Original Article

Investigation of the Antiangiogenic Properties of Zoledronic Acid by Using Chorioallantoic Membrane Model

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Abstract

Objective: Zoledronic acid (ZA) is a bisphosphonate-derived agent used in osteoporotic clinical pathologies to prevent the development of complications such as fractures and hypercalcemia by regulating bone metabolism. Studies have been conducted on the antiangiogenic efficacy of this agent, which also has other systemic side effects. In this study, the dose-dependent antiangiogenic activity of ZA was investigated on the chorioallantoic membrane model (CAM).

Methods: Three doses (10^{-4}, 10^{-5}, and 10^{-6} M concentrations) of drug pellets were prepared with ZA and another pellet was prepared as the positive control group with vascular endothelial growth factor (VEGF) inhibitor agent bevacizumab (10^{-6} M concentration). Thereafter all pellets were placed on chorioallantoic membranes on the fourth day of egg incubation. All eggs were evaluated for capillary development four days after the drug application.

Results: The highest antiangiogenic effect was detected in the positive control group. Moreover, incremental antiangiogenic effects were detected with average scores of .9, 1.1, 1.2 in 10^{-6}, 10^{-5}, and 10^{-4} M concentrations of ZA groups respectively.

Conclusion: Our findings supported that ZA has dose-dependent antiangiogenic effects. This result suggests that different dosing may be required in cases where angiogenesis is therapeutic.

Keywords

zoledronic acid, dose-dependent effects, antiangiogenesis

Introduction

Zoledronic acid (ZA) is a potent bisphosphonate that is used for osteoporosis treatment via the intravenous route. Previous clinical results indicate that this agent is well tolerated and an effective method for prohibiting osteoporosis-related bone fractures.[1] By the way some side effects were reported in patients during ZA utilization. Some reported side effects can be listed as acute fever, influenza-like symptoms, renal toxicity, osteonecrosis, and cardiac arrhythmias.[2]

Zoledronic acid regulates higher blood calcium levels by decreasing calcium released from bone to blood and also it has metabolic effects on the organism. Therefore, the asymptomatic or symptomatic hypocalcemia cases were reported related with it is potent osteoclastic inhibitory effects.[1,3] However, its effects on the body are not limited to this. It has been reported that affected acute phase reactants such as neutrophils, lymphocytes, procollagen type 1 N-terminal propeptide, which show systemic effects due to ZA usage.[4] There are reports of the use of ZA in various diseases in children as well, and its advantages in preventing fractures that may occur in adolescent group diseases have been emphasized.[5] As well as these reactions, the antiangiogenic properties of ZA are reported in different kinds of cancer cell

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lines.[6,7] However, there is limited basic descriptive study investigating the effects of ZA on physiological or embryological angiogenesis. Although some studies have investigated the antiangiogenic effects of ZA in vivo and in vitro on the endothelium and chorioallantoic membrane model (CAM), dose-dependent effects have not been evaluated.[8,9]

Materials and Method

CAM model was created for evaluation of antiangiogenic effects with different doses of ZA according to previous reports.[10,11] Although the CAM model did not require ethical approval due to its in-vitro nature, all steps were performed in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals.

Designation of Study

Groups. Group 1: The negative control group was created with drug-free pellets for investigation of physiologic angiogenesis during embryo development with 10 eggs.

Group 2: The positive control group was formed by using Bevacizumab (Avastin™; Roche, Grenzach, Germany) (well known vascular endothelial growth factor inhibitor agent) embed pellets (10^{-6} M) as stated in previous reports[12] to determine standard angiogenesis inhibition. (n:10 eggs)

Three study groups were created using different dosages of ZA (Zoledronic acid-Zometa®, Novartis Pharmaceuticals Corp, East Hanover, NJ) as follows:

Group 3: The 10^{-6} M concentrations ZA embed pellets were administered to 10 eggs

Group 4: The 10^{-5} M concentrations ZA embed pellets were administered to 10 eggs

Group 5: The 10^{-4} M concentrations ZA embed pellets were administered to 10 eggs

Preparation of Pellets

The pellets were prepared by mixing agarose (2.5%) and distilled water. The obtained pellets were sterilized by using autoclave and sterilized pellets were cooled into sterile plates after the addition of evaluated drugs with different dosages.

The drug dosages were settled with the addition of the targeted drug to cooled agarose by 1 IU/10 µL dilutions until reaching wanted molar (M) concentration. Agar drops for application to each egg were prepared using a micropipette to obtain micropellets after reaching the expected concentration.

Arrangement of the Chorioallantoic Membrane (CAM)

The fertilized Ross 308 eggs were used for model creation. All eggshells were cleaned with disinfectants before placement to incubator. Thereafter, eggs were stored in an incubator under controlled humidity (80%) and temperature (37.5°C). The eggs were cleaned again on the fourth day and the viability of the embryos was checked by making an oval hole on the eggshell. Those who were not alive were excluded from the study. The viable ones were grouped into 10 and micropellets were placed on the CAM. Then the eggs were covered with sterile tape and placed back in the incubator. After 4 days of micropellet application, eggs were removed again and angiogenesis was evaluated.

The evaluation of angiogenesis was evaluated according to surrounding capillary density (as described previously) and the antiangiogenic scores were determined according to scoring system that described in previous literature.[12-14]

The Scoring System

Normal capillary development: score 0 (Figure 1A)

Partially negligible change in capillary bed: score .5 (Figure 1B)

Less than twice the pellet area in the capillary bed, but significant reduction: score 1 (Figure 1C)

Significant reduction of twice or more of the pellet area in the capillary bed: score 2 (Figure 1D)

After determination of antiangiogenic scores in each egg, the average score was calculated according to following formula for each group: ([2x score 2 eggs] + [1x score 1 eggs])/Number of total eggs

Average score lower than .5 accepted as insignificant antiangiogenic effect

Average score between .5 and 1 accepted moderate antiangiogenic effect

Average score higher than 1 accepted as strong antiangiogenic effect

Statistical Analysis. We used to calculate score 2, score 1, and lower score (<.5) eggs separately. As a result, we obtained a sum of values. To calculate an average, we used the formula that mentioned above and got a general average.

Results

Normal capillary development was detected in the control group. The reference antiangiogenic effects were determined in Bevacizumab 10^{-6} M group. The dose-dependent incremental antiangiogenic effects were detected in zoledronic acid 10^{-6} M, 10^{-5} M, 10^{-4} M groups, respectively. The antiangiogenic score distribution for each group was demonstrated in Figure 2.

The average antiangiogenic score of bevacizumab 10^{-6} M was found as 1.3 (strong antiangiogenic effect) and closest average score was observed in zoledronic acid 10^{-4} M group (1.2: strong antiangiogenic effect). It was found that the mean score decreased at lower doses (the lowest score was detected in zoledronic acid 10^{-6} M group as .9:moderate antiangiogenic effect). The average scores were listed in Table 1.

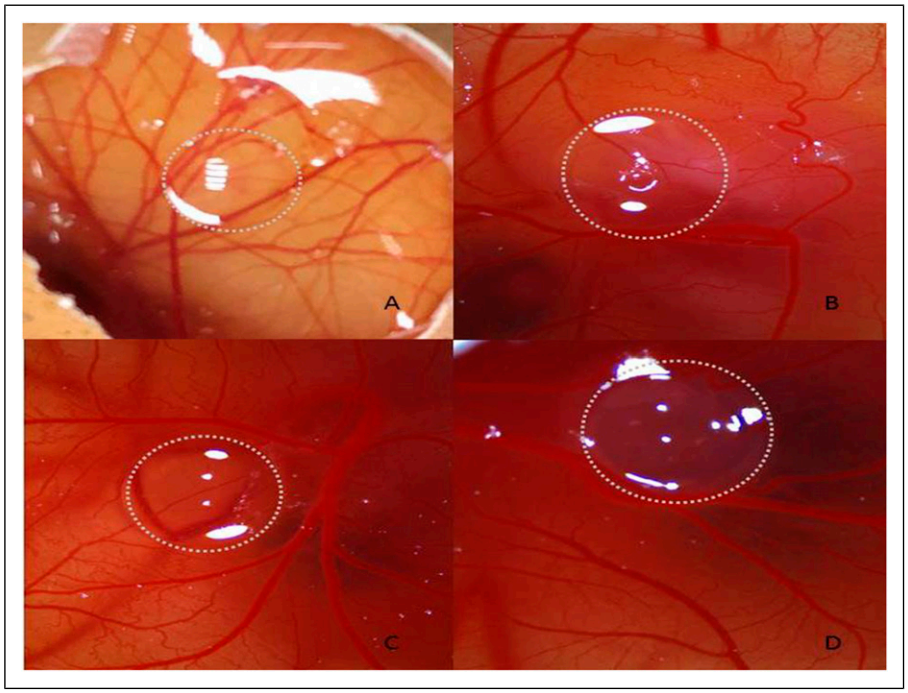

Figure 1. A. Normal capillary development of chorioallantoic membrane, B. Score .5: Minimal changes o capillary bed, C. Score 1.0: The decreased capillary growth of almost all areas of drug pellet, D. Score 2: The marked reduction on the capillary bed covered more than the drug pellet area, dotted circles demonstrate the drug pellet area.

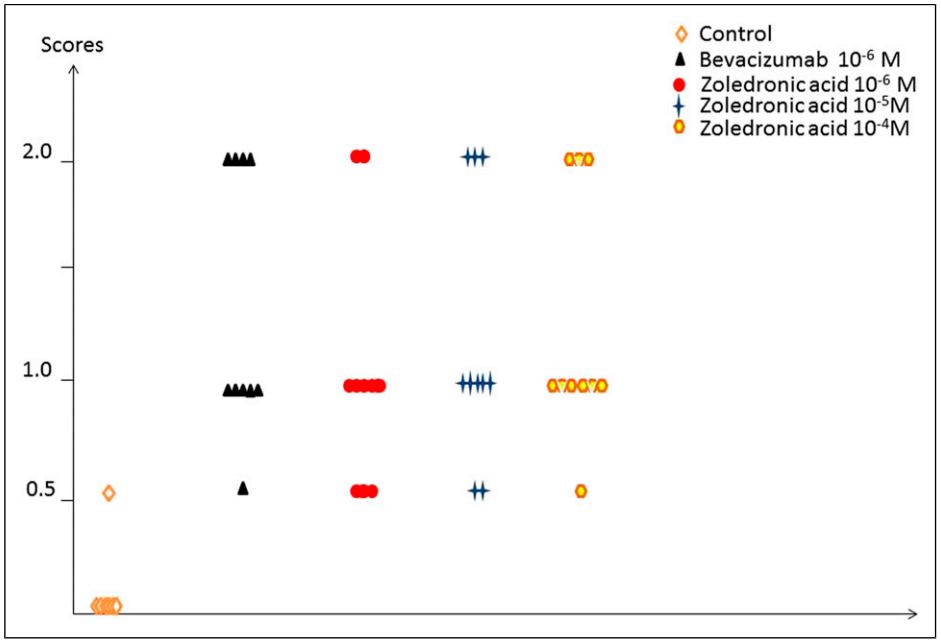

Figure 2. Scores distribution and comparison of chorioallantoic membranes in each group.

Discussion

Our results support that zoledronic acid shows the dose depended on antiangiogenic effects. To the best of our knowledge, although studies are showing the antiangiogenic effects of zoledronic acid, there is no study showing a dose-dependent effect.

Actually, ZA is an actively used molecule in the treatment of conditions that result in bone loss, such as metastatic bone diseases, but side effects such as osteonecrosis limit its use.

Table 1. Average scores of drug groups.

Groups	Average Scores	Antiangiogenesis
Bevacizumab 10^{-6} M	1.3	Strong
Zoledronic acid 10^{-6} M	0.9	Moderate
Zoledronic acid 10^{-5} M	1.1	Strong
Zoledronic acid 10^{-4} M	1.2	Strong

Although it has been stated that conditions such as myeloma, previous surgical procedures, incidence of additional diseases, and smoking increase the risk of side effects; it has been emphasized that one of the most important factors is dose-related.[14] In studies on subchondral bone resorption and turnover, which contributes to the pathogenesis of osteoarthritis, it has been stated that treatment with bisphosphonates reduces cartilage loss by inhibiting bone resorption in a dose-dependent manner. For this reason, it has been emphasized that different doses may have different effects in terms of treatment efficacy and incidence of side effects.[14,15] There are also reports that ZA has other metabolic effects. In studies on increasing muscle strength, it has been mentioned that it may have anabolic or catabolic effects on cells. It has been reported to inhibit smooth muscle cell proliferation, migration, and adhesion and induce membrane damage in in vitro studies. They stated that this effect may cause muscle or joint complaints and even fever in humans.[16] In studies on stem cells, it has been reported that continued ZA exposure causes over-regulation of genes that affect alkaline phosphatase activity. According to the results obtained, it was revealed that while low doses were effective only on osteogenic differentiation, it negatively affected root cell viability and induced apoptosis at high doses.[17] In studies on bone cancer, the effects of ZA and estrogen on the bone microenvironment were examined and it was stated that ZA prevents bone metastases by preventing tumor growth. In the same study, it was reported that ZA also regressed vascular development by negatively affecting vascular endothelial growth factor (VEGF) concentrations.[18] Similar to our study, in the study of Yamada et al., the antiangiogenic effects of ZA were investigated on cell culture and CAM model.[19] In this study, ZA was shown to affect cell proliferation at low doses, and it was reported to induce apoptosis at high doses. The hypothesis that this effect may be due to the inhibition of prenylation of small G-proteins has been discussed. However, ZA has been shown to inhibit angiogenesis on the CAM model, but it has not been determined to what extent different doses affect angiogenesis.[19] Our results showed that similar to other dose-dependent cellular effects, ZA has varying degrees of antiangiogenic activity in a dose-dependent manner.

In conclusion, according to our findings, ZA has different degrees of antiangiogenic potential in a dose-dependent manner. Therefore, different doses can be evaluated for different clinical conditions. For instance, lower doses can be safer in the patient who needs critical follow-up for circulatory events and higher doses might be beneficial in the patient group where it is aimed to keep tumorigenesis under control.

Limitations of Study: The main limitation of the study was that the dose-dependent antiangiogenic effects of ZA were demonstrated without the biological effects to elucidate its mechanism of action were not investigated. Another main limitation is due to the in-vitro nature of the study. Therefore, the results should be made more consistent with animal and human studies.

Declaration of Conflicting Interests

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