JO JOURNAL OF OSSEOINTEGRATION periodontics and prosthodontics

Effect of topical ellagic acid application on titanium implant osseointegration and oxidative stress

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TO CITE THIS ARTICLE
Gul M, Dündar S, Koyuncu İ, Yiğin A, Tanik A, Bahattin Bingül M. Effect of topical ellagic acid application on titanium implant osseointegration and oxidative stress. J Osseointegr 2022;14():.

DOI 10.23805/JO.2022.14.23

ABSTRACT

Aim The aim of our study was to biomechanically evaluate the effect of topical ellagic acid (EA) on implant osseointegration before dental implants were placed in the tibia of rats, its effect on the bone tissue in the dental implant area, and its effect on oxidative stress.

Materials and methods In the study, a total of 16 Wistar albino rats each weighing 250–300 g were divided into two equal groups. All rats were placed in standard cages in groups of two animals and fed ad libitum with a normal diet and water. Control group: One 2.5 mm-diameter, 4 mm-long titanium implant was placed in the metaphyseal part of the right tibia bone of each subject (n = 8). Test group (EA): The same surgical procedure as for the controls was applied to each subject (n = 8), and additionally 0.325 mg / kg EA was applied topically to the implant cavity before the implants were placed.

Results The biomechanical analysis showed that, although higher values were obtained in bone implant fusion in the EA group than among the controls, no statistically significant difference was found. Oxidative stress index (OSI) values were compared, and statistically significantly lower values were obtained in both blood and bone tissue around the implant.

Conclusions It was found that the topical use of EA in dental implant applications has positive effects on implant osseointegration and bone mechanism, thanks to its antioxidant, antibacterial, and anti-inflammatory properties.

KEYWORDS Dental implants, Ellagic acid, Oxidative stress, Osseointegration.

INTRODUCTION

Dental implant applications are an effective and predictable treatment method to restore dental function in patients by replacing missing teeth. Despite high implant survival and success rates, failures still occur. Failures of dental implants may be early or late, depending on whether they occur before (early) prosthesis or after occlusal loading (late) with prosthetic restoration (1). Premature failure of an implant results from the inability to establish close contact between the bone and the implant. In this case, bone healing is impaired after the implant is placed. This situation may be caused by local or systemic factors (2).

The osseointegration and performance of dental implants are highly dependent on the early mechanisms at the implant–issue interface. When there is no adverse event in bone-implant healing, a stable integration of the implant with the surrounding bone tissue occurs, thereby guaranteeing the long-lasting function of the implant. However, bone type, implantation location, size of the trauma during the surgical procedure, and the degree of primary implant stabilization are factors affecting the bone-healing process (3). Systemic diseases, smoking, and exposure to radiation create unfavorable conditions for bone healing (4-6). Additionally, bacteria that form in a biofilm layer may adversely affect implant success (7-9).

Various procedures are performed to functionalize the surfaces used in implants (10,11). For this purpose, rich plant-derived compounds are used. Many of the polyphenols and phenolic compounds used have been reported to form multifunctional adhesive coatings on various materials through auto-oxidative surface polymerization (12-14).

Phenolics are the main antioxidant compounds found in foods. In particular, flavonoids commonly found in fruits and vegetables show strong antioxidant activity. Clinical trials and epidemiological studies show an inverse relationship between fruit and vegetable consumption

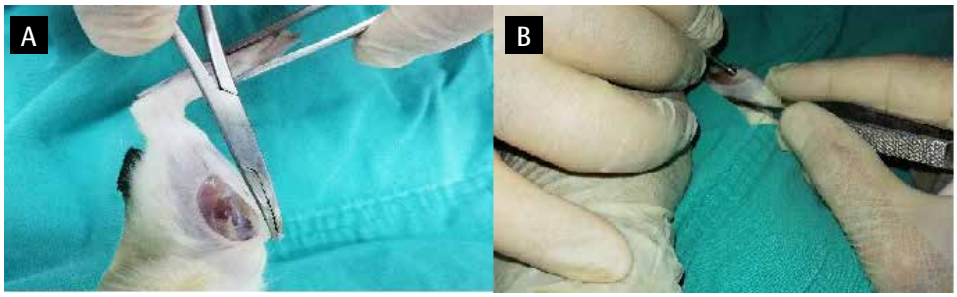

FIG. 1 Surgical preperation of the titanium implant sockets (a) and insertion of the titanium implants in the bone sockets (b).

and the occurrence of cardiovascular diseases, cancer, and some other chronic conditions. Phenolic compounds, vitamins (vitamin C and vitamin E), and carotenoids, which are found in fruits and vegetables and have antioxidant activity, stand out as effective compounds in the prevention of these oxidative stress-related diseases (15). Antioxidants are divided into enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants include superoxide dismutase (SOD), catalase, and glutathione peroxidase. Some of the non-enzymatic antioxidants are minerals, especially zinc and selenium; vitamins such as vitamin A, vitamin C, vitamin E, vitamin K, and phenolic acids. Ellagic acid (EA), one of the non-enzymatic antioxidants, is a polyphenolic component (16,17). EA is found in most red fruits, such as ahu berry, blackberry, strawberry, cranberry, pomegranate, and walnut (18). Some food antioxidants may be beneficial in atherosclerosis, malaria, rheumatoid arthritis, and diabetes, by inhibiting oxidation; they also have antitumoral, antimutagenic, antimetastatic, antithrombotic, anti-ulcer, anticarcinogenic, and antihypertensive, as well as antibacterial and antifungal properties (19-21). It has been determined by *in vivo* studies that they also have anti-aging effects (22). Pomegranate extract containing EA scavenges free radicals and reduces macrophage oxidative stress and lipid peroxidation (23).

Many activities of EA in the organism have been examined, but there is no study in the literature examining its effectiveness in dental implant applications. The aim of our study was to biomechanically evaluate the effect of topical EA on implant osseointegration before dental implants were placed in the tibia of rats, its effect on the bone tissue in the dental implant area, and its effect on oxidative stress.

MATERIALS AND METHODS

This work was conducted at the Harran University Experimental Research Center (Turkey). Ethical approval was obtained from the Harran University Animal Experiments Local Ethics Committee (Approved no: 2019/001/03).

This study was supported by Harran University Scientific Research Projects Unit, project no: 19349.

The rats used in the experiment were obtained from Fırat University Experimental Research Center, and all the recommendations in the Helsinki Declaration were followed during the experiment. In the study, a total of 16 Wistar albino rats each weighing 250–300 g were divided into two equal groups. The rats were kept in a humidity- (55%) and temperature-controlled room (22 ± 2 °C) on a 12 hour light / 12 hour dark cycle. All rats were placed in standard cages in groups of two animals and fed ad libitum with a normal diet and water. Control group: One 2.5 mm-diameter, 4 mm-long titanium implant was placed in the metaphyseal part of the right tibia bone of each subject (n = 8). Test group (EA): The same surgical procedure as for the controls was applied to each subject (n = 8), and additionally 0.325 mg / kg EA was applied topically to the implant cavity before the implants were placed.

Surgical procedure

The placement of dental implants into the bone was performed in accordance with asepsis and antisepsis rules. Ketamine hydrochloride (Ketalar, Eczacıbaşı, Turkey) 50 mg / kg and xylazine (Rompun; Bayer AG, Germany) 5 mg / kg were injected intramuscularly for anesthesia in all subjects. Povidone-iodine (Batticon, Adeka, Turkey) was applied to sterilize the operation area. Articain (Ultraca DS; Aventis, Istanbul, Turkey) containing 0.5 cc epinephrine was applied for local hemostasis. In the study, the metaphyseal part of the tibia bone of the animals in which dental implants were to be applied was exposed after a crestal incision was made in the bone. One 2.5 mm-diameter and 4 mm-long standard implant cavity was opened on the corticocancellous part of the right tibia bone of each rat with the help of a drill (Fig 1a). Then, 0.325 mg / kg EA was dissolved in 0.1 ml of water and applied to the cavity of the EA group. A 4 mm-long implant with a diameter of 2.5 mm was placed in each cavity at bone level (Fig. 1b). After the implants were placed, all tissues were sutured in their original positions with 4-0 dissolvable suture. All subjects were given 50 mg / kg cefazolin sodium antibiotic intramuscularly for three days postoperatively to prevent infection. In addition, a pain reliever, 1 mg / kg tramadol hydrochloride, was administered intramuscularly for three days. The subjects were sacrificed at the end of 28 days by

administering a lethal dose (60 mg/kg) of anesthetic. Blood was collected from the hearts of the subjects by cardiac puncture. Tissues were stored at -80 °C until biochemical analyses were performed.

Biomechanical analysis

To evaluate the osseointegration of the implants, the reverse torque test was performed on rats sacrificed after the 28-day recovery period. Evaluation was carried out immediately after sacrification to prevent dehydration. All implants were placed in polymethylmethacrylate blocks for analysis. After the reversing parts of the implants were screwed in, a digital torque tool (MARK-10 MTT01-12, Copiague, New York, USA) was fixed for each implant. Next, a counterclockwise ejection force was slowly and progressively applied manually. Reverse torque application was terminated when the implant rotated in the bone socket. When the stabilization of the implants was completed, the highest torque value (N-cm) displayed on the digital torque screen was automatically recorded (Fig. 2).

Enzyme-linked immunosorbent assay (ELISA)

Osteonectin (MyBioSource, Inc., San Diego, USA), osteopontin (Shanghai Korain Biotech Co., Ltd, Shanghai, China), and HSP70 (Sunred Biological Technology Co., Lt., Shanghai, China) were analyzed according to the commercial ELISA kit method. This kit is based on sandwich enzyme-linked immunosorbent assay technology. For the analysis, 96-well plates were precoated with anti-Osteonectin (ON) antibody, which, when conjugated with biotin, served as a detection antibody. Washing was performed by adding test samples and the biotin-conjugated detection antibody to the wells and washing with a buffer.

After the addition of HRP-streptavidin, unbound conjugates were washed with a buffer. Substrates of 3,3',5,5'-tetramethylbenzidine (TMB) assisted in visualizing the HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue-colored product that turned yellow after the acid-stopping solution was added. The density of the yellow was evaluated in proportion to the amount of ON in the sample captured on the plate. In a microplate reader (BioTek Instruments, USA), the absorbance at 450 nm, followed by the ON concentration, was calculated.

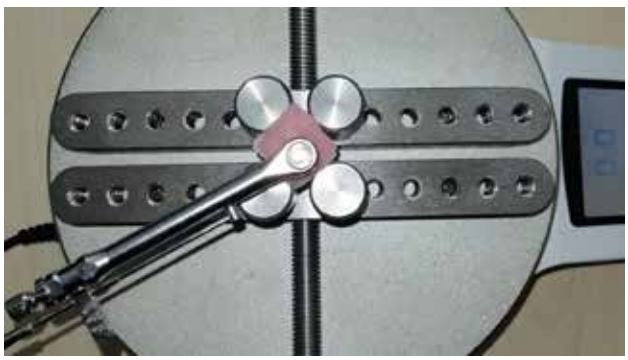

FIG. 2 Reverse torque analysis of the titanium implants (MARK-10 MTT01-12, Copiague, New York, USA).

Oxidative stress assay

Total oxidant status (TOS) and total antioxidant status (TAS) (Rel Assay Diagnostics kit, Mega Tip, Gaziantep, Turkey) were read by a commercial kit protocol microplate reader (BioTek Instruments, USA). Calculation of the oxidative stress index (OSI); The TOS values of the samples were divided into percentage TAS values and OSI values were calculated. (OSI (arbitrary unit, AU) = (TOS, µmol H_2O_2 equiv. / L) / (TAS, µmol Troloxeq / L)) (24). Trolox, a water-soluble analog of vitamin E, was used as the calibrator. Results are expressed in mmol trolox equiv. / l. TOS was measured using a Rel Assay commercial kit (Rel Assay Diagnostics, Turkey), with hydrogen peroxide as a calibrator. Results are expressed as µmol H_2O_2 equiv. / l. OSI is expressed as the percentage of the ratio of TOS levels to TAS levels. In the TAS test, the mmol value was converted into µmol units as in the TOS test. Results are expressed in AU. TOS, µmol H_2O_2 equivalent. / l OSI = TAS, mmol trolox equivalent. / l X 1 (24).

Statistical analysis

Statistical analysis values were calculated using SPSS 20 windows (IBM, USA) statistical program. Analysis values were evaluated as mean and standard deviation (SD). The normal distribution of the data was determined by the Kolmogorov-Smirnov test. Therefore, independent T test was used to compare the data between the two groups. A p value of <0.05 was considered significant in all analyses.

RESULTS

The biomechanical analysis showed that, although higher values were obtained in bone implant fusion in the EA group than among the controls, no statistically significant difference was found (Table 1).

The analysis compared HSP 70 values, showing lower results in the group in which EA was applied in the bone tissue around the implant, but no statistically significant difference was found ($p > 0.05$) (Table 1). In the comparison of HSP 70 in blood serum, a statistically significant difference was found between the groups ($p < 0.05$) (Table 2). Comparison of osteonectin and osteopontin values was performed both in the blood and in the tissue around the implant. Higher values were obtained in the EA group, and statistically significant differences were obtained between the groups ($p < 0.05$). In the evaluation of oxidative stress parameters TAS, TOS, OSI, and TAS values in both blood and bone fill around the implant were found to be higher in the EA group. The

Parameters	Control Group(N)	Test group(N)	P* value
BIC	0.68±0,62(8)	0.74±0,63(8)	0.868
HSP-70	1330.58±198.80(8)	1125.33±261.73(8)	0.101
OPN	244.29±80.15(8)	363.99±109.43(8)	0.026*
ON	546.37±130.03(8)	873.71±160.42(8)	0.001**
TAS	1.10±0.14(8)	1.51±0.17(8)	0.001**
TOS	16.40±1.36(8)	12.43±1.61(8)	0.001**
OSI	1.50±0.15(8)	0.82±0.09(8)	0.001**

TABLE 1 Changes in the Elisa test analysis values of the markers in the bone tissue between test and control groups after the 28th day.

*Values are given as mean ± SD (standard deviation), BIC: Bone implant contact, HSP-70: The 70 kilodalton heat shock proteins, OPN: Osteopontin, ON: Osteonectin, TAS: Total antioxidant status, TOS: Total oxidant status, OSI: oxidative stress index , N: Number of rats, P *: significant difference between groups according to independent T test, P * value: significance, P ** value: multi-understanding*

Parameters	Control Group(N)	Test group(N)	P* value
HSP-70	1024.40±58.45(8)	816.14±72.88(8)	0.001**
OPN	131.70±12.16(8)	207.49±35.90(8)	0.001**
ON	172.36±30.51(8)	220.71±45.11(8)	0.027*
TAS	1.19±0.06(8)	1.56±0.11(8)	0.001*
TOS	16.97±1.98(8)	13.19±3.88(8)	0.033*
OSI	1.43±0.20(8)	0.87±0.30(8)	0.001**

TABLE 2 Changes in biochemical values in serum between test and control groups after the 28th day.

*Values are given as mean ± SD (standard deviation), HSP-70: The 70 kilodalton heat shock proteins, OPN: Osteopontin, ON: Osteonectin, TAS: Total antioxidant status, TOS: Total oxidant status, OSI: oxidative stress index, N: Number of rats, P *: significant difference between groups according to independent T test, P * value: significance, P ** value: multi-understanding*

difference was found to be statistically significant ($p< 0.05$). TOS values were calculated both in the blood and in the bone tissue around the implant, and lower values were obtained in the EA group, the difference being statistically significant ($p < 0.05$). In addition, OSI values were compared, and statistically significantly lower values were obtained in both blood and bone tissue around the implant (Table 1, 2).

DISCUSSION

Phenolic compounds are bioactive compounds known to be effective in bone health and responsible for bone metabolism (25). Ellagic acid (EA) is a flavonoid, usually produced by plants (26). EA consists of two lactone groups and four hydroxyl groups. It is known that the hydroxyl group increases antioxidant activity in lipid peroxidation and protects cells from oxidative damage (27). In our study, evaluation was conducted of the biomechanical osseointegration of dental implants that were placed in rat tibia to which EA was applied, and of osteonectin and osteopontin cytokines, HSP70 protein, and oxidative stress parameters, which are involved in the bone healing mechanism in the tissue and blood around the implant.

One study has demonstrated that EA accelerates bone formation after tooth extraction in normal rats (28). Jamil Al-Obaidi et al. (29) reported a positive effect on trabecular bone formation after tooth extraction in nicotinic rats treated with EA. They suggested that this is due to the antioxidant activity of EA, which leads to the upregulation of osteocalcin and alkaline phosphatase (ALP) proteins responsible for osteogenesis. In our study, osseointegration between implant and bone tissue was evaluated biomechanically. Although we obtained higher values in the EA applied group, no statistically significant difference was obtained. We think that this is due to the limited biomechanical effect of EA during the 28-day early implant bone-fusion process. It has been emphasized in studies that the protein osteopontin has an important role in bone formation and resorption (30-32). It is found in high concentrations on the bone surface and the cementum layer during new bone formation (33). Osteopontin reportedly has different effects in various concentrations during bone formation (34). Osteopontin is also an important component of bone tissue, but it is similarly found in non-mineralized tissues (30). During the early bone-formation period, osteopontin levels reach the highest value in preosteoblastic cells. This protein thus clearly has an important role in bone resorption and mineralization (35). In our study, osteopontin levels in both the blood and the tissue surrounding the implant were evaluated and found to be higher in the EA group. We think that this is due to the positive effects of EA on bone metabolism, thanks to its antioxidant, antibacterial, and anti-inflammatory properties.

As Ram et al. (35) confirmed, osteonectin is a calcium-

binding glycoprotein consisting of 25% of non-collagen protein. Osteonectin plays a role in the collagen mechanism, and it has been reported to cause an increase in the mineralization of the collagen matrix. Its level is also reportedly high during wound healing. Implant osseointegration involves a process similar to bone healing, and therefore osteonectin has an important place in implant stability. In our study, osteonectin levels in both the blood and the implant-surrounding tissue were evaluated, revealing statistically high values in the EA applied group. This shows that EA has a positive effect on bone metabolism and thus on implant stability. In the study of Kara et al. (36) the rats exercised and the oxidative stress that developed afterwards was measured. They found that EA can reduce oxidative stress. In addition, it was determined that proinflammatory cytokine levels were accompanied by increased oxidative stress and that EA exerted an anti-inflammatory effect that lowered the increased cytokine levels. Uzar et al. (37), in their study on the effect of EA on brain and sciatic nerve tissue in diabetic rats, reported that Malondialdehyde (MDA), TOS, OSI, and nitric oxide (NO) levels significantly decreased in the diabetic group treated with EA compared to the untreated diabetic group ($p < 0.05$). Thus, EA appeared to exhibit neuroprotective effects against oxidative damage in diabetic rats. Böyük et al. (38) reported that they observed an increase in TOS and MDA levels in the ischemia reperfusion group compared to a control group, as well as a decrease in Total Antioxidant Capacity (TAC) levels, which is an indicator of lipid peroxidation. In addition, they detected a significant decrease in TOS and MDA levels and an increase in TAC levels with EA application, which may be related to the antioxidant and free radical-scavenging effect of EA. They suggested that EA prevents the increase in lipid peroxidation caused by ischemia reperfusion. EA has been found to reduce oxidative stress in osteoblasts, thanks to its antioxidant effects, and increases osteocalcin production by stimulating osteoblast activation. Similarly, EA has been demonstrated to increase the expression of osteocalcin and osteopontin (28,39). EA can also hamper bone resorption by inhibiting the production of cytokines and proteins that trigger osteoclast differentiation. EA reportedly inhibits the production of cytokines by limiting the transcription of NF-kB, thus increasing the differentiation of osteoblasts and osteocytes, increasing osteoclast apoptosis, and preventing bone resorption by suppressing osteoclast formation (40). In our study, we evaluated the effect of topical EA application on oxidative stress in dental implants, as measured by TAS, TOS, and OSI levels. TOS and OSI values decreased in the groups that received dental implants plus EA application, compared to the groups that received only dental implants, while TAS values increased. This difference was found to be statistically significant. This indicates that the antioxidant properties of EA are also at work in dental implant applications.

CONCLUSION

It was found that the topical use of EA in dental implant applications has positive effects on implant osseointegration and bone mechanism, thanks to its antioxidant, antibacterial, and anti-inflammatory properties. We think that EA increases the production of osteocalcin by reducing oxidative stress in osteoblasts and stimulating osteoblast activation, owing to its antioxidant effects. This in turn positively affects the implant–bone connection, thus enhancing the likely success of the implant. More studies are needed for more accurate and reliable results.

Acknowledgments

The authors wish to thanks Implance Dental Implant System, AGS Medical Corporation, Istanbul (Turkey), for manufacturing and providing the titanium implants.
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Authors' contributions

Mehmet Gul, Serkan Dundar and Akın Yiğin made his study design. Mehmet Gul, Serkan Dundar, Akın Yiğin and İsmail Koyuncu worked in the experimental stage, in the collection and analysis of data, Mehmet Gul ve Abdulsamet Tanik conducted statistical analysis. Mehmet Gul and Muhammet Bahattin Bingul worked in the writing of the manuscript. The authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing of interests.

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