ORIGINAL RESEARCH

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The Effects of the Angiotensin Converting Enzyme Inhibitor Enalapril and the Angiotensin II Type 1 Receptor Blocker Losartan on Fracture Healing in Rats

Abstract

Purpose: Angiotensin converting enzyme inhibitors (ACEI) and type I angiotensin receptor blockers (ARB) have been shown to exert significant effects on bone tissue via a local renin-angiotensin-aldosterone system (RAS). The aim of our study was to delineate their influences on fracture healing process.

Methods: Sixty adult male Wistar Albino rats were divided into three groups. After undergoing surgical femoral fracture and fixation, the ACEI group received 10 mg/kg of Enalapril, the ARB group received 10 mg/kg of Losartan and the Control group did not receive any medication. Fracture healing was evaluated at second and fifth postoperative weeks by the Lane-Sandhu radiological staging system and by histological scoring system of Huo *et al.* ACE expression in fracture callus was studied by immunohistochemistry.

Results: Both ACEI and ARB groups showed less fibrous tissue in the fracture area at the second week, but the histologic score differences were significant only between Control and ARB groups. At the fifth week, however, both radiological and histological scores for the ACEI group were significantly higher than both ARB and Control groups, while the scores for ARB and Control groups were similar. The presence of ACE expression in fracture callus was also observed.

Conclusion: ACEIs had significant positive effects on fracture repair. Losartan failed to display these stimulatory effects, which suggests that local RAS in bone tissue exerts its actions via alternative receptors or pathways than the AT1 receptor.

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In addition to the classical understanding of the renin-angiotensin-aldosterone system (RAS), in that RAS is a circulating endocrine system that controls blood pressure changes[1], local RASs have been identified in many organs including heart, kidney, bone marrow and blood vessels[2] and display local effects like mediating inflammation, angiogenesis, cell proliferation and apoptosis[3].

The detection of expression of angiotensin-converting enzyme (ACE) and type 1 and type 2 angiotensin II receptors (AT1 and AT2) in cell cultures and *in vivo* studies has proven the presence of components of a local RAS in bone tissue[4,5,6,7,8]. This awareness prompted researchers to study the effects of widely-used RAS inhibiting drugs, especially ACE inhibitors (ACEIs) and type 1 angiotensin receptor blockers (ARBs), on bone mass. The results were contradictory but the general assumption is that angiotensin II (Ang II) exerts its detrimental effects on bone by 1) regulating blood flow in the bone marrow, which increases bone resorption, 2) activating osteoclasts via the AT1 receptor on osteoblasts and 3) affecting calcium metabolism by lowering ionized calcium and increasing parathyroid hormone levels[9].

The possible effect of this local RAS on bone tissue during fracture healing is a more recent question. Garcia *et al.*[8] have found that ACEIs have a stimulatory effect on fracture union and periosteal callus formation. They also showed the expression of RAS components for the first time during fracture healing, and that ACEIs decreased fibrosis in the early phase of a fracture. Based on these findings, we have designed a study to compare the influences of ACEIs and ARBs on fracture healing and hypothesized that, owing to the substantial data that AngII exerts its effects on bone mainly via AT1 receptor[6,7,10,11,12], ARBs should be as successful as ACEIs for stimulating fracture healing.

Methods

This experimental study was carried out at the Bülent Ecevit University (Zonguldak, Turkey) animal research laboratories, after the approval of the Ethics Committee for Animal Research. Sixty adult male Wistar Albino rats, with an average weight of 300 grams, were used. The rats were divided into three groups: ACEI Group, ARB Group and Control Group; each consisting of 20 rats. All rats were anesthetized by 50mg/kg of Ketamine HCl (Ketalar, Pfizer, USA), injected intraperitoneally, prior to surgery. Rats were placed in right lateral decubitus position and a surgical transverse osteotomy was performed to their left femora with an osteotome, which were then fixed by single intramedullary Kirschner wires. Fifteen

mg/kg of Tramadol (Ultrameks, ADEKA, Turkey) was used for postoperative analgesia. After the procedure, rats were put into their own separate cages with no restriction of weight-bearing. While the rats in ACEI Group received 10 mg/kg Enalapril (Enapril, Sandoz, Turkey), rats in ARB Group received 10 mg/kg of Losartan (Cozaar, Merck, USA), which are prepared daily as 30 ml solutions for each of the rats as their initial drinking water. After this solution had been finished, they were allowed water *ad libitum*. Rats in the Control Group did not receive any further drugs.

Rats were sacrificed by cervical dislocation at the second postoperative week. After X-rays had been taken, specimens were prepared for histologic examination. Remaining rats continued their drug regimen until they were sacrificed at the fifth week.

Radiological analyses were performed by two observers (both orthopedic surgeons), who were not otherwise involved in the study and who were blinded about the group assignments and the study weeks) according to the Lane-Sandhu Scoring System[13] as follows: 0-no callus tissue, fracture line clear; 1-25% callus tissue, fracture line still clearly visible; 2-50% callus tissue, fracture line blurred; 3-75% callus tissue, fracture line barely visible; and, 4-100% callus tissue, no remaining fracture line visible. The mean of the scores given by the two observers were accepted to be the final score.

For histological analysis, whole specimens were initially fixed in 10% formaldehyde for 2 weeks. Afterwards, samples were decalcification in 10% EDTA solution for 2 weeks. Samples were then embedded in paraffin blocks and longitudinal sections of 4-5 μ m of thickness were obtained from fracture zones. For routine light microscopic analyses, the sections were stained with Hematoxylin-Eosine and Masson's trichrome stains. Histological evaluation was performed according to fracture healing scoring system reported by Huo *et al.*[14] (Table 1).

For immunohistochemistry studies, paraffin sections (5 μ m) of the specimens were deparaffinized in xylene and rehydrated in graded alcohol. Antigen retrieval was performed by boiling sections in 0.1 M sodium citrate, pH 6.0, followed by 0.1% Triton X-100 PBS for 5 minutes. Slides were treated with H₂O₂ in deionized water for 10 minutes to quench endogenous peroxidase activity. After blocking with 10% normal serum for 1 hour at room temperature, sections were incubated with primary antibody at RT for 1 hour. The following primary antibody (1:100) was used: ACE (N-20; sc-12184, Santa Cruz Biotechnology Inc, Santa Cruz). The specimens were then incubated with biotinylated secondary antibody for 30 minutes at 37°C, followed by incubation with ABC reagent (VECTA-

SElite ABC kit; Vector Laboratories, Burlingame, CA) for 10 minutes at 37°C. Sections were developed using a DAB substrate kit (Vector Laboratories) for 5 minutes and counter-stained with hematoxylin.

Statistical analysis was done using SPSS for Windows version 19. Descriptive statistics were given as mean±standard deviation. Kruskal-Wallis variance analysis was used to determine the presence of differences among the three groups; after which the Mann-Whitney U test with Bonferroni correction was performed to compare the groups with each other. Significance was assigned to $p < 0.05$.

Results

Two rats from each of the three groups were excluded in first two weeks due to their death or implant failure and one rat from ARB group died between two and five weeks; so a total of seven rats out of 60 were lost during the study period. The study was completed with 53 rats, of which 24 were sacrificed at the second week and 29 at the fifth week.

Radiologic studies at the second week revealed that some samples of the ACEI and ARB groups had early signs of callus formation with apparent fracture lines, while most specimens of the Control group did not display any callus at all. Radiological scores at the 2nd week are depicted in Table 2. There was no significant difference among scores of groups at the 2nd week ($p = 0.334$). At the 5th week following fracture, healing was most prominent in the ACEI group with disappearance of

fracture lines in most samples. Radiological scores at the 5th week are shown in Table 3. There was a significant difference among the groups at the 5th week ($p = 0.001$). The score of the ACEI group was found to be significantly higher than both the Control ($p = 0.001$) and ARB ($p = 0.006$) groups, while the scores of Control and ARB groups were similar ($p = 0.356$).

Histologic scores at the 2nd week are given in Table 4. The difference among the groups was found to be significant ($p = 0.015$). Both ARB and ACE groups showed less fibrous tissue at the 2nd week, but the difference was significant only between histologic scores of Control and ARB groups, with the Control group having the lower score ($p = 0.007$). The scores at the 5th week are shown in Table 5. There was also significant differences in the histologic results among the three groups at the 5th week ($p = 0.001$): the ACEI group showed significantly higher mean scores than both the Control ($p < 0.001$) and ARB ($p = 0.016$) groups, while the scores of the Control and ARB groups were similar. Typical histologic appearances of the groups at 2nd and 5th weeks are shown in Figure 1. Immunohistochemistry studies revealed expression of ACE in the fracture calluses (Figure 2).

Discussion

This study showed that, the ACEI drug, Enalapril, exerted substantial positive effects on the fracture healing process; however, contrary to our hypothesis, the ARB drug, Losartan, failed to exhibit comparable inductive effects.

The role of RAS components in bone tissue and the effects of the drugs that inhibit the action of Ang II on bone physiology has been a matter of interest for the last two decades; however, the consequences of using these drugs fracture repair have not been widely studied. Garcia *et al.* [8] were the first to show that ACEIs improved fracture healing and periosteal callus formation. Our study is the first to determine the effects of ARBs on fracture healing and to compare the effects of ARBs with those of ACEIs.

TABLE 1. Scoring system for histological evaluation of fracture healing

Score	Histological findings
Grade 1	Fibrous tissue
Grade 2	Mainly fibrous and less cartilage tissue
Grade 3	Equal fibrous and cartilage tissue
Grade 4	Cartilage tissue
Grade 5	Mainly cartilage and less immature bone tissue
Grade 6	Equal cartilage and immature bone tissue
Grade 7	Mainly immature bone and less cartilage tissue
Grade 8	Whole immature bone
Grade 9	Immature bone and less mature bone
Grade 10	Mature (lamellar) bone

From Huo *et al.*, [14]

TABLE 2. Radiological scores at the 2nd postoperative week.

	Group Control (n=8)	Group ACEI (n=8)	Group ARB (n=8)
Radiological Scores (Mean±SD)	0.63±0.74	1.0±0.76	1.13±0.64
p*	0.334		

* Significance levels with Kruskal-Wallis test

Various antihypertensive drugs have been studied for their effects on bone metabolism, based on the knowledge that hypertension has detrimental effects on bone physiology[15]. Thiazide diuretics were shown to decrease calciuria[16] and fracture risk in elderly women[17]. β -blockers were also reported to decrease osteoporotic fractures[18,19]. Despite some early reports stating that Ang II has stimulatory effects on osteoblasts[5,20], it is generally been shown to have negative effects on bone structure. It has been shown to be a potent stimulator of osteoclastic bone resorption[4,6] and to decrease osteoblastic cell differentiation, osteocalcin mRNA synthesis, alkaline phosphatase activity and calcium precipitation in osteoblasts[11]. The study of the potential effects of Ang II and

its inhibitors on bone mineral density (BMD) has been an area of intense interest and has yielded conflicting results. While Losartan increased bone mass and strength in ovariectomized osteoporotic female rat femurs[12], it failed to improve bone mass loss in orchietomized male rats[21]. Broulík *et al.* found that the use of Losartan and Enalapril was not associated with increased bone mass in intact female rats [22]. Izueta *et al.*[7]found that Losartan did not improve bone mass in mice, but that a positive association was seen with the use of Type II ARB. Lynn *et al.*[9]reported that ACEI use was associated with higher BMD in Chinese elderly; however, this association could not be shown in another large prospective cohort, which indicated that ACEI use caused a slight but significant bone

TABLE 3. Radiological scores at the 5th postoperative week.

	Group Control (n=10)	Group ACEI (n=10)	Group ARB (n=9)	P** Control- ACEI	P** Control- ARB	P** ACEI- ARB
Radiological Scores (Mean $\pm$ SD)	2.00 $\pm$ 0.67	3.50 $\pm$ 0.71	2.33 $\pm$ 0.71	0.001	0.356	0.006
P*	0.001					

* Significance levels with Kruskal-Wallis test

** Significance levels with Bonferroni corrected Mann Whitney U test

TABLE 4. Histological scores at 2nd week.

	Group Control (n=8)	Group ACEI (n=8)	Group ARB (n=8)	P** Control- ACEI	P** Control- ARB	P** ACEI- ARB
Histological Scores (Mean $\pm$ SD)	1.38 $\pm$ 0.74	2.13 $\pm$ 0.83	2.63 $\pm$ 0.51	0.105	0.007	0.279
P*	0.015					

* Significance levels with Kruskal-Wallis test

** Significance levels with Bonferroni corrected Mann Whitney U test

TABLE 5. Histological scores at the 5th post-operative week.

	Group Control (n=10)	Group ACEI (n=10)	Group ARB (n=9)	P** Control- ACEI	P** Control- ARB	P** ACEI- ARB
Histological Scores (Mean $\pm$ SD)	5.00 $\pm$ 0.75	7.70 $\pm$ 0.48	5.89 $\pm$ 1.64	<0.001	0.195	0.016
P*	0.001					

* Significance levels with Kruskal-Wallis test

** Significance levels with Bonferroni-corrected Mann Whitney U test

FIGURE 1. Typical histological appearances of specimens from groups in second and fifth post-operative weeks with Masson's trichrome stain. The letter G and numbers stand for grades according to histological grading system of Huo *et al.* [14]: G1: Control group, second week, fibrous tissue. G2: ACEI group, second week, mainly fibrous and less cartilage tissue. G3: ARB group, second week equal fibrous and cartilage tissue. G5: Control group, fifth week, mainly cartilage and less immature bone tissue. G6: ARB group, fifth week, equal cartilage and immature bone tissue. G8: ACEI group, fifth week, whole immature bone. b: bone, ft: fibrous tissue, ct: cartilage, *: fracture area, ib: immature bone. Bar:200 µm.

FIGURE 2. Immunohistochemical analysis of angiotensin-converting enzyme (ACE) expression in femora of rats after fracture and stabilization. a) Sample from ACEI group at fifth week, which shows that ACE is expressed in osteoblasts (arrows) attached to trabecular bone close to fracture area. b) Negative control for ACE. There was no positive staining for ACE in osteoblasts (arrows). Bar:50 µm

loss[23].

Fracture healing involves the basic steps of initial inflammatory response, soft callus formation, which then turns into hard callus, and remodeling[24]. Transition from cartilaginous tissue to bone tissue is the key event and occurs by coordination of chondrocyte apoptosis, matrix degradation and removal, vascularization and osteogenesis[25]. ACEIs and ARBs have numerous effects in various systems that might influence the abovementioned steps of fracture healing. Of intense interest is their anti-inflammatory action, and this action may be the rationale for their use by protecting organs like the heart and kidneys[26,27]. In an animal experiment, 10mg/kg of Losartan decreased neutrophil migration, local cytokine production and leucocyte rolling and adhesion, thus ameliorating the local signs of inflammation in arthritis[28]. ARBs and ACEIs were also shown to decrease levels of IL-6, IL-8 and some other inflammatory cytokines[29,30,31]. RAS inhibitor drugs have been shown to have differential effects on metalloproteinases (MMPs)[32]. ACEIs, but not ARBs, were found to decrease levels of MMP 1, 2, 8 and 9[32,33,34,35]. ACEIs were shown to inhibit MMP activity directly and also through the induction of the synthesis of plasminogen activator inhibitor-1[32]. From the perspective of fracture healing, both the direct anti-inflammatory effects of ARBs and the MMP inhibitory effects of ACEIs would have been expected to be deleterious; but this was not the case -both for our study and for the report of Garcia *et al.*[8]. MMP activity has been shown to have pivotal importance in the endochondral phase of fracture

healing[24,36]. In animal studies, MMPs 9,13 and 14 were shown to be highly expressed during fracture healing, and stimulation of MMPs led to chondrocyte apoptosis, allowing the ossification process to take place[24,25,37,38]. The clinical study by Henle *et al.*[36] showed that MMP 1 and 2 increased during fracture healing and McDonald *et al.*[24] have suggested that although early fracture union may occur without osteoclast activity, MMP activity is of vital importance.

RAS inhibitors may affect fracture union by the reduction of fibrous tissue formation via the reversal of the fibrogenesis action of RAS in various systems [39,40,41,42]. ACE inhibition also increased serum levels of *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP), which has been shown to have a significant antifibrotic effect, regardless of its hemodynamic influences [43]. We agree with Garcia *et al.*[8] that administration of ACEIs resulted in less fibrous tissue formation in early fracture healing, and we also found that ARBs demonstrated this effect in the second week.

Another possible explanation for the improvement of fracture healing by ACEIs might be their ability to decrease apoptosis[3,18], which was also shown by decreased levels of cleaved caspase-3 in callus tissue[8]. ACEIs, but not ARBs, have also been shown to increase levels of bradykinin, leading to an increase in nitric oxide production, which causes arteriolar vasodilatation and increases vascular leakage[44].

Our study is one of the pioneering studies on the influence of RAS-inhibiting drugs on fracture healing. RAS-inhibiting drugs are widely used throughout the world with many indica-

tions. We have shown that ACEIs led to significant improvement in fracture union and callus formation. Rats in the ARB group, although they yielded better histological results and less fibrous tissue in the second week, failed to display an improved fracture union comparable to animals in the ACEI group. Although the scores of the ARB group were not as high as those of the ACEI group at 5th week, they were better than the controls; i.e., ARBs showed no detrimental effects on fracture healing.

This differential effect of both drugs should be emphasized, because most of the effects of Ang II on bone are believed to be carried out via the AT1 receptor[11,45]. Consistent with our finding that ACE is expressed in the fracture callus, Garcia *et al.*[8] have shown both the presence of AT1 and AT2 receptors and a higher expression of AT2 receptors after ACE inhibition, which may suggest a role of both receptors in fracture healing. Considering that inhibition of ACE and AT1 receptor yields variances in fracture union, unopposed action of Ang II, exerted either via AT2 receptor (like increased apoptosis [3,18,46]) or due to an alternative counter-regulatory mechanism, may have a decelerating effect on the process of fracture healing. Ang-(1-7)-Mas receptor axis, which showed increased expression after AT1 inhibition, has vasodilator, antiproliferative, anti-inflammatory and anti-fibrotic effects in different systems[47].

Izu *et al.*[7] have pointed out the significance of AT2 receptors *in vivo* and reported that inhibition of AT2 receptor both enhances osteoblastic activity and suppresses osteoclastic activity, which explain the difference between the actions of ACEI and ARB in our study.

Lack of biomechanical testing and measuring of mean arterial pressure (MAP) in rats are limitations of our study. Radiologically and histologically, we have shown that all specimens demonstrated healing by the 5th week of the study, with differences evident between groups, but measurement of maximal torque and torsional stiffness would have added useful data about fracture healing processes. MAP, on the other hand, was evaluated in the study of Garcia *et al.*[8] and they found decreased MAP in the ACEI-treated group. They had expected that decreased MAP would impair fracture healing, because of decreased interstitial blood flow, but their results showed the opposite.

In conclusion, our study has shown that ACEIs significantly stimulated fracture healing and that ARBs, although they did not adversely affect the union process, had only minor positive effects, including the reduction of fibrous tissue formation in the early phase. Although the exact mechanisms of the invigorating influences of ACEIs are not understood, the data

provided by this study will contribute to a more complete explanation of the effects of antihypertensive RAS-blocking drugs on bone repair. Given the huge number of fracture patients taking antihypertensive treatment, this subject warrants more detailed study. We suggest that, together with clinical studies in these patient groups, animal studies should be directed to the study of expression of anti-inflammatory actions of ACEIs, especially inhibition of various metalloproteinases, in fracture healing process.

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