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Research Report

Cytotoxicity difference of 316L stainless steel and titanium reconstruction plate

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ABSTRACT

Background: *Pure titanium is the most biocompatible material today and used as a gold standard for metallic implants. However, stainless steel is still being used as implants because of its strength, ductility, lower price, corrosion resistant and biocompatibility.* ***Purpose:*** *This study was done to revealed the cytotoxicity difference between reconstruction plate made of 316L stainless steel and of commercially pure (CP) titanium in baby hamster kidney-21 (BHK-21) fibroblast culture through MTT assay.* ***Methods:*** *Eight samples were prepared from reconstruction plates made of stainless steel type 316L grade 2 (Coen's reconstruction plate®) that had been cut into cylindrical form of 2 mm in diameter and 3 mm long. The other one were made of CP titanium (STEMA Gmbh®)) of 2 mm in diameter and 2,2 mm long; and had been cleaned with silica paper and ultrasonic cleaner, and sterilized in autoclave at 121° C for 20 minutes.[9] Both samples were bathed into microplate well containing 50 µl of fibroblast cells with 2 x 10^5 density in Rosewell Park Memorial Institute-1640 (RPMI-1640) media, spinned at 30 rpm for 5 minutes. Microplate well was incubated for 24 and 48 hours in 37° C. After 24 hours, each well that will be read at 24 hour were added with 50 µl solution containing 5mg/ml MTT reagent in phosphate buffer saline (PBS) solutions, then reincubated for 4 hours in CO_2 10% and 37° C. Colorometric assay with MTT was used to evaluate viability of the cells population after 24 hours. Then, each well were added with 50 µl dimethyl sulfoxide (DMSO) and reincubated for 5 minutes in 37° C. the wells were read using Elisa reader in 620 nm wave length. Same steps were done for the wells that will be read in 48 hours. Each data were tabulated and analyzed using independent T-test with significance of 5%.* ***Results:*** *This study showed that the percentage of living fibroblast after exposure to 316L stainless steel reconstruction plate was 61.58% after 24 hours and 62.33% after 48 hours. And after exposure to titanium reconstruction plate, the percentage of living fibroblast was 98.69% after 24 hours and 82.24% after 48 hours. Based on cytotoxicity parameter ($CD_{50\%}$), both reconstruction plate made of 316L stainless steel or titanium showed as a non-toxic materials to fibroblast.* ***Conclusion:*** *Both reconstruction plate made of stainless steel and CP titanium were non-toxic to fibroblast, although the stainless steel plate showed lower cytotoxicity level compared to titanium. Therefore a reconstruction plate made from stainless steel type 316L can be used as a safe material for mandibular reconstruction.*

Key words: *316L Stainless steel plate, titanium plate, cyototoxicity, MTT assay*

ABSTRAK

Latar belakang: *Titanium murni adalah bahan yang paling biokompatibel saat ini dan digunakan sebagai standar emas implan logam. Saat ini stainless steel masih digunakan karena kekuatan, ductility, harganya yang murah, tahan terhadap korosi dan cukup biokompatibel.* ***Tujuan:*** *Penelitian ini dilakukan untuk mengetahui perbedaan sitotoksisitas antara plat rekonstruksi yang terbuat dari titanium murni komersial dan plat rekonstruksi yang terbuat dari stainless steel pada kultur sel fibroblas baby hamster kidney-21 (BHK-21) menggunakan MTT assay.* ***Metode:*** *Delapan sampel yang masing-masing tipe 316L terbuat dari stainless steel 316L grade 2 (Coen's reconstruction plate®) yang dipotong berbentuk silinder diameter 2 mm dan panjang 3 mm, serta yang terbuat dari titanium murni komersial (STEMA Gmbh®) diameter 2 mm dan panjang 2,2 mm; dan dibersihkan dengan kertas silika dan pembersih ultrasonik serta disterilkan dengan autoclave pada suhu 121° C selama 20 menit. Kedua sampel dimasukkan ke dalam sumur mikroplat yang mengandung 50 µl sel fibroblas dengan kepadatan 2 × 10^5 dalam media Rosewell Park Memorial Institute-1640 (RPMI-1640), diputar dengan kecepatan 30 rpm selama 5 menit. Sumur mikroplat diinkubasi selama 24 dan 48 jam pada suhu 37° C. Setelah 24*

jam, pada tiap sumur yang akan dibaca pada jam ke 24 ditambahkan 50 µl cairan yang mengandung 5mg/ml MTT dalam phosphat buffer saline (PBS), kemudian diinkubasi kembali selama 4 jam dalam CO_2 10% pada suhu 37° C. Assay kolorimetri dengan MTT digunakan untuk mengetahui viabilitas populasi sel setelah 24 jam. Setiap sumur ditambahkan pelarut dimetil sulfoksida (DMSO) dan diinkubasi kembali selama 5 menit pada suhu 37° C. sumur-sumur tersebut kemudian dibaca dengan Elisa reader dengan panjang gelombang 620 nm. Langkah yang sama dilakukan pada sumur-sumur yang akan dibaca pada jam ke 48. Data kemudian ditabulasi dan dianalisis dengan menggunakan independent T-test dengan signifikansi 5%. ***Hasil:*** *Penelitian ini menunjukkan presentase fibroblas hidup setelah terpapar plat rekonstruksi yang terbuat dari stainless steel adalah 61,58% setelah 24 jam dan 62,33% setelah 48 jam. Dan setelah paparan dengan plat rekonstruksi yang terbuat dari titanium murni adalah 98,69% setelah 24 jam dan 82,24% setelah 48 jam. Berdasarkan pada parameter sitotoksisitas ($CD_{50\%}$) kedua plat rekonstruksi baik yang terbuat dari titanium murni maupun yang terbuat dari stainless steel tipe 316L merupakan bahan yang tidak bersifat toksik terhadap fibroblas.* ***Kesimpulan:*** *Kedua plat rekonstruksi baik yang terbuat dari stainless steel maupun CP titanium tidak bersifat toksik terhadap fibroblas, walaupun plat stainless steel menunjukkan level sitotoksisitas yang lebih rendah daripada titanium murni. Dengan demikian plat rekonstruksi yang terbuat dari stainless steel 316 L aman digunakan sebagai bahan untuk rekonstruksi mandibula.*

Kata kunci: *Plat stainless steel, plat titanium, sitotoksisitas, MTT assay*

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INTRODUCTION

Implant materials can be classified into biotolerant, bioinert, and bioactive materials. Stainless steel is a biotolerant implant material, that characterized by the presence of a thin fibrous layer overlying implant surface in contact to bone. Titanium is a bioinert material with a characteristic of direct contact to bone or osseointegration, osseointegration can be achieved because there is no chemical reaction between material surface to surrounding tissue or to body fluid.[3] metallic materials implanted in the human body rarely induce serious conditions. Metallic materials conventionally used in medicine and dentistry does not show toxicity. However, some elements of the alloys show toxicity. The toxicity of a metallic material is governed not only by the elements content of the material but also by its corrosion and wear resistance.[4]

Before implantation, every materials should pass compatibility study. In vitro citotoxicity study is the primary study to determined a material or material component's biocompatibility. In this study, an unprocessed material or material component's are placed directly into tissue cell culture.[5,6] Cell culture can be used to evaluate material's citotoxicity through microscopic examination or quantitatif analysis. Cell morphology and characteristics in the adhesion process are evaluated to determine the effect of cellular citotoxicity, adhesion mechanism changes, cell atypia, or cell damage. Fibroblast and osteoblast are the common cell type used in the study of implant biocompatibility.[7] One of the citotoxicity study that commonly used is the MTT assay or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. This study are based on the reduction of yellow tetrazolium salt into purple formazan crystal by hidrogenase enzyme secreted from the mitochondria of the metabolically active cells. Amounts of purple formazan crystal define amounts of living cells.[8]

Until these days, stainless steel and titanium alloys commonly used as implant material in orthopedics and dentistry. Since 2003 until 2008, there were 51 patients with benign mandibular tumor at the department of oral and maxillofacial surgery, Airlangga University/Dr. Soetomo hospital Surabaya were being treated with mandibular resection and immediate reconstruction wether using stainless steel reconstruction plate, bone graft, and combined bone graft and stainless steel plate as stabilizer. Airlangga University Dental Hospital use stainless steel plate because it's cheaper than titanium plate but have a good strength, ductility, resistant to corrosion, and compatible. Whereas pure titanium is considered as the most biocompatible material, so commonly used as a gold standard in metallic implant. Inexpensive reconstruction material are still needed in Indonesia because of the social economic level of the Indonesian people which mayoritas could not afford expensive materials.

The aim of this study was to evaluate the cytotoxicity of mandibular reconstruction plates made from stainless steel type 316L and the one made from pure titanium in BHK-21 cell culture through MTT assay in 24 and 28 hours measurements.

MATERIALS AND METHODS

This was an experimental laboratory study conducted in December 2009 at Pusat Veterenaria Farma (PUSVETMA) Surabaya. Eight samples were prepared from reconstruction plates made stainless steel type 316L grade 2 (Coen's reconstruction plate®) that had been cut with wire cutter into cyllindrical form of 2 mm in diameter and 3 mm long; and from one made of commercially pure (CP) titanium (STEMA Gmbh®) that had been cut into cylindrical form of 2 mm in diameter and 2.2 mm long. After cutting, all

samples were cleaned with silica paper to remove cutting debris, soaked into ethanol 70% in ultrasonic cleaner for 60 minutes, and sterilized in autoclave at 121° C for 20 minutes.[9]

Fibroblast cells from BHK-21 cell line were cultivated in a roux bottle until confluent, and harvested with trypsine versene solution. Harvested fibroblast were cultured in Rosewell Park Memorial Institute-1640 (RPMI-1640) media which contain 10% fetal bovine serum albumin, incubated for 24 hours in 37° C, cells were transported into small roux botle dan cultured in the density of 2×10^5 into each 96 well microplate until confluent. Each microplate well contain 50 µl of cells with 2×10^5 density in RPMI media, spanned at 30 rpm for 5 minutes. There was also cell control contains cells in culture media as a positive control which assumed as 100% living cells, and media control contains culture media without cells which assumed as 0% living cells.

Each microplate well was examined under light microscope to ensure that the incubation time was enough to form crystals. Both samples made from stainless steel and titanium bathed into the well containing fibroblast cells and RPMI. Microplate well was incubated for 24 and 48 hours in 37° C. after 24 hours, each well that will be read at 24 hour were added with 50 µl solution containing 5 mg/ml MTT reagent in PBS, then reincubated for 4 hours in CO_2 10% and 37° C. Colorimetric assay with MTT was used to evaluate viability of the cells population after 24 hours. Then, each well were added with 50 µl DMSO and reincubated for 5 minutes in 37° C. the wells were read at Elisa reader in 620 nm wave length. Same steps were done for the wells that will be read in 48 hours. Each data were tabulated and analyzed using independent T-test with significance of 95%. Percentage of the living fibroblast cells were calculated with the following formula, according to experimental study by Meizarini *et al*:[10]

$$\text{Percentage of the living cells} = \frac{\text{treatment + media}}{\text{cell + media}} \times 100\%$$

RESULTS

Cell in control group had the highest mean value of optical density and media control group had the lowest mean value of optical density (Table 1). Normality study with nonparametric test using Kolmogorof Smirnov test, showed that all study groups had $p > 0.05$ which mean that all study groups had a normal distribution that mean values of the data lies between standard deviation and significance test was done with Independent t-test, showed in table 2 and 3.

Table 1. Average of optical density and standard deviation of each study groups in 24 and 48 hours measurements

Groups	n	$\overline{X}$ + SD	
		24 hours	48 hours
Titanium	8	0.42 ± 0.10	0.55 ± 0.05
Stainless steel	8	0.23 ± 0.05	0.39 ± 0.05
Cell control	8	0.42 ± 0.14	0.69 ± 0.12
Media control	8	0.09 ± 0.02	0.10 ± 0.02

Table 2. Difference test of the optical density from each study groups in 24 hours measurement using Independent t-test

	Titanium	*Stainless steel*	Cell control	Media control
Titanium	-	0.001**	0.915	0.001**
Stainless steel	-	-	0.002**	0.001**
Cell control	-	-	-	0.001**
Media control	-	-	-	-

Table 3. Difference test of the optical density from each study groups in 48 hours measurement using Independent t-test

	Titanium	*Stainless steel*	Cell control	Media control
Titanium	-	0,001**	0,013**	0,001**
Stainless steel	-	-	0,001**	0,001**
Cell control	-	-	-	0,001**
Media control	-	-	-	-

There was a significant difference of the formazan density in the 24 and 48 hours measurement, showing there was a significant increased number of living fibroblast following exposure time to titanium and stainless steel plate (Table 2 and 3). There was no significant difference between the titanium group and the cell control group in the 24 hours measurement with $p > 0.05$ (Table 2).

Table 4. Percentage of the living fibroblast cells in each study groups in 24 and 48 hours measurement

Cell group	n	24 hours (%)	48 hours (%)
Titanium	8	98.69	82.24
Stainless steel	8	61.58	62.33

The percentage of the living fibroblast cells in each study groups were more than 50% (Table 4). This results showed both plate made of stainless steel and CP titanium were non-toxic to fibroblast based on $CD_{50\%}$.[11]

DISCUSSION

Biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local and systemic effects in the recipient or beneficiary of that therapy, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy.[12] Biocompatibility is important because implant surface in contact with the tissue can undergo corrosion in vivo. Implant corrosion can cause lost of load bearing strength and can undergo degradation into toxic substances in the tissue.[13] Main criteria in choosing metallic implants is its biocompatibility.[14] Materials can be classified as a biocompatible material if culture cells remain living and metabolically active in long term culture. There are 2 quick and simple quantitative assay to test biocompatibility in vitro, these are cell viability based on physical uptake of neutral red (NR) and based on cell's metabolic activity through MTT assay, which based on cellular enzyme activity. Both test are widely accepted as a biocompatibility and cytotoxicity study to evaluate cell viability and growth.[15] In this study MTT assay was performed to evaluate the biocompatibility of stainless steel 316L and titanium reconstruction plates, because this method is a simple, accurate measure, and can be done in a large scale study.[16–18]

This study showed that there was a significant optical density difference of treatment group exposed to stainless steel in 24 and 48 hours measurement, and in treatment group exposed to titanium in 48 hours measurement. Whereas in 24 hours measurement, there was no significant optical density difference between group exposed to titanium and cell control group. This was consistent with the reference that stated titanium is a bioinert material because there is no chemical reaction between material surface to surrounding tissue or to body fluid.[3] There were increased optical density of the cell control group from 0.4223 in 24 hours into 0.6860 in 48 hours measurement, optical density of the group exposed to titanium only increased from 0.4156 in 24 hours into 0.5470 in 48 hours measurement. This study showed that reconstruction plates made from stainless steel 316L and CP titanium had a good in vitro biocompatibility to fibroblast cell which indicated from there was no cytotoxic effect to BHK-21 cells. That was concluded from the percentage of living fibroblast cell after exposure to stainless steel 316L in 24 hours was 61.58%; after 48 hours was 62.33%; after exposure to CP titanium was 98.69% in 24 hours; and 82.24% after 48 hours. All measures showed there was no cytotoxicity based on $CD_{50\%}$, the cytotoxicity parameter.[11] The difference caused by increasing number of living fibroblast cell from both exposure time, showing increasing living fibroblast cell, because fibroblast were able to anchor and adapt accordingly to metal particle,[9] so that cells can replicate.

This study showed that CP titanium plates had a higher biocompatibility level compared to stainless steel 316L plates, this consistent with the classification of material which categorize CP titanium into bioinert material, whereas stainless steel 316L is a biotolerant material.[19] Biocompatibility of implant material also affected by their resistance and their alloy to corrosion process in body fluid that was known as electrolyte. After implantation there was changes in neutral pH that depend on implantation time and appropriate healing process, tissue pH were between 6.8–7.4.[20] Titanium and its alloy are materials with the best corrosion resistance, because of their passive nature and an unreactive passive film formed on titanium surface. Stainless steel 316L had an acceptable corrosion resistance. Stainless steel 316L implant was mostly manufactured in India to be used for orthopedic application because of its lower price, easier welding and processing compared to cobalt-chromium alloy and titanium or titanium alloy. Other properties of stainless steel 316L are biocompatible, good tensile strength, fatigue resistance, with appropriate density for weight bearing, made it a preferable surgical implant material. The disadvantage of titanium are expensive, easily wear off, and brittle.[21]

It can be concluded that both reconstruction plate made of stainless steel and CP titanium were non-toxic to fibroblast, although the stainless steel plate showed lower cytotoxicity level compared to titanium. Therefore a reconstruction plate made from stainless steel type 316L can be used as a safe material for mandibular reconstruction.

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