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Bone regeneration in a mouse model of type 1 diabetes: Influence of sex, vitamin D3, and insulin

Natália P. Cignachi[a,b], Alice Ribeiro[a,b], Gustavo D.B. Machado[b], Amanda P. Cignachi[b], Luiza W. Kist[d], Maurício R. Bogo[c,d], Rodrigo B.M. Silva[b], Maria M. Campos[a,b,c,*]

[a] Programa de Pós-Graduação em Odontologia, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS 90619-900, Brazil
[b] Centro de Pesquisa em Toxicologia e Farmacologia, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS 90619-900, Brazil
[c] Programa de Pós-Graduação em Medicina e Ciências da Saúde, Escola de Medicina, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre 90619-900, Brazil
[d] Laboratório de Genômica e Biologia Molecular, Escola de Ciências da Saúde e da Vida, Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Porto Alegre, RS 90619-900, Brazil

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ABSTRACT

Aim: This study set forth a question: are there any differences in bone responses to insulin and/or vitamin D3 treatment in female and male type 1 diabetic (T1D) mice?
Main methods: To address this issue, a non-critical sized femur defect was created in streptozotocin (STZ)-T1D mice. Control non-diabetic and T1D female and male mice received: saline; vitamin D3; insulin; or vitamin D3 plus insulin, for 21 days.
Key findings: Female and male T1D mice showed impaired bone healing, as indicated by histological and micro-computed tomography (micro-CT) analysis. Vitamin D3 or insulin improved the bone regeneration in T1D mice, irrespective of sex. Vitamin D3 plus insulin did not exhibit any additional effects. There were no differences regarding the numbers of TRAP-stained osteoclasts in either evaluated groups. The osteoblast-related gene *osterix* was upregulated in vitamin D3-treated male T1D mice, as revealed by RT-qPCR. Female T1D mice treated with vitamin D3, insulin, or vitamin D3 plus insulin presented an increased expression of insulin growth factor-1 (*IGF-1*) mRNA. Conversely, *IGF-1* mRNA levels were reduced by the same treatments in male TD1 mice.
Significance: Altogether, the results suggested that T1D similarly delayed the osseous healing in female and male mice, with beneficial effects for either vitamin D3 or insulin in T1D mice of both sexes. However, data indicated marked sex differences regarding the expression of genes implicated in bone formation, in T1D mice treated with vitamin D3 and/or insulin.

1. Introduction

Diabetes mellitus (DM) is a metabolic disease affecting a great number of individuals of both sexes globally. According to the International Diabetes Federation (IDF), 425 million people were estimated to have DM in 2017, while 629 million cases are predicted for 2045 [1]. DM is classified as type 1 (insulin-dependent) or type 2 (obesity-related). Type 1 diabetes (T1D) is characterized by autoimmune destruction of the pancreatic β-cells, resulting in hyperglycemia. The patients with T1D can develop many complications, such as retinopathy, neuropathy, nephropathy and cardiovascular diseases [2]. DM also affects the cicatrization of soft and bone tissues [3,4]. Noteworthy, there is an increased risk of bone fractures, in association with low bone turnover [5]. The changes in bone metabolism related to DM might include alterations in the activity and viability of osteoblasts, osteoclasts or even osteocytes, although this matter requires further investigation [6].

The mechanisms underlying bone-related problems in T1D are not currently clear, and the management of this condition remains a clinical challenge. The major goal of T1D treatment is to control the glycemic levels by insulin replacement therapy. An adequate insulin treatment postpones the main T1D-related complications, also contributing to the stabilization of bone metabolism, likely via bone formation [7,8]. The classic role of vitamin D in the endocrine system is to stimulate the

* Corresponding author at: Av. Ipiranga 6681, 90619-900, Brazil.
E-mail address: maria.campos@pucrs.br (M.M. Campos).

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Table 1
The sequences of reverse and forward primers used in the present study.

Gene	Forward primer	Reverse primer
Hprt1	5′-CTCATGGACTGATTATGGACAGGAC-3′	5′-GCAGGTCAGCAAAGAACTTATAGCC-3′
Runx2	5′-AAATGCCTCCGCTGTTATGAA-3′	5′-GCTCCGGCCCACAAATCT-3′
Osterix	5′-AGCGACCACTTGAGCAAACAT-3′	5′-GCGGCTGATTGGCTTCTT-3′
IGF-1	5′-CAAGCCCACAGGCTATGGC-3′	5′-TCTGAGTCTTGGGCATGTCAG-3′

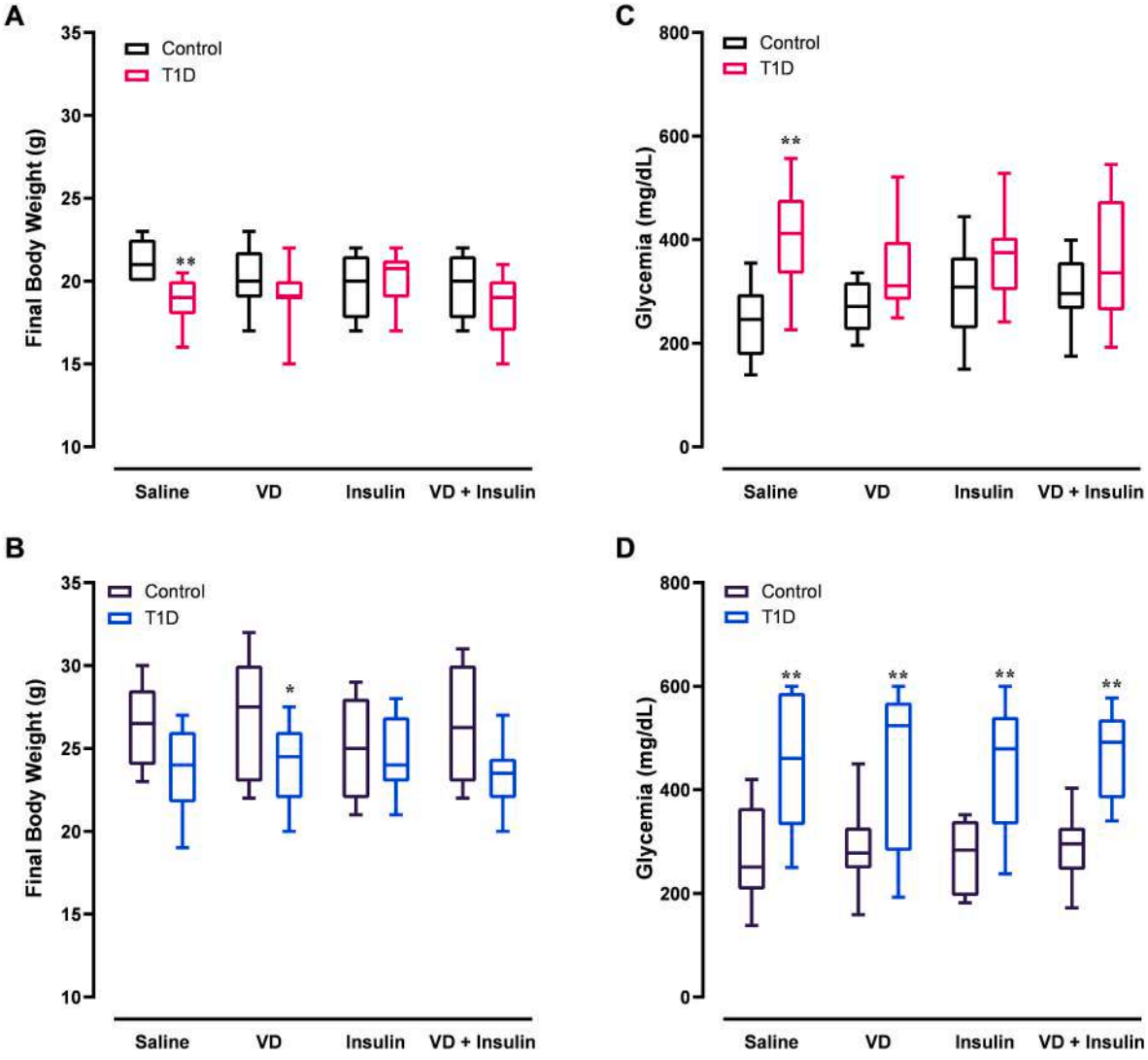

Fig. 1. T1D induction by STZ led to hyperglycemia in female and male mice, regardless of treatment with vitamin D3 and insulin. Effects of T1D induction by STZ in glycemia levels of female (panel A) or male (panel B) mice that had been treated with saline, vitamin D3 (VD), insulin, or vitamin D3 plus insulin (VD + insulin). The box plots show the median of 9–12 experiments with the upper and lower quartiles. The whiskers indicate the maximal and the minimal values. **P < 0.01 in comparison with non-diabetic control mice.

absorption of calcium in the intestine, secondarily regulating the bone mineralization [9]. Furthermore, vitamin D can also affect the bone turnover, via direct modulation of inflammatory pathways [10]. Its ability to induce osteogenic differentiation indicates a great potential for bone tissue engineering [11]. Nonetheless, the effects of vitamin D3 supplementation on diabetes complications, including bone changes, require further investigation [12]. Of note, an experimental study by Wu et al. showed that the combined treatment of insulin plus vitamin D3 had additional effects in improving the implant osseointegration in T1D rats [13].

The present study evaluated the bone regeneration in a femoral defect model, comparing the outcomes in male and female T1D mice. Attempts have also been made to assess whether the insulin replacement, alone or combined with vitamin D3 supplementation, might influence the bone healing in this experimental paradigm.

2. Materials and methods

2.1. Ethical approval

The experimental procedures were carried out in agreement with the Guidelines for the Use and Care with Laboratory Animals from the National Institutes of Health (2011). The Ethics Committee on Animal Use of Pontifícia Universidade Católica do Rio Grande do Sul (CEUA-

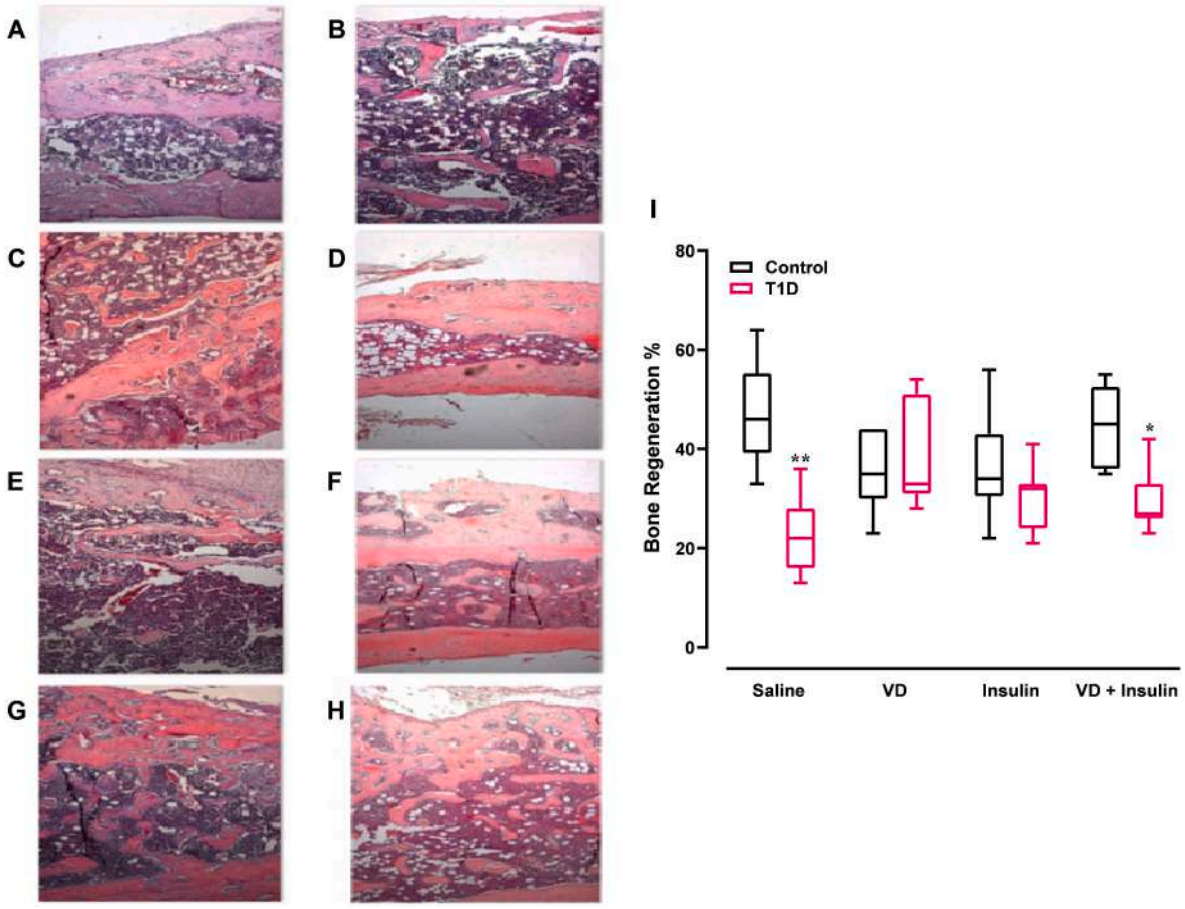

Fig. 2. Impaired bone regeneration in female T1D mice is restored by vitamin D3 or insulin treatment. Representative images of H&E-stained slides in the area corresponding to the center of the bone defect: A, saline-treated non-diabetic mice; B, saline-treated T1D mice; C, vitamin D3-treated non-diabetic mice; D, vitamin D3-treated T1D mice; E, insulin-treated non-diabetic mice; F, insulin-treated T1D mice; G, vitamin D3 + insulin-treated non-diabetic mice; H, vitamin D3 + insulin-treated T1D mice. Panel I, percentages of bone formation in the different experimental groups. The box plots show the median of 5–8 experiments with the upper and lower quartiles. The whiskers indicate the maximal and the minimal values. *P < 0.05; **P < 0.01 in comparison with non-diabetic control mice.

PUCRS) evaluated and approved the research protocols (15/00433). The number of animals was the least necessary to demonstrate the consistent effects of interventions.

2.2. Experimental animals

Male and female C57BL/6J mice (two months old, 18–25 g, total final N = 186) were obtained from the Center of Experimental Biological Models (CeMBE/PUCRS). The animals were housed under standard conditions of temperature (22 ± 2 °C), in a 12:12 h light-dark cycle (lights on 07:00 AM), and humidity (50–70%), in ventilated cages, with autoclaved wood chip bedding. Standard rodent chow (Nuvilab® (g/kg), protein: 220; total fat: 40; fiber: 80; mineral mix: 100 and carbohydrates: 560) and tap water were provided ad libitum. The initial total number of animals at the beginning of the study was 192, with 12 animals per group, to be divided for histological, micro-CT and RT-PCR analysis. From the initial sample, six animals were lost after anesthesia to induce the bone defect, resulting in the number of 186 animals.

2.3. Induction of type 1 diabetes

The animals (male and female) were randomly divided into two major groups: type 1 diabetes (T1D) and non-diabetic (control). T1D was induced by 5-daily injections of streptozotocin (STZ; 50 mg/kg; Sigma–Aldrich, St. Louis, MO), given intraperitoneally (i.p.), dissolved in citrate buffer (50 mM; pH 4.5). Control animals received citrate buffer vehicle alone (i.p.), at the same schedule of administration. The glucose levels were measured at the end of experiments (after 28 days of completing the induction protocol with STZ) and expressed in mg/dL (OneTouch; Johnson & Johnson Medical Brazil; São Paulo, Brazil).

2.4. Femur defects

Seven days after the last STZ injection, which corresponds to the 12th day of the experimental protocol, a single operator created a non-critical size bone defect in the mouse femur, as described before [14]. The surgical procedures were performed under aseptic conditions. The animals were anesthetized through a mixture of saline, ketamine (50 mg/mL) and xylazine (20 mg/mL) – in a ratio of 3:2:1 respectively – by i.p. application. An access to the left mouse femur was made through a skin incision (6–8 mm in length), followed by a muscle retraction and detachment of the periosteum. The osteotomy was carried out under irrigation, by using a surgical contra-angle handpiece (800 rpm) to create a mono-cortical bone defect (4-mm in length and 2-mm in diameter). Following the bone defect creation, the soft tissues were sutured in separated layers, and the animals received cephalexin (60 mg/kg; p.o.; single dose) and acetaminophen (50 mg/kg; i.p.; 6/6 h; for 48 h) as post-operative medication.

2.5. Treatments

Control or T1D male and female mice were randomly distributed into four subgroups, according to the treatment: (i) vehicle; (ii) vitamin

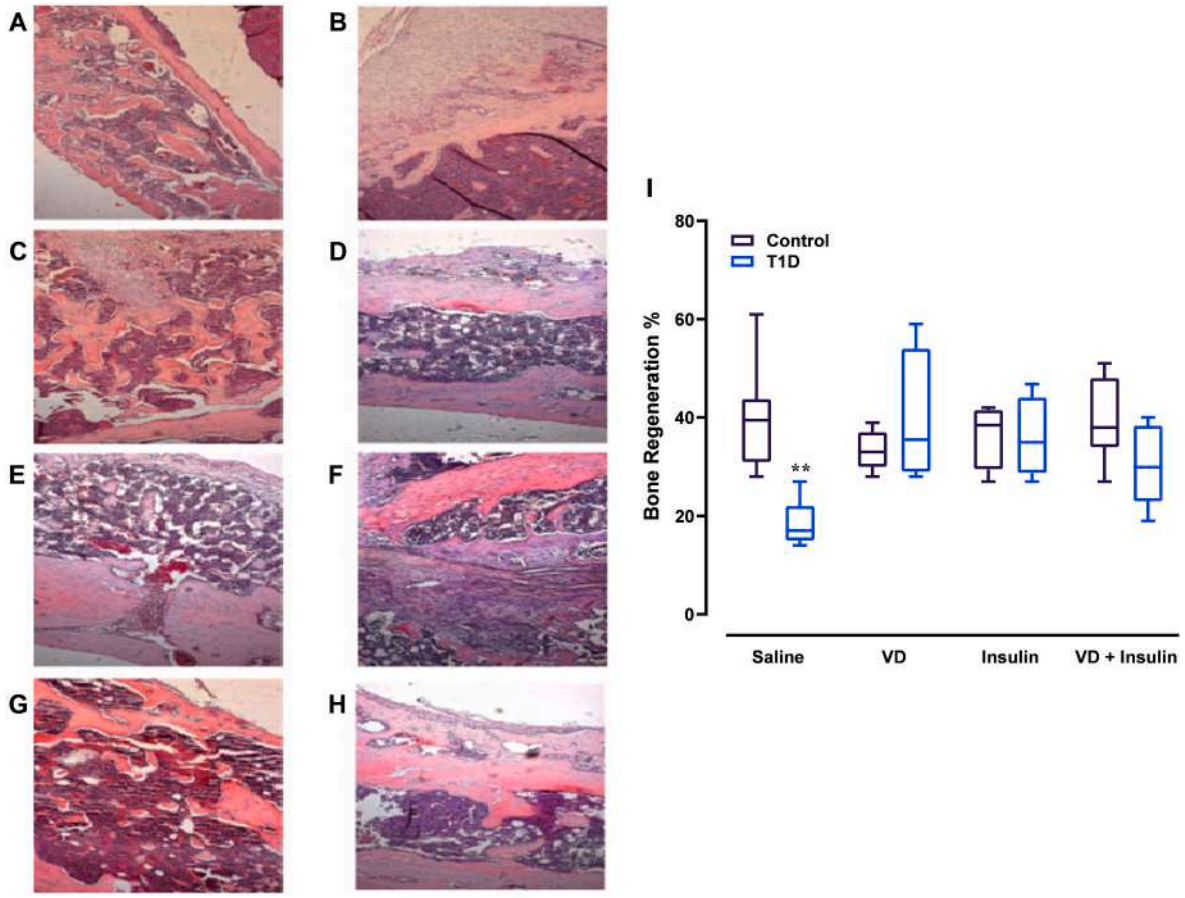

Fig. 3. Impaired bone regeneration in male T1D mice is restored by vitamin D3 or insulin treatment. Representative images of H&E-stained slides in the area corresponding to the center of the bone defect: A, saline-treated non-diabetic mice; B, saline-treated T1D mice; C, vitamin D3-treated non-diabetic mice; D, vitamin D3-treated T1D mice; E, insulin-treated non-diabetic mice; F, insulin-treated T1D mice; G, vitamin D3 + insulin-treated non-diabetic mice; H, vitamin D3 + insulin-treated T1D mice. Panel I, percentages of bone formation in the different experimental groups. The box plots show the median of 5–8 experiments with the upper and lower quartiles. The whiskers indicate the maximal and the minimal values. **P < 0.01 in comparison with non-diabetic control mice.

D3; (iii) insulin and (iv) vitamin D3 plus insulin. Vitamin D3 (4 μg/kg; orally) and/or insulin (3 UI/kg; by subcutaneous route; Humulin R, Eli Lilly and Co., Indianapolis) were administered daily, for 21 days. The doses of vitamin D3 and insulin were selected based on previous publications [15,16]. The tested doses did not cause any unspecific central effect or deaths during the treatment period.

2.6. Sample collection

On day 22 post-surgery (33 days after the onset of T1D induction), the animals were euthanized by sevoflurane overdose, after overnight fasting (between 10 and 12 h). The blood was collected from the abdominal aorta for analysis of glycemia. The femurs were cleaned from connective tissues, and fixed in 4%-buffered formaldehyde solution, to subsequent histological procedures or micro-computed tomography (micro-CT) analysis. Separately, samples corresponding to the defect area were stored in TRIzol® Reagent (Life Technologies) for quantitative polymerase chain reaction (PCR) analysis.

2.7. Micro-CT analysis

Micro-CT is a useful tool to measure the microstructural changes in the process of bone regeneration [17,18]. The bone regeneration was further evaluated by micro-CT scanning using Skyscan 1172 (Bruker Micro-CT, Belgium) before decalcification, at 89 Kv and 112 μA, with 6-μm thickness, in a resolution of 1336 × 2000 pixels. For each femur, a specific volume-of-interest (VOI) was selected considering the size of the defect (4-mm × 2-mm). A scheme showing the VOI used for the analysis is provided in Supplementary Fig. 1. The Software CTAn V1.16 (Bruker) was used for 3D-morphometric analysis. The following parameters were evaluated: bone volume fraction (BV/TV in percentage), trabecular thickness (Tb.Th in mm), and trabecular separation (Tb.Sp in mm) [19].

2.8. General histological procedures

After fixation, the samples were decalcified in a 17% ethylenediaminetetraacetic acid (EDTA) solution, for 10 days (with daily changes of fresh solution) and embedded in paraffin. Consecutive four μm-thick longitudinal sections were obtained from each femur for different sets of analysis. The slides were stained with hematoxylin-eosin (H&E), Mallory (Accustain Mallory's stain kit, Sigma-Aldrich, St. Louis, MO), or tartrate-resistant acid phosphate (Leukocyte TRAP kit, Sigma-Aldrich, USA). The histological images were taken with a microscope (Axio Imager A1, Jena, Germany) coupled to an image capture system (Axio Vision Rel. 4.4 Software Multimedia, Jena, Germany), from Carl Zeiss (Hallbergmoos, Germany). The methods for image analysis regarding H &E, Mallory and TRAP-stained sections has been published elsewhere [14]. The NIH Image J 1.36b software program was used to determine the percentage areas of bone formation in relation to the total area in H &E-stained slides. The same software was used for analyzing the blue-colored collagen fibers in Mallory-stained slides. The numbers of TRAP-positive cells presenting osteoclast morphology were counted manually under 400-× magnification.

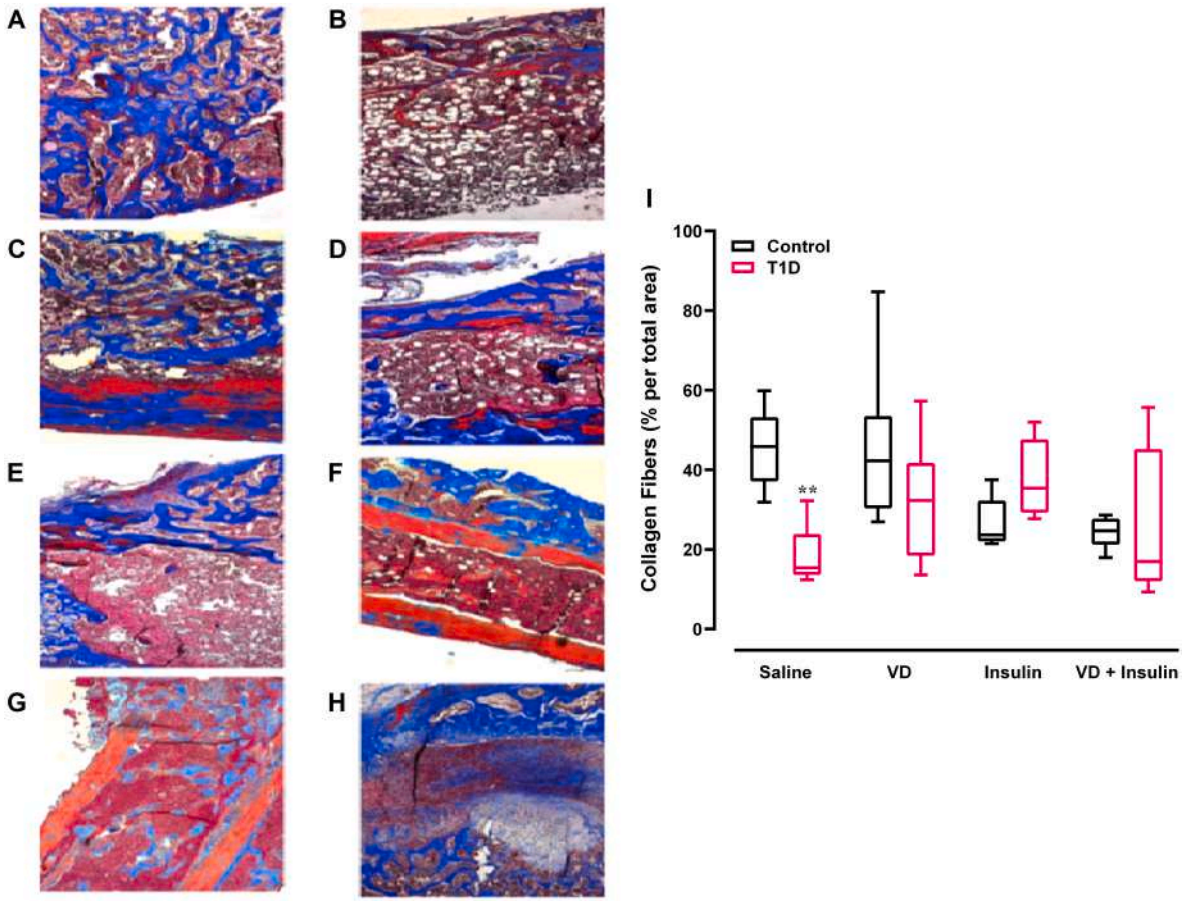

Fig. 4. Vitamin D3 or insulin treatment rescued the reduced collagen contents in female T1D mice. Representative images of Mallory-stained slides in the area corresponding to the center of the bone defect: A, saline-treated non-diabetic mice; B, saline-treated T1D mice; C, vitamin D3-treated non-diabetic mice; D, vitamin D3-treated T1D mice; E, insulin-treated non-diabetic mice; F, insulin-treated T1D mice; G, vitamin D3 + insulin-treated non-diabetic mice; H, vitamin D3 + insulin-treated T1D mice. Panel I, percentages of blue-colored collagen fibers in the different experimental groups. The box plots show the median of 5–8 experiments with the upper and lower quartiles. The whiskers indicate the maximal and the minimal values. **P < 0.01 in comparison with non-diabetic control mice. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.9. RNA isolation and real-time (RT)-qPCR

The gene expression of *Runx2*, *osterix* and *IGF-1* was determined by RT-qPCR. Bone samples were crushed in liquid nitrogen, and total RNA was isolated from bone with TRIzol® Reagent (Life Technologies) in accordance with the manufacturer's instructions. RNA purity (Abs 260/280 nm ~2.0) and concentration was determined by L-Quant (Loccus Biotecnologia). Deoxyribonuclease I (Sigma-Aldrich) was used to eliminate genomic DNA contamination. The cDNA was synthesized with ImProm-II™ Reverse Transcription System (Promega) from one μg of the total RNA, following the manufacturer's instructions. Quantitative PCR was performed using SYBR® Green I (Invitrogen) to detect double-strand cDNA synthesis on the 7500 Real-time PCR System (Applied Biosystems). The PCR cycling conditions were: an initial polymerase activation step for 5 min at 95 °C, 40 cycles of 15 s at 95 °C for denaturation, 35 s at 60 °C for annealing and 15 s at 72 °C for elongation. At the end of cycling protocol, a melting-curve analysis was included, and the fluorescence was measured from 60 to 99 °C to confirm the specificity of primers and the absence of primer-dimers; in all cases, one single peak was obtained. All RT assays were carried out in quadruplicate, and in all experiments, a reverse transcriptase negative control was included. *Hprt1* was used as the reference gene for normalization. The sequences of reverse and forward primers are provided in Table 1. The efficiency per sample was calculated using LinRegPCR 2016.1 Software (http://LinRegPCR.nl). GeNorm 3.5 Software (http://medgen.ugent.be/genorm/) was used to assess the stability of the reference gene, and the optimal number of reference genes according to the pairwise variation (V). Relative mRNA expression levels were determined using the 2-ΔΔCq method [20].

2.10. Statistical analysis

Data are depicted as Box and Whiskers graphs, permitting to observe the medians, interquartile ranges, besides minimal and maximal values. Statistical analysis was performed by two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test. We compared the responses to treatments in control versus T1D groups. For this purpose, a group factor with two levels was included in the analysis. For glycemia levels, a one-way ANOVA followed by Bonferroni's post-hoc test was used to assess the differences between saline-treated control mice and T1D mice treated with vitamin D, insulin, or vitamin D + insulin. P values less than 0.05 were considered significant. The experimental N for each protocol is indicated in each legend to figure. GraphPad Prism® software version 8.02 (San Diego, USA) was used for statistical analysis and for creation of graphs. All the qualitative and semi-quantitative analysis were performed by trained experimenters in a blinded manner. The raw data regarding the present study is available as supporting information.

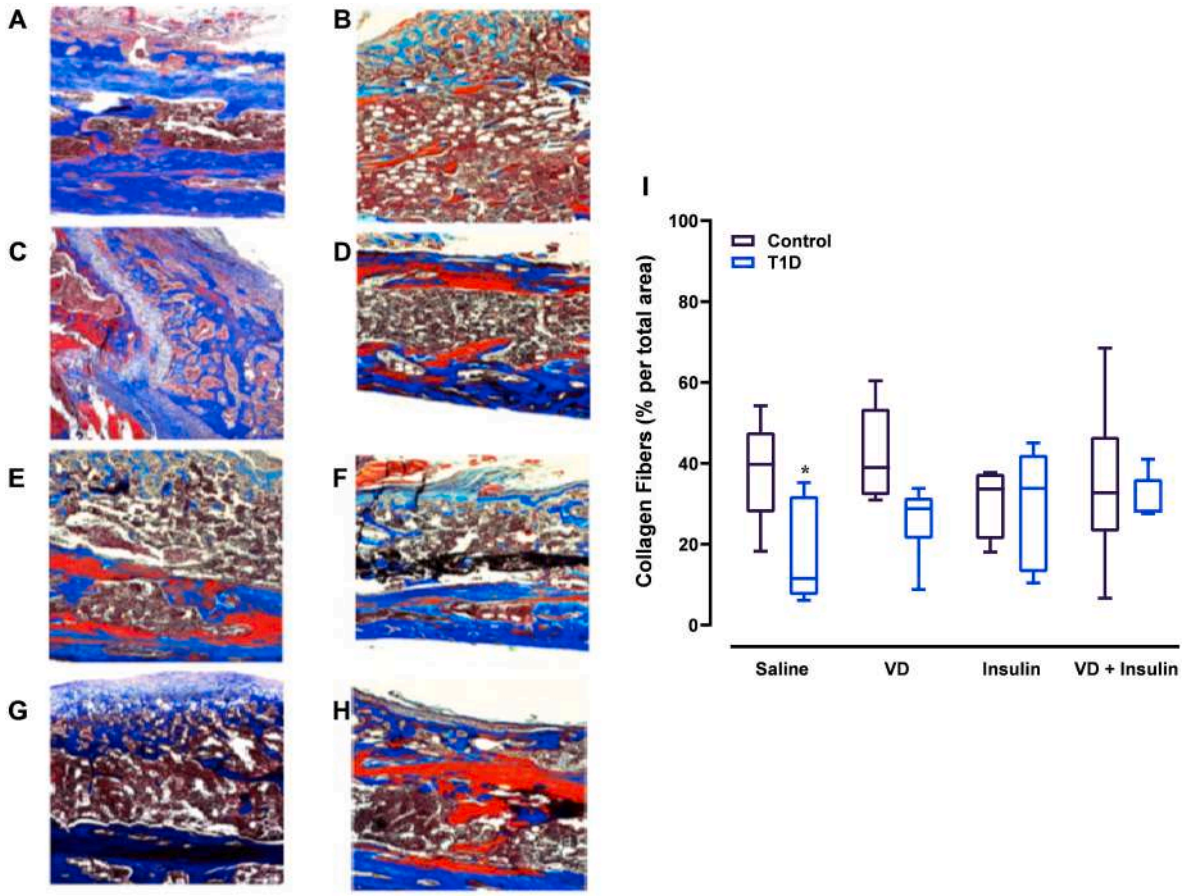

Fig. 5. Vitamin D3 or insulin treatment rescued the reduced collagen contents in male T1D mice. Representative images of Mallory-stained slides in the area corresponding to the center of the bone defect: A, saline-treated non-diabetic mice; B, saline-treated T1D mice; C, vitamin D3-treated non-diabetic mice; D, vitamin D3-treated T1D mice; E, insulin-treated non-diabetic mice; F, insulin-treated T1D mice; G, vitamin D3 + insulin-treated non-diabetic mice; H, vitamin D3 + insulin-treated T1D mice. Panel I, percentages of blue-colored collagen fibers in the different experimental groups. The box plots show the median of 5–8 experiments with the upper and lower quartiles. The whiskers indicate the maximal and the minimal values. *P < 0.05 in comparison with non-diabetic control mice. The arrows indicate TRAP-positive osteoclasts. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Glycemic levels and bone regeneration in female and male T1D mice

As expected, T1D induction by STZ injection led to hyperglycemia, confirming the development of diabetes in female and male mice (Fig. 1A and B, respectively). For males, a two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc multiple comparison test revealed a significant difference ($P < 0.01$) when comparing control and T1D mice in all groups, irrespective of treatment (Fig. 1B). As for females, by using a two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc multiple comparison test, there was a significant difference when comparing control and T1D mice treated with saline ($P < 0.01$), but not for the other treatments (Fig. 1A). However, when comparing saline-treated control mice with T1D mice treated with vitamin D, insulin, or vitamin D + insulin, by using one-way ANOVA followed by Bonferroni's test, the P values were: 0.1283; 0.0101; and 0.0230, respectively. This led us to imply a trend for higher glucose levels in all of T1D groups, regardless of the treatment. The last treatment with vitamin D3 and/or insulin was performed one day before the euthanasia, justifying the elevated glycemia levels in all groups, even in insulin-treated mice.

Based on the evaluation of H&E staining, female (Fig. 2A–I) and male (Fig. 3A–I) T1D mice showed an impairment of bone healing, with the presence of a disorganized loose connective tissue within the defects. The percentages of newly formed bone areas were reduced by 52.2 ± 6% and 53.4 ± 5%, in female and male T1D mice, respectively, in comparison with non-diabetic control animals. The supplementation with vitamin D3 or insulin recovered the bone regeneration to the values seen in control non-diabetic mice, in either T1D females (Fig. 2I) or males (Fig. 3I). No further increase in the areas of newly formed bone was observed by the combined treatment with vitamin D3 plus insulin, in male mice (Fig. 3I). Nonetheless, this combination showed unfavorable results in female mice (Fig. 2I). The assessment of Mallory-stained slides showed a marked diminishment of blue-colored collagen contents, with reduction percentages of 60.3 ± 8% and 55.7 ± 12%, in T1D females (Fig. 4A–I) and males (Fig. 5A–I), respectively, when compared with non-diabetic control mice. A recovery of collagen contents was observed for vitamin D3 and insulin treatment, in female and male mice, when administered alone or in a combination scheme (Figs. 4I and 5I, respectively). We also performed an analysis of tartrate-resistant acid phosphate (TRAP) in the areas of the defect, as an indication of osteoclast activity, in female (Fig. 6A–J) and male (Fig. 7A–J) T1D animals. This staining did not reveal significant differences of osteoclast numbers among the experimental groups, regardless of the sex or treatments.

3.2. Micro-CT evaluation

The quantitative analysis of bone volume density (BV/TV) revealed that either female (Fig. 8I) or male (Fig. 9I) T1D mice displayed lessened bone formation (BV/TV) in relation to control animals. The

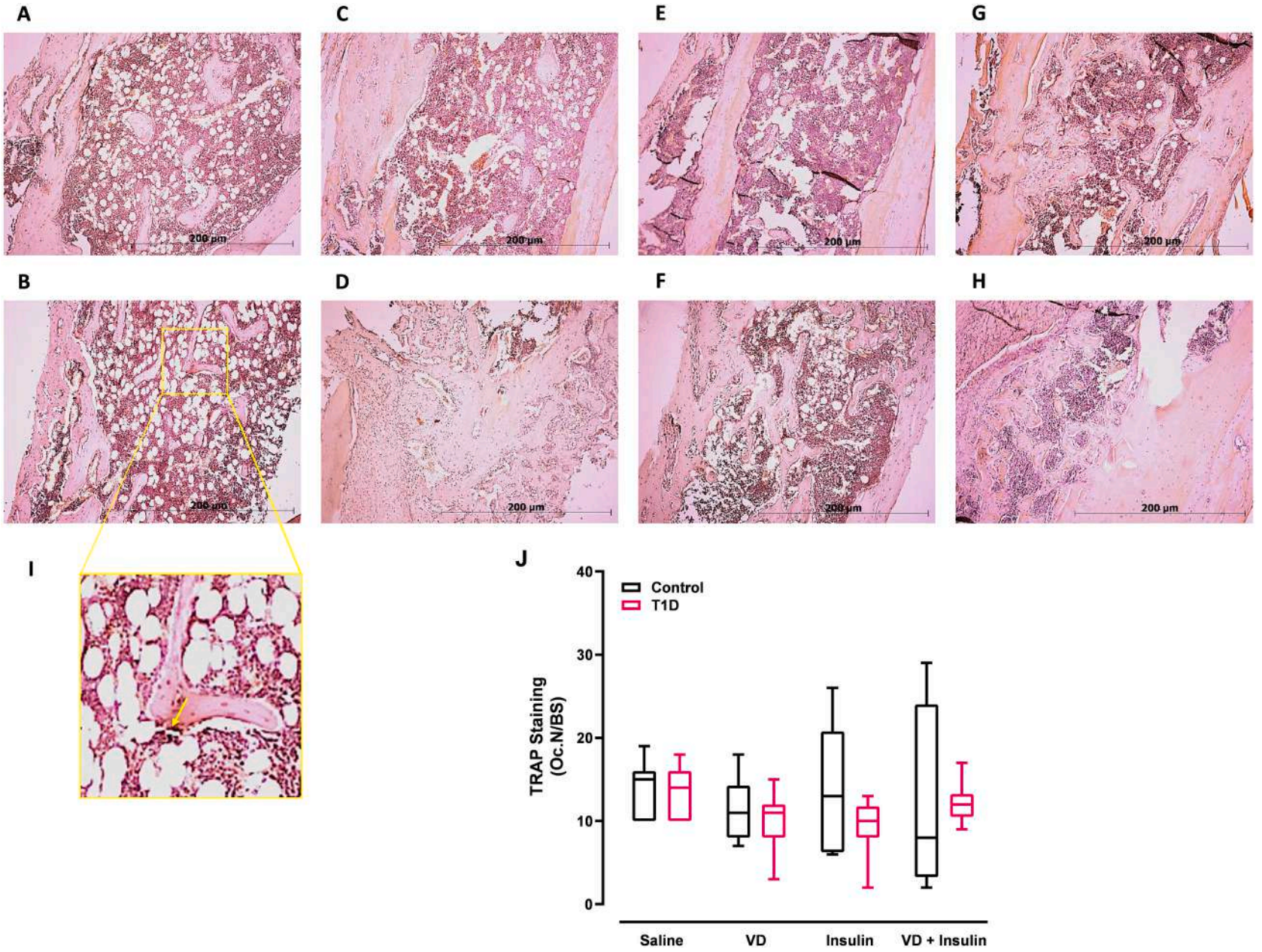

Fig. 6. TRAP staining and number of osteoclasts in female T1D mice treated with vitamin D3 and/or insulin. Representative images of TRAP-stained slides in the area corresponding to the center of the bone defect: A, saline-treated non-diabetic mice; B, saline-treated T1D mice; C, vitamin D3-treated non-diabetic mice; D, vitamin D3-treated T1D mice; E, insulin-treated non-diabetic mice; F, insulin-treated T1D mice; G, vitamin D3 + insulin-treated non-diabetic mice; H, vitamin D3 + insulin-treated T1D mice; I, zoomed image (yellow square) from panel B showing a TRAP-positive osteoclast (yellow arrow). All of the images were modified with the following parameters: −20% bright; +40% contrast. Panel J, number of osteoclasts per total bone area (Oc.N/BS) in the different experimental groups. The box plots show the median of 4–8 experiments with the upper and lower quartiles. The whiskers indicate the maximal and the minimal values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

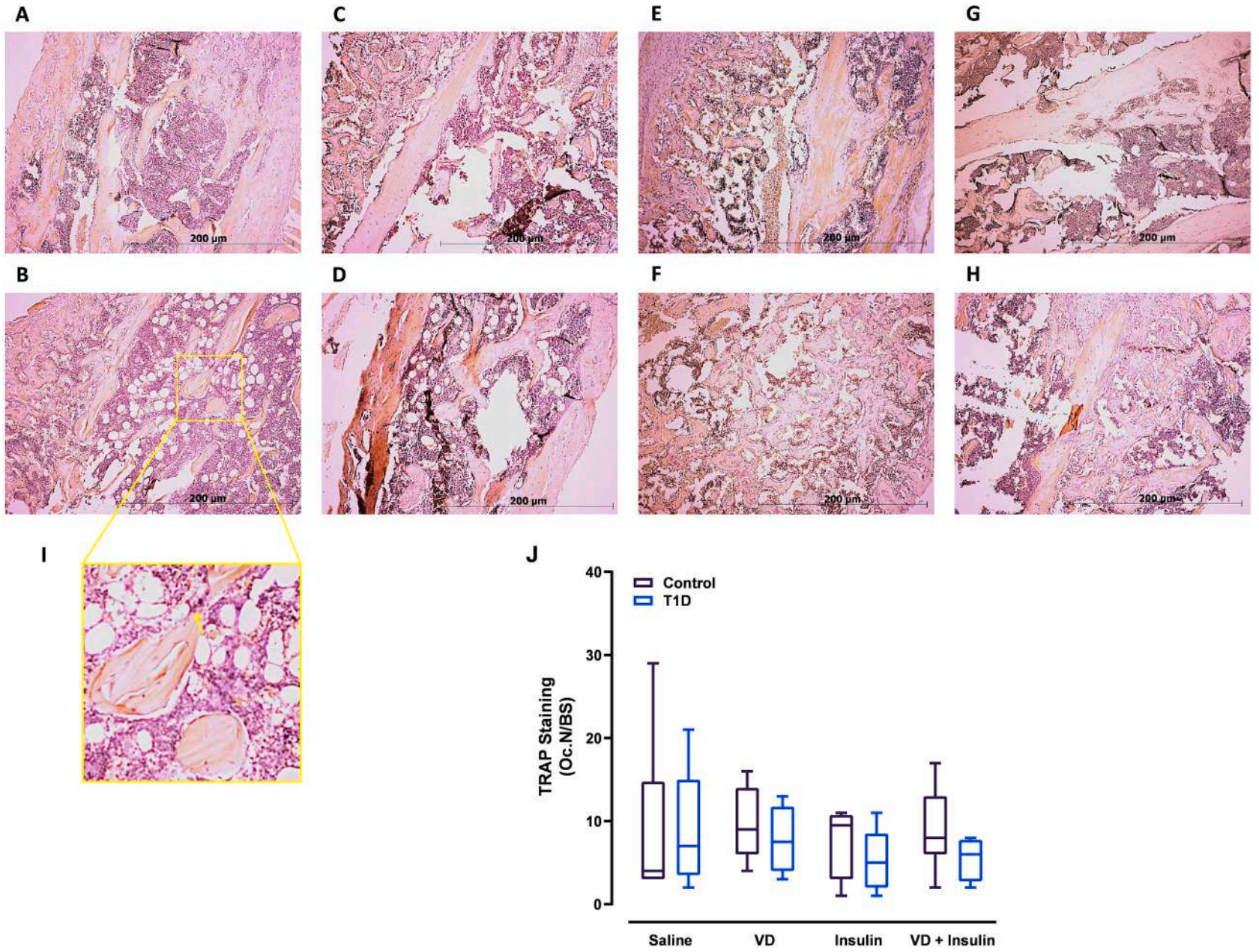

Fig. 7. TRAP staining and number of osteoclasts in male T1D mice treated with vitamin D3 and/or insulin. Representative images of TRAP-stained slides in the area corresponding to the center of the bone defect: A, saline-treated non-diabetic mice; B, saline-treated T1D mice; C, vitamin D3-treated non-diabetic mice; D, vitamin D3-treated T1D mice; E, insulin-treated non-diabetic mice; F, insulin-treated T1D mice; G, vitamin D3 + insulin-treated non-diabetic mice; H, vitamin D3 + insulin-treated T1D mice; I, zoomed image (yellow square) from panel B showing a TRAP-positive osteoclast (yellow arrow). All of the images were modified with the following parameters: −20% bright; +40% contrast. Panel J, number of osteoclasts per total bone area (Oc.N/BS) in the different experimental groups. The box plots show the median of 4–8 experiments with the upper and lower quartiles. The whiskers indicate the maximal and the minimal values. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

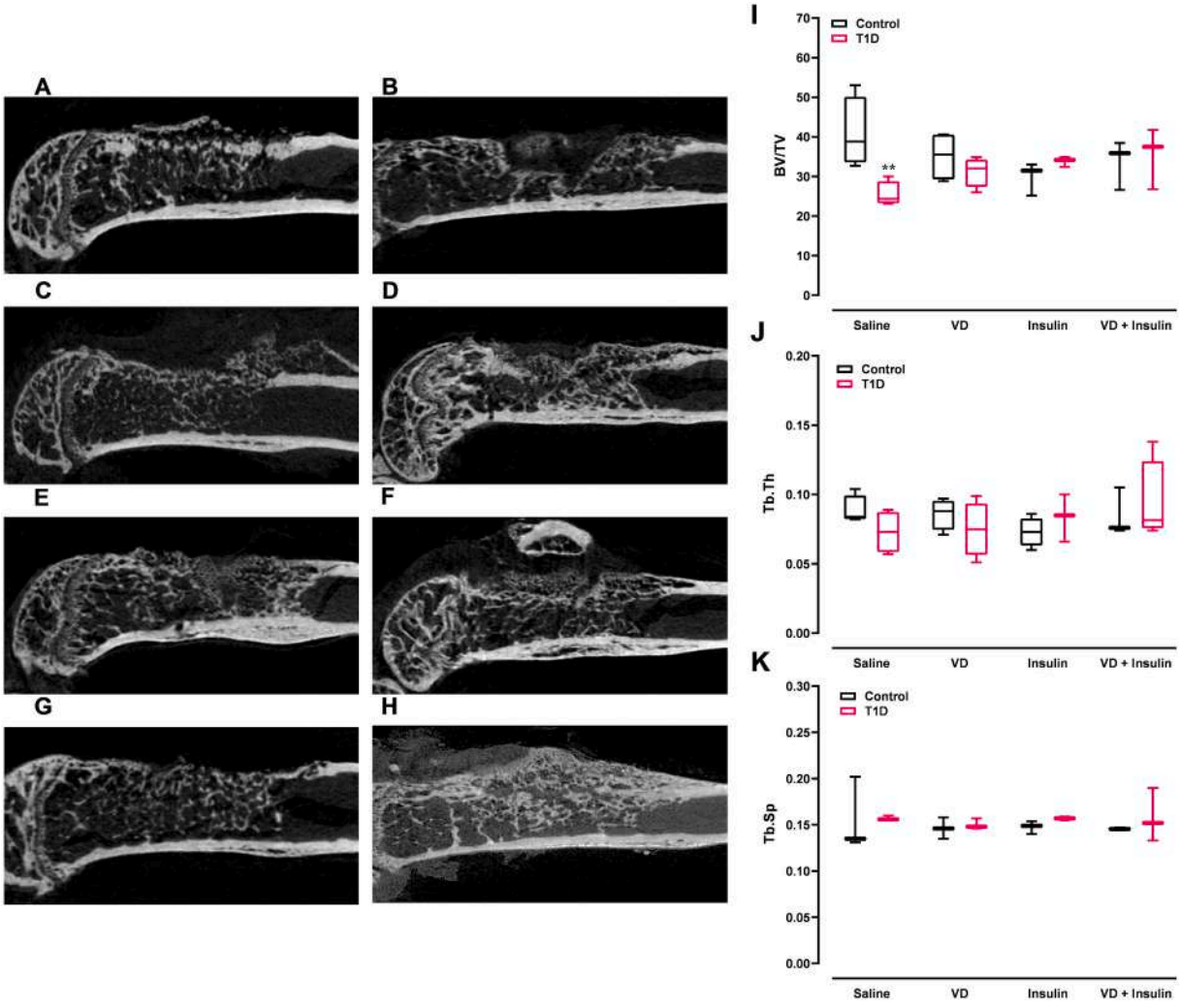

Fig. 8. Bone micro-CT evaluation in female T1D mice treated with vitamin D3 and/or insulin. Representative images of micro-CT images in the different experimental groups: A, saline-treated non-diabetic mice; B, saline-treated T1D mice; C, vitamin D3-treated non-diabetic mice; D, vitamin D3-treated T1D mice; E, insulin-treated non-diabetic mice; F, insulin-treated T1D mice; G, vitamin D3 + insulin-treated non-diabetic mice; H, vitamin D3 + insulin-treated T1D mice. Panel I, bone volume fraction (BV/TV in percentage); panel J, trabecular thickness (Tb.Th in mm), panel K, trabecular separation (Tb.Sp in mm). The box plots show the median of 3–4 experiments with the upper and lower quartiles. The whiskers indicate the maximal and the minimal values. **P < 0.01 in comparison with non-diabetic control mice.

percentages of reduction caused by T1D induction were 37 ± 4% and 45 ± 2%, in female and male mice, respectively. The supplementation with vitamin D3 and/or insulin improved BV/TV values in female (Fig. 8I) or male (Fig. 9I) T1D mice, towards the levels seen in control non-diabetic animals. Other parameters were assessed in micro-CT analysis, such as trabecular thickness (Tb.Th) and trabecular separation (Tb.Sp), without any significant differences among the treatment groups in female (Fig. 8J–K) or male (Fig. 9J–K) mice. Representative micro-CT images for female and male mice in the different experimental groups are depicted in Figs. 8A–H and 9A–H, correspondingly.

3.3. Runx2, Osterix and IGF-1 expression in bone

Next, we evaluated the bone mRNA expression of two osteoblast-related transcription factors, namely *Runx2* and *osterix*, in addition to *IGF-1*. The evaluation of *RunX2* mRNA expression did not reveal any significant difference among the treatment groups, in either female (Fig. 10A) or male (Fig. 10B) mice. In male T1D mice, the treatment with vitamin D3 led to a significant increase of *osterix* mRNA levels (Fig. 10D), an effect that was not observed in female T1D mice (Fig. 10C). In female T1D mice, the treatment with vitamin D3 and insulin, dosed alone or in combination, triggered an upregulation of *IGF-1* mRNA expression (Fig. 10E). Alternatively, the same treatments caused a diminishment in the expression of *IGF-1* mRNA, in male T1D mice (Fig. 10F).

4. Discussion

The present study evaluated the bone healing in a femoral defect model in T1D mice, comparing the outcomes in males and females. Additionally, we investigated whether insulin replacement and/or the vitamin D3 supplementation might influence the bone regeneration in this experimental model.

The repeated administration of STZ resulted in hyperglycemia, confirming T1D induction [21]. According to the International Diabetes Federation, DM has no predilection for men or women, similarly affecting both sexes, as demonstrated herein. Clinical evidence has indicated sex differences regarding bone mineral density, according to the evaluation of T1D adult patients [22,23]. Of interest, a meta-analysis study conducted by Chrcanovic et al. proposed that implant failure rates are superior in males, in relation to females [24]. Hence, it is reasonable to ponder that sex has an influence on bone metabolism in

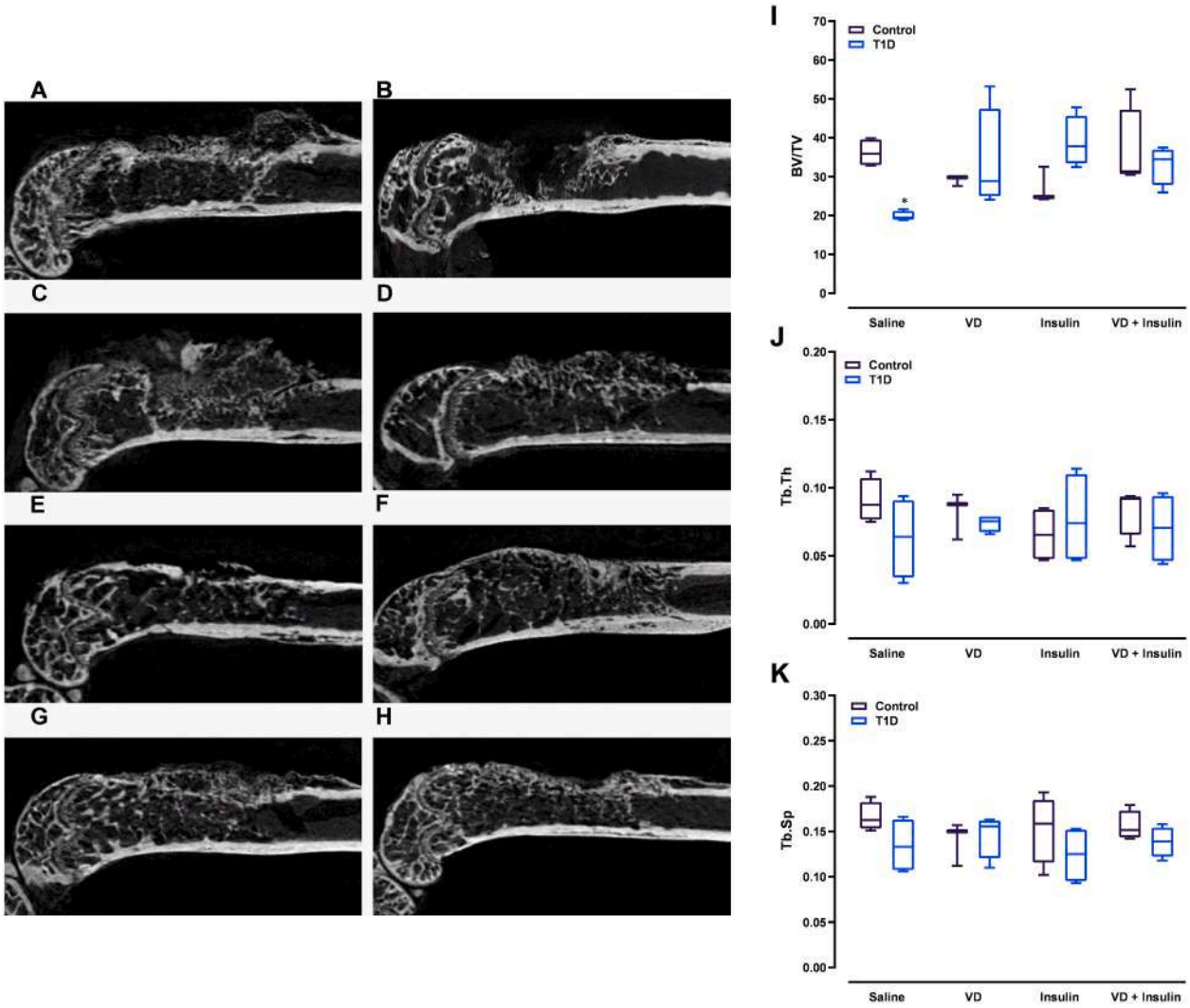

Fig. 9. Bone micro-CT evaluation in male T1D mice treated with vitamin D3 and/or insulin. Representative images of micro-CT images in the different experimental groups: A, saline-treated non-diabetic mice; B, saline-treated T1D mice; C, vitamin D3-treated non-diabetic mice; D, vitamin D3-treated T1D mice; E, insulin-treated non-diabetic mice; F, insulin-treated T1D mice; G, vitamin D3 + insulin-treated non-diabetic mice; H, vitamin D3 + insulin-treated T1D mice. Panel I, bone volume fraction (BV/TV in percentage); panel J, trabecular thickness (Tb.Th in mm), panel K, trabecular separation (Tb.Sp in mm). The box plots show the median of 3–4 experiments with the upper and lower quartiles. The whiskers indicate the maximal and the minimal values. *P $<$ 0.05 in comparison with non-diabetic control mice.

T1D, especially when considering the complexity of bone biology, justifying the use of males and females in the present study. Our data demonstrated marked histological bone changes in vehicle-treated T1D mice. These animals showed an impaired bone regeneration, according to the evaluation of H&E-stained sections, with the presence of a disorganized cancellous bone within the area of the defect, regardless of the sex. Similarly, the collagen contents were diminished in T1D animals, as revealed by Mallory's staining, without sex differences. Some experimental studies showed a significant negative impact of T1D on bone formation in rodent models of calvarial or femur critical defects [14,25,26]. In addition, previous evidence indicated that T1D-related osteopenia is not sex dependent [27], corroborating our data.

There is a clear relationship between vitamin D3 insufficiency and diabetic bone disease. However, only a few studies investigated the effects of vitamin D3 supplementation on bone healing under T1D [28,29]. Indeed, there is no consensus regarding the beneficial effects of vitamin D3 therapy on major chronic diseases, such as DM [30]. We provide novel evidence showing that vitamin D3 treatment was able to recover the bone regeneration in female and male T1D animals, to the values seen in control non-diabetic mice. A similar favorable effect for vitamin D3 on bone healing under T1D was also demonstrated by analysis of Mallory-stained slides, indicating a positive modulation of collagen contents towards bone regeneration, in animals of both sexes. Supporting the present results, Li et al. (2013) showed that administration of vitamin D3 ameliorated alveolar bone loss in a periodontitis model in STZ-T1D mice [26].

Insulin replacement is imperative for T1D management. However, the real benefits of insulin treatment on bone recovery in DM still require further investigations. We report that insulin therapy generally improved the bone regeneration in male and female mice, as indicated by H&E, besides Mallory's collagen staining. The insulin therapy recovered the bone regeneration in T2D rats, by using a subcritical size defect model [31]. Of high interest, it was demonstrated that continuous infusion of insulin favored osseous healing in T1D mice, an effect that was positively correlated with the insulin doses, but independent on the retrieval of glucose homeostasis [32]. It was demonstrated that insulin increased the proliferation and survival of mouse and human primary osteoblasts, via stimulation of nitric oxide/cGMP pathway [33]. Another study indicated that insulin therapy in T1D mice led to increased expression of collagen type 1 [34], what

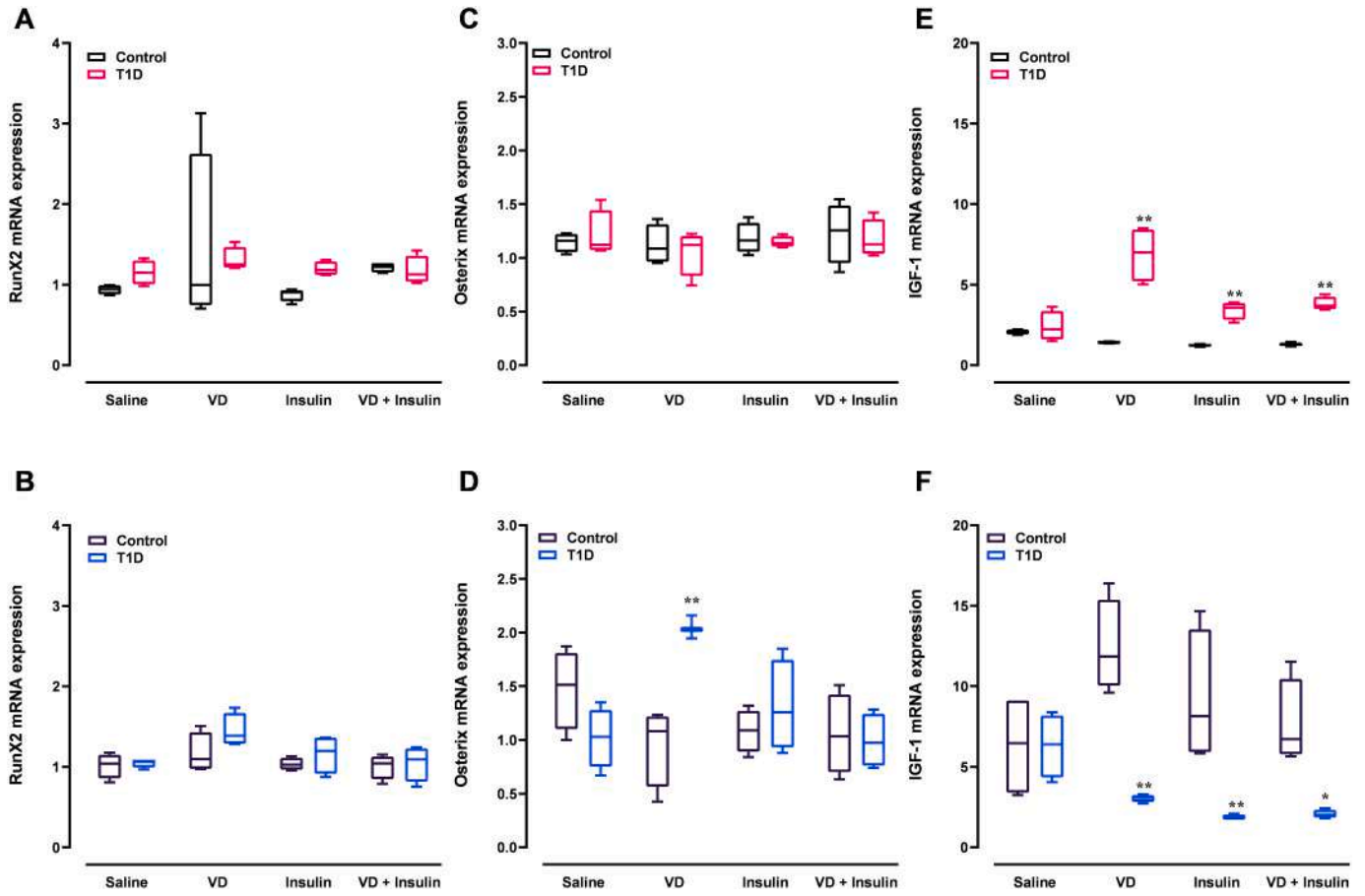

Fig. 10. Bone gene expression in female and male mice submitted to different treatments. Real time-qPCR for *Runx2* (A–B), *osterix* (C–D) and *IGF-1* (E–F) in T1D female (A, C and E) or male (B, D and F) mice that had been treated with saline, vitamin D3 (VD), insulin, or vitamin D3 plus insulin (VD + insulin). The area corresponding to the bone defect was collected for the analysis. The box plots show the median of 4 experiments (run in triplicate) with the upper and lower quartiles. The whiskers indicate the maximal and the minimal values. **P < 0.01 in comparison with non-diabetic control mice.

might support our data on the recovery of collagen levels by insulin.

Since insulin therapy is indispensable for the treatment of T1D, we investigated whether vitamin D3 combination might increase the favorable effects of insulin on bone healing. A study conducted by Wang and colleagues showed that the administration of vitamin D3, prior to induction with STZ, increased insulin secretion in mice [16]. We found that combined treatment with vitamin D3 plus insulin failed to present superior bone healing or collagen levels, when compared to the separate treatments, in female or male T1D animals. On the other hand, Wu et al. demonstrated that vitamin D3 and insulin combination synergistically stimulated titanium implant osseointegration in male T1D rats [13]. Different from our approach, the authors evaluated the mechanisms of osseointegration, but not the bone healing, what might justify the unlike results. In the present study, we evaluated TRAP staining, as an indicative of osteoclast activity.

Herein, the counts of active osteoclasts in the defect, as measured by TRAP staining, did not reveal significant differences among the experimental groups. This data set indicates that either the negative impacts of TD1 induction on bone healing, or the beneficial effects of vitamin D3 or insulin treatment, did not rely on the modulation of osteoclast activation, irrespective of the sex.

The micro-CT scanning revealed decreased BV/TV values in female and male T1D animals, when compared to non-diabetic control mice, an effect that was prevented by insulin treatment. Picke and colleagues observed a similar result in the values of BV/TV, using male T2D ZDF rats [31]. Of note, insulin infusion dose-dependently recovered the BV/TV in male DBA/2J STZ-T1D mice [32], supporting somewhat our data. In the present study, the vitamin D3 supplementation, alone or in combination with insulin, mirrored the effects of insulin therapy on BV/TV, in either female or male animals. A study conducted by Wu and coworkers showed an increase of BV/TV and Tb.Th ratios in the implant osseointegration, when male T1D rats were treated with vitamin D3 plus insulin [13]. Additionally, it was verified that vitamin D3 administration increased the BV/TV levels in male mice, leading to improved implant osseointegration, likely by inactivation of FoxO1 in osteoblasts [29]. Herein, the values of Tb.Th and Tb.Sp did not exhibit marked differences when comparing females and males in the different experimental groups. This contrasts somewhat with previous evidence showing an elevation of Tb.Sp in male T1D mice, without beneficial effects for vitamin D3 therapy on this parameter [28]. Whereas the authors tested the osseointegration of titanium implants, we evaluated the bone healing in a non-critical sized bone defect.

It was previously demonstrated that mRNA expression of either *Runx2* or *osterix*, besides other osteoblast-related factors, such as osteocalcin, was reduced in the tibial bone of STZ-T1D female rats [35]. In addition, the exposure to high glucose levels led to *Runx2* and *osterix* downregulation, according to assessment of osteoblast-like cells obtained from T2D diabetic patients submitted to total hip replacement [36]. A recent study indicated that expression of bone-related genes, such as *Acp5*, *osteocalcin* and *Runx2* was downregulated in $Ins2^{Akita}$ T1D male mice [37]. Strikingly, in our study, vitamin D3 supplementation enhanced the mRNA expression of *osterix* in T1D males, but not in females, when compared with vehicle-treated T1D mice. Corroborating partly our results, Xiong et al. demonstrated that vitamin D3 treatment rescued T1D-induced *Runx2* and *osterix* mRNA downregulation in male mice, in a protocol for studying femur implant osseointegration [29].

Experimental data suggested that IGF-1 signaling in immature osteoblasts is crucial for bone fracture repair [35]. Furthermore, a clinical study conducted with young T1D patients correlated low bone density and poor glycemic control with a reduced expression of *IGF-1* and its receptor [38]. Low expression of *IGF-1* and *Col2* has been correlated with reduced bone growth in T1D $Ins2^{Akita}$ mice [37]. Our data revealed an upregulation of *IGF-1* mRNA in female T1D mice treated with vitamin D3, insulin, or vitamin D3 plus insulin. On the contrary, the same protocols of treatment led to a downregulation of *IGF-1* mRNA in male T1D mice. Altogether, these results suggest sex-related differences for *osterix* and *IGF-1* mRNA expression in bone of T1D mice that had been treated with vitamin D3 or insulin. Further studies using immunohistochemistry or western blot techniques are still required to verify whether differences of osterix and IGF-1 protein expression are observed when comparing T1D females and males, under vitamin D3 and/or insulin treatment.

5. Conclusion

The present results bring novel evidence on the mechanisms underlying T1D bone disease. T1D mice presented a similar detriment in bone regeneration, and the administration of insulin or vitamin D3 improved T1D-impaired osseous healing, regardless of the sex. Nonetheless, the combination of vitamin D3 with insulin did not provide additional benefits in this experimental paradigm. Of note, the regulation of genes relevant for bone formation, such as osterix and IGF-1, was distinctly modulated in female and male T1D mice, under treatment with vitamin D3 and/or insulin, revealing sex-related differences regarding bone healing mechanisms under T1D.

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CRediT authorship contribution statement

Conception and design of the study: Natália P. Cignachi, Maria M. Campos. Sample and data collection: Natália P. Cignachi, Alice Ribeiro, Gustavo D. Barroso, Amanda P. Cignachi, Luiza W. Kist, Rodrigo B. M. Silva. *Statistical analysis*: Natália P. Cignachi, Luiza W. Kist, Rodrigo B.M. Silva, Maria M. Campos. *Data review and interpretation*: Natália P. Cignachi, Alice Ribeiro, Luiza W. Kist, Rodrigo B. M. Silva, Maria M. Campos. *Drafting of manuscript*: Natália P. Cignachi and Maria M. Campos. *Critical revision of manuscript*: Natália P. Cignachi, Alice Ribeiro, Luiza W. Kist, Maurício R. Bogo, Rodrigo B. M. Silva, Maria M. Campos. *Approving final manuscript version*: Natália P. Cignachi, Alice Ribeiro, Gustavo D. Barroso, Amanda P. Cignachi, Luiza W. Kist, Maurício R. Bogo, Rodrigo B. M. Silva, Maria M. Campos.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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