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In-Vitro Biocompatibility Study and Comparison of Magnesium AZ31 and PEEK 450G Biomaterials used as Cardiovascular Stent Implants

Vasantha Kumar[1*], C. M. Ramesha[2], V. Sharanraj[3], Sadashiva M[4] and Kavya K[5]

[1]Department of Mechanical Engineering, Bearys Institute of Technology, Visvesvaraya Technological University, Mangalore, Karnataka, India. E-mail: mr.vasis@gmail.com*

[2]Department of Mechanical Engineering, MSRIT, Visvesvaraya Technological University, Bengaluru, Karnataka, India.

[3]Department of Mechanical Engineering (W&SM), Sri Jayachamarajendra (Govt.) Polytechnic, Bengaluru, Karnataka, India.

[4]Department of Mechanical Engineering, PES College of Engineering, Visvesvaraya Technological University, Mandya, Karnataka, India.

[5]Department of Zoology, St. Joseph's University, Bengaluru, Karnataka, India.

Abstract

This research article intended to study and comparison of cytotoxicity effects of Magnesium AZ31 and PEEK 450G biomaterials. L-929 mouse fibroblast cell line was used to measure cytotoxicity effect of Magnesium AZ31 and PEEK 450G biomaterials by extraction method. Biocompatibility in-vitro cytotoxicity test was performed on L-929 mouse fibroblast cell line for Magnesium AZ31 and PEEK 450G samples. In extraction process, leachates take out from the test samples were used for measurements of cytotoxicity. Since there was reactivity and the reactivity grade was greater than '2' in Magnesium AZ31 biomaterial, test sample was measured as non-toxic, where as in PEEK 450G biomaterial there was no reactivity, no reduction in cell growth and no cell lysis, the grade was zero, and test sample was measured as non-toxic. Hence PEEK 450G biomaterial reveals an outstanding cytotoxicity behaviour than Magnesium AZ31. This is an added advantage for cardiovascular stent implant applications.

Keywords: *Cytotoxicity; Biomaterials; PEEK 450G; In-Vitro; Biocompatibility; Cardiovascular stent implant*

1.0 Introduction

Cardiovascular disease is the leading cause of death in the world today. In the United States alone, the number of deaths is anticipated to increase from 17.9 million per year to 23.6 million by 2030[1]. To treat cardiovascular disease, there are several procedures used. Comparing to other potential therapies, the stenting operation has several advantages, including reduced pain and a quicker recovery time. Therefore the use of coronary stents improved from 10% in 1994 to over 80% in current practice[2]. Stent is basically a medical device having a tube scaffold, to deploy within the human body to enlarge to unlock the narrowed artery and permit blood to flow normally. Cardiovascular stents are manufactured by using various types biomaterials based on requirements. A biomaterial is essentially a substance that is utilised and transformed for the purposes of medical treatment. The implantation of cardiovascular

[*]Author for correspondence

devices, dental implants, heart valves, coated hip implants, surgery, and drug delivery are just some of the common applications for these biomaterials[3].

Primarily, biomaterial should be very good biocompatible and should not draw out unpleasant reaction from the human body, and vice-versa. Furthermore, it must be safe because of nontoxic and no carcinogenic. These necessities reduce the use of several engineering materials.

Biomaterials are expected to work efficiently once implantation is carried out, and to interact with the biological system without any problems. This is achievable by the proficient and the consistent characteristics of the bio-materials[4]. The characteristics features require an appropriate grouping of physical, mechanical, chemical, and the biological properties[5]. The bio-materials could be prepared from the metals and alloys, polymers, ceramics and composite materials[6,7]. Biomaterials should possess excellent functional performance coupled with superior biocompatibility.

Biocompatibility indicates the interface between living system and the medical device or constituent material and their interaction. A biocompatible device or material does not harm the patient. In general, biocompatibility is testing to decide the possible toxicity resultant from physical contact with a medical device or material. Biocompatibility is essential for medical devices. The reason of performing the biocompatibility test is to find out the suitability of a biomaterial for human being and to examine whether the biomaterial can have any possible damaging physiological effect. Before doing any kind of biological test to establish a biomaterial's cytotoxicity, material testing and research of the component of the biomaterial are often carried out first. The potential of a biomaterial to kill living cells is measured using a technique called cytotoxicity. The in vitro test and the in vivo test are the two varieties of biocompatibility testing that are available. In-vitro testing can be done in test tubes or on living creatures obtained from the outside. Once the in-vitro study has been done satisfactorily, the in-vivo biological test can be carried out based on the biomaterial's proposed use to check biological reaction like skin irritation and some other medical testing[8].

In the present work, L929 (mouse fibroblast) cell line biocompatibility was evaluated in vitro using the direct contact test technique. Conventionally, the in-vitro a determination of toxic effects of indefinite compound is done by counting feasible cells after discoloration with a critical colour[9,10].

Every coronary stent material should exhibit outstanding biocompatibility and excellent corrosion resistance also as radio opacity. The SS 316L is the most frequently used material for manufacturing of balloon expanding stents, however being slowly replaced by cobalt-chromium alloys, Titanium alloys and Tantalum alloys. Further, Nickel-Titanium is the very frequent alternative for self expandable stents because of the shape memory effect. Newly, Polyether ether ketone (PEEK)[2,11], PLLA and Mg alloys are used to produce the bio-resorbable stents.

The present problem in cardiovascular field is that, Magnesium AZ31 alloy exhibits the cytotoxic effect caused due to allergic reaction between the implant material and surrounding tissues. Hence PEEK 450G has been selected as alternate material for coronary stent implant which may overcome the problems encountered with Magnesium AZ31 implants.

The main objective of this research work is to identify suitable stent material which enables to achieve biocompatibility properties sufficient to convince the cardiovascular disease patient to accept it as an alternate stent implant to replace for Magnesium AZ31 biomaterial for cardiovascular stent applications.

2.0 Materials and Methods

2.1 Biocompatibility Test

Biocompatibility test was carried out by using Magnesium AZ31 and PEEK 450G biomaterials, and samples were prepared by turning operation as per ISO 10993 standards[12]. Figure 1 shows the prepared biomaterials samples.

Figure 1: Biocompatibility test samples

2.2 In-vitro Cytotoxicity Study

The main purpose of this examination was to measure the cytotoxic potential of the extract of the test item, Magnesium AZ31 and PEEK 450G biomaterial using L-929 mouse fibroblast cells as indicated by morphological

Table 1: Extraction ratio and quantity

Treatment	Extraction ratio	Quantity used for extraction	Volume of media used for extraction (mL)
Blank	-	-	4
Test item	0.2 g of sample/mL	5.66 g	28.3
Negative control	3 cm^2 sample/mL	12 cm^2	4
Positive control	6 cm^2 sample/mL	24 cm^2	4

examination of cell lines after treatment. L-929 mouse fibroblast cells are the most commonly used cell line in in-vitro mammalian cell culture studies. Traditionally, L-929 murine fibroblast cells have been utilised to determine the cytotoxicity of medical devices and biomaterials. Minimum Essential Medium Eagles by the addition of L-Glutamine, 10% Fetal Bovine Serum, and 1% Penicillin-Streptomycin. 7.35 was the pH of the medium used for culturing. The media was stored at 2 - 8ºC till use and thawed to room temperature before use.

L-929 mouse fibroblast cells were permitted to proliferate and be maintained in MEM-containing culture flasks. For the experiment, 1105 cells/mL of cell suspension were used. Each well of six-well plates was seeded with one millilitre of cell suspension. The cells were maintained at 37°C for 27 hours and 40 minutes in the presence of 0.25 per cent CO_2 in order to obtain a sub-confluent monolayer prior to usage. Elution Method was chosen in accordance with ISO 10993 Part 5:2009(E) recommendations [13].

In the current investigation extraction method was used for In vitro cytotoxicity study of Magnesium AZ31 and PEEK 450G biomaterials used as cardiovascular stent applications.

2.3 Extraction Procedure

The test item extraction was carried out in a container with MEM (supplemented with 10% Fetal Bovine Serum and 1% Penicillin-Streptomycin) with extraction ratio as mentioned in the below table. The contents were maintained at 37ºC for 23 hours 55 min in an incubator shaker.

Similarly, the media control without test item as blank, negative control and positive control were maintained with extraction ratio and quantity as indicated in Table 1.

Negative control and positive control were made into pieces and used for extraction. The extraction was done as per the following procedure:

1. As the test item is an irregular shaped solid medical device, 0.2 g/mL was used for extraction as per ISO 10993 Part 12 specification [12], 5.66 g of the test item was added to 28.3mL of MEM addition with FBS 10% and

Table 2: Treatment details

Treatment	Details
Blank wells	1 mL of cell culture media without any treatment maintained similar to extraction procedure.
Test item	1 mL of test item extraction mixture.
Negative control	1 mL of high density polyethylene (HDPE) sheet extraction mixture.
Positive control	1 mL of polyurethane sheet extraction mixture.

Table 3: Cytotoxicity Scoring

Grade	Reactivity	Description of Reactivity Condition
0	Nothing	Granules inside the intracytoplasm that are easily distinguishable, no cell lysis, and no suppression of cell development.
1	Minor	The percentage of cells that are spherical, loosely linked, and devoid of intracytoplasmic granules, as well as those that display morphological abnormalities, does not exceed twenty per cent; Even though there is just a moderate amount of growth inhibition, there are some occasional lysed cells.
2	Mild	There is no major cell lysis and less than fifty per cent of the cells have a spherical shape. Additionally, there is no evidence of considerable cell death. Finally, the growth inhibition is less than fifty per cent.
3	Modest	The cell layers are not completely destroyed, but growth is impeded by more than 50 per cent. Not more than 70 per cent of the cell layers have rounded cells or are lysed.
4	Extreme	Cell membranes that have nearly completely or completely broken down.

Penicillin-Streptomycin1%. The medium without any treatment was used as blank.

2. As the thickness of negative control was greater than 0.5 mm, 3 cm^2/mL of extraction ratio was required; hence 12cm^2 of HDPE was cut into pieces and extracted in 4mL of MEM with 10% FBS and 1% Penicillin-Streptomycin for extraction at the ratio of 12cm^2 : 4mL (surface area/volume).
3. As the thickness of positive control was less than 0.5 mm, 6 cm^2/mL of extraction ratio was required; hence 24 cm^2 of Polyurethane sheets was cut into pieces and extracted in 4mL of MEM with FBS 10% and Penicillin-Streptomycin 1% extraction at the ratio of 24cm^2 : 4mL (Surface area/volume).

2.4 Testing Procedure

Selecting the culture wells with a subconfluent cell monolayer. All procedures were performed in duplicate. Wells were maintained by changing 1 mL of growth medium with the corresponding extraction mixes of the test item, the positive control, and the negative control. As demonstrated in Table 2, blank wells were maintained by adding 1 mL of extraction growth medium without any treatment. The wells were labelled with the study number, the number of replicates, the associated treatment, and the treatment date.

For 47 hours, cells were maintained heated at 37°C in the presence of 0.25 per cent CO_2. After 47 hours of incubation, the cultures were inspected microscopically for any

Table 4: Mg AZ31 Cytotoxicity study results

Treatment	Microscopic observations			
	Replicate	Grade	Reactivity	Condition of the culture
Blank	1	0	Nothing	Granules inside the intracytoplasm that are easily distinguishable, no cell lysis, and no suppression of cell development.
	2	0	Nothing	Granules inside the intracytoplasm that are easily distinguishable, no cell lysis, and no suppression of cell development.
	3	0	Nothing	Granules inside the intracytoplasm that are easily distinguishable, no cell lysis, and no suppression of cell development.
Negative control (HDPE)	1	0	Nothing	Granules inside the cytoplasm that are easily distinguishable, no lysis of the cell, and no inhibition of cell development.
	2	0	Nothing	Granules inside the cytoplasm that are easily distinguishable, no lysis of the cell, and no inhibition of cell development.
	3	0	Nothing	Granules inside the cytoplasm that are easily distinguishable, no lysis of the cell, and no inhibition of cell development.
Positive control (Polyurethane)	1	4	Extreme	The cell membranes have almost completely or completely broken down.
	2	4	Extreme	The cell membranes have almost completely or completely broken down.
	3	4	Extreme	The cell membranes have almost completely or completely broken down.
Test item - Magnesium AZ31	1	4	Extreme	The cell's outermost layers have almost completely or completely disintegrated into their constituent parts.
	2	4	Extreme	The cell's outermost layers have almost completely or completely disintegrated into their constituent parts.
	3	4	Extreme	The cell's outermost layers have almost completely or completely disintegrated into their constituent parts.

alterations in cell morphology caused by the test substance.

When a test item had a numerical score of 0 (no response), it was deemed non-cytotoxic.

Cytotoxicity scoring was done based on the following criteria as represented in Table 3.

3.0 Results

Table 4 provides a summary of the in vitro cytotoxicity data for magnesium AZ31 biomaterial. Subconfluent monolayers of L-929 murine fibroblast cells were treated with the test substance, a negative control, a positive control, and a blank for 47 hours at 37 degrees Celsius and 5% CO_2. L-929 murine fibroblast cells were analyzed microscopically for changes in cell shape and cell death following treatment with extracts of the test item, positive control, negative control, and blank. Treatment of L-929 murine fibroblast cell lines with the test substance and a positive control extract resulted in nearly full or whole cell layer breakdown and severe reactivity (Grade 4).

Table 5 presents the in vitro cytotoxicity data for PEEK 450G biomaterial. Subconfluent monolayers of L-929 murine fibroblast cells were treated with the test substance, a

Table 5: PEEK 450G Cytotoxicity study results

Treatment	Microscopic observations			
	Replicate	Grade	Reactivity	Condition of the culture
Blank	1	0	Nothing	Granules inside the intracytoplasm that are easily distinguishable, no cell lysis, and no suppression of cell development.
	2	0	Nothing	Granules inside the intracytoplasm that are easily distinguishable, no cell lysis, and no suppression of cell development.
	3	0	Nothing	Granules inside the intracytoplasm that are easily distinguishable, no cell lysis, and no suppression of cell development.
Negative control (HDPE)	1	0	Nothing	Granules within the intracytoplasm that are distinct, no lysis of the cells, and no slowing of cell growth.
	2	0	Nothing	Granules within the intracytoplasm that are distinct, no lysis of the cells, and no slowing of cell growth.
	3	0	Nothing	Granules within the intracytoplasm that are distinct, no lysis of the cells, and no slowing of cell growth.
Positive control (Polyurethane)	1	4	Extreme	The cell membranes have almost completely or completely broken down.
	2	4	Extreme	The cell membranes have almost completely or completely broken down.
	3	4	Extreme	The cell membranes have almost completely or completely broken down.
Test item - PEEK 450G	1	0	Nothing	Granules inside the intracytoplasm that are easily distinguishable, no cell lysis, and no suppression of cell development.
	2	0	Nothing	Granules inside the intracytoplasm that are easily distinguishable, no cell lysis, and no suppression of cell development.
	3	0	Nothing	Granules inside the intracytoplasm that are easily distinguishable, no cell lysis, and no suppression of cell development.

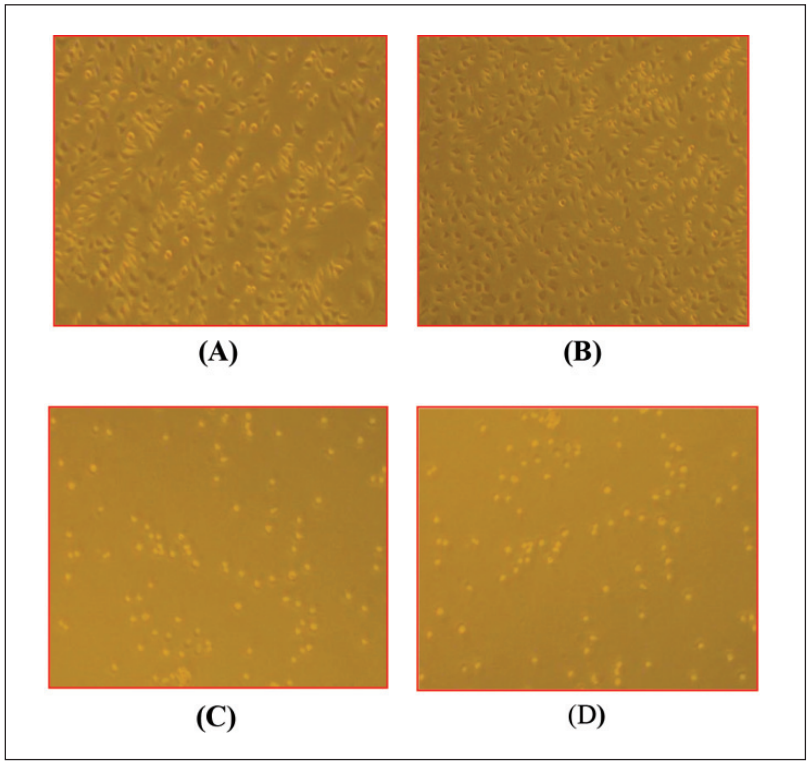

Figure 2: Representative images of the test system post treatment (A) Blank, (B) Negative Control (C) Positive control and (D) Magnesium AZ31

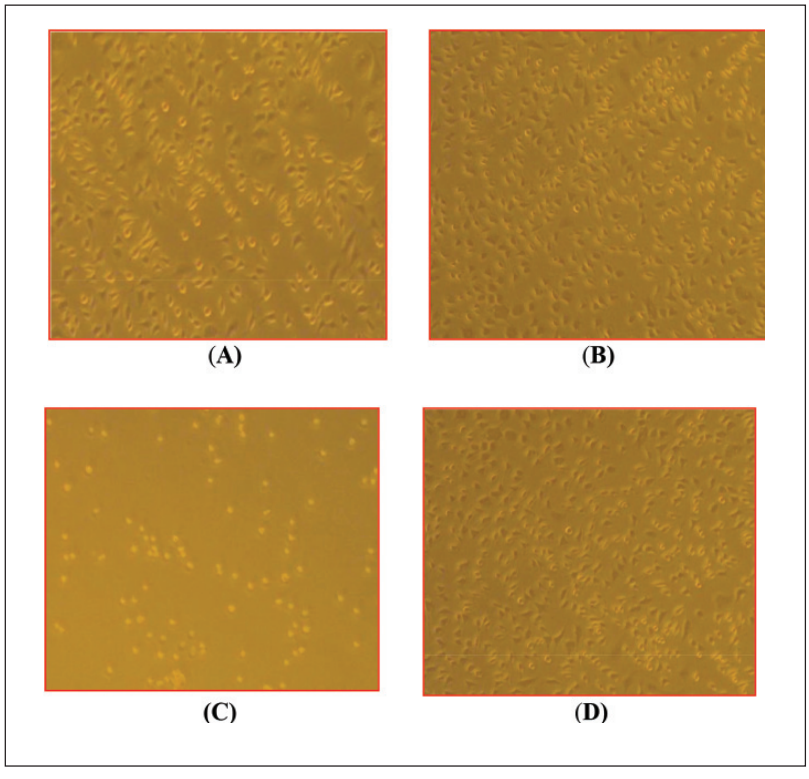

Figure 3: Representative images of the test system post treatment (A) Blank, (B) Negative Control (C) Positive control and (D) PEEK 450G

negative control, a positive control, and a blank for 47 hours at 37°C and 0.25 per cent CO_2. L-929 murine fibroblast cells were analysed microscopically for changes in cell lysis and morphology following treatment with extracts of the test item, positive control, negative control, and blank. L-929 mouse cell lines treated with the test item extract exhibited distinct intracytoplasmic particles, without a decrease in cell growth or cell lysis (Grade 0).

4.0 Discussion

As shown in Table 4, since there was reactivity and the reactivity grade was more than 2, the test item is determined to be cytotoxic according to the Magnesium AZ31 cytotoxicity research results. As seen in Figure 2, cells in the blank wells and negative control exhibited distinct intracytoplasmic particles, no loss in cell growth, no cell lysis, and no reactivity (Grade 0).

In contrast, according to Table 5 and the findings of the PEEK 450G cytotoxicity investigation, since there was no response and the grade was not larger than 2, the test item was deemed non-cytotoxic.

As depicted in Figure 3, cells in the blank wells and negative control exhibited separate intracytoplasmic particles, no reduction in cell growth, no cell lysis, and no reactivity (Grade 0), whereas cells in the positive control exhibited almost total or total destruction of cell layers and severe reactivity (Grade 4).

Biocompatibility in-vitro cytotoxicity testing shows that PEEK 450G demonstrates the best cell proliferation with zero grade cytotoxicity compared to Magnesium AZ31.

5.0 Conclusion

Biocompatibility L-929 murine fibroblast cells were used to conduct an extraction-based in vitro cytotoxicity assay. It is evident that the PEEK 450G biomaterial demonstrates the highest cell growth with no cytotoxicity. Whereas there was reactivity in the magnesium AZ31 biomaterial and the reactivity grade was more than 2, the test

item is judged cytotoxic. Therefore, PEEK 450G biomaterial is considered to have higher biocompatibility and can be utilised as an alternative cardiovascular stent implant and in other medical implant applications.

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