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RESEARCH ARTICLE

An *in vitro* Study to Evaluate the Bioactivity of Osteoblast Cells on the Titanium Disk Coated with the Hydro Gel formulated from Acemannan and Curcuminoids

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ABSTRACT

Background: Recently, increasing attention has been paid to the potential of hydrophilic implant surfaces to further stimulate osseointegration.

Purpose: The main purpose of this study was to evaluate the cell viability with the Hydro gel formulated from acemannan and curcuminoids coated on titanium disks.

Materials and methods: Hydro gel formulated from acemannan and curcuminoids in different proportions were prepared and coated on the titanium disk and the bioactivity of Osteoblast cells (MG-63) was evaluated. The hydrogel was formulated in to 3 groups. Sample 1 (75% acemannan and 25% curcuminoid), sample 2 (50% acemannan and 50% curcuminoid) and sample 3 (25% acemannan and 75% curcuminoid). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done to evaluate the cell viability. To exclude material's effect on the test and to see how the cells react during the assay, the MTT assay was also performed for the disks which were not seeded with the cell culture.

Results: Curcumin and acemannan had good cell viability from day 1, but then, the viability decreased. Acemannan showed less than 10% inhibition at 10 µg/mL when compared with curcumin and so acemannan in the formulation 10 µg/mL can be used. Osteoblastic differentiation (OD) of sample 1 at the end of 24 hrs was 0.0837 and over all % of cell inhibition was 75.65;for sample 2 at the end of 24 hrs OD was 0.2402 and overall % cell inhibition was 57.67%; for sample 3 at the end of 24 hrs OD was 0.1643 and over all % of cell inhibition was 75.62%.

Conclusion: Changes in concentration of hydrogel could not create any favourable condition for the proliferation of the cells, secondly the other condition was the use of cancerous cell line,turmeric previously showed to have tremendous effect on killing cancer cells which also might have contributed for the negative outcome of the result.

Keywords: Acemannan, Curcuminoids, Hydrophilic gel, MTT assay, Osseointegration, Titanium.

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INTRODUCTION

Currently, dental implants have become an integral part of dentistry.[1-3] Enhancing of dental implant osseointegration has been an area of research for more than four decades now. Researchers have tried from macrogeometry to microgeometry, change in materials, drill sequences, etc., to enhance osseointegration.[4-6] It is well acknowledged that the overall success and survival of implants depend on the quality and quantity of host bone, presence of sufficient primary stability at the time of implant placement, and formation of a direct BIC. However, osseointegration and BIC are mainly enhanced by implant surface characteristics, such as surface topography, chemistry, roughness, and energy.[7-10]

Recently, increasing attention has been paid to the potential of hydrophilic implant surfaces to further stimulate osseointegration.[9] It was well documented that in an attempt to improve implant surface activity and osteopromotive activity, implants were coated with localized organic and inorganic osteogenic coatings on their surfaces.[11] It was suggested that hydro gels should be incorporated in the gel, as they are hydrophilic in nature and can give a sustained release of bone-proliferating medicine.[2]

Previously, herbal extracts were used in bone-healing process. Acemannan is extracted from aloe vera gel, and it is a biodegradable polysaccharide consisting of beta-acetylated polymannose. *In vitro* studies had shown that acemannan can stimulate gingival fibroblast, cementoblasts, dental pulp fibroblasts and lead to bone marrow-stromal cell proliferation and differentiation.[12-14] Since centuries, turmeric has been used as a dietary spice and as a traditional Indian medicine. Extracts obtained from the rhizome of turmeric (*Curcuma longa*), have been used as anti-inflammatory agents. In various experimental conditions and clinical settings, it was found that curcumin exhibits antibacterial, antioxidant, antifungal, antiviral, and anticancer activities.[15-17]

JAYPEE

The main aim of the present study was to formulate a hydrophilic gel to be used along with the dental implant placement to create a hydrophilic surface for the dental implant, to enhance implant-to-blood contact, but would also enhance the BIC. The study was a basic microbiological study and the aim of this study was to evaluate the influence of direct exposure of gel applied and nongel applied to titanium disks on the bioactivity of osteoblast cells (MG-63).

MATERIALS AND METHODS

The study was approved by the institutional animal ethics committee of RajaRajeswari Dental College & Hospital, Bengaluru, India.

Basic Microbiological Study

The study was done to find the long-term direct effect of hydrophilic gel on different cell lines. Viability of the cells and their differentiation were analyzed so that biocompatibility of the tested materials can be assessed. *In vivo* situation was simulated as closely as possible to evaluate the long-term effects of the gel on osteoblast cells. The hydrogel was formulated into three groups. Sample 1 (75% acemannan and 25% curcuminoid), sample 2 (50% acemannan and 50% curcuminoid), and sample 3 (25% acemannan and 75% curcuminoid) (Fig. 1). Disks of commercially pure titanium grade IV (ASTM F67) with diameter of 5 mm and thickness of 2 mm were used (Fig. 2). The oxide layers formed on the surface of the disks were removed by subjecting it to an acid treatment. Titanium disks were coated with the hydrophilic gel made from acemannan and curcuminoid in different combinations. For proper coating of gel around the disks and controlled release, the disks were immersed in gel for around 45 to 60 seconds.

Procedure

Trypsinized (70–80%) confluent cell lines (MG-63) were centrifuged and checked for the viability. The formulated hydrogel samples 1, 2, and 3 were put on each titanium disk. After half an hour, samples with titanium disks were placed in each 96-well plate. Seeded 50,000 cells/well in 96-well plate were incubated for 24 hours at 37°C in 5% CO_2 incubator. After 24 hours of incubation, the medium was removed and replaced with fresh medium and kept for next 24 hours for incubation and for cells to proliferate. After 24 hours, the medium was removed from the wells and 100 µL/well of the MTT (5 mg/10 mL of MTT in 1X PBS, the solution is filtered through a 0.2 µm filter and stored at 2 to 8°C for frequent use or frozen for extended periods) working solution was added and incubated for 3 to 4 hours. After incubation with MTT reagent, the medium was removed from the wells and 100 µL of dimethyl sulfoxide (DMSO) was added to rapidly solubilize the formazan. The absorbance was measured at 590 nm.

$$\text{\% of inhibition} = 100 - \left(\frac{\text{sample}}{\text{control}}\right) \times 100$$

Cell Viability

An MTT assay was conducted to determine cell viability; 12-well plates containing gel applied and non-gel applied disks were seeded with 10,000 cells per cm^2 and F12K (Kaighn's modification of Ham's F-12 medium) medium with 20% fetal calf serum (FCS) and 100 µg/gm streptomycin. During the experiment, the cell media were changed once a week. Nine wells per specimen in total at different time intervals were tested. The MTT solution was added to the cell medium after 24 hours, 7 days, 14 days, and 21 days. The cells were then incubated in the dark for 4 hours at 37°C. The cell medium

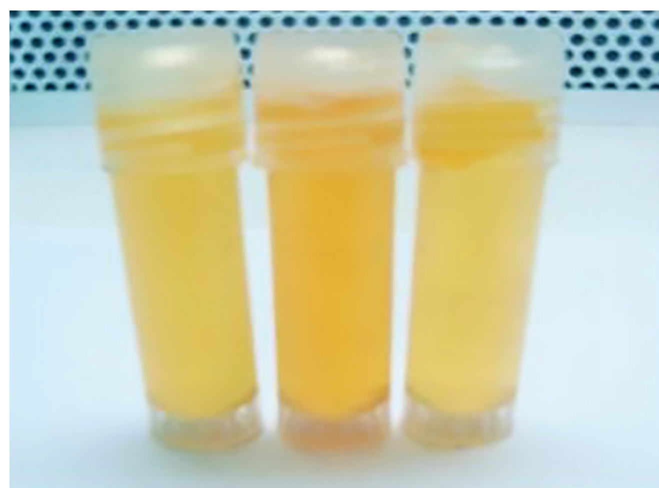

Fig. 1: Hydrophilic gel formulated from acemannan and curcuminoids in three different proportions

Fig. 2: Titanium disks 5 mm in diameter and 2 mm in width

was discarded subsequently and the cells were lysed with 0.004 N HCl in isopropanol. The cell lysates were centrifuged and supernatants were transferred as triplets to a 96-well plate. The adsorption was measured at 570 and 630 nm using a Synergy HT microplate reader (BioTek, Bad Friedrichshall, Germany).

In order to exclude the material's effect on the test and to see how the cells react during the assay, the MTT assay was also performed for the disks which were not seeded with the cell culture. Additionally, cell morphology was studied by inverted light microscopy using a microscope type 090-135.002 (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a Nikon Ds-Fi1 digital camera (Nikon, Duesseldorf, Germany).[18]

Alkaline Phosphatase Content

Senso Lyte pNPP alkaline phosphatase (ALP) assay (Ana Spec, Fremont, CA), which acts as an indicator of changes in the differentiation behavior of the bone-forming cells caused by the test substances, was applied after 24 hours, 7 days, 14 days, and 21 days of culturing in Dulbecco's modified Eagle's medium, low glucose with l-glutamine, 10% FCS, 100 U/mL penicillin, 100 μg/gm streptomycin, 0.1 μM dexamethasone, 0.005 μM ascorbic acid, and 10 mM β-glycerolphosphate to induce osteogenic differentiation. During the experiment, the cell media were changed in once a week. Nine wells per specimen in total at different time intervals were tested.

The cells were washed and frozen at –80°C. After thawing, the cell number was measured according to the manufacturer's protocol, using a PicoGreen dsDNA quantitation assay (Invitrogen, Eugene, OR); 1% Triton X-100 in phosphate-buffered saline was used to lyse the cells. The cell lysates were centrifuged in a 96-well plate, and the supernatants were mixed with the PicoGreen® working solution. The excitations of the samples were done at 485 nm and the fluorescence emission intensity was measured at 528 nm. The cells lysed for the PicoGreen assay were centrifuged, and in specific assay, buffer dilution of the supernatants was done. The diluted samples were coated with ALP and the absorbance was measured at 405 nm. The absolute amounts of ALP were correlated with the cell numbers obtained from the PicoGreen assay. In order to exclude the material's effect on the test and to see how the cells react during the assay, both ALP and PicoGreen assays were also performed for the disks which were not seeded with the cell culture.[18]

Statistical Analysis

Statistical Package for the Social Sciences (version 22, IBM, Corp.) software was used for statistical analysis. Mean and standard deviation were done to find the frequency distribution for continuous variables. Student's unpaired t-test was used to compare mean values between the two groups. The level of significance was set at $p < 0.05$.

RESULTS

Basic Microbiological Study

Curcumin and acemannan had good cell viability from day 1, but then the viability decreased (Tables 1 and 2). Osteoblastic differentiation (OD) of sample 1 at the end of 24 hours was 0.0837 and the overall cell inhibition was 75.65%; for sample 2, at the end of 24 hours, OD was 0.2402 and the overall cell inhibition was 57.67%; for sample 3, at the end of 24 hours, OD was 0.1643 and the overall cell inhibition was 75.62% (Table 3). Changes in concentration of hydrogel could not create any favorable

Table 1: Individual analysis of curcuminoids

Plant name	*Concentration (μg/mL)*	*Absorbance 590 nm*	*% inhibition*	*IC_{50}*
Control	0.0	0.495	0.00	36.13 μg/mL
Curcumin	1.5	0.482	2.70	
	3.1	0.449	9.37	
	6.3	0.403	18.65	
	12.5	0.346	30.16	
	25.0	0.279	43.68	
	50.0	0.164	66.90	

Table 2: Individual analysis of acemannan

Plant name	*Concentration (μg/mL)*	*Absorbance 590 nm*	*% inhibition*	*IC_{50}*
Control	0.0	0.347	0.00	Not applicable
Aloe vera	1.5	0.341	1.64	
	3.1	0.334	3.66	
	6.3	0.329	5.11	
	12.5	0.312	10.01	
	25.0	0.295	14.91	
	50.0	0.243	29.91	
	100.0	0.207	40.29	

Table 3: Absorbance values of effect of sample with titanium disk on MG-63 cells by MTT assay

Sample	*OD (n = 1)*	*OD (n = 2)*	*OD (n = 3)*	*Average*	*% inhibition*	*Comment*
(Control) only titanium disk	0.7288	0.6067	0.5061	0.6138	0.00	
Sample 1 + titanium disk	0.0837	0.195	0.1696	0.1494	75.65	No proliferation
Sample 2 + titanium disk	0.2402	0.3032	0.2361	0.2598	57.67	No proliferation
Sample 3 + titanium disk	0.1643	0.1334	0.1511	0.1496	75.62	No proliferation

condition for the proliferation of the cells, and secondly, the other condition was the use of cancerous cell line; turmeric previously showed to have tremendous effect on killing cancer cells which also might have contributed for the negative outcome of the result.

DISCUSSION

Osseointegration results in structural and functional contact between implant surface and living bone.[20] Since the beginning of implant dentistry, attention has been paid to the design of implant surface topography for enhancing osseointegration.[2,21,22] Microsurface supports the transition from mechanical to biological stability, helping to minimize the risk of early complication.[23] Hydrophilic implant surface has also been used.[24] *In vitro* studies have shown that hydrophilic surface supports the adhesion of monocytes, platelet activation, and blood clot formation, which are the initial events in osseointegration.[25,26] Hydrophilic surfaces are favorable for the osteogenesis differentiation of mesenchymal stem cells and they demonstrated anti-inflammatory properties.[27,28]

This study looked at the direct long-term effect of hydrophilic gel on different cell lines. Cell viability, differentiation, and morphology as well as pH and calcium uptake were analyzed to assess the overall biocompatibility of the tested materials. We evaluated the long-term effects of the gel on osteoblast cells to simulate them in *in vivo* situation as closely as possible.

It was also shown in previous studies that acemannan polysaccharides extracted from aloe vera have negligible cytotoxicity and sound cytocompatibility.[12] Pure acemannan had high viability at the very first day, but then the viability decreased. Turmeric which has excellent anti-inflammatory and bone-healing properties[15] was added as a second ingredient to the gel.

An important drawback of tetrazolium-based tests is that the difference between cytotoxic (cell death) and cytostatic (reduced growth rate) effects cannot be distinguished.[29] We thus looked at cell morphology under light microscopy and transmission electron microscopy (TEM). After examination under TEM and light microscopy, it was revealed that the number of cells decreased in the presence of hydrophilic gel. Later, individual analysis of both the ingredients was done; in this, it was shown that curcumin had a very high percentage of cell inhibition, whereas acemannan had significantly better results, as it can be used until 10 µg/mL.

Degradation particles were also found in the cytoplasm. The presence of high amounts of degradation products inside the cells could explain the lower cell viability values for hydrophilic gel. It was shown in previous studies that the uptake of material particles leads to induction of cell stress which triggers cytotoxicity. Here in the present study, we showed that the treatment of MG-63 cells with hydrophilic gel inhibited their proliferation. This result is consistent with previous studies performed in human[30] or rat[31] osteoblast and with others, indicating that curcumin inhibited the proliferation of several cell types, most likely due to an apoptosis-dependent mechanism.[32] This mechanism has not been completely elucidated, but it may be, at least in part, due to the ability of curcumin to suppress the activation of activator protein 1, a dimeric transcription factor consisting of a Fos-related osteoblast differentiation. During their developmental sequence, osteoblasts express genes associated with differentiation protein and a Jun-related protein. Osteoblast differentiation comprises three distinct processes: proliferation, maturation of the extracellular matrix, and mineralization.[33-36] We observed changes in the early stages, with increased ALP expression in curcumin-stimulated cells at 7 days postculture. However, there was a significant decrease in the late stages of osteoblast differentiation at day 21, evidenced by the decelerating accumulation of calcium in osteoblasts.

In previously reported studies, curcumin has been reported to cause the arrest of cell cycle and the upregulation of expression of p21WAF1/CIP1.[37-39] Furthermore, there is a report that p21WAF1/CIP1 mediates G1 arrest. Thus, the inhibitory effects of curcumin on proliferation of ROB cells might be due, in part, to the arrest of cell cycle progression via expression of p21WAF1/CIP1. In addition, curcumin inhibited the mineralization by ROB cells. Recently, p21WAF1/CIP1 has been reported to act as a brake in osteoblast differentiation by using p21WAF1/CIP1 null mice.[40] Furthermore, transient overexpression of p21WAF1/CIP1 from an adenovirus vector delayed the onset of differentiation both in wild-type and in p21WAF1/CIP1 null osteoblasts.[39]

Turmeric inhibition for cell proliferation could be supported by studies done by Notoya et al,[31] wherein they concluded that curcumin might be an inhibitor for metabolism of both cultured osteoblast and osteoclasts. Hence, this combination was prepared for the animal trials.

Future studies can be carried out which might include implant placement with hydrogel coating in patient with compromised wound healing (e.g., diabetes mellitus, osteoporosis, and postradiation therapy) and in clinical situation, such as immediate implant placement or implant placement with guided bone regeneration.

CONCLUSION

Changes in concentration of hydrogel could not create any favourable condition for the proliferation of the cells, secondly the other condition was the use of cancerous cell line,turmeric previously showed to have tremendous effect on killing cancer cells which also might have contributed for the negative outcome of the result.

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