applied sciences

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# Effect of Systemic Zoledronic Acid Dosing Regimens on Bone Regeneration in Osteoporotic Rats

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**Abstract:** The aim of this study was to evaluate the regeneration of bone defects created in the femoral condyle of osteoporotic rats, following intravenous (IV) zoledronate (ZA) treatment in three settings: pre-bone grafting (ZA-Pre), post-bone grafting (ZA-Post), and pre- plus post-bone grafting (ZA-Pre+Post). Twenty-four female Wistar rats were ovariectomized (OVX). After 12 weeks, bone defects were created in the left femoral condyle. All defects were grafted with a particulate inorganic cancellous bovine bone substitute. ZA (0.04 mg/kg, weekly) was administered to six rats 4 weeks pre-bone graft placement. To another six rats, ZA was given post-bone graft placement creation and continued for 6 weeks. Additional six rats received ZA treatment pre- and post-bone graft placement. Control animals received weekly saline intravenous injections. At 6 weeks post-bone graft placement, samples were retrieved for histological evaluation of the bone area percentage (BA%) and remaining bone graft percentage (RBG%). BA% for ZA-Pre (50.1 ± 3.5%) and ZA-Post (49.2 ± 8.2%) rats was significantly increased compared to that of the controls (35.4 ± 5.4%, *p*-value 0.031 and 0.043, respectively). In contrast, ZA-Pre+Post rats (40.7 ± 16.0%) showed similar BA% compared to saline controls ($p = 0.663$). For RBG%, all experimental groups showed similar results ranging from 36.3 to 47.1%. Our data indicate that pre- or post-surgical systemic IV administration of ZA improves the regeneration of bone defects grafted with inorganic cancellous bovine-bone particles in osteoporotic bone conditions. However, no favorable effect on bone repair was seen for continued pre- plus post-surgical ZA treatment.

**Keywords:** osteoporosis; zoledronic acid; bone graft; bone regeneration; animal model



**Citation:** Shaheen, M.Y.; Basudan, A.M.; Niazy, A.A.; van den Beucken, J.J.J.P.; Jansen, J.A.; Alghamdi, H.S. Effect of Systemic Zoledronic Acid Dosing Regimens on Bone Regeneration in Osteoporotic Rats. *Appl. Sci.* **2021**, *11*, 1906. https://doi.org/10.3390/app11041906

Academic Editor: Susana Santos Braga

Received: 20 December 2020
Accepted: 18 February 2021
Published: 22 February 2021



## 1. Introduction

Skeletal conditions such as osteoporosis, which further accentuate bone resorption and healing, are a major challenge for bone regenerative processes. The physiological process of bone healing is more complex in situations associated with metabolic disorders compared to healthy condition [1,2]. Osteoporosis decreases both cortical and trabecular bone mineralization and is often diagnosed in postmenopausal women. It is characterized by decreased bone mass and strength, altered bone microstructure, and reduced regenerative capacity [3,4]. Medication is prescribed for osteoporosis treatment to improve bone health. The mostly prescribed drugs are bisphosphonates (BPs), which decrease bone turnover by inhibiting osteoclastic cells [5,6] through the disruption of the intracellular processes required for osteoclast function [7].

The chemical structure of BPs is characterized by two phosphate groups covalently linked to carbon [8]. Further, the phosphorus–carbon–phosphorus backbone contains two side chains (called R1 and R2), which determine the mode of action and strength of BPs.

Considering the R-groups, two classes of BPs can be discerned, i.e., non-nitrogenous (no nitrogen in R2) and nitrogenous (nitrogen in R2). Non-nitrogenous BPs affect cellular energy metabolism and initiate osteoclast apoptosis, resulting in a reduction of bone breakdown. Nitrogenous BPs affect osteoclastogenesis and cytoskeletal dynamics and inhibit the formation of the ruffled border. Non-nitrogenous-BPs are associated with increased negative effects compared with nitrogenous BPs. Zoledronic acid (ZA) is a nitrogenous BP, generally administered by intravenous (IV) infusion [9]. It is the most potent BP, demonstrating a very high affinity for hydroxyapatite crystals and the longest retention into bone mineral in comparison to other BPs [10]. It has been hypothesized that ZA might interfere in bone turnover, which can result in improvement of bone healing around bone substitutes through reduction of the hyperactivity of osteoclastic cells in osteoporosis. Recent animal studies have investigated the effects of IV and subcutaneously ZA administration in a healthy animal model [7,10–13], but there are no published data regarding the long-term effect of systemically administered ZA on bone healing in bone defects grafted with a bone substitute in an osteoporotic animal model.

Besides, BP drugs can cause a rare but serious condition called bisphosphonate-related osteonecrosis of the jaw (BRONJ) [14]. Despite the risk posed by BP administration of development of a jaw disease, studies have also shown that BPs can promote bone regeneration [6,15,16]. In dental clinics, bone regeneration is commonly carried out by several bone grafting procedures to restore alveolar bone loss [17]. Bone grafting involves using natural or synthetic bone substitutes to stimulate bone healing through three different mechanisms: osteogenesis, osteoconduction, and osteoinduction [18]. However, the regeneration of alveolar bone is still a major clinical challenge in dentistry.

Alveolar bone loss can result from trauma, pathology, chronic/acute dental infections, or severe periodontal disease. The most frequently reported cause of clinical discrepancies in edentulous residual alveolar ridges is the loss of mechanical function associated with the early loss of teeth and a lack of immediate replacement. This physiological bone loss after tooth extraction has been corroborated both in clinical and in experimental studies, which reported both vertical and horizontal bone resorption of the residual alveolar ridge [19–21].

Bone defect healing after dental extraction occurs through regeneration and reconstruction of lost bone tissue and is essentially similar to fracture healing [20]. However, new bone formation could be impaired or delayed due to several external factors such as decreased blood supply, surgical site infection, and deficiency of essential micronutrients such as calcium and phosphorous [22]. This may further be influenced by factors like the large size of the defect, unfavorable healing conditions, underlying metabolic diseases, overall nutrition, and even suboptimal surgical techniques [19,20,22]. An important factor determining the spontaneous healing ability is the size of the bone defect. Large bone defects incapable of spontaneous healing are considered as critical-size bone defects, and these defects require adjunctive measures to achieve healing [23].

Several therapeutic strategies involving bone grafts and substitute biomaterials have been used successfully for bone augmentation and bone defect regeneration in the fields of orthopedics, oral and maxillofacial surgery, and periodontology [24]. Depending upon their origin, bone grafts and substitutes may be classified as autografts, allografts, xenografts, or synthetic alloplasts [25,26]. In order to be a clinically utilizable bone substitute, a material needs to fulfill requirements such as biocompatibility, ability to provide mechanical support and volume for bone regeneration, and biodegradability [27,28]. Autografts (derived from the same individual) are considered the gold standard among substitute biomaterials for bone regeneration, because of their ability to promote osteoinduction, osteoconduction, and osteogenesis [24]. Nevertheless, their routine use is limited by inadequate availability in intraoral sites, requirement for a second surgical site for harvesting (e.g., iliac crest), and post-operative morbidity at the donor site [29,30]. Bone allografts obtained from banked donor bone is easily available and has similar properties to those of autograft bone [20]. However, when used as a bone substitute for regeneration, allografts need to be

deproteinized and are only capable of osteoconduction, in addition to carrying a significant risk of infectious disease transmission [26].

An alternative to allograft bone is xenograft bone, obtained routinely from animal sources. Xenograft bone is usually deproteinized by means of a chemical or low-heat process, which preserves the original bone micro-architecture and the inorganic bone mineral composition, but removes the organic component, thereby preventing the possibility of immunogenic reactions [24,28]. Anorganic bovine bone graft (ABBG) or deproteinized bovine bone mineral (DBBM) have shown good biocompatibility and osteoconductivity in preclinical studies when used in conjunction with the principles of guided bone regeneration (GBR) [31]. In addition to the afore-mentioned types of bone grafts, synthetic bone-graft substitutes or alloplasts such as calcium phosphate (CaP) ceramics, bioactive glass and polymers are also used for bone defect augmentation and regeneration [32].

In spite of the availability of several biomaterials, it is still a challenge to achieve successful bone defect regeneration in patients suffering from diseases that affect bone metabolism. For example, osteoporosis is one of the most frequently occurring bone disorders and is characterized by a progressive decrease in bone mineral density [33,34]. Bone regeneration in osteoporotic patients could be affected by inadequate mineralization, which is essential for strengthening the new bone. Both experimental and clinical data have reported that osteoporosis negatively influences the healing process of a bone defect, either in the presence or in the absence of a suitable bone substitute material [34–36].

Clinically, the mission of regenerative dentistry is restoring damaged alveolar bone in both healthy and medically compromised patients. The key step in alveolar bone regeneration is to stimulate a cascade of healing events that can promote bone quantity and quality [33]. One of the greatest challenges associated with alveolar bone augmentation is bone resorption, which is further potentiated by skeletal disorders such as osteoporosis. The proven effectiveness of anti-osteoporotic drugs in increasing the biomechanical properties of bone tissue suggests a possible role of these molecules in promoting and/or accelerating bone healing. The use of anti-osteoporotic drugs has been reported as an approach to favor early bone healing in bone defect regeneration in osteoporotic conditions [37]. A meta-analysis assessed the data available from preclinical studies to quantify literature evidence of anti-osteoporotic drug efficacy, in adjunction with bone grafting, in bone regeneration, in healthy and osteoporotic conditions. It showed an increase in overall histological bone area percentage and concluded that using anti-osteoporotic drugs in adjunction with bone grafting stimulated bone regeneration [38]. BPs are the preferred pharmacotherapy for osteoporosis treatment. ZA is a new-generation intravenous BP that counteracts osteoporosis on two fronts by decreasing bone resorption and encouraging bone anabolism. BPs reduce bone turnover, largely by decreasing the activation frequency and the recruitment of osteoclast precursors [39], but they also reduce the amount of bone removed during bone remodeling by increasing apoptosis [40].

The effect of BPs on bone regeneration in osteoporotic animal models has already been evaluated in multiple studies. Takahata et al. studied the impact of systemic alendronate treatment on autologous bone graft in a vehicle for spinal fixation in ovariectomized (OVX) rats [41]. They showed BPs to inhibit endochondral ossification and to reduce graft as well as newly formed bone resorption in osteoporotic rats compared to untreated animals due to the suppression of osteoclastic activity [41]. Using an osteoporotic sheep model, Verron et al. reported that local BP treatment of calcium phosphate-grafted vertebral bone defects counteracted symptoms of osteoporosis by promoting new bone formation and increasing bone area [15].

Accordingly, we hypothesized that an initial treatment of an osteoporotic condition before bone defect preparation and bone substitute installation could enhance bone regeneration compared to a treatment started after this procedure. Therefore, the aim of the present study was to evaluate bone regeneration in bone defects grafted with a bone substitute material in an osteoporotic rat model treated with intravenous ZA using three different treatment regimens (pre-bone grafting, post-bone grafting, and pre- and post-bone

grafting) compared to non-treated controls. The obtained data can be used as a prelude for clinical steps focused on the regeneration of maxillofacial bone defects.

## 2. Materials and Methods

### *2.1. Animals and Ovariectomized Rat Model*

This study followed ethical standards as defined by the Committee for Ethical Animal Use at King Saud University, College of Dentistry, Kingdom of Saudi Arabia (Approval # 4/67/389683). A total of 24 skeletally mature female Wistar rats (12 weeks of age and weighing approximately 250 g) were used in this study. The animals were housed in standardized rat cages (3–4 animals per cage) maintained in a laboratory environment with controlled temperature (22–24 °C) and humidity (45%–55%) and 12 h light and dark cycles with free access to a standard rat chow diet and water.

All animals were subjected to a bilateral ovariectomy procedure under general anesthesia (GA) to induce osteoporosis, which has been described and reported before [42]. As in Figure 1, the animals were divided and randomly distributed into four equal groups (n = 6 rats per group), according to the protocol for ZA administration, as following:

- ZA-Pre: weekly ZA administration (0.04 mg/kg body weight) in the 4 weeks prior to bone graft placement.
- ZA-Post: weekly ZA administration in the 6 weeks after bone graft placement until the end of study.
- ZA-Pre+Post: weekly ZA administration starting 4 weeks prior to bone graft placement and continuing until the end of the study.
- Saline: weekly intravenous injection of 1 mL of physiological saline solution starting 4 weeks prior to bone graft placement until the end of the study; this group was considered the non-treated control.

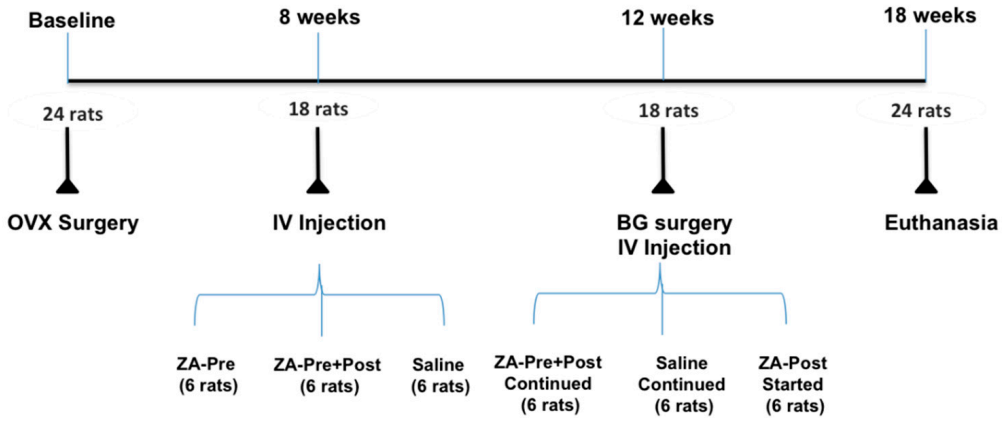


**Figure 1.** Experimental animal groups and timeline of intravenous injections, surgical procedures, and euthanasia. OVX; ovariectomized, IV, intravenous, BG, bone graft, ZA, zoledronic acid.

### *2.2. Surgical Procedure for Bone Graft Placement and Study Groups*

Twelve weeks after ovariectomy, all animals underwent surgery under general anesthesia for bone graft placement in the left femoral condyle, as described previously [43]. Once anesthetized, the left hind limb of the rats was shaved and disinfected with a Povidone–iodine 10% solution (Alphadine; Riyadh Pharma, Riyadh, Saudi Arabia). The knee capsule was incised longitudinally, and the patellar ligament was gently elevated laterally. At the femoral intercondylar notch, a bone defect (3 mm in diameter and depth) was prepared using a surgical bur in a slow-speed rotary drill (800 rpm) (Elcomed 100, W&H Dentalwerk Burmoos GmbH, Austria). The bone defect was grafted with particulate anorganic bovine cancellous bone graft granules (particle size: 0.25–1 mm (InterOss®, SigmaGraft Inc., Fullerton, CA, USA)). No membrane was used to cover the bovine-bone granules into the bone defect. Following the placement of the bone graft, the soft tissue lay-

ers and skin were closed with VICRYL™ (4–0) polyglactin 910 resorbable Sutures (Ethicon, Johnson & Johnson, New Brunswick, NJ, USA). After surgery, the animals were allowed to move unrestrictedly.

Zoledronic acid injection (0.04 mg/kg body weight) (Zometa 4 mg/5 mL, Novartis, Basel, Switzerland) was prepared by mixing 0.05 mL of the drug with 0.95 mL of normal saline (0.04 mg zoledronic acid) [44]. The prepared solution was injected IV into the tail vein, as per animal weight and according to the study protocol. This dose of zoledronic acid was selected to mimic the cumulative clinical dose administered to human patients treated for osteoporosis [44]. Animals in the control group received 1 mL of saline solution through tail vein injection.

### 2.3. Sample Retrieval and Specimen Preparation for Analysis

At the experimental endpoint (6 weeks after bone graft placement and 18 weeks since the beginning of the experiment), all rats were euthanized by $CO_2$ suffocation in accordance with ethical standards. The left femoral bones were harvested and dissected from the surrounding soft tissues. Subsequently, bone specimens were fixed in 10% neutral buffered formalin solution for 24 h and then transferred to 70% isopropanol for further histological preparation and histomorphometric analysis.

### 2.4. Specimen Preparation for Hard-Tissue Sectioning and Histomorphometry

The femoral condyle specimens grafted with bone substitute (n = 6 specimens per protocol) were embedded in freshly prepared poly(methyl methacrylate) (pMMA) resin. After polymerization, thin non-decalcified serial transversal sections (perpendicular to the long axis of the cylindrical bone defect) were cut at a thickness of ~10 μm using a sawing microtome (Leica SP-1600, Leica Microsystems Nussloch GmbH, Heidelberger, Germany), based on a technique described previously [45]. The non-decalcified sections were stained with methylene blue and basic fuchsin.

### 2.5. Histomorphometric Examination

Digital images of all stained sections were obtained and recorded using a light microscope. The Aperio ImageScope software was used to examine images taken by a Leica biosystem scanner (Aperio ImageScope, Leica Biosystems, Buffalo Grove, Illinois, USA), used for descriptive and quantitative histological analysis. For histomorphometric analysis, a 3 mm-diameter circular shape area (7.069 $mm^2$), representing the region of interest (ROI), was superimposed onto the images of the bone defect. Using ImageJ software (image processing and analysis in java freeware–Ver. 1.4; National Institutes of Health, Bethesda, Maryland, USA), the percentage areas of newly formed bone (BA%) and remaining bone graft (RBG%) were measured, as depicted in Figure S1. Always, three sections per tissue specimen were analyzed, and the mean values of BA% and RBG% of these three sections were calculated.

### 2.6. Statistical Analysis

Descriptive statistics including mean values and standard deviations were calculated for the quantitative variables BA% and RBG%. The SPSS Statistical Program (Version 26, IBM, USA) was used for statistical analysis of the data. One-way analysis of variance (ANOVA) with Dunnett post-hoc test was used for comparison of the variables in each treatment group, with the saline group as the control. The level of statistical significance was set at $p < 0.05$.

## 3. Results

### 3.1. Animal Model

Healing progressed uneventfully in all study animals, and no postoperative complications were observed during the experimental period and at the time of euthanasia.

At the end of the study, six specimens of each experimental group were retrieved for histological preparation. Unfortunately, one of the specimens of the ZA Pre+Post group was lost due to problems with the embedding in pMMA (Table 1).

**Table 1.** Number of samples used for histological evaluation and histomorphometric measurements.

| | Osteoporotic Condition | | |
|---|---|---|---|
| **Groups** | **Samples Numbers** | **Included in Analysis** | **No. Histological Sections** |
| ZA-Pre | 6 | 6 | 18 |
| ZA-Post | 6 | 6 | 18 |
| ZA-Pre+Post | 6 | 5# | 13 |
| Saline | 6 | 6 | 18 |

#one sample was excluded due to microscopic scan failure.

### *3.2. Descriptive Histological Evaluation of Bone Defects*

Saline group: the light-microscope images shown in Figure 2 confirmed the osteopenic character of the femoral condylar bone, which was characterized by the presence of sparse and discontinuous bone trabeculae, with bone marrow in between these trabeculae. The remaining bovine bone particles could easily be recognized. Bone ingrowth into the defect area was very limited and mainly restricted to the edge of the defect. At the defect boundary, bone had grown into the spaces between the particles, and these particles were surrounded for their major part by newly formed bone. Bone ingrowth did not proceed into the center of the defect, which was filled with fibrous tissue between the bovine bone particles. Osteoclast-like cells were observed in close contact with the bovine bone particles (Figure 3).

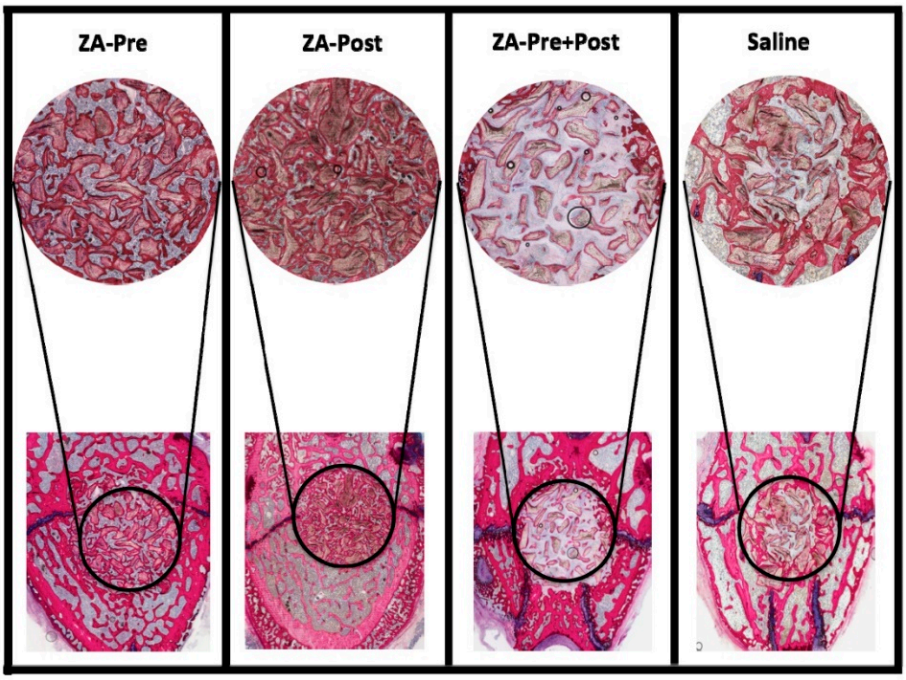


**Figure 2.** Representative histological sections of specimens from different study groups showing a transverse section through the femoral condyle, perpendicular to the long axis of the cylindrical bone defect. A circular region depicting the region of interest (ROI) is shown separately as an offset image for each group.

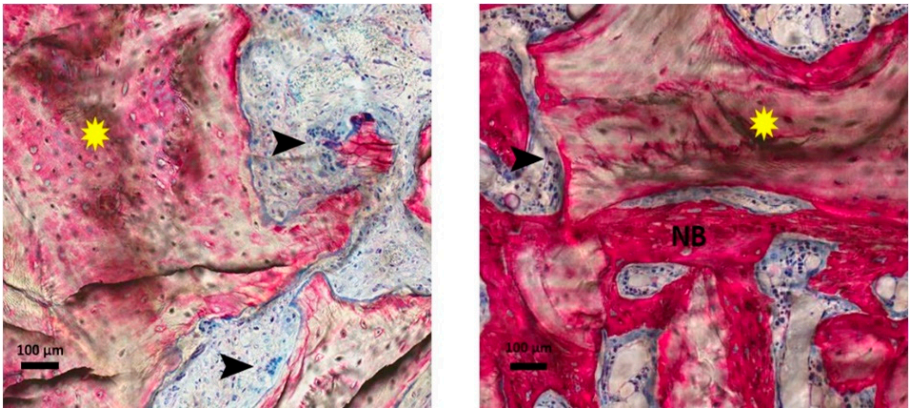


**Figure 3.** Representative histological images at high magnification showing bovine bone granules (yellow stars) well integrated into and completely covered by newly formed bone (NB) and osteoclast-like cells (black arrows). The left image represents the saline group, and the right image represents the ZA-post group.

ZA-Pre group and ZA-Post group: the histological sections in Figure 2 of ZA-Pre and ZA-Post specimens showed a very similar appearance. At low magnification, the femoral condylar bone was denser and more uniform compared to that of the saline control group (Figure 2). In addition, the size of the intra-trabecular space was reduced. Bovine bone particles could still easily be recognized (Figure 3). In contrast to the saline control group, bone had grown throughout the bone defect in these treated groups. The majority of the bovine bone particles was surrounded by a layer of bone, which was in tight contact with the surface of the bovine bone particles. Bone marrow-like tissue was present within the newly deposited bone. Only very few osteoclast-like cells were observed (Figure 3).

ZA-Pre+Post group: light-microscopy analysis of specimens of the ZA-Pre+Post group showed that the density of the femoral condylar bone was increased compared with specimens of the saline control group. On the other hand, histological examination revealed a wide variation in bone defect healing compared to the ZA-Pre and ZA-Post groups. In three specimens, bone formation into the defect space was similar to that observed in ZA-Pre and ZA-Post specimens. Bone had grown from the defect border between the bovine bone particles and was surrounding these particles. However, in two specimens, no bone ingrowth into the defect space had occurred. The bovine bone particles were still present but were surrounded by fibrous tissue.

### *3.3. Histomorphometric Analysis*

BA% and RBG% were determined for the prepared histological specimens. One sample of the ZA-Pre+Post group was not included into the measurements due to the loss of this specimen in the histological preparation procedure.

Data showed mean BA% of 50.1 ± 3.5% for ZA-Pre, 49.2 ± 8.2% for ZA-Post, 40.7 ± 16.0% for ZA-Pre+Post, and 35.4 ± 5.4% for saline control group. A repeated-measures analysis of variance with Dunnett's multiple comparison test indicated a significant difference in BA% between ZA-Pre and saline control ($p = 0.031$) and ZA-Post and saline control ($p = 0.043$). ZA-Pre+Post group and saline control group showed similar BA% ($p = 0.663$; Figure 4).

RBG% is considered e a measure for the osteoclastic activity and dimensional stability of a bone substitute material. RBG% values were 44.5 ± 8.8% for ZA-Pre, 47.1 ± 10.4% for ZA-Post, 36.3 ± 7.6% for ZA-Pre+Post, and 42.3 ± 7.5% for saline control group. No significant differences in RBG% existed between the various experimental groups ($p > 0.5$; Figure 5).

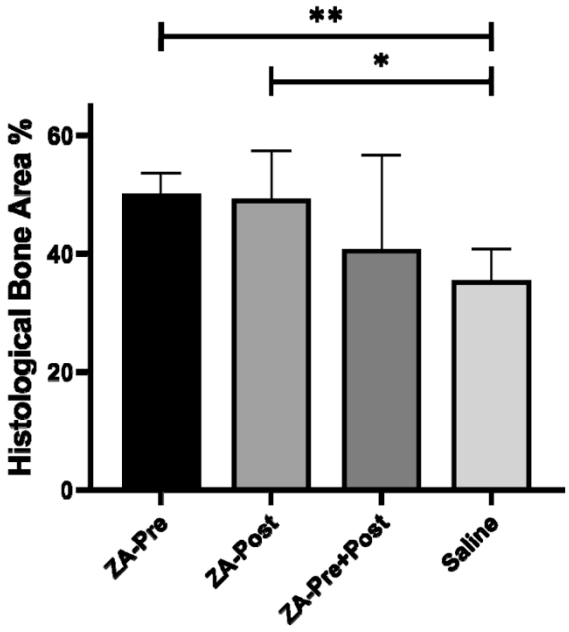


**Figure 4.** Bar graph with standard deviation indicating the mean quantitative histomorphometric values of bone area percentage (BA%) for the different study groups. Statistically significant difference between the mean values: ** indicates $p = 0.031$, * indicates $p = 0.043$.

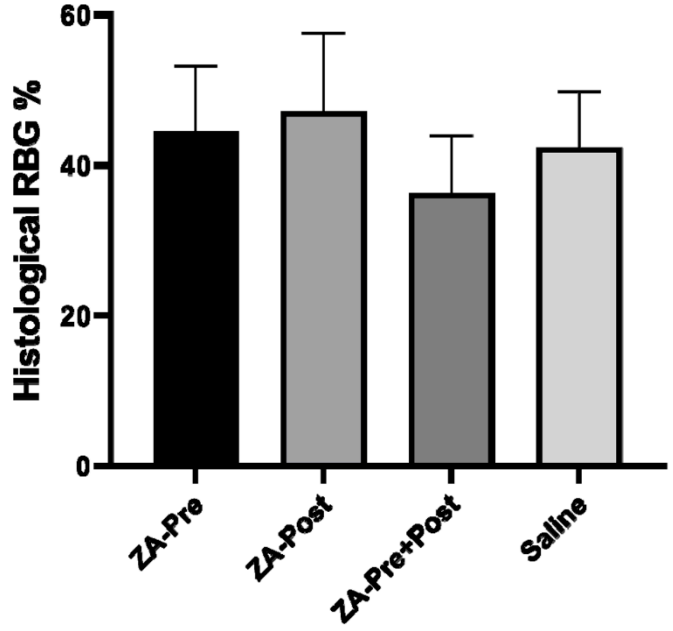


**Figure 5.** Bar graph with standard deviation indicating the mean quantitative histomorphometric values of remaining bone graft percentage (RBG%) for the different study groups.

## 4. Discussion

The present study aimed at evaluating bone regeneration in bone defects grafted with a bone substitute material in an ovariectomized rat model treated with intravenous ZA via three different dosing regimens (pre-bone grafting, post-bone grafting, and pre+post-bone grafting). Ovariectomized rats receiving weekly saline injections served as the control group. The study was conducted over a period of 18 weeks, from ovariectomy with beginning of ZA injection until bone graft placement and finally euthanasia. The study time included a six-week healing period for the grafted bone defects. The harvested bone specimens were prepared for descriptive and quantitative histological examination. The

data showed that ZA-Pre and ZA-Post treatments resulted in a significant gain in BA% compared to saline control treatment. No evident effect of the ZA-Pre+Post treatment was found on BA%. Additionally, RBG% showed similar values for all experimental groups.

Experiments performed with animal species such as rabbits, cows, dogs, mice, and rats are often used to study bone regeneration with the aid of bone substitutes. For mimicking osteoporotic bone conditions, predominantly rats are used, in which osteoporosis is induced via gonadectomy (i.e., orchidectomy for male and ovariectomy for female animals). Physiologically, gonadectomized rats possess an increased rate of bone turnover when compared to humans with osteoporosis [46,47]. Nevertheless, they are excellent preclinical models for studying osteoporotic changes, as they closely emulate pharmacotherapeutic responses and allow studying the effect of estrogen depletion on the skeleton [42]. However, it should be highlighted that sometimes, there is a disconnection between animal research and clinical research [48]. The relevance of animal models to human health is questioned because of differences between the species. Many studies in animal models are of poor methodological quality. Lack of concordance between animal experiments and clinical trials may be due to bias, random error, or the failure of animal models to adequately represent human diseases. These drawbacks should be kept in mind before clinical translation and while constructing animal model to test a hypothesis. Systematic reviews of animal experiments could promote closer collaboration between research communities and support an iterative approach to improve the relevance of animal models to clinical trial design. When models do not represent the clinical context, they could be adjusted accordingly [49]. For the current study, our published animal systematic review and meta-analysis about anti-osteoporotic drugs in adjunction with bone grafting helped construct the experiment design [38].

Currently, ZA is considered the most potent intravenous BP available for the treatment of osteoporotic patients and is approved for use by the U.S. Food and Drug Administration (USFDA) [50]. Guidelines for the required dose and administration route of ZA have been devised on the basis of data from pre-clinical models and clinical practice [37]. Consequently, the current protocol for the administration of ZA to osteoporotic patients follows a once-yearly dosing regimen (5 mg IV) for the maintenance of therapeutic drug levels and to facilitate patient compliance [51]. The duration of this therapy is limited to no more than three years. ZA is also administered in a 4 mg dose to patients suffering from hypercalcemia and with advanced malignancies involving bone. ZA treatment by infusion for these patients is every 3–4 weeks. All patients receiving ZA infusion are advised to drink two glasses of water before and after IV infusion to ensure adequate hydration when receiving ZA in order to prevent adverse effects. The rats in the current study were administered 0.04 mg/kg body weight ZA IV every week for a duration of, respectively, 4 (ZA-Pre), 6 (ZA-Post), and 10 weeks (ZA-Pre+Post). No specific measures were taken for the hydration of the rats of before and after infusion. The posology and method of administration for the ZA-Pre and ZA-Post groups was based on experience from previous studies [37,52]. However, the dosage for the ZA-Pre+Post group was obtained by combining the doses of the ZA-Pre and ZA-Post groups. It is known that the serum concentrations of ZA after IV infusion decrease rapidly due to the high affinity of ZA for bone mineral [53]. Additionally, the effect of ZA on osteoclasts is reported to be determined by ZA dosage and administration frequency [53,54]. This was confirmed by a clinical study involving patients with Paget's disease, which reported a reduced therapeutic effect with ZA doses above 200 μg [55]. The serum alkaline phosphatase (SAP) level, which is an indicator for the therapeutic efficacy of ZA treatment, was significantly higher in patients treated with a high dose of ZA (>200 μg) compared with patients treated with a lower dose of ZA (<200 μg). The histomorphometric analysis of our bone specimens revealed no significant difference in BA% between the ZA-Pre+Post and the saline control treatment groups. Consequently, we suppose that the lack of effect of ZA infusion on BA% with this dosing regimen was due to an issue with the total administered dose. As previous work has shown that high doses of ZA (i.e., 66 mg/kg body weight; 3 times weekly intraperitoneally during the 3 weeks

prior to surgery) drastically decrease implant osseointegration [56], it seems likely that already the ZA-Pre+Post dosing regimen in the current study was too high. Therefore, we recommend that in future studies with ZA (as well as other BPs), the SAP level in the experimental animals is determined to assess the efficacy of the treatment.

The beneficial effect of BPs, including ZA, on bone regeneration in an osteoporotic condition corroborates the existing literature [57]. The effect of these agents is known to be independent of the route of administration (i.e., systemically or locally) [58]. Additionally, several animal studies showed that BP therapy can also enhance bone volume and density in healthy animals. In these studies, bone defects in healthy animals were grafted with bone substitutes, followed by the administration of ZA either locally or systemically [10,11]. Multiple studies reported that ZA treatment of a bone graft, locally and systemically, increased osteogenesis of the graft material and enabled bone formation [59]. Local ZA administration was found to be a highly efficient method of concentrating BPs at the site of bone graft placement and supported early bone formation as well as facilitated the biomechanical fixation of the bone substitute to the native bone [11,60]. Further, Harding et al. used their rat bone chamber model to investigate the beneficial effects of systemic ZA administration [61]. They showed that a single subcutaneous injection of 0.1 mg/kg ZA in rats significantly increased the volume of cancellous bone grafts installed in the bone chamber and protected the bone grafts against resorption [61]. Also, experiments demonstrated that just the soaking of an allograft in a 4 µg/kg ZA solution increased bone volume and bone mineral density significantly over a 6-week healing period compared with a sodium chloride-soaked allograft [11,60]. Our data from the ZA-Pre and ZA-Post groups are in agreement with all these previous studies. This observation may be explained by the capacity of ZA to promote bone anabolism and prevent osteoclastic activity. In addition, BPs can increase the proliferation of osteoblasts and the synthesis of collagen and osteocalcin by bone cells at the cellular level [62]. Myoung et al. studied the effect of a BP on the expression of specific bone formation genes after autogenous free bone grafting in rats. They found that the BP reduced osteoclastic activity and induced osteoblasts to secrete an inhibitor of osteoclast-mediated resorption [63]. Also, in a different animal study, BP was given systemically for 8 weeks, and the authors found that alendronate stimulated bone formation in autogenous bone grafts [64]. The histomorphometric analysis of the remaining bone graft material showed that between the various treatment groups and the saline control group, statistically significant difference was not present. This observation is not in line with the supposed mechanism of action of ZA, which acts as an inhibitor for osteoclastogenesis. A reduced number of osteoclasts will result in a decreased degradation rate of the applied bone substitute [65]. Histology indeed confirmed the scarce presence of osteoclasts in the bone specimens of the ZA-Pre and ZA-Post groups. On the other hand, light-microscopic examination of the saline control group samples revealed the presence of osteoclasts, but the resorption of the bovine bone particles was not pronounced after 6 weeks of implantation. In view of the limited biodegradation capacity of bovine bone particles as reported in several publications [66,67], the current data confirm that inorganic bovine bone substitute is very stable and not easily replaced by newly formed bone [68].

Although the results of the current animal study demonstrate the efficacy of ZA administration as a therapeutic strategy for bone regeneration in artificially created bone defects in an osteoporotic condition, it has to be noticed that ovariectomized rats have a faster bone turnover than patients with osteoporosis. This limitation must be considered prior to the clinical translation of the present study results. Therefore, further in vivo studies are necessary to clinically validate the effect of long-term ZA administration on bone substitutes healing.

## 5. Conclusions

In conclusion, our data show that intravenous administration of ZA pre- as well as post-surgery improved and positively influences bone regeneration of bone defects grafted

with bone substitute in osteoporotic bone conditions. On the other hand, no favorable effect on the bone repair process was found for continued (pre- plus post-surgical) ZA treatment.

Further, we observed that anti-osteoporotic drugs, i.e., BPs, can favorably support bone regeneration, depending on the timing of administration.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/2076-3417/11/4/1906/s1, Figure S1: Steps in histomorphometric analysis of non-decalcified sections using ImageJ software.

**Author Contributions:** Conceptualization, A.M.B., M.Y.S., A.A.N., J.J.J.P.v.d.B., J.A.J., and H.S.A.; data curation, M.Y.S.; formal analysis, A.M.B.; funding acquisition, H.S.A.; investigation, M.Y.S., and A.M.B.; methodology, M.Y.S., A.M.B., A.A.N., J.J.J.P.v.d.B., and H.S.A.; project administration, A.A.N.; resources, A.A.N. and J.J.J.P.v.d.B.; validation, J.J.J.P.v.d.B. and J.A.J.; visualization, J.A.J.; writing—original draft, M.Y.S.; writing—review and editing, J.J.J.P.v.d.B., J.A.J., and H.S.A. All authors have read and agreed to the published version of the manuscript.

**Funding:** The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through research group No. (RG-1439-026).

**Acknowledgments:** The authors are grateful to the technical support of Vincent Cuijpers, Martijn Martens, Natasja van Dijk in histological preparation and sectioning. The authors are also grateful to Mary Grace Baysa Vigilla and Rhodanne Nicole Lambarte for their support during animal experimental work. Finally, the authors would like to thank Terrence Sumague for his assistance during the histomorphometric measurements.

**Conflicts of Interest:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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