

Research article

The effect of mofettes and natural carbonated mineral water on accelerating bone healing in a femoral defect model in rats

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Abstract: (1) Background: The aim of the study was to investigate the effects of mofettes and carbonated mineral water baths on bone healing using a rat femoral defect model; (2) Methods: A 2mm diameter unicortical defect in the left femoral diaphysis in 40 Wistar Rats was surgically created. Furthermore, the subjects were divided into 4 treatment-groups: control, mofette therapy, mofette therapy and carbonated mineral water bath, and carbonated mineral water bath. At the end of the 2-week treatment and at 4 and 6 weeks, the animals were evaluated through Micro-CT analysis of the bone defect and histological analysis of bone tissue and skin; (3) Results: The processes of bone consolidation and repair are not completed at 6 weeks in all groups. However, comparing the proliferated bone tissue in the created orifice and the degree of thickening of the femoral wall, it can be affirmed that at 6 weeks, the best results are present in Group 4, treated with carbonated mineral water baths, followed by the combination of mofettes and carbonated water. (4) The use of these treatments could open a new possibility for shortening the healing time in patients with bone defects, as it is non-invasive and accessible.

Keywords: mofette; carbonated mineral water; bone defect; bone healing

1. Introduction

The carbon dioxide (CO₂) baths and mofette therapy, being natural, are known for their CO₂ concentration to be beneficial as a treatment for cardiovascular diseases, peripheral arterial diseases, arterial hypertension, due to their strong vasodilatory effect [1,2]. They are also recognized for their potential in rheumatic, neurological, and dermatological conditions. Previous studies have concluded that the improvement in blood flow depends on the skin vasodilatory effect caused by the high CO₂ content, which diffuses into the skin tissue through its layers [3]. Experimental studies have shown that baths with carbonated mineral waters and mofette from Băile Tușnad, Romania, increase peripheral arterial blood flow during repeated immersions [4]. Moreover, they have led to a statistically significant reduction in total oxidative stress and improvement in antioxidant status in experimentally induced myocardial ischemia [5,6].

Additionally, a study demonstrated that transcutaneous application of CO₂ can enhance blood flow in cutaneous flaps and promote angiogenesis [7]. Bone defects may arise due to inadequate bone consolidation, severe trauma, infections, or tumor resection. However, treatments for bone defects are often challenging and not fully established. Basic and clinical research on bone healing, which could improve current treatments for bone defects, is still necessary. Currently, the topical application of CO₂ is gaining attention in various fields such as health, dermatology, and sports.

The therapeutic effects of CO₂ are attributed to increased blood flow, microcirculation, and nitric oxide-dependent neocapillary formation, as well as an increase in local oxygen partial pressure known as the Bohr effect. CO₂ can improve both cutaneous microcirculation and arterial macrocirculation [8-11]. Furthermore, it has been shown that CO₂ application significantly increased the gene expression of vascular endothelial growth factor (VEGF) in rat muscle [12]. The effect of topical application of CO₂ in accelerating fracture repair through promoting angiogenesis, increasing blood flow, and endochondral ossification has been demonstrated in a rat femoral fracture model [13,14]. A similar effect can be expected to be effective for bone defects. However, until now, only the effect of topical application of CO₂ has been studied.

The therapeutic effects of baths with carbonated mineral waters rely more on the action of CO₂ and less on the pharmacodynamic action of mineral salts in the water composition. In a full bath, between 10 and over 80 ml/min/m² of CO₂ are absorbed through the skin surface, averaging 30 ml/min/m² of CO₂ (equivalent to 1.8-4.5 l/h), depending on concentration, measurement method, and temperature [15-17]. Therefore, in this study, we hypothesized that baths with natural carbonated mineral water and mofette therapy from Băile Tușnad, which first act on the skin, could accelerate bone healing due to the activation of microcirculation. Our objective was to investigate the natural CO₂ application's effect on bone healing using a rat femoral defect model.

2. Material and Methods

2.1. Animal preparation

The experimental study was approved by the Ethics Committee of the "Iuliu Hațiega-nu" University of Medicine and Pharmacy in Cluj-Napoca and the Veterinary Health Committee (approval no. 332/17.09.2022). The study included male Wistar rats of the *Rattus Norvegicus* species, 16 weeks old, clinically healthy, weighing between 250-300 g. These animals originated from the university's authorized sanitary-veterinary biobase and were housed in cages meeting European standards. Their housing space was equipped with adequate ventilation, with continuous air refreshment at a rate of 10-20 air changes per hour. The optimal temperature in their housing area was maintained between 20-24°C, with humidity at 55% ± 10%.

2.2. Experimental model

Forty animals were randomly divided into four groups, and a unicortical defect with a diameter of 2 mm was created at the left femoral diaphysis level. Surgical procedures were performed under general anesthesia with Ketamine 10% (90 mg/kg) and Xylazine 2% (10 mg/kg) administered intramuscularly, in sterile conditions. The groups were organized as follows:

- Group 1 (C): Control group – no treatment applied
- Group 2 (M): Mofette therapy – animals exposed daily to the Băile Tușnad mofette for 20 minutes/day over two weeks
- Group 3 (M-CMW): Mofette therapy and carbonated mineral water bath – animals underwent both treatments daily for two weeks
- Group 4 (CMW): Carbonated mineral water bath – animals underwent this treatment for 20 minutes/day over two weeks

The carbonated mineral water composition included chlorine, bromine, sulphates, bicarbonate, sodium, potassium, calcium, magnesium, iron, carbon dioxide, with a pH of 5.8. The total mineralization of the water was 122.036 mmol/L.

At the end of the two-week treatment and at 4 and 6 weeks, the animals were evaluated through Micro-CT analysis of the bone defect and histological analysis of bone tissue and skin. Euthanasia was performed using an overdose of general anesthesia with Xylazine and Ketamine.

2.3. Micro-CT analysis

Bone tissue (femur) samples were examined using the Bruker Skyscan 1172 apparatus, and the region of interest was analyzed for bone volume, tissue volume, bone surface, trabecular number, trabecular thickness, trabecular separation, intersection surface, and bone surface density.

2.4. Histological analysis of skin

Skin samples perpendicular to the incision line were fixed, dehydrated, and paraffin-embedded. Sections were stained with Goldner's trichrome for microscopic examination.

2.5. Histological analysis of bone tissue

Transverse femur sections containing the induced bone defect were fixed, decalcified, dehydrated, paraffin-embedded, and stained with Goldner's trichrome for microscopic examination.

2.6. Data analysis

Descriptive exploratory analysis was conducted, reporting minimum and maximum values for each measured parameter. Graphical representations were created for the time evolution of markers in each treatment group. Statistical analysis was performed using Microsoft Excel.

3. Results

3.1. Histological analysis results for skin

At 2 weeks after inducing the defect (incision) in the control group animals (Group 1) (Fig. 1-1A), epidermal reepithelialization is observed. The thickness of the proliferated epithelium in the incision area is greater than the epithelium located laterally. In both the dermis and hypodermis, collagen proliferation is observed, but the degree of consolidation is still low. Collagen fibers are generally thin, and their density is lower compared to areas located laterally to the incision. At 4 weeks, the conjunctive consolidation degree in the control group (Fig. 1-1B) is quite close to the lateral areas of the incision, although small differences in the thickness and density of proliferated collagen fibers are observed in both the dermis and hypodermis.

For Group 2, at 2 weeks post-incision (Fig. 1-2A), the proliferated epithelium is comparable to that present laterally. Proliferated collagen in the dermis and hypodermis is more abundant than in the control group, leading to a slight thickening of the skin. At 4 weeks, the epithelium along the incision line is similar to that located laterally, while there are no differences in the thickness of the layers in the dermis and hypodermis compared to lateral areas. However, the degree of collagen consolidation is lower compared to the control group, where the skin is slightly thicker at 4 weeks post-incision.

In most animals from Group 3, at 2 weeks post-incision (Fig. 1-3A), a crust with cellular debris is present on the epidermis. Below the crust, the existing epithelium varies in thickness, with areas where the proliferated epithelium is thicker than laterally and other areas where it is thinner. In the superficial half of the dermis, there is a discontinuity zone approximately along the incision line. Around this zone, a chronic inflammatory infiltrate is present, including rare neutrophils. In the deep dermis and hypodermis, the degree of collagen consolidation is somewhat higher compared to Group 1. At 4 weeks into the experiment, in Group 3 (Fig. 1-3B), the proliferated epithelium is similar to that laterally. Proliferated collagen in the dermis and hypodermis has a higher density and is also thicker than the lateral areas. Additionally, it can be observed that at 4 weeks post-incision, the skin in this area is somewhat thinner than in laterally located zones.

For Group 4 at the first harvest (Fig. 1-4A), reepithelialization is also present. Compared to the control group, the proliferated epithelium is not as thickened, and the degree of collagen proliferation in the dermis and hypodermis is higher. At 4 weeks post-incision for Group 4 (Fig. 1-4B), the proliferated epithelium is almost identical to that located laterally. In the dermis and hypodermis, there are areas where the proliferated collagen is comparable to that in lateral areas, but there are also zones where the density is much lower. Comparing the density of proliferated collagen fibers in animals from Group 4 with that of Group 1, it is slightly lower.

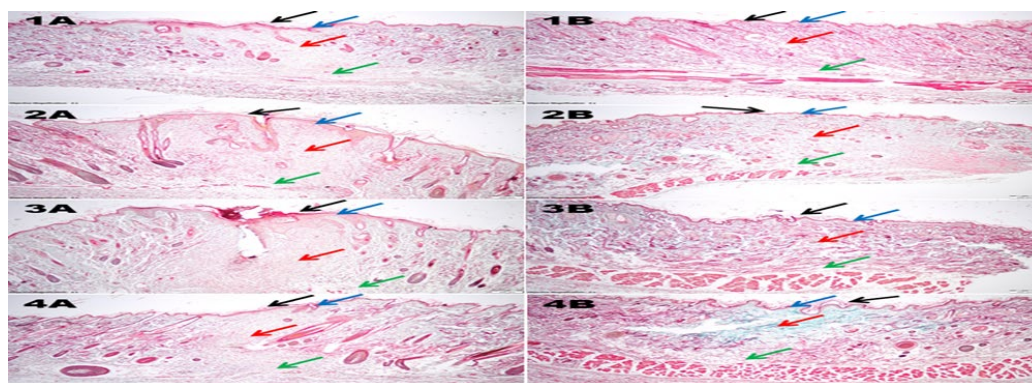


Figure 1. Microscopic Appearance of the Skin in the Experimental Induced Defect Area (Incision); Goldner's Trichrome Method. 1 – control; 2 – mofette; 3 – mofette + mineral water; 4 – mineral water; A – microscopic aspects at 2 weeks post-induction of the experimental defect; B – 4 weeks post-induction of the experimental defect; black arrow – site of the induced defect; blue arrow – epidermis; red arrow – dermis; green arrow – hypodermis.

3.2 Histological analysis results for bone tissue

In the control group (C) (1) after 2 weeks (harvest 1 - A) from inducing the bone defect (Fig. 2-1A), reparative processes are observed, represented by the proliferation of branched trabecular bone, typically consisting of 6-8 bone lamellae on average. Bone proliferation processes occur both from the periosteum and endosteum. The proliferated trabecular bone at 2 weeks has not completely fused in the central area of the bone defect. There is also a slight proliferation of subperiosteal bone lamellae over a short distance above the remaining existing bone at the bone-defect interface. At 4 weeks (Fig. 2-1B), the degree of bone proliferation is in a more advanced stage, with the proliferated trabeculae from the periphery of the bone defect towards the central area now fused. On average, the trabeculae are composed of 12-14 bone lamellae, and the delimited areolae are much smaller than at 2 weeks. Additionally, the proliferated subperiosteal lamellae are more numerous (16-18 lamellae) and extend from the bone defect boundary to approximately half of the femur's circumference. Some bone lamellae (5-7) have also proliferated into the medullary canal with concentric arrangement, doubling the existing bone wall internally, but they extend over a much smaller distance compared to those arranged subperiosteally.

At 6 weeks (Fig. 2-1C), the appearance of the proliferated bone tissue at the bone defect is much more compact than at 4 weeks due to the formation of new lamellae in the areolae, resulting in few and very small existing areolae with a diameter of up to 50 μm at this point. In the proliferated bone at the bone defect's outer half, the lamellae are arranged plexiformly but quite compact, while in the inner half, there are some branched trabeculae. By proliferating new lamellae arranged concentrically to the femur's diaphysis, both subperiosteally and subendosteally, at the bone-defect interface, the thickness of the femoral wall has almost doubled.

In Group 2 (M) at 2 weeks (Fig. 2-2A) post-bone defect induction, branched trabecular bone is present in the created hole. In terms of thickness, these are comparable to those from Group 1, but they extend more deeply towards the medullary cavity. The proliferated bone over the femoral wall, both subperiosteally and in the medullary cavity, is thicker and extends in circumference compared to Group 1. At 4 weeks (Fig. 2-2B), the existing bone in the created hole consists of compactly arranged bone lamellae delimiting several small areolae with an average diameter of up to 50 μm . On average, the proliferated bone in the created hole has a thickness ranging from 80 to 100 μm . Branched trabecular bone also detaches from its inner face. The proliferated bone tissue, both subperiosteally and on the inner face towards the medullary canal, over the existing bone is comparable in thickness and extent to that of Group 1.

At 6 weeks, the proliferated bone in the induced hole (Fig. 2-2C) is thinner than that of animals in Group 1, but the arrangement of the lamellae is much more compact. Regarding the proliferated bone, both on the external and internal face of the femoral wall, it is somewhat better represented in terms of thickness and extension compared to Group 1.

At 2 weeks into the experiment in Group 3 (M-CMW) (Fig. 2-3A), the trabecular bone thickness in the created hole is comparable to that of Groups 1 and 2, but here, they extend more towards the medullary cavity. Regarding the proliferated bone both subperiosteally and subendosteally, it is less represented compared to Groups 1 and 2. At 4 weeks, the proliferated bone in the created hole in the femoral wall (Fig. 2-3B) is comparable in thickness to that of Group 1 at 6 weeks, but the lamellae's density is not as high. However, towards the medullary cavity, numerous branched trabeculae are present, providing additional strength. It is also observed that at 4 weeks, the proliferated bone both subperiosteally and subendosteally is thinner than in Groups 1 and 2. At 6 weeks (Fig. 2-3C), the existing bone in the created hole is slightly thicker than at 4 weeks; however, the lamellae's density is much higher. It is also noted that the femoral wall is thicker than at 4 weeks due to the proliferation of bone lamellae predominantly subperiosteally. The thickness of the femoral wall at 6 weeks is thinner than in animals from Groups 1 and 2.

For animals in Group 4 (CMW), at the first harvest (Fig. 2-4A), the proliferated bone in the created hole is comparable to that of Group 3 in both density and extension. The only difference is that in the blood vessels within the areolae, the blood quantity is higher. At 4 weeks (Fig. 2-4B), the bone proliferated in the hole is comparable to that existing in animals from Group 3 at 4 weeks and Group 1 at 6 weeks. Also, the thickness of the femoral wall is smaller than in Groups 1 and 2 at 6 weeks. At 6 weeks (Fig. 2-4C), in the created hole, the present bone is more consolidated than at 4 weeks. In the outer part, the bone lamellae are more compactly arranged, and additionally, towards the medullary cavity, numerous branched trabeculae are present. Also, there is no significant thickening of the femoral wall.

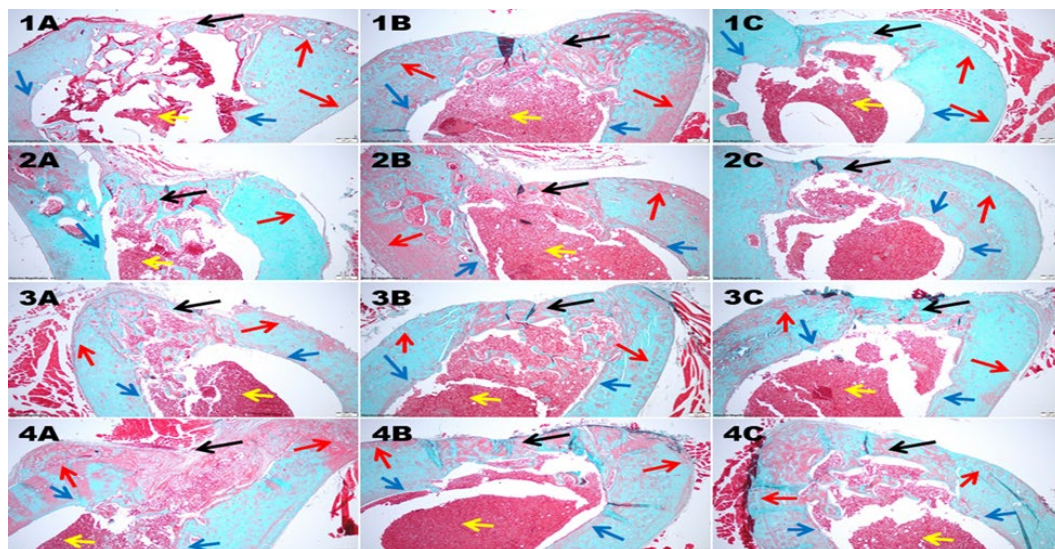
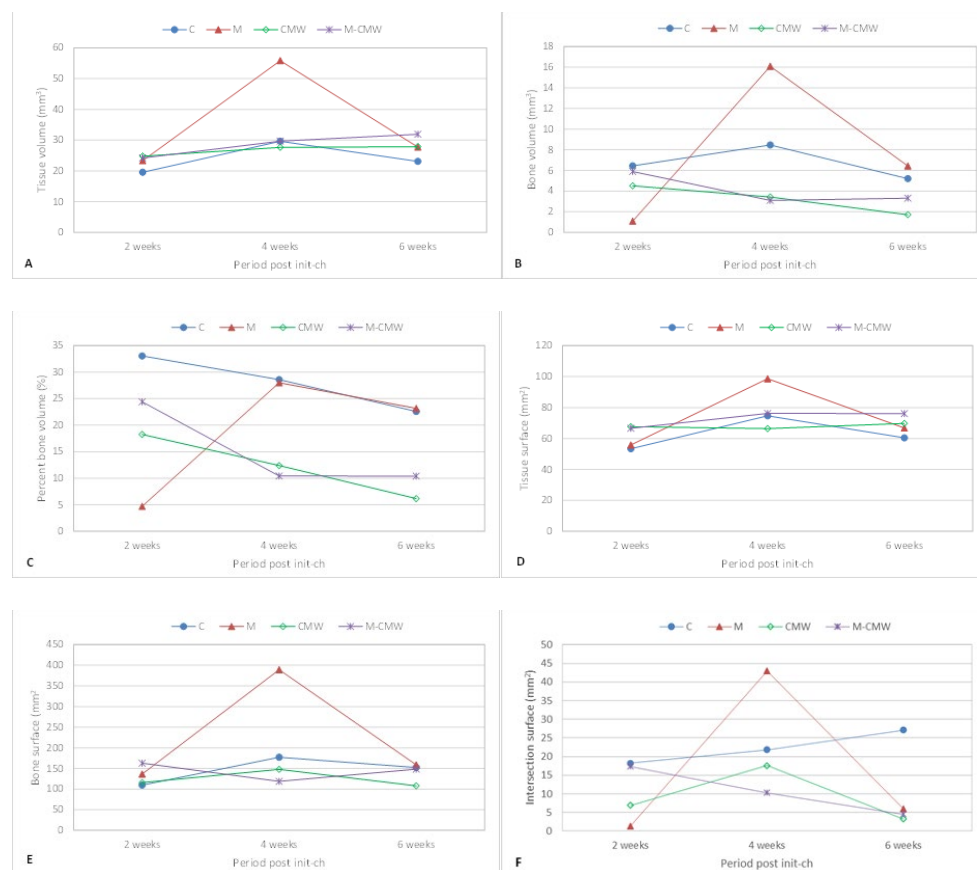


Figure 2. Cross-Sections at the Femoral Level in the Area of the Experimentally Induced Bone Defect; Groups: 1 – control; 2 – mofette; 3 – mofette + mineral water; 4 – mineral water; A – microscopic aspects at 2 weeks from bone defect induction; B – 4 weeks from bone defect induction; C – 6 weeks from bone defect induction; black arrow – proliferated bone in the created hole; red arrow – boundary between the bone proliferated subperiosteally over the femoral wall; blue arrow – boundary between the bone proliferated subendosteally over the femoral wall; yellow arrow – hematogenous marrow in the diaphyseal canal; Goldner's Trichrome Method.

3.3 Results of Micro-CT examination

The applied treatments (Mofette – M, therapy in carbonated mineral water – CMW, and combination of M with CMW) demonstrated different effects on micro-CT measurements (Figure 3).



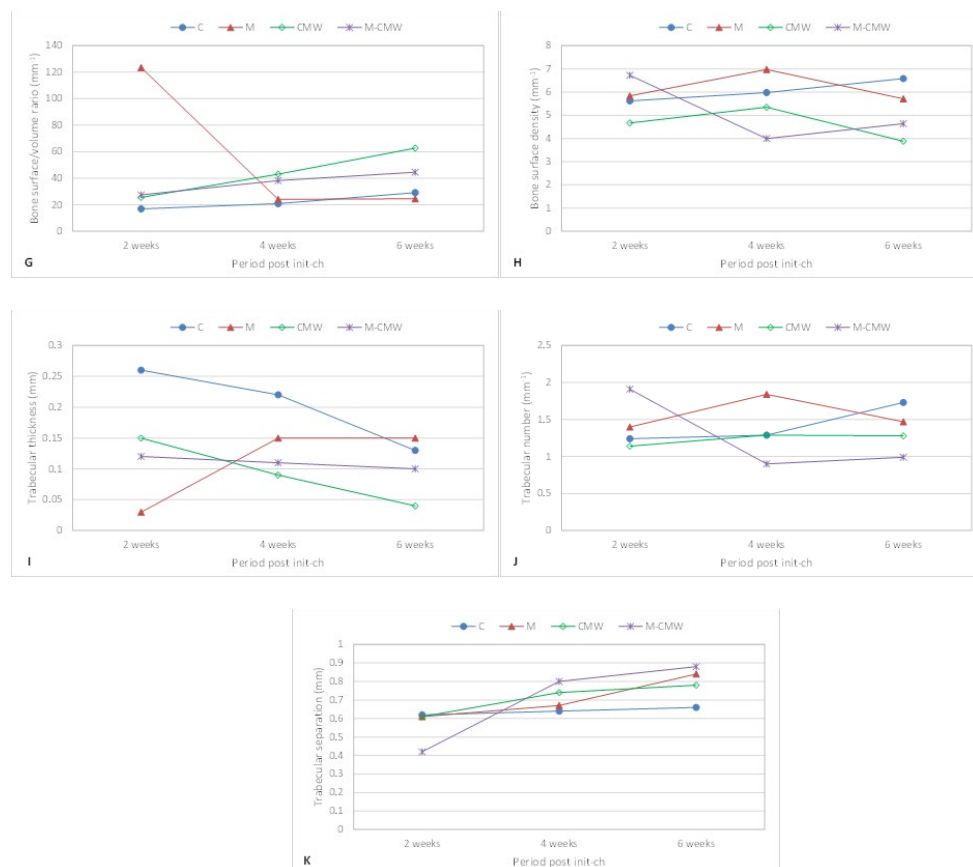


Figure 3. Pattern of micro-CT measurements by group (C – control group, M – Mofette, CMW – physical exercises in carbonated mineral water, M-CMW - Mofette and physical activity in CMW)

The evaluation of the whole sample regardless of the treatment indicates the C and M group in 4/11 micro-CT measurements with the lower values of evaluated parameters at the first follow-up evaluation (2 weeks), M-CMW (6/11) at the evaluation performed at 4 weeks and CMW (6/11) at the last evaluation (4 weeks). The higher values were observed in the C group (5/11) at 2 weeks, M (7/11) at 4 weeks and M (4/11) at 4 weeks (Table 1, Figure 4-7).

Table 1. The minimum and maximum values obtained regardless of the group on measured micro-CT values

	2 weeks	4 weeks	6 weeks
Tissue volume (mm ³)	{19.53 to 24.77}	{27.65 to 55.83}	{23.1 to 31.91}
	{C CMW}	{CMW M}	{C M-CMW}
Bone volume (mm ³)	{1.1 to 6.45}	{3.1 to 16.08}	{1.72 to 6.43}
	{M C}	{M-CMW M}	{CMW M}
Percent bone volume (%)	{4.73 to 33.06}	{10.45 to 28.59}	{6.18 to 23.18}
	{M C}	{M-CMW C}	{CMW M}
Tissue surface (mm ²)	{53.43 to 67.67}	{66.38 to 98.64}	{60.43 to 76.09}
	{C CMW}	{CMW M}	{C M-CMW}
Bone surface (mm ²)	{109.92 to 162.8}	{118.89 to 389.45}	{108 to 158.67}
	{C M-CMW}	{M-CMW M}	{CMW M}
Intersection surface (mm ²)	{1.39 to 18.2}	{10.24 to 42.99}	{3.21 to 27.12}
	{M C}	{M-CMW M}	{CMW C}

Bone surface/volume ratio (mm ⁻¹)	{17.01 to 123.51} {C M}	{20.92 to 43.15} {C CMW}	{24.66 to 62.8} {M CMW}
Bone surface density (mm ⁻¹)	{4.67 to 6.72} {CMW M-CMW}	{4 to 6.97} {M-CMW M}	{3.88 to 6.58} {CMW C}
Trabecular thickness (mm)	{0.03 to 0.26} {M C}	{0.09 to 0.22} {CMW C}	{0.04 to 0.15} {CMW M}
Trabecular number(mm ⁻¹)	{1.14 to 1.91} {CMW M-CMW}	{0.9 to 1.84} {M-CMW M}	{0.99 to 1.73} {M-CMW C}
Trabecular separation (mm)	{0.42 to 0.62} {M-CMW C}	{0.64 to 0.8} {C M-CMW}	{0.66 to 0.88} {C M-CMW}

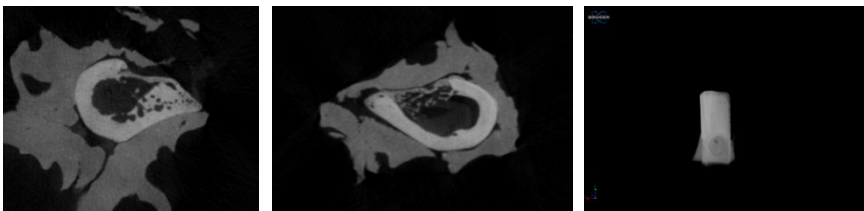


Figure 4. Control group 1 (C) - Micro-CT aspects - degree of bone consolidation at 4 and 6 weeks, the osteosynthesis process continues.

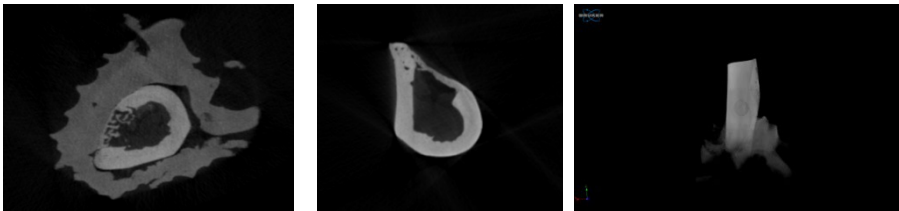


Figure 5. Group 4 (carbonated mineral water) CMW - Micro-CT aspects - degree of bone repair and consolidation at 4 and 6 weeks. At 4 weeks, the degree of bone repair and consolidation in the CMW group is similar to that in groups C and M at 6 weeks.

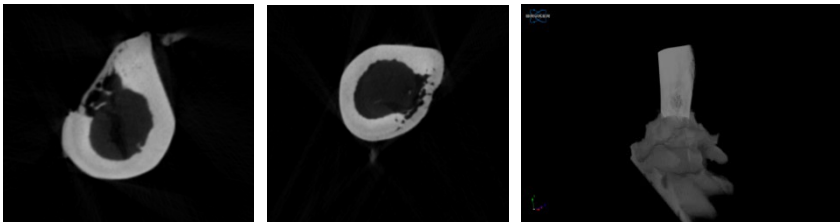


Figure 6. Group 3 (carbonated mineral water and mofette) M-CMW - Micro-CT aspects - degree of bone repair and consolidation at 4 and 6 weeks. At 4 weeks, the degree of bone repair and consolidation in the M-CMW group is similar to that in groups C and M at 6 weeks.

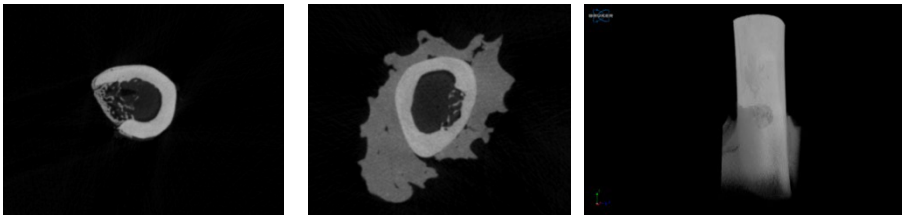


Figure 7. Group 2 (mofette) M - Micro-CT aspects - degree of bone consolidation at 4 and 6 weeks.

3. Discussion

Comparing the results obtained from the histological analysis of the skin at 4 weeks after the incision, for all four groups, it can be stated that in Group 2, the formed scar is the finest, even though the degree of collagen consolidation is lower compared to the control group. On the other hand, in Groups 1 and 4, the skin in the incision area is thicker, while in Group 3, the skin appears thinner and slightly congested.

From the histological analysis of the bone tissue, comparing the results obtained, at 2 weeks after inducing the bone defect, no major differences are observed in the bone repair process among the four groups. However, at 4 weeks, there are differences in the degree of bone consolidation between Groups 1 and 2, and Groups 3 and 4. Thus, in Groups 3 and 4, the bone repair processes are more advanced than in Groups 1 and 2. It can be affirmed that at 4 weeks, the degree of bone repair and consolidation in Groups 3 and 4 is similar to that in Groups 1 and 2 at 6 weeks.

At 6 weeks, the best results in bone proliferation and consolidation are present in animals from Group 4. Regarding the proliferated bone at the orifice, there are no significant differences between Groups 1 and 3, but in Group 2, the bone present in the outer part of the orifice is somewhat thinner than in Groups 1 and 3.

Regarding the thickening of the femoral wall through the proliferation of bone lamellae over the existing bone, both subperiosteally and subendosteally, there are no significant differences between Groups 1 and 2. However, in Groups 3 and 4, the femoral wall is not as thickened.

The proliferation of new bone lamellae, with circumferential arrangement both externally and internally in the diaphyseal bone, serves to increase bone resistance to pressures along the femur's long axis. However, it represents a disadvantage in terms of locomotion due to the increased weight of the femur, placing additional strain on regional muscles.

Based on the obtained results, it can be stated that the processes of bone consolidation and repair are not completed, as ongoing processes of osteosynthesis and bone remodeling are still occurring in all groups. Comparing the proliferated bone tissue in the created orifice and the degree of thickening of the femoral wall, it can be affirmed that at 6 weeks, the best results are present in Group 4, followed by Groups 3, 1, and 2.

In the micro-CT analysis, regarding tissue volume, better results were obtained at 4 weeks in Group M (Fig. 3-A), but at 6 weeks, the tissue volume in the region of the bone defect was larger in Group M-CMV, followed by Groups CMW, M, and C. The same results were obtained for the indicator Tissue Surface (mm^2) (Fig. 3-D).

When calculating bone volume (mm^3) (Fig. 3-B), percent bone volume (%) (Fig. 3-C), bone surface (mm^2) (Fig. 3-C), intersection surface (mm^2) (Fig. 3-E), the results at 2 weeks showed higher values in Group M, while at 6 weeks after completing the results, they remained higher in Group M, followed by Groups C, M-CMW, CMW.

Regarding the ratio Bone Surface/Volume (mm^2) (Fig. 3-F), at 2 weeks, Group M had higher values. However, at 4 weeks, the ratio of bone surface/volume in the bone defect area was higher in Group CMW, followed by Group M-CMW, Group C, and Group M, in accordance with the histological analysis of bone consolidation and repair processes (Table 1).

This is the first experimental study in which the bone consolidation process and accelerated bone healing were analyzed in a rat femoral defect model using natural therapeutic factors, namely carbonated mineral water and mofette from Băile Tușnad. In Romania, natural mofettes exist in the balneary resorts Covasna, Băile Tușnad, Balványos, Balnaș, Harghita Băi, Buziaș, Borșa, Slănic Moldova, Sangeorz Băi. These are gases resulting from natural post-volcanic emanations, of which carbon dioxide is present in a proportion of 95-98% and is used as a therapeutic factor [16]. Alongside carbon dioxide, mofette gas contains small amounts of other volcanic emission gases: ammonia, sulfur, helium, and radon (18,19), which induce the pulverization of CO_2 molecules, increasing

the penetration power of CO₂ [20-23]. Mofettes also contain positive and negative air ions, 2,000-15,000 ions/cm³, have a radioactivity of 0.3 µCi/l, without a cancer risk, and a radon concentration of less than 1% [18]. The biological mechanisms by which natural factors act and induce immunological and neuroendocrine responses are not yet completely understood, but according to some studies, they determine analgesic, anti-inflammatory, antioxidant, anabolic, chondroprotective effects, and neuroendocrine-immune regulation [24]. Also, the results of some studies suggest that a CO₂ laser equipped with a water spray function can be beneficial for reducing thermal damage and could contribute to the development of bone regeneration therapies [25,26,27], but transcutaneous application of CO₂ accelerates bone generation in a rabbit tibia osteogenesis model by promoting angiogenesis, blood flow, and endochondral ossification [14].

5. Conclusions

This is the first experimental study conducted on the effectiveness of carbonated mineral waters and natural mofette in a rat femoral defect model. The obtained results show that the processes of bone consolidation and repair are not completed at 6 weeks, as ongoing processes of osteosynthesis and bone remodeling are still occurring in all groups. However, comparing the proliferated bone tissue in the created orifice and the degree of thickening of the femoral wall, it can be affirmed that at 6 weeks, the best results are present in Group 4 (CMW), followed by Groups 3 (M-CMW), 1 (C), and 2 (M). The use of these treatments could open a new possibility for shortening the healing time in patients with bone defects, as it is non-invasive and accessible. Nevertheless, more in-depth studies of the CO₂ mechanism in local tissue and translational studies from animals to humans are needed for future clinical practice.

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Institutional Review Board Statement: The study was approved by the Ethics Committee of “Iuliu Hațieganu” University of Medicine and Pharmacy Cluj-Napoca and the Sanitary Veterinary Authority (approval no. 332/17.09.2022).

Informed Consent Statement: “Not applicable.”

Data Availability Statement: The data can be provided on reasonable request from the corresponding author.

Conflicts of Interest: “The authors declare no conflict of interest.”

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