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Biomechanical Effects of Angiotensin 1-7 in Diabetes Rats Femur

Efectos Biomecánicos de la Angiotensina 1-7 en el Fémur de Ratas Diabéticas

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SUMMARY: It is known that diabetes mellitus has late complications, including microvascular and macrovascular diseases. Diabetes can affect bones through biochemical markers of bone structure, density, and turnover. This study aimed to biomechanically investigate the bone-protective effects of angiotensin 1–7 (Ang 1-7), one of the active peptides in the renin-angiotensin system, in rats with diabetes. Thirty male Wistar albino rats, three months old and weighing 250–300 g, were divided into four groups: diabetes, Ang 1–7, diabetes plus Ang 1–7, and control. One month later, diabetes developed in rats; the rats were sacrificed, and their right femur was removed. Three-point bending biomechanical tests were performed on the femurs. The diabetic group had significantly higher bone fragility than the other groups (Pr >.05). Bone fragility was lower, and bone flexibility was higher in the Ang 1-7 groups (Pr>F value 0.05). As a result of our study, the effect of Ang 1–7 on the bones of rats with diabetes was investigated biomechanically. Ang 1–7 has a protective impact on the bones of rats with diabetes.

KEY WORDS: Diabetes mellitus; Angiotensin 1-7; Three point bending test; Bone; Renin-angiotensin system.

INTRODUCTION

Diabetes mellitus is a common disease across the World (Darenskaya *et al*., 2021). The studies show that oxidative stress increases in diabetes, which may lead to long-term complications observed in diabetic patients (Mordwinkin *et al*., 2012). Well-known late complications of diabetes are microvascular diseases, including nephropathy, retinopathy, neuropathy, and macrovascular diseases such as acute coronary syndrome claudication intermittent. Decreased nitric oxide presence and increased oxidative stress are widely accepted as the main molecular mechanisms that accelerate diabetic vascular disease (Vasam *et al*., 2017). Along with common complications, fragility fractures are recognized as an essential complication of both type 1 diabetes (T1D) and type 2 diabetes (T2D) (Napoli *et al*., 2017). In addition, diabetes mellitus is associated with an increased risk of fractures not explained by bone mineral density (BMD) (Starup-Linde & Vestergaard, 2016).

Low BMD is consistently observed in T1D, whereas BMD in T2D is usually normal or slightly higher compared to a control population of the same age (Napoli *et al*., 2017). Recent meta-analyses and cohort studies have confirmed that T1D and T2D are associated with a higher risk of fracture (Isidro & Ruano, 2010; Drake *et al*., 2012) due to the bone fragility common in T1D and T2D (Goswami & Nair, 2019).

The renin-angiotensin system (RAS), which is well known for its roles in blood pressure regulation and fluid homeostasis, has recently been reported to play a role in metastatic bone disease, including inflammation, angiogenesis, tumor cell proliferation, and migration (Passos *et al*., 2013; Forte *et al*., 2016). Remarkable experimental and clinical data suggest that RAS components are active inflammatory mediators. Angiotensin II (ANG II), one of the main components of the renin-angiotensin system (RAS), exerts its biological effects by binding to its receptor ANG II type 1 receptor (AT1R) in physiological and pathological conditions (Zhang *et al*., 2012; Namsolleck *et al*., 2014). High angiotensin (Ang) II expression induces proinflammatory cytokine and ROS production, apoptosis,

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endothelial dysfunction, and increased platelet activation and coagulation (Sha *et al*., 2021). Besides ANG II, several other peptides are now considered biologically vital. The endogenous heptapeptide Ang-(1-7), which emerged as a new metabolite of RAS decades ago, is particularly important (Gomes *et al*., 2012). The physiological effects of Ang-(1-7) were initially defined as its protective effects on the cardiovascular system and kidney by acting on its receptors (Gomes *et al*., 2012; Wong *et al*., 2012). Ang-(1-7) supplementation improves structural and mineral bone lesions in ovariectomy-induced osteoporosis in rats (Abuohashish *et al*., 2017a) and reduces osteolytic damage in an intratibial tumor model (Krishnan *et al*., 2013; Queiroz-Junior *et al*., 2019). RAS components have also been characterized as mediators associated with bone remodeling. Another study found that Ang-(1-7) treatment reversed diabetic mobilopathy in physiological conditions or response to ischemic injury.

Previous studies mentioned that RAS has a significant effect on bone tissue among the pathophysiological mechanisms underlying bone fragility in DM. However, few evidence-based studies have been reported on the effects of drugs targeting the RAS on the bone density of patients with diabetes (Zhang *et al*., 2017).

The primary purpose of bone biomechanics; is to determine the load on the bone and the resulting deformity. The mechanical properties of bone tissue are considered the basic parameters that reflect the structural and functional characterization of the bone. Bone biomechanics provides information about bone quality, flexibility, and durability. With bone biomechanics, especially bone fragility, and structural and material properties of a bone in pathological conditions, information can be obtained about what changes in the structural and material dimensions facilitate fracture and the treatment procedures associated with the pathology.

The bending test is an important test used to determine the mechanical properties of the long bones of experimental animals such as mice, rats, and guinea pigs. In the bending test, the bone is used as a whole. The tissue is fixed on two abutment points, and the load is applied perpendicular to the tissue surface. Due to this load position, the bone bends and breaks after a certain period. Due to bending, stretching stress is observed on one side of the bone, and compression stress is celebrated on the other. Bone is more resistant to compression stress than stretching. Therefore, fracture in the bending test is usually observed on the surface where the tensile stress occurs (Drake *et al*., 2012; Napoli *et al*., 2017).

The biomechanical definition of bone fragility includes at least three components: strength, fragility, and energy absorption. It can be derived from a biomechanical test in which the bone sample is loaded until it breaks. Decreased elastic modulus and ultimate strain values with increasing elongation determine the increased fragility of a bone in which biomechanical tests are applied (Turner, 2002).

Previous studies have revealed convincing evidence that Ang-(1-7) has positive effects on bone marrow and bone mineral density in experimentally established diabetes models. However, the biomechanical effects of Ang-1-7 on long bones in diabetes have not been studied yet. Therefore, this experimental study aimed to determine the biomechanical changes in the femurs of rats with diabetes.

MATERAL AND METHOD

Animal preparations and experimental design. Three months old male Wistar rats (n:29) weighing 280 to 330 g were used in this study. The animals were kept at room temperature (25±1 ?) and humidity-controlled room maintained on a 12h standard light/dark cycle with free access pellet feed and water through the experimental period. Diabetes was induced by a single intraperitoneal injection of STZ (50 mg kg-1), (STZ sigma ldrich product with code S0130-1g) in citrate buffer (0.1 M, pH 4.5), while control group received citrate buffer alone. Blood glucose levels were determined using a glucose reagent strip and a standard automated glucometer. A week after injection of STZ, blood glucose levels were measured and only the rats having 300 mg dL-1 or higher blood glucose level were used in experiments. The rats in the treatment groups with or without Diabetes Mellitus received Ang-(1-7) (0.6 mg kg-1d-1) subcutaneously for 28 days. All animals were used five weeks after the first day of the experiment. In this context, animals were separated into four groups: control group © n:7, STZ-induced diabetes (DM) n:7, STZ-induced diabetes and administration of Ang-(1-7) (DM+Ang 1-7) n:8 and administration of Ang-(1-7) n:8. Experimental design and animal groups are summarized in Figure 1. Animal handling and experiments were approved by the Akdeniz University Faculty of Medicine and performed in accordance with its ethics guidelines for the care and use of laboratory animals (Akdeniz University Animal Experiments Local Ethics Committee approval number: 2017.01.12).

Surgical Procedure. Rats were anesthetized with a combination of xylazine (10 mg/kg) and ketamine (50 mg/kg) intraperitoneally (IP). After anesthesia, the leg to be operated on was shaved with electric clippers, and the fur was removed entirely. The area was washed using chlorhexidine scrub and 70 % ethanol, and then Povidone

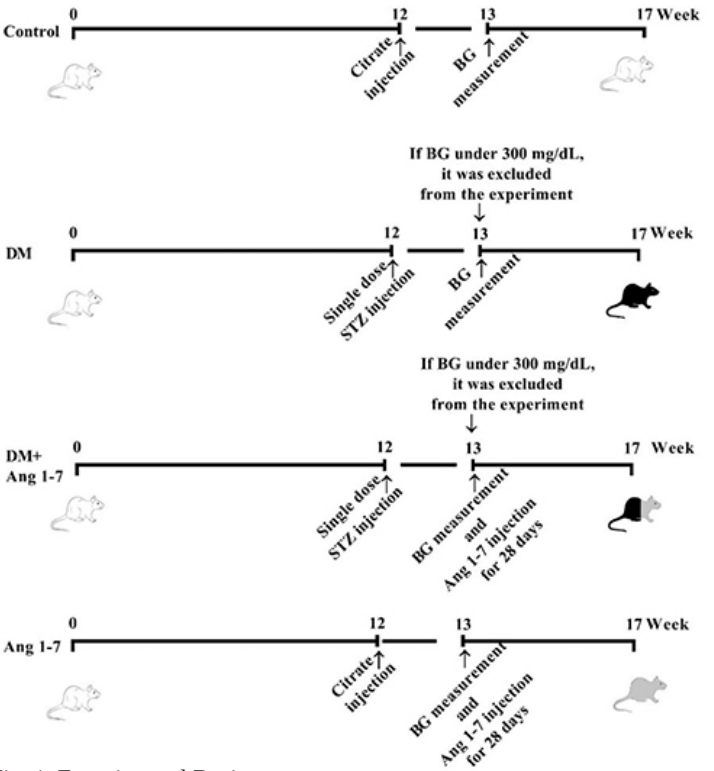

Fig. 1. Experimental Desing.

Iodine was applied to the area to disinfect the skin. The right femur of each animal was then removed. Muscle tissue on the femur was dissected. To calculate the biomechanical parameters, the lengths of the femurs were measured with digital caliper.

Biomechanical tests. The three-point bending test (3PB) is one of the commonly applied technique for the loading condition in fracture mechanics. The 3PB test was carried out by the (BESMAK BMT-100E Ankara, Turkey) universal test device. In all tests, the loading speed was 2 mm/sec, and the distance between the two end supports was set to 15 mm according to the femur length of the rats and fixed. Femur diameters were measured for each rat. The femur was positioned so the loading point was at the center of the femoral diaphysis, and bending occurred at the medial-lateral axis. The parameters analyzed were bone stiffness, work to failure, and ultimate force.

Statistics. SPSS 20 program was used for statistical analysis. The Kolmogorov-Smirnov test was applied to check whether the data were normally distributed. Descriptive statistics were expressed as mean ± standart deviation for continuous variables. A one-way ANOVA test was used to compare the means of the groups. Duncan's Multiple Range Test was used to compare the groups. Statistical significance levels were accepted as 5 % (Pr>F value).

RESULTS

The experimental diabetes model results (First body weight, Last body weight, First blood glucose, Last blood glucose) are shown in Table I. As a result of biomechanical tests, maximum load, maximum stress, duration, yield point, and displacement of maximum load data of the femurs in the groups were obtained. According to these data, group means values and standard deviations are given in Table II and Figure 2.

The mean and SD values of biomechanical parameters obtained from femur samples in all groups were compared in Table III. Maximum stress and maximum load showed a significant difference between the groups (Pr>F value<0.05).

According to the data, rats with the highest maximum stress and maximum load values were observed in Ang 1-7 and Diabetes+Ang 1-7 groups. In the diabetes group, the maximum stress and maximum load were lower than the control group. Although there was no significant difference between groups in Duration, Displacement of maximum load values and Young's modulus, Duration values were higher in Ang 1-7 group. There was no significant difference between yield point groups, but these values were higher in the Ang 1-7 groups, especially in the DM+Ang 1-7 group.

Table I. Results of experimental diabetes model.

	C	DM	DM+Ang-(1-7)	Ang-(1-7)
FBW (g)	286.8 ± 5.87	283.4 ± 6.36	299.2 ± 13.84	307.9 ± 7.13
LBW (g)	317.6 ± 5.67	245.9 ± 9.14 *	264.5 ± 13.53*	351.9 ± 7.49*
FBG (mg/dL)	102.7 ± 4.23	116.1 ± 4.13	111.4 ± 5.70	111.9 ± 4.24
LBG (mg/dL)	134.0 ± 6.36	552.1 ± 22.33 *	530.0 ± 33.25 *	137.4 ± 4.95

One-way ANOVA test was applied statistically. *Control vs. calculated as p<0.05.
FBW: First body weight, LBW: Last body weight, FBG: First blood glucose, LBG: Last blood glucose

Table II. 3PB test results obtained from all groups.

Grup	Max. Load (N) (N)	Max. Stress (Mpa) (MPA)	Duration (s)	Yield point (MPa)	Disp.maxload (mm)
C1	96,1	226,59	132,4	214,92	4,24
C2	96,1	213,63	54,4	104,6	1,66
C3	92,19	217,36	68,1	55,36	2,12
C4	104,42	246,22	54,5	77,53	1,81
C5	105,73	249,3	47,8	245,66	1,58
C6	111,48	262,86	88,5	259,14	2,93
C7	112,35	264,91	65,5	245,41	2,17
DM1	73,13	172,44	78,9	66,06	2,32
DM2	102,5	241,73	74,6	237,17	2,48
DM3	116,9	275,84	41	49,79	1,36
DM4	120,05	283,06	89,9	66,19	2,99
DM5	104,17	245,62	64	239,67	2,12
DM6	103,62	244,33	40	226,3	1,2
DM7	60,75	143,23	57,7	141,26	1,42
Ang-(1-7)-1	95,71	225,66	67,4	51,9	1,98
Ang-(1-7)-2	119,82	282,53	70,2	278,54	2,33
Ang-(1-7)-3	143,75	338,93	108,9	337,67	3,62
Ang-(1-7)-4	133,4	314,56	51,6	238,4	1,71
Ang-(1-7)-5	95,86	226,02	74,4	56,93	2,16
Ang-(1-7)-6	115,9	273,27	70,1	268,12	2,33
Ang-(1-7)-7	121,06	285,44	72,3	276,19	2,39
Ang-(1-7)-8	146,14	344,58	96	339,22	3,19
DM+ Ang-(1-7)-1	152,49	359,54	66	340,54	2,19
DM+ Ang-(1-7)-2	135,5	319,49	78,9	316,23	2,62
DM+ Ang-(1-7)-3	102,33	241,29	39,2	53,65	1,3
DM+ Ang-(1-7)-4	108,19	255,1	73,3	234,38	2,2
DM+ Ang-(1-7)-5	90,15	212,56	93,9	204,78	2,86
DM+ Ang-(1-7)-6	141,02	332,5	42,3	291,06	1,4
DM+ Ang-(1-7)-7	115,03	271,23	52,5	265	1,74
DM+ Ang-(1-7)-8	135,92	320,49	77,9	303,61	2,58

Table III. Comparison of the biomechanical parameters of the groups.

Group	Max. load (N)	Max.stress (MPa)	Duration (s)	Disp.maxload (mm)	Yield point (MPa)	Young's modulus (MPa)
Control	102,6±7,9	240,1±21,0	73,0±29,3	2,3±0,9	171,8±88,8	3194±1702
Diabet	97,3±22,1	229,4±52,1	63,7±18,9	1,9±0,6	146,6±87,1	2689±1428
Diabet+Ang-(1-7)	122,5±21,7	289,0±51,2	65,5±19,2	2,1±0,5	251,1±91,18	5341±1870
Ang-(1-7)	121,4±19,2	286,3±45,4	76,3±17,8	2,4±0,6	230,8±114,1	4003±1882
Pr>F value	0,02*	0,02*	0,69	0,55	0,15	0,14

Pr>F value <0.05 was accepted for significance

DISCUSSION

Deteriorated bone quality and increased risk of fractures have become known complications of diabetes mellitus (Janghorbani *et al*., 2006). Previous studies have found an increased incidence of bone fractures in individuals with diabetes mellitus compared to the general population, with those living with type 1 diabetes mellitus having a higher incidence than those with type 2 diabetes mellitus (Murray & Coleman, 2019). In addition, the risk of diabetic fractures is higher in

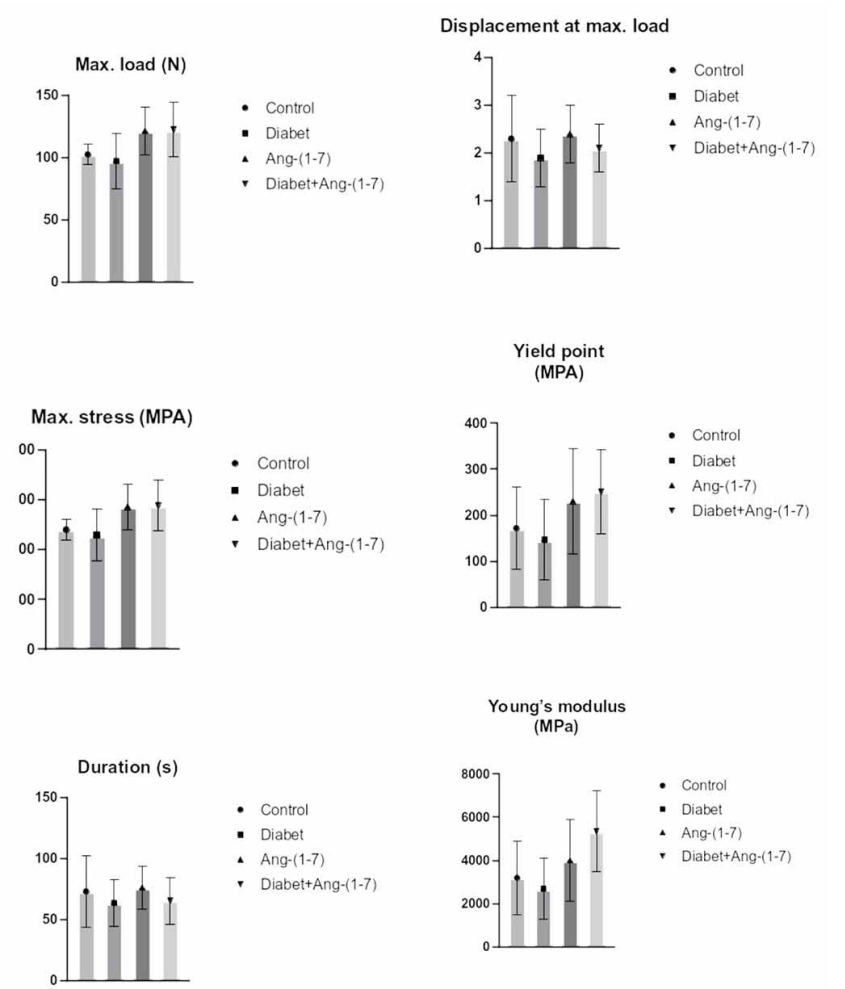

Fig. 2. Mean Values.

diabetes mellitus with poor glycemic control than in poorly controlled diabetes mellitus (Leanza *et al*., 2019). The increased risk of fractures in individuals living with diabetes mellitus is combined with impaired fracture healing. Specifically, changes in bone metabolism and the development of microvascular disease can prolong healing time by 87 %. Given the higher incidence of diabetes and the significant socioeconomic burden of fragility fractures, these findings highlight the need for a better understanding of bone health and fracture risk in patients with diabetes mellitus (Murray & Coleman, 2019).

Our study aimed to show the increase in bone fragility in diabetes by biomechanical test and to examine the effect of Ang-(1-7) on bone in bone fragility developing with diabetes. At the same time, this study is the first to evaluate Ang-(1-7) biomechanically on bone fragility in rats with this model diabetes.

Bone fragility can be defined by biomechanical parameters, including ultimate force (a measure of strength), ultimate displacement (the equivalent of fragility), and work to failure (energy absorption). Bone fragility is affected by bone size, shape, architecture, and tissue "quality" (Turner, 2002).

The term "bone quality" includes both material and geometrical properties of bone. Material properties are intrinsic properties that determine the durability of the bone (Burr, 2002). Material properties are determined by biomechanical tests applied in laboratory conditions. With these tests, the strength and durability of the bone are evaluated by using mechanical loads that cause tensile, pressing, rotational, and bending forces (Okyay *et al*., 2014).

According to the results of previous studies, it has been shown that there is a strong interaction between glucose levels and bone metabolism, and new ideas have been put forward to explain the increased risk of fracture in patients with diabetes mellitus (Napoli *et al*., 2017). Further studies comparing BMS in T2D patients with and without fragility fractures are needed (Sha *et al*., 2021).

Insulin signaling and IGF signaling in osteoblasts have been suggested to play an essential factor in bone quality, as demonstrated in animal models (Pramojanee *et al*., 2014), emphasizing the need to differentiate the effects of insulin and glucose in experimental human studies. Based on previous studies, it can be stated that insulin increases the secretion of osteocalcin in osteoblasts (Ferron *et al*., 2010), which may increase insulin sensitivity (Ferron *et al*., 2012) and increase pancreatic insulin production (Pramojanee *et al*., 2014). It has been stated that there may be a bone-glucose axis mediated by osteocalcin and insulin with bidirectional effects (Startup-Linde & Vestergaard, 2016). In addition, studies show that glucose uptake in osteoblasts and osteoclasts mediated by the current insulin and glucose combination reduces bone turnover markers (BTM) (Starup-Linde & Vestergaard, 2016). As a result of our study, the femurs of rats with diabetes mellitus are more fragile and less flexible than the control and angiotensin groups. This confirms the bone fragility that occurs in diabetes.

Activation of the ANG II 2/Ang-(1-7)/MasR pathway stimulates the functions of bone marrow progenitor cells (BMPCs) related to vascular repair (Jarajapu *et al*., 2013; Singh *et al*., 2015). According to the study results, the fact that the force applied to the bones was significantly higher in Ang-(1-7) and DM+Ang-(1-7) group shows the protection of Ang-(1-7) on bone tissue. Importantly, Ang-(1-7) has been found to reverse bone marrow oxidative stress and increase NO bioavailability in BMPCs in experimental diabetes (Mordwinkin *et al*., 2012) or CD34+ cells from diabetic individuals (Jarajapu *et al*., 2013; Vasam *et al*., 2017). The results of our study are similar to previous biochemical studies. In addition, although bone flexibility was not significant, the fact that Ang-(1-7) was found to be high in the groups given Ang-(1-7) indicates that Ang-(1-7) is effective in bone fragility. At the same time, the time to bone fracture was higher in the Ang 1-7 group. Young's modulus was lower in the diabetes group than in the other groups, showing that bone stiffness decreased in the diabetes group and increased bone stiffness when Ang-(1-7) was applied. This indicates that Ang-(1-7) administration has a protective effect on bone fragility. Results from previous studies suggest that osteoblasts and osteoclasts express ACE2 and MasR, and that activation of the ACE2/Ang-(1-7)/MasR axis reduces the bone resorption environment, at least in part, by modulating the bone cells phenotype for their anti-inflammatory properties (Donmez *et al*., 2012; Abuohashish *et al*., 2017b). Querioz *et al*., (2019) found that ACE2/Ang-(1-7)/MasR took an active role in remodeling the alveolar bone. When the data of this study were evaluated with other studies, it was concluded that Ang-(1-7) effectively increased the force applied for bone fragility, broke the bone in a long time, and gave flexibility to the bone.

Dramatically recovered trabecular BMD and increased mechanical strength (ultimate force) of the cortical bone in diabetic spontaneously hypertensive rats. Ang-(1-7) consistently ameliorated the structural and biochemical alterations of rats with ovariectomy-induced osteoporosis (16). So far, only a few studies have been reported on the beneficial health effects of Ang-(1-7) on the skeletal system. One of them is Sha *et al*. (2021) study. Ang's effects of Ang-(1-7) on bone in diabetic and hypertensive rats were examined in vivo and in vitro. They concluded that Ang-(1-7) could stimulate osteogenesis, inhibit osteoclastic resorption, and consequently protect against osteoporosis in diabetic spontaneously hypertensive rats with hyperglycemia. Sha *et al*. (2021) in their study, max. load was low in diabetic rats and increased in Ang-(1-7) group. These results are consistent with our results.

CONCLUSION

It should not be forgotten that the biomechanical quality of the bone is more important than the mineral content in determining fragility. The results of our study showed that bone fragility increased in rats with experimental diabetes mellitus, and bone strength was significantly improved when Ang-(1-7) was administered. Further investigation of the molecular mechanism of the interaction between the biaxial systems of RAS [ANG II/AT1R and Ang-(1-7)/Mas receptor] is needed to discover new drug candidates for the treatment of metabolic bone diseases and osteoporosis.

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RESUMEN: Se sabe que la diabetes mellitus tiene complicaciones tardías, incluyendo enfermedades microvasculares y macrovasculares. La diabetes puede afectar los huesos a través de los marcadores bioquímicos de la estructura, la densidad y el recambio óseo. Este estudio tuvo como objetivo investigar biomecánicamente los efectos protectores en los huesos de la angiotensina 1-7 (Ang 1-7), uno de los péptidos activos en el sistema renina-angiotensina, en ratas con diabetes. Treinta ratas albinas Wistar macho, de tres meses de edad y con un peso de 250-300 g, se dividieron en cuatro grupos: diabetes, Ang 1-7, diabetes más Ang 1-7 y control. Un mes después, se desarrolló diabetes en ratas; se sacrificaron los animales y se extrajo su fémur derecho. Se realizaron pruebas biomecánicas de flexión de tres puntos en los fémures. El grupo diabéticos tenía una fragilidad ósea significativamente mayor que los otros grupos (Pr > 0,05). La fragilidad ósea fue menor y la flexibilidad ósea fue mayor en los grupos Ang 1-7 (valor Pr>F 0,05). Como resultado de nuestro estudio, se determinó biomecánicamente el efecto de Ang 1–7 en los huesos de ratas con diabetes. Se concluye que Ang 1–7 tiene un impacto protector en los huesos de ratas diabéticas.

PALABRAS CLAVE: Diabetes mellitus; Angiotensina 1-7; Prueba de flexión de tres puntos; Hueso; Sistema renina-angiotensina.

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