not cause this effect, showing that it is only attributable to MTX. The whole crude BV was reported to have analgesic properties at higher doses, 1 mg/kg by acupoint (Kwon et al., 2001) or 0.8–1.2 mg/kg s.c. (Chen et al., 2006). Unfortunately, there are still no uniform guidelines for BV standardization. Furthermore, the dose control and administration route present challenges to the experimental and therapeutical use of a protein/peptide mixture as BV, given the known heterogeneity in the crude venom's composition (Peiren et al., 2005). Additionally, the most serious limitation for the therapeutic use of whole BV seems to be that 20.7% of the overall population develops a hypersensitivity reaction of type 1, which may result in local skin edema, or in more severe cases, to life threatening systemic anaphylactic shock (Peiren et al., 2005). Furthermore, impaired anti-edematogenic and stabilizer of BM gain responses of MTX and BV when administered in association are also important restrictions to indicate the combined therapeutic use.

4.2.3. Peptidase activities

The present study highlights changes on peptidases that might have major pathophysiological significance. The existence of DPPIV in BV (Lee et al., 2007) was confirmed in the present study. Thus, the increased DPPIV in plasma of control and arthritic individuals, and in SF of arthritic rats could be explained by this fact. Considering this exception, BV and MTX had marked effects on altered peptidases in arthritic rats. In general, the combination therapy of MTX and BV does not overcome the effects of BV or MTX alone on the peptidase activities. Altered APB could alter antigen processing (Hattori and Tsujimoto, 2004) and formation of angiotensin (Ang) IV (Martínez-Martos et al., 2011), a peptide related to renin-Ang system (RAS) that causes upregulation of genes associated with NF κ B (Ruiz-Ortega et al., 2007). RAS is strongly involved in increased vascular oxidative stress and endothelial dysfunction in animal models of inflammatory and autoimmune diseases (Sakuta et al., 2010). Furthermore, two zinc-dependent metallopeptidases (Penning et al., 2002) are known to concomitantly exhibit APB and leukotriene-A4 hydrolase (LTA4H) activities (Mantle et al., 1999; Mendes and Silveira, 2013). Recently, APB was reported to

facilitate the resolution of inflammation by hydrolyzing neutrophil chemoattractant Pro-Gly-Pro (PGP) (Snelgrove et al., 2010). Acting on APB, MTX can upregulate NF κ B and RAS, via Ang IV (Cutolo et al., 2001), and LTA4H, via bifunctionality, can also downregulate neutrophil chemoattraction, via PGP (Snelgrove et al., 2010). APN hydrolyses Ang III, forming Ang IV (Ruiz-Ortega et al., 2007), but altered levels of APN were restored by BV without concomitant amelioration of pain. Thus, both effects of MTX, the antinociceptive and APB enhancer in S-PBMCs, should be correlated only through their indirect consequences on LTA4H and PGP. Thus, the amelioration of algnesia caused by MTX might be partly attributed to its anti-inflammatory effect, but both effects are not associated only with the effects upon APN and DPPIV, which were shared with BV. Immunohistochemical evidences have shown an increase of APN in blood vessels of ST in RA patients (Haringman et al., 2006) and thus they have started an intense search for inhibitors for APN and for dual inhibitors for APN and DPPIV (Reinhold et al., 2007; Thanawala et al., 2008) that have already been reported to be beneficial for treatment of autoimmune diseases (Reinhold et al., 2007; Ansorge et al., 2009). A major constraint to interpret these data is that aminopeptidases have many overlapping activities on synthetic substrates and there may be differences in the level of enzymatic activity according to the substrate used (Olivo et al., 2005). Another obvious aggravating issue is that only the immunohistochemical detection is not sufficiently indicative of the existence of enzymatic activity. Regarding DPPIV activity, its increase in blood cells of arthritic individuals has been reported (Sromova et al., 2010), but little is known about its changes under treatment with MTX. Elicited peritoneal macrophages from healthy mice treated with MTX have higher DPPIV than resident macrophages (Olivo et al., 2008). In the present study, in arthritic and healthy individuals treated with MTX, DPPIV increased in M-PBMCs and decreased in plasma. The combined effects of BV on DPPIV and the impaired anti-edematogenic and anti reduction of BM gain responses of MTX and BV when those drugs are associated strongly suggest that increased plasma cytokines and proinflammatory chemokines in arthritis (Pavkova et al., 2012), which are reported to be substrates of DPPIV (de Meester et al., 2003), might be regulated by MTX and BV not only through DPPIV. Plasma levels of these cytokines and chemokines, when kept under control by MTX or BV, might lead to decreased cell proliferation (except PBMCs) that release DPPIV in plasma. Increased number of peripheral blood T lymphocytes was found in RA (Ospelt et al., 2010), and DPPIV was reported to be secreted by these activated T cells after induction of arthritis (Williams et al., 2003). MTX and BV might also have direct cytotoxic effect on these cells. Another relationship among MTX and DPPIV, that seems unlikely in this case, is via adenosine deaminase (ADA). DPPIV of various mammalian species when coupled to the ADA makes it more effective (Sedo et al., 2005) and MTX inhibits ADA (Cutolo et al., 2001), but rat DPPIV does not exhibit this binding ability (Iwaki-Egawa et al., 1997).

5. Concluding remarks

As illustrated in Fig. 7, many beneficial effects of BV by non-acupoint in arthritic individuals are shared with MTX, but they are insufficient to relieve pain. Among examined peptidases, APN in synovial fluid and soluble fraction from synovial tissue and DPPIV in membrane-bound-fraction from PBMCs could be related to anti-edematogenic and anti reduction of BM gain effects of BV and MTX, but only APB in soluble fraction from PBMCs could be a possible target of MTX effects associated with pain relief. It is noteworthy that BV by non-acupoint exhibits against reduction of BM gain, as well as being anti-edematogenic and anti-TNF- α in arthritic, and a source for obtaining DPPIV enzyme.

Ethical statement

We declare that the work described has not been published previously (except in the form of an abstract or as part of a published lecture or academic thesis), that it is not under consideration for publication elsewhere, that the animal protocols have been reviewed and approved by the appropriate ethics committees, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright-holder.

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