LUCIANA MARQUEZ

Enhanced bone healing of rat tooth sockets after administration of epidermal growth factor (EGF) carried by liposome

Trabalho apresentado ao Programa de Mestrado em Odontologia da Universidade de Uberaba – UNIUBE, área de concentração em Biomateriais., para a obtenção do título de Mestre em Odontologia.

Orientador: Prof. Dr. José Bento Alves

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Ata da Sessão Pública de defesa de dissertação para obtenção do título de Mestre em Odontologia, área de concentração em Biopatologia, a que se submeteu a Luciana Marquez – matrícula 5087320, orientada pelo Prof. Dr. José Bento Alves

Aos vinte e quatro dias do mês de maio do ano de dois mil e doze, às 14h, na sala plenária de reuniões - PROPEPE da Universidade de Uberaba, reuniu-se a Comissão Julgadora da defesa em epígrafe indicada pelo Colegiado do Programa de Mestrado em Odontologia da Universidade de Uberaba, composta pelos Professores Doutores: José Bento Alves - **Presidente**, Maria Angélica Hueb de Menezes Oliveira e Andréa Dias Neves Lago, para julgar o trabalho da candidata Luciana Marquez, apresentado sob o título: **"Enhanced Bone Healing of Rat Tooth Sockets After Administration of Epidermal Growth Factor (EGF) Carried by Liposome"**. O Presidente declarou abertos os trabalhos e agradeceu a presença de todos os Membros da Comissão Julgadora. A seguir a candidata dissertou sobre o seu trabalho e foi argüida pela Comissão Julgadora, tendo a todos respondido às respectivas argüições. Terminada a exposição, a Comissão reuniu-se e deliberou pelo seguinte resultado:

APROVADO
REPROVADO (anexar parecer circunstanciado elaborado pela Comissão Julgadora)

Para fazer jus ao título de MESTRE EM ODONTOLOGIA ÁREA DE CONCENTRAÇÃO BIOPATOLOGIA, a versão final da dissertação, considerada Aprovada devidamente conferida pela Secretaria do Mestrado em Odontologia, deverá ser entregue à Secretaria dentro do prazo de 30 dias, a partir da data da defesa. O aluno Aprovado que não atender a esse prazo será considerado Reprovado. Após a entrega do exemplar definitivo, o resultado será homologado pela Universidade de Uberaba, conferindo título de validade nacional aos aprovados. Nada mais havendo a tratar, o Senhor Presidente declara a sessão encerrada, cujos trabalhos são objeto desta ata, lavrada por mim, que segue assinada pelos Senhores Membros da Comissão Julgadora, pelo Coordenador do Programa de Mestrado em Odontologia da UNIUBE, com ciência do aluno. Uberaba, aos vinte e quatro dias do mês de maio de dois mil e doze.

Prof. Dr. José Bento Alves. _____

Profa. Dra. Maria Angélica Hueb de Menezes Oliveira _____

Profa. Dra. Andréa Dias Neves Lago _____

Prof. Dr. José Bento Alves _____
Coordenador do Programa de Mestrado em Odontologia da UNIUBE

Poliana Gomes da Silva Alves _____
Secretária do Programa de Mestrado em Odontologia da UNIUBE

Ciência da Aluna: _____

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Fig 2. Histological sections of sockets after 7 days of tooth extraction. (a) Histological aspect of bone healing at the groups BC, L, P and EGF-P. Note the development of the first bone trabeculae at the apical portion (A) of the alveoli (*). Localized inflammatory infiltrated regions can be observed at the cervical portion (C) (arrows). (b) Bone healing process on sockets from group EGF-L. There are more developed bone trabeculae at the apical (A) and medium (M) portions of the alveoli. Bar 80 µm.

Fig 3. Histological sections representing groups BC, L, P and EGF-P at 14 days after tooth extraction. (a) the connective tissue is highly organized with neoformed trabeculae (*) at the apical portion (A). (b) healing process on group EGF-L, showing bone trabeculae (*) at both apical (A) and medium (M) portions. Bar 100µm.

Fig 4. Histological characteristics of alveolar healing process at 21 days (a) alveoli from groups BC, L, P and EGF-P. Note the fine trabeculae (T) with wide medular spaces (m). (b) alveoli from group EGF-L, where more organized trabeculae (T) and smaller medular spaces (m) rather than in the earlier groups are observed. Note the trabeculae rearrangement to form osteons (arrows). Bar 100 µm.

Figure 5. Quantification of bone trabeculae percentage in tooth sockets at 7, 14 and 21 days after surgical procedure. Data are shown as mean ± S.D. *$p < 0.05$.

Fig 6. Representative photomicrographs of Fibronectin (FN) immunoreactivity in sockets at 3 days after surgical procedures: a – filled with blood clot (BC) and treated with L, P and EGF-P; b – treated with EGF-L; and after 7 days of surgery: c - filled with blood clot (BC) and treated with L, P and EGF-P; d – treated with EGF-L. Bar 50 µm.

Fig 7. Representative photomicrographs of Collagen III (Col III) immunoreactivity in sockets at 3 days after surgical procedures: a – filled with blood clot (BC) and treated with L, P and EGF-P; b – treated with EGF-L; and after 7 days of surgery: c - filled with blood clot (BC) and treated with L, P and EGF-P; d – treated with EGF-L. Bar 50 µm.

Table 1. Control and experimental groups.

Table 2. Staining levels of fibronectin and type III collagen at 7 days.

Enhanced bone healing of rat tooth sockets after administration of epidermal growth factor (EGF) carried by liposome

Luciana Marquez [a]
Fernando Antônio Mauad de Abreu [b]
Cynthia Lopes Ferreira [b]
Guilherme Dias Alves [a]
Melissa Nunes Miziara [a]
José Bento Alves [a,b]

Author's affiliations:

[a] Laboratory of Biopathology and Molecular Biology, University of Uberaba, MG, Brazil.
[b] Laboratory of Oral and Development Biology, Department of Morphology, Biological Sciences Institute, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil.

Corresponding author:

José Bento Alves, PhD
Laboratory of Biopathology and Molecular Biology - University of Uberaba
Av. Nenê Sabino, 1801 – Bairro Universitário. Uberaba, MG. Cep 38.055-500.
Uberaba MG, Brazil - Phone: +55 034 3319 8915 Fax: +55 034 3314 8910
E-mail: propg.pr@uniube.br

ABSTRACT

Considering the potential use of growth factors carried by liposomes for bone repair, this study aimed to assess the progress of bone healing process in injured alveoli of rats after administering EGF within liposomes. For this assessment we used 48 male Wistar rats that had their maxillary second molar extracted and separated into 5 groups: sockets filled with blood clot (BC), treated with empty liposome (L), PBS (P), EGF in PBS (EGF-P) and EGF in liposome (EGF-L). The animals were sacrificed after 3, 7, 14 and 21 days after surgery. Histological, histomorphometric and immunohistochemistry analysis were performed to evaluate new bone and blood vessels formation as well as the expression of fibronectin and collagen type III, two determinant proteins for early wound regeneration. Our analysis showed a continuous transformation of sockets during all stages of wound healing. Nevertheless, groups BC, L, P and EGF-P followed a regular time for regeneration significantly different from the EGF-L group, which showed faster recovering. A higher expression of fibronectin and type III collagen in the group EGF-L after 3 and 7 days of surgery was observed and might be explained by the ability of the liposome to deliver EGF in a controlled manner, stimulating mesenchymal cells migration and osteoblast differentiation. As liposome efficiently regulated the availability of EGF without risks for its function and protected the factor from early absorption and degradation, the present work indicates that liposomes can be successful used as carriers for controlled delivery of growth factors in bone healing.

Keywords: bone regeneration, liposomes, epidermal growth factor.

INTRODUCTION

The understanding of the basic events of bone healing process is one of the most important steps for developing new techniques and strategies to stimulate it[1]. Such knowledge depends on deciphering the molecular pathways that surround the bone formation, and in particular, the relationship between progenitor cells and the environment where they will differentiate themselves as well as the interaction with biomaterials that have been usually applied for filling the bone defects.

Many strategies have been developed to accelerate bone repair, including treatment with exogenous growth factors, the use of tentative bone grafts, scaffolds and the development of new methods and alternatives for administering inducing agents and osteogenic drugs enabled to promote bone regeneration[2-6]. These methods aim to improve wound healing by accelerating bone growth focusing on each stage of the repair process: inflammation, vascularization, remodeling, osteoinduction or osteoconductivity[7].

The tissue repair process is a complex cascade of biological events controlled by numerous cytokines and growth factors (GF) that provide local signals to mediate migration of osteoprogenitor cells and subsequently differentiation towards specific cell lineages, cell proliferation, revascularization and production of extracellular matrix. Several growth factors, such as FGF (fibroblast), EGF (epidermal) and VEGF (vascular endothelial), are currently expressed during development and bone repair and their local availability in the wound might collaborate for faster and successful clinical procedures for bone healing, as they affect regeneration, chemo attraction, proliferation and differentiation of cells[8].

Thus, therapies with growth factors have been applied for the promotion and/or induction of new osseous formation in bone defects, fracture sites and adjacent areas of metallic implants[9-14], allowing Tissue Engineering to clarify the details concerning the growth factor roles in the different steps of wound healing[15].

Present in several tissues[16], the epidermal growth factor (EGF) is a protein that owns the ability to stimulate DNA synthesis and is directly associated with rapid tissue regeneration after injuries[17,18]. This factor has wide biological action and it is involved with the induction of growth and proliferation of mesenchymal and epithelial cells, stimulus for granulation tissue formation and angiogenesis, when the invasion of blood vessels has a decisive role in tissue repair[19-22].

EGF has been demonstrated to stimulate vascular permeability and tissue growth, suggesting that this growth factor is a path by which angiogenesis and osteogenesis are linked or dependent during bone repair[23]. The administration of these stimulating molecules in bone wounds aims to make possible their action as healers. However, most of the injected drug is usually eliminated before any additional enhanced activity and sometimes a high and unsafe (physiologically) concentration of the protein is needed to obtain the desired results which, in some cases, may incite unwanted side effects[6,15,24-25]. Thus, the development of methods that induce vascularization in a physiologically sustainable way is critical[14].

Considering the knowledge about cell roles of growth factors, many researchers have developed strategies to promote healing using a managed delivery system set, e.g., vehicles that allow GF to be transported while speed, time and amount of the delivery are controlled[5,13,26-31]. However, the current systems do not permit the factors to reach their targets properly and the biological feedback may not be the expected[14].

Thus, the ideal is to get a system enabled to release therapeutic compounds in a gradual, localized and constant way during the process of alveolar healing. In this context, liposomes have been described as a choice for carrying and distributing a great variety of active compounds, such as small drug molecules, proteins, nucleotides and plasmids[23]. They are microscopic spherical particles produced from cholesterols, non toxic surfactants, sphingolipids, glycolipids, long chain fatty acids and even membrane proteins[32]. Liposome membranes, developed with one or more lipid bilayers, encapsulate a portion of solvent that is kept suspended within it.

Compared with other carriers, liposomes have several advantages, such as biocompatibility, biodegradability and toxicological and immunological safety. This mechanism of molecular transport increases efficiency and solubility of the drug, reduces side effects and toxicity, releases drugs directly to specific locations and in a controlled manner, reduces toxicity, improves bioavailability and alters the pharmacokinetics as well[23,25,26,33].

During wound healing, fibroblasts produce several proteins, such as osteocalcin, osteonectin, proteoglicans, fibronectin, some growth factors, morphogenetic proteins and different types of collagen. All these molecules are involved in the formation and/or organization of the extracellular matrix (ECM), including cell adhesion and migration as well as vascularization of the matrix.

Fibronectin is an important and abundant protein of the granulation tissue[34,35], as it contributes for organizing this tissue by assisting the binding of cells and fibril components

and mediating the migration and attachment of osteo-progenitor cells to the collagenous matrix, which results on a dense network around fibroblasts. This protein is also associated to collagen deposition on the ECM[34,35].

Type III collagen is one of the fibrilar collagens present on the connective tissue. It has been found in high concentrations at the early stages of bone regeneration[36,37], when migration of osteoprogenitor cells and vascularisation occurs to form the new tissue. At the final stages, collagen type III is replaced by collagen type I, which lead the tissue calcification[38].

As fibronectin and type III collagen stimulates the beginning of the wound healing, these two proteins have been used as indicatives of bone regeneration progress[33].

Considering the potential use of growth factors carried by liposomes for bone repair, this study aimed to assess, by using histological, histomorphometric and immunohistochemical procedures, the progress of bone healing in injured alveoli of rats after administration of EGF carried by liposomes. In addition, immunohistochemical analysis was carried out to evaluate the expression of fibronectin and collagen type III, two determinant proteins for early wound regeneration.

MATERIAL AND METHODS

EGF Liposome preparation

The EGF liposomes were prepared according to Alves et al.[23], as follows: EGF was dissolved in 1mL of 10mM acetic acid containing 0.1% (w/v) bovine serum albumin (BSA) in a 0.2 µg/ml final concentration and stored at −20 °C. The liposomes were obtained by sonication using a solution of 3.0mg/mL of dipalmitoyl fostatidil choline (DPPC), 0.3 mg/mL lisofosfatidil choline and 10µL of EGF solution, producing homogeneous unilamellar vesicles containing EGF with ~100 nm of diameter.

Animal sampling and surgery

Wistar male rats weighing 250-300g were conditioned at room temperature and light-dark cycle of 12 hours into plastic cages with acess to food and water ad libitum. All animal handling procedures followed the Brazilian College of Animal Experimentation Guidelines and the research was approved by the Ethics Committee in Animal Experimentation of University of Uberaba under Protocol nº 082/2009. Forty-eight randomly chosen animals were anesthetized by intramuscular injection of 2% xylazine and 10% ketamine (1:1; 0.1 ml/100g body weight) solution and had both right and left second upper molars extracted.

Samples distribution

The right sockets were occasionally chosen to be filled with clot and constituted the control side (BC group); the left sockets were used to perform the EGF-liposome administration procedure (experimental side) and were divided into four groups as follows: group P: 1uL of phosphate buffered saline (PBS); group L: 1uL of empty liposomes; group EGF-P: 20ng/uL of EGF in PBS and group EGF-L: 20ng/uL of EGF in liposomes (Table 1). The rats were sacrificed at 3, 7, 14 and 21 days after surgery by anesthetic overdose and subsequent cervical dislocation. The sockets were removed and instantly fixed in 10% buffered formalin for 48 hours and demineralized in 10% buffered EDTA solution for 40 days. Finally the demineralized sockets were then embedded in paraffin.

Histological analysis

The paraffin blocks were serially sectioned at 6μm in the sagittal plane and stained with hematoxylin-eosin (HE) for histological and morphometric analysis or processed for immunohistochemistry. The sections were first observed in a light microscope (Axiostar Plus – Carl Zeiss) and analyzed by considering the patterns of organization and maturation of the connective tissue that fills the alveoli, the presence of bone cells (osteoblasts), neoformed bone tissue, blood vessels and inflammatory cells on the control and experimental sockets.

Morphometric analysis

The morphometric analysis was performed according to previous work[23,40], as follows: 12 randomly chosen sections were evaluated for both experimental and control sides. Representative areas of each sample on different stages were captured and the observed trabecular area were contoured and determined. The total area was also registered. The percentage of trabecular bone area of each captured field was obtained by dividing the trabecular area by the total area using the AxioVision Release 4.8 software (Carl Zeiss).

Immunohistochemical and histoquantitative analysis

Additional sections were mounted on glass slides and processed for immunohistochemistry as previously described[24] to evaluate EGF expression. Briefly, slides were pre-treated with 3-aminopropyltriethoxy-silane (Sigma), immersed for 3 min in xylene to eliminate the paraffin, dehydrated in absolute alcohol and rehydrated with Tris-buffered saline (TBS). The sections were then rinsed in TBS and immersed in 3% hydrogen peroxide in methanol for 10 min and incubated for 30 min at 90 °C for antigen detection. Slides were incubated in 2% bovine serum albumin for 30 min at room temperature to reduce non-specific binding. The slides were then incubated with polyclonal antibodies anti-collagen type III and anti-fibronecftin at 1:50 dilution (Santa Cruz Biotechnology) for 2h at 37 °C and then rinsed with TBS three times for 3 min each. Sections were incubated with the appropriated secondary biotinylated antibody from Link System (DAKO, EUA) for 30 min at 37°C. After being washed, the sections were incubated with streptavidin–peroxidase conjugated (DAKO) for 30 min. Next,

slides were treated with a solution containing H2O2 (0.05%) and 1mg/mL DAB (1,4-dideoxy-1,4-imino-D-arabinitol-diaminobenzidine; Sigma), incubated for 30 min, and then rinsed in Tris–HCl buffer (pH=7.4). Slides were counterstained with Mayer's hematoxylin, dehydrated and mounted. Staining specificity was tested by omission of primary antibody. In order to determine the amount of immunostained fibroblasts, randomly chosen sections were measured for both experimental and control sides. The slide images were obtained by using a capture plate and a microscope (Zeiss Axiostar Plus) interfaced with a personal computer and AxioVision Release 4.8 software (Carl Zeiss).

The immunostaining intensity was assessed individually in a blind fashion by two calibrated examiners (kappa index 0.91)[41]. At least 10 representative sections of each specimen were analyzed under light microscope (Zeiss Axiostar Plus). Relative staining intensity was assessed for each molecule at the extracellular matrix and fibroblasts cells. Samples were scored as follows: 0 (zero) - no immunoreactivity; 1 - weak but visible staining intensity; 2 - moderate staining intensity; and 3 - strong staining intensity.

Statistical analysis

The collected data were statistically tested by using Tukey test according to Dun analysis. All groups were evaluated three times and significance was considered when $p<0,05$.

RESULTS

Histological and histomorphometric analysis

Our results followed the healing process on sockets of rats killed at 3, 7, 14 and 21 days after tooth extraction. The sockets from the groups BC, L, P and EGF-P are described here as the same set (control groups – CG), since their histological features were very similar and were compared with histological results of the EGF-L group. All the histological analyses considered the healing process on the three portions of the socket: apical (maxilla bottom), middle and cervical (top).

During the period of the study the healing process on the tooth sockets followed a well-defined course, showing typical histological features of the bone healing stages: inflammation, formation of granulation tissue and primary bone tissue as well as its replacement by lamellar bone. Blood clot was consistently replaced by granulation tissue (and intense inflammation), which gave place to a provisional, vascularized and cell-enriched matrix.

In specimens representing 3 days of bone healing, the blood clot was found in the cervical region of the socket. On the apical and middle portions the coagulum had been replaced by a granulation tissue (GT), characterized by the presence of large number of vascular structures within a connective tissue comprising mesenchymal cells and inflammatory infiltrates (Fig. 1A). In the sockets of the EGF-L group, the process of healing was more pronounced, including well organized fibers and the first events of osteoblasts differentiation (Fig. 1B).

After 7 days of extraction, the wound had undergone a remarkable change when compared to the 3-day specimen. In CG sockets (BC, L, P and EGF-P), the granulation tissue was replaced by a vascularized provisional matrix and new bone formation (woven bone). Connective tissue associated with inflammatory infiltrate was observed on the cervical region (Fig. 2A). In the EGF-L group, the woven bone formation was more organized where dilated vessels and localized infiltrate cells have merged (Fig. 2B), and groups of osteoblasts closely associated with bone neoformation suggested a greater number of trabeculae than in CG set.

Histological analyses of sockets from rats killed at 14 and 21 days after tooth extraction revealed a progressive replacement of the immature woven bone by lamellar bone, characterized by a few number of osteocytes, the presence of early osteons and reduction of

marrow spaces. These alterations were less evident on the CG set and more pronounced on the EGF-L group (Fig. 3A and B; Fig. 4A and B), where the histomorphometric evaluation registered a statistically significant difference ($p<0.5$) in the number of trabeculae (Fig. 5).

Immunohistochemistry analyses

Immunohistochemical procedures were used to investigate the time-dependent changes on fibronectin and collagen type III expression involved in post-extraction wound healing. Our results revealed a progressive increase in the intensity of immunostaining at 3 and 7 days in the sockets from group EGF-L (Table 2; Fig. 6 and Fig. 7).

DISCUSSION

Healing process of a tooth socket involves chemotaxis of undifferentiated cells, differentiation, synthesis of osteoid, mineralization, maturation and remodeling of bone. These processes are regulated by growth factors and cytokines. Furthermore, local factors as blood supply, mechanic stimuli and microenvironment play an important role in repair and regeneration[42].

It has been reported that during the process of healing a series of events such as formation and maturation of a blood clot, infiltration of fibroblast to replace the coagulum, establishment of a provisional matrix that allowed for bone tissue formation[43]. These events are supported by many cell components and molecules, e. g., cytokines, mesenchymal cells and growth factors, including VEGF, EGF, BMP and others.

The role of these factors consists on stimulating proliferation, differentiation and migration of cells, resulting on filling the socket with all machinery and cells that had been lost after extraction. However, the external administration of such factors has been frequently shown inefficiency on promoting healing, which has required a higher dosage that brings about undesirable side effects.

EGF has been demonstrated to activate the angiogenesis process[44]. Thus, this study followed the healing process occurring in rats sockets treated with EGF within liposome, with the aim to evaluate whether the carrier could influence on time and progress of bone repair, since the growth factor has been gradually released by the liposomes, which might allow the EGF to achieve its signaling role during all phases of regeneration.

Considering the early stages of bone repair, which were the focuses of the present work, fibronectin and collagen type III have been selected for immunohistochemistry to confirm their role in healing process and to indicate the progress of regeneration.

Our results showed that after three days of tooth extraction, a blood clot has formed within the cavity and consequently the healing process has started, as a granulate tissue is formed as well as a provisional matrix filled with primary blood vessels and inflammatory cells. This is in accordance to literature, which also identified these first steps of the regeneration[42]. In this stage, fibronectin and collagen type III were immunohistochemically identified in a high concentration, confirming their intensive achievement during the early stage of bone regeneration.

At seven days, primary trabeculae have emerged in an immature bone tissue (woven bone), quite vascularized at the apical portion of the socket. This condition was even more pronounced in the group EGF-L. Immunoanalyses showed fibronectin and collagen type III expressing on the matrix in a way to follow the progress of maturation of the bone: as more advanced is the process, less expression is observed, indicating that the apical portion has evolved from inflammatory stage to immature tissue.

In the sockets analyzed after 14 and 21 days of surgery, an increased number of trabeculae from all groups was observed, but in a higher level on group EGF-L. From these stages on, fibronectin and collagen type III were completely replaced and no immunohistochemical results were observed.

Considering the performance during all stages of wound healing, there was a continuous transformation of the socket, from a blood clot to mature bone. Nevertheless, the groups which have not received EGF, as well as the group EGF-P, followed a regular time for regeneration that was significantly different from the EGF-L group. The most prominent finding of the current study is that EGF carried by liposomes accelerated the healing process of rat sockets after upper second molar extraction. At 3 and 7 days after surgery, sockets treated with EGF-L showed a more pronounced expression of fibronectin and collagen type III and an increased new bone formation. The higher expression of fibronectin and type III collagen sockets treated with EGF-L could be due to the ability of the liposome to continuously deliver the growth factor to stimulate the migration of mesenchymal cells and consequently the osteoblasts differentiation.

The comparative analysis of groups EGF-P and EGF-L showed a very interesting and clarifying behavior. After three days of surgery we could expect similar morphometric results for the two groups, but our experiments indicated that free exogenous EGF in PBS had a localized, limited and rapid performance that was not sufficient to cause enhanced differentiation and influence regeneration from that stage until tissue maturation. The presented data showed that EGF-P behavior was very similar to those groups with no EGF injected, suggesting that the factor might be rapidly flushed by blood circulation and has not participated of the next stages of bone healing.

Similar results were described by Alves and co-workers[23] during experimental orthodontic movement in rats treated with EGF carried by liposome compared to EGF administered directly in PBS solution.

In the EGF-L group at this present work, the liposomes retained EGF for a period sufficient to stimulate continuous new bone formation, thereby providing a carrier of growth factor for further bone growth. As EGF had been gradually released by liposomes, it achieved since the earlier steps of regeneration, including the first differentiation events, until the differentiation of lamellar bone tissue, influencing resorption.

CONCLUSION

The present study indicates liposomes as successful carriers for controlled releasing, which enhance bone healing by regulating the availability of the growth factor on the socket without jeopardizing its function and protecting it from early absorption as well as degradation by proteases.

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Tables

Table 1.

groups	description
BC	blood clot
P	1uL of PBS
L	1uL of empty liposomes
EGF-P	20ng/uL of EGF in PBS
EGF-L	20ng/uL of EGF in liposomes

Table 2.

Fibronectin					
Cell	0.7±0.3[a]	0.6±0.5[a]	0.4±0.1[a]	0.7±0.5[a]	1.21±0.15[b]
Extracellular matrix	0.8±0.4[a]	0.6±0.7[a]	0.5±0.2[a]	0.4±0.3[a]	1.15±0.17[b]
Collagen III					
Cell	0.2±0.08[a]	0.16±0.07[a]	0.21±0.10[a]	0.16±0.09[a]	1.30±0.17[b]
Extracellular matrix	0.3±0.05[a]	0.14±0.04[a]	0.17±0.11[a]	0.13±0.06[a]	1.32±0.15[b]

Data are presented as mean and standard deviation of the mean from all sections analyzed per area.
a,b ***Letters represent intra-group analysis. Different letters differ statistically (p<0.05)***

Figures

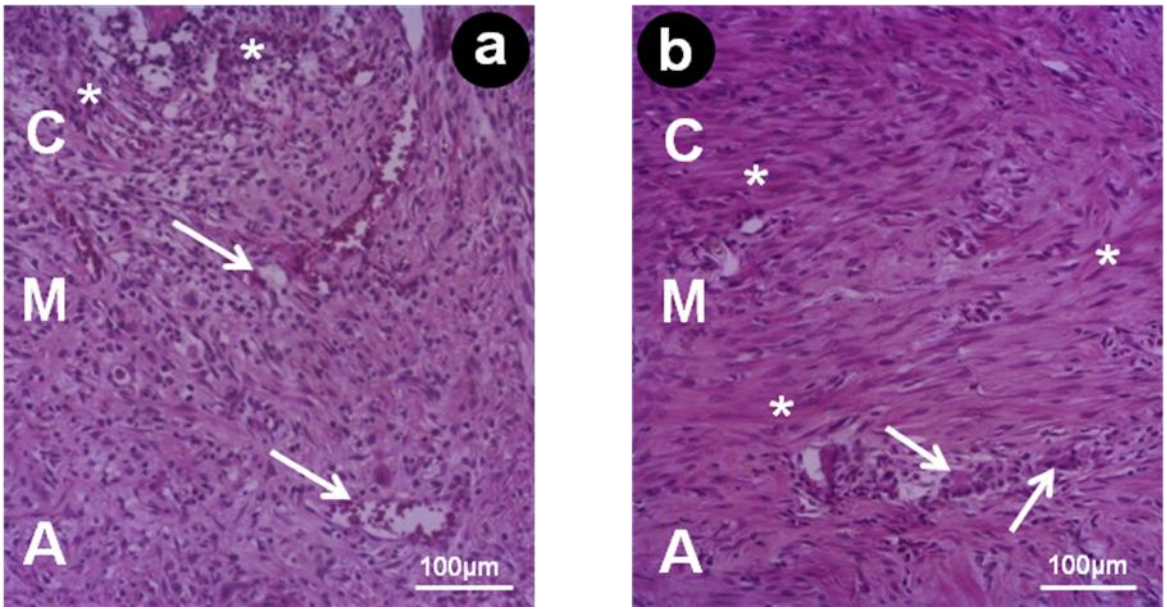

Figure 1

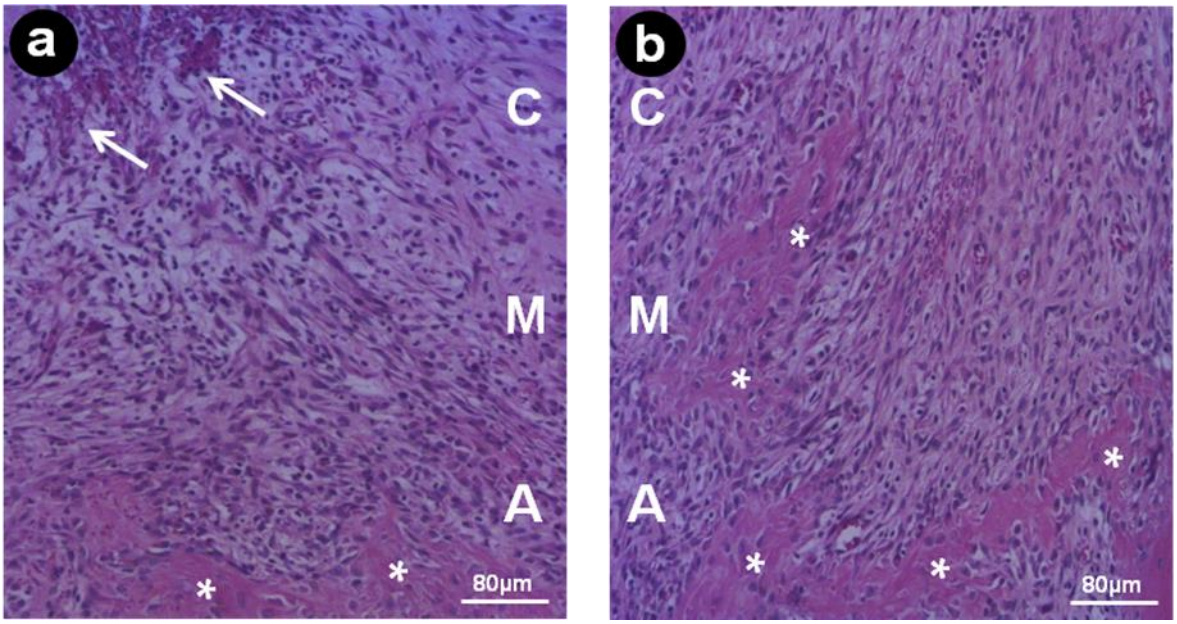

Figure 2

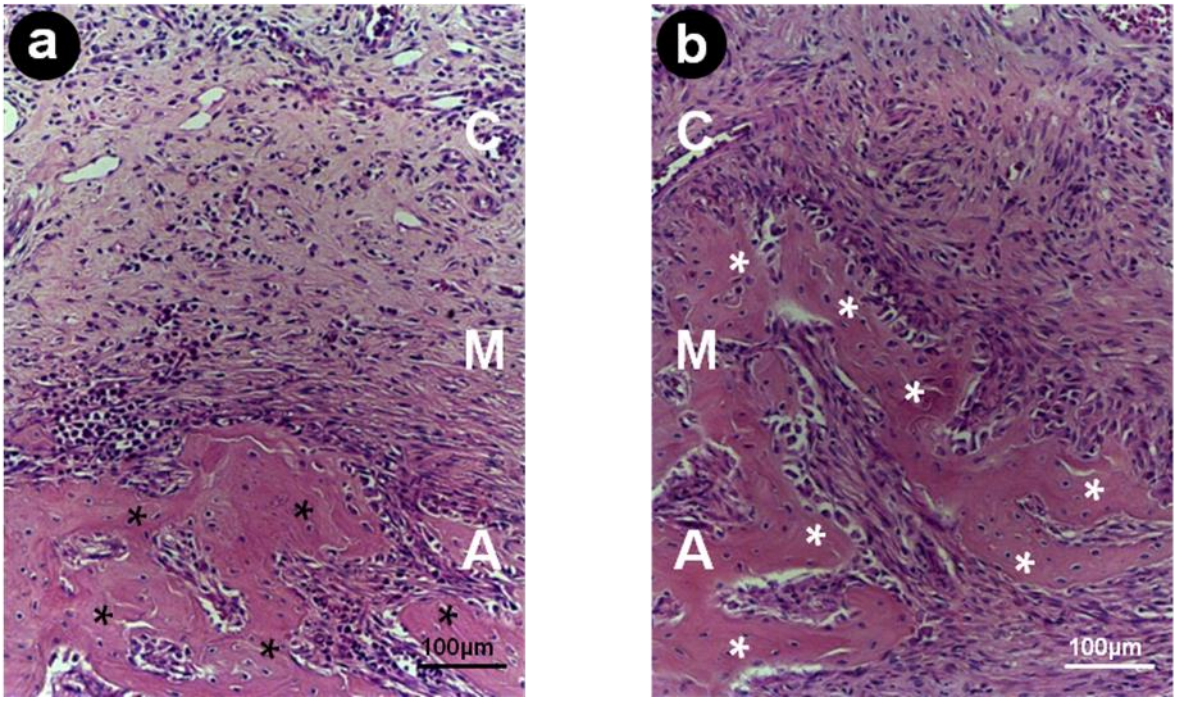

Figure 3

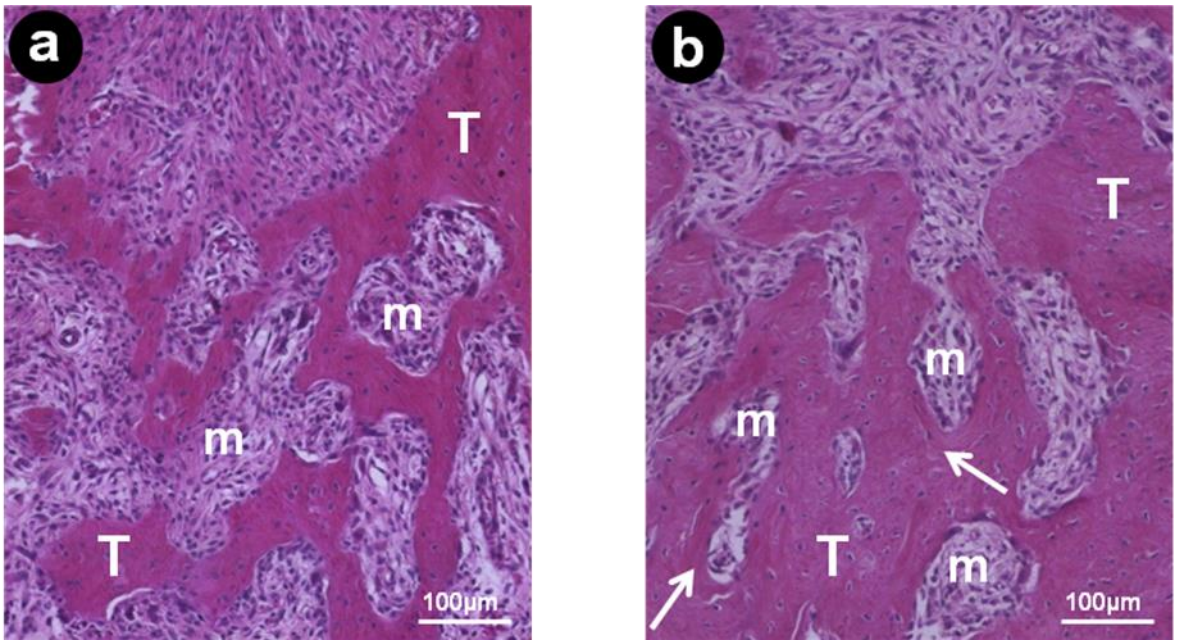

Figure 4

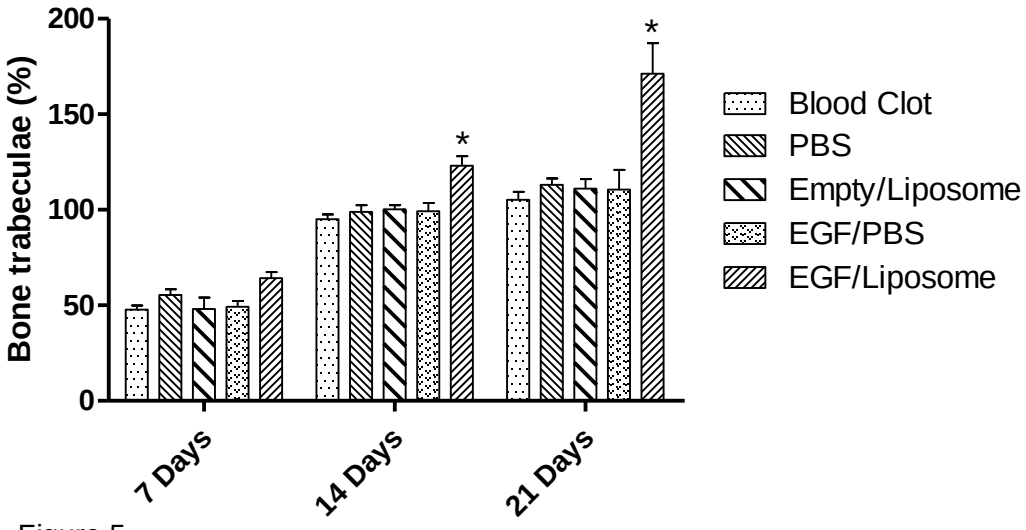
Bone trabeculae (%)
200
150
100
50
0
*
*
7 Days
14 Days
21 Days
Blood Clot
PBS
Empty/Liposome
EGF/PBS
EGF/Liposome

Figure 5

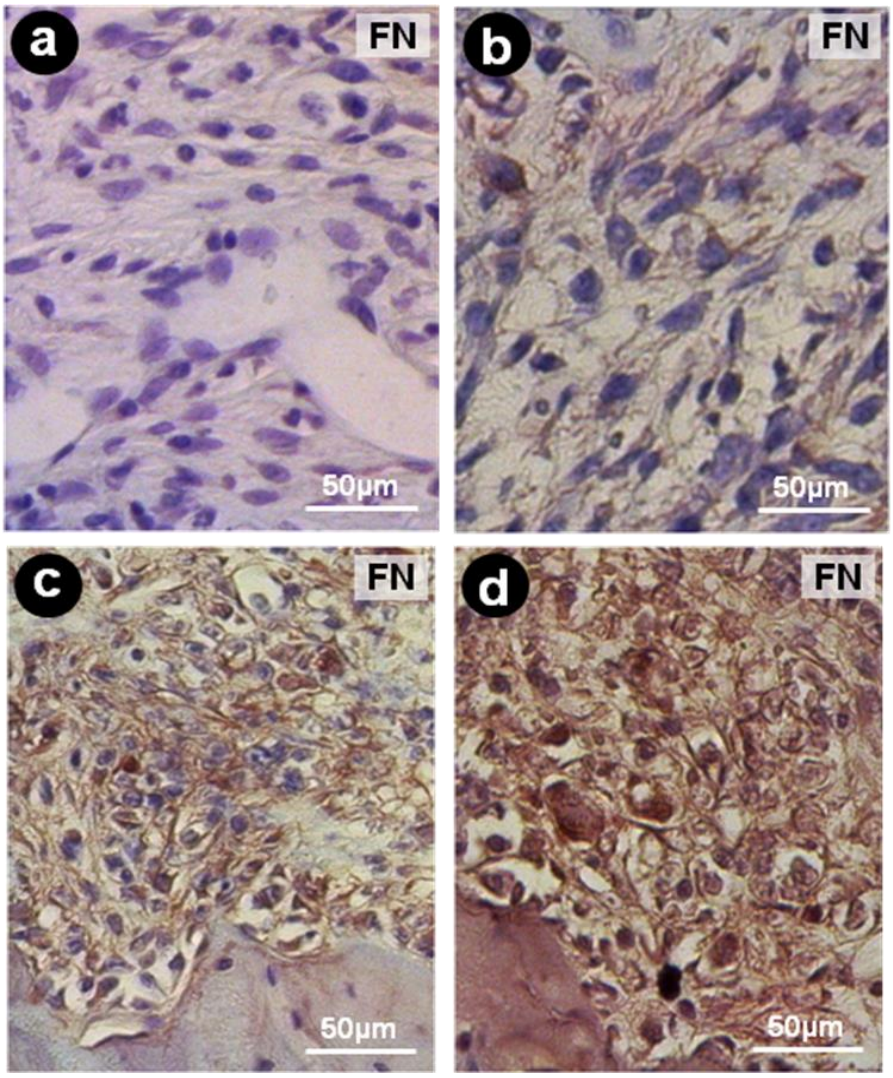

Figure 6

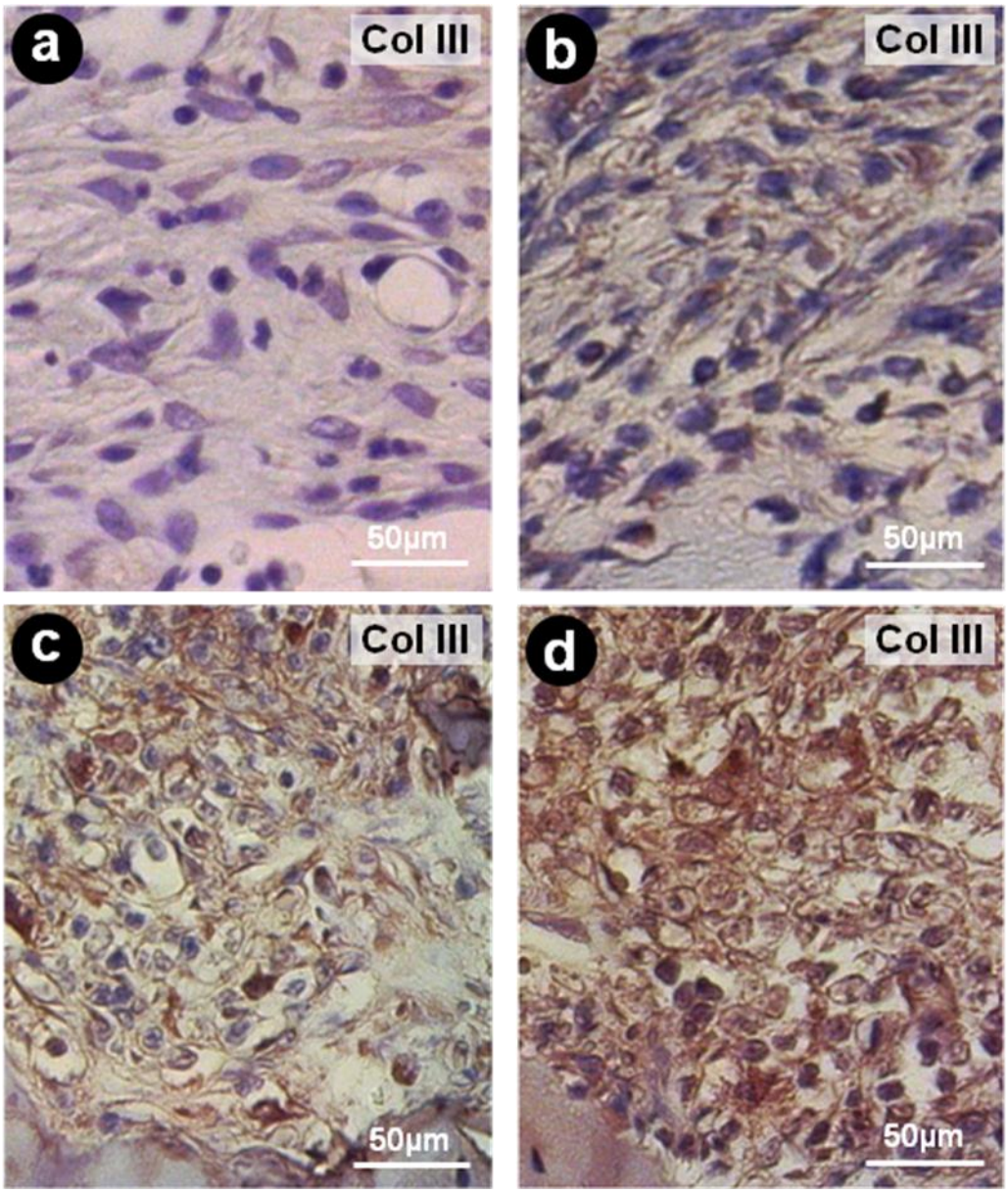

Figure 7