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Evaluation and comparison of tissue toxicity induced by common mini-plates used in craniomaxillofacial surgery in Iran (In Vitro Assessment)

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ABSTRACT

Objective: Good agents that can act on biologic system should be biocompatible. Biocompatible Products are the products that have the least negative effect on the body's tissues. The aim of this study was to evaluate the in vitro tissue toxicity induced by common mini-plates used in craniomaxillofacial surgery.

Materials and Methods: This study was conducted In vitro. In this study, mitochondrial coloring of living cell (MTT assay) and Annexin V/PI binding assay were measured in 5 treatments with 3 replications. to have mini plate extract, mini plates were pour in DMEM media for 30 days and used it as mini-plate extract. Experimental groups were group 1: control group (in MTT assay control group was cells treated by fresh media as negative control and incubated media as positive control and in Annexin V/PI binding assay control was cells which treated by high dose dexamethasone as positive control and cells with treated by fresh media), group 2: AO Synthes mini-plates (Tehran Arkak), group 3: General-Implants mini-plates (Behin Idea orthopedic "Biomed"), group 4: JEIL mini-plates (Pouya Teb) and group 5: Imen Ijaz mini-plates. A number of 1×104 cells was cultured on culture plate in the 96 pits for 24 and 48 hours and 7 days with each group. ANOVA test was used to compare group means. SPSS23 statistical software was used for data analysis.

Results: The results of MTT assay in the time periods studied was significantly different between the studied groups (p value <0.05). The results of flowcytometry showed that the number of necrotic and apoptotic cells in experimental groups was significantly different. The maximum amount of apoptosis was seen in General mini-plate and the lowest was observed in AO Synthes and JEIL.

Conclusion: The results of this study showed that the toxicity of titanium based mini-plates such as JEIL, General-Implants, Imen Ijaz and AO Synthes mini-plates had minimal toxicity and they were biocompatible.

Key words: Toxicity, Mini-Plate, Titanium alloys, Apoptosis, Necrosis.

Introduction

Biological compatibility is defined as the ability of a substance with a specific application which is accompanied by a favourable reaction from the host. Therefore a substance must be biologically compatible in order to act effectively in biological environments. An absolute biological compatibility of a substance is almost impossible because there may be no substance available which is completely effective on biological tissues. When the substance is biologically compatible, the amount of reaction between the substance and the living tissue is neg-

In order to understand the biological compatibility of biomaterials, it is important to recognize the reaction between biomaterials and the host biological system at the contact surfaces. In these contact surfaces the molecular structure of the biological system reacts with the biomaterials; these primary reactions happen on a molecular level and in a very narrow contact area of less than 1 nanometer width (Guizzardiet al, 2004). In biomaterials-tissue interactions debate, the effect of biomaterials on blood, their toxicity, tissue restoration effects, inflammation, infection and malignancy on tissues are taken into consideration (Zibowicz A & Marciniak J, 2006).

Titanium is a metal with a relatively low density; it is highly resistant to corrosion and has high strength compared to its weight and it has a suitable physical and mechanical behaviour in high temperatures. The adhesive and protective layer of titanium oxide on the surface of this metal provides an excellent resistance to corrosion (Ishizava H & Ogino M, 1995). The fashions of cellular interaction with titanium play an essential role in clinical success of bone grafting and dental implants (Rajaei A, Daliri joupari M, 2011). The tissue reaction in contact with titanium and titanium alloy is very low; therefore the bone grafting and growth can be made effectively (Ishizava H & Ogino M, 1995).

Tissue reaction as a consequence of corrosion and release of metal particles in surrounding tissue; is the most important supportive factor for removal of the stainless steel miniplates after repairing of a fracture (Dugal A& Thakur G, 2010). In field of Craniomaxillofacial surgeries; the debate of choosing different brands and materials of miniplates with regards to existence of broad advertisement and large scale prices; evaluation of amount of possible tissue toxicity (toxicity results from the interaction of a specific substance with the living tissue) which can be caused by each brands; comparison, price evaluation and the effectiveness of different brands of miniplates, can be a good way in reduction of excessive costs for patients and determining of one or more ideal brands and materials for using in surgeries.

The biological reaction of substances in contact with tissue is reconstructed in vitro in order to evaluate tissue compatibility. The cell culture experiments provide convenient, controllable and repeatable methods for primary evaluation of biological responses. Indeed, these experiments, mainly evaluate the cytotoxicity of substances used in the experiment; also they may be helpful in the differentiation of pathogenicity effects of some substances (Caughman et al, 1990).

The studies in dentistry profession are mainly focused on evaluation of tissue toxicity results from metals and other substances which are used in orthodontic appliances (Kerosuo E & Moe G, Kleven E, 1995; Locci et al, 2000; Mockers et al, 2002). It seems there is not much research and studies on quantity of tissue toxicity results from miniplates used in surgeries; therefore the present study was done. The purpose of this study was to provide a clear picture of the amount of tissue toxicity result from 4 different brands of titanium miniplates which currently used in craniomaxillofacial surgery in Iran (Imen Ijaz, (Pooya Teb) JEIL, (Behin Idea Orthoped "Biomed") General-Implants and (Tehran-Arkak) AO Synthes miniplates). For this purpose two standard tests, including MTT assay (mitochondrial colouring of a living cell) (Rajaei A, Daliri joupari M, 2011; Ortiz et al, 2011) and flowcytometry using Annexin V/PI binding assay (Dalili et al, 2012; Xu et al, 2012; He et al, 2012) on human osteoblasts were used.

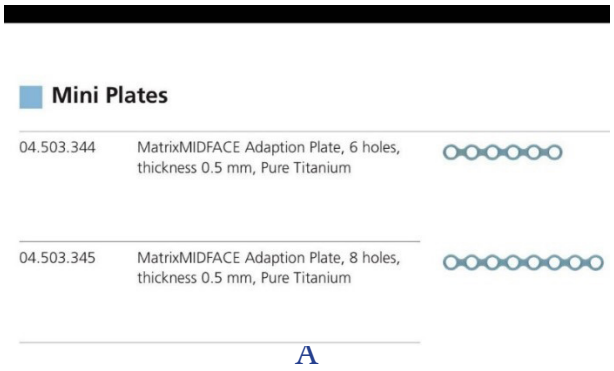
Materials and Methods

The present study was carried out in vitro. By taking the nature of the experiment into account (In vitro), the experimental substances and miniplates (Fig. 1) costs, 6 samples in each group (3 samples in each experiment), and total number of 30 samples were evaluated. In the present study the MTT assay (mitochondrial colouring of living cell) and Annexin V/PI binding assay tests were carried out three times for each following group. These experiments were done in three periods include 24 and 48 hour and 7 days after treatment for MTT assay. MG-63 cell line was used to evaluate the toxicity of the mini-plates on them. To evaluate the effect of mini plate toxicity, these miniplates poured in DMEM media for 30 days and used this extract as mini-plate extract. The number of 1×10^4 cells was cultured on culture plate mini-plates in the 96-well plate. The samples which were evaluated include titanium based mini-plates such as JEIL, General-Implants, Imen Ijaz and AO Synthes mini-plates. Negative control in MTT assay and flow cytometry was fresh media and positive control in flow cytometry test was high dose dexamethasone. In MTT assay two dose of treatment were analysed illustrated as 50% and 100%; 50% was defined as a treatment including 50% fresh media and 50% extracted media but in 100% whole analysed sample was extracted media.

MTT assay for determining the viability and number

of cells 50 mg of MTT (3-[4,5-dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide) was solved in 10 ml of Phosphate-Buffered Saline (PBS) to produce the MTT 5mg/ml stock solution (Hughes D&Mehmet H, 1985). 400µl of MTT solution was transferred into each well of the 96-well culture plate (the culture media contain collagen and other cells under the study). The 96-well culture plate was incubated for 7 hours at 37 °C with 5% CO_2.

After incubation and seeing the colour of cells (turning dark) using an inverted microscope, the MTT solution was taken out and 400µl of isopropanol was added into each well of the culture plate and was left for 30 minutes. In the next step the content of each well was transferred into a 96-well culture plate; by using Spectrophotometric ELISA Plate Reader from Lab-Systems Multiscan RC Company with a wavelength of 570nm and a certain optical density, the cells viability and number of cells was determined.



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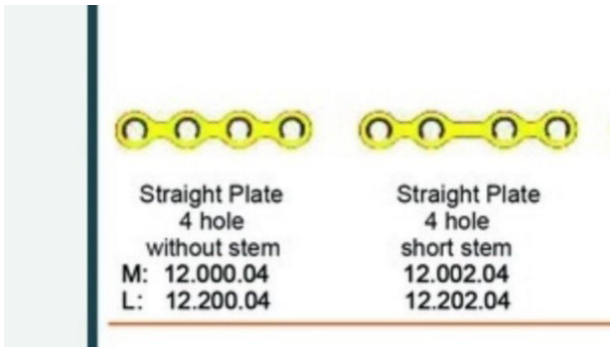

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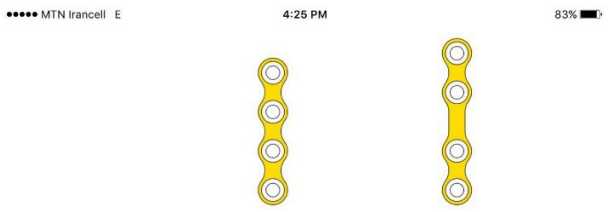

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Figure 1: Miniplats: AO Synthes (a), Imen Ijaz (b), General-Implants (c) and JEIL (d).

Flow Cytometer

The cells were extracted from the culture media using (0.1 mm) Trypsin-EDTA (0.25%) solution. The cells were transferred into a 2 ml Eppendorf tube and then were centrifuged for 10 minutes with an acceleration of 200g. Then the top layer of liquid was discarded and 2ml of PBS was added into the Eppendorf tube and the solution was centrifuged for another 10 minutes again. This process was repeated in order to washing the remaining culture media away. Then the top layer of liquid was discarded and ethanol (70%) was added into the tube and the tube stored in a fridge at -20 °C for 2 hours. The tube was centrifuged for 10 minutes with an acceleration of 200g and ethanol was discarded after centrifugation. The cells were washed again using 2 ml of PBS and centrifuged; the top layer of liquid was discarded and then RNase 13 kunitz solution was added into the tube. The tube was stored in water with a temperature of 37 °C for 15 minutes. Then 20 µl of the Annexin V solution and 20 µl of PI solution were added into the tube. The tube was then stored in water with a temperature of 37 °C for 20 minutes. After this time the tubes containing pigmented cells were stored

in an ice flask and then the samples were analysed using flow cytometer machine (Becton Dickinson brand) with a wavelength of 488 nm (Hughes D&Mehmet, 1982).

Statistical Analysis

In the present study, descriptive parameters such as mean, standard deviation, minimum and maximum values were used in order to describe the findings. ANOVA test was used in order to compare the mean value of the experimental groups. A significance level of 0.05 was used for determining the statistical significance values (statistically significant as $P < 0.05$ and statistically non-significant as $P > 0.05$). SPSS 23 Statistical software was used in order to analyse the data.

Results

The descriptive results of quantity of cell necrosis, primary cell apoptosis, final cell apoptosis and MTT of the 4 different types of mini-plates and control group are illustrated in table 1. Figure 1 demonstrate the flow cytometry graphs.

MTT results were compared by ANOVA test after each period of incubation. The results were illustrated in table2. To compare the effect of 50% and 100% treatment dose, T-test was used. The results of this test were demonstrated in table 3. Table 4 demonstrates the results of ANOVA test to compare the cytotoxicity of mini- plate that analysed by flow cytometry.

Groups		*Necrosis*	*Initial apoptosis*	*Final apoptosis*
JEIL	*Minimum*	*4.47*	*4.62*	*4.78*
	Maximum	*6.22*	*6.73*	*6*
	Mean	*5.62*	*6.81*	*5.23*
	St.D.	*0.81*	*0.94*	*0.61*
General-Implants	*Minimum*	*4.62*	*6.02*	*3.05*
	Maximum	*6.73*	*12.3*	*3.74*
	Mean	*5.84*	*8.85*	*3.25*
	St.D.	*1.09*	*3.18*	*0.35*
Imen Ijaz	*Minimum*	*5.88*	*5.35*	*3.95*
	Maximum	*7.27*	*12.28*	*7.02*
	Mean	*6.57*	*8.82*	*5.7*
	St.D.	*0.69*	*3.46*	*1.5*
AO Synthes	*Minimum*	*3.78*	*8.75*	*3.05*
	Maximum	*5.76*	*11.86*	*3.74*
	Mean	*4.67*	*10.79*	*0.35*
	St.D.	*1*	*1.7*	*3.35*
Negative control	*Minimum*	*0.04*	*0.07*	*0.01*
	Maximum	*0.05*	*0.09*	*0.01*
	Mean	*0.43*	*0.08*	*0.01*
	St.D.	*0.01*	*0.01*	*0*
Positive control	*Minimum*	*5.99*	*11.27*	*1.12*
	Maximum	*8.38*	*13.28*	*4.62*
	Mean	*7.47*	*12.12*	*3.35*
	St.D.	*1.29*	*1.98*	*1.9*

Table 1: Descriptive data of cell necrosis, primary cell apoptosis, final cell apoptosis and MTT values for the 5 groups.

Incubation time	Dose of incubation	P-value
24 Hour	50%	.005
24 Hour	100%	.0017
48 Hour	50%	.000
48 Hour	100%	.000
7 Days	50%	.000
7 Days	100%	.000

Table 2: ANOVA test to compare the cytotoxicity effect of mini-plate analysed by MTT assay.

Compare 50% and 100% treatment dose		P-value		
		24 Hour	48 Hour	7 Dyas
Pair 1	Jeil	.288	0523	.833
Pair 2	Imen ijaz	.180	.145	.894
Pair 3	AO	.629	.042	.256
Pair 4	General	.474	.017	.997
Pair 6	Fresh media	.216	.365	.323

Table 3: T-test to evaluate the effect of dose treatment in MTT assay.

Cells	P-value
Necrotic cells	.000
Early apoptotic cells	.000
Late apoptotic cells	.000

Table 4: ANOVA test to compare the cytotoxicity effect of mini-plate evaluated by flow cytometry assay.

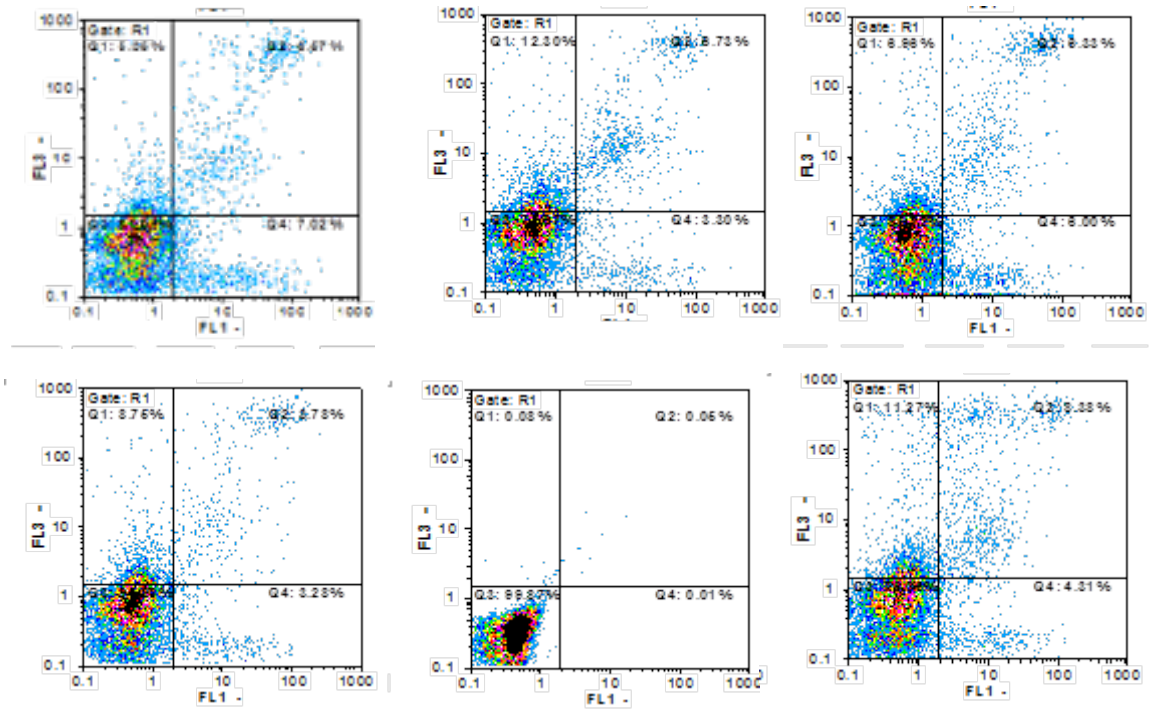

Figure 1: The results of flow cytometry.1: Imen Ijaz 2: General 3: Jeil 4: AO 5: Negative control 6: Positive control.

Discussion

The use of titanium and related alloys in medical and dental work in recent years has become widespread. special features of this metal led to use it in medical industry as material in implants and prostheses (Wang RR & Fenton A, 1996). Different cytological studies in the medical industry have been done to evaluate the toxicity of alloys based on titanium. The results of many studies showed no toxicity in mouse and human fibroblasts has been cast on macrophages (Wang RR & Fenton A, 1996;Rae T, 1975). However, there are still conflicting results in this regard such as one research that show titanium oxide can have carcinogenic effect on body (Rae T, 1981). So the aim of the present study was to evaluate and compare the quantity of tissue toxicity results from the common miniplates used in the cranium, jaw and facial surgeries (JEIL, Imen Ijaz, General-Implants and AO Synthes) and the control group. MTT assay and flow cytometry was used for this purpose. MG-63 Cell line was used to evaluate the toxic effect of mini-plates. ANOVA statistical analysis test was used to compare the MTT results in different incubation time. In all incubation times (24,48 hours and 7 days) significantly different toxicity was observed but at first 24 hours of incubation, toxicity of JEIL mini-plate was higher than others and after 48 hours and 7 days higher toxicity were seen in General mini-plate. To evaluate the effect of mini- plate in inducing apoptosis and necrosis pathway in cell flow cytometry was done. In evaluation of the average number of apoptotic and necrotic cells in the 5 groups, this study showed that there was a statistically significant difference between the control group and the 4 different brands of miniplates ($P<0.05$).

By evaluating toxicity of metal and non-metal orthodontic appliances (stainless steel, gold plated steel, pure titanium, titanium-molybdenum alloy, solder alloy with silver base and non-metal substances of polycarbonates and ceramic), Mockers et al (Mockers et al, 2002), reported the absence of cytotoxicity of these substances; their finding was in accordance with the result of the current research as it was shown that the toxicity of studied samples is significantly less than the positive control but unlike the results Mockers significant difference between the cytotoxicity of mini-plates and negative control group are shown.

With evaluating cytotoxicity and morphological changes of human fibroblasts of orthodontic appliances, Locci et al (Locci et al, 2000), reported that the 304,316 stainless steel was more biologically compatible compared to solder alloy; furthermore they reported that solder alloy component (Ag and Pd) caused the most cytotoxicity.

The Lin et al (Lin, 2006) finding was in accordance with the result of the current research. By evaluating the clinical outcomes of skull bone flap fixation using a new bioresorbable system (Bonamates) and titanium plate, Lin et al, reported that healing without incident occurred during the entire follow-up period for all 4 patients (100%) using Bonamates and for only 3 of 4 patients (75%) using titanium plate. None of the patient experienced postoperative side effects such as infection, soft tissue dehiscence, bone flap sink and implant related tissue reaction.

With evaluating the sensitivity of liver, kidney and lung for cytotoxicity following implantation of internal fixture materials composed of titanium, Piozzi et al (Piozzi et al ,2009), reported the absence of any toxic; their finding was in accordance with the result of the current research.

The results of MTT assay of Rajaei et al (Rajaei A, Daliri joupari M, 2011) with the aim of evaluating the effect of surface preparation of Sandblasted and Acid-Etched titanium implants on cellular adherence and survival of osteoblasts showed that the recorded viable cells in Sandblasted group were significantly more than that in Acid-Etched pure titanium. However the number of recorded viable cells in comparison with the control group was not evaluated in this study. Therefore it cannot be accurately stated whether this study is in accordance with the result of the current research or not. The aim of Ortiz et al (Ortiz et al, 2011) study was to determine the amounts of metal ions released from nickel-free, stainless steel and titanium which are used in orthodontic appliances. They reported that the titanium alloy had increased cell viability and the least toxicity compared with the control group. Their finding was in accordance with the results of the current research.

With evaluating the increased strength and adhesion of metal to bone tissue, Raji et al (Raji AR, Yamashita k, 2004) showed that the usage of calcium deposits along with titanium increased the adhesion of titanium to collagen fibbers, this also increased the strength of bone and furthermore there was no sign of necrosis in this experiment. In the present study, in comparison of the average value of primary apoptosis to final apoptosis among the 5 groups, a statistically significant difference was identified between the 4 dif-

ferent brands of miniplates and the control group. The apoptosis value in miniplates was significantly higher than the control value ($p<0.05$).

Therefore this can be concluded that the miniplates which are mostly composed of titanium did not cause cytotoxicity; hence the cellular necrosis value was too low and similar to the control group. Some differences were identified while evaluating the results of resources and the present study; this can be due to the type of plates used in the experiments, materials, and the amount of alloy and concentration of different components used in the plates. The methods used in the experiments (In vitro or In vivo) can also affect the outcomes of the studies. The methods used for measuring the dependent variables such as quantity of necrosis, cellular apoptosis and amount of toxicity can also affect the outcomes of the study. Difference in brand of devices and their manufacturing company leads to obtaining different results in this study.

Conclusion

The present study demonstrated that the toxicity amount of JEIL, Imen Ijaz, General-Implants and AO Synthes miniplates with titanium base had the least toxicity and the most biological compatibility.

Conflict of Interest

There is no conflict of interest to declare.

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