EUROPEAN JOURNAL OF EMERGING MEDICINE AND PUBLIC HEALTH

ENHANCING MAXILLOFACIAL BONE REGENERATION: AN IN VIVO CANINE MODEL ASSESSMENT OF NANOSTRUCTURED IRON-MANGANESE ALLOYS DOPED WITH COPPER, TUNGSTEN, AND COBALT

Author Details:

Dr. Nelira D. Vossan
Department of Biomedical Engineering, University of Malaya, Kuala Lumpur, Malaysia

Dr. Terun A. Clevoy
Faculty of Dentistry, Cairo University, Egypt

Dr. Vimlesh K. Dornet
Centre for Biomaterials and Tissue Engineering, Indian Institute of Technology (IIT) Kanpur, India

V0LUME01 ISSUE01 (2024)

Published Date: 08 December 2024 // Page no.: - 06-24

ABSTRACT

The development of biodegradable metallic alloys for medical applications, particularly in maxillofacial and orthopedic surgery, represents a significant leap forward in implantology. These materials offer the promise of providing robust mechanical support during tissue healing while gradually degrading, thereby obviating the need for secondary removal surgeries {2, 45}. This investigation delves into the osteogenic capabilities of novel nanostructured iron-manganese (Fe-Mn) based alloys individually doped with copper (Cu), tungsten (W), and cobalt (Co). The study was conducted using a rigorous in vivo canine model to closely mimic human physiological conditions. Four distinct alloy compositions were fabricated using advanced mechanical alloying and selective laser melting techniques: a baseline FeMn35 alloy (M0), and three variants, FeMn32Cu3 (M1), FeMn32W3 (M2), and FeMn32Co3 (M3). These alloys were implanted into surgically created critical-sized mandibular defects in ten mongrel dogs, with an empty defect group serving as a control (M). After a 12-week healing period, a comprehensive multi-modal analysis was performed, including cone-beam computed tomography (CBCT), scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDX), detailed histological and histomorphometric evaluations, and quantitative real-time polymerase chain reaction (qRT-PCR) for key osteogenic gene markers (osteopontin and osteocalcin). The results demonstrated that all alloy-implanted defects exhibited enhanced bone formation compared to the control group, which primarily formed immature woven bone. Quantitative analysis revealed a clear hierarchy of osteogenic potential among the alloys. The cobalt-doped alloy (M3) showed a statistically significant superior performance across all metrics, including the highest percentage of new bone area, the greatest degree of bone maturation, and the most pronounced upregulation of osteopontin and osteocalcin expression. The tungsten-doped alloy (M2) and copper-doped alloy (M1) also showed significant improvements over the baseline Fe-Mn alloy and the control group. SEM-EDX analysis post-implantation confirmed the onset of biodegradation and the deposition of calcium and phosphorus on the alloy surfaces, indicating active biomineralization. The collective findings strongly suggest that the incorporation of cobalt, tungsten, and copper into a nanostructured Fe-Mn matrix significantly enhances its biocompatibility and osteogenic potential {18}. These results underscore the promise of these novel biodegradable alloys for clinical use in maxillofacial reconstruction and other load-bearing orthopedic applications, paving the way for improved patient outcomes and reduced healthcare burdens {19}.

Keywords: Biodegradable Metals, Iron-Manganese Alloys, Maxillofacial Surgery, Osteogenesis, In Vivo Canine Model, Cobalt, Copper, Tungsten.

INTRODUCTION

Broad Background and Historical Context

The field of biomedical implantology has undergone a profound evolution, driven by the persistent need to repair and regenerate tissues damaged by trauma, disease, or congenital defects {1}. For centuries, the primary challenge in skeletal reconstruction, particularly within the complex anatomy of the maxillofacial region, has been to find materials that can provide stable mechanical support while being harmoniously integrated by the host's biological systems. The earliest forays into surgical implants were fraught with complications, primarily related to infection, material rejection, and mechanical failure. The 20th century heralded the era of modern biomaterials, with the widespread adoption of biocompatible but non-resorbable metals such as stainless steel and titanium alloys {3}. These materials became the gold standard in orthopedics and

dentistry due to their excellent mechanical strength, corrosion resistance, and proven track record of biocompatibility {3}. Their use in fracture fixation plates, screws, dental implants, and joint prostheses revolutionized patient care, enabling functional recovery on an unprecedented scale.

However, the very permanence of these traditional metallic implants gives rise to a significant clinical drawback: the frequent necessity of a second surgical procedure for their removal once the tissue has healed {2, 44}. This additional surgery is not a trivial matter; it exposes the patient to renewed risks of anesthesia, infection, and surgical trauma, while also imposing a considerable economic burden on healthcare systems {2}. Furthermore, long-term implantation of these rigid materials can lead to stress-shielding, a phenomenon where the implant carries an excessive portion of the physiological load, leading to reduced bone density and potential weakening of the surrounding bone over time. In pediatric patients, permanent implants pose an even greater challenge, as they can interfere with the natural growth and development of the skeletal system.

In response to these limitations, the last few decades have seen a paradigm shift in biomaterial science, focusing on the development of biodegradable materials {45, 47}. The concept is elegantly simple: an ideal implant should provide the necessary mechanical support for tissue regeneration and then gradually and safely degrade and be absorbed by the body, leaving behind fully healed, native tissue {45}. This approach completely eliminates the need for removal surgeries, aligning perfectly with the natural healing process {2, 5}. Initial research in this domain focused on biodegradable polymers and ceramics. While successful in certain non-load-bearing applications, these materials often lack the requisite mechanical strength and toughness for demanding orthopedic and maxillofacial procedures. This mechanical mismatch led researchers to turn their attention back to metals, this time with a focus on biodegradability. The exploration of biodegradable metals (BMs) began with magnesium (Mg) and its alloys, which offered excellent biocompatibility and promising resorption profiles {7}. However, the extremely rapid and often uncontrolled degradation of Mg alloys, coupled with the production of hydrogen gas, presented significant hurdles to their clinical translation {7}. This shifted the focus towards iron (Fe) and its alloys, which offer a more controlled and slower degradation rate, coupled with superior mechanical properties that closely resemble those of stainless steel {7, 28}.

1.2. Critical Literature Review

The journey to optimize iron-based biodegradable alloys is a story of meticulous material engineering. Pure iron, while mechanically robust, was found to degrade too slowly in the physiological environment, potentially leading to its encapsulation by fibrous tissue rather than its replacement by bone {5, 28}. This slow degradation rate prompted researchers to explore alloying as a strategy to modulate the material's electrochemical properties and biological response. One of the most promising strategies to emerge was the development of iron-manganese (Fe-Mn) binary alloys {4}. The addition of manganese (typically in the range of 20-35% by weight) was shown to significantly enhance the corrosion rate compared to pure iron, bringing it closer to the ideal timeline for bone healing {4, 27}. Manganese is not merely a passive element in this process; it is a vital trace element in human biology, playing a crucial role as a cofactor in enzymes essential for bone matrix synthesis and development {31}. Studies have shown that manganese ions can activate integrin signaling pathways, which are critical for osteoblast attachment, proliferation, and survival {32, 33}. Furthermore, biomaterials containing manganese have been linked to increased expression of osteogenic genes and enhanced collagen deposition, directly contributing to the quality of newly formed bone {34, 35}. Some manganese-containing bioceramics have even demonstrated the ability to scavenge harmful reactive oxygen species (ROS) and inhibit the activity of osteoclasts (cells that resorb bone), thereby shifting the local environment to favor bone formation over resorption {36}.

Building upon the promising Fe-Mn base, the next logical step was the introduction of tertiary alloying elements to further enhance the material's bioactivity and osteogenic potential {44}. The selection of these elements is a highly strategic process, guided by their known biological functions and potential synergistic effects.

Copper (Cu): Copper has long been recognized for its potent antimicrobial properties, a highly desirable trait for any surgical implant to prevent post-operative infections {9}. Beyond this, copper plays a direct and active role in bone metabolism. It has been shown to stimulate the proliferation of osteoblasts, the primary bone-forming cells, and to enhance the overall process of osteogenesis, leading to faster healing of bone defects {29, 30}. The molecular mechanisms behind these effects are gradually being elucidated. For instance, copper ions have been shown to modulate the mammalian target of rapamycin (mTOR) signaling pathway, a key regulator of cell growth, by suppressing the phosphorylation of AMP-activated protein kinase (AMPK) {37}. This action effectively promotes osteoblast proliferation. Moreover, the presence of copper in biomaterials like bioactive glass can positively influence the expression of a suite of genes critical for bone formation, including Runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), collagen type I, osteocalcin, and osteopontin {38, 39, 40}. Copper also appears to modulate the local inflammatory environment in a way that is conducive to bone healing {41}. It can steer macrophages towards a pro-inflammatory M1 phenotype, a process

which, when properly regulated, can initiate the cascade of healing signals required for robust osteogenesis {42, 43}.

Tungsten (W): Tungsten is a unique candidate for alloying. It is known to accumulate in bone, and its incorporation into biodegradable iron alloys was initially proposed due to its biocompatibility and its ability to slow down the degradation rate, offering another lever to control the implant's lifespan {7, 44}. However, its direct role in bone homeostasis is complex and somewhat controversial {12}. Some *in vitro* studies have suggested that tungsten may have an inhibitory effect on the differentiation of mesenchymal stem cells (MSCs) into osteoblasts, while simultaneously promoting their differentiation into adipocytes (fat cells) {13}. This would suggest a potentially negative impact on bone formation. However, the translation of these *in vitro* findings to a complex *in vivo* environment is not straightforward. The same study that reported these inhibitory effects *in vitro* found no clear evidence that tungsten impeded osteogenesis in their *in vivo* model {13}. It has been suggested that tungsten may integrate into the inorganic hydroxyapatite component of the bone matrix as phosphotungstate, which could explain its slow release and persistence in bone tissue {10}. The ultimate biological effect of tungsten likely depends on its form, concentration, and the specific cellular context, making *in vivo* investigation essential to clarify its true potential as an alloying element for bone regeneration {11, 12}.

Cobalt (Co): Cobalt is another essential trace element, most famously known as a component of vitamin B12 {14}. In the context of bone regeneration, cobalt has garnered significant interest for its ability to mimic a hypoxic (low oxygen) state within cells {15}. It achieves this by stabilizing Hypoxia-Inducible Factor 1-alpha (HIF-1α), a transcription factor that is normally degraded in the presence of oxygen {25}. The stabilization of HIF-1α triggers a cascade of downstream signaling that is profoundly pro-regenerative. Most notably, it leads to the upregulation of Vascular Endothelial Growth Factor (VEGF), a potent signaling molecule that stimulates angiogenesis—the formation of new blood vessels {15, 45}. A robust vascular supply is an absolute prerequisite for bone healing, as it delivers oxygen, nutrients, and progenitor cells to the injury site {16}. By promoting angiogenesis, cobalt directly supports the metabolic demands of new bone formation {25}. Consequently, biomaterials incorporating cobalt, such as bioactive glasses and hydroxyapatite scaffolds, have been shown to significantly enhance osteoblast proliferation, upregulate the expression of bone-related genes, and promote the formation of vascularized bone in both ectopic and orthotopic sites {14, 15, 16}. However, the use of cobalt is not without its challenges. At high concentrations, cobalt ions can be cytotoxic, making it critical to control their release rate from the implant to maintain a safe and therapeutic window {46, 47}.

1.3. The Identified Research Gap

While the individual biological effects of manganese, copper, tungsten, and cobalt have been explored in various biomaterial contexts, a significant knowledge gap exists. To the authors' knowledge, there has been no comprehensive *in vivo* investigation into the combined effects of incorporating Cu, W, and Co as individual tertiary elements into a nanostructured Fe-Mn alloy platform specifically designed for the demanding environment of maxillofacial reconstruction. The "nanostructured" aspect is also critical, as creating materials with grain sizes in the nanometer range can drastically alter their mechanical properties and degradation behavior, often for the better {1}. The synergistic or antagonistic interactions between the base Fe-Mn alloy and these bioactive ions in a living organism remain largely uncharacterized. Preliminary *in vitro* work has shown that these novel alloys are biocompatible with oral epithelial cells and even exhibit anticancer activity against osteosarcoma cell lines, but such studies cannot replicate the complex interplay of biological systems involved in bone healing {21}. Therefore, a well-controlled, preclinical animal study is imperative to validate their performance {22}.

1.4. Study Rationale, Objectives, and Hypotheses

The rationale for this study is to bridge the aforementioned research gap by systematically evaluating the osteogenic potential of these three novel nanostructured alloys (FeMn32Cu3, FeMn32W3, and FeMn32Co3) against a baseline Fe-Mn alloy and an empty control defect in a clinically relevant large animal model. The dog model was specifically chosen because its bone architecture, density, and metabolic turnover rate are remarkably similar to those of humans, making it an ideal preclinical platform for assessing bone regeneration technologies {23, 24}.

The primary **objective** of this investigation was to conduct a multi-modal, quantitative assessment of the biocompatibility, biodegradability, and osteogenic capacity of these alloys when implanted in critical-sized mandibular defects in dogs.

The specific **aims** were to:

1. Compare the quantity and quality of new bone formation induced by each of the four alloy types and the control group using radiographic and histological methods.
2. Analyze the surface of the alloys after implantation to confirm the onset of degradation and biomineralization.
3. Quantify the expression of key osteogenic gene markers (osteopontin and osteocalcin) to assess the level of cellular activity and bone matrix maturation.

Based on the existing literature, we formulated the following **hypotheses**:

1. All Fe-Mn based alloy implants (M0, M1, M2, M3) will induce significantly more bone regeneration than the empty control defects (M).
2. The alloys containing bioactive dopants (M1, M2, M3) will demonstrate superior osteogenic potential compared to the baseline Fe-Mn alloy (M0).
3. Among the doped alloys, the cobalt-containing alloy (M3) will exhibit the most robust bone formation, owing to its dual pro-angiogenic and osteogenic properties.

METHODS

2.1. Research Design

This study was executed as a randomized controlled experimental trial, a design chosen for its strength in minimizing bias and establishing causal relationships between the implanted materials and the observed biological outcomes. The *in vivo* phase of the study was conducted on ten skeletally mature male mongrel dogs. The choice of a large animal model was deliberate and critical; the dog mandible provides a surgical site of sufficient size to create "critical-sized defects." A critical-sized defect is defined as a bone void of a size that will not heal spontaneously on its own over the animal's lifetime. This is an essential feature of the study design, as it ensures that any bone regeneration observed within the defect can be directly attributed to the osteogenic properties of the implanted material rather than the animal's innate healing capacity {23, 24}.

Five distinct experimental groups were established. A control group (Group M) consisted of empty critical-sized defects that were created and then covered by the preserved bone disc, serving as a baseline for natural (and expectedly incomplete) healing. Four implant groups were created to test the different alloy compositions: Group M0, implanted with the baseline biodegradable FeMn35 alloy; Group M1, implanted with the copper-doped FeMn32Cu3 alloy; Group M2, implanted with the tungsten-doped FeMn32W3 alloy; and Group M3, implanted with the cobalt-doped FeMn32Co3 alloy.

To ensure an unbiased distribution of treatments, five cylindrical mandibular defects were surgically created in each of the ten dogs, and the allocation of each defect to one of the five groups was performed randomly. This randomization helps to control for any potential confounding variables related to individual animal physiology or anatomical location of the defect. The study duration was set at 12 weeks post-implantation. This timeframe was selected to be sufficiently long to allow for the advanced stages of bone healing and remodeling to occur, yet short enough to be a practical duration for a preclinical study. At the conclusion of the 12-week period, the animals were humanely euthanized, and the mandibular tissues were harvested for a comprehensive panel of analyses. All procedures involving animals were conducted in strict accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines and received full ethical approval from the Institutional Animal Care and Use Ethical Committee of the Faculty of Veterinary Medicine, Cairo University (Approval # Vet CU 01122022549).

2.2. Study Animals

The study utilized ten male mongrel dogs, selected for their skeletal maturity to ensure that active bone growth would not be a confounding factor in the results. The animals had a mean weight of 23.7±1.9 kg and a mean age of 19.9±2.7 months. The selection of male dogs helps to eliminate potential hormonal variations associated with the female estrous cycle that could influence bone metabolism. Prior to inclusion in the study, each dog underwent a thorough clinical and hematological examination to confirm its health status and rule out any pre-existing systemic or bone-related diseases.

Upon arrival at the research facility, the dogs were subjected to a two-week quarantine period for acclimatization and observation. During this time, they received standard prophylactic anti-parasitic medication. Throughout the entire study period, the animals were housed individually in separate, clean cages to prevent cross-contamination and allow for individual monitoring. They were provided with a standard canine diet, fed twice daily, and had free access to drinking water at all times. This standardized care regimen was crucial for maintaining the overall health and well-being of the animals and ensuring consistency across the study cohort.

2.3. Materials and Apparatus

2.3.1. Alloy Fabrication

The biodegradable alloys at the heart of this study were synthesized using a multi-step, high-technology manufacturing process {17, 18}. The raw materials consisted of high-purity (99.99%) elemental powders of iron (Fe), manganese (Mn), copper (Cu), tungsten (W), and cobalt (Co).

The synthesis process began with **mechanical alloying**. The mixed elemental powders were placed in a Pulverisette 5/2 planetary ball mill. This high-energy milling process involves the repeated fracturing and cold-welding of powder particles, which is essential for creating a homogenous alloy with a refined, nanostructured grain size. The milling was conducted for 5 hours at a rotational speed of 300 rpm, with a ball-to-powder mass ratio of 10:1 to ensure efficient energy transfer. Toluene was used as a

process control agent during milling to prevent excessive heat generation and agglomeration of the powders {17}.

Following mechanical alloying, the resulting nanostructured alloy powders were subjected to a **stress relief heat treatment**. This step was performed under a vacuum in a tube furnace at 150°C for 1 hour. The purpose of this heat treatment is to relieve the internal stresses induced during the high-energy milling process, which improves the stability and handling of the powders {17}.

The final step was **consolidation**. The heat-treated powders were consolidated into dense, solid samples using a state-of-the-art selective laser melting (SLM) process {18}. SLM is an additive manufacturing (3D printing) technique where a high-power laser selectively fuses thin layers of the alloy powder, building the final part layer by layer. This allows for the creation of complex geometries with high precision. For this study, solid cylindrical samples with a 15 mm diameter were fabricated. These cylinders were then sectioned into discs of 1 mm thickness to create the final implants for the *in vivo* study. The detailed specifics of the alloy fabrication, microstructural characterization, and mechanical properties were published in separate, dedicated studies {17, 18}.

2.3.2. Analytical Equipment

A suite of advanced analytical instruments was employed for the comprehensive evaluation of the samples.

- **Microstructural and Elemental Analysis:** An Apreo field emission gun high-resolution scanning electron microscope (Apreo FEG-HR-SEM) was used to examine the microstructure and elemental composition of the alloys both before and after implantation. This instrument was equipped with backscattered electron (BSE) and energy-dispersive X-ray (EDX) detectors, allowing for both imaging and quantitative elemental analysis. The EDX system was operated using TEAM software for data acquisition and spectral analysis.
- **Radiographic Imaging:** A Planmeca CBCT machine (Helsinki, Finland) was used for three-dimensional radiographic evaluation of the mandibular defects. The imaging parameters were standardized at 10 mAs, 86 kVp, and an 11.2-second exposure time, with a voxel size of 150 µm. The machine's integrated Romexis software (version 4.4.1.R) was used for image reconstruction and for the quantitative measurement of bone density in Hounsfield Units (HU).
- **Histological Processing:** Standard laboratory equipment was used for histological sample processing, including microtomes for sectioning the paraffin-embedded tissue blocks. The staining was performed using Hematoxylin and Eosin (H&E) and Masson's Trichrome (MT) stain kits (ab245880 and ab150686, respectively; Abcam). A Leica DM300 light microscope equipped with a digital camera was used for microscopic examination.
- **Gene Expression Analysis:** Quantitative real-time PCR (qRT-PCR) was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Total RNA was extracted using a Direct-zol RNA Miniprep Plus kit (Zymo Research Corp., USA). RNA quantity and purity were assessed using a Beckman dual spectrophotometer. The reverse transcription and PCR amplification were conducted using a One-Step RT-PCR reagent kit (Thermo Fisher Scientific).

2.4. Experimental Procedure / Data Collection Protocol

2.4.1. Anesthetic and Surgical Protocol

On the day of surgery, a standardized anesthetic protocol was administered to ensure the safety and comfort of the animals. Dogs were first tranquilized with an intramuscular injection of xylazine HCl (1 mg/kg). Atropine sulfate (0.1%) was given subcutaneously 15 minutes prior to anesthesia to prevent bradycardia and reduce salivary secretions. Anesthesia was induced with intravenous ketamine HCl (10 mg/kg) and maintained with intravenous thiopental sodium (25 mg/kg) as needed throughout the procedure.

With the dog fully anesthetized, the skin over the right and left mandibles was shaved and prepared for aseptic surgery. An extra-oral approach was used, involving a 6-cm skin incision made approximately 1 cm below the inferior border of the mandible to provide access to the mandibular body. The underlying deep fascia and periosteum were carefully incised and elevated to expose the bone surface.

A specialized 15 mm diameter trephine drill was used to create five cylindrical, critical-sized defects (15 mm diameter x 4 mm depth) in the mandible of each dog. Throughout the drilling process, continuous sterile saline irrigation was provided to prevent thermal necrosis of the surrounding bone tissue. Extreme care was taken to avoid damaging the inferior alveolar canal, which houses the nerve and blood vessels supplying the lower teeth. The bone disc removed by the trephine bur was carefully preserved for later use. The biodegradable metal alloy discs were then inserted into the defects according to the randomized allocation plan. Finally, the preserved bone disc was press-fitted back into place over the implant, creating a biological cover. The surgical site was then closed in layers, with the subcutaneous tissue and skin being sutured using 2-0 Vicryl.

2.4.2. Post-operative Care and Euthanasia

Following the surgery, a rigorous post-operative care regimen was implemented. The surgical wounds were dressed daily for 10 days with 2% povidone-iodine to prevent infection. A systemic antibiotic (Ceftriaxone, 1g/dog) was administered intramuscularly for 7 days as a prophylactic measure. The animals' general health,

including their behavior, appetite, body weight, and masticatory function, was monitored daily. The surgical sites were closely evaluated for any signs of adverse reactions, such as edema, hyperemia, swelling, or allergic responses. Skin sutures were removed 10 days after the surgery.

At the 12-week endpoint, the dogs were humanely euthanized using an overdose of sodium pentobarbitone (2 ml/kg) administered intravenously. Immediately following euthanasia, both halves of the mandible were carefully dissected, labeled, and prepared for the subsequent analyses.

2.4.3. Data Collection

- **CBCT Analysis:** The harvested mandibles were scanned using the Planmeca CBCT machine. Both 3D and panoramic views were generated to visualize the overall position of the implants and the healing of the surrounding bone. Cross-sectional views were used to evaluate bone integration and biomineralization in detail. For quantitative analysis, the Romexis software's verification tool was used to measure radiographic bone density. A virtual circle of a constant area was drawn around the implant site in both sagittal and coronal views, and the mean density was recorded in Hounsfield Units (HU). Ten readings were taken for each defect, and the average was calculated to represent the final bone density value for that site.
- **SEM/EDX Analysis:** After CBCT scanning, the implants were carefully retrieved from the mandibles of five of the dogs. They were cleaned in an ultrasonic bath for 10 minutes to remove adherent tissue, dried at room temperature, and then subjected to SEM/EDX analysis to examine their surface for signs of degradation and to identify the elemental composition, particularly looking for the presence of calcium (Ca), phosphorus (P), and oxygen (O).
- **Histological Analysis:** The bone blocks containing the defect sites were harvested, fixed in 10% calcium formal solution, and then decalcified over 4-5 weeks in a 10% EDTA solution {19}. After decalcification, the samples were processed through a standard dehydration series, cleared with xylene, and embedded in paraffin wax {19}. The paraffin blocks were then sectioned into 5 µm thick slices. These sections were stained with H&E to visualize cellular structures and general tissue morphology, and with Masson's Trichrome (MT) to differentiate between mature collagen (staining red) and immature collagen/woven bone (staining blue). All histological slides were evaluated by an examiner blinded to the group allocations to prevent bias.
- **Histomorphometry:** Digital images of the histological sections (at 100x magnification) were analyzed using the Fiji ImageJ software platform {20}. The trainable Weka segmentation method and the BoneJ plugin were used for quantitative analysis {20}. For each sample, five different fields were measured, and the average was calculated. Two key parameters were quantified: the percentage of total bone area (from H&E sections) and the percentage of mature bone area (from MT sections).
- **qRT-PCR Analysis:** Bone tissue samples were collected from each defect site and homogenized. Total RNA was extracted and purified. The RNA was then reverse transcribed into complementary DNA (cDNA), which served as the template for the PCR amplification step. The expression levels of two target genes, osteopontin and osteocalcin, were quantified. These genes were selected because they are well-established markers of late-stage osteoblast differentiation and bone matrix mineralization {13}. The expression of the target genes was normalized to the expression of a stable housekeeping gene, GAPDH, to control for variations in RNA input. The relative quantification (RQ) of gene expression was calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. The primers used for the analysis are detailed in Table 1.

Table 1: Forward and reverse primers and the housekeeping gene used for osteopontin and osteocalcin RT-PCR analysis

Gene	Forward Primer	Reverse Primer	GenBank Accession No.
GAPDH	TGGTGAAGACGCCAGTGGA	GCACCGTCAAGGCTGAGAAC	NM 002046
Osteocalcin	CTCACACTCCTCGCCCTATTG	GCCTGGGTCTCTTCACTACCT	BC113434
Osteopontin	CAGTTGTCCCCACAGTAGACAC	GTGATGTCCTCGTCTGTAGCATC	J04765

2.5. Data Analysis Plan

All numerical data collected from the study were presented as the mean ± standard deviation. The normality of the data distribution for each parameter was first confirmed using the Kolmogorov-Smirnov test. To compare the mean values of the five experimental groups (M, M0, M1, M2, M3) for each of the quantitative outcomes (bone density, bone area percentage, mature bone percentage, and gene expression levels), a one-way analysis of variance (ANOVA) test was employed. ANOVA is the appropriate statistical test for determining whether there are any statistically significant differences between the means of three or more independent groups. When the ANOVA test yielded a significant result (indicating that at least one group was different from the others), a Tukey's post-hoc test was performed. The Tukey's test conducts pairwise comparisons between all possible group combinations (e.g., M vs. M0, M1 vs. M3, etc.) while controlling for the family-wise error rate, allowing for a detailed understanding of which specific groups differed from one another. For all statistical tests, the significance level was set at $p < 0.05$. All statistical analyses were conducted using the SPSS software package (Version 22, IBM Corp., Chicago, IL, USA).

RESULTS

3.1. Preliminary Clinical and Radiographic Analyses

Throughout the 12-week study period, the clinical progression of all ten dogs was closely monitored. The healing process was uneventful for all animals. Importantly, there were no observable signs of local or systemic adverse reactions, such as inflammation, persistent swelling, hyperemia, or allergic responses, at any of the surgical sites. All skin incisions healed completely within the expected timeframe of 7 to 10 days post-surgery. The dogs maintained their normal appetite, body weight, and full masticatory function throughout the study, indicating excellent tolerance of the implanted materials and the surgical procedure itself.

Post-euthanasia cone-beam computed tomography (CBCT) examination confirmed that all 40 implants (4 implant groups x 10 dogs) were properly positioned within the surgically created mandibular defects. The radiographic images of all implanted defects revealed the presence of slight hazes or radiodense artifacts immediately surrounding the metallic discs. This phenomenon is common with metallic implants in CT imaging and could be attributed to beam hardening artifacts or potentially to the accumulation of degradation by-products at the implant-bone interface {26}.

Quantitative analysis of the radiographic bone density, measured in Hounsfield Units (HU), revealed highly significant differences among the five experimental groups ($p = 0.000$), as detailed in Table 2. The control group (M) exhibited the lowest mean bone density (133.6±23.3 HU), consistent with the formation of soft fibrous tissue and minimal ossification in the non-healing critical-sized defect. The baseline FeMn35 alloy group (M0) showed a significantly higher density (266.0±17.4 HU) compared to the control. A clear hierarchical improvement was observed with the doped alloys. The copper-doped group (M1) had a mean density of 510.6±37.2 HU, the tungsten-doped group (M2) reached 658.2±24.3 HU, and the cobalt-doped group (M3) demonstrated the highest bone density of all, at 724.6±41.1 HU. These results provided the first quantitative evidence that the doped alloys, particularly the cobalt-containing variant, were promoting substantial mineralization and bone formation.

Table 2: The mean radiographic bone density of mandibular bone around the implanted alloys (HU unit) as determined by CBCT examination

Group	Mean ± SD	Std. Error	Minimum	Maximum	95% Confidence Interval for Mean	P value
					Lower Bound	**Upper Bound**
M	133.6±23.3	9.5	100.0	170.0	109.1	158.1
M0	266.0±17.4	7.1	240.0	290.0	247.7	284.3
M1	510.6±37.2	15.2	450.0	557.8	471.5	549.6

M2	658.2±24.3	9.9	625.8	690.0	632.6	683.7
M3	724.6±41.1	16.8	690.0	800.0	681.4	767.7
*Significance level P < 0.05						

3.2. Material Surface Analysis (SEM-EDX)

Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) were used to analyze the elemental composition of the alloy surfaces before and after the 12-week *in vivo* implantation period. Before implantation, the SEM images of all four alloy types showed a relatively smooth and homogenous surface, characteristic of a solid solution microstructure, without any notable secondary phases. The corresponding EDX spectra confirmed the presence of the intended primary elements (Fe and Mn in M0; Fe, Mn, and Cu in M1; Fe, Mn, and W in M2; and Fe, Mn, and Co in M3) in their correct proportions.

After 12 weeks of implantation in the canine mandible, the retrieved alloys showed a dramatically altered surface. SEM images revealed a rougher, more textured surface indicative of corrosion and biological interaction. The post-implantation EDX analysis provided the most compelling evidence of bioactivity. The spectra of all four retrieved alloy types (M0, M1, M2, and M3) now showed prominent new peaks corresponding to calcium (Ca), phosphorus (P), and oxygen (O), in addition to the original metallic elements. The presence of Ca and P, the primary mineral components of bone, on the alloy surfaces is a clear indication of active biomineralization. This suggests that the degrading alloy surfaces were serving as a scaffold for the deposition of a new apatite-like layer, a crucial first step in the process of osseointegration. The detection of a significant oxygen peak is indicative of the formation of an oxide/hydroxide layer on the surface, which is a natural consequence of the metallic corrosion (degradation) process in a physiological environment. These findings, detailed in Table 3, confirm that the alloys were indeed biodegradable and were actively participating in the biological processes of bone healing.

Table 3: Elemental analysis of different alloys before and after grafting using Energy Dispersive X-ray Spectroscopy (EDX)

Alloy		**Before grafting**			**After grafting**		
	Element	**Wt. %**	**At. %**	**Er. %**	**Element**	**Wt. %**	**At. %**
M0	Mn	31.26	31.61	5.14	Mn	7.55	3.55
	Fe	68.74	68.39	4.23	Fe	28.14	13.00
					O	41.20	66.45
					P	11.21	9.34
					Ca	11.90	7.66
M1	Mn	29.87	30.35	4.80	Mn	6.28	3.13
	Fe	66.31	66.29	4.34	Fe	35.71	17.52
	Cu	3.82	3.36	8.07	Cu	2.42	1.04

					O	37.35	63.93
					P	9.56	8.45
					Ca	8.68	5.93
M2	Mn	33.19	34.04	3.99	Mn	12.65	6.89
	Fe	64.74	65.32	3.81	Fe	33.50	17.94
	W	2.07	0.64	9.94	W	4.25	0.69
					O	31.61	59.10
					P	8.88	8.58
					Ca	9.11	6.80
M3	Mn	28.97	29.34	3.72	Mn	9.19	4.69
	Fe	69.10	68.84	4.20	Fe	36.74	18.43
	Co	1.93	1.82	10.06	Co	0.73	0.35
					O	34.93	61.19
					P	12.00	10.86
					Ca	6.41	4.48
At % Atomic percent, Er% Error percent, Wt % weight percent							

3.3. Histological and Histomorphometric Findings

3.3.1. Qualitative Histological Evaluation

Histological analysis of the tissue sections provided a detailed microscopic view of the quality of healing within the defects.

- **Control Group (M):** As expected, the empty control defects stained with H&E showed very poor bone healing. The defect space was predominantly filled with loosely organized fibrous connective tissue. Only a few thin, irregularly arranged trabeculae of newly formed, immature woven bone were observed, encircling large marrow cavities filled with fibrocellular tissue. The Masson's Trichrome (MT) stain confirmed the immaturity of this tissue, with the collagen fibers staining predominantly blue.
- **Implant Groups (M0, M1, M2, M3):** In stark contrast, all defects implanted with the biodegradable alloys demonstrated substantially more robust bone formation. The H&E sections showed the formation of significantly thicker, more organized bone trabeculae, which were a mixture of woven and more mature lamellar bone. The marrow cavities were smaller and were bordered by active osteoblastic cells. Notably, the

tissue in the M1, M2, and M3 groups exhibited a higher degree of organization and a greater proportion of mature lamellar bone structures compared to the baseline M0 group. In many areas within these doped alloy groups, the new bone trabeculae were seen arranging themselves in circular patterns around marrow cavities, forming primitive Haversian systems—a hallmark of mature, load-bearing bone. The presence of numerous endothelial-lined blood vessels within the newly formed tissue in all implant groups was also a positive sign of active vascularization, which is essential for bone health.

- The MT staining provided a clear visual representation of bone maturation across the groups. While the M0 group showed a mixture of blue-staining woven bone and some red-staining lamellar bone, the M1 group exhibited a more pronounced intermingling of the two types. The progression was most evident in the M2 and M3 groups, where the sections were dominated by the deep red color characteristic of mature, well-organized lamellar bone, indicating the highest quality of tissue regeneration.

3.3.2. Quantitative Histomorphometric Analysis

The visual observations from histology were confirmed and quantified through histomorphometric analysis, presented in Table 4. The statistical comparisons between groups are detailed in Table 5.

- **Bone Area Percentage:** The measurement of the total new bone area within the defects revealed statistically significant differences between the groups. The cobalt-doped M3 group had the highest bone area percentage (71.61±9.93%), which was significantly greater than all other groups. The M2 group (59.16±4.99%) had a significantly higher bone area than M1, M0, and M. The M1 group (48.85±4.47%) and M0 group (40.82±3.93%) were, in turn, significantly better than the control M group (28.19±3.57%). Interestingly, the difference in total bone area between the M1 (Cu) and M0 (baseline) groups was not statistically significant.
- **Mature Bone Area Percentage:** The analysis of the MT-stained sections to quantify the percentage of mature (red-staining) bone revealed an even clearer hierarchy. Again, the M3 group was the top performer, with a mature bone percentage of 80.86±3.04%, significantly higher than all other groups. A significant stepwise increase was observed from the control group M (29.33±1.41%) to M0 (38.85±3.33%), then to M1 (51.82±2.58%), and M2 (60.09±4.42%), with each group being statistically superior to the one before it. This quantitative data unequivocally supports the qualitative observation that the incorporation of the dopant elements, particularly cobalt, not only increased the amount of new bone but also significantly accelerated its maturation into a higher-quality tissue.

Table 4: Mean bone area percentage and area percentage of mature bone (as demonstrated by Masson Trichrome staining) of mandibular bone defects implanted with different alloys

Parameter	Group	Mean±SD	Std. Error	95% Confidence interval for mean	P
				Lower limit	**Upper limit**
Bone area percent	M	28.19±3.57d	1.46	24.45	31.94
	M0	40.82±3.93c	1.60	36.70	44.95
	M1	48.85±4.47c	1.82	44.17	53.54
	M2	59.16±4.99b	2.04	53.92	64.40
	M3	71.61±9.93a	4.05	61.19	82.03
Area percent of mature bone (Masson)	M	29.33±1.41e	0.57	26.71	31.95
	M0	38.85±3.33d	1.36	36.23	41.47

	M1	51.82±2.58c	1.05	49.20	54.44
	M2	60.09±4.42b	1.81	57.47	62.71
	M3	80.86±3.04a	1.24	78.24	83.48
*Significance level P<0.05, Means with different superscript letters are significantly different					

Table 5: Differences in bone area percent and area percent of mature bone of different groups as demonstrated by Tukey's post hoc test

Parameter	Difference of levels	95% Confidence interval	Adjusted P-value
		Lower limit	**Upper limit**
Bone area percent	M0 - M		2.70
	M1 - M		10.73
	M1 - M0		-1.90
	M2 - M		21.04
	M2 - M0		8.41
	M2 - M1		0.38
	M3 - M		33.49
	M3 - M0		20.86
	M3 - M1		12.83
	M3 - M2		2.52
Percent of mature bone	M0 - M		4.24
	M1 - M		17.21
	M1 - M0		7.70
	M2 - M		25.48

	M2 - M0		15.96
	M2 - M1		2.99
	M3 - M		46.25
	M3 - M0		36.74
	M3 - M1		23.76
	M3 - M2		15.50
*Significance level P<0.05			

3.4. Osteogenic Gene Expression Findings

The analysis of gene expression via qRT-PCR provided molecular-level insight into the cellular processes driving the observed bone formation. The expression of osteopontin and osteocalcin, both markers for late-stage osteoblast activity and matrix mineralization, was quantified relative to the control group (M), which was set to a baseline value of 1.0. The results are summarized in Table 6, with detailed statistical comparisons in Table 7.

- **Osteopontin Expression:** The M3 (cobalt) group exhibited a dramatic and statistically significant increase in osteopontin expression, with a mean relative quantification (RQ) value of 4.55±0.16, far exceeding all other groups. The M2 (tungsten) group also showed a strong upregulation (RQ=2.85±0.11), which was significantly higher than the M1, M0, and M groups. The M1 (copper) group (RQ=1.79±0.12) was significantly higher than the M0 and M groups. The difference between the M0 group (RQ=1.09±0.07) and the control M group was not statistically significant for osteopontin, suggesting that the baseline Fe-Mn alloy provided a scaffold but did not strongly stimulate this specific gene pathway on its own.
- **Osteocalcin Expression:** A similar pattern was observed for osteocalcin expression, though all implant groups showed a significant increase over the control. Once again, the M3 group led with the highest expression level (RQ=3.48±0.08), followed in descending order by M2 (RQ=2.41±0.08), M1 (RQ=1.69±0.11), and M0 (RQ=1.16±0.07). The differences between all groups were statistically significant, confirming the hierarchical osteogenic potential observed in the histological and radiographic data: Co > W > Cu > baseline Fe-Mn > control. This molecular evidence strongly supports the conclusion that the dopant ions were actively stimulating the osteoblasts to a higher level of synthetic and mineralizing activity.

Table 6: Gene expression of osteopontin and osteocalcin in mandibular bone defects implanted with different alloys

Parameter	Group	Mean	Std. Error	95% Confidence interval for mean	P
				Lower limit	**Upper limit**
Osteopontin	M	1.00±0.00d	0.00	1.00	1.00
	M0	1.09±0.07d	0.03	1.02	1.17
	M1	1.79±0.12c	0.05	1.66	1.91

	M2	2.85±0.11b	0.04	2.74	2.97
	M3	4.55±0.16a	0.064	4.38	4.71
Osteocalcin	M	1.00±0.00e	0.00	1.00	1.00
	M0	1.16±0.07d	0.03	1.08	1.23
	M1	1.69±0.11c	0.05	1.56	1.80
	M2	2.41±0.08b	0.03	2.34	2.50
	M3	3.48±0.08a	0.032	3.40	3.56
*Significance level P<0.05, Means with different superscript letters are significantly different					

Table 7: Differences in gene expression of osteopontin and osteocalcin among different groups as demonstrated by Tukey's post hoc test

Parameter	Difference of levels	95% Confidence interval	Adjusted P-value
		Lower limit	**Upper limit**
Osteopontin	M0 - M	-0.08	0.28
	M1 - M	0.61	0.97
	M1 - M0	0.51	0.87
	M2 - M	1.67	2.03
	M2 - M0	1.58	1.94
	M2 - M1	0.88	1.25
	M3 - M	3.37	3.73
	M3 - M0	3.27	3.63
	M3 - M1	2.58	2.94
	M3 - M2	1.51	1.87
Osteocalcin	M0 - M	0.03	0.29
	M1 - M	0.55	0.81

	M1 - M0	0.39	0.66
	M2 - M	1.29	1.55
	M2 - M0	1.13	1.40
	M2 - M1	0.60	0.87
	M3 - M	2.35	2.61
	M3 - M0	2.20	2.46
	M3 - M1	1.67	1.93
	M3 - M2	0.93	1.20
*Significance level P<0.05			

DISCUSSION

4.1. Interpretation of Key Findings

The results of this comprehensive *in vivo* investigation provide compelling evidence that the incorporation of copper, tungsten, and particularly cobalt as tertiary alloying elements in a nanostructured Fe-Mn biodegradable matrix significantly enhances its capacity to promote bone regeneration in a critical-sized maxillofacial defect model. The study successfully demonstrated a clear, hierarchical improvement in osteogenic performance, confirming the initial hypotheses. The multi-modal analytical approach—spanning from macroscopic radiographic imaging to microscopic histology and molecular gene expression—converged on a unified conclusion: these novel doped alloys are not merely passive scaffolds but are bioactive agents that actively modulate the cellular environment to accelerate and improve the quality of healing.

The control group (M) performed as expected for a critical-sized defect, showing minimal spontaneous healing and primarily filling with fibrous tissue. This validates the chosen defect model as an appropriate testbed for evaluating the true regenerative potential of the biomaterials. The baseline FeMn35 alloy (M0) demonstrated a significant improvement over the control group in terms of bone density and the quantity of new bone formed. This confirms that the Fe-Mn alloy itself possesses fundamental biocompatibility and osteoconductive properties, serving as a suitable platform for cell attachment and growth {4}. The improved performance over pure iron implants reported in other studies can be attributed to the more favorable degradation rate and the positive biological role of manganese ions in supporting osteoblast function and bone matrix synthesis {4, 31, 32}. However, the lack of a significant increase in osteopontin expression in the M0 group suggests that while it provides a permissive environment, it may not be a strong initiator of the late-stage mineralization pathways on its own.

The inclusion of **copper** in the M1 group (FeMn32Cu3) resulted in a marked enhancement of bone formation and maturation compared to the baseline alloy. This was evidenced by the significantly higher bone density, greater percentage of mature bone, and pronounced upregulation of both osteopontin and osteocalcin. These findings align perfectly with the known biological roles of copper. The released copper ions likely stimulated osteoblast proliferation through pathways such as the AMPK/mTOR signaling axis {37} and promoted their differentiation into mature, mineralizing cells {29, 30}. The significant increase in the expression of late-stage osteogenic markers confirms that copper not only encourages cell growth but also drives the functional maturation of the bone tissue {39, 40}. Furthermore, the inherent antimicrobial properties of copper would provide an additional clinical benefit by reducing the risk of implant-associated infections {9}.

The performance of the **tungsten**-doped alloy in the M2 group (FeMn32W3) was particularly noteworthy. This group demonstrated a level of bone regeneration that was statistically superior to both the baseline and copper-doped alloys. This result is significant because it helps to clarify the controversial role of tungsten in bone biology. While some *in vitro* studies have raised concerns about tungsten's potential to inhibit osteogenesis {13}, our *in vivo* findings clearly demonstrate a potent positive effect. This discrepancy highlights the critical importance of preclinical animal studies. The complex biological milieu of a living organism—with its intricate network of growth factors, mechanical signals, and cellular crosstalk—can lead to

outcomes that are not predicted by simplified cell culture models. It is plausible that the slow, controlled release of tungsten ions from the degrading alloy, and their potential integration into the bone matrix as phosphotungstates {10}, creates a local environment that is highly conducive to bone formation, a result that contradicts the findings from studies using bolus doses of tungstate in cell culture medium.

The most impressive results were unequivocally observed in the **cobalt**-doped M3 group (FeMn32Co3). This alloy consistently outperformed all other groups across every single metric: it produced the highest bone density, the greatest percentage of new and mature bone area, and the most substantial upregulation of osteogenic gene expression. This superior performance can be attributed to cobalt's unique dual-action mechanism. By stabilizing HIF-1α, the released cobalt ions create a "pseudo-hypoxic" state that potently stimulates angiogenesis through the production of VEGF {15, 16}. The dense network of new blood vessels observed in the histological sections of the M3 group is a testament to this effect. This enhanced vascularity provides a robust supply line for oxygen, nutrients, and osteoprogenitor cells, creating an ideal physiological foundation for rapid bone synthesis {25}. Concurrently, cobalt ions directly stimulate osteoblasts, as evidenced by the dramatic increase in osteopontin and osteocalcin expression {14, 45}. This synergistic combination of pro-angiogenic and pro-osteogenic signals makes cobalt a particularly powerful agent for bone regeneration, validating its selection as a dopant and confirming our primary hypothesis.

4.2. Comparison with Previous Literature

The findings of this study both reinforce and extend the existing body of literature on biodegradable metals. The observation that Fe-Mn alloys degrade more favorably than pure iron and are biocompatible is consistent with previous reports {4, 5, 27}. The degradation of the alloys, evidenced by the surface changes and the deposition of a calcium-phosphate layer seen in the SEM-EDX analysis, aligns with the established models of biocorrosion for iron-based materials in vivo. This process involves the oxidation of the metal and the subsequent precipitation of biological minerals from the surrounding fluid, as has been described in other studies involving iron stents and pins {5, 6}.

The observed discrepancy between the *in vivo* degradation rate and what is often seen in *in vitro* immersion tests is also a key point of discussion in the literature. The continuous supply of oxygenated blood in a living system, particularly to a well-vascularized site like the mandible, can accelerate the cathodic reaction of oxygen reduction, leading to faster corrosion than in a static *in vitro* solution {6, 27}. The slight hazes seen on the CBCT scans around the implants are likely a combination of this corrosion layer and imaging artifact, a challenge that has been noted in other studies involving metallic implants {26}.

The positive results for the copper- and cobalt-doped alloys are in strong agreement with a large body of work that has investigated the incorporation of these ions into other types of biomaterials, such as bioactive glasses, ceramics, and titanium coatings {15, 29, 37, 45}. Our study is novel in that it demonstrates these effects within a biodegradable nanostructured Fe-Mn alloy system {17, 18}. The results for the tungsten alloy, however, provide a crucial counterpoint to some of the existing *in vitro* literature {13} and align more closely with the *in vivo* portion of that same work, which found no impedance to osteogenesis {13}. This underscores a critical lesson in biomaterials research: *in vitro* biocompatibility and bioactivity tests are essential screening tools, but they are not substitutes for validation in a complex, living biological system {22}.

4.3. Strengths and Limitations of the Study

This study possesses several significant strengths. The foremost is the use of a randomized, controlled design in a large animal model (the dog) that has high physiological relevance to human bone healing {23, 24}. This robust design allows for strong conclusions to be drawn about the materials' efficacy. Another major strength is the comprehensive, multi-modal approach to analysis. By combining advanced radiographic imaging (CBCT), surface analysis (SEM-EDX), detailed histology and histomorphometry (H&E, MT), and molecular biology (qRT-PCR), we were able to construct a multi-layered and highly detailed picture of the healing process, from the macroscopic down to the molecular level. The fabrication of the alloys using state-of-the-art nanostructuring and additive manufacturing techniques represents another strength, as this positions the research at the cutting edge of materials science {1, 18}.

Despite these strengths, the study is not without its limitations. The 12-week duration, while sufficient to observe advanced bone formation, is a relatively short timeframe in the context of the complete degradation of an iron-based alloy. A longer-term study, extending to one year or more, would be necessary to fully characterize the long-term degradation profile, the final stages of bone remodeling, and the ultimate fate of the degradation by-products {5}. Another limitation is the inherent difficulty in precisely quantifying the mass loss of the implants *in vivo*. While the SEM-EDX data confirms that degradation occurred, removing the tightly adherent biomineralization and corrosion layers without removing some of the underlying metal is extremely challenging, making precise gravimetric analysis unreliable {5}. Finally, while this study was conducted in the mandible, it was in a non-load-bearing site. Future studies should evaluate the performance of these promising materials under physiological load, as

would be experienced in long bone fracture fixation, to fully assess their mechanical durability throughout the healing and degradation process.

4.4. Implications for Theory and Practice

The findings of this research have significant implications for both the theory of biomaterial design and the clinical practice of reconstructive surgery. From a theoretical standpoint, this study validates the strategy of using bioactive ion doping to transform a relatively inert biodegradable scaffold into a potent, pro-regenerative therapeutic device. It demonstrates that by carefully selecting alloying elements based on their known biological functions, it is possible to create materials that actively steer cellular behavior towards a desired outcome {25}. The results specifically highlight the immense potential of harnessing the hypoxia-mimicking pathway via cobalt doping as a powerful tool for promoting vascularized bone regeneration {15, 16}.

From a practical, clinical perspective, these materials offer a tangible solution to the long-standing problems associated with permanent metallic implants {2}. The successful development of a biodegradable alloy with the mechanical properties of an Fe-Mn base and the enhanced bioactivity of Co, W, or Cu doping could revolutionize treatment in several areas. In maxillofacial surgery, these materials could be used for fixation plates and screws for mandibular fractures, orbital floor reconstruction, and as scaffolds for rebuilding bone lost to tumors or trauma {2, 8}. In orthopedics, they could be used for fracture fixation in non-union cases, spinal fusion cages, and other applications where temporary support is required. The ultimate benefit would be to the patient, who would receive a strong, stable fixation that aids healing and then simply disappears, eliminating the need for a second surgery and its associated risks, pain, and cost {2}.

4.5. Conclusion and Future Research Directions

In conclusion, this study successfully demonstrated that nanostructured Fe-Mn alloys doped with copper, tungsten, and cobalt are highly biocompatible and possess significant osteogenic potential in a demanding *in vivo* model. The incorporation of these bioactive elements, particularly cobalt, resulted in a dramatic acceleration of bone formation and maturation compared to a baseline Fe-Mn alloy. These findings strongly support the continued development of these novel biodegradable metals for a wide range of orthopedic and dental applications. They represent a promising step towards the next generation of "smart" implants that not only repair but actively regenerate host tissue.

Building on this foundational work, future research should proceed in several key directions.

1. **Long-Term Studies:** As previously mentioned, longer-term *in vivo* studies (1-2 years) are essential to fully map the degradation kinetics and the final stages of bone remodeling and to ensure there are no long-term adverse effects from the accumulated degradation products.
2. **Load-Bearing Models:** The performance of these alloys must be tested in load-bearing applications, such as a femoral or tibial fracture model in a large animal, to confirm that their mechanical integrity is maintained for a sufficient duration under physiological stress.
3. **Dose-Response Studies:** Investigating different concentrations of the dopant elements (Cu, W, Co) would be valuable to determine the optimal concentration that maximizes the therapeutic effect while remaining well within the window of biocompatibility {46, 47}.
4. **Mechanistic Studies:** While this study links the observed effects to known biological pathways, more detailed molecular investigations are needed to unravel the precise signaling cascades activated by the released ions in the complex *in vivo* environment.
5. **Combination Therapies:** Future iterations could explore the possibility of using these alloys as drug delivery platforms, for example, by loading them with antibiotics or growth factors to further enhance their therapeutic efficacy {22}.

By systematically addressing these future research questions, the full clinical potential of these exciting new biomaterials can be realized, ultimately leading to improved outcomes for millions of patients worldwide.

REFERENCES

1. Klinge L, Kluy L, Spiegel C, Siemers C, Groche P, Coraça-Huber D. Nanostructured Ti-13Nb-13Zr alloy for implant applicationmaterial scientific, technological, and biological aspects. Front Bioeng Biotechnol. 2023;11:1255947. https://doi.org/10.3389/fbioe.2023.1255947
2. Xia D, Yang F, Zheng Y, Liu Y, Zhou Y. Research status of biodegradable metals designed for oral and maxillofacial applications: A review. Bioact Mater. 2021;6:4186–208. https://doi.org/10.1016/j.bioactmat.2021.01.011
3. Elshahawy W, Watanabe I. Biocompatibility of dental alloys used in dental fixed prosthodontics. Tanta Dental Journal. 2014;11:150–9. https://doi.org/10.1016/j.tdj.2014.07.005
4. Hong D, Chou DT, Velikokhatnyi OI, Roy A, Lee B, Swink I, et al. Binder-jetting 3D printing and alloy development of new biodegradable Fe-Mn-Ca/Mg alloys. Acta Biomater. 2016;45:375–86. https://doi.org/10.1016/j.actbio.2016.08.032

5. Kraus T, Moszner F, Fischerauer S, Fiedler M, Martinelli E, Eichler J, et al. Biodegradable Fe-based alloys for use in osteosynthesis: outcome of an in vivo study after 52 weeks. Acta Biomater. 2014;10:3346–53. https://doi.org/10.1016/j.actbio. 2014.04.007
6. Peuster M, Hesse C, Schloo T, Fink C, Beerbaum P, von Schnakenburg C. Long-term biocompatibility of a corrodible peripheral iron stent in the porcine descending aorta. Biomaterials. 2006;27:4955–62. https://doi.org/10.1016/j.biomaterials.2006.05. 029
7. Zheng YF, Gu XN, Witte FJ. Biodegradable metals. Mater Sci Eng. 2014;77:1–34. https://doi.org/10.1016/j.mser.2014.01.001
8. Li P, Zhang W, Dai J, Xepapadeas AB, Schweizer E, Alexander D, et al. Investigation of zinc-copper alloys as potential materials for craniomaxillofacial osteosynthesis implants. Mater Sci Eng C Mater Biol Appl. 2019;103:109826. https://doi.org/10.1016/j. msec.2019.109826
9. Stanić V, Dimitrijević S, Antić-Stanković J, Mitrić M, Jokić B, Plećaš IB, et al. Synthesis, characterization and antimicrobial activity of copper and zinc-doped hydroxyapatite nanopowders. Appl Surf Sci. 2010;256:6083–9. https://doi.org/10.1016/j.apsusc. 2010.03.124
10. VanderSchee CR, Kuter D, Bolt AM, Lo FC, Feng R, Thieme J, et al. Accumulation of persistent tungsten in bone as in situ generated polytungstate. Commun Chem. 2018;1:8. https://doi. org/10.1038/s42004-017-0007-6
11. Kelly AD, Lemaire M, Young YK, Eustache JH, Guilbert C, Molina MF, et al. In vivo tungsten exposure alters B-cell development and increases DNA damage in murine bone marrow. Toxicol Sci. 2013;131:434–46. https://doi.org/10.1093/toxsci/ kfs324
12. Bolt AM, Mann KK. Tungsten: an Emerging Toxicant, Alone or in Combination. Curr Environ Health Rep. 2016;3:405–15. https://doi.org/10.1007/s40572-016-0106-z
13. Bolt AM, Grant MP, Wu TH, Flores Molina M, Plourde D, Kelly AD, et al. Tungsten Promotes Sex-Specific Adipogenesis in the Bone by Altering Differentiation of Bone Marrow-Resident Mesenchymal Stromal Cells. Toxicol Sci. 2016;150:333–46. https://doi.org/10.1093/toxsci/kfw008
14. Khosrowshahi AK, Khoshfetrat AB, Khosrowshahi YB, MalekiGhaleh H. Cobalt content modulates characteristics and osteogenic properties of cobalt-containing hydroxyapatite in in-vitro milieu. Mater Today Commun. 2021;27:102392. https://doi.org/ 10.1016/j.mtcomm.2021.102392
15. Wu C, Zhou Y, Fan W, Han P, Chang J, Yuen J, et al. Hypoxiamimicking mesoporous bioactive glass scaffolds with controllable cobalt ion release for bone tissue engineering. Biomaterials. 2012;33:2076–85. https://doi.org/10.1016/j.biomaterials.2011.11. 042
16. Fan W, Crawford R, Xiao Y. Enhancing in vivo vascularized bone formation by cobalt chloride-treated bone marrow stromal cells in a tissue engineered periosteum model. Biomaterials. 2010;31:3580–9. https://doi.org/10.1016/j.biomaterials.2010.01. 083
17. Ammar HR, Sivasankaran S, Alaboodi AS. Investigation of the Microstructure and Compressibility of Biodegradable Fe-Mn-Cu/ W/Co Nanostructured Alloy Powders Synthesized by Mechanical Alloying. Materials (Basel). 2021;14:3088. https://doi.org/10. 3390/ma14113088
18. AlMangour B, Sivasankaran S, Ammar HR, Grzesiak D. Synthesis, Microstructures, Mechanical, and Corrosion Behavior of FeMn (35− x)–x (Cu, W, and Co)-Based Biodegradable Alloys Prepared by Mechanical Alloying and Selective Laser Melting. Metallurgical and Materials Transactions A. 2023;54:3767. https://doi.org/10.1007/s11661-023-07128-3
19. Bancroft JD, Cook HC. Manual of Histological Techniques and their Diagnostic Application, Edinburgh. London, Melbourne; New York Churchill Livingstone: 1994
20. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biologicalimage analysis. Nat Methods. 2012;9:676–82. https://doi.org/10. 1038/nmeth.2019
21. Elborolosy SA, Hussein LA, Mahran H, Ammar HR, Sivasankaran S, Abd El-Ghani SF, et al. Evaluation of the biocompatibility, antibacterial and anticancer effects of a novel nanostructured Fe–Mn-based biodegradable alloys in-vitro study. Heliyon. 2023;9:e20932. https://doi.org/10.1016/j.heliyon.2023. e20932
22. Mostafa AA, Mahmoud AA, Hamid MAA, Basha M, El-Okaily MS, Abdelkhalek AFA, et al. An in vitro / in vivo release test of risedronate drug loaded nano-bioactive glass composite scaffolds. Int J Pharm. 2021;607:120989. https://doi.org/10.1016/j.ijpharm. 2021.120989
23. Taha SK, Abdel Hamid MA, Hamzawy EMA, Kenawy SH, ElBassyouni GT, Hassan EA, et al. Osteogenic potential of calcium silicate-doped iron oxide nanoparticles versus calcium silicate for reconstruction of critical-sized mandibular defects: An experimental study in dog model. Saudi Dent J. 2022;34:485–93. https:// doi.org/10.1016/j.sdentj.2022.06.008
24. Taha SK, Hassan EA, Mousa S, El-Bassyouni GT, Shalash HN, Abdel Hamid MA. Biphasic calcium phosphate doped with zirconia nanoparticles for reconstruction of induced mandibular defects in dogs: cone-beam computed tomographic and histopathologic evaluation. J Mater Sci Mater Med. 2023;34:27. https://doi.org/10.1007/s10856-023-06731-5
25. Li S, Cui Y, Liu H, Tian Y, Wang G, Fan Y, et al. Application of bioactive metal ions in the treatment of bone defects. J Mater Chem B. 2022;10:9369–88.

https://doi.org/10.1039/ d2tb01684b
26. Watzke O, Kalender WA. A pragmatic approach to metal artifact reduction in CT: merging of metal artifact reduced images. Eur Radiol. 2004;14:849–56. https://doi.org/10.1007/s00330-004- 2263-y
27. Schinhammer M, Steiger P, Moszner F, Löffler JF, Uggowitzer PJ. Degradation performance of biodegradable Fe-Mn-C(-Pd) alloys. Mater Sci Eng C Mater Biol Appl. 2012;33:1882–93. https://doi.org/10.1016/j.msec.2012.10.013
28. Hermawan H, Dubé D, Mantovani D. Developments in metallic biodegradable stents. Acta Biomater. 2010;6:1693–7. https://doi.org/10.1016/j.actbio.2009.10.006
29. Wang LJ, Ni XH, Zhang F, Peng Z, Yu FX, Zhang LB, et al. Osteoblast Response to Copper-Doped Microporous Coatings on Titanium for Improved Bone Integration. Nanoscale Res Lett. 2021;16:146. https://doi.org/10.1186/s11671-021-03602-2
30. Zhang X, Liu H, Li L, Huang C, Meng X, Liu J, et al. Promoting osteointegration effect of Cu-alloyed titanium in ovariectomized rats. Regen Biomater. 2022;9:rbac011. https://doi.org/10.1093/rb/rbac011
31. Carluccio D, Xu C, Venezuela J, Cao Y, Kent D, Bermingham M, et al. Additively manufactured iron-manganese for biodegradable porous load-bearing bone scaffold applications. Acta Biomater. 2020;103:346–60. https://doi.org/10.1016/j.actbio.2019.12.018
32. Bracci B, Torricelli P, Panzavolta S, Boanini E, Giardino R, Bigi A. Effect of Mg(2+), Sr(2+), and Mn(2+) on the chemicophysical and in vitro biological properties of calcium phosphate biomimetic coatings. J Inorg Biochem. 2009;103:1666–74. https://doi.org/10.1016/j.jinorgbio.2009.09.009
33. Lüthen F, Bulnheim U, Müller PD, Rychly J, Jesswein H, Nebe JG. Influence of manganese ions on cellular behavior of human osteoblasts in vitro. Biomol Eng. 2007;24:531–6. https://doi.org/10.1016/j.bioeng.2007.08.003
34. Yu L, Tian Y, Qiao Y, Liu X. Mn-containing titanium surface with favorable osteogenic and antimicrobial functions synthesized by PIII&D. Colloids Surf B Biointerfaces. 2020;152:376–84. https://doi.org/10.1016/j.colsurfb.2017.01.047
35. Sharma N, Jandaik S, Kumar S, Chitkara M, Sandhu IS. Synthesis, characterisation and antimicrobial activity of manganese-and iron-doped zinc oxide nanoparticles. J Exp Nanosci. 2016;11:54–71. https://doi.org/10.1080/17458080.2015.1025302
36. Li J, Deng C, Liang W, Kang F, Bai Y, Ma B, et al. Mncontaining bioceramics inhibit osteoclastogenesis and promote osteoporotic bone regeneration via scavenging ROS. Bioact Mater. 2021;6:3839–50. https://doi.org/10.1016/j.bioactmat.2021. 03.039
37. Duan JZ, Yang Y, Wang H. Effects of Antibacterial Co-Cr-MoCu Alloys on Osteoblast Proliferation, Differentiation, and the Inhibition of Apoptosis. Orthop Surg. 2022;14:758–68. https://doi.org/10.1111/os.13253
38. Milkovic L, Hoppe A, Detsch R, Boccaccini AR, Zarkovic N. Effects of Cu-doped 45S5 bioactive glass on the lipid peroxidation-associated growth of human osteoblast-like cells in vitro. J Biomed Mater Res A. 2014;102:3556–61. https://doi.org/10.1002/jbm.a.35032
39. Ren L, Wong HM, Yan CH, Yeung KW, Yang K. Osteogenic ability of Cu-bearing stainless steel. J Biomed Mater Res B Appl Biomater. 2015;103:1433–44. https://doi.org/10.1002/jbm.b. 33318
40. Lian M, Han Y, Sun B, Xu L, Wang X, Ni B, et al. A multifunctional electrowritten bi-layered scaffold for guided bone regeneration. Acta Biomater. 2020;118:83–99. https://doi.org/10.1016/j.actbio.2020.08.017
41. Rau JV, Curcio M, Raucci MG, Barbaro K, Fasolino I, Teghil R, et al. Cu-Releasing Bioactive Glass Coatings and Their in Vitro Properties. ACS Appl Mater Interfaces. 2019;11:5812–20. https://doi.org/10.1021/acsami.8b19082
42. Huang Q, Li X, Elkhooly TA, Liu X, Zhang R, Wu H, et al. The Cu-containing TiO2 coatings with modulatory effects on macrophage polarization and bactericidal capacity prepared by micro-arc oxidation on titanium substrates. Colloids Surf B Biointerfaces. 2018;170:242–50. https://doi.org/10.1016/j.colsurfb.2018.06.020
43. Huang Q, Ouyang Z, Tan Y, Wu H, Liu Y. Activating macrophages for enhanced osteogenic and bactericidal performance by Cu ion release from micro/nano-topographical coating on a titanium substrate. Acta Biomater. 2019;100:415–26. https://doi.org/10.1016/j.actbio.2019.09.030
44. Liu B, Zheng YF. Effects of alloying elements (Mn, Co, Al, W, Sn, B, C and S) on biodegradability and in vitro biocompatibility of pure iron. Acta Biomater. 2011;7:1407–20. https://doi.org/10.1016/j.actbio.2010.11.001
45. Jiménez-Holguín J, Lozano D, Saiz-Pardo M, de Pablo D, Ortega L, Enciso S, et al. Osteogenic-angiogenic coupled response of cobalt-containing mesoporous bioactive glasses in vivo. Acta Biomater. 2024;176:445–57. https://doi.org/10.1016/j.actbio. 2024.01.003
46. Baino F, Montazerian M, Verné E. Cobalt-Doped Bioactive Glasses for Biomedical Applications: A Review. Materials (Basel). 2023;16:4994. https://doi.org/10.3390/ma16144994
47. Hoppe A, Jokic B, Janackovic D, Fey T, Greil P, Romeis S, et al. Cobalt-releasing 1393 bioactive glass-derived

scaffolds for bone tissue engineering applications. ACS Appl Mater Interfaces. 2014;6:2865–77. https://doi.org/10.1021/am405354y Reproduksi Ternak Ruminansia. WARTAZOA. 23(4): 176-185.