ORIGINAL ARTICLE

In Vitro Osteogenic Analysis of a Novel Scaffold for Bone Regeneration

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ABSTRACT

Objective: This study aimed to analyse the osteogenic potential of a novel experimental material comprising of nano-hydroxyapatite grafted glass fiber scaffolds using murine osteoblast MC3T3-E1 cell lines through gene expression of biomarkers for osteogenesis.

Study Design: In vitro experimental study.

Place and Duration of Study: Bone Marrow Transplant Centre and Genetic Resource Centre, Rawalpindi from 10th May 2021 to 17th June 2022.

Materials and Methods: Two types of nano-apatite grafted E-glass fiber scaffolds i.e., 10 wt% E-glass/90wt% Hydroxyapatite (E-10), 20% E-glass/80% Hydroxyapatite (E-20) were used, whereby pure nanohydroxyapatite (E-0) was used as control group. A comparative gene expression of Osteopontin (OP) and Collagen (Col) Type 1 was measured through reverse transcription polymerase chain reaction (RT-PCR).

Results: Both experimental groups showed expression of the osteogenic proteins. The level of OP expression was higher in E-10 compared to E-20, whereas the level of Col expression was higher in E-20. Only for E-0, Col expression was almost same as OP whereas for the E-10 and E-20, OP expression was greater.

Conclusions: Based on the findings, both experimental scaffolds supported the growth of cells and showed an osteogenic nature. It is anticipated that the experimental scaffolds have potential to be used for bone regeneration.

Key Words: *Bone Regeneration, E-glass fibres, Gene expression, Hydroxyapatite, Reverse Transcription Polymerase Chain Reaction.*

Introduction

Tissue engineering concepts have promising prospects as they combine the use of scaffolds in combination with techniques at a molecular level to replace missing or diseased tissues.[1] These procedures not only restore the non-functioning tissues, but also utilize specific materials that can be used to create a fully functional, three dimensional (3D) and structurally sound tissue. These regenerative procedures are now being explored to replace bone defects and loss of bone seen in periodontal diseases. The essence of the treatment aims at eliminating inflammation and promoting periodontal regeneration.[2]

As tissue engineering has evolved with growing research, new materials are now being explored to develop an ideal scaffold possessing an interlinked structure with porosities, reliable mechanical properties and biocompatibility.[3] Hydroxyapatite (HA) has also been widely researched due to its excellent biocompatibility owing to structural similarities to the inorganic or HAP bone phase. It has the remarkable capacity to bond directly to the bone and provide a structural base for cellular attachment and proliferation.[4]

For bone tissue engineering HA scaffolds are primarily opted in non-load bearing areas owing to their inferior physico-mechanical characteristics and slow degradation.[4] To overcome this disadvantage, a new amalgamation of nano Hydroxyapatite (nHA) with E-glass fibres was developed in which the fibres were added at different concentrations to impart

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strength and rigidity to the otherwise brittle nHA. An in vitro trial was conducted to document the osteogenic potential of this novel material.

Our study involves the analysis of the experimental scaffold using murine osteoblast MC3T3-E1 cell lines through gene expression of biomarkers for osteogenesis. Gene expression for two biomarkers namely (Col) Type 1 Collagen and Osteopontin (OP) will be observed through PCR. Expression of the Col Type 1 gene signifies the initial phase of bone formation whereas expression of OP gene comes in the later stages which is consistent with mineralization. [5] OP gene expression is a marker for mature osteoblasts signifying an advanced stage in bone formation.[5]

The prospect of studying the behaviour of the cells through gene expression is very promising as the complete information on the cells 'genetic code' can be obtained with polymerase chain reaction (PCR).[6] The study of expression of the genetic code through quantitation of messenger RNA (mRNA) is called gene expression study. The Pfaffl method was used for relative quantification in which change in expression is expressed as a ratio as it can provide useful information about the physiological and pathological processes.[7] In this study real time PCR was employed which is considered a gold standard for quantitative PCR and gene expression studies.[8]

RT-PCR is considered a powerful tool for amplification of small amounts of mRNA due to its high sensitivity, accuracy and quick reading of gene expression.[8] As this technique directly measures the amount of bone proteins being formed by the cells, it leaves no doubt or need for any other analysis as the 'genetic code' of the cell can be read directly and information regarding any function of the cell can be determined. In case of bone tissue engineering, it is an authentic method to determine the progress of tissue formation and identify the stages based on expression of different proteins.

This in vitro study aimed at determining the suitability of the experimental scaffold to be utilized for bone tissue engineering, using murine osteoblasts by measuring the gene expression of biomarkers expressed during different stages of bone formation. The prospects of tissue engineering are promising as the tissue heals naturally with better clinical outcomes.

Materials and Methods

This In vitro experimental study was conducted in Bone Marrow Transplant Centre and Genetic Resource Centre, Rawalpindi from 10th May 2021 to 17th June 2022. Permission from the Ethical Review Board of National University of Medical Sciences was obtained on 5th April 2021. Murine Osteoblasts MC3T3-E1 (RIKEN Bio Resource Centre, Japan) were used for the osteogenic analysis of the materials. The culture media and related consumables were purchased from GIBCO®. The cell line was cultured in α-Minimum Essential Medium (α-MEM), where 10% Fetal Bovine Serum (FBS) and 2 mM Glutamine were added. The cells were stored at 37°C and 5% CO_2. The osteoblasts were trypsinized using 0.25% trypsin and seeded on to the test materials (E-0, E-10, and E-20 discs) at a concentration of 3×10^4 /well. Composition of the materials is given in Table I. The size of the sample discs was kept at 6×2mm. According to the ISO Standard for Direct Contact Test ISO 10993-5, it should cover at least one tenth of the cell layer surface. The experiment was done for Triplicates (n=3), for each sample group (ISO Standard ISO 10993-5). The control plate (E-0) was given a similar treatment, and each well plate was stored at 37°C with 5% CO_2 for 5 days. The experimental cells (cells with discs) and control cells (cells without discs) were detached, for RNA extraction towards the end of the 5-day experiment. The discs were removed from the well plate and 220 µL of cell lysis buffer was used. The RNA was extracted from the lysed cells using the RNA extraction machine. A DNAase (1 µL) treatment was given to the obtained sample of pure RNA.[9] The confounding variable in this study was the DNA sample in the extracted RNA as that may be responsible for false positive results. Incubation of the sample was done at 37°C for 10 min and DNase-1 was inactivated at 75°C for 10 min.

After decontamination, cDNA synthesis was done by using the enzyme, MMLV Reverse transcriptase (RT) (Thermo Fisher Scientific, USA).[10] Primer synthesis was done by Integrated DNA Technologies (IDT), USA. The primers and their sequences are given in Table II. A real-time PCR (Rotor Gene-Q machine, Qiagen, USA) was run for 35 cycles with initial denaturation at 95°C for 5 min and denaturation at 95°C for 15s followed by annealing/extension at 60°C for 50 s. The control, housekeeping gene was

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GAPDH. The cycle threshold (Ct) values of both the target genes, Type 1 Collagen and Osteopontin as well as GAPDH was used for data analysis. Quantification of mRNA expression was done and compared with the relative levels of GAPDH.

The gene expression was measured from an amplification plot generated from the PCR software in which a comparison of cells with and without exposure was done. The Ct values of the target and the reference genes were used for mathematical calculations. The first significant amount of fluorescence in real-time PCR correlated with the amount of template RNA. The target genes in the test (exposed) and the control (unexposed) samples were amplified and their Ct values were noted. A reference gene (GAPDH), unrelated to the target gene, was also amplified to control for the variation in the RNA concentration between the test and the control samples. The difference in the Ct values of the control and the test is called ΔCt. The relative expression ratio (R) of the target gene was calculated based on the ΔCt of the target and the reference gene as follows:

$\Delta Ct = Ct^{control} - Ct^{test}$

$Ratio\ (R) = 2.0^{\Delta Ct(target)} / 2.0^{\Delta Ct(reference)}$

Statistical analysis was done using Statistical Package for Social Sciences (SPSS) version 21.0. For comparable variables between groups one-way analysis of variance (ANOVA) was done and for a pair-wise comparison post hoc Tuckey's test was done.

Table I: Composition of test materials and sample group

Sample Group	Composition	
E0	No E glass	100 wt% Hydroxyapatite
E10	10 wt% E-glass	90 wt% Hydroxyapatite
E20	20 wt% E-glass	80 wt% Hydroxyapatite

Table II: F (forward) R (reverse) Primer Sequences for PCR

Gene	Primer Sequence
Mur-Col-1a1-F	5'-GAGAGGTGAACAAGGTCCCG
Mur-Col-1a1-R	5'-AAACCTCTCTCGCCTCTTGC
Mur GAPDH-F	5'-AAGGTCATCCCAGAGCTGAA
Mur GAPDH-R	5'-CTGCTTCACCACCTTCTTGA
Mur OP-F	5'-TCTGATGAGACCGTCACTGC
Mur OP-R	5'-AGGTCCTCATCTGTGGCATC

Results

Results of Collagen Type 1 and Osteopontin gene expression include the ratio of relative expression among the test samples (exposed) and control samples (unexposed). The relative expression ratio of OP in the experimental groups was 15.4 (E-0), 10.2 (E-10) and 9.3 (E-20) respectively. The mathematical calculations of the Pfaffl method for OP gene expression are summarised in Table III. The presence of 10% E-glass/90% nHA reduced the level of OP expression from 15.4-fold to 10.2-fold (p=0.009). The presence of 20% E-glass/80% nHA reduced the level of OP gene expression from 15.4-fold to 9.30-fold (p=0.001).

The relative expression ratio of Col in the experimental groups was 15.69 (E-0), 6.59 (E-10) and 7.22 (E-20) respectively. The mathematical calculations of the Pfaffl method for Col gene expression are summarised in Table 4. The presence of 10% E-glass/90% nHA reduced the level of Col expression from 15.69-fold to 6.59-fold ($p < 0.001$). The presence of 20% E-glass/80% nHA reduced the level of Collagen gene expression from 15.69-fold to 7.22-fold ($p < 0.001$). A Comparison of the relative expression ratio of OP and Col measured and compared in each sample group is shown in Figure 1**.** Only for E-0, Col expression was almost same as OP whereas for all the other experimental materials, OP expression was greater in comparison to Col.

Table III: Ct and ratio (R) of OP gene expression in murine osteoblasts exposed to E-10, E-20 E-0 and significance of post hoc Tuckeys test.

Groups	*Gene*	*Cycle threshold test*	*Cycle threshold control*	*ΔCt#*	*$2.0^{\Delta Ct}$*	*Ratio (R)@*	*Expression%*	*Significance*
E-0	Target OP	27.15	16.81	-10.34	0.00077	15.4	100%	
	Ref GAPDH	31.41	17.23	-14.18	0.00005			-
E-10	Target OP	27.74	16.81	-10.93	0.00051	10.2	66.2%	
	Ref GAPDH	31.61	17.23	-14.38	0.00005			0.009
E-20	Target OP	22.90	16.81	-6.09	0.01470	9.30	60.3%	
	Ref GAPDH	26.54	17.23	-9.31	0.00158			0.001

 # (Ct control – Ct test) **@$2.0^{\Delta Ct(target)}/2.0^{\Delta Ct(reference)}$**

Table IV: Ct and ratio (R) of Col gene expression in murine osteoblasts exposed to E-10, E-20 and E-0 and significance of post hoc Tuckeys test

Groups	*Gene*	*Cycle threshold test*	*Cycle threshold control*	*ΔCt#*	*2.0^ΔCt*	*Ratio (R)@*	*Expression %*	*Significance*
E-0	Target Col	27.16	19.92	-7.24	0.0066	15.69	100%	
	Ref GAPDH	31.40	20.18	-11.22	0.0004			-
E-10	Target Col	28.50	19.92	-8.58	0.0026	6.59	42.03%	
	Ref GAPDH	31.51	20.18	-11.33	0.0003			p < 0.001
E-20	Target Col	27.16	19.92	-7.24	0.0066	7.22	46.02%	
	Ref GAPDH	30.27	20.18	-10.09	0.0009			p < 0.001
	Ref GAPDH	30.22	20.18	-10.04	0.0009			

 # (Ct control – Ct test) **@ $2.0^{\Delta Ct(target)}/2.0^{\Delta Ct(reference)}$**

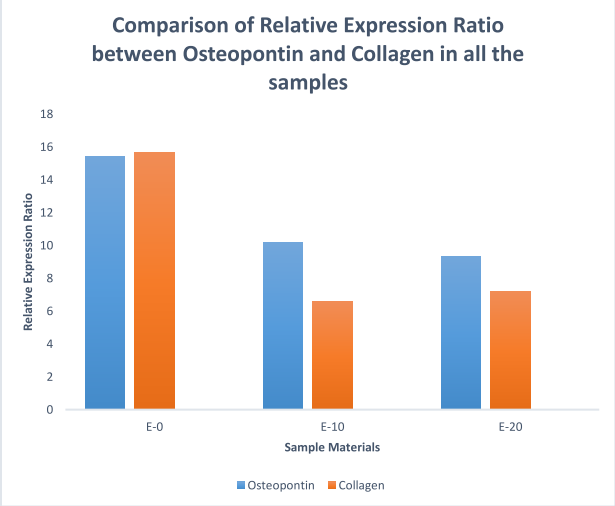

Fig 1: Comparison of the Relative Expression Ratio of Osteopontin and Collagen measured and compared in each sample group separately

Discussion

Periodontal therapy after loss of periodontal tissue, is aimed at repairing the damaged tissue and encourage regeneration of new bone tissue as treatment therapies based on regeneration have proved to be more effective than limiting infection alone.[11,12] Nano-hydroxyapatite has been used in the past with multiple other components to study its most efficient combinations. In a previous study, n-HA was used in combination with calcium and strontium substitutes at varying proportions and the results showed that not only the material exhibited osteoblastogenicity but was also non-cytotoxic.[13] This experiment studied the osteogenic potential of the novel blend of nHA with E-glass fibres designed for use in the clinical applications of tissue engineering. Hydroxyapatite is known for its ability to form bone bonds and in addition to allowing cell attachment and proliferation.[14] The current study reaffirmed the ability of nHA to augment bone formation and assist in bone regeneration.

PCR is a highly sophisticated and accurate diagnostic tool which enables monitoring all the activities within the cells.[15] A comparison of gene expression between exposed and unexposed cells was done using relative quantification method. Amount of fluorescence was measured against the cycle number. There was a clear difference between the Ct of OP and GAPDH genes in the test (exposed) samples as compared to the same in the control (unexposed) indicating increased level of OP gene expression in the test as compared to the control. Similar difference was noted for Col as well indicating increased level of Col gene expression in the test as compared to the control.

The control disc containing only nHA had the highest expression ratio for both collagen and osteopontin, 15.69 and 15.4 respectively. The relative expression ratio for OP was found to be inversely proportional to concentration of E-glass fibres. For E-10 the ratio dropped to 10.2 and then a slight decrease in expression to 9.30 folds in E-20. For Col 1 gene expression results in all the materials were similar to OP. The highest ratio was expressed by E-0 (15.69) followed by E-20 (7.22) and then E-10 (6.59). Col Type 1 expression proves that the cells were proliferating on the scaffolds due to the presence of nHA while being able to maintain their phenotype. This finding is similar to previous studies in which

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composites of HA have up regulated the Col Type 1 gene expression.[16-18] The difference between the discs could be attributed to their difference in composition as addition of E-glass fibres reduced the osteogenic potential of the materials.

Only for E-0, Col expression was almost same as OP whereas for all the other experimental materials, OP expression was higher in comparison with Col. The discs were able to up regulate the gene expression of both proteins attributing to the natural osteogenic tendency of HA which allows murine osteoblasts to attach, proliferate and differentiate. It has been documented that early expression of OP is consistent with formation of bone matrix and expression later in bone development is associated with remodelling.[19] OP also promotes the osteoblastic attachment to the extracellular matrix so that these cells can play their role during osteogenesis.[20] The five-day cell culture experiment generated optimum results for OP as it's up regulation as early as 3 days has been demonstrated in another study, in which PLGA scaffolds were coated with biomimetic apatites and osteogenic gene expression was measured after seeding the scaffolds with MC3T3 E-1 cells.[21]

Nano Hydroxyapatite is known for its osteoconductive and osteoinductive behaviour and allows the integration of bone tissue to the material surface.[22] HA is an osteoconductive biomaterial.[23] Since natural tissues of the body are on a nanometer scale, the development of nanostructured materials can replicate the extracellular matrix and therefore cells can interact directly with nanometer grain sizes.[24] Therefore these nanoceramics can induce osteointegration, osteogenesis, osteoinduction and osteoconduction owing to a higher surface area-volume ratio. [24] Nano hydroxyapatite can regulate the expression of both Col Type1 and OP in this experiment effectively. MC3T3 E-1 cells were expressing these genes even in the absence of the materials and through relative quantification, thus, establishing that the presence of this novel material increased the expression even more.

The major limitation of the study was the absence of a three-dimensional structure of the scaffold but even the 2D structure had still up regulated the expression of both genes owing to the osteogenic nature of nHA. Collagen expression during the initial stages of extracellular matrix deposition has been extensively documented and has been proved by this experiment as well. The expression of OP as early as five days also signifies that the cells had reached a stage where mineralization of the extracellular matrix was possible. OP gene expression is a marker for mature osteoblasts signifying an advanced stage in bone formation. This information is of paramount importance to a clinician who aims to use this osteogenic biomaterial for bone augmentation. Based on these results the authors recommend the use of this material to conduct animal studies to see the extent of bone formation preferably using 3D scaffolds in the future.

Conclusion

It was found that all the experimental materials and control materials supported the growth of MC3T3 E-1 cells. The material was also able to demonstrate the induction of mineralization associated gene expression in MC3T3 E-1 cells. The novel material was osteogenic and cells grown on this scaffold were capable of producing an extracellular matrix containing the Col Type 1 and OP bone proteins. This material has the potential to be used as a reinforcing agent and implant material because it will impart strength as well as play a bioactive role. With new research being done on a massive scale, tissue engineering which was just an idea three decades ago, has promising prospects in the future.

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Conflict of Interest

The authors have no conflict of interest to disclose, and no financial assistance was taken. Data has not been presented previously in any conference.

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CONFLICT OF INTEREST

Authors declared no conflicts of Interest.

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DATA SHARING STATMENT

The data that support the findings of this study are available from the corresponding author upon request.