Effects of Zinc Ions Released from Ti-NW-Zn Surface on Osteogenesis and Angiogenesis *in Vitro* and in an *in Vivo* Zebrafish Model

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Research

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Abstract

Objective: Zinc-modified titanium materials have been widely applied in oral implants, among them, our previous studies have also successfully prepared a novel acid-etched micro-structured titanium surface modified with zinc-containing nanowires (Ti-NW-Zn). However, there are very few reports concerned about the biological safeties of zinc ions released from material surface and the appropriate concentration range of released zinc ions which was more conducive to angiogenesis and bone regeneration. *Danio rerio* (Zebrafish) represents a powerful alternative *in vivo* model to study both angiogenesis and osteogenesis. Then, the aim of this study is to investigate the effects of zinc ions released from Ti-NW-Zn surfaces on angiogenesis and osteogenesis via zebrafish model, and further revealed the relationship between angiogenesis and osteogenesis via HUVECs and MC3T3-E1s *in vitro* models.

Materials and methods: Tg (Fli-1:EGFP)^{y1} zebrafish embryos were used to investigate the *in vivo* angiogenesis exposure to zinc ions, Wild-type Zebrafish (*Danio rerio*) adult fish and their embryos were used to investigate the *in vivo* bone regeneration and survival rate. Cell experiments including proliferation, adhesion, osteogenic differentiation and signaling pathway experiments were carried out by CCK-8, Laser scanning confocal microscope and Western blot, etc. Zinc ion release assay was detected by a Zinc assay kit.

Results: The zinc ions released from Ti-NW-Zn surfaces were far lower than median lethal concentrations (LCs) of both embryos and adult fish, which was beneficial to osteogenesis and angiogenesis promotion of zebrafish model. Moreover, the appropriate concentration range of zinc ions was 1-2 ppm *in vitro* models, which could induce HUVECs proliferations, and the conditioned medium (CM) collected from the supernatant of 1-2 ppm zinc ions-induced HUVECs medium could obviously promote MC3T3-E1 osteoblasts behaviors via activated the MAPK/ERK signaling pathway.

Conclusion: Zinc ions released from Ti-NW-Zn surfaces was at a biological safe and appropriate concentration, which could promote the angiogenesis and osteogenesis *in vivo* and *vitro*. The positive effects of the appropriate concentration of zinc ions on osteoblast behaviors might be regulated by activating the MAPK/ERK signaling pathway.

1. Introduction

Titanium (Ti) and its alloys as bio-implants have excellent biocompatibilities and osteogenic properties after modification of chemical composition and topography via various methods[1–3]. Among them, zinc coated titanium materials have been widely used in biomedicine and have been proved to improve osteoblastic differentiation[4, 5]. Our previous studies have successfully prepared a novel acid-etched micro-structured titanium surface modified with zinc-containing nanowires (Ti-NW-Zn)[6]. We also proved that this Ti-NW-Zn surface has excellent biocompatibilities and osteogenic capacities and exhibits excellent corrosion resistance under the complex oral electrochemical environment. However, the internal

mechanism of this surface on osteogenesis has not yet been clarified, especially the effect of zinc ions introduced into the Ti-NW-Zn surface on bone development is urgently to be ascertained.

Zinc is an essential trace element, required for the functional integrity of many organ systems, as well as for development, growth, and tissue repair[7, 8]. It has been demonstrated that zinc ions are able not only to induce and stimulate the expression of genes related to osteoblastic differentiation and bone formation but to stimulate angiogenesis *in vitro* and *in vivo*[9, 10]. Studies have shown that bone regeneration is inseparable from angiogenesis[11]. The new blood vessels in bone are not only transport channels for oxygen, nutrients and metabolites, but also important pathways involved in the transport of related cell signaling molecules and maintain the metabolism of bone regeneration and repair as a whole microenvironment[12, 13]. Studies on zinc-modified titanium surface has mainly focused on promoting bone formation, while the exploration of the surface to regulate the direction of vascularization has not been reported. In the process of implant osseointegration, osteogenesis and angiogenesis are complementary and indispensable[14]. Therefore, it is crucial for in-depth research on the relationship between osteogenesis and angiogenesis under zinc ions exposure, especially the role of angiogenesis in bone regeneration performance both *in vivo* and *in vitro*.

In vivo experimental models especially rabbits and dogs have been used to simulate the oral implantation process and investigate the characteristics and the basic molecular mechanisms of osseointegration[15–17]. However, the complexity of metabolic and physical-chemical regulations of higher vertebrate makes it difficult to dissect the early phase of vascularized bone regeneration within a short period under zinc ions exposure. A simpler animal model in lower vertebrate could be very helpful to extend the knowledge in this specific field of study.

Zebrafish has emerged as an alternative *in vivo* model to study angiogenesis, a tissue-specific germ line-induced transgenic line promotes Enhanced Green Fluorescence Protein (EGFP) expression in all endothelial cells under the Friend leukemia integration-1 (Fli-1) promoter. The Tg (Fli-1:EGFP)^{y1} transgenic zebrafish can express EGFP in endothelial cells during early embryonic vascular development, making it possible to capture images of vascular development and adult blood vessels in real time[18, 19]. Recently, the adult zebrafish is also gaining importance as innovative and readily available resource for studying skeletal system and bone metabolism both at cellular and molecular level[20, 21]. In particular, the caudal fins represent a unique anatomical feature because they are formed from a type of dermal bone and include osteoclasts, osteoblasts, and other characteristics of bone tissue, which is like to human lamellar bone[22, 23]. Undoubtedly, caudal fins take advantages of high regenerative capacity. These characteristics allows zebrafish as an ideal model to visualize angiogenesis and easily observe mineral matrix deposition and resorption. The same approach cannot be applied to internal bones in other higher vertebrate animals.

In this study, we aim to investigate the positive effects of zinc ions released from Ti-NW-Zn surfaces on angiogenesis via the Tg (Fli-1:EGFP)^{y1} zebrafish embryo assay and on osteogenesis via adult zebrafish caudal fins model of bone regeneration. Based on the *in vivo* results, we will further use endothelial cells

and osteoblasts as *in vitro* models to explore the cellular and genetic regulatory mechanisms of the angiogenesis effects on osteogenesis under zinc ions exposure. This study will assess the appropriate zinc ion concentration released from Ti-NW-Zn surfaces for osteogenesis and angiogenesis promotion and expected to provide a scientific basis for the functional optimization design of zinc-modified titanium surface and its potential transformation applications.

2. Results

2.1 Promoting effect of Ti-NW-Zn surface on angiogenesis

After pretreated with 75 nM vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor (VRI) to inhibit the normal embryos angiogenesis for 8 hours and incubated on cp-Ti (commercially pure titanium) surface or Ti-NW-Zn surface for 24 hours, the intersegmental blood vessels (ISVs) of Tg (Fli-1:EGFP)^{y1} zebrafish were observed via an inverted fluorescent microscope. As shown in Fig. 1, the number of ISVs in the medial region of Tg (Fli-1:EGFP)^{y1} zebrafish on cp-Ti surface obviously decreased compared to the control group, and the ISVs are thinner and a bit morphological distortion, but Tg (Fli-1:EGFP)^{y1} zebrafish on Ti-NW-Zn surface showed more ISVs compared to the cp-Ti surface group while less than the control group. The subintestinal vessels (SIVs) of the yolk sac region gross morphological changes were also observed in the 72 hpf, and we found that SIVs of zebrafish on cp-Ti surface are nearly absent after pretreated with VRI, while SIVs of zebrafish on Ti-NW-Zn surface showed some new vessel branching, these results indicating that Ti-NW-Zn surface can promote the zebrafish angiogenesis.

2.2 Promoting effect of Ti-NW-Zn surface on osteogenesis

Since blood vessels and bone development are closely connected, and we have found that Ti-NW-Zn surface can promote the zebrafish angiogenesis, we further attempted to explore the effect of Ti-NW-Zn surface on osteogenesis *in vivo* and constructed the zebrafish caudal fin regeneration model. 9 days after caudal fins amputation, the regeneration of the caudal fins was already visible to the naked eye. Then, Alizarin Red Stain was used to detect the skeletal mineralization of zebrafish. As shown in Fig. 2a, we can clearly find the fracture healing lines in the fin rays. Interestingly, in the control group, the width of the new fin rays at the fracture was significantly reduced compared to the original parts, while zebrafish on Ti-NW-Zn surface showed obvious bulges in the fractured area. Moreover, staining of the fracture on Ti-NW-Zn surface was also deeper than the control group, suggesting that the mineralization was more adequate on Ti-NW-Zn surface. These results indicated that Ti-NW-Zn surface can promote the zebrafish bone regeneration and mineralization.

2.3 Zinc ions release from Ti-NW-Zn surface

In order to clarify the promoting effects of Ti-NW-Zn surface on osteogenesis and angiogenesis, we further detect the amount of zinc ions released from Ti-NW-Zn surface. Figure 4 shows the concentrations of zinc ions released from Ti-NW-Zn surfaces into PBS. It was found that samples released 0.9 ppm of zinc ions within 24 h, respectively. After the burst release in the initial 24 h period,

concentrations of zinc ion released from samples nearly reached to peaks. Then, Zn ions were gradual released into PBS over the ensuing time.

2.4 Effects of different zinc ion concentrations on osteogenesis

To find out the most suitable zinc ion concentration to promote osteogenesis *in vivo*, we further studied the effects of different concentrations of zinc ions on the regeneration and development of zebrafish caudal fins. In Fig. 2b, compared with the control group, 1 ppm zinc ions significantly promoted bone bulges in the fractured area, which appeared a better trend of bone healing. However, the 2 ppm zinc ions seemed to inhibit the fracture healing of the fin rays, where the width of the new fin rays at the fracture was significantly reduced, the structure at the fracture was abnormal, and the bone mineral density at the fracture was reduced. These results revealed that different concentrations of zinc ion showed different effects on bone development, and 1 ppm zinc ions in this study was more conducive to bone development.

2.5 Mortality Curve and Median Lethal Concentration for zinc ions exposure

The survival test of embryos was performed at different concentrations (0, 2, 4, 8, 16, 24 and 32 ppm) zinc ions from low to high concentrations last for 120 h of exposure (Fig. 3a). No deaths occurred in the control group throughout the exposure experiments. Embryos incubated at zinc ion concentrations of 1 ppm or lower showed mortality similar to the control group, while embryos treated with 32 ppm had 100% mortality after 24 h of incubation. Median lethal concentration (LC) was calculated from the percentage of embryos mortality for each zinc ion concentration with different exposure times (Fig. 3a). The 120 h - LC50 of zinc ions was estimated around 10 ppm. Similarly, we also explored the survival test of adult fish exposure to different concentrations of zinc ions (0, 0.5, 1, 1.5, 2, 2.5 and 3 ppm). As shown in Fig. 3b, adult fish exposed to zinc ion concentrations of 1 ppm or lower showed no deaths, while adult fish treated with 3 ppm had 100% mortality after 24 h of incubation. These results indicated the effect of zinc ion concentration was dose-dependent and time-dependent, which had different effects on individuals at different developmental stages. Interestingly, the reason why embryos showed higher resistance of zinc ion exposure needed further exploration.

2.6 Cell proliferation

The results of cell proliferation assessed by the CCK-8 assay, was shown in Fig. 5. On the first day, no obvious proliferation changes in HUVECs and MC3T3-E1 treated with zinc ions was observed. After culturing for 2 and 3 days, zinc ions had a positive effect on HUVECs, especially 1 and 2 ppm. As time went on, 1 and 2 ppm zinc ions significantly promoted the proliferation of MC3T3-E1 cells. However, when the concentration of zinc ions was up to 4 and 8 ppm, zinc ions promoted the proliferation of MC3T3-E1 unapparently, or even negatively. These results demonstrated that an appropriate dose of zinc ions was biologically safe for cells, whereas excessive zinc ions were cytotoxic, leading to apoptosis.

2.7 Cell adhesion and spreading

Exposing to different doses of zinc ions, differences in the cell morphology could be observed under the laser scanning confocal fluorescence microscopy (Fig. 6). Compared with 0 ppm, more adherent cells, larger cell spreading area and more pseudopodia could be observed under 1 and 2 ppm zinc ions, which was opposite of 4 and 8 ppm. There were no obvious differences in 1 and 2 ppm. After treating with CM, the number of adherent cells and the cell morphology were better than that of control groups without CM, in other words, CM could facilitate the cell adhesion significantly (Fig. 7).

2.8 Western blotting analysis

The result of western blotting was performed to examine the osteogenic-related protein expression levels. Runx2 and OSX play a key role in the early stage of osteogenic differentiation and bone formation. Besides, OCN serves as a late marker of osteogenic differentiation and directly participates in the mineralization process. As was shown in Fig. 8, different doses of CM had different influence on cells. In this study, protein expression of Runx2, OSX and OCN of experimental groups (1, 2, 4, 8 ppm) were significantly higher than that of control group (0 ppm). The conditioned medium, achieved from endothelial cells treating with 2 ppm zinc ions, showed the best osteogenic capacity. Hence, 2 ppm CM was chosen to culture osteoblast to observe the expression of p-ERK. As one of the MAPK protein members, the expression of phosphorylated ERK protein peaked at 10 min. In Fig. 9, as the concentration of conditioned medium increased, the expression of ERK was found to be increased, indicating that conditioned medium activated the MAPK/ERK signaling pathway.

3. Discussion

Surface modification of titanium implants can significantly promote osseointegration around implants. In recent years, there have been more and more studies on zinc-modified implants, mainly focusing on its excellent ability to promote bone regeneration[24]. In addition, our previous studies have also found that zinc-modified titanium surface has good corrosion resistance and antibacterial ability. The excellent performance of zinc-modified implants mainly benefits from the introduction of zinc ions[25]. Zebrafish share 70 percent of their genetic homology with humans[26]. Nowadays, transgenic zebrafish model is commonly used to observe the early development of blood vessels[19]. Also, the physiological structure of the zebrafish's caudal fin is similar to that of human dermal bone, while it has strong regeneration ability[27]. Therefore, the zebrafish models can be simultaneously applied in the study of blood vessel development and bone regeneration *in vivo*. In this study, we established a Tg (Fli-1:EGFP)^{y1} zebrafish model to study the effects of zinc ions on angiogenesis and bone regeneration *in vivo*, and further analyzed the effects of vascular endothelial cells on osteoblasts under zinc ions through the *in vitro* cell models to reveal the specific mechanism between angiogenesis and osteogenesis under zinc ions exposure.

Our previous study proved that the modified titanium surfaces accelerated dental implant osseointegration, showing the good biocompatibility. In this study, Ti-NW-Zn could promote not only

angiogenesis which pretreated with VRI in Tg (Fli-1:EGFP)^{y1} zebrafish model (Fig. 1), but also osteogenesis in zebrafish fin amputation model (Fig. 2a), demonstrating that zinc ions played a vital role in angiogenesis actually. We also found that these positive effects depend on the zinc ion concentration, as the 1 ppm concentration of zinc ion seemed better to promote the regeneration of fin amputation compared with the control group and 2 ppm zinc ions group (Fig. 2b). In order to more accurately identify the amount of zinc ions released from Ti-NW-Zn surface, the zinc assay kit was used to find that the concentration of zinc ions released from Ti-NW-Zn surface was about 1 ppm (Fig. 4), which was consistent with the appropriate zinc ion concentration range that could promote the zebrafish fin regeneration. To further evaluate the biological safety of the zinc ion surround zebrafish and find out the best concentration range of released zinc ions which was benefit to zebrafish angiogenesis and bone regeneration, we assessed the survival curve of embryos and adult fish under zinc ions exposure. The results in Fig. 3 showed that, after exposed to zinc ions, median lethal concentration (LC) in 24 hpf-zebrafish embryos was more than 30 ppm, while that in adult fish was about 2 ppm. Although it was found that the survival rates in different periods were quite different, zebrafish embryos chronically exposed to zinc ions developed more degree of tolerance than adult fish, the concentration of zinc ions released from Ti-NW-Zn surface detected above was much lower than median lethal concentrations (LCs) of both embryos and adult fish. These results suggested that the zinc ion released from Ti-NW-Zn surface was at an appropriate and safe concentration range that could promote angiogenesis, osteogenesis and the survival rate of zebrafish. It was also proved that the zebrafish model was successfully applied in the study of blood vessel development and bone regeneration exposure to zinc ions.

Although studies have shown that zinc ions can not only affect the mineralization of osteoblasts by participating in the formation of bone salt[28], but also promote the migration of vascular endothelial cells, maintain proliferation and the growth and active state of apoptotic balance promotes its skeleton remodeling[29, 30], which suggests that one of the important ways for zinc ions to promote bone regeneration may be to induce the development of blood vessels in the bone. However, how did zinc ions affect angiogenesis and mineralization in vitro? And what is the relationship between angiogenesis and osteogenesis under zinc ions exposure? In our vitro cell models, we further explored the effects of vascular endothelial cells on osteoblasts under the exposure of zinc ions. As shown in Fig. 5, our results showed that appropriate concentration of zinc ions under 1 and 2 ppm both had positive effects on the proliferation of endothelial cells and osteoblast compared with the control group. It is known that cell adhesion and spreading is of vital importance in regulating cell behaviors[31]. In Fig. 6, we explored the relationship between zinc ions and adhesion morphology of osteoblast, finding that the concentration of zinc ions at 1–2 ppm could better promote the cell spreading and pseudopodia extending. Interestingly, the number and morphology of MC3T3 treated with conditioned medium (CM) were better than that of control groups without CM (Fig. 7), in other words, CM could improve the cell adhesion significantly. More and more studies have reported that some cytokines and growth factors the endothelial cells secreted had impacts on osteoblasts in some way, such as the most important pro-angiogenic factor, vascular endothelial growth factor (VEGF) and the key regulatory factor of VEGF, hypoxia inducing factor (HIF)[32, 33]. It could be inferred from these results and reports that appropriate zinc ions could promote

osteoblast behavior via induce the secret of beneficial substances from endothelial cells. We further found the promotion of cell differentiation under the exposure of 2 ppm CM as the expressions of osteogenic markers were all up-regulated (Fig. 8). These data suggested that low concentration of zinc ions positively regulated osteoblast behaviors while the condition was opposite as the concentration of zinc ions rising up.

MAPK signaling pathways have been recognized as important regulators of bone mass and osteoblast differentiation[34]. MAPK can be activated by different stresses, and influence apoptosis either positively or negatively[35]. In many cells, ERK inhibits apoptotic processes, whereas JNK and p38 signaling pathways contribute to the induction of apoptosis[36–38]. In Fig. 9, the expression of ERK increased as the concentration of Zn ions rising up. In this study, we found that 2 ppm CM had the best osteogenesis ability. Hence, the CM containing 2 ppm Zn ions, was selected for subsequent experiments to explore the role of MAPK/ERK signaling pathway in osteoblast behaviors and noticed the expression of p-ERK under Zn ions exposure. Interestingly, p-ERK is rapidly phosphorylated and activated peaked at 10 min before decreasing during this process. Generally speaking, MAPK maintains normal level[39]. It was zinc ions, acting as an extracellular, that stimulated the upstream of the MAPK signaling axis, activated the phosphorylation of MAPK and facilitated the downstream cascade reaction. The phosphorylation process was transient within 60 min and would return to normal rapidly. Once activated, the MAPK signaling pathway would respond and induce further reaction, which was like a cascade, and affected the regulation of osteogenic markers in the end[40, 41]. These results indicated that MAPK/ERK signaling pathway might participate in the osteoblast behavior under zinc ions exposure, but the specific mechanism still needs further study.

4. Conclusions

In summary, we developed an innovative zinc-modified titanium surface and showed good capacity of angiogenesis and osteogenesis. It was zinc ions that played a key role in this improved titanium surface. The results revealed that the appropriate concentration of zinc ions could promote the angiogenesis and osteogenesis in vivo and vitro, and could activate the MAPK/ERK signaling pathway, which affect the osteoblast differentiation. These data provided a theoretical basis for us to further improve the new titanium surface that how much zinc ions it released was suitable.

5. Materials And Methods

5.1 Materials preparation and characterization

The preparation method of Ti-NW-Zn surfaces and the identification of surface element was described previously[6]. After being washed and dried, the obtained samples were randomly assigned to 6-well plates with 2 ml ddH₂O.

5.2 Zinc ion release assay

Ti-NW-Zn samples were immersed in 6-well plates (2 ml PBS/well) at 37°C for 1 h, 1 day and 10 days. The concentrations of the released zinc ions in PBS solution were quantified using a Zinc assay kit (E011-1-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The absorbances were measured using a microplate reader (SpectraMax 190, MD, USA) at 630 nm wave-length. Three samples of each group were used in this assay.

5.3 Zebrafish embryo collection

Wild-type Zebrafish (*Danio rerio*) embryos were obtained from Wild-type AB strain adult zebrafish (*Danio rerio*), while Tg (Fli-1:EGFP)^{y1} zebrafish embryos were obtained from outcrosses of Tg (Fli-1:EGFP)^{y1} parents[18]. The spawning adults were offspring of parents obtained from Model Animal Research Center of Nanjing University and maintained in an aquatic animal breeding and reproduction system (HAISHENG, Shanghai, China), under standard conditions. All zebrafish studies were approved by the Institutional Animal Care and Use Committee at Nanjing Medical University. Groups of 1 male and 2 females were mated in translucent plastic tanks and embryos were obtained within 30 min after the onset of light in the morning. The eggs were collected immediately after fertilization and washed several times for 1 min using a 0.5 % bleach solution for disinfection. After this, clean eggs were incubated in 6-well plates at 28.5°C.

5.4 Zebrafish Rearing

Wild-type AB strain adult zebrafish (*Danio rerio*) were maintained in a recirculating aquatic system at 28.5°C with a 10/14-h dark/light cycle according to standards[18]. Circulating water in the aquarium was filtered by reverse osmosis (pH 7.0–7.5). All zebrafish was fasted for 1 day before starting the experiment. Male and female zebrafish were randomly used in experiments.

5.5 Angiogenesis observation of transgenic Zebrafish embryo

24 hpf (hours-post-fertilization) Tg (Fli-1:EGFP)^{y1} zebrafish embryos were pretreated with 75 nM vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor to inhibit the normal angiogenesis for 8 hours, then co-cultured on Ti-NW-Zn surfaces randomly pre-assigned to 6-well plates (10 embryos per well with 2 ml medium) at 28.5°C for 3 days to detect the defective vessels regeneration. In the 48 hpf, zebrafish were anesthetized (4% Tricaine) and the intersegmental blood vessels (ISVs) were observed under the inverted fluorescent microscope; In the 72 hpf, the subintestinal vessels (SIVs) of the yolk sac region gross morphological changes were observed under the inverted fluorescent microscope. Three parameters indicative of angiogenesis or vasodilation were measured: variation in the number of vessels, vessel thickness and the subintestinal venous plexus (SIVP) branching (angiogenic phenotype).

5.6 Survival test in embryos and adult fish

According to the concentration of zinc ion release from Ti-NW-Zn surfaces, all embryos were cultured in 6-well plates (10 embryos per well with 2 ml medium) at 28.5°C with different concentrations of zinc ions, including a nominal concentration of 0 (control group), as well as concentrations of 2, 4, 8, 16, 24 and 32

ppm zinc ions, which last for 120 hpf ($n = 10$ for each testing concentration), respectively. LC50 tests were then carried out according to these series groups. Adult fish were exposed to ddH₂O with different concentrations of zinc ions, including a nominal concentration of 0 (control group), as well as concentrations of 1 and 2 ppm zinc ions for 7 days for chronic exposures ($n = 5$ for each testing concentration), respectively. LC50 tests were also carried out according to these series groups. The exposure water was changed daily. The zebrafish were fasted during the entire experiment's duration.

5.7 Zebrafish fin amputation and regeneration test

7–10 months-old adult zebrafish with body weights of 0.3–0.5 g were initially anesthetized with tricaine (160 mg/L) for 5 minutes, then caudal fins were partially amputated using a #11 blade. All fish were then allowed to recover in open tank for 2 hours and randomly assigned to 500 ml culture vessels with different concentrations of zinc ions, where one was placed in each vessel and the solution was changed every 3 days and fed twice a day. Then, Alizarin Red Stain was used to detect the skeletal calcification of Zebrafish fin after executed. The experiment was carried out for 9 days and repeated three times.

5.8 Cell culture

Commercially available osteoblast-like cell line MC3T3-E1 (Cell Bank of Chinese Academy of Science, Shanghai, China) and human umbilical vein endothelial cells (HUVECs, ATCC, USA) were used in this study. MC3T3-E1 cells were cultured in α -Minimum Essential Medium (α -MEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA). HUVECs were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA), which containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA). Both MC3T3-E1 and HUVECs were maintained in an incubator containing 5% CO₂ and 95% air at 37°C. The fresh complete medium changed every two days. When reaching 80% cell confluence, the cells were passaged every three or four days.

5.9 Collection and preparation of conditioned medium (CM)

HUVECs were seeded in the 6-well plates and incubated with Zn ions at a range of doses (0, 1, 2, 4, 8 ppm). When the cell confluence was up to 80%, the culture medium of each group was collected and centrifuged (1000 rpm, 15 min) under sterile conditions. After collecting the supernatant, it was mixed with α -MEM containing 10% FBS and 1% penicillin/streptomycin at a ratio of 1:1 to get conditioned medium (CM). CM was placed in a -20°C refrigerator for later use.

5.10 Cell proliferation assay

CCK-8 kit was used to assess the cell proliferation. MC3T3-E1 cells (3×10^3 cells/well) and HUVECs (3×10^3 cells/well) were seeded in the 96-well plates and treated with Zn ions at different concentrations (0, 1, 2, 4, 8 ppm). MC3T3-E1 cells were cultured for 1, 3 and 6 days, while HUVECs were cultured for 1, 2 and 3 days. Afterwards, 100 μ l fresh medium which containing 10 μ l of CCK-8 solution (Beyotime, Shanghai,

China) was added in each well, incubating for another 2 hours at 37°C. The absorbance of each well was measured by a microplate spectrophotometer (Spectramax 190, CA, USA) at the wavelength of 450 nm.

5.11 Cell adhesion and spreading assay

Commercially pure titanium (99.5 wt% purity, Alfa Aesar, USA) disks were polished with 600, 800, 1200 and 1500-grit silicon carbide abrasive papers. MC3T3-E1 cells (5×10^3 cells/well) were seeding on the surface of polished titanium disks in the 96-well plates, treating with or without CM as described above for 8 hours. Afterwards, each sample was washed with PBS and fixed with 4% paraformaldehyde at room temperature for 10 minutes. To observe cell morphology on titanium, cells were stained with 100 nM Rhodamine phalloidin (Cytoskeleton, USA) for 30 minutes and 4', 6' -diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China) for 2 minutes in the dark. The cell morphology was observed under a laser scanning confocal fluorescence microscopy (LSM710NLO; Zeiss, Jena, Germany) at 100 x and 200 x magnification.

5.12 Western blotting

MC3T3-E1 cells (2×10^5 cells/well) were seeded in 6-well plates and cultured with CM described above, after which cells were washed with pre-cooled PBS and lysed with RIPA buffer containing 1% PMSF. Protein samples were separated after electrophoresis, transferred to PVDF membranes (Millipore, Billerica, MA, USA), blocked in protein free rapid blocking buffer (EpiZyme, Shanghai, China) for 10 minutes, and incubated with primary antibodies specific for Runx2 (12556, CST, USA), OSX (ab22552; Abcam, USA), OCN (ab93876, Abcam, USA), ERK (4695, CST, USA), p-ERK (4370, CST, USA) and GAPDH (BM0627, Boster, China) at 4°C overnight. Afterwards, PVDF membranes were incubated with secondary antibodies (ZB-2301; Goat anti-Rabbit IgG, ZSGB-BIO, China) for 2 h at room temperature and exposed to ECL substrate solution (NCM Biotech, Suzhou, China). GAPDH served as a loading control. The cell experiments were performed in triplicate.

5.13 Statistics

Statistical analysis was performed on SPSS 22.0 software (SPSS, Inc., Chicago, IL, USA) using one-way analysis of variance (ANOVA) with Student-Newman-Keuls (SNK) method for multiple comparisons. The significant changes were set as * $P < 0.05$, ** $P < 0.01$.

Abbreviations

CM conditioned medium

Ti-NW-Zn titanium surface modified with zinc-containing nanowires

cp-Ti commercially pure titanium

Data availability statement

The data that support the findings of this study are available upon reasonable request from the authors.

Declarations

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Conflict of interest

All authors declare that they have no conflicts of interest with the contents of this article.

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Author contributions

WQZ contributed to design, data acquisition and analysis, and drafted the manuscript. KL and SS contributed to data acquisition and analysis. WC and YL contributed to design and data analysis. The corresponding author JQ contributed to conception, design, data interpretation, and critically revised the manuscript. All authors give final approval and agree to be accountable for all aspects of the work.

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Figures

Figure 1

Angiogenesis of transgenic Zebrafish in the group of control, cp-Ti and Ti-NW-Zn.

A

B

Figure 2

Zebrafish fin amputation and regeneration test. a) Effect of control group and Ti-NW-Zn surface on osteogenesis, b) Effect of different concentrations of zinc ions on osteogenesis: 0, 1 and 2 ppm.

Embryos Acute Toxicity

A

Adult Fish Acute Toxicity

B

Figure 3

Survival rate in a) embryos and b) adult fish.

Zn ion release

Figure 4

Concentrations of Zn ion released from Ti-NW-Zn samples into PBS after 4 h, 1 days, 4 days and 7 days.

A

B

Figure 5

a) Proliferation of human umbilical vein endothelial cells HUVEC with different concentrations of zinc ions for 1, 2 and 3 days, b) Proliferation of osteoblast-like MC3T3-E1 cells for 1, 3 and 6 days. Results are presented as Mean $\pm$ SD (* $P < 0.05$, ** $P < 0.01$).

Figure 6

Cell adhesion and spreading of osteoblasts cultured with different concentrations of zinc ions. Actin (red), and cell nucleus (blue). Upper panel displays at 200× magnification. Lower panel displays at 400× magnification.

Figure 7

Cell adhesion and spreading of osteoblasts cultured with different concentrations of CM. Actin (red), and cell nucleus (blue). Upper panel displays at 200× magnification. Lower panel displays at 400× magnification.

Figure 8

Protein expression levels of osteogenic markers Runx2, OSX and OCN of osteoblast-like MC3T3-E1 cells in different concentrations of CM (Runx2, Runt-related transcription factor 2, OSX, Osterix, OCN, Osteocalcin).

Figure 9

Activation of MAPK/ERK Signaling by different concentrations of CM. a) Protein expressions of vital members for MAPK/ERK signaling pathway in MC3T3-E1 cells treated with different concentrations of CM, b) Protein expressions of vital members for MAPK/ERK pathway in MC3T3-E1 cells exposed to CM of 2 ppm at different time points, c) Histogram showed normalized ratios of p-ERK/ERK. Results were presented as Mean $\pm$ SD (* $P < 0.05$, ** $P < 0.01$).

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