Plates made from magnesium alloy with a long period stacking ordered structure promote bone formation in a rabbit fracture model

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Supplementary Material and Methods

***In vitro* osteoblastic differentiation with or without Mg^{2+}, Zn^{2+}, Y^{3+} or Al^{3+}**

Human adipose-derived stem cells (ADSCs) were cultured in a culture dish in DMEM containing 10% FBS. After a 5-day incubation, cells were resuspended in osteogenic medium (Osteoblast-Inducer Reagent, Takara Bio Inc.) and seeded in 24-well plates (2.0×10^4 cells per well). These media to which nothing was added were used as controls. Magnesium chloride ($MgCl_2$) solution was added to control medium and Mg^{2+} concentration was adjusted to 10 mMOL (Mg 10mMOL). Based on this concentration, according to the ratio Mg-0.56Zn-1.5Y-0.15Al (at.%), zinc sulphate (ZnSO4) solution, yttrium (Y) or aluminium chloride ($AlCl_3$) was added to the control medium to bring Zn^{2+}, Y^{3+}, or Al^{3+} concentration to 0.05 mMOL (Zn 0.05mMOL), 0.15 mMOL (Y 0.15mMOL) or 0.015 mMOL (Al 0.015mMOL), respectively. Culture medium was changed every 4 days. After 20 days of culture, cells were washed with PBS three times, and fixed in 100% methanol at -20°C. After washing with dH_2O, cells were stained with Alizarin Red S staining solution (pH6.3). Staining solution was washed away with dH_2O, and stained cells were air-dried and observed under a microscope. To quantitate mineralization, Alizarin Red S was extracted in 5% formic acid and assessed for absorbance at 450 nm using an iMark Microplate Absorbance Reader (BIO-RAD). Values were normalized to those seen in untreated samples.

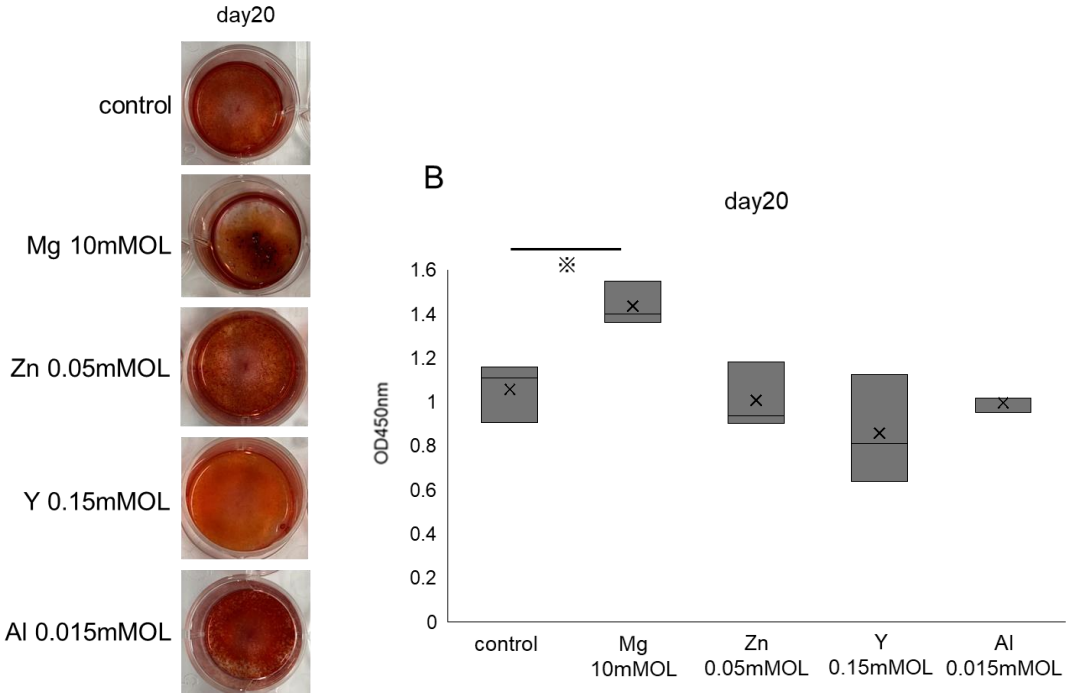
Evaluation of hydrogen gas in the fracture model

Internal fixation was performed on the right tibia of six-month-old female Japanese white rabbits in either the Ti or Mg plate. Ultrasonography was performed at 1 week postoperatively to examine the presence of the free air on the plate in the tibial long-axis image.

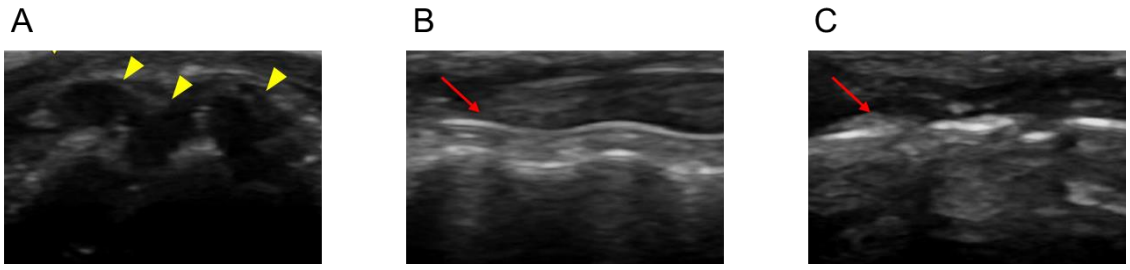
3 ml of air was injected subcutaneously into the lower leg of another rabbit with a needle to see how the free air would appear on ultrasonography.

The testing equipment was a Venue Go (GE healthcare Japan, Hino, Tokyo, Japan) and ultrasound probe was high-frequency linear type probe with a central frequency of 12MHz.

Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure Legend

Supplementary Figure 1. Treatment with low dose Zn, Y or Al ions dose not stimulate mineralization of human adipose-derived stem cells (ADSCs).

ADSCs were cultured in osteogenic medium in 24-well plates (2.0×10^4 cells per well) in the presence or absence of 10 mMOL Mg^{2+}, 0.05 mMOL Zn^{2+}, 0.15 mMOL Y^{3+} or 0.015 mMOL Al^{3+}. Culture medium was changed every 4 days. Alizarin Red S staining was performed on day 20, and cells were observed microscopically (A). Subsequently, Alizarin Red S in each culture well was extracted in 5% formic acid and assessed for absorbance at 450 nm using an iMark Microplate Absorbance Reader (BIO-RAD) to quantitate mineralization. Values are normalized to untreated samples. Data represents mean relative absorbance ± SD at day 20 (B) (each $n = 3$, $*p < 0.05$).

Supplementary Figure 2. Rabbits with fractures treated with Mg plates shown no evidence of hydrogen gas generation at 1 week postoperatively, based on ultrasonography.

Internal fixation was performed on the right tibia of six-month-old female Japanese white rabbits using either Ti or Mg plates. In a control rabbit that had not undergone any kind of surgical procedure, we subcutaneously injected 3 ml of air into the lower leg to check how the free air would appear on ultrasonography (A). Ultrasonography was performed at 1 week postoperatively to confirm the presence of free air on plates in the tibial long-axis image. None of the rabbits harboring Ti or Mg plates showed a free air image on ultrasonography at 1 week postoperatively (B and C).

(A) Shown is free air (yellow arrowheads) detected by ultrasonography.

(B) Postoperative ultrasonography showing no free air on the Ti plate (red arrow) at 1 week.

(C) Postoperative ultrasonography showing no free air on the Mg plate (red arrow) at 1 week.