Int. J. Morphol.,
43(2):535-540, 2025.

Oral Intake of Transfer Factor Accelerates Bone Consolidation in Adult Wistar Rats

La Ingesta Oral de Factor de Transferencia Acelera la Consolidación Ósea en Ratas Wistar Adultas

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PADULA, E. O. C.; FORTUNA-COSTA, A.; SAMPAIO, F. J. B. & GREGORIO, B.M. Oral intake of transfer factor accelerates bone consolidation in adult Wistar rats. *Int. J. Morphol., 43(2)*:535-540, 2025.

SUMMARY: Bone fracture healing is a complex regenerative process that aims to restore damaged bone to its pre-injury state. To date, no natural substance has been described that accelerates and/or promotes bone consolidation. Transfer factor (TF), by acting on the immune system, may have benefits in this type of injury, as it can modulate the post-fracture inflammatory response. So, we aimed to investigate the histological effects promoted by oral ingestion of TF on the bone consolidation process in adult rats. Adult Wistar rats were divided into two groups: control (C, n=10) and transfer factor (TF), which were followed for two (C2 and TF2) and six weeks (C6 and TF6) post-fracture. Both groups had fractured tibias and received, by gavage, water, or TF, respectively. Body mass and food intake were monitored weekly and daily, respectively. At the end of these times, the animals were euthanized, and the tibias were removed for histomorphometric analysis. Data were analyzed using Student's t-test. Body mass did not differ between the groups, although the food intake of the TF2 group was lower than that of the C2 group. The density of the mature bone matrix was higher in the TF2 and TF6 groups when compared to the respective controls. In the groups two weeks post-fracture (TF2 and C2) there was no difference in the densities of chondrocytes, vessels, and cartilaginous and intermediate bone matrices. In contrast, the TF6 group showed a reduction in the densities of cartilaginous, intermediate bone matrix and vessels when compared to C6. Chondrocyte density was not different. Treatment with TF showed positive results in bone consolidation, and can be an adjuvant in the treatment of bone consolidation, as well as in preventing the delay in this process.

KEY WORDS: Transfer factor; Fracture healing; Rats; Bone regeneration.

INTRODUCTION

Bone fractures are one of the most common medical events. Failure in the bone repair process leads to non-union in 5 to 10 % of long bone fractures. This leads to a significant economic impact due to increased hospital stays and overuse of health resources such as drugs and surgeries (Ekegren *et al*., 2018). It is known that the bone repair process involves different stages, from the occurrence of the fracture to the end of bone remodeling. Initially (first days post-fracture), there are signs of an acute inflammatory process, with the presence of inflammatory cells, such as neutrophils and macrophages, at the fracture site. Then (one to two weeks post-fracture), there is the formation of a soft callus, made up predominantly of cartilage and collagen. Subsequently (three to four weeks post-fracture), the hard callus (bone tissue) originates, in which the ossification of the soft callus can now be seen. Finally, the last stage, which can take months after the fracture, is the bone tissue remodeling phase, in which the callus is replaced by newly formed bone that will be remodeled (Umiatin *et al*., 2021).

In both humans and rats, this organization and alignment of bone tissue during bone repair are accompanied by the presence of blood vessels and cells responsible for bone synthesis (osteoblasts) and reabsorption (osteoclasts) (Udupa & Prasad, 1963; Umiatin *et al*., 2021). However, some factors, such as nutrition, age, presence of comorbidities, can negatively affect the bone consolidation process, altering the composition of the extracellular matrix (Umiatin *et al*., 2021). To date, little has been discussed in the literature about the use of natural substances that can act directly on bone repair, modulating it. Transfer factors (TF), discovered in 1949 by the American immunologist Dr. Henry Sherwood Lawrence at New York University, are some of them. By definition, they are natural peptides that are non-species specific, small in size, non-allergenic, and act as immunomodulators (Lawrence, 1974). Some studies show their applicability as a dietary supplement, not only in medicine but also in global nutrition, as they appear to act directly on the immune response, providing prophylactic and therapeutic benefits to users (Krishnaveni, 2013).

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Received: 2024-10-18 Accepted: 2024-12-12

Experimental studies correlating TF and the immune system have contributed to a better understanding of the pathophysiological mechanisms involved in this relationship (Krishnaveni, 2013). However, nothing is described about the role of this substance in bone repair. Thus, this work aims to identify the histological effects promoted by the oral ingestion of TF on the bone consolidation process in adult Wistar rats submitted to fractures.

MATERIAL AND METHOD

Ethical aspects. The study was carried out at the Urogenital Research Unit of the State University of Rio de Janeiro (UERJ). Animal handling occurred according to the principles described in The Guide for the Care and Use of Laboratory Animals, CIOMS code of ethics for animal experimentation (Howard-Jones, 1985; Bayne, 1996), and Use of animals in experimental surgery, being approved by the local Ethics Committee for the Care and Use of Laboratory Animals (CEUA/020/2021).

Sampling and experimental protocol. Twenty-one adults male Wistar rats (90 days old), obtained from the animal facility of the Urogenital Research Unit, were divided into two experimental groups, according to the post-fracture time and respecting the initial and final stages of the bone consolidation process: two weeks (n=10) and six weeks (n=11). The rats were anesthetized with ketamine hydrochloride (Pfizer Ltda., São Paulo, SP, Brazil) at a dose of 40 mg/kg/weight associated with xylazine (Virbax® 2 %, Virbac do Brasil, Jurubatuba, SP, Brazil) at a dose of 5 mg/kg/weight, intramuscular injection in the medial surface of the animal's right thigh. Animals that presented regular breathing, flaccid skeletal muscles, and absence of reflexes were considered anesthetized. A metallic wire of 1 mm in diameter was introduced into the medullary canal of the tibias (Padula *et al.*, 2003), and subsequent production of the fracture in the middle third of the two tibias of the rats mechanically with a guillotine. According to An *et al*. (1994) we used three support points on the diaphysis, similar in terms of trait and without exposure to the external environment, after realigning the fragments, thus facilitating the histological cuts. After recovery from anesthesia, the animals were placed back in the previously identified boxes, with water and food ad libitum.

Throughout the first-week post-fracture, all animals received 50 mg of tramadol by gavage. The radiographic exams were performed and processed in the Radiology Department of the Pedro Ernesto University Hospital (UERJ, Rio de Janeiro, Brazil) in compliance with all radiological safety standards. Images were analyzed using the RadiAnt DICON 2021.2.2 viewer.

All animals were submitted to gavage, half with TF, and the other half with placebo (water, in the same volume), forming four groups: C2 (n=5, control animals, two weeks, which received water); TF2 (n=5, animals treated with TF, two weeks); C6 (n=5, control animals, six weeks, which received water); and TF6 (n=6, animals treated with TF, six weeks). The TF was provided by the company 4life (USA, Utah, West Sandy), administered daily from the time of the fracture; and its preparation followed the methodology described by Vetvicka *et al*. (2019).

Food intake and body mass were assessed daily and weekly, respectively. Sacrifice occurred at two and six weeks, using ketamine (50 mg/kg/weight) associated with xylazine (10 mg/kg/weight), intraperitoneally. The tibias were removed, x-rayed (same methodology as described above), and processed for histological analysis.

Histochemical and histomorphometric analysis. The tibias were removed by disarticulation at the ankles and knees and dissected, keeping the periosteum around the fracture focus. The pieces were fixed for 48 hours in 10 % formalin. Next, the material was washed in running water and demineralized with EDTA (ethylene diamine tetra-acetic acid) for 48 hours. After this time, the ends of the tibias were removed and sectioned longitudinally, and calluses were obtained from the fracture sites to perform histomorphometric analyses. They were then immersed in a decalcifying solution composed of EDTA (0.07 %), sodium tartrate (0.014 %), sodium and potassium tartrate (0.8 %) and hydrochloric acid (10 %). The decalcification period varied between seven (minimum) and 15 days (maximum), according to the decalcification speed of each sample. After seven days in the decalcifying solution, the solidity of the part was tested with a disposable needle. Transfixing the needle to the bone fragment (outside the bone callus region) determined the interruption of the decalcification process.

Subsequently, the material followed the routine histological protocol established by Pessoa (de Campos Pessoa *et al*., 2021). The material was sectioned using a microtome (Leica, Heerbrugg, St.Gallen, Switzerland) obtaining consecutive 5mM sections, which were stained with Masson's Trichrome to quantify the elements of the bone matrix.

The fields were captured with the aid of a camera (Olympus DP70-Tokyo, Japan) attached to an Olympus BX51 optical microscope. The quantification of the bone callus was carried out using the Image J Software. The surface densities (Sv) of the vessels, chondrocytes, cartilaginous bone matrix (blue color), intermediate (blue to pink transition color), and mature (pink color) were determined with the help of the “cell counter” and “grid”

tools. The test grid, containing 100 points, was superimposed on the images, and each structure that was touched by a point was counted. These analyses were performed with a 40× objective (20 fields/animal) and the results were expressed as percentages. The total region of the bone callus was captured by an Axiocam 506 color digital camera (Carl Zeiss, Gottingen, Germany) coupled to a Stereo DiscoveryV8 stereomicroscope (Carl Zeiss), at 8x magnification.

Statistical analysis. Data were tested for the normality curve and were reported as mean ± standard deviation (SD). Differences between groups were assessed using the Student's t-test. A $p<0.05$ was considered statistically significant.

RESULTS

The evaluation of the radiological images of the fractures showed that the animals treated for two weeks (TF2) presented a more abundant bone callus, in the anterior part of the tibia, compared to their counterpart. In the six-week groups, we noticed the presence of a mature callus, mainly on the posterior surface of the tibia of the control animals, not being seen in the treated groups, suggesting a more advanced stage of bone remodeling in the treated group (Fig. 1). There was no difference in body mass between the groups at two and six weeks, although the food intake of the TF2 group was lower compared to the control group (p=0.0079) (Table I). Figure 2 illustrates the bone calluses of the different groups, a region that we used in morphometric quantification. Regarding the histomorphometry of the bone tissue of the animals two weeks post-fracture, the amount of mature bone matrix in the TF2 group was approximately five times greater in comparison to the C2 group (p=0.0232), corroborating the radiological findings. On the other hand, the other parameters evaluated (chondrocytes, vessels, cartilaginous bone matrix, and intermediate bone matrix) did not differ between the groups (Table I, Fig. 3).

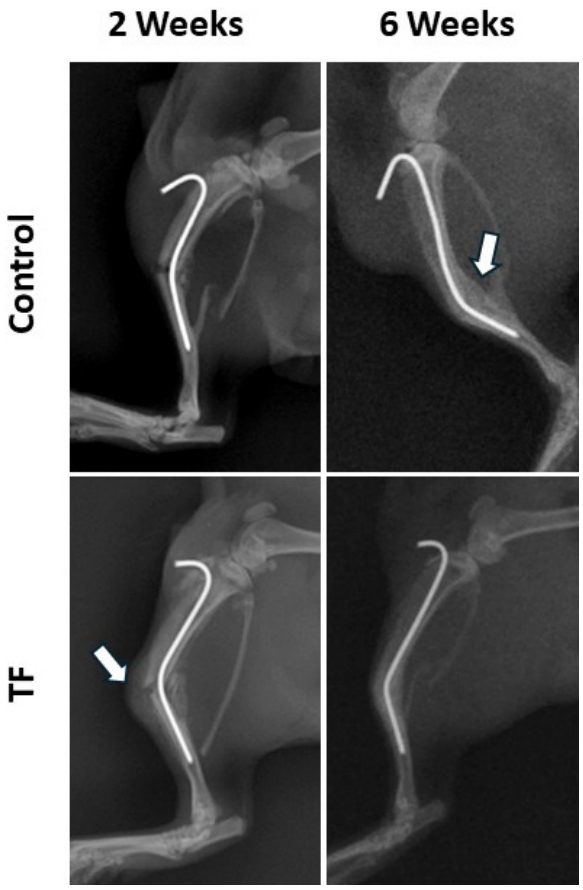

Fig. 1. Tibia radiological images of the different experimental groups, two and six weeks post-fracture. TF2 animals presented a more abundant callus in the anterior surface of the tibia when compared to C2 (arrow, bottom image). C6 animals showed a more mature callus on the posterior surface of the tibia (arrow, upper image), which was not observed in the TF6 group; which suggests a more advanced stage of bone remodeling.

When analyzing the sixth week of the experiment (Table I, Fig. 3), the Svs of cartilaginous and intermediate bone matrix, as well as the Sv of blood vessels, was lower in animals treated with transfer factor (FT6) compared to animals in the C6 group (p=0.0001; p=0.0042; p=0.0199, respectively). As for the Sv of the mature bone matrix, the FT6 group showed an increase in this parameter when compared to its counterpart ($p<0.0001$), being approximately four times higher. Regarding the Sv of chondrocytes, there was no difference between the groups.

Table I. Biometric and morphometric data of the experimental groups.

	C2 Group	TF2 Group	P value
Body Mass (g)	367.20 ± 38.31	368.00 ± 44.60	0.9765
Diet intake (g/day/animal)	23.09 ± 0.67	20.54 ± 1.51	0.0079*
Sv Cartilaginous bone matrix (%)	47.14 ± 5.64	49.41 ± 7.88	0.6133
Sv Intermediate bone matrix (%)	20.25 ± 10.83	15.25 ± 5.41	0.3829
Sv Mature bone matrix (%)	1.32 ± 1.89	6.15 ± 3.36	0.0232*
Sv Vessels (%)	1.73 ± 0.80	1.35 ± 0.52	0.4046
Sv chondrocytes (%)	29.56 ± 9.60	27.84 ± 8.11	0.7667
	C6 Group	TF6 Group	P value
Body mass (g)	396.90 ± 21.40	373.30 ± 23.19	0.1153
Diet intake (g/day/animal)	24.37 ± 0.40	22.79 ± 2.06	0.0737
Sv Cartilaginous bone matrix (%)	19.19 ± 3.66	8.77 ± 1.39	0.0001*
Sv Intermediate bone matrix (%)	63.96 ± 6.94	48.38 ± 6.63	0.0042*
Sv Mature bone matrix (%)Sv	8.69 ± 3.47	35.45 ± 6.43	<0.0001*
Sv Vessels (%)	1.64 ± 0.39	0.99 ± 0.37	0.0199*
Sv chondrocytes (%)	6.53 ± 0.56	6.42 ± 1.01	0.8396

Data presented as mean ± standard deviation. For each parameter, * indicates p-values with statistical significance. Note: Sv = Surface density

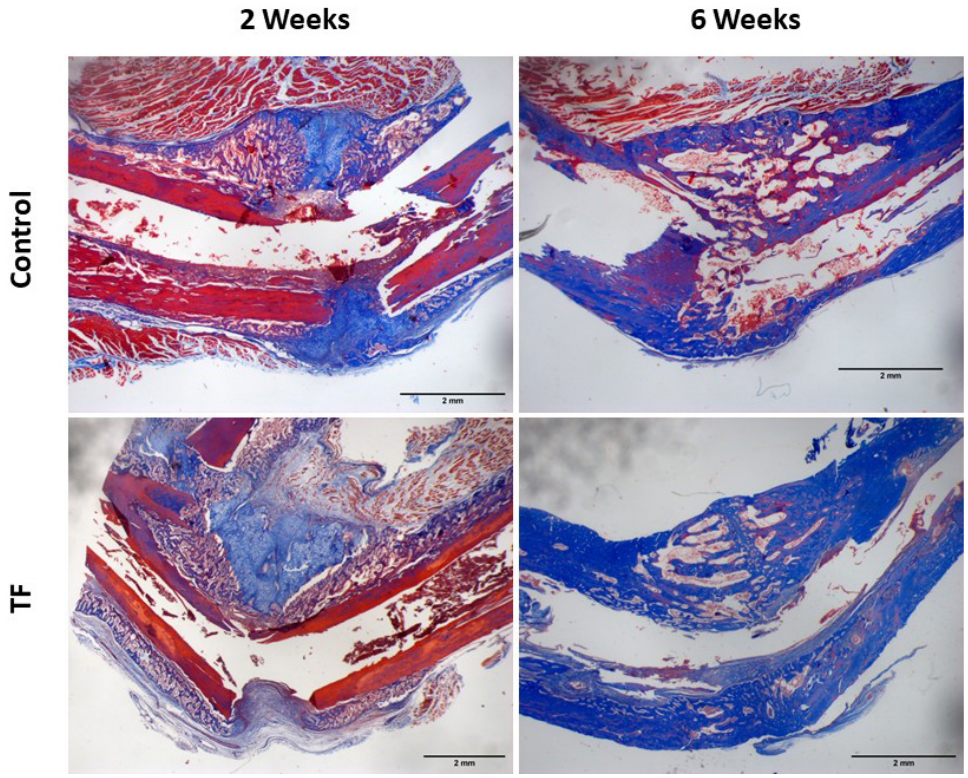

Fig. 2. Photomicrograph of the total region of the bone callus of the different experimental groups, two and six weeks post-fracture.

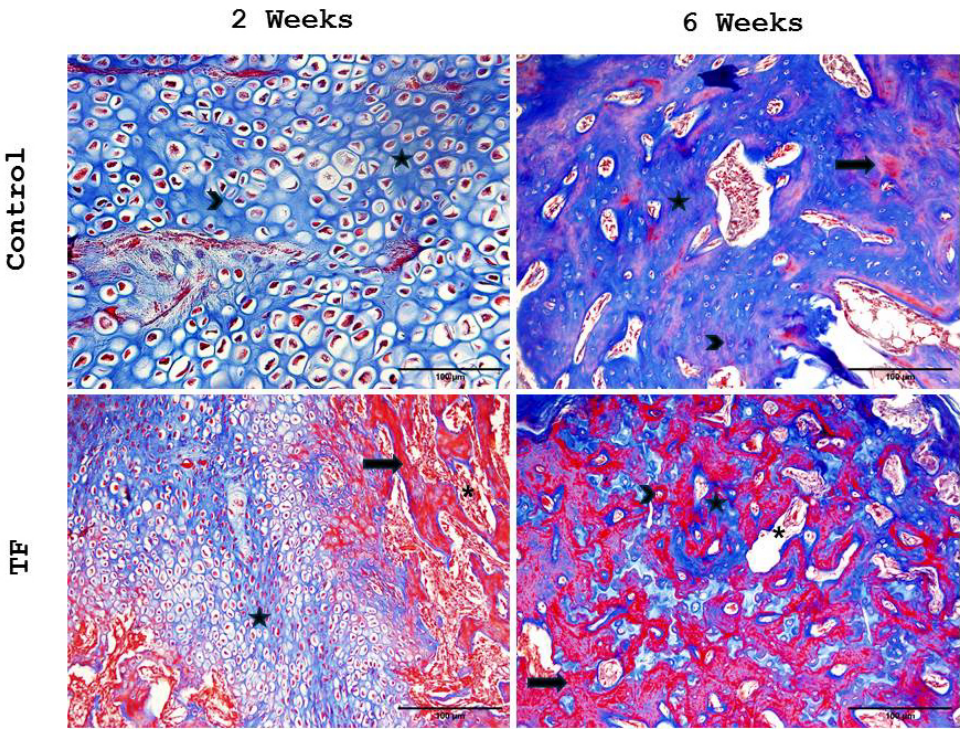

Fig. 3. Photomicrograph of the region of the tibia bone callus of the different experimental groups, two and six weeks post-fracture (x40, Masson's Trichrome). Arrowheads indicate the presence of chondrocytes; the stars illustrate the cartilaginous bone matrix; arrows show mature bone matrix; while asterisks indicate vessels.

DISCUSSION

Recent epidemiological data show that bone fractures have increased, as have their complications (Ekegren *et al*., 2018). Bone consolidation is a well-known and described process, which is affected by several factors: nutrition, age, some drug types, and comorbidities (de Campos Pessoa *et al*., 2021). The literature points out that some natural substances modulate the inflammatory process during bone consolidation (Singh, 2017). However, there is little to none information regarding the TF, which is a small natural peptide that has strong immunomodulatory and anti-inflammatory potential (Krishnaveni, 2013). Here, we found that ingesting TF for two weeks favored the bone consolidation process, in addition to increasing the animals' food intake. On the other hand, the use of this substance for six weeks only improved bone parameters.

It is known that metabolic diseases associated with obesity delay bone consolidation, which can lead to nonunion (Ekegren *et al*., 2018). Although we did not obtain a difference in body mass, food intake in the TF2 group was lower than in the control group, which did not interfere with the consolidation process. Trauma associated with fractures promotes an acute metabolic response with an increase in cortisol levels, directly impacting anabolic and catabolic processes (Simsek *et al*., 2014). Here, regardless of the animal's food consumption, those that were supplemented with TF showed an advance in bone regeneration, as the percentage of mature bone matrix was higher after two weeks.

Naturally, bone tissue regeneration has several phases: inflammatory (fracture hematoma), soft callus/non-mineralized cartilage (one to two weeks post-fracture), hard callus (three to four weeks post-fracture), and remodeling (four to six weeks) (Sant'Anna *et al*., 2017). The second phase (corresponding to two-week-old animals) is characterized by the proliferation of blood vessels, chondrocytes, and deposition of cartilaginous extracellular matrix, intermediate and beginning of the mature bone matrix. Supplementation with TF in the two-week group (FT2) did not affect the quantity of the aforementioned bone elements, except for the increase in the percentage of mature bone matrix, which was pivotal for the next phases, as it accelerated bone consolidation. Although this work does not include the hard callus formation phase, which was one of the limitations, studying the beginning and end of the bone consolidation phase is sufficient to elucidate this entire process.

One of the likely explanations focuses on the fact that TF has immunomodulatory properties. Studies carried out in rats show that this substance increases the activity of Natural Killer cells, modulating the production and activity of interferon-gamma and macrophages, which may favor the initial stages of bone regeneration (Viza *et al*., 2013; Habar *et al*., 2021).

Corroborating this finding, supplementation with TF until the sixth-week post-fracture maintained the upward curve in this process of maturation of the bone matrix. Intermediate bone matrix density was reduced in this group compared to control animals. It is notable that during the progression of consolidation, the bone callus gradually becomes denser and more abundant as it is replaced by new bone tissue, which explains the decrease in the intermediate bone matrix (Sant'Anna *et al*., 2017).

It is suspected that the administration of TF accelerates the absorption of the intermediate bone matrix, associated with an increase in phagocytic activity, enhancing the maturation of the bone matrix. The last phase of bone consolidation, the remodeling phase, is long and characterized by a process of bone reabsorption and deposition, simultaneously with the appearance of new vessels (de Campos Pessoa *et al*., 2021; Sheen *et al*., 2024). Here, interestingly, animals that received TF for six weeks showed a decrease in the density of blood vessels, which may infer that this substance enhanced the bone regeneration process since the progression of bone remodeling shows a reduction in angiogenesis (Sant'Anna *et al*., 2017). The cartilaginous bone matrix did not follow this process, which justifies additional studies of this substance to fully understand its action.

In short, the present study demonstrated that the oral use of TF can positively influence the bone consolidation process, accelerating remodeling and new bone formation. However, more studies are needed to fill some gaps, as it is a substance that has not yet been evaluated in this context. Its mechanisms of action, as well as the dose administered and follow-up time, may vary and influence the results.

ACKNOWLEDGMENTS. We are grateful to the Ana Lucia Rosa Nascimento and Gustavo Miranda for their technical assistance.

PADULA, E. O. C.; FORTUNA-COSTA, A.; SAMPAIO, F. J. B. & GREGORIO, B. M. La ingesta oral de factor de transferencia acelera la consolidación ósea en ratas Wistar adultas. *Int. J. Morphol., 43(2)*:535-540, 2025.

RESUMEN: La curación de fracturas óseas es un proceso regenerativo complejo que tiene como objetivo restaurar el hueso dañado a su estado previo a la lesión. Hasta la fecha, no se ha descrito ninguna sustancia natural que acelere y/o promueva la consolidación ósea. El factor de transferencia (TF), al actuar sobre el sistema inmunológico, puede tener beneficios en este tipo de

lesiones, ya que puede modular la respuesta inflamatoria post-fractura. Por lo tanto, nos propusimos investigar los efectos histológicos promovidos por la ingestión oral de TF sobre el proceso de consolidación ósea en ratas adultas. Las ratas Wistar adultas se dividieron en dos grupos: control (C, n=10) y factor de transferencia (TF), que fueron seguidos durante dos (C2 y TF2) y seis semanas (C6 y TF6) posteriores a la fractura. Ambos grupos tenían tibias fracturadas y recibieron, por sonda, agua o TF, respectivamente. La masa corporal y la ingesta de alimentos se controlaron y diaria y semanalmente, respectivamente. Al final de estos tiempos, los animales fueron sacrificados y las tibias fueron extraídas para el análisis histomorfométrico. Los datos se analizaron utilizando la prueba t de Student. La masa corporal no difirió entre los grupos, aunque la ingesta de alimentos del grupo TF2 fue menor que la del grupo C2. La densidad de la matriz ósea madura fue mayor en los grupos TF2 y TF6 en comparación con los respectivos controles. En los grupos dos semanas después de la fractura (TF2 y C2) no hubo diferencias en las densidades de condrocitos, vasos y matrices óseas cartilaginosas e intermedias. Por el contrario, el grupo TF6 mostró una reducción en las densidades de la matriz ósea intermedia, cartilaginosa y de los vasos en comparación con C6. La densidad de condrocitos no fue diferente. El tratamiento con TF mostró resultados positivos en la consolidación ósea, y puede ser un coadyuvante en el tratamiento de la consolidación ósea, así como en la prevención del retraso de este proceso.

PALABRAS CLAVE: Factor de transferencia; Curación de fracturas; Ratas; Regeneración ósea.

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