

## Osteocalcin expression during autogenous onlay bone grafts with or without resorbable collagen membrane in diabetic rats

Expressão da osteocalcina no reparo de enxertos ósseos autógenos onlay recobertos ou não por membrana colágena reabsorvível em ratos diabéticos

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### ABSTRACT

**Objective:** This study aimed to quantify through immunohistochemistry the expression of bone formation marker osteocalcin during the repair process of autogenous onlay bone graft, associated or not to the resorbable collagen membrane and to compare these findings with the presence of Diabetes *Mellitus*. **Material and Methods:** Sixty rats (*Rattus norvegicus*, albinus variation, Wistar) aged 90 days, were divided into two groups with 30 animals: test group – rats with induced diabetes; control group – normoglycemic rats. All rats received grafts on the left and right hemi-mandible with or without collagen membrane coverage, respectively. The animals were euthanized at the following periods: 0h, 7, 14, 21, 45, and 60 days. Immunohistochemistry analysis was performed by the marker osteocalcin at receptor site-graft interface. To analyze osteocalcin immunohistochemical expression, a panoramic view photograph of the graft was taken followed by two photographs at larger magnification. **Results:** No statistically significances at 5% level were observed between diabetic and control group with and without membrane; and diabetic and control groups with membrane coverage. **Conclusion:** Within the limits of this present study, it can be concluded that the osteocalcin marker might be influenced by diabetes so that it was late expressed during this condition. However, the association of the graft with the membrane could improve this delay by reaching expression values similar to those of control group.

### KEYWORDS

Bone grafts; Diabetes; Osteocalcin; Collage membrane; Bone repair.

### RESUMO

**Objetivo:** O objetivo deste estudo foi quantificar, através da técnica imunoistoquímica, a expressão do marcador de formação óssea Osteocalcina no processo de reparo do enxerto ósseo autógeno onlay, associado ou não à membrana colágena reabsorvível e comparar esses achados com a presença da Diabetes *Mellitus*. **Material e Métodos:** Foram utilizados 60 ratos (*Rattus norvegicus*, variação albinus, Wistar) com 90 dias de idade, divididos em dois grupos, cada um contendo 30 animais: Grupo Teste com Diabéticos, composto por ratos com diabetes induzida e Grupo Controle composto por ratos normoglicêmicos. Todos receberam enxertos na hemi mandíbula esquerda com recobrimento de membrana colágena e na hemi mandíbula direita sem recobrimento. Os animais foram eutanasiados nos períodos 0h, 7, 14, 21, 45 e 60 dias. A análise imunoistoquímica foi realizada com o marcador Osteocalcina na interface leito-enxerto. Para análise da expressão imunoistoquímica da Osteocalcina, realizou-se uma fotografia com visão panorâmica do enxerto e duas fotografias em maior aumento. **Resultados:** Os resultados mostraram que houve diferença estatisticamente significativa ao nível de 5% intragrupo para Diabético e Controle com e sem membrana e também quando comparamos o grupo Diabético com o Controle com a presença da membrana. **Conclusão:** Dentro dos limites do presente estudo, pode-se concluir que a marcação da osteocalcina pode sofrer alguma influência do diabetes, sendo expressa de forma mais tardia na presença dessa condição. Porém, a associação da membrana ao enxerto pode melhorar esse atraso e deixar a expressão semelhante ao grupo controle.

### PALAVRAS-CHAVE

Enxertos ósseos; Diabete; Osteocalcina; Membrana colágena; Reparo ósseo.

## INTRODUCTION

The increasing in life expectancy is followed by the increasing in advanced age-related diseases, such as hypertension, osteoporosis and diabetes. Associated with the population aging, a greater demand to esthetic and functional reconstructive procedures, such as osseointegrated implants, has been observed.

One of the requirements for osseointegrated implant success is that the receptor site has enough bone amounts, which is not always possible. In these situations, the dentist might employ techniques aiming at increasing the bone tissue. Among them, autogenous bone graft is considered very effective [1], because of the osteogenic properties and lack of large rejection risks [2]. Guided Bone Regeneration technique has been associated to autogenous bone grafts, with great results, as demonstrated by many studies [3,4].

Diabetes *Mellitus* is an evolutionary chronic disease characterized by the alterations of carbohydrate, fat, and lipid metabolism. Many studies have shown that type 1 diabetes is associated with a decreasing in bone mass, and some authors have still considered Diabetes a predisposing factor to developing Osteoporosis [5,6].

It has been suggested that early development of type 1 Diabetes might compromise bone mineralization at long term, and the prevalence of osteopenia might be higher than 50% in these patients [7].

Lee et al. [8] described a new function of the skeleton on the energetic metabolism. They especially demonstrated that osteocalcin, non-collagen protein produced by the osteoclasts, is involved in the glucose metabolism, increasing the insulin secretion and the proliferation of pancreatic  $\beta$ -cell.

Given the above, this present study quantified through immunohistochemistry

the expression of the bone formation marker osteocalcin during the repair process of autogenous onlay bone graft, associated or not to the resorbable collagen membrane and to compare these findings with the presence of Diabetes *Mellitus*.

## MATERIAL AND METHODS

### *Animals and experimental groups*

This study was submitted and approved by the Ethical Committee in Research of the School of Dentistry of São José dos Campos – UNESP under protocol n° 01/2011-PA/CEP, and received funding support of the São Paulo Research Foundation – FAPESP (process n° 2010/52697-7).

The sample comprised 60 adult rats (*Rattus norvegicus*, albinus variation, Wistar) aged 90 days, weighing approximately 300 g, kept in cages at environment temperature, fed with ration (Guabi Nutrilabor) and water ad libitum, provided by the vivarium of the School of Dentistry of São José dos Campos - UNESP.

The animals were randomly divided into two groups containing 30 animals each:

- test group (D), composed by rats with induced diabetes through alloxan administration;
- control group (C) composed by normoglycemic rats.

### *Induction of experimental diabetes*

Monohydrated alloxan (Sigma Chemical Co., St. Louis, MO, USA) was diluted in sterile saline solution at dose of 150 mg/kg of rat weigh. This dilution was injected intraperitoneally to induce diabetes. After 12 h of the alloxan application, glucose solution was administered at the concentration of 10% per rat weigh, to prevent hypoglycemia in the rats.

Elapsed 72 h after alloxan administration, a blood sample was collected through caudal vein of the animals to evaluate the glycaemia with the aid of Accu-Chek Advantage device

(Boehringer Mannheim, IN, USA). The animals showing glycaemia higher than 200 mg/dl were considered as diabetic.

This measure was repeated at every week to assure that the animals continued with diabetes. The rats that exhibited glycaemia lower than 200 mg/dl, at any period, were excluded from the study and replaced by animals that were again submitted to diabetes induction.

### ***Surgical procedures***

To accomplish the graft and euthanasia surgeries, the animals were anesthetized with a solution of 13 mg/Kg of 2-(2,6-xylidine)-5-6-dihydro-4H-1,3 thiazine chlorhydrate (Rompum – Bayer of Brazil), a substance of sedative and analgesic properties and muscle relaxant and 33 mg/Kg of ketamine base (Dopalen – Agribands of Brazil), general anesthetic, intramuscularly. After the surgical procedures to perform the bone grafts, all animals received a single dose of 1 mg/Kg of antibiotics (Veterinary Pentabiotic – Fort Dodge) intramuscularly.

All the animals were submitted to grafting on right and left hemi-mandible [1,4]. The grafts on left hemi-mandible were covered by resorbable collagen membrane (BioGuide, Geistlich Pharmaceutical, Wollhusen, Switzerland), enabling the coverage of all grafted block, in the diabetic group with graft and membrane (DEM) and control group with graft and membrane (CEM). The grafts on the right hemi-mandible were carried out similar to those on the left hemi-mandible, but without collagen membrane coverage, in diabetic groups with graft (DE) and control group with graft (CE).

The grafts were performed 15 days after diabetes induction. The animals were anesthetized and bilateral temporal/parotid masseteric and bilateral parietal/frontal regions trichotomized with the aid of razor blades. The antiseptis of these areas was performed with 0.2% chlorhexidine gluconate. The parietal bone was the autogenous bone donor area.

On the mandible skin, a linear incision measuring approximately 1.5 cm was executed parallel to the zygomatic arch. Thus, the masseter muscle could be visualized and divulsed with the aid of instruments developed especially for this purpose up to reach the lateral surface of the mandible. The next step was to displace the lingual anatomic structures until exposing the mesial mandibular surface with the aid of a spatula, designed for this purposed, to enable the bone graft and collagen membrane fixation according to the experimental group.

To obtain the bone grafts, a linear incision measuring approximately 2.0 cm was executed on the center of the animal's calvarium, exposing the parietal bones, bilaterally, but one graft was removed from each side. The grafts were removed with the aid of a trephine bur with external diameter of 4.1 mm (Neodent – Brazil) under copious irrigation with saline solution. After the removal, this block was perforated on the center with a spiral bur of 1.2 mm in diameter (Neodent – Brazil) at low speed, under refrigeration with saline solution aiming at the future fixation on the same receptor site. The blocks were temporally stored in a cube containing saline solution.

At that moment, on the receptor site, a perforation with 1.2 mm bur (Neodent – Brazil) at low speed and under copious irrigation was perfumed enabling to stabilize the bone block to the receptor site with the aid of titanium mini-implant (1.5 mm of head diameter, 1.4 mm of body diameter, and 2.5 mm in length). These procedures enabled the graft placement in close contact with the mandibular bone surface, stabilizing it. After the fixation of the grafts on the left hemi-mandible of each rat, the collage membrane was placed, covering them (groups DEM and CEM).

To cut the collagen membranes uniformly, an aluminum matrix measuring 7 x 7 mm obtained from radiographic films on the occlusive portion of the collagen membrane.

To remove the bone graft and to execute the perforations, an electric motor (Driller BLM 600 Plus) was used, enabling the speed control at 960 rpm, and refrigeration with constant sterile saline solution flow.

The sutures were carried out at each plane, starting firstly on the muscle with absorbable polyglactin 910 size 5.0 (Ethicon – Johnson & Johnson) followed by the skin with silk thread size 4.0 (Ethicon – Johnson & Johnson). The donor area was also sutured with this latter.

Next, the operated areas were cleaned with 0.2% chlorhexidine. After the surgery, the animals received normal diet and water “*ad libitum*”.

The animals were anesthetized and the cardiac perfusion with 4% formalin was initiated at the following periods: 0 hour, 7, 14, 21, 45, and 60 days. At each period, 5 animals were sacrificed per experimental groups.

### ***Histological processing***

The hemi-mandibles were removed and stored for fixation in 10% buffered formaldehyde solution for at least 48 h. Each specimen was registered.

The pieces were demineralized with 10% EDTA, pH 7.8 and cross-sectionally cut on the central area of the bone graft. Each fragment was totally included in the paraffin block.

### ***Immunohistochemical processing***

For the immunohistochemical analysis, the paraffin blocks were submitted to serial cuts measuring 3  $\mu$ m in width placed in laminas treated by 3-aminopropyltriethoxysilane (Sigma Chemical CO., St.Louis, USA).

After this, a lamina from each specimen was chosen to perform the immunohistochemical reactions.

The used immunohistochemical marker was osteocalcin (Ab13420, Abcam, Inc., Cambridge, MA, USA).

The immunohistochemical reactions were carried out according to the protocol of Immunohistochemistry Laboratory of the School of Dentistry of São José dos Campos - ICT/UNESP:

a) deparaffinization in two xylol baths for 5 min each.

b) rehydration in an ethanol decreasing series (absolute, 70% and 50%, twice) for 3 min each. Washing in running water followed by washing in distilled water twice

c) antigenic recovery. At first, proteinase K (DAKO CO., California, USA), as suggested by the antibody manufacturer. For the antibody standardization, citrate pH 6.0 and pepsin pH 1.8 were tested. For citrate tests, the laminas were stored and immersed in flasks containing the solution and put in microwave oven to be submitted to three consecutive cycles of 3 min at power 7, followed of cooling at environmental temperature. For pepsin tests, the laminas were immersed into solution and put in an incubator at 60 °C for 10 min, and then in an incubator at 37 °C for 50 min.

Next, the laminas were washed for 10 min in running water followed by distilled water twice;

d) peroxidase blockage of Endogenous tissue peroxidase with 50% methyl alcohol solution and hydrogen peroxide 20 volumes, at 1:1 ration, for two cycles of de 10 min each;

e) washing for 10 min in running water followed by distilled water twice;

f) immersion into TRIS hydroxymethyl aminomethane buffering solution, pH 7.4, for two cycles of 5 min each;

g) storage with bovine serum albumin (BSA) for 1 h, into moist chamber, to eliminate the nonspecific antibodies;

h) incubation of primary antibody;

i) washing and immersion in TRIS



hydroxymethyl aminomethane solution for two cycles of 5 min each;

j) incubation of secondary antibody (Universal LSAB TM Kit/HRP, Rb/Mo/Goat – DAKO CO., California, USA) for 30 min;

k) washing and immersion into TRIS hydroxymethyl aminomethane solution for two cycles of 5 min each;

l) incubation of the streptavidin-peroxidase tertiary complex (Universal LSAB TM Kit/HRP, Rb/Mo/Goat – Dako) for 30 min;

m) washing and immersion into TRIS hydroxymethyl aminomethane solution for two cycles of 5 min each;

n) development of the reaction with diaminobenzidine (DAB) (DAKO CO., California, USA) for 10 min;

o) washing in TRIS hydroxymethyl aminomethane solution twice;

p) washing for 10 min in running water followed by running water twice;

q) counter staining with Mayer's hematoxylin for 2 min;

r) washing for 10 min in running water followed by distilled water twice;

s) the cuts were dehydrated in ethanol increasing series (50%, 70%, 95% and 3 times in

absolute alcohol) for 1 min each, diaphanized and mounted in permount resin (Fisher Scientific®, New Jersey, NY, USA) for microscopy.

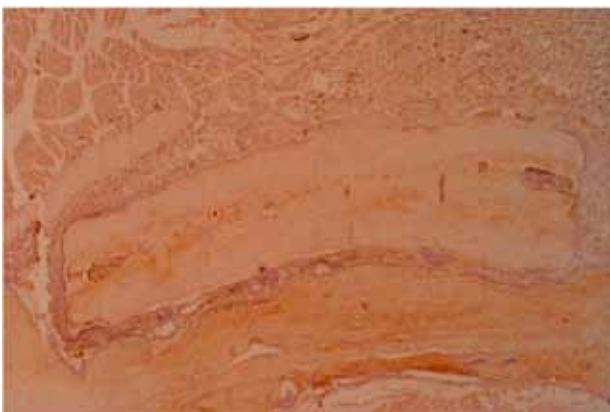
### *Analysis of the results*

The microscopic analysis was performed by capturing the images of the laminas with the aid of a light microscope Axiophot 2 (Carl Zeiss, Oberkochen, Germany) linked to a digital camera AxioCam MRc 5 (Carl Zeiss Oberkochen, Germany) to be analyzed by AxioVision Release 4.7.2 software.

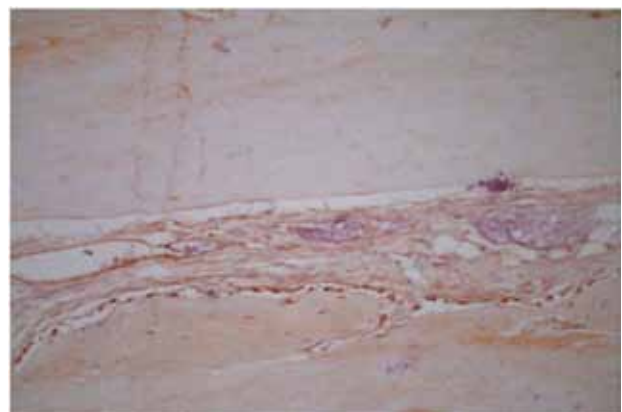
To analyze the immunohistochemical expression of osteocalcin, three photographs were taken per specimen. A panoramic view photograph of all receptor site-graft interface at x4 magnification was obtained (Figure 1) and two photographs at X100 magnification, positioned between the center and the lateral surfaces of the graft and in the receptor site-graft interface (Figure 2). The counted cells were present in the two higher magnification photographs (X100), and counted with the aid of ImageJ 1.x software developed by Wayne Rasband at NIH, as follows osteoblasts, osteocyte and osteoclast with intracytoplasmic marking. One lamina was analyzed per specimen.

### *Statistical analysis*

The obtained data were submitted to statistical analysis through the following software:



**Figure 1** - Panoramic view photomicrography of receptor site-graft interface. (Immunohistochemical staining, X4 magnification).



**Figure 2** - Photomicrography of receptor site-graft interface. (Immunohistochemical staining, X100 magnification).

MINITAB (Minitab, version 14.12, 2004) and STATISTICA (StatSoft, version 5.5, 2000).

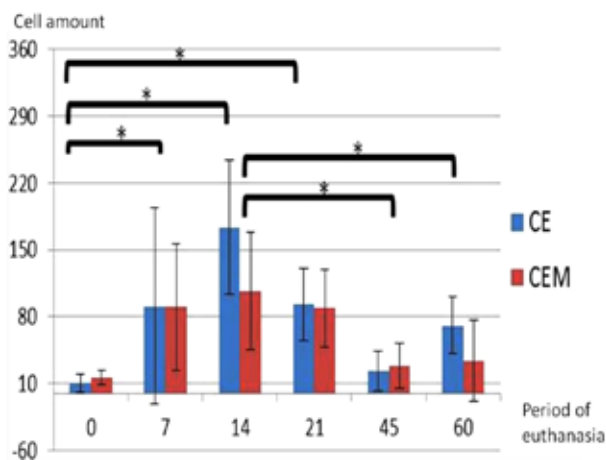
The descriptive statistics comprised mean and standard deviation, while inferential statistics was performed ANOVA and Tukey test, at significance level of 5%. The study of the interaction effect condition x membrane x sacrifice period was conducted through mean graph made in Prism software (version 4.00, 2003 Graph Pad Software Inc.).

## RESULTS

Figures 3 to 6 show the obtained results. By analyzing the osteocalcin expression in the group of normoglycemic rats, the periods of 7, 14 and 21 days exhibited the higher expression, regardless of the presence of the membrane; the final periods showed the smaller expression, with statistically significant differences (Figure 3).

In the group of diabetic rats, the expression was similar for all periods and subgroups (with or without membrane) without statistically significant differences ( $p > 0.05$ ); the final periods demonstrated high level of expression (Figure 4).

With regard to the effects of the membrane in the repair process of the groups, the collagen



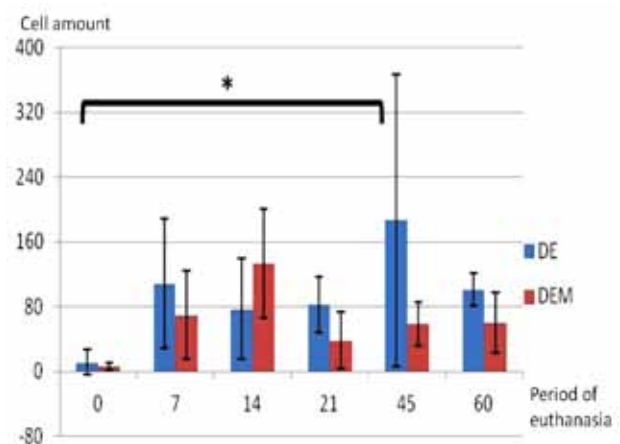
**Figure 3** - Mean and standard deviation of osteocalcin expression on receptor site-graft interface in control group, for the condition with or without membrane.

\*Statistically significant difference. Intragroup Comparison. ANOVA.

membrane presence covering the bone graft tend to generate similar osteocalcin expression both in diabetic and normoglycemic rats (Figure 5).

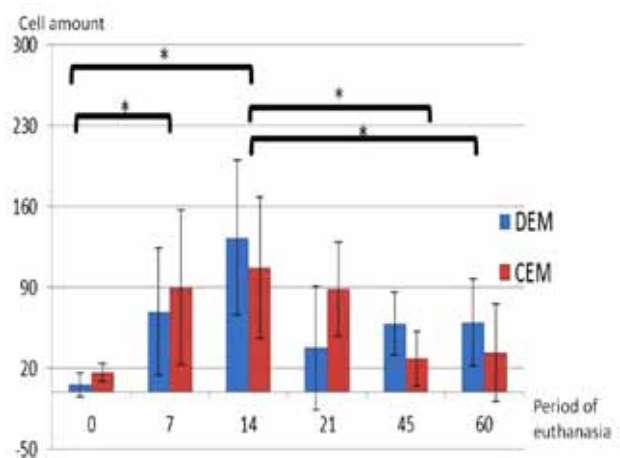
Conversely, in the blocks without membrane coverage, the osteocalcin expression pattern was more delayed in the group of diabetic rats than normoglycemic rats (Figure 6).

Thus, the presence of the membrane covering the bone graft might compensate the negative effects of diabetes in osteocalcin expression.



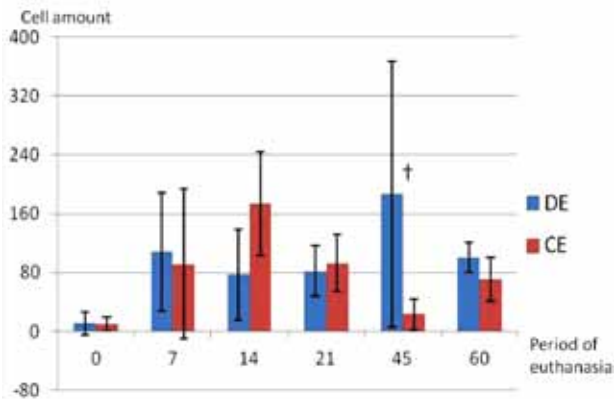
**Figure 4** - Mean and standard deviation of osteocalcin expression on receptor site-graft interface in diabetic group, for the condition with or without membrane.

\*Statistically significant difference. Intragroup Comparison. ANOVA.



**Figure 5** - Mean and standard deviation of osteocalcin expression on receptor site-graft interface between the diabetic group and control group, for the condition graft with membrane.

\*Statistically significant difference. Intragroup Comparison. ANOVA.



**Figure 6** - Mean and standard deviation of osteocalcin expression on receptor site-graft interface between the diabetic group and control group, for the condition graft.

\* Statistically significant difference. Intragroup Comparison. ANOVA.

## DISCUSSION

Considering the importance of Diabetes *Mellitus* in public health and the increasing of life expectancy of the population as well as the search for rehabilitative procedures, this present study is part of a large study on evaluating the influence of Diabetes *Mellitus* on the repair of autogenous bone grafts associated with or not resorbable collagen membrane in an experimental model of Diabetes *Mellitus* induction in Wistar rats. In this present study, we evaluated the immunohistochemical expression of osteocalcin.

Osteocalcin is associated to the initial phases of osteoid matrix mineralization and regulation of the growth of crystals [9], thus it is an important maker of late osteoblastic activity [10,11]. A study showed association of osteocalcin with mineralized areas of bone and cartilage extracellular matrices and accumulation of these proteins in tissue surfaces, which is consistent with the hypothesis that these proteins have fundamental role in the extracellular mineralization process and/or might mediate cellular adhesion. Studies [12,13] evaluated through immunohistochemistry, the guided tissue regeneration with PTFE-e barrier in created bone defects. Osteocalcin was more intensely marked in mature bone than in

the bone suffering regeneration process. The immunolocation pattern of the macromolecules of extracellular matrix suggests that the population of heterogeneous cells filling the bone defect created a favorable environment to periodontal regeneration.

It is known that Diabetes *Mellitus* delays bone repair due to the lack of fibroblastic and osteoblastic proliferation consequently reflecting on the production of amorphous fundamental substance, collagen fibers, and organic matrix [14].

The results of this present study showed that, in the diabetic group, when the presence or absence of the membrane on the receptor site/graft interface was compared, significant differences were seen regarding the period from zero to 45 days. The rationale behind this fact is that at baseline there is no remodeling activity which produces osteocalcin, while at 45 days, the remodeling process is in an advanced stage. No statistically significant differences were observed at the initial periods of 0, 7, 14 and 21 days, corroborating the affirmation that this protein is apparently unnecessary at the initial stage of mineralization process [15]. In control group, statistically differences were seen from the period zero in relation to 7, 14 and 21 days; and from day 14 in relation to days 45 and 60. This result probably occurred because at 45 and 60 days the repairing process is at final stage in rats, thus showing decreased osteocalcin activity. Accordingly, one can infer that the repairing process of normoglycemic animals seems to occur prior to that of diabetic animals, with statistically significant differences after 7 days. Moreover, both subgroups (with and without membrane), day 14 seems to be osteocalcin expression peak for normoglycemic rats, because both groups showed a similar expression pattern.

By analyzing the influence of the membrane in the diabetic versus control group statistically significant higher expression were seen regarding the period of 14 days in relation

to 0 and 7 days; and in relation to 45 and 60 days. This demonstrates that the osteocalcin expression peak in the groups receiving the membrane occur at day 14, an expression pattern similar to that of control group (with or without the membrane). This same peak could not be observed in the subgroups receiving only the graft. This might be explained by the fact that the membrane presence might have favored early remineralization, even in the diabetic group (DGM). The literature has affirmed that resorbable membranes have been associated with in-block grafts aiming at decreasing the resorption maintaining bone contour and preventing the migration or proliferation of less specialized tissues towards the bone repair process area [16]. Thus, it seems that the presence of the membrane associated with the graft in the group of diabetic rats favors the repair by matching the process speed with that of control group.

By comparing diabetic and control group without the membrane, and analyzing the factors time and condition, statistically significant differences were seen regarding the condition, at day 45 in diabetic group in relation to control group, which is expected since the repairing process in the diabetic animal is delayed compared with the normoglycemic animal. In this comparison, it could be observed that the osteocalcin peak expression in normoglycemic rats occurred at day 14 while it occurred at day 45 in diabetic rats. This might mean a delay in relation to the graft repair in the group of diabetic animals.

Still regarding to the membrane, by comparing diabetic and control group without membrane and the same groups with the membrane, it could be observed that the expression is more disordered without the membrane, while with the membrane this expression is more predictable, that is, at the initial and final stages of the repair when it is so necessary. This result corroborates the prior

finding that the membrane could improve the repair process in diabetic rats.

However, despite of the interesting findings, this present study has some limitations. The main limitation is the small number of rats per group (n). The small sample size may significantly influence the results in relation to the final mean and standard deviation by decreasing the chances of differences between groups can be detected by the statistical analysis. Thus, further studies with larger samples are necessary.

## CONCLUSION

Within the limits of this present study, it can be concluded that the osteocalcin marker might undergo some influence of diabetes, by exhibiting a late expression. However the association of the membrane with the graft may improve this delay, so that the osteocalcin expression is similar to that of normoglycemic animals.

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