due to superior biocompatibility and corrosion resistance compared to other metallic alloys. The Ti alloys, however, have a disadvantage in wear resistance due to lower elastic modulus/hardness [12] and alumina ceramics are attractive as an implant material for the bearing surface of prosthetic arthroplasty due to their high abrasion resistance and hardness. Vickers hardness of Ti alloy is approximately 340 HV and that of alumina ceramics is approximately 2000 HV [13,14]. On the other hand, fracture toughness of the alumina ceramics (less than 4 MPa $m^{1/2}$) is poor when compared to that of metals such as CoCr alloy (less than 100 MPa $m^{1/2}$) [13,15] and fabrication of complex shapes of the ceramics is difficult. As to the Young's modulus, the modulus of CoCr alloys (220–230 GPa) is about twice than that of Ti alloys (100–110 GPa) [16]. Thus, CoCr based alloys possess superior stiffness and toughness, furthermore, a recent method [17] significantly improved the mechanical property of CoCr alloys. Therefore, the alloys have been used as an alternative to Ti alloys and alumina ceramics in the orthopedics industry. However, several studies of CoCr alloy implantation have reported the possibility of risks to successful arthroplasty when using such alloys [4].

Soluble ions such as cobalt, which is a major component of CoCr alloy implants, are known to promote bone resorption [18] and inhibit proliferation / mineralization of bone marrow cells [19]. The metal ions also stimulate inflammatory cytokines and have a cytotoxic effect on cells surrounding the implants [4,20–24]. Thus, released metal ions from the CoCr implants might disturb local bone homeostasis at the bone-implant interface, leading to bone loss and thus resulting in aseptic loosening of the implant. If we control the bone homeostasis to promote bone formation around the implants, the bone loss could be prevented. One possible way is to supply osteogenic function to the implants prior to their implantation, because Co ions are reported to inhibit the osteogenic differentiation capability of marrow mesenchymal cells [19]. We thus culture-expanded the number of mesenchymal cells from bone marrow and then loaded the cells on the CoCr alloys. We further cultured the cells loaded CoCr alloys in the presence of ascorbic acid, β-glycerophosphate and dexamethasone and then implanted into rabbit bone defects.

As shown in Figure 1, positive staining for Alizarin red S on the CoCr alloy implants with mesenchymal cells loading indicated the appearance of osteoblasts and bone matrix formation on the alloy surface [25]. Thus, we succeeded in generating thin layer of tissue engineered bone on one side of the CoCr alloy. The alloys were implanted with due consideration given to the differences of the triangular cross-sectional geometry of the tibia as described later in the methods section. After 3 weeks, all the implants were analyzed. In mechanical testing, the non-cell loaded sides in all the implants detached first even though the tibia surface area facing the implants was larger. There was a significant difference between the pull-out force of the non-cell loaded surface and that of the cell loaded surface (Figure 2). Furthermore, histological and electron microscope images of the cell loaded surface exhibited abundant new bone formation (Figures 3,4). These findings suggest that the newly formed bone on the cell loaded CoCr surface interlocked the implants and, importantly, tight fixation could be obtained just 3 weeks after implantation. We reported that the tissue engineered bone on the ceramics surface well integrated to host bone [26] and also experienced good clinical cases using alumina ankle arthroplasty [6], therefore prolonged stable fixation between the CoCr surface and host bone could be expected.

While the use of poly(methyl methacrylate) (PMMA) bone cements may show good implant fixation, their disadvantages have been reported. They include toxicity of PMMA [27], decreased bone stock at

the time of revision, difficulty in the treatment after infection around the implants and weakening of the fixation over time [28]. As a result, various authors have advocated cementless fixation, especially for young and active patients [29,30], and some retrospective studies have reported better results for cementless fixation compared to cemented [31,32]. The main disadvantage of the cementless fixation, however, is poor fixation in the early period after the implantation. To promote early fixation, rough surfaces of the implants were adopted. One type of surface is the metal-bead coated implant as used in the present study. However, bony ingrowth into the rough surface may take some months so the issue with the cementless fixation is how to obtain stable early fixation [33–35]. Therefore, the use of CoCr alloy implants is inappropriate for the purpose of cementless fixation because they impair the osteogenesis around their implants as discussed above. In this study, loading and culturing the cells on the CoCr implants prior to implantation achieved stable early bone fixation. These findings suggest that the tissue engineered CoCr implants might be used for cementless fixation in joint arthroplasty.

Coating the metal implants with the chemically synthetic hydroxyapatite (HA) using plasma spray has also been introduced in an attempt to provide early as well as long-term fixation [36]. However, uniform coating of HA in the rough surfaces of the prosthesis is difficult. Our tissue engineering approach shows the appearance of bone forming osteoblasts as well as bone matrix on the surface of CoCr implants. The approach needs seeding of mesenchymal cells on the rough surface of the implants. As the cells are suspended in a culture medium, they can easily inhabit even deep surface areas. Therefore, the mesenchymal cells are distributed uniformly, and further differentiate into osteoblasts which fabricate bone matrix. Importantly, the matrix contains low crystallized carbonate containing HA, therefore so called biological HA exist in the tissue engineered implants [9]. Biological HA is known to show greater biocompatibility compared to synthetic HA. These facts indicate that our approach enabled us to coat the implant surface uniformly with a viable HA layer. Moreover, active osteoblasts with osteogenic function are present in the implant surface. Thus, our method is unique with regard to coating HA concomitant with active osteoblasts on the CoCr alloy implant surface. That is to say, we can generate an HA coating layer with osteogenic function.

In this paper, we described the tissue engineering approach using mesenchymal cells on metallic implants intended for orthopedic applications; however we believe this approach might also be appropriate for other applications such as maxillofacial prosthesis. Further studies are needed to provide evidence of suitability in other clinical applications.

3. Materials and Methods

3.1. Preparation of Marrow Mesenchymal Cells and Implants

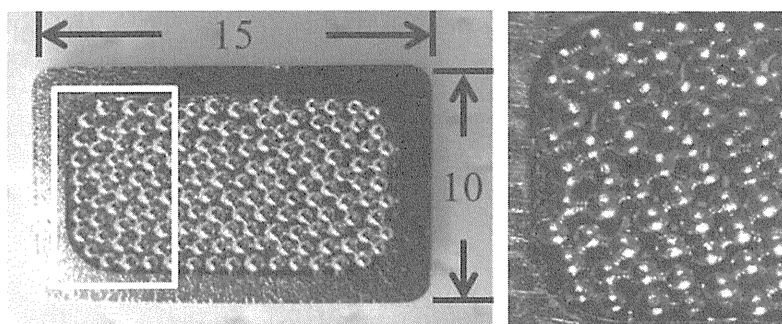
Approval from the animal experimental review board of Nara Medical University was obtained prior to the start of the study. Mature male Japanese white rabbits weighing about 3.0 kg were used. Six rabbits were anesthetized and 2 mL of bone marrow was aspirated from the humerus of each rabbit. The bone marrow aspirates were placed in a T-75 flask (Coster Co., Cambridge, MA) and mixed with 15 mL of Eagle mimimum essential medium (MEM; Nacalai Tesque, Inc., Kyoto, Japan) containing 15% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS) and a mixture of antibiotics (100 Unit/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B; Sigma Chemicals Co.,

St. Louis, MO). The primary cultures were maintained in a 5% CO_2 atmosphere at 37 °C. After 2 days of culture, non-adherent cells were removed and 20 mL of the culture medium was added. The following medium change was done at 3 times per week. Floating cells (red blood cells and hematopoietic cells) were removed during these medium changes. After 2 weeks of the primary culture, the number of adherent cells with fibroblastic shape increased. The adherent cells were termed mesenchymal cells in the present study.

The cultured mesenchymal cells were then released with 0.25% trypsin, centrifuged at 900 rpm for 5 min at room temperature and condensed at a cell density of 1×10^6 cells/mL. Cell number was counted by CDA-1000 (Sysmex, Kobe, Japan). Two milliliter of the cell suspension (2×10^6 cells) was applied on one side of an implant surface and incubated overnight, then subcultured. The subcultures were done on six-well plates for 2 weeks with MEM containing 15% FBS, 10^{-8} M dexamethasone, 10 mM β-glycerophosphate, and 0.07 mM ascorbic acid. This allowed differentiation of the mesenchymal cells to osteoblasts and formation of bone matrix on the implant surface [7,9–11].

The implants used in the present study were Cobalt-Chromium (CoCr) based alloy (Japan Medical Materials Co., Osaka, Japan) measuring 15 × 10 × 2 mm. The surfaces were coated with a single layer of 710 to 850 μm diameter CoCr based alloy beads (Figure 5).

Figure 5. Cobalt-Chromium (CoCr) based alloy. The surfaces were coated with a single layer of 710 to 850 μm diameter CoCr based alloy beads. Right figure is a magnified image of the rectangular area in the left figure. The size of the alloy is 15 × 10 × 2 mm.

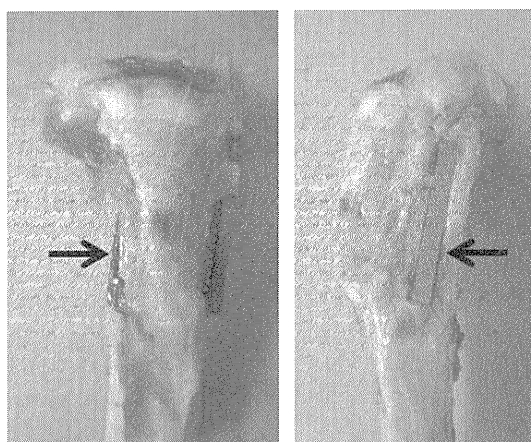


3.2. Implantation

After the subculturing, the CoCr alloys were implanted under general anesthesia bilaterally in the tibias of the rabbits from which bone marrow had been aspirated; therefore we used autogeneic marrow mesenchymal cells. Using sterile surgical technique, a 2-cm longitudinal skin incision was made on the anteromedial aspect of the proximal metaphysis of the tibia. The fascia and periosteum were incised and retracted to expose the tibial cortex. Using a 2-mm electric steel dental burr, a 15 × 10 × 2 mm opening was made from the medial cortex to the lateral cortex parallel to the longitudinal axis of the tibial metaphysis. After irrigating the opening with saline, the CoCr alloy was implanted in the frontal direction, perforating the tibia, and protruding from the medial-to-lateral cortex symmetrically with respect to the longitudinal axis of the tibial metaphysis [37,38]. The cell loaded side was implanted facing the anterior surface of the tibia in the right leg, and facing the posterior surface in the left leg. The interface between the implant and bone was not the same on both sides due to the triangular cross-sectional geometry of the tibia; the anterior side had a smaller facing area than the posterior side.

Therefore the CoCr alloys were implanted in opposite directions in the right and left tibias to offset differences in test sites (Figure 6). Three weeks after implantation, each rabbit was sacrificed, and the tibias with implants were extracted to evaluate the pull-out force in the mechanical test.

Figure 6. Implantation of the CoCr alloy in rabbit tibial bone defect. Left and right figure are anterior-posterior and lateral view, respectively. Arrows indicate the implant which inserted into the bone defect.



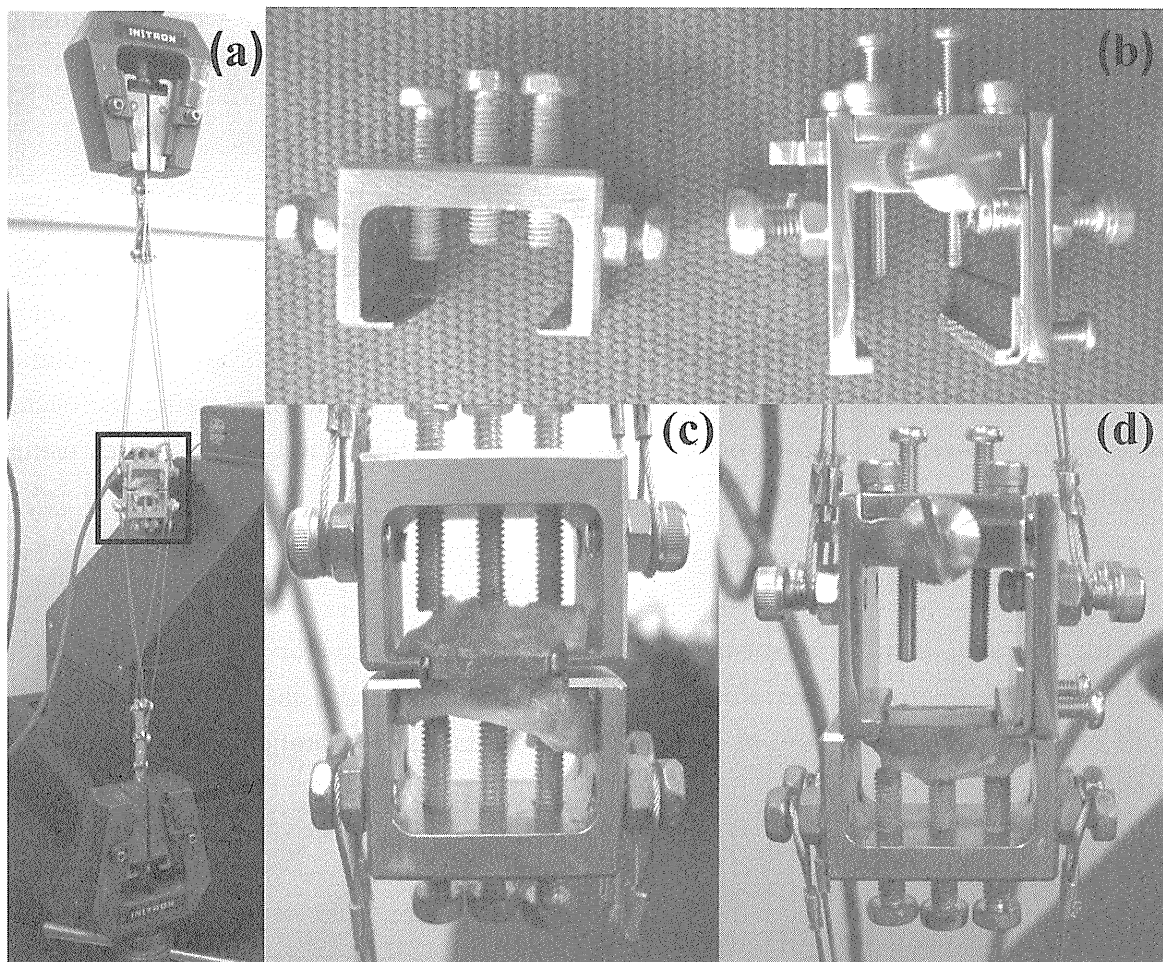
3.3. Alizarin Red S Staining

For the Alizarin Red S staining, the subcultured cell layers were washed twice with PBS (−) (phosphate-buffered saline without Ca^{2+} and Mg^{2+}). After fixing with 95% ethanol (4 °C, 15 min), they were washed with deionized water and then 0.4 mL of Alizarin Red S (Nacalai Tesque Inc.) solution dissolved in the PBS (5 mg/mL) was added to the culture well. After 1 min, the wells were washed several times with deionized water to remove the remaining stain.

3.4. Measurement of Pull-out Force

Eight of the 12 limbs with implants were examined by mechanical test. Two implants were excluded because of loosening due to infection in one limb. Thus, tibias from 6 limbs were used for this test. The specimens were trimmed with the implants sandwiched above and below the tibia, and were prepared for the detachment test using the method of Nakamura [37]. These specimens were positioned horizontally, and the upper and lower bone cortex was placed in a special grip. Mechanical pull-out force was evaluated under tension at a crosshead speed of 0.5 mm/min (Figure 7). First, there was detachment of the implant from the side with weaker bonding. Maximum pull-out force was measured at detachment of the implant from bone. After the first pull-out test, a second pull-out test between the CoCr implant which was held directly with another grip and remaining cortex bone on other side was performed.

Figure 7. Mechanical test (pull-out force). (**a**) Whole image of the mechanical test; (**b**) Left photo shows a grip for first pull-out test and right shows a grip for the second pull-out test. The grip for the second test was used to grasp the implant directly; (**c**) Image of first pull-out test. This is a magnified image of the rectangular area in Figure a; (**d**) Image of the second pull-out test.



3.5. Histological Examination and Electron Microscope Analysis

Four of the 12 limbs with implants were examined by histological examination and electron microscope analysis. These specimens were fixed with 10% neutral buffered formalin, and then embedded in polyester resin (Ohken, Tokyo, Japan). The resin-embedded tissue blocks were sectioned into midportion perpendicularly to the long axis of the implants using a sawing machine (BS3000N, Exakt, Norderstedt, Germany), and ground to final thickness of about 100 μm using a microgrinding machine (MG4000, Exakt, Norderstedt, Germany). The undecalcified sections were then stained with toluidine blue [11].

Each block was sectioned at the implantation site and the surface of the section was polished using waterproof-paper. After setting polished sections in the chamber, sections were examined using a scanning electron microscope (SEM, S-3400N, HITACHI, Japan) and SEM images were acquired.

3.6. Statistical Analysis

Pull-out forces for the cell-loaded and noncell-loaded sides of each implant were compared using a Wilcoxon test. Differences with a p-value of <0.05 were considered statistically significant.

4. Conclusions

We have succeeded in differentiating the mesenchymal cells into active osteoblasts concomitant with bone matrix formation on the CoCr based alloy implant surface using rabbit bone marrow culture. The culture of the mesenchymal cells on the CoCr alloy implant surface prior to implantation resulted in a stable interface between the implant surface and host bone just 3 weeks after implantation. The present findings indicate early fixation of CoCr based alloy by our tissue engineering approach, which might lead to the desired solution of cementless fixation in various joint arthroplasties using CoCr alloy implants.

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Conflict of Interest

The authors declare no conflict of interest.

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