The Effect Of Osteogenesis on Newly Fabricated Cu-bearing Stainless Steel

Ling Ren, PhD¹, Hoi Man Wong, PhD²,³, Ke Yang, PhD¹, Kelvin Yeung, PhD²,³.
¹Institute of Metal Research, Chinese Academy of Sciences, Shenyang, China, ²The University of Hong Kong, Hong Kong, Hong Kong, ³Shenzhen Key Laboratory for Innovative Technology in Orthopaedic Trauma, The University of Hong Kong Shenzhen Hospital, Shenzhen, China.

Disclosures:

Introduction: Stainless steel has a long history in orthopaedic implantations e.g. prosthesis and implant for fracture fixation due to its high strength and toughness as well as ease of processing [1]. However, its osteoconductivity does not satisfy the demands in bone-implant integration, thereby resulting to aseptic implant loosening and eventually implant failure [2,3,4]. In addition, higher rate of implant-related osteomyelitis was occurred in stainless steel rather than titanium alloy [5]. Therefore, our team has fabricated a new type of stainless steel incorporating with cupper nano-sized cupper-rich participations in the matrix. In previous studies, this newly fabricated steel had demonstrated its superior antimicrobial effect in-vitro and in-vivo [6-8]. The present study aims at investigating the adhesion and proliferation as well apoptosis of osteoblastic cells while culturing with Cu-bearing stainless steel, the osteogenic differentiation ability of osteoblasts against to the release profile of cupper ions, and the bone formation abilities as well as inflammatory response under in-vivo condition.

Methods: The 317L-Cu stainless steel (Cu-SS) with nominal chemical compositions (wt.%): Cr 19, Ni 13, Mo 3.5, Cu 4.5 and Fe in balance was successfully developed in our previous work. Samples for in-vitro tests were prepared from rods in 4.4 and 13.9 mm diameter to disks with 1 mm in thickness and followed by mechanical polishing until mirror surface. For in-vivo testing, samples were machined into cylindrical shape of 2mm in diameter and 6mm in length and followed by electrochemical polishing. All the experimental samples were sterilized prior to the biological tests and conventional 317L stainless steel (SS) served as control. To determine the amount of Cu²⁺ ions released from the Cu-SS, samples were submerged to physiological saline and incubated under 37°C bio-incubator from D0 until 1 month. The extracts were then examined by inductively coupled plasma atomic emission spectrometer. Material characterizations also included electrochemical corrosion analysis, scanning electron microscopy and energy dispersive spectrometry. In order to investigate the cell viability, adhesion and proliferation and apoptosis as well as the expression of osteogenic differentiation markers e.g. alkaline phosphatase (ALP), type I collagen (Col1-a1), runt-related transcription factor 2 (Runx2) and osteopontin (Opn), the samples together with control were cultured against SaOS-2 human osteoblasts and MC3T3-E1 mouse pre-osteoblasts for various periods of time, respectively. Lastly, all the samples were implanted into the distal femur of rat models according to the approved ethical protocol until post-op 15 days. Post-op in-vivo analyses included bone quality evaluation and new bone formation examined by micro-computed tomography at D3, 7 and 15, separately. After sacrificed, histological and immunohistochemical analyses were applied to examine the new bone formation and inflammatory response of tissues adjacent to the implants. Specially designed push-out biomechanical test was conducted to the bone blocks harvested from animals in order to assess the mechanical strength of bone-implant interface. All data were analyzed by Student’s t-test using ANOVA and statistical significance would only considered when level of p was < 0.05.

Results: In Fig. 1, more actin filaments were found on Cu-SS sample than that of SS control in the morphology examination after 2-day culture. It seems that the surface of Cu-SS sample can significantly promote the adhesion and proliferation. The cell proliferation and the expression of alkaline phosphatase (ALP), type I collagen (Col1a1), osteopontin (Opn) and runt-related transcription factor 2 (Runx2) of osteoblasts in Cu-SS group were significantly higher as compared with the conventional stainless steel (SS) (Fig.2a-c). In addition, the LDH assay suggested that the cytotoxicity was even lower than that of SS (data not shown). Furthermore, higher volume of new bone formation adjacent to the implant and bone-to-implant contact ratio were reported in the Cu-SS sample under in-vivo condition (data not shown). The bone mineral density of newly formed bone on Cu-SS sample was better than that of conventional SS (Fig.3a-b). Also, the expression of TNF-α on Cu-SS sample was significantly less than that of SS sample (Fig.3c) at Day 3 and 7, indicating that the release of small amount of cupper ions could help suppress inflammatory response. In the post-operative biomechanical testing, the push-out force of Cu-SS group was much higher than that of SS group. This result suggested that the osseointegration at the bone-implant interface in Cu-SS group was superior to that of conventional SS. Lastly, the corrosion testing result indicated that the release of Cu²⁺ ions was triggered by pitting corrosion at the Cu rich phases of the new stainless steel matrix. The amount was approximately 1.4 ppb per day, while its corrosion resistance generally maintained.

Discussion: The concept of bio-functional design for metal implant is to introduce bioactive factors e.g. metallic ions and growth factorsto the currently used bio-inert metallic biomaterials. The current approach is to incorporate the nano-sized cupper-rich precipitations to the stainless steel matrix during alloying rather than coat a layer of cupper ions on material surface. Therefore, the release of cupper ions upon the degradation of cupper-rich phases in the matrix is expected to be long lasting than the conventional coatings. Furthermore, delamination of coating is no longer a concern in our study. Additionally, the use of cupper...
ions in biology and medicine has been studied for long time. For instance, copper ions is important to the development of connective tissues, nerve covering and bone growth. It also plays a unique catalytic action in the bio-system [9-12]. In addition to the antimicrobial effect, our current study has demonstrated that small amount of copper ions can not only help promote morphogenesis of osteoblasts and up-regulate the major osteogeneic differentiation markers, but also enhances the osseointegration and suppresses the TNF-α expression in-vivo.

Significance: The newly fabricated copper bearing stainless steel is able to up-regulate cellular activities as well as to enhance osteogenesis in-vivo. Combining with its intrinsic antimicrobial property, this new metallic material is very promising to apply in various orthopaedic implantations.

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Fig. 2 (a) The plot of cell viability of SaOS-2 human osteoblasts cultured on the surfaces of 317L SS and Cu-SS. (b) The plot of ALP activity of MC3T3-E1 pre-osteoblasts cultured on 317L SS and Cu-SS for 3 and 7 days, respectively. (c) The plot of osteogenic differentiation markers including alkaline phosphatase (ALP), type I collagen (Col1a1), osteocalcin (Oc), and osteopontin (Opx) as well as related transcription factors 2 (Runx2) and 3 (Runx3) of MC3T3-E1 pre-osteoblasts cultured on 317L SS and Cu-SS for 3 and 7 days, respectively. The mRNA level was normalized with the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Fig. 3 (a) Micro-CT reconstruction images of the lateral epicondyl containing 317L SS and Cu-SS after 15 days of operation. Green arrows represent the newly formed bone. (b) Bone mineral density (BMD) of the bone formed around the implant after 15 days of operation. (c) Percentage change of the bone volume during the implantation period. (d) Average maximum push-out forces of 317L SS and Cu-SS after 13 days of implantation. (e) Immunohistochemical staining of TNF-a of bone tissue around the implants after 3 and 7 days of implantation. Brown color represents positive staining and 'T' represents the implant location.