Bone putty bioimplant improvement: evaluation of the influence of surface area and shape of the DBM (demineralised bone matrix) component on bone induction and biocompatibility properties of carrier (polymer)/demineralised bone matrix

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Abstract

Using demineralised bone matrix (DBM) grafts is one of common treatments for bone end stage disease.

One of the best products in this field is putty created by adding a carrier to the DBM powder which makes it malleable. However, this can lead to reduced osteoinductivity of the graft, as compared with the original DBM. Other disadvantage of currently used water soluble carriers is their water solubility, which can result in release of DBM particles from the graft site or disaggregation of DBM before regeneration.

The aim of this project is to increase the osteoinductivity of DBM putty and to improve its mechanical properties, in order to prevent DBM wash-out and allow better graft fixture to the bone. This project will formulate and test polymeric bi-component carriers (Alginate base) capable of hardening *in situ and* becoming insoluble. Their performance will be compared with Carboxymethyl Cellulose base carrier.

In addition, the project wills trial DBM samples of varying particle sizes and shapes.

- powder with particle size 150-500 μm
- powder with particle size 500-1000 μm
- fibres 600 micron thick of variable lengths

In this project by using in-vitro osteoblastic like cell culture model, the samples was tested for its ability to induce bone cell growth and assessed with qPCR for osteonectin, osteopontin and osteocalcin mRNA quantitative expression.

The samples were tested in different group in accordance to DBM and carrier type.

The cytotoxicity test results ($p < 0.05$) show that all newly formulated bone putty samples are biocompatible. The data also confirms that all groups are capable of supporting the in vitro

growth and maturation of osteoblasts-like cells. There is up-regulation of bone formation specific mRNA especially in the powder particle sizes 150-500 μm and in fibre samples.
Also, the carrier had an effect on qPCR results although more assessment needs to be done in order to confirm this finding.

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List of Abbreviations

OI: Osteoinduction

qPCR: Quantitative Polymerase chain reaction

Cq: Quantification cycle in qPCR

ALG: Alginate

OC: Osteocalcin

OP: Osteopontin

ON: Osteonectin

B2M: Beta-2 micro globulin

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

PGK1: Phosphoglycerate Kinase1

TUBB: Tubbolin

Cp: Crossing point in qPCR light cycler

MW: Molecular Weight

TSB: Tryptic soy broth

FTM: Fluid Thioglycollate Medium

List of definitions:

Allogenic: relating to or denoting tissues or cells which are genetically dissimilar and hence immunologically incompatible, although from individuals of the same species.

Allograft : a tissue graft from a donor of the same species as the recipient but not genetically identical.

Osteoinductive: is the process by which osteogenesis is induced. It is a phenomenon regularly seen in any type of bone healing process.

Osteoinduction: implies the recruitment of immature cells and the stimulation of these cells to develop into preosteoblasts.

Osteoinductivity: to have osteoinductive property

Sterility assurance level (SAL): is the probability of a single unit being non-sterile after it has been subjected to sterilization.

Xenograft: a tissue graft from a donor of the other species as the recipient but not genetically identical.

Chapter 1

GENERAL INTRODUCTION and Literature Review

1.1 INTRODUCTION

Lack of adequate bone is a common complication in different surgeries such as implant dentistry[1]. Autogenous bone is still the gold standard in bone augmentation procedures but its low availability and donor site morbidity necessitates the development of alternative products for it.[2, 3] Many bone substitutes are introduced every day such as allografts, xenografts and synthetically produced ones. One of the commonly used substitute is allogenic bone graft.[4]

Bone grafts have been used in clinical applications such as oral and maxillofacial orthopaedic procedures and also in spinal and neurosurgery for decades. The bone scaffold formed by ground cortical bone particles and cancellous chips creates a favourable environment required for bone-forming cells to be able to generate new bone [5, 6]

Demineralised bone matrix (DBM) is widely used as a bone graft substitute. It has been used successfully as a grafting material in many clinical indications including long bone defects[7, 8] , spinal fusion[9] , and craniofacial reconstruction [10]. In addition, DBM in combination with local bone has been shown to perform as well as auto graft and eliminates the need for Autogenous bone harvest[9] . Allogenic DBM possesses inherent osteoconductive and osteoinductive properties [11]. In this context, osteoconductivity is defined as the ability to provide a 3D configuration for in-growth of host capillaries, per vascular tissue, and osteoprogenitor cells into the graft and osteoinduction is defined as the ability to encourage the host to synthesize new bone. DBM consists of both insoluble collagen and non-collagenous proteins [12].

In spite of their widespread use, ground human bone matrix grafts, and most specifically particulate demineralised bone matrix (DBM), can be difficult to handle in the operating room. To overcome issues with handling, many companies offer DBM in combination with

a natural or synthetic carrier. The term “osteobiologics” has been introduced to refer to these manufactured bone graft substitutes.

The carrier is usually an inert gel-like material, and it varies between 50% and 70% of total volume of putty or gel. Commonly used materials include natural polysaccharides like alginate, cellulose, hyaluronic acid and / or proteins (such as collagen). With increasing amounts of DBM part, the handling and malleability properties of the putty decreases but increasing of DBM part is valuable as it increases the osteoinductivity of the product that is necessary for new bone formation in clinical use.

Also, current gel carriers are soluble in the physiological (aqueous) conditions encountered inside the body. This can lead to premature dissolution of the putty, with consequent release and / or dislocation of DBM particles from the implant area. Consequences of this process include unreliable bone induction and formation of bone in unwanted areas. Stability of the host-graft interface is also important to allow vessels to grow into the graft, so ideally the carrier should keep its original shape during 2-3 weeks. The clinical usage of DBM (Demineralised Bone Matrix) is well established (as mentioned in the following articles) but studies on physiopathology of DBM / carrier putties in connection with bone regenerative medicine are still pertinent, and improvement of bone putty bio implant could lead to a better material for bone regeneration in clinical use.

1.2 DBM (demineralised bone matrix)

1.2.1 Bone induction properties of DBM (demineralised bone matrix)

The following section cites data from relevant published references, as well as reporting on own findings from CenoBiologics.

Loss of bone and connective tissue follows a range of diseases or due to accidents can result in a bone defect that the body cannot repair through normal healing processes. For example, loss of alveolus bone leads to clearance, tooth loss and decreases the available bone for implant placement. In order to prevent these complications, complete and predictable regeneration of lost tissues are the main goal of regenerative therapy. Several methods and

materials have been applied including application bone grafts.

Demineralised bone matrix (DBM) is an important therapeutic option for the appendicular, axial and craniofacial skeletons. In these skeletal locales, DBM has osteoconductive and osteoinductive properties that prompt bone regeneration. Consequently, about 20% (or about 108,000 procedures per year) of the $1billion per year bone grafting market [13] focuses on using DBM products in bone repair and regenerative strategies.

Context for DBM use was prompted by clinical recognition of the value of bone grafting that alone has substantial history. Senn [14] used decalcified bone to treat osseous defects [14]. He soaked tibiae from oxen in hydrochloric acid (HCl), using logic that HCl had antiseptic effects on the xenografts and would benefit patients with osteomyelitis. Deaver [15] , Curtis [16] and Mackie [17] validated Senn's work. An initial alternative to auto grafting was offered by Huggins [19,20][18]. He described a propitious finding where transitional epithelium from the urogenital system promoted ectopic osteo genesis: the formation of bone in a connective tissue site. These reports were similar to Neuhof's observation [21], where fascia used to augment bladder in dogs elicited an ectopic osteogenic outcome. Interestingly, in 1998 Urist and colleagues reported detection of messenger RNA for bone morphogenetic proteins (BMPs) 2, 4 and 5 in bladder tissue, producing an important causal connection[19]. Important to DBM development, these reports on ‘ectopic osteo genesis’ were followed by two provocative papers in 1934 and 1938 describing the response in muscle to alcohol extracts prepared from bone [23,24]. The alcohol extracts injected into skeletal muscle produced osteogenesis. These studies were phenomenological, and it was not until 1945 and 1947 that a biological explanation was offered by Lacroix [25,26] who thought that bone contained ‘substances’ that enabled osteogenesis. His 1947 paper in Nature stated that ‘osteogenin’, speculated to be in bone, initiated its growth [26]. Ray and Holloway [27] and then Urist in his landmark paper in Science [28] determined ectopic osteogenesis occurred when demineralised bone was implanted into a non-bony site. This elegantly detailed scientific explanation of ectopic osteogenesis coined the term ‘auto induction’. Urist posited that the substratum (the non-mineralized matrix of bone) was key, providing morphogenetic signals that prompted osteogenesis. It is highly noteworthy that in 1971, Urist and Strates introduced ‘bone

morphogenetic protein' (BMP) and 'osteoinduction' to the scientific and clinical communities [29]. Urist systematically and lucidly presented a visionary process—still highly relevant today—of bone morphogenesis promoted by the demineralised component of bone: the organic substratum (as Urist called it).

Preliminary studies on bone grafts and mineralisation were reported in 1965 by Urist. In this study bone derived demineralised matrix from bovine cortical bone was used in lab mouse and guinea pig muscles and it led to formation of bone structure. Urist showed that demineralization of allogenic cortical bone increases its osteogenic potential resulting from exposed bone morphogenic proteins that induces the differentiation of host cells into osteoblasts.

Grafts are divided into 4 groups: auto graft, allograft, xenografts and alloplastic materials which lead to bone regeneration and creation of new periodontal connections through osteogenesis, osteoinduction and osteoconduction. Using allograft is common practice in order to overcome obvious limitations present in using patient auto grafts. DBM allograft has been widely used in regenerative therapy in past three decades. Urist`s empirical studies revealed the oestrogenic potential of DBM in 1965 that has led to bone growth in dental implant loss treatment. Using of DBM is based on its osteoactive characteristics, as the demineralisation processes exposes inducing proteins involved in new bone formation, and are present in the bone matrix. These proteins called BMP (Bone Morphogenic Proteins) and can lead to the differentiation of progenitor cells and finally to bone formation. On the other hand, some studies have reported that the successful treatment using DBM follows a similar pathway to those using mineralised allograft bone matrix like FDBA (freeze-dried bone allograft) which could indicate that demineralisation is not the unique factor in bone induction process. Osteoinductivity characteristics of these materials is however variable, depending upon the manufacturer preparation techniques, donor characteristics and DBM particle sizes. This was mentioned as early as 1968, when Urist [20] stated that the activity and function of bone morphologic proteins decreases in the fourth decade of life.

In 1976, Reddi [21] and Anderson [22] refined and polished Urist's work on ‘induced osteogenesis’ and then provided the first credible and compelling explanation of the functional role of purified organic bone matrices (i.e., the bone's demineralised matrix)[23] Reddi's comprehensive and pioneering work on DBM enabled the cloning and expressing of recombinant human BMPs. These BMP molecules are part of the ‘soluble extract’ Reddi first identified in his original demineralised bone matrix (DBM) (reviewed in [24]).

This demineralised component was proven to contain the biological drivers essential for ontogenesis. This history provides a conceptual foundation to develop several issues germane to the biological activity of DBM and its clinical utility, including procurement, donor profile (e.g., gender, age), processing, production issues, sterilization, and its combinations with carrier biomaterials.

With a robust clinical demand for DBM in human patients, tissue banks and companies continue to commit remarkable resources to produce a diverse array of DBM-containing products—to the point where clinicians are often confused by the DBM product diversity. Moreover, some uncertainty exists clinically about the validity of various claims made by commercial vendors about DBM-containing products. Additionally, combinations of DBMs with other biomaterials in several composite forms increase the versatility of this clinical human tissue-derived product to expand its capabilities for bone repair. Several factors regarding DBM as a human-derived tissue product are important to understand in guiding use as a bone repair matrix and vehicle for delivering bioactive agents; for example, bone procurement techniques from human donors; donor age and gender, and DBM composition and properties [2–5]. Differences in preparation and processing methods for bone can impact properties and clinical performance. Biological testing of processed bone and the outcome measures to validate biological activity (i.e., the so-called osteoinductive index, OI, described herein) are not uniform among tissue banks, and as a result DBM products have variable composition and properties. This variability has obvious significance for clinicians and patients, but also issues for understanding performance in research studies. In addition, DBM products consist of bone-derived particle sizes and particle size ranges, even protein fibres. DBM sterilization protocols are also variable. Finally, the composition of various carriers combined with the DBM, and DBM combined with various bioactive substances will

influence clinical and research outcomes.

1.2.2 DBM procurement and process

The origin for all DBM clinical products is the human donor. Donor bone, by contemporary jargon, is referred to as an allograft. However, the removal of bone from the donor, as well as the processing of that donor bone, renders the tissue void of viable cells. Therefore, by definition the DBM product is an alloimplant: it does not contain viable cells.

By contrast the auto graft contains viable cells. The procurement and processing of the donor bone tissue that will become DBM uses the term allograft despite the fact that the bone allograft is a cell-free matrix of bone containing the inorganic and organic matrices; allogenic bone grafts such as DFDBA (demineralised freeze dried bone allograft) can be obtained from tissue banks and allograft machining companies. As a consequence of donor procurement to obtain the graft, the primary risk from the allograft bone products is transmission of infectious diseases from the donor to the recipient. Since the implementation of rigorous product testing began over 10years ago, only rare, isolated instances of disease transmission are reported that may have resulted as a consequence of unscrupulous tissue banking procedures. Notably, these events occurred when irresponsible groups did not adhere to the tissue procurement guidelines advocated by the American Association of Tissue Banks (AATB) Methods to prepare DBM from donor bone generally sterilize the product and eliminate potential infectious agents. Nevertheless, the first line of defence against disease transmission is donor screening. Donor screening begins at the procurement level. To avoid transmission of infectious diseases, potential donors are screened for specific exclusionary risk factors. The procurement process is highly regulated by the FDA and specific requirements are found in Title 21 Code of Federal Regulations (CFR 21) Part 1270. Compliance with these tissue processing guidelines requires the cooperation of independent organizations, including hospital-based medical staff, recovery and screening organizations, tissue processing facilities and sales and distribution organizations. The starting point is an assessment of the suitability of a donor conducted by a recovery or screening organization

based on general criteria followed by an initial medical screening. The family of the deceased donor is consulted to obtain an informed consent for tissue donation. If the triage and consent steps are successful, the tissue processing facility is contacted and takes responsibility for final screening. A number of guidance documents, continuously updated to account for newly identified risk factors, are available from the FDA with recommendations for screening potential donors for exclusionary risk factors. For example, the current FDA guidance document includes potential exposure to newly identified zoonotic diseases such as West Nile Virus (WNV) and Severe Acute Respiratory Syndrome (SARS) as exclusionary risk factors. FDA mandates that a detailed medical and social history is obtained to uncover social and behavioural risk factors. The information is obtained through interviews with family and other close contacts of the donors. In some instances identification of a behavioural risk factor disqualifies a donor and in other cases it triggers further investigation. The next assessment is a physical examination that may reveal evidence of risk factors or signs of pathologies not otherwise detected through serological testing and could lead to donor exclusion. Finally, a series of clinical tests are completed to rule out Human Immunodeficiency Virus (HIV) types 1 and 2, Hepatitis B virus (HBV), Hepatitis C virus (HCV), and *Treponema pallidum* (syphilis). The human donor qualification process for DBM sourcing is rigorous and stringent. The intent is to ensure procurement of disease-free tissue and DBM biomaterials with requisite safely and efficacy for patient use. As a direct product of donor allograft bone, DBM is a composite of collagens (mostly type I with some types IV and X), non-collagenous proteins and growth factors, a variable percent of residual calcium phosphate mineral (1–6%) and some small percent cellular debris. In general, the DBM preparation protocol includes donor bone debridement of adherent soft tissues and removal of blood and lipids.

At this point, often an antibiotic soak is used to initiate the sterilization process. Subsequently, cleansed donor bone is morselized to defined particles or fibres (and milling to obtain particles with size ranging between 150-1000 μm) and subjected to acid demineralization followed by one or more rounds of freeze-drying. The mineral phase is extracted from the particulate whole donor bone with 0.5–0.6N HCl, leaving the organic matrix intact. Upon freeze-drying, the resulting demineralised bone powder(synonymous with DBM) can be formulated into putties, pastes and more recently, flexible, pre-formed

strips for implant use. The freeze-drying as one step in the processing has led to the alternative DBM term: Demineralised Freeze-Dried Bone Allograft (DFDBA). DBM contains abundant bone morphogenetic proteins known to be essential for bone growth and regeneration [28, 29]. Currently the sterility assurance level (SAL) required for DBM and all other implants are set at SAL 10−6. This SAL nomenclature signifies that no more than one unit out of one million devices sterilized would fail sterility testing. While there is evidence that demineralisation processes can inactivate certain viruses [51], tissue banks and industries that process and prepare DBM products rely on additional steps to ensure that the DBM is free of bacterial and viral contamination. Sterility is a challenge for producers of DBM-based products. The sterilization protocol may inactivate or attenuate the BMPs in DBM that confer its clinically important biological activity. Terminal sterilization of DBM was reported 20 years ago [25]. Munting and co-workers prepared rat DBM preparation and sterilized it using different protocols: gamma irradiation, Merthiolate, Glutaraldehyde, Formaldehyde and ethylene oxide (ETO)[26]. Glutaraldehyde, Formaldehyde and ETO abolished OI while Merthiolate and gamma radiation were less damaging to the OI. Other reports document the impact of sterilizing BMPs derived from DBM [27, 28] However, it is unclear whether the BMP within the DBM will be affected differently than BMPs extracted from the DBM and sterilized alone. Suitable conditions were also identified for radiation-based methods. Radiation doses of approximately 30–50 KGy are considered sufficient to sterilize medical implants and these doses are within a range shown not to degrade DBM [29, 30] and also capable of inactivating certain viruses [31, 32]

Given the potential for traditional and non-traditional sterilisation methods to adversely impact DBM OI, many manufacturers have resorted to the more expensive option of aseptic processing. Sterility is achieved by aseptic processing through a combination of manufacturing controls and standard operating procedures. The FDA provided a guidance document in September 2004 entitled “Guidance for Industry, Sterile Drug Products Produced by Aseptic Processing-Current Good Manufacturing Practice to help manufacturers achieve sterility through aseptic processing. The document provides information on the engineering and human controls that can be used to ensure sterility. Compliance with the

guidelines to ensure a sterile product depends on adequate manufacturing spaces called “clean rooms” with sufficient air quality controls. Clean rooms must have High-Efficiency Particulate Air (HEPA) filtration and allow entry and exit of staff and materials without the introduction of infectious agents. Chemical and physical conditions typical of other sterilization methods that could degrade or alter biologically derived substances are avoided in the aseptic processing of DBM. Several DBM manufacturers have endorsed the cost effectiveness of aseptic processing in preserving the DBM OI Donor-recipient gender and age have been mentioned as variables that could affect the activity of DBM. Work by Schwartz and colleagues found no evidence for gender related differences in DBM OI[4]. Moreover, Zhang's group reported OI of DBM that suggested males between 41 and 50years of age and females between 51 and 60 years of age had a better OI than DBM prepared from young donors [5]. In contrast, Lohmann and co-workers reported that increasing donor age decreased OI[88]. They prepared DBM from donors with an average age of 32.8years and 75.6years and found the 32.8year-old average-aged cohort had a higher OI than the older group. Additional work from the Lohman group suggested a decrease in OI with an increase in age of the donor. Data from this group has not been validated by others. For example, Traianedes and colleagues determined the OI of human DBM prepared from 133 male and 115 female donors [89].Data indicated DBM from donors as old as 85 had an OI comparable to younger donors.

1.2.3 Influence of DBM particle size and properties on new bone formation

The vast majority of the DBM particles possess random, irregular geometries with bone particles size ranging from about 110 to 850 microns If the extent of bone demineralization is constant, then DBM particle size remaining after morselization in the powder, or DBM fiber geometry produced by processing the bone-derived collagen proteins then defines DBM surface area as a clinical variable. Different surface geometries may impact host cellular interactions as well as diffusion rates of DBM-resident biological molecules and endogenous agents such as BMPs or growth factors in and out of DBM. Consequently, some discussion regarding the optimal size and size range of particle for DBM preparations suggests that particles less than 250μm are not as osteoinductive as larger-sized (420–840μm) particles [33-35]. Generally, preclinical data are inconsistent regarding DBM compositions as

particles and their size ranges, as well as for fibre, and DBM formulations in sheets and gels. Inconsistency is due to different animal models and outcome measures, many of which are highly subjective and are unique to one laboratory and not universally accepted. Moreover, human data on DBM are weak due in part to the emphasis on DBM as a bone graft extender rather than as a stand-alone therapy. Consequently, the efficacy for different formulations for DBM has not been clearly elucidated. Formulation design features are largely empirically ascertained in arbitrary test beds.

An article published by Schwartz [36] reported on the ability of different commercial DFDBA in relation to the induction of new bone regeneration. DFDBA with a particle size ranging from 200 to 500 μm were taken from 6 different bone banks which used different methods for their respective product preparation. 14 packs of DFDBA were studied totally. 10mg of each pack placed percutaneous and in muscle in 3 different mice and after 4 weeks a biopsy was obtained.

The results of this study showed that different commercial types of DFDBA have different in bone induction ability and that there are significant differences between different tissue banks production methods, affecting the osteoinductivity of the final product. It was also found that presence of sufficient quantity and quality of proteins responsible for bone induction is the main factor in obtaining an effective product with the desired therapeutic properties.

Schwartz [37] examined the osteoinductive effects of adding rhBMP-2 (human bone morphogenetic protein, part of a family of inducing factors present in DBM) to inactive commercial DFDBA. In the study 2 packages of different active DFDBA were used as positive control, 2 packages of inactive DFDBA as negative control and 2 packages of inactive DFDBA with 5 and 20 mg rhBMP-2 as test group. It was found that the effect of rhBmp-2 in inducing bone formation is dependent on the dose. Diagnosis of active and inactive DBM packages was based on Schwartz`s previous study Schwartz [36] where it became clear that some commercial products have high (normal) inductive function while some others lack this effect. Once more, the conclusion is that there is high variability in the

osteoinductivity of commercial allografts, and presence of inducing factors is necessary for formation of bone.

1.2.4 Effect of residual calcium on DBM bone-inducing properties

The effect of residual calcium on DBM and its influence on cell differentiation was investigated by Herold [38] For this, allografts with different levels of calcification were used to induce bone formation by osteoclast cells.

Bone marrow were obtained from pig was then cultured for 3 weeks and hematopoietic stem cells were permitted to differentiate to mature polykaryons and marker characteristics of Osteoclasts were observed. Osteoclasts showed a dense actin bar at the edge of the cytoplasm under light microscope. These were then incubated with DBM samples with calcium amounts of 1.44%, 2.41% and 30%. Controls were done in absence of DBM, and incubated during one week. TRAP (tartrate-resistant acid phosphatase) is expressed by osteoclasts, macrophages, dendritic cells and a number of other cell types. TRAP is able to degrade skeletal phosphoproteins including osteopontin (OPN) and has a critical role in many biological processes including skeletal degradation. Results showed that more TRAPT cells were produced in absence of DBM, and importantly, the osteogenic activity in the presence of DBM with 2.41% calcium increased significantly when compared to the other groups.
Also Melloning [39] examined the clinical effects of different sizes of DFDBA on formation and growth of bone. They found that the optimum particle size for bone formation is 200 to 500 microns. The biological and subsequent clinical impact of bone demineralization on DBM is that residual calcium may influence its osteoinductivity [40]
The removal of the mineral content of the graft increases its osteoinductivity by exposing more biologically active bone morphogenetic proteins (growth factors), in comparison with mineralised grafts. Surgeons use DBM (demineralised bone matrix) grafts on a wide range of bone regeneration disorders, commonly caused by cancer, infection or accidents. DBM has osteoinductive effects, providing induction and growth of bone tissue in specific areas as required repairing defects [41, 42]. DBM is produced by acid-extraction of the mineral content of bone, and it is essentially constituted of collagen plus various other proteins, including osteogenic agents. It is usually produced from cortical bone (produced from diaphysis of long bones).

Induction of new bone growth by DBM is correlated with the amount which is present at the graft site, and its ability to recruit and stimulate differentiation of progenitor cells into osteoblasts.

The demineralisation of bone matrix exposes bone morphogenetic protein (BMP) and other bone growth promoting factors. Because of this, when used as a graft, demineralised bone matrix (DBM) not only provides a scaffold for bone formation, it also promotes differentiation of osteoprogenitor cells into viable bone-forming cells, a process called osteoinductivity [43].

1.3 Osteobiologics DBM carrier and its properties

In spite of their widespread use, ground human bone matrix grafts, and most specifically particulate demineralised bone matrix (DBM), can be difficult to handle in the operating room. To overcome issues with handling, many companies offer DBM in combination with a natural or synthetic carrier. The term "osteobiologics" has been introduced to refer to these manufactured bone graft substitutes.

The carrier is usually an inert gel-like material, and it varies between 50% and 70% of total volume of putty or gel. Commonly used materials include natural polysaccharides like alginate, cellulose, hyaluronic acid and / or proteins. With increasing amounts of DBM part, the handling and malleability properties of the putty decreases but increasing of DBM part is valuable as it increases the osteoinductivity of the product that is necessary for new bone formation in clinical use. The relatively low amount of DBM in common graft putty leads to actual reductions in osteoinductivity when compared with pure DBM. Also, current gel carriers are soluble in the physiological (aqueous) conditions encountered inside the body. This can lead to premature dissolution of the putty, with consequent release and / or dislocation of DBM particles from the implant area. Consequences of this process include unreliable bone induction and formation of bone in unwanted areas. Stability of the host-graft interface is also important to allow vessels to grow into the graft, so ideally the carrier should keep its original shape during 2-3 weeks. The current most popular clinical DBM format is mouldable putty that can be packed into bone defects and resists dispersion from irrigation

and blood during surgery. Conversion of DBM powder to putty involves formulation with a biocompatible viscous carrier that provides a stable suspension of DBM powder particles. The viscous carriers can be classified as water-soluble polymers such as sodium hyaluronate or carboxymethyl cellulose, or anhydrous water-miscible solvents such as glycerol. In some cases, the carrier selection has implications for other processing steps, compatibilities, applications, and even sterilisation. Further, DBM can be mixed with these carriers to produce flexible sheets that may contain both DBM and cortical bone chips as a composite biomaterial. Additionally, the polymer carrier Pluronic (BASF product, synonymous with poloxamer), is a temperature-sensitive biomedical copolymer carrier used with DBM. The composition becomes firmer as it warms to body temperature. Another carrier is a thermoplastic, porcine collagen-based hydrogel that is non-water-soluble. The DBM-porcine collagen can be extruded through a syringe after it is heated to 46–50°C. In situ at body temperature the composition becomes firm.

In order to address the lack of cohesiveness of DBM at implant sites, some binders, such as high molecular weight hydrogels or other polymers as carrier vehicles have been utilised. However, these binders can negatively affect the bio compatibility and osteoinductivity of the DBM composition.

Furthermore, these binders provide cohesiveness to the composition only prior to its implantation; following implantation, these binders are eroded or dissolved from the implant site and, consequently, the implant does not retain its shape in vivo.

An issue remains of how to optimally immobilize and deliver osteoinductive agents and bone-forming cells to the site of a bone defect that is unlikely to heal spontaneously and how to retain an adequate concentration of graft material and factors at the site of new bone formation. Because the desired proteins to be delivered to the bone healing site are water soluble, direct placement of the devices would be unacceptable due to rapid diffusion and resorption. In addition, carriers must not interfere with bone production or the biomechanical integrity of the repaired defect (Table 1.1). The purpose of this review is to discuss the types of commonly used carrier systems for delivery of bone graft substitutes to a critical sized defect. The ideal carrier materials may vary accordingly with the specific osteogenic or osteoinductive factors used[44-46] the location and placement of bone graft, inter transverse or inter the body [47, 48]

Table 1.1: Ideal characteristics of a delivery carrier for bone graft substitutes

Ideal characteristics of a delivery carrier for bone graft substitutes
• Maximize osteoinductive and osteogenic effects of agents delivered to a bone defect site • Retain DBM at the defect site for the optimal time of release • Function as an osteoconductive scaffold for bone ingrowths with appropriate sized porous nature for cellular and vascular passage • Limit local effects of space occupying delivery systems and not compete with or limit bone formation • Limit inflammatory response by biocompatibility • Allow for timed removal of residual carrier structure by biodegradability and limiting deleterious effects of biomechanical nature of new bone formation

The most commonly used delivery systems available for use are shown in Table 1.2. The carrier must support bone formation by direct interaction with the target area and cells by providing an attachment substrate and binding and delivering the appropriate agent (growth factors/ Autogenous cells) to the local environment for "release" at the most opportune time. The ideal release pattern of bound to free growth factors from the carrier is a challenge that remains unsolved.

Table 1.2: Commonly used carriers or delivery vehicles for ostegenic and osteoinductivity DBM

Commonly used carriers or delivery vehicles for DBM
• Soluble Allograft/ Demineralised bone matrix • Natural polymers – collagen • Natural and synthetic calcium phosphate compounds – ceramics • Collagen–calcium phosphate composites • Biodegradable polymers

Studies designed to discriminate the effectiveness of various carriers on DBM (pre)clinical efficacy are limited. Wang implanted athymic rats with commercially available DBMs Dynagraft putty, Grafton putty, or Osteofil allograft bone paste [45] for spinal fusion. Most of the segments implanted with Grafton and Osteofil fused and none of the segments implanted with Dynagraft fused. Sassard and colleagues reported on a retrospective review of patients who had undergone instrumented posterolateral lumbar spinal fusion with an Autogenous bone graft and Grafton gel[46]. Age-, gender-, and procedure-matched group of patients were involved. There were no differences between treatments groups (auto graft vs. auto graft plus Grafton) based on radiographs up to 24months after surgery. The fusion rates in the auto graft with Grafton group and the auto graft-only groups were only 60% and 56%, respectively. The most important predictor of 24-month bone mineralization was a correlation between the type of instrumentation and fusion success. Bostrom and co-workers implanted Grafton subcutaneously into athymic rats and reported acute tubular necrosis [47].It was speculated the glycerol component in Grafton led to the nephrotxoicity. However, the dose was 10 times the human clinical dose. Nevertheless, glycerol-containing products should be used with caution in paediatrics patients and in those at risk of renal disease [48]. Acarturk and Hollinger determined in a pre-clinical model in an orthopaedic critical-sized defect site that treatment with either Grafton or DBX promoted significantly more bone regeneration than other DBM-carrier products[49]They concluded that differences in osteogenic activity among commercial DBM products may be related to differences in the carrier, the amount of DBM in the carrier and ability of the carrier to localize the DBM particulate to the bone defect site for a sufficient period to promote bone regeneration. An important variable inherent to these products is that the DBM content among different commercial composites (i.e., DBM plus carrier) is non-standardized and inconsistent. Therefore, different DBM doses will be delivered to tissue sites by different products. This could explain the variability in responses reported by Acarturk and Hollinger [49]. Moreover, individual ‘DBM lots’ processed by the same tissue bank may possess different osteoinductive capacities, and these capacities may vary among different donors. While biological activity is measured by osteoinduction and is reported as the osteoinductive index “OI”, regulatory standards are enacted across DBM products to understand or control these

differences. It is also noteworthy to emphasize the significance of shelf life and carrier stability for DBM composite products. If precise control of conditions is not maintained, then endogenous osteogenic proteins in the DBM, most importantly, BMPs might be susceptible to chemical and physical degradation. The OP-1 implant (3.5 mg OP-1/g collagen carrier) is combined with carboxymethyl cellulose to improve handling characteristics. This OP-1 carrier composite has the consistency of putty and is currently being evaluated in prospective, randomized controlled clinical trials in posterior lumbar fusion[49]

1.4 Biocompability property of Osteobiologics\bone putty

Cell viability and cytotoxicity assays are used for drug screening and cytotoxicity tests of chemicals. Fig. 1.1 indicates various reagents used for cell viability detection. They are based on various cell functions such as enzyme activity, cell membrane permeability, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity. Many have established methods such as Colony Formation method, Crystal Violet method, Tritium-Labelled Thymidine Uptake method, MTT, and WST methods, which are used for counting the number of live cells.

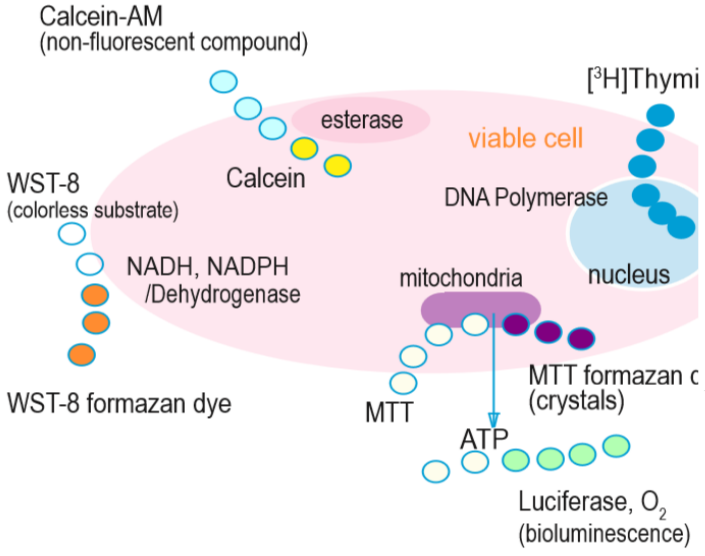


Figure 1.1: Various reagents used for cell viability detection **(**from Dojindo Cell Counting

Kit-8 instructions).

Trepan Blue is a widely used assay for staining dead cells. In this method, cell viability must be determined by counting the unstained cells with a microscope or other instruments. However, Trepan Blue staining cannot be used to distinguish between the healthy cells and the cells that are alive but losing cell functions. In the Colony Formation method, the number of cell colonies is counted using a microscope as a cell viability indicator. In the Tritium-Labelled Thymidine Uptake method, [3H]- thymidine is involved in the cell nucleus due to the cell growth, and the amount of the tritium in the nucleus is then measured using a scintillation counter. Though the Tritium- labelled thymidine uptake assay is sensitive to determine the influence on the DNA polymerization activity, it requires radioisotope which causes various concerns.

Enzyme-based methods using MTT and WST rely on a reductive colouring reagent and dehydrogenase in a viable cell to determine cell viability with a colorimetric method. This method is far superior to the previously mentioned methods because it is easy-to-use, safe, has a high reproducibility, and is widely used in both cell viability and cytotoxicity tests. Therefore, this method is suitable for those who are just beginning such experiments. Among the enzyme-based assays, the MTT as- say is the best known method for determining mitochondrial dehydrogenase activities in the living cells. In the method, MTT is reduced to a purple Formazan by NADH. However, MTT Formazan is insoluble in water, and it forms purple needle- shaped crystals in the cells. Therefore prior to measuring the absorbance, an organic solvent is required to solubilise the crystals. Additionally, the cytotoxicity of MTT Formazan makes it difficult to remove cell culture media from the plate wells due to floating cells with MTT Formazan needles, giving significant well-to-well error.

Dojindo developed highly water-soluble tetrazoliun salts called WSTs. WSTs produce water-soluble Formazan and are suitable for cell proliferation and cytotoxicity assays. WSTs receive two electrons from viable cells to generate a yellow, orange, or purple Formazan dye. WST-8, a highly stable WST, is utilized in Cell Counting Kit-8 (CCK-8). The electron mediator used in this kit, 1-Methoxy PMS, is also highly stable. Therefore, CCK-8 is stable for at least 6 months at the room temperature and for one year at 0-5 ˚C. Since WST-8, WST-

8 Formazan, and 1-Methoxy PMS have no cytotoxicity in the cell culture media, additional experiments may be carried out using the same cells from the previous assay.

Dehydrogenase-based assays reflect cell conditions with more sensitivity than the other assays because they depend on several elements including dehydrogenase, NAD (H), NADP (H), and mitochondrial activity. The major difference between CCK-8 and the MTT assay, other than MTT's toxicity, is the enzymes involved. The CCK-8 assay involves most of the dehydrogenase in a cell. On the other hand, MTT only involves mitochondrial dehydrogenase. Therefore, the MTT assay depends on mitochondrial activity, not the cell itself. Additionally, CCK-8 is far more sensitive than the MTT assay. Since WST-8 formazan is water soluble, it does not form crystals like MTT. Therefore, after 1-4 hours of incubation with the CCK-8 solution, measurement of O.D. at 450 nm gives the number of viable cells. No extra steps are required.

The Quick Cell Proliferation Assay Kit provides all reagents and detailed instructions for a fast and sensitive quantification of cell proliferation and viability. The assay is based on the cleavage of the tetrazolium salt WST-1 to Formazan by cellular mitochondrial dehydrogenases. Expansion in the number of viable cells resulted in an increase in the activity of the mitochondrial dehydrogenases, which leads to the increase in the amount of Formazan dye formed. The Formazan dye produced by viable cells can be quantified by multi-well spectrophotometer (microtiter plate reader) by measuring the absorbance of the dye solution at 440 nm. The assay can be used for the measurement of cell proliferation in response to growth factors, cytokines, mitogens, and nutrients, etc. It can also be used for the analysis of cytotoxic compounds like anticancer drugs and many other toxic agents and pharmaceutical compounds. The new method is so simple, requiring no washing, no harvesting, and no solubilisation steps, and is faster and more sensitive than MTT, XTT, or MTS-based assays. The entire assay can be performed in a microtiter plate.

1.5 In vitro osteoinductivity evaluation tests

Histological studies of influence of bone marrow in bone ectopic induction by SaOs2 (sarcoma oestrogenic cells) cells and determination of which category of major hematopoietic cells was involved in this process was studied by Rodan and Nahar Rodan [50]; Nahar[51]

First SaOs2 implants derived from 10 mg of freeze derived SaOs2 and 3 mg cellular components with micro size SaOs2 cells were prepared and implanted subcutaneously adjacent to mouse muscle. After 14 days the area was removed with surgery. H&E staining was used for identifying the bone marrow cell types and immunohistochemical localisation of specific antigens was used to determine the presence of both types of main hematopoietic cells as follows:

- Glycophorin for erythropoietin cells
- Neutrophil elates for granulopoietic cells
- CD79a for lymphocyte B cells
- Factor VIII related antigen for megakaryocyte cells

Study results were as follows:

1- Standard H & E staining showed presence of normally organized apparently complete bone marrow within 3 weeks after implantation.

2- Immunohistochemistry confirmed the presence of all 4 cell groups in ectopic marrow.

The researchers concluded that SaOs2 cells which were cultivated subcutaneously in mice, led to normal marrow induction and all 4 main groups of hematopoietic cells are involved, therefore induced marrow by SaOs2 can recover the bone defects and produce healthy bone. Therefore, it was concluded that SaOs2 cells are also suitable models for *in vitro* osteoinduction tests.

The comparative osteoinductive activity of various commercial demineralised freeze dried bone allografts was studied *in vitro* by Vaziri [52] . For this purpose, SaOs2 cells were treated with two concentrations of 8 and 16 mg/ml from 3 different commercially available DFDBA powders after 24 hours. These included Cenobone (150-1000 μm particle size), Allo-oss (250-1000 μm particle size) and Ossocot (200-850 μm particle size).

The osteoinductivity properties of the different products were assessed qualitatively, but the effect of different concentrations of DFDBA on the osteoinduction was not qualitatively investigated. The researchers concluded that all three commercial DFDBA are able to reduce the proliferation and lead to an increase the osteogenic differentiation of SaOs2 cells, so the three commercial DFDBA have osteoinductivity properties *in vitro*, and again confirmed the suitability of SaOs2 cells for such studies.

In a separate study conducted by Markopoulou [53] the response of human PDL cells (specialised connective tissue cells) to rhBMp2 in presence and absence of bone allografts was evaluated. Particle size of allograft ranged from 250 to 425 μm. It was initially found that rhBMP-2 reduced the proliferation of PDL cells, up to the 4^{th} day of incubation. However, rhBMP-2 was also responsible for early PDL cell differentiation into osteoblasts. Osteoinductive effects observed were correlated with the presence of rhBMP-2 up to the 4^{th} day, but from then on, the presence of the allograft was sufficient to induce cell differentiation into osteoblasts. Given these findings, the researchers concluded that rhBMP-2 leads to osteoblastic differentiation of human PDL cells and reduces cell proliferation. Also, the presence of extra rhBMP-2 in DBM can accelerate cell differentiation in *vitro*.

The effects of other bone growth factors were evaluated by Mott [54]. Studies were carried out in the presence of DBM, and focused on the assessment of differentiation and proliferation of osteoblasts derived from mouse calvarias cultured with one or a combination of more extracellular signalling molecules (growth factors). These were:

- Transforming growth factor beta (TGFB)

- Bone morphogenic proteins (BMPs)
- Platelet-derived growth factor (PDGF)
- Insulin like growth factor (IGFs)
- Fibroblast growth factor (FGFs)

Increased activity and proliferation of osteoblastic cells was observed in presence of DBM containing 2% calcium, or combinations of growth factors (TGFB, PDGF) or (TGFB, IGFs) or (PDGF, IGFs), as compared to a control group which did not have any growth factor or DBM present. This shows that DBM contains active bone morphogenetic proteins, and can act as a growth factor for bone induction.

1.6 The aims and scope of the present investigation

Aims

In order to address the issues mentioned in the Introduction, the aim of the current research project is to achieve a biocompatible and stable osteobiologic material with highest amount of osteoinductivity in order to obtain a rapid and predictable regeneration of bone. These aims are expected to be achieved by developing the subsequent research hypothesis and associated project objectives as described below.

1.7 Research hypothesis

To try to improve the osteoinductivity of DBM / carrier putty without compromising its malleability, the influence of surface area and shape of the DBM component will be investigated. Different sizes, as well as shapes (fibres and particulate) will be tested, the rational being that different effective surface areas will have different cell signalling and osteoinductive properties. Also, the grinding process used to obtain particulate DBM leads to damage in the microstructure of the bone tissue (Fig. 1.2), So an added advantage of shaping the DBM in fibres (lamination by cutting vs. grinding) will possibly be better preservation of this structure, and so obtain also better osteoinductivity. It is anticipated that by combining these two approaches could lead to maximisation of the osteoinductive properties of the DBM content whilst maintaining an adequate malleability.

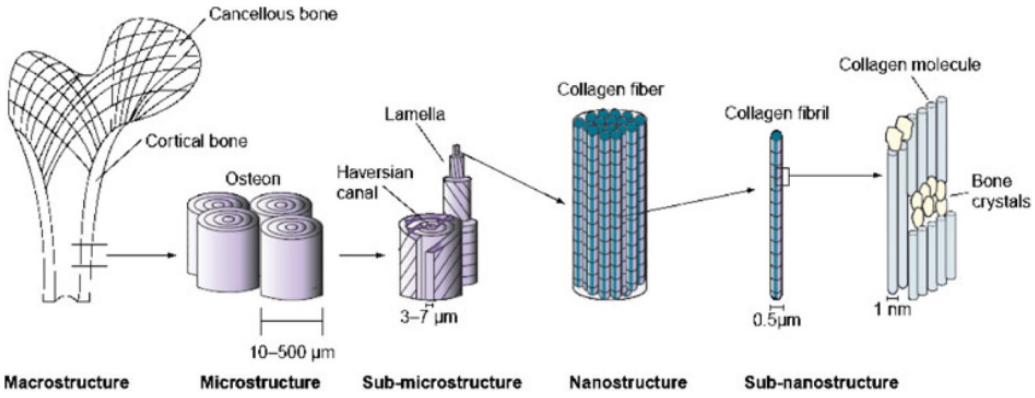


Figure 1.2: Bone sub-micron and sub-nanostructure.(from Sato, Webster et al.,2004 [55]) .

Bone powder particle produced from osteon microstructure normally damages the regular fibre structure (microstructure) due to the grinding process. With the DBM fibre shaping technique it might be possible to preserve the microstructure and produce equivalent surface area as compared with a similar weight of powder type DBM.

Currently, at the facilities of the sponsor (CenoBiologics Ltd.), DBM putty is prepared with carboxymethyl cellulose (CMC) with MW of 250000 as the binder. Initially, tests of osteoinductivity will be done with this carrier, as it is already approved for medical use. Different molecular weights of CMC will be evaluated, in order to find the one with ideal plastic properties in conjunction with the different DBM sizes to be tested. However, as mentioned above (in the Introduction), CMC carriers have some drawbacks, mainly due to water solubility. A possible solution is to use bi-component carrier mixtures which harden upon mixing, prior or post to application to the bone defect. The proposed solution is to use biocompatible sodium alginate, which hardens by forming cross-links with calcium ions. In this project the diffusion setting is considered for gel formation so setting-time could be considered about 10 to 20min. The further setting methods like PH related setting or thermal modulated setting would be evaluate in further study. The two component putty will comprise CMC gel containing the DBM and free calcium and a

second gel of sodium alginate. The two parts will be stored separately, and be mixed just before use. The cellulose gel will function as plasticiser, imparting flexibility to the putty, and the alginate will function as hardener, allowing it to maintain its shape once applied to the bone defect. Previous studies indicate that alginate gels have a lifespan of up to 80 days in the body, making the proposed approach suitable for the development of more stable carrier which could then be compared with CMC for generation of osteobiologic products. At this stage (during the current research project), no detailed (*in vivo*) optimisation of the composition of bicomponent carriers will be performed. Instead, the focus will be on its plastic, malleability and osteoinductive / toxicological properties, which, if found promising, will be developed into a future research programme.

1.8 Objectives

- Development of a technique to reliably produce DBM fibres and filaments, suitable for subsequent manufacture of osteobiologic products. The evaluation will also cover different aspects of production like the effectiveness of sterilization by gamma irradiation and freeze drying time. Gamma sterilization effectiveness will be verified by microbial culture tests according to Cenobiologics`s validated gamma sterilization procedure.
- Use of DBM with larger effective surface area or different physical shape like fibres or filaments so the cell signalling for osteoinductivity could be increased. For this, DBM fibres will be produced (0.6mm thickness and 4cm length) together with two different powders DBM, with different particle size. For this propose two particle size group that currently more used for bone graft from CenoBiologics products basket was chosen (150-500 micron and 500 -1000 μm).
- Evaluate the amount of residual calcium present in the different types of DBM as described above. For best osteoinductivity, residual calcium in the DBM should be below 8 %, according to The American Association of Tissue Banking(AATB) guidelines, (AATB Standards for Tissue Banking, 2012)
- Optimisation of the DBM-carboxymethyl cellulose gel putty composition using the different shape and size of DBM and different molecular weights of carboxymethyl cellulose (range 90,000 to 700,000).

- Explore the osteoinductive properties of carboxymethyl cellulose gel putty containing different size and shapes of DBM *in vitro*. This will include qualitative and quantitative study of morphologic, phenotypic, and genotypic differentiation induction of different concentrations / shapes of putty on SaOs2 cells. (Mansur Ahmad et al,1999) Studies will be performed by real-time PCR, for determination of quantitative osteogenic gene expression (osteonectin, osteocalcin, osteopontin) in the presence of different concentrations and types of bone putty products.
- Development and optimisation of the new bi-component DBM gel. The best type of DBM as identified above will be used for the production of the bi-component gel. Different ratios of carboxymethyl cellulose to alginate and free calcium will be tested, in order to produce a material which hardens to a soft insoluble gel within 10 min, according to the needs of CenoBiologics.
- Biocompability evaluation by Cytotoxicity assay of new carrier and bone putty according to ISO 10993.

1.9 The research questions

- What type of production methods for DBM yields more effectively osteoinductive graft putty?
- Is the variation in osteoinductivity related with the surface area or shape of the DBM component of putty?
- What are the differences in malleability of the DBM putty with different shape or size of particulate DBM part?
- Can the new bi-component carrier gel be rated for medical device according to ISO 10993 for cytotoxicity?

Summary

Bone grafts have been used in clinical applications such as oral and maxillofacial orthopaedic procedures and also in spinal and neurosurgery for decades. The bone scaffold formed by ground cortical bone particles and cancellous chips creates a favourable environment required for bone-forming cells to be able to generate new bone (Nandi [5] Brydone [6]. The removal of the mineral content of the graft increases its osteoinductivity by exposing more biologically active bone morphogenetic proteins (growth factors), in comparison with mineralised grafts. Surgeons use DBM (demineralised bone matrix) grafts on a wide range of bone regeneration disorders, commonly caused by cancer, infection or accidents. DBM has osteoinductive effects, providing induction and growth of bone tissue in specific areas as required to repair defects Tiedeman [41], Peterson [42]. DBM is produced by acid-extraction of the mineral content of bone, and it is essentially constituted of collagen plus various other proteins, including osteogenic agents. It is usually produced from cortical bone (produced from diaphysis of long bones).

Induction of new bone growth by DBM is correlated with the amount which is present at the graft site, and its ability to recruit and stimulate differentiation of progenitor cells into osteoblasts.

The demineralisation of bone matrix exposes bone morphogenetic protein (BMP) and other bone growth promoting factors. Because of this, when used as a graft, demineralised bone matrix (DBM) not only provides a scaffold for bone formation, it also promotes differentiation of osteoprogenitor cells into viable bone-forming cells, a process called osteoinductivity [43].

In spite of their widespread use, ground human bone matrix grafts, and most specifically particulate demineralized bone matrix (DBM), can be difficult to handle in the operating room. To overcome issues with handling, many companies offer DBM in combination with a natural or synthetic carrier. The term “osteobiologics” has been introduced to refer to these manufactured bone graft substitutes.

The carrier is usually an inert gel-like material, and it varies between 50% and 70% of total volume of putty or gel. Commonly used materials include natural polysaccharides like alginate, cellulose, hyaluronic acid and / or proteins. With increasing amounts of DBM part, the handling and malleability properties of the putty decreases but increasing of DBM part is valuable as it increases the osteoinductivity of the product that is necessary for new bone

formation in clinical use. The relatively low amount of DBM in common graft putty leads to actual reductions in osteoinductivity when compared with pure DBM. Also, current gel carriers are soluble in the physiological (aqueous) conditions encountered inside the body. This can lead to premature dissolution of the putty, with consequent release and / or dislocation of DBM particles from the implant area. Consequences of this process include unreliable bone induction and formation of bone in unwanted areas. Stability of the host-graft interface is also important to allow vessels to grow into the graft, so ideally the carrier should keep its original shape during 2-3 weeks.

Chapter 2
Materials and methods of sample preparation

In order to achieve the objectives initially set out for the project, an experimental research plan was designed, and depicted in the following diagram, Fig. 3.1 .

The research was design to be done on different group regards to bone DBM size and shape and also the carrier type that it is summarize in Table 2.1.

Table 2.1: Research group regards to bone DBM size and shape and also the carrier type

Group code	**Sample description**	**Carrier**
Group1	Fibre DBM with CMC base carrier	CMC base
Group 2	small particle powder(<500 micron) DBM with CMC base carrier	CMC base

Group 3	Large particle powder (500< <1000 micron) DBM with CMC base carrier	CMC base
Group 4	Fibre DBM with Alginate base carrier	Alginate base
Group 5	small particle powder (<500 micron) DBM	Alginate base
Group 6	Large particle powder (500< <1000 micron) DBM	Alginate base

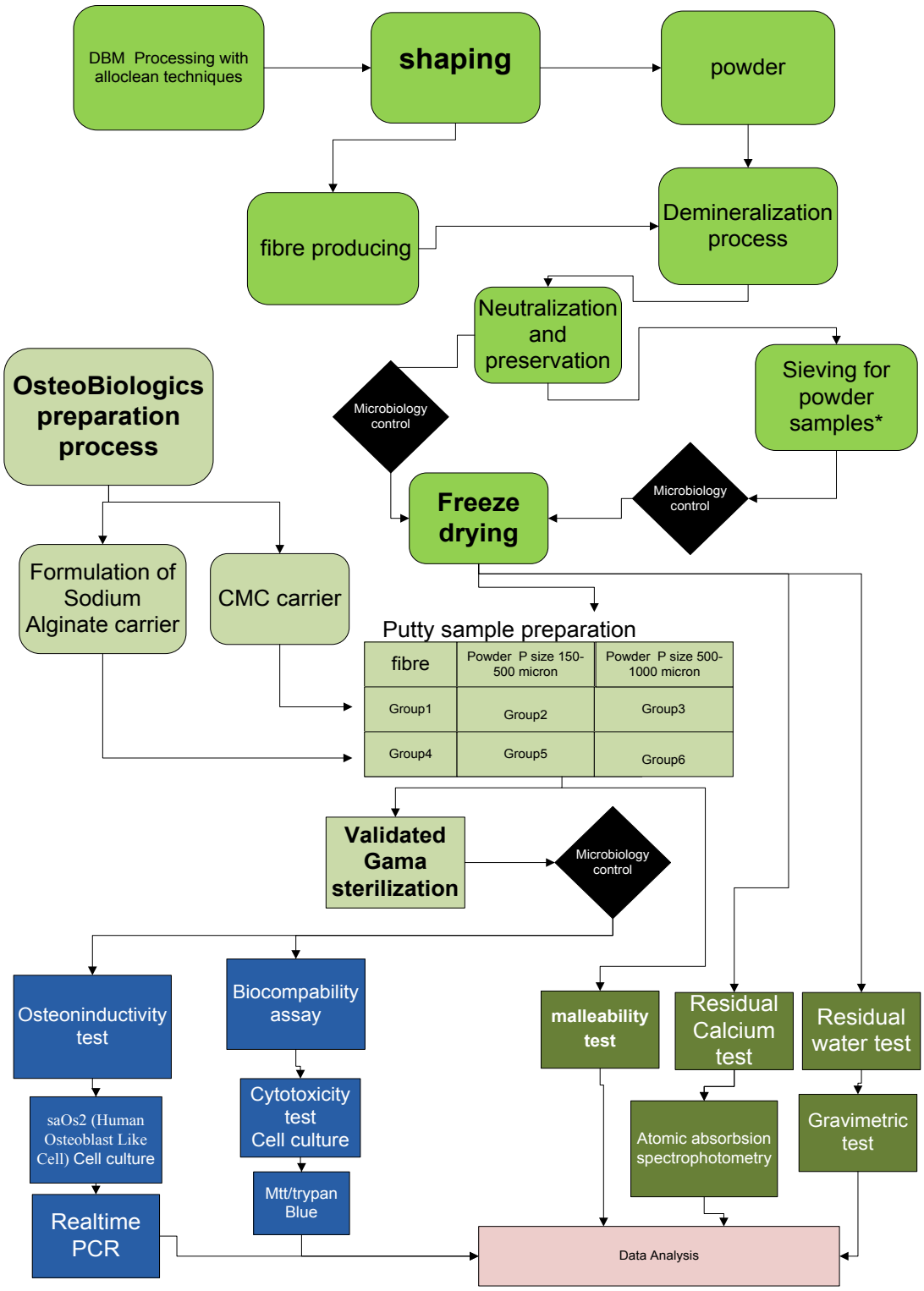


Figure 2.1: Work-flow diagram of current research project**.**

2.1 Aseptic DBM production and processing

As the sample is classed as a clinical product, All sample has been prepared by me in respect to all aspects and standards of production were strictly adhered to at CenoBiologics Ltd. DBM allograft matrix is prepared and processed according to related standards of Cell and Tissue base products. For this purpose it is compulsory to have a research license from the UK Authority (HTA); all work was done according to CenoBiologics Ltd. license.

Bioimplant bone grafts were procured using aseptic techniques and processed in sterile aseptic conditions. The work on the samples was carried out in a validated clean room (ISO 14644: class 5). The purpose of this practice is to process and produce the starting bone material in a manner that minimizes or prevents contamination or decomposition of the tissue.

To maximize allograft safety and osteoinductivity, numerous tissue banks have developed processes to clean and disinfect human allograft tissue with the objective of reducing the potential of disease transmission, in particular emerging infectious diseases that were not known yet. It is critical that the methods used to disinfect the bone tissue will not affect the osteoinductivity or osteoconductivity of the allograft and will not cause an inflammatory response at the implantation site. The samples were prepared by a controlled process to remove infective agents, but at same time ensuring that the bone is not stripped of all of the protein elements that contribute to the material's osteoinductive potential.

Technology developed by CenoBiologics was used to prepare the bone for graft production. The process includes an intensive decontamination, disinfection, and cleaning regimen that use chemical and biomechanical steps to remove virtually all of the biological material that can harbour pathogens. During this process, the vast majority of bone marrow and blood elements are removed from the internal bone matrix. This step, along with a subsequent chemical sterilising treatment, has been shown to substantially reduce the bacterial and fungal bio burden and inactivate viruses. These processes have been done in Cenobiologics production clean rooms and according to their standard operating protocols. To avoid effects of uncontrolled variables on the osteoinductive properties if the final product (DBM putty), all samples were processed under similar conditions, and using the same batch of bone tissue

as starting material.

Treated bone tissue was then processed into three sample groups, according to CenoBiologics techniques and procedures:

- Powder with particle size 150-500 μm
- Powder with particle size 500-1000 μm
- Fibres, thickness below 600 micron with variable length up to 100000 μm

All three groups were treated to remove mineral component of the bone tissue (demineralisation), to final calcium content below 5 ppm. During the demineralisation, samples were processed with dilute HCl at a constant bone weight to solution ratio. Afterwards, samples were neutralised and freeze-dried. Also to minimise the effect of extraneous variables, affecting later on the sample osteoinductivity, a similar drying program was used with the three sample types.

2.1.1 Determination of residual water

Residual water was determined by gravimetric techniques. It was found that residual water content of all samples was below 6 %, and acceptable for this type of material. The shape and size of the DBM had no appreciable effect on the drying process.

2.1.2 Determination of residual calcium

Residual Ca was evaluated by atomic absorption. DBM samples were dissolved (hydrolysed) in concentrated HCl and the amount of Ca in solution determined. Initially there were considerable differences in the amount present in the fibre-shaped DBM, as compared with powder samples. This was possibly due to reduced penetration of the acid solution in the fibre-shaped bone samples. The Demineralization process is acceptable as all sample contain less than 8% amount of Ca in the three sample types, as values above 8% can have an adverse effect on the osteoinductivity. Parameters evaluated included the influence of demineralisation time and volume HCl demineralisation solution to bone mass ratio on DBM

residual calcium. The results of all DBM samples was in acceptable range (less than %8.0) but as in Fig. 2.2 is obvious the residual calcium in fibre samples is about 5% and for powder less than 1%.

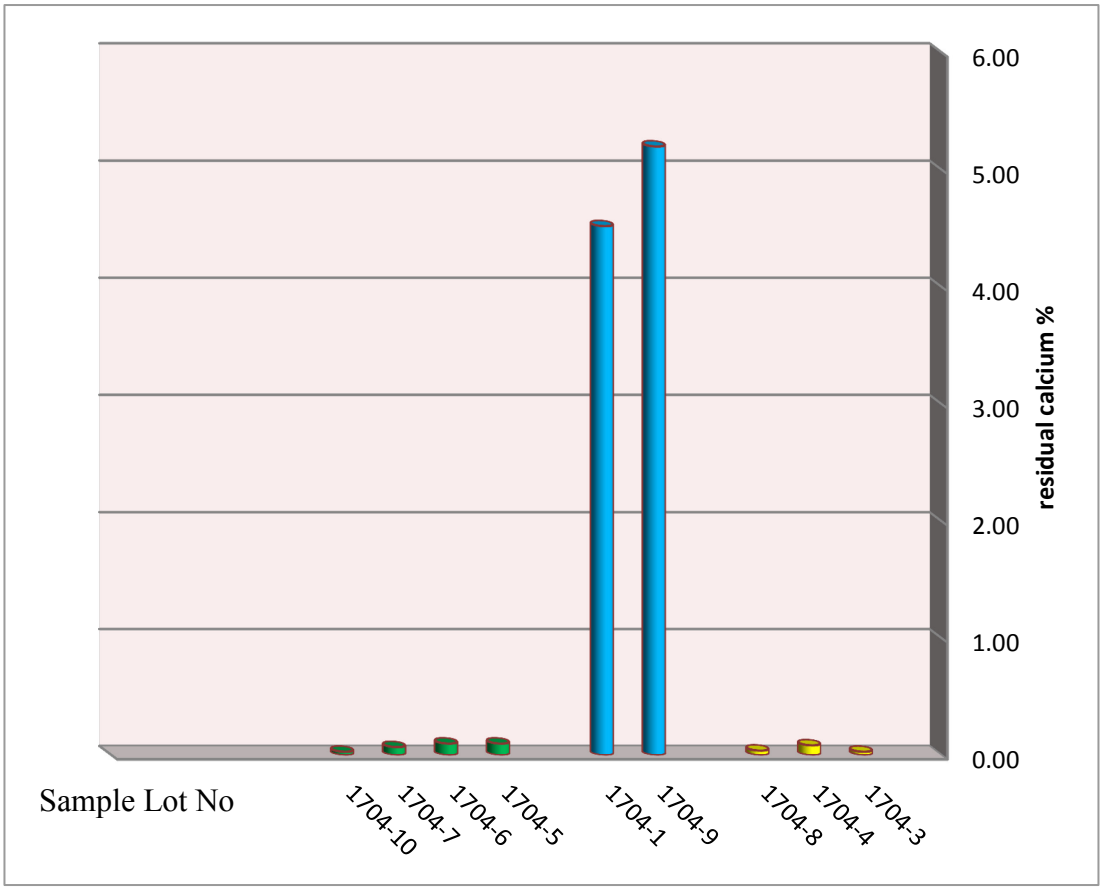


Figure 2.2: Residual calcium content in DBM samples. Yellow: Powder with particle size 150-500 µm; Blue: Fibre; Green: Powder with particle size 500-1000 µm

2.2 Preparation of osteobiologic material

Osteobiologic carrier was prepared in similar conditions and processed in clean room using the all aseptic procedure. In this study different features of carrier composition and its effects on its plastic properties were evaluated. Malleability and stability in time of implantation were the two main features analysed. Osteobiologics carrier was prepared according to CenoBiologics procedure and formulation. To achieve the proper malleability results, for this

part of study, six different carrier formulations prepared and compared for their malleability. Malleability was evaluate by two blinded expert and related score is considered for each group (see Table 3.1)

2.3 Quantitative PCR

Quantitative PCR Quantitative, or real time, PCR (qPCR) is standard PCR with the advantage of detecting the amount of DNA formed after each cycle with either fluorescent dyes or fluorescently-tagged oligonucleotides probes. qPCR results can be obtained faster and with less variability than standard PCR due to sensitive fluorescent chemistry and elimination of post-PCR detection procedures. qPCR is useful for the investigation of gene expression, viral load, pathogen detection, and numerous other applications.

The intensity of the fluorescence emitted during qPCR correlates to the amount of DNA product formed. Fluorescence exponentially increases as the DNA template is amplified. After a few cycles of qPCR, fluorescence surpasses a threshold level set above background fluorescence and starts to increase exponentially. Eventually the fluorescence signal levels off because the fluorescence saturates the detector of the real time PCR machine. Fluorescence is no longer related to the starting template copy number (Fig. 2.1).As starting template quantity is important for quantification by Determining the Cq value, process would be check and read for decision making absorbance . The Cq value: Quantification cycle (Cq) is the metric used for analyzing qPCR results (Fig. 2.3).

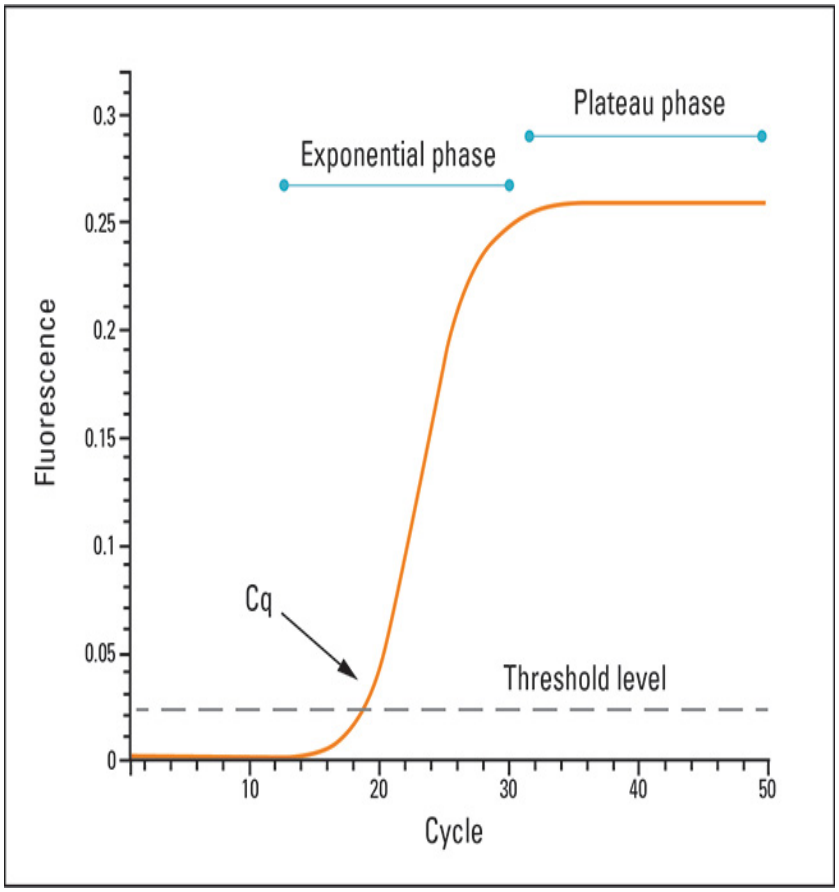


Figure 2.3: Fluorescent signal exponentially increases in qPCR. Threshold Level: the point at which fluorescence emitted from PCR product formation is significant from background fluorescence. Exponential Phase: the fluorescence increases exponentially as the DNA target sequence is amplified. Plateau Phase: the fluorescence signal levels off and any changes in DNA concentration can no longer be recognized.(from thermo scientific)

It needs to consider following point in qPCR:

1-Subtract background fluorescence from raw data. 2. Choose a fluorescence threshold value, either manually or using an instrument-specific algorithm. 3. The data analysis searches data curves for each sample and estimates a Cq value that represents where that sample crossed the threshold. The exact level used for this threshold should be chosen so that it captures data during the exponential phase, when reaction efficiency is still stable and hence the results are most reliable. The threshold value should be the same for all samples analyzed in a run

Fluorescence Detection Chemistries Fluorescence Detection Chemistries There are two types of detection chemistries utilized in qPCR: SYBR-based chemistry and probe-based

chemistry.

1. SYBR-based chemistries use fluorophores or fluorescent dyes that can intercalate and bind within double-stranded DNA. The fluorescent signal of intercalated dye is several orders of magnitude higher than that of unbound dye. The most common fluorophore used is SYBR Green, but other suitable fluorophores include BEBO, BOXTO, SYTO9 and EvaGreen™.

2. Probe-based chemistries utilize fluorescently-tagged oligonucleotide probes that are specifically designed to detect and target the DNA sequence. These probes rely on the principles of Fluorescence Resonance Energy Transfer (FRET) to generate an increase in fluorescence. The most common target specific probe used is a hydrolysis probe, TaqMan, but Basic Principles of qPCR

2. 4. Validated gamma sterilisation

As a final step in ensuring the utmost safety of samples, many tissue banks have adopted sterilisation procedures that do not negatively affect the performance of implanted allograft tissue. SAL of 10^{-6} (Sterility assurance level) is acceptable definition for sterility of medical devices. **Sterility assurance level** (**SAL**) is the probability of a single unit being non-sterile after it has been subjected to sterilization. The validated process of CenoBiologics Ltd for Gamma sterilization was used in this project. It is a Controlled-dose terminal gamma irradiation sterilisation at low temperatures that results shows in a sterility assurance level (SAL) of 10^{-6} without compromising the biomechanical or biochemical properties of the tissue as needed for its intended surgical application. Gamma irradiation's bactericidal characteristic is due to its direct and indirect effects on nucleic acids which lead to genome dysfunction and destruction. Samples for subsequent tests (cytotoxicity and Genotoxicity) were sterilised by gamma irradiation at the facilities of Ionises-France, who was used for this propose as third party contractor.

The samples irradiated at range 18.3 – 25.5 kGy and reference sample microbiologically tested after radiation had shown that sterilisation was done successfully and completely eliminated bacterial contamination from samples. The transferred gamma dose is acceptable as it more than minimum dose that defined by Cenobiologics validation documents. Sterility

was re-confirmed on irradiated DBM material after two weeks. The sterilisation protocol has previously been validated by CenoBiologics Ltd. Who have demonstrated that apart from being effective in elimination of microbial contaminants, it has negligible effects on the osteoinductivity of DBM.

2.4.1 Final microbial contamination check

All samples were further tested for microbial contamination before being processed into DBM putty. Standard microbiological assays under aerobic and anaerobic conditions were used to culture and identify bacteria and fungi. . For Microbiological evaluation TSB and FTM was used in 37 and 22`C incubation for at least 10 days. All samples microbiology test result before final sterilisation was negative.

2.5. METHODS OF DATA ANALYSIS

A similar test to MTT assay was used for cytotoxicity test. The cytotoxicity assay is a colorimetric assay for assessing cell viability. The calorimetric assay of viability was represented by mean ± SD of dye colorimetric in groups. The results of the RT-PCR is analysed by **Kruskal–Wallis** Test. As the quantity of samples in different group is restricted the Non-parametric method **Kruskal–Wallis one-way** analysis of variance was used for testing for quantitative results, scoring will be used for analysis of qualitative data. Since it is a non-parametric method, the Kruskal–Wallis test does not assume a normal distribution of the residuals, unlike the analogous one-way analysis of variance. P values below 0.05 will be considered significant.

Data obtained in this study was analysed using SPSS statistical software 11.5, descriptive data was calculated as mean, median and standard deviation in different groups. The statistical analysis has been done by a cenobiologics`s statistical analyst associate.

Chapter 3
DBM Putty formulation and Malleability properties

A variety of synthetic and naturally derived materials may be used to form hydro gels for carrier. Typical DBM / carrier ratios used for manufacture of osteobiologics were used. Ideally, a carrier performs several important functions in addition to binding the protein.

The OI inducing proteins must be released into the surrounding cell population in such a way that it is available at an opportune time in the progression of healing or is able to stay within the site long enough to recruit CMC base carrier the appropriate cells and accommodate each step of the cellular response during bone formation. The carrier should protect the bone protein from nonspecific proteolysis and provide a substrate for the attachment of recruited osteoprogenitor cells. The material must also be biocompatible and biodegradable, must not evoke a deleterious chronic response, and should act as a temporary scaffold until replaced by new bone. CMC base carrier has been used for DBM carrier and there are very successful clinical results and outcomes according to Cenobiologics end user feedback.

Carboxymethyl cellulose (CMC) is a water-soluble, nontoxic polymer that has been widely used as a pharmaceutical additive[56]. In the Code of Federal Regulations, Title 21, section 1821745, CMC is classified as “Substances That Are Generally Regarded as Safe (GRAS).” Studies in rats and rabbits have demonstrated CMC to be biocompatible with no negative effect on bone formation[57, 58].

CMC carrier viscosity is decrease in aqueous environment so it could decrease the suitability of putty. So it is the disadvantage of currently used carrier .Its water solubility, which can result in release of DBM particles from the graft site (or disaggregation of the graft putty) before regeneration of bone tissue is underway. For improvement of carrier looking for any carrier that could be more stable in equal condition was important. As biomaterials, alginates can easily be formulated into a variety of soft, elastic gels, fibres, foams, Nanoparticles, multi layers etc. at physiological conditions ensuring the preservation of cell viability and function. Alginates are generally biocompatible and ultrapure alginates have become available, avoiding the toxicity and immunogenicity of minor contaminants present in conventional, industrial alginates. As Calcium alginate prepare hardening and water insolubility it could

prepare advantage and improve the carrier regards to this specifications. For this study two different formulation of carrier was considered and also making bone putty bioimplants in two groups: CMC and alginate.

3.1 Carrier Formulation:

3.1.1CMC (carboxymethyl cellulose)

Carboxymethyl cellulose (CMC) is a water-soluble, nontoxic polymer that has been widely used as a pharmaceutical additive and CenoBiologics has successful clinical results with CMC base formulation. For this propose, used different CMC according to their average molecular weight (Mw) of 90000, 250000 and 700000 and in 2 different DBM percentages (see Table 3.1) to achieve proper malleability and resistance to dissolving in aqueous environment. The CMC Mw 700000 was unsuitable to prepare putty with DBM at concentrations higher than 50%. The effect of hydro gel concentration on malleability was studied on group 1, 2, 3 as other groups did not possess good handling properties. For this study different concentration of CMC were also used (ranging from 0.4 g/10 ml to 0.8 g/10 ml water); samples were rejected for malleability tests if gel was unsuitable (such as in group 1 at concentrations 0.4 and 0.55 g/10 ml)

Table 3.1: Malleability studies were performed on the different groups of putty carrier with different Mw CMC and DBM/carrier percentage.

Putty ball	Carrier hydro gel	Ratio of DBM to carrier	Malleability result
A (Group1)	CMC Mw: 90000	50 % w/w	homogeneous (+3) / sticky(2+)
B (Group2)	CMC Mw: 250000	50 % w/w	Homogeneous(+1), sticky(1+)
C (Group3)	CMC Mw: 700000	50 % w/w	homogeneous(2), sticky(0)
D (Group4)	CMC Mw: 90000	60 % w/w	Un Suitable : Homogeneous(+1), sticky(1+)
E (Group5)	CMC Mw: 250000	60 % w/w	Un Suitable : homogeneous(2), sticky(0)
F (Group6)	Sodium Alginate	50 % w/w	homogeneous (+3) / sticky(2+)

3.1.2 Alginate base carrier

To achieve proper malleability and hardening Alginate base putty was prepared as two compartments that would be hardening when mixing them:

part 1: sodium alginate - It was making ball by mixing different amount of Alginate with 20ml water (10%, 20%,30% and 40% see Table 3 to find and choose suitable malleability) and adding bone powder (50%of gel volume)

Sodium alginate specification: Sigma-Aldrich W201502, Alginic Acid sodium salt from brown algae

Part 2: Calcium chloride with CMC carrier (23.73 gram CMC in 15 cc calcium chloride solution with different concentration see Table 3-2)

Different percentage of alginate (Table 3.2) was tested to assess its effect on texture and malleability. Sodium alginate was used in group 6 CMC 50% w/w DBM, before using the putty for the assays the two parts was properly mix by hand as double-component self-hardening putty.

Table 3.2: Malleability studies were performed on the different mixtures of putty carrier with different alginate and calcium chloride percentage. The texture is examined to recover the best one for further tests; any putty with 40% of alginate was too dry. Score from 0 to 5:
0: non homogeneous, 5: completely homogeneous
0: dry and non-sticky, 5: too sticky hard to handle. 3: best stickiness

	Calcium chloride (%)					
Sodium alginate (%)		0.5	1.0	2.0	5.0	Malleability result
	10.0				*	homogeneous (+3) / sticky(2+)
	20.0			*		homogeneous(+1), sticky(1+)
	30.0	*				homogeneous(0) sticky(1+)
	40.0	*				homogeneous(0), sticky(0)

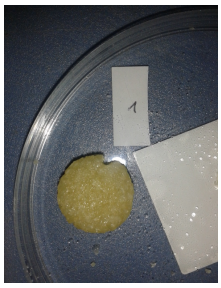
3.2 Malleability tests of CMC and alginate gel carriers

As there is no standard test to assess the malleability and handling characteristics of different putty and gel carriers, in this study the materials were compared against the combined following empirical criteria:

i Handling (stickiness and particle release during manipulation)

ii Consistency, as an indication of suitability for implantation

Double blind tests were scored by an observer and the user, and were scored from 0 to 5. All samples scored between 3 and 5, and were considered suitable for further testing.. Tests on the capacity of the putty to hold its shape were also performed. For this, putty was shaped into balls and left standing for 3 minutes under specific 300 gr weight. The increase in diameter was then measured; see Fig. 3.1 that shows the appearance of the putty after 3 minutes. In Fig. 3.2 this test results is comparing in different molecular weight CMC.



3.1.1. Group A putty

3.1.2. Group B putty

3.1.3. Group C putty

3.1.4. Group D putty

3.1.5. Group E Putty

3.1.6. Group F Putty

Figure 3.1: Photo of putty ball after weight applied to them in different groups.

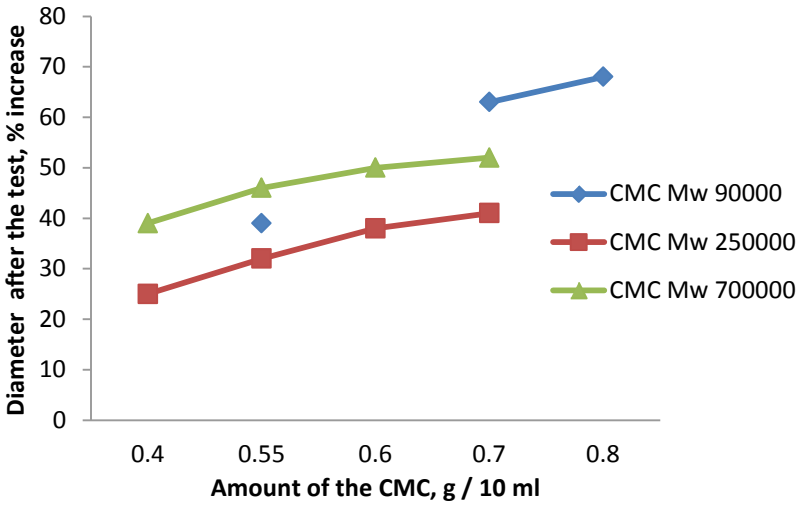


Figure 3.2: Effect of hydro gel concentration on malleability and shape holding capacity. When using CMC 90000, no gel could be formed below a concentration of 0.7 g / 10 ml.

3.3 Final formulation of putty:

Several putty samples from same lot no of DBM is prepared for testing. The samples specifications and lot no is summarized in Table 3.3 .

Table 3.3: Final Putty formulation in different samples bone powder and gel making:

Lot no.	Bone Type	Volume	Carrier	% CMC (medium viscosity) in carrier w/w	% Carrier in putty v/v	% DBM in putty v/v	Quantity
1704-1	Fibre (diff. size)	1 cc	CMC	%16.67	42%	58%	14
1704-3	DM powder (150-500)	0.5 cc	CMC	%16.67	50%	50%	30
1704-5	DM powder (500-1000)	0.5 cc	CMC	%16.67	50%	50%	21
1704-7	DM powder (500-1000)	0.5 cc	CMC	%16.67	48%	52%	41
1704-9	Fibre (diff. size)	1 cc	CMC	%16.67	72%	28%	20

Lot no.	Bone Type	Volume	Carrier	CMC	% Carrier in putty v/v	% DBM in putty v/v	Quantity
1704-4	DM powder (150-500)	1 cc (2*0.5)	CMC with CaCl2	30 gr (MW)	57%	43%	8
1704-6	DM powder (500-1000)				50%	50%	20

Lot no.	Bone Type	Volume	Carrier	% ALG in carrier	% Carrier in putty v/v	% DBM in putty v/v	Quantity
1704-8	DM powder (150-500)	1 cc (2*0.5)	ALG	%10.5 (MW)	50%	50%	4
1704-10	DM powder (500-1000)						6

Chapter 4

Biocompability assay

The *in vitro* cytotoxicity test is done according to the ISO 10993-5:2009 and ISO 10993-12:2012 procedures.

4.1 In vitro cytotoxicity test

The test was set up, and performed in the cell culture unit at the Genetics Department of the University of Leicester. Materials and cell lines have been ordered, and necessary laboratory inductions arranged. The experiments were carried out for different groups of the gel carrier and corresponding bone putty.

The Extract Test (also called "Elution Test" in USP29 <87>) was used for evaluation of cytotoxicity. The following sections describe the necessary materials and methodology identified that performed the cytotoxicity tests according to the relevant ISO standards.

4.1.1. Equipment and materials

- -80^{o} freezer
- Vapour or liquid phase liquid nitrogen tank and refill
- pH meter
- Incubator (37±1 ^{o}C) with/without carbon dioxide
  (Presence of 5% carbon dioxide recommended)
- Phase contrast microscope
- Laminar flow cabinet
- Agitator
- Centrifuge

- HT1080 Human fibro sarcoma Cell (storage temperature −80°C)
- Minimum Essential Medium with serum (5%) and antibiotics (2%)
  Culture medium pH must be kept between 7.2 and 7.4

Type and concentration of medium, serum and antibiotics will depend in the cell line; they should be stated in the cell line description

- Phenol, as a positive material
- Aluminium oxide ceramic rods, as a negative material
- Glycerol, as a cryoprotectant
- Borosilicate glass tubes with caps having an inert liner
- Cry tubes
- Glass Petri dishes
- Plastic culture flasks
- Plastic multiwall plates
- Plastic microtiter plates
- Glass drain-out pipettes
- Manual/Automatic pipettor
- Micropipettes

4.1.2 Protocol

Minimum of three replicates was done for test samples and controls. Test was performed on the original extract and a dilution series of the extracts of DBM putty in culture media up to 1/8 original. As carrier materials dissolve under conditions of the test, complete dissolution was used, and solids removed by centrifugation. The extraction was done in aseptic conditions according to EN ISO 10993−12. According to it, while there are no standardized methods available at present for testing absorbents and hydrocolloids, the following is a suggested protocol: Determine the "absorption capacity" of the material, i.e. the amount of extract liquid absorbed per gram of the material. The test sample shall be 0.1 g/ml beyond the absorptive capacity of the material

4.1.3 Sample extraction:

- Add a certain quantity of sample (DBM putty, at 0.2 g/ml to culture media with serum
- Incubate the extract 24±2 hours at 37±1 ^{o}C with shaking

- Centrifuge the extract at 3500rpm for 10 min
- Use the extract immediately after preparation and do not store it more than 24 hours

4.1.4 Cell Culture:

Pipette an aliquot of the cell suspension into each of a sufficient number of vessels for exposure of extracts *Distribute those cells over the surface of each vessel by rotation*

- Incubate the cells in culture media with serum at 37±1 ^{o}C in air with carbon dioxide for 24 h

  Verify the sub-confluence and the morphology of the cultures with a microscope before starting the test. *Cells should be creating a sub confluent monolayer or freshly suspended*
- Add the extract or dilution to the culture
- When using **monolayer**, remove and discard the culture medium and add an aliquot of the extract or the dilution

  Using of **suspended cells**, need to add the extract or dilution immediately after the preparation of the cell suspension. *Extract should be tested at the highest physiologically compatible concentration after dilution in culture medium (2x or 5x)*
- Add aliquots of the blank, negative and positive controls
- Incubate all vessels at 37±1 ^{o}C in air with/without carbon dioxide for 48h

4.1.5 Qualitative determination of cytotoxic effects

After incubation, the viability and cell count is assessed with tryptophan blue and microscopically, to look for changes in general morphology, vacuolization, detachment, and cell lyses and membrane integrity. Table 4.1 below was used to grade test samples. Grade greater than 2 is considered cytotoxic effect.

Table 4.1: Qualitative morphological grading of cytotoxicity of extracts

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth
1	Slight	Not more than 20 % of the cells are round, loosely attached and without intracytoplasmatic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
2	Mild	Not more than 50 % of the cells are round, devoid of intracytoplasmatic granules, no extensive cell lysis; not more than 50 % growth inhibition observable.
3	Moderate	Not more than 70 % of the cell layers contain rounded cells or are lysed; cell layers not completly destroyed, but more than 50 % growth inhibition observable.
4	Severe	Nearly complete or complete destruction of the cell layers.

4.1.6 Quantitative evaluation

Quantitative determination includes measurement of cell death by tryptophan blue. Reduction of cell viability by more than 30% is considered a cytotoxic effect.

4.2-Cell culture for Cytotoxicity test

- **The Cells that was used is: HT1080** is a fibro sarcoma cell line which has been used extensively in biomedical research. The cell line was created from tissue taken in a biopsy of a fibro sarcoma present in a 35 year old human male.
- The formulation of complete growth media (R10) is given in Table 4.2
- deplete the cells was done according Table 4.3. Corning® Co-star® cell culture plates,96 well, flat bottom (individually wrapped), was used .

Table 4.2: Formulation of growth media

General example using DMEM media:

DMEM - Remove 50 ml from 500 ml bottle then add the other constituents.	450 ml
10% FBS	50 ml
2 mM glutamine	5 ml
100 U penicillin / 0.1 mg/ml streptomycin	5 ml

Table 4.3: Depletion and seeding density that used in experiment

	Surface Area (mm^2)	Seeding Density	Cells at Confluency[1]	Versene (ml of 0.53 mM EDTA)	Trypsin (ml of 0.05% trypsin, 0.53 mM EDTA)	Growth Medium (ml)
Dishes						
35 mm	962	0.3×10^6	1.2×10^6	1	1	2
60 mm	2,827	0.8×10^6	3.2×10^6	3	2	3
100 mm	7,854	2.2×10^6	8.8×10^6	5	3	10
150 mm	17,671	5.0×10^6	20.0×10^6	10	8	20
Cluster Plates						
6-well	962	0.3×10^6	1.2×10^6	2	2	3–5
12-well	401	0.1×10^6	0.4×10^6	1	1	1–2
24-well	200	0.05×10^6	0.2×10^6	0.5	0.5	0.5–1.0
Flasks						
T-25	2,500	0.7×10^6	2.8×10^6	3	3	3–5
T-75	7,500	2.1×10^6	8.4×10^6	5	5	8–15
T-160	16,000	4.6×10^6	18.4×10^6	10	10	15–30

4.2.1 Splitting cells for cytotoxicity test:

The culture medium was removed and discarded. Then:

- 1.0 to 2.0 ml of Trypsin solution was added to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 10 minutes). **Note:** To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach was placed at 37 °C to facilitate dispersal.

- 4.0 to 5.0 ml of complete growth medium and aspirate cells was added by gently pipe ting.
- Appropriate aliquots of the cell suspension were added to new culture vessels.
- Cultures were incubated at 37 °C.

4.3 Sub-cultivation Ratio:

A sub-cultivation ratio of 1:4 to 1:8 was done

4.4 Medium Renewal: Every 2 to 3 days

Procedure for splitting:

It was spited one T75 with 90% confluence:

- Throw out the liquid
- Washing with 8ml PBs
- Trypsin (1cc trypsin with 2 ml PBS, because of concentration of trypsin)
- Shaking 2 sec
- Incubator for 2 min(5 ml all together)
- 18ml medium (completed) in each of 2 T75
- Checking with microscope,
- Need to shake and hit the table, to all cell be free
- 18ml medium (completed) in each of 2 T75
- 5ml cell liquid, divided to 2 T75
- labelling
- incubator

4.5 Cells Freezing technique:

The cells were cryopreserved according to following steps for future experiments:

- Flask T75 with 80% confluence (after 20 hours culture)
- PBS WASHIMG
- PBS 2 ml+ tripsin1 ml
- 2min incubator

- Microscope checking
- 3 ml medium to neutralization
- Centrifuge, and discard the liquid

4.6 Cytotoxicity assay Method:

For cytotoxicity test the Quick cell Proliferation Assay Kit from Source Bioscience Life Sciences was used. Catalogue ABE2415.

Recommended procedure:

- use 1000 to 50000 cell per each well
- Maximum volume of medium and compound was 100 micro litres
- preparing the micro plates,
- Adding the Cells in to each well
- Incubation for different times
- Adding the cell Proliferation Assay kit reagent in each well
- Absorbance control with ,micro plate reader after 3hours

4.7The agent extraction:

1- Dissolve the agent in (R10 cell culture medium) until solution is homogeneous.

A : Alginate base (2 part), sample code: 1704-8 (150-500), 1704-10 (500-1000)

G : CMC base, sample code: 1704-7 (500-1000) N=2, 1704-3 (150-500)

Medium: complete medium, 1.5 ml

A: Sterile jar Weight: 13.244 gr, pot+ putty A: 14.972 gr putty weight: 1728 mg, medium weight: 19.682 gr,

G: Sterile pot, Weight: 13.264 gr pot+ putty G: 15,017gr putty weight1753 mg medium weight: 19.930 gr

Sample G: 2.02gr solid, water content: 55.3 % and Sample A: 1.74 gr solid, water content: 56.4 %

4.7.1 Adding the component to the microplate series1:

1- If solids were present, sample was vortexed to complete dissolution.
2- If sample had bone powder, then centrifugation was used, (500 g, 10 min) to separate the powder,.
3- Make serial dilution based on 1:2 until 4 different concentrations for 1, 1:2, 1:4.
4- Put 90 ml of each concentration into each well.
5- Add agent based on this chart, the 4 first raw was for gel with CMC base and next 4 raw was for gel with alginate base.
6- The Rows are: a,b,c,d for CMC and e,f,g,h for Alginate.

7- The design of the 96 wells micro plate is (series 1):
    - A1 –D1: pour only culture media
    - A2- D2: pour cells + culture media as negative control
    - A3- D3 :pour dilution 1 of the agent C
    - A4- D4: pour dilution 1:2 of the agent C
    - A5-D5: ; pour dilution 1:4 of the agent C
    - A6-D6: pour dilution 1:8 of the agent C
    - A7-D7: another sample
    - A8-D8: pour dilution 1 of the agent C+ neutral red as positive control

    - E1-H1: pour only culture media
    - E2- H2: pour cells + culture media as negative control
    - E3- H3: pour dilution 1 of the agent A
    - E4- H4: pour dilution 1:2 of the agent A
    - E5-H5: pour dilution 1:4 of the agent A
    - E6-H6: pour dilution 1:8 of the agent A
    - E7-H7: another sample
    - E8-H8: pour dilution 1 of the agent A + neutral red as positive control

4.7.2 Adding the Cells in to each well series1:

Before conducting any cytotoxicity experiments the viability of cells was assessed using cell counter with trepans blue with following procedure.

Cell count was done by cell counter :

- Transfer 0.1 ml of cell suspension l to Eppendorf.
- Add 0.1 trepan blue in to the falcon on special counter lamp
- Count cells present.

Total yield: 2.4 * 10^6

Live: 2.3 * 10^6

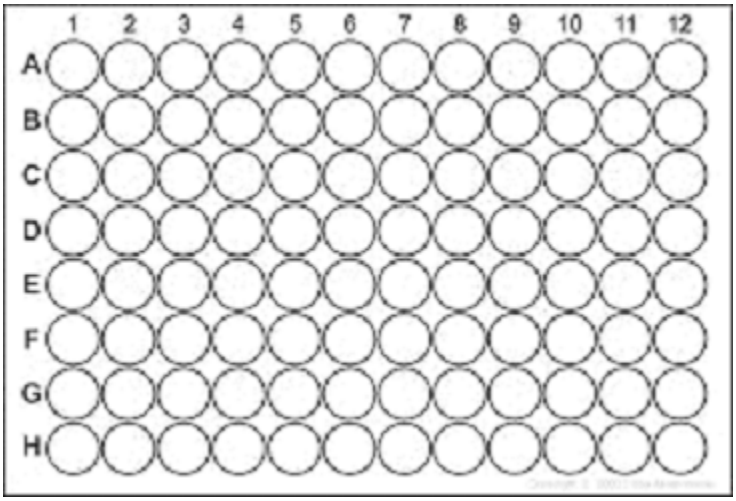
Dead: 1* 10^4

Viability read by cell counter: 100%

The cell yield was 2,400,000 cell per ml, 350,000 cell was need, so 0.2 ml of this medium with cells was used per well.

Add this 0.2 ml of this cell suspension to 0.5 ml of medium and split it to 96 well plates by 0.01 ml, 0.02 ml in to the 2 central well row.

Second Colum remained empty for blank. The plate cell count and test group is shown in Fig. 4.1 . The A is stand for co-culture cells with Alginate base and C with CMC. The cell count is shown for each row that it is in two cell count 5000 and 10000.



A5000
A5000
A5000
A10000
C10000
C5000
C5000
C5000

Figure 4.1: The 96 well cytotoxicity kit arrangement, series1

4.7.3 Cytotoxicity Incubation times for series1:

The test kits were incubated for 1, 2, 5 days.

4.7.4 Results:

The result was rejected as both carrier compounds has turbidity and affect the absorption in micro reader so series 2 was designed to be read in two wavelengths: 480/690 nm.

4.8 Cytotoxicity experiment Series2

4.8.1 Calculation the agent weight series2:

CMC base putty-:
Sterile jar, Weight: 13.160,
Jar+ putty A: 13.270
Putty weight: 1100 mg,
Medium weight: 19.80,

Alginate base putty:
Sterile jar, Weight: 13.160,
Jar+ putty G: 13,330
Putty weight 1700 mg
Medium weight: 19.970
Sample CMC: 2.02gr solid, water content: 55.3 % and Sample Alginate: 1.74 gram solid, water content: 56.4 %

4.8.2 Adding the Cells in to each well (series2):

On the second series the different dilution of compound, was used:
1, 1/2, 1/4, 1/8, 1/16, each in 2 different Colum, and then was added 50 000 cell in to each second Colum. 4 first raw was for gel with CMC base, and 4 raw on the bottom was for gel

with Alginate base, C is co-culture with CMC and A is for Alginate base samples

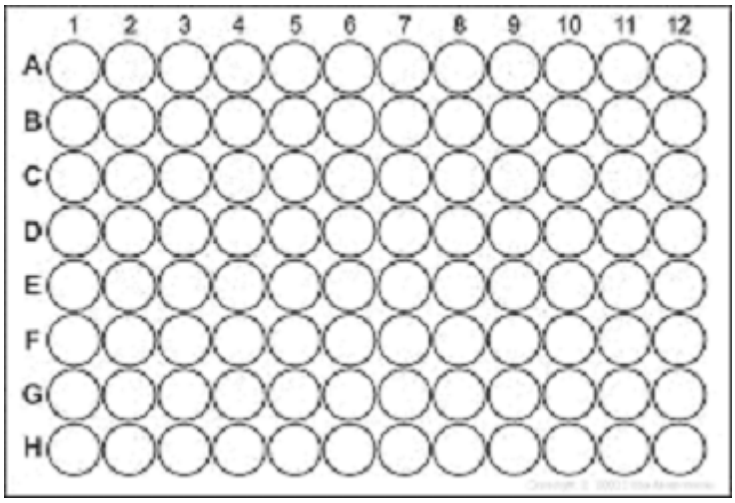
In test series 2 , 50 000 cell was added to mentioned well on defined Columns (see Fig. 4.2)

Column 1, 2, 4, 6, 8, 10, 12: Cell+ medium

Column 3.5.7.9.11: medium+ compound

- A1 –D1: dilution 1 of the agent C+ cell+ neutral red as positive control
- A2- D2: culture media + cell as negative control:
- A3- D3 : dilution 1 of the agent C
- A4- D4: dilution 1of the agent C + cell
- A5-D5: dilution 1:2of the agent C
- A6-D6: dilution 1:2 of the agent C+ cell
- A7-D7: dilution 1:4 of the agent
- A8-D8: dilution 1:4 of the agent C+ cell
- A9- D9: dilution 1:8 of the agent
- A10-D10: dilution 1:8 of the agent C+ cell
- A11-D11: dilution 1:16 of the agent C
- A12-D12: dilution 1:16 of the agent C+ cell

- E1-H1: dilution 1 of the agent A+ cell+ neutral red as positive control
- E2- H2: culture media + cell as negative control:
- E3- H3: dilution 1 of the agent A
- E4- H4: dilution 1of the agent A + cell
- E5-H5: dilution 1:2of the agent A
- E6-H6: dilution 1:2 of the agent A+ cell
- E7-H7: dilution 1:4 of the agent
- E8-H8: dilution 1:4 of the agent A+ cell
- E9- H9: dilution 1:8 of the agent
- E10-H10: dilution 1:8 of the agent A+ cell
- E11-H11: dilution 1:16 of the agent A
- E12-H12: dilution 1:16 of the agent A+ cell



C

Figure 4.2: The 96 well cytotoxicity kit arrangement, series2.

4.8.3 Compound Incubation for series2:

The evaluation was done after plates were incubated for 2 and 6 days.

Test kit: Product Quick Cell Proliferation Assay Kit

Catalogue Number ABE2415

Description WST-1 Reagent (lyophilized) Electro Coupling Solution (ECS

The assay is based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. Expansion in the number of viable cells resulted in an increase in the activity of the mitochondrial dehydrogenases, which leads to the increase in the amount of formazan dye formed. The formazan dye produced by viable cells can be quantified by multi-well spectrophotometer (microtiter plate reader) by measuring the absorbance of the dye solution at 420-480 nm. In this measurement the absorbance of the treated and untreated samples was tested using a microtiter plate reader at 440 nm according to the filter available for the plate reader. The reference wavelength was 650 nm.

4.8.4 Results:

In both group CMC and Alginate there is not a significant difference between the test and negative control (cell only tests) Although there is some reduction in concentrated and ½ diluted samples but viability is just reduced by 25% and so still in the acceptable range (viability more than 30% is acceptable). Also there is slightly better viability for CMC base putty in compare to Alginate base putty but difference is not significant. The effect of cell count also is negligible as for both 10,000 and 50,000 cell there is similar results. Following Tables (and graphs) depict the results for different exposure time and cell concentrations.

The results of cytotoxicity effect of CMC base putty can be found in Table 4.4 and Fig. 4.3 . There is considerable higher absorbance in all groups compared to media without cells that show good activity of mitochondrial dehydrogenases and so the viability of cells. The effect of CMC base putty extract in different concentration is shown minimal effect specially in ½, ¼ and 1/8 concentration of original extract as in these concentration there is no significant difference with control group (Media+ cell). Also the more concentrated group (Media + C1) show about 10% reduction in absorbance . Similar study was done for alginate base putty (see Table 4.5 and Fig. 4.4)

Table 4.4: Cytotoxicity results after 48h culture with extract of CMC base putty for 50000 cell seeding.

	media	media+cell	cell+c1	cell+c/2	cell+c/4	cell+c/8
	0.18	0.79	0.68	0.81	0.72	0.80
	0.18	0.71	0.66	0.69	0.68	0.72
	0.17	0.79	0.72	0.65	0.73	0.71
	0.18	0.77	0.690	0.72	0.71	0.75
STD	0.0045	0.046	0.029	0.08	0.027	0.048

First seeding : 50000	CMC putty
Evaluation Time: 48h	
Wavelength : 480/690	

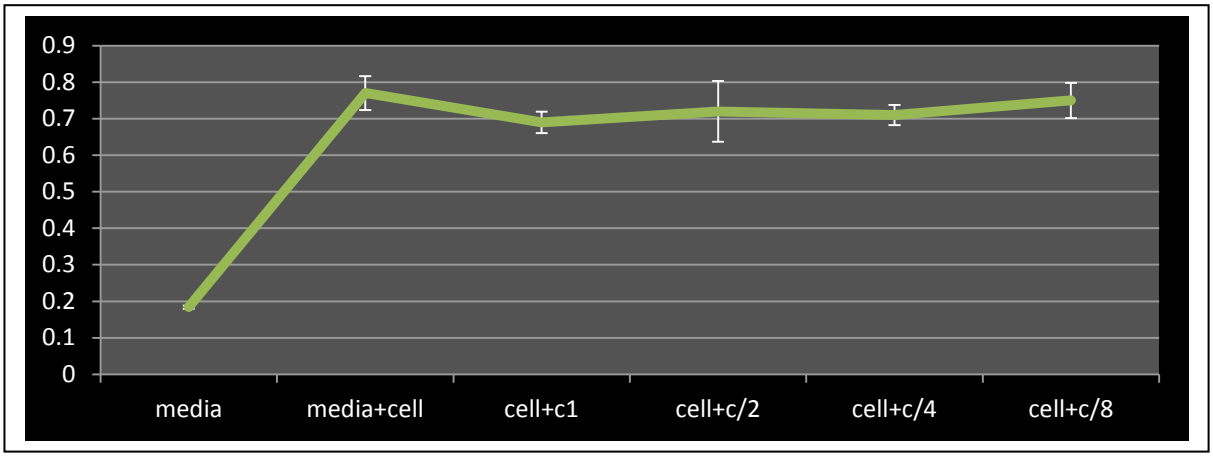


Figure 4.3: Cytotoxicity results after 48h culture with extract of CMC base putty for 50000 cell seeding.

In Table 4-5 the results show reduction of absorbance in all concentration of alginate compound but this is less than 20% so can be considered as a slight effect and so non cytotoxic according to ISO 10993. There is similar pattern for both compounds so this effect could be negligible.

Table 4.5: Cytotoxicity results after 48h culture with extract of Alginate base putty for 50000 cells

	media	media+cell	cell+c1	cell+c/2	cell+c/4	cell+c/8
	0.17	0.75	0.72	0.66	0.65	0.73
	0.18	0.78	0.63	0.65	0.73	0.73
	0.18	0.79	0.55	0.61	0.70	0.73
AVE	0.17	0.77	0.64	0.64	0.70	0.73
STD	0.0047	0.019	0.085	0.029	0.043	0.0017

Alginate base putty
First seeding : 50000
Evaluation Time: 48h
Wavelength : 480/690

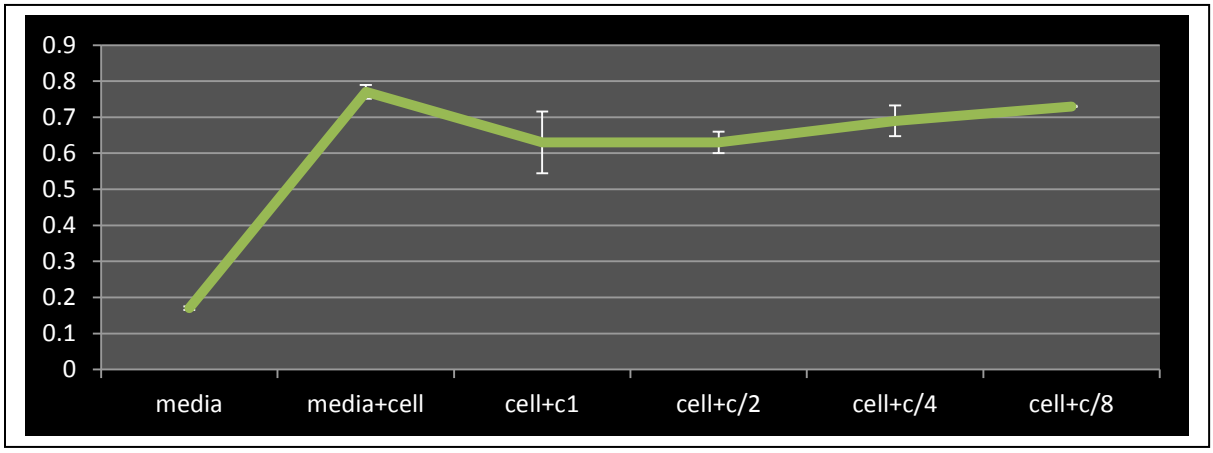


Figure 4.4: Cytotoxicity results after 48h culture with extract of Alginate base putty for 50000 cell seeding.

The Cytotoxicity study was done for 6 days culture for 50,000 cell seeding experiments as well. The results of cytotoxicity effects of CMC base putty are presented in Table 4.6 and Fig. 4.5. There is 20% absorbance reduction in concentrated sample group (Cell+c1) but diluted compound shows no reduction in cell viability. Also similar results were found for Alginate putty for 50,000cell in 6 day culture (see Table 4-7 and Fig. 4.6).

Table 4.6: Cytotoxicity results after 6 days culture with extract of CMC base putty for 50000 cells

	media	media+cell	cell+c1	cell+c/2	cell+c/4	cell+c/8
	0.279	1.081	0.876	1.475	1.059	1.171
	0.306	1.227	0.937	1.323	1.212	1.327
	0.333	1.12	0.928	1.825	1.372	1.389
AVE	0.306	1.1426666	0.9136666	1.541	1.214333	1.295666
STD	0.027	0.0755932	0.0329292	0.257425	0.156513	0.112326
	First seeding : 50000 CMC Evaluation Time: 6 days Wavelength : 450/690					

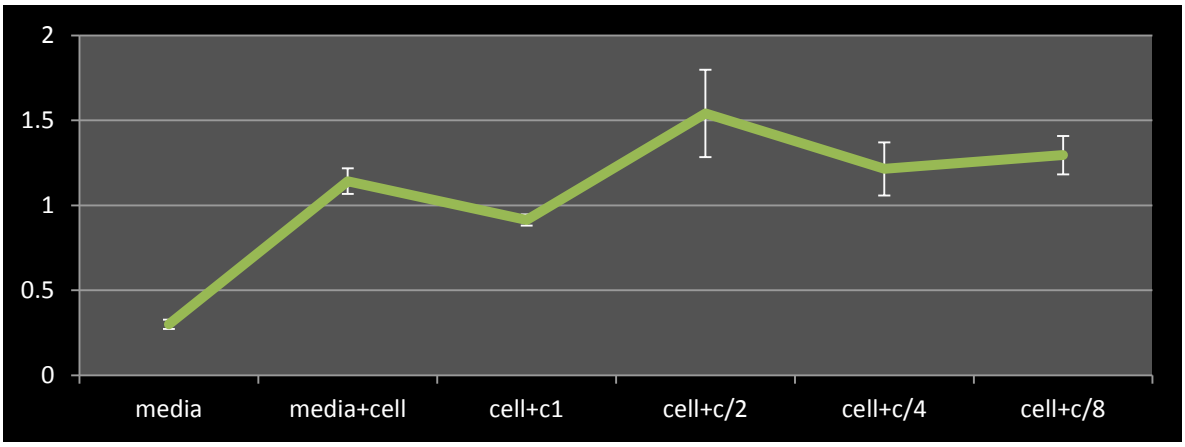


Figure 4.5: Cytotoxicity results after 6 days culture with extract of CMC base putty for 50000 cell seeding.

Table 4.7: Cytotoxicity results after 6days culture with extract of Alginate base putty for 50000 cells

	media	media+cell	cell+c1	cell+c/2	cell+c/4	cell+c/8
	0.323	1.172	1.085	1,222	1.321	1.302
	0.288	1.338	0.981	1.209	1.195	1.362
	0.325	1.242	1.035	1.1	1.205	0.966
AVE	0.312	1.2506666	1.033666	1.1121	1.24033	1.21
STD	0.020808652	0.083338	0.052012	0.013455	0.07003	0.2134291

First seeding : 50000 Alginate
Evaluation
Time: 6 days
Wavelength : 450/690

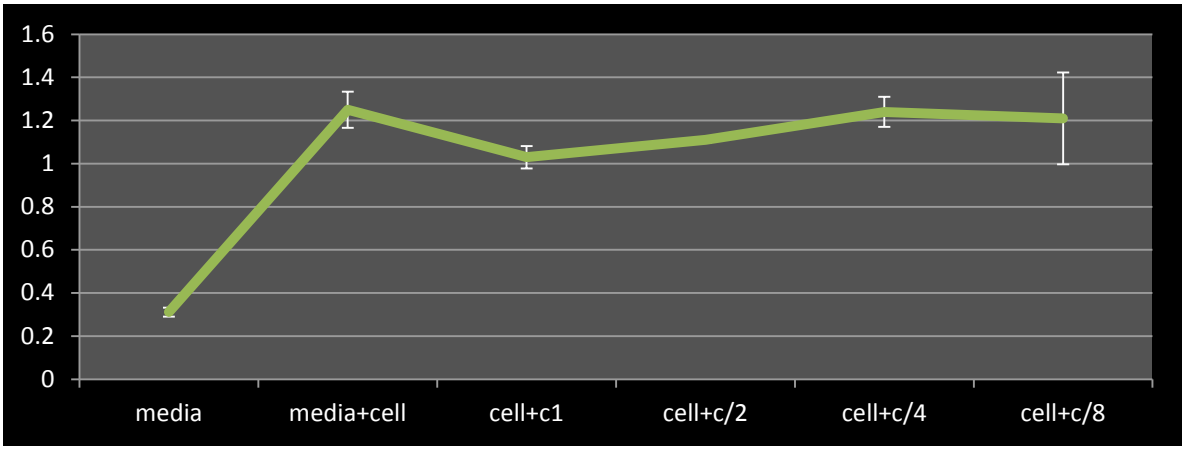


Figure 4.6: Cytotoxicity results after 6 days culture with extract of Alginate base putty for 50000 cell seeding.

Chapter 5
In vitro osteoinductivity assessment

5.1 Preparation of cells

SaOS2 (Human Osteoblasts like Cell) cell line purchased from Cell Bank was cultured with DMEM Dulbecco Modified Eagles medium in a Cell culture Incubator with 5% carbon dioxide and 95% humidity and a temperature of 37 ºC. To evaluate the effect of different types of putty, gene expression will quantitatively evaluated by mRNA production of associated with proteins involved on osteogenesis. In this study, SaOs2 cells are cultured in 6-well plates in 3 ml DMEM medium which after 24 hours will be replaced with the FBS 1% to stop the proliferation of cells. After 24 hours, cells will be treated as follows:

Negative control: DMEM Medium or FBS 1% as a negative control for cell proliferation

Control (positive control): DMEM Medium with FBS 10% as a positive control for cell proliferation

SB: DMEM or FBS 1% plus small size (<500 micron) particle powder DBM from CenoBiologics Ltd.

SG: DMEM or FBS 1% plus putty2 (CMC with small size particle powder DBM) from CenoBiologics Ltd.

LB: DMEM or FBS 1% plus large size (500< <1000 micron) particle powder DBM from CenoBiologics Ltd.

LG: DMEM or FBS 1% plus large size (500< <1000 micron) particle powder DBM from CenoBiologics Ltd.

FB: DMEM or FBS 1% plus fibre DBM from CenoBiologics Ltd.

FG: DMEM or FBS 1% plus putty4 (Alginate with fibre DBM) from CenoBiologics Ltd.

5.2 Qualitative investigation of osteogenic gene expression by RT-PCR

For PCR, two oligonucleotides primers that flank the DNA sequence in question were used. The primers hybridise to opposite strands of denatured DNA and allow synthesis by DNA

polymerase to begin. The principle of the PCR reaction is based on the fact that DNA molecules denatures and anneals at different temperatures. These temperature changes occur in cycles to produce many copies of the DNA sequence. The first step is denaturation of double stranded DNA at 94 ºC. Then the primers anneals to their complementary sequences at about 55 ºC (depending on the primer base composition and length). Extension by the DNA polymerase from the two primers has an optimal temperature of 72 ºC. This cycle is repeated 25 to 40 times to give exponential amplification of the desired DNA sequence. After 30 cycles, theoretically about 268 million copies are created. The DNA polymerase used in PCR is the thermo-stable Taq polymerase isolated from the bacteria *Thermus aquaticus*. This enzyme can survive the high temperatures and the temperature changes during the reaction.

To assess the type of gene expression involved in osteoblastic differentiation, including osteonectin, osteocalcin and osteopontin as an indication of osteoinductivity of DBM putties. SaOs2 cells will be cultured in a number of 20,000 cells in 60 ml plates in 5 ml DMEM medium. After 24 hours the current medium (containing FBS 10% serum) will replaced with FBS 1% to stop the proliferating of cells. Cells are treated after 24 hours, according to the sample groups mentioned above. They are extracted using RNA extraction kits and according to the manufacturer instructions after 3 and 5 days and its purity and integrity are investigated by spectrophotometer (wavelengths of 260 and 280 nm) and the agarose gel. Then target mRNA will used for the real time PCR.

5.3 Cell specifications:

Cell: Saos-2

Catalogue No: 89050205

HUMAN PRIMARYOSTEOGENIC SARCOMA

Reactivity: not applicable

Lot No: 10C019

Passage No. P+13

Mode: Adherent

Epithelial like

Cells/ml × 10^6: 6.70.

Viability: 100%

Medium: McCoy's 5A Medium/ Sigma

5.4 Method of cell preparation:

A: Thawing: adding FBS to freeze cell on the falcon, and add it to ready medium into the flask 1 ml adds to 5ml medium (named cell, 6ml):

1- T25, 3ml medium + 2ml cell

after 24 h: total number of cell was 4.1* 10^5 with 98% viability, adding 1ml freezing medium (FBS,8ml+ R10, 1ml+DMSO,1ml) and freeze with code: 1p/31/7

2- T25, 5ml medium +1 ml cell

after 48 h: total number of Cell was 1.5*10^5 with 83% viability, (named cell 2, 6ml)

3- T75, 15ml medium + 3ml cell

Second Passage was done as follow:

1- T25, 3ml medium + 1.5ml cell 2

2- T25,5ml medium+ 0,5 ml c3ll 2

3- T75, 15ml medium+ 4ml cell 2

5.5Method of designing the 6 well plate and cells treatment:

Aim: comparing of the amount of 3 different RNA between 3 different samples, (150-500, 500-1000, fibre).

1- Take the samples on the 6 wells plate as a duplicate each well, and 2 empty well as the control. sample was weighted and the location of cell culture was summarized in Table 5-1.

2- Adding 200 000 cell per 0.6ml medium into each well

3- Adding 3ml medium in each well (the fibres absorbed all medium, and then was added 5 ml more medium in each of the fibres well)

4- 4 days incubation(fibres absorbed most amount of medium and the colour change to yellow and there wasn't any space for growth the cell, then was removed almost 95% of the fibres in each well, to provide space for cell growth)

5- Removing the medium and bones from each well

6- Using trypsin to removing the Cells

7- Collect the cell and trypsin in to the special nylon meshed (Cell strainers)

8- Spin down

9- Collecting the Cell with trypsin in to the falcon, and counting the Cell in each group:

- FB 5×10^4/ml Viability 72%
- LB 1.3×10^5/ml Viability 85%
- SB 1×10^5/ml Viability 93%
- FG 1×10^4/ml Viability 30%
- LG 1.4×10^5/ml Viability 81%
- SG 3.8×10^5/ml Viability 94%
- Control (only cells) 3×10^5/ml Viability 94%

10- Freezing the cells (6 tube / see , 1.5 ml per well)(for three different protein)(3 different samples)/ (4* 2= 8 falcon) And 1 falcon with the cell without any treatment, with freezing medium.

11- The qPCR according to mentioned method in chapter 3.

Table 5.1: DBM sample weight for qPCR test.

Sample description	Sample code	Weight of Sample with jar	Weight of jar	Plate Well No	DBM weight mg
small particle powder DBM with CMC base carrier	SG1	6.93	6.74	10	0.19
small particle powder DBM with CMC base carrier	SG2	6.97	6.72	9	0.25
Large particle powder DBM with CMC base carrier	LG3	7.05	6.78	11	0.27
Large particle powder DBM with CMC base carrier	LG4	7.05	6.74	8	0.31
Fibre DBM with Alginate base carrier	FG5	7.15	6.74	12	0.41
Fibre DBM with Alginate base carrier	FG6	7.44	6.72	7	0.72
small particle powder DBM	SB7	1.605	1.575	3	0.03
small particle powder DBM	SB8	1.582	1.546	4	0.04
Large particle powder DBM	LB9	1.71	1.587	2	0.12
Large particle powder DBM	LB10	1.738	1.563	5	0.18
Fibre DBM	FB12	6.96	6.78	6	0.18
Fibre DBM	FB13	7.1	6.87	1	0.23

5.6 RNA extraction:

RNA was eluted in 50ul, used the Maxwell 16 from Promega (which is a robot) in combination with the Maxwell® 16 Cell LEV Total RNA Purification Kit

5.7 The cDNA Synthesis:

was used, Strand cDNA Synthesis Kit , K1611& K1612, The First Strand cDNA Synthesis Kit that it is a complete system for efficient synthesis of first strand cDNA from mRNA or total RNA templates.

5.8 Primer design:

House Keeping Genes:

Hs-TUBB_F	GCGCTTGTGGAATTAAAATGGG
Hs-TUBB_R	AGGAACATATTTGCCACCTGTG
Hs B2M-F	GTGCTCGCGCTACTCTCTCT
Hs B2M-R	GTCAACTTCAATGTCGGATGG
GAPDH-F	AGGTCGGTGTGAACGGATTTG
GAPDH-R	TGTAGACCATGTAGGTGAGGTCA
HS_PGK1_F	AAGTGAAGCTCGGAAAGCTTCTAT
hs_PGK1_R	AGGGAAAAGATGCTTCTGGG

Target special Genes:

hs_SPP1_F (Osteopontin)	ACTGATTTTCCCACGGACCT
hs_SPP1_R (Osteopontin)	CTCGCTTTCCATGTGTGAGG
hs_BGLAP_F (Osteocalcin)	GCAGAGTCCAGCAAAGGTG
hs_BGLAP_R (Osteocalcin)	TCACAGTCC GGATTGAGCTC
OSTEONECTIN_F	GGAAGAAACTGTGGCAGAGGTGAC
OSTEONECTIN_R	TGTTGTCCTCATCCCTCTCATACAG

Osteocalcin, also known as bone gamma-carboxyglutamic acid-containing protein (GAPDH), is a non collagenousprotein found in bone and dentin. In humans, the osteocalcin is encoded by the GAPDH gene
http://www.ncbi.nlm.nih.gov/gene/632

Osteopontin (OPN), also known as bone sialoprotein I (BSP-1 or BNSP), early T-lymphocyte activation (ETA-1), secreted phosphoprotein 1 (SPP1), 2ar and Rickettsia resistance (Ric),[1] is a protein that in humans is encoded by the SPP1 gene (secreted

phosphoproteins)

http://www.ncbi.nlm.nih.gov/gene/6696

Osteonectin is a glycoprotein in the bone that binds calcium. It is secreted by osteoblasts during bone formation, initiating mineralization and promoting mineral crystal formation. Osteonectin also shows affinity for collagen in addition to bone mineral calcium

http://www.ncbi.nlm.nih.gov/gene/6678

5.9- Termocycler, qPCR specification:

Was used Roche Light Cycler 480, Achieved efficiency: achieved an efficiency of 1.9-2 almost 99% of the time.

Maxima Sybro Green, Q pcr master mix, K0221

5-10- Normalization:

Normalization was done according to housekeeping Genes and so cell count variation could be ignored. The starting amount of RNA when doing the cDNA can affect the results. 4 different housekeeping genes were chosen to find out what is the more constant housekeeping gene in all groups.

B2m: Beta-2 micro globulin
GAPDH: Glyceraldehydes 3-phosphate dehydrogenise
PGK1: Phospho glycerate Kinase1
TUBB: Tubule

5.11 *In vitro* osteoinductivity assessment results :

The qPCR was done for 3 separate runs from each group for all housekeeping and specific target mRNA except for spp1(osteopontin) the test was repeated that series 2 was done for 5 runs. Cp and Abs Quantity was calculated with Roche software. The group abbreviation and composition is summarized in Table 5.2

Table 5-2: Sample codes in RT-PCR test

Sample code	**Sample description**	**Carrier**
Control	Control –cell culture without any compound	No
SG	small particle powder(<500 micron) DBM with CMC base carrier	Yes- CMC base
LG	Large particle powder (<1000 micron) DBM with CMC base carrier	Yes- CMC base
FG	Fibre DBM with Alginate base carrier	Yes- Alginate base
SB	small particle powder (<500 micron) DBM	No
LB	Large particle powder (<1000 micron) DBM	No

The housekeeping mRNA results is considered for housekeeping genes B2M and GAPDH in Table 5.3 and mean Cp is comparing for B2M (Fig. 5.1) and GAPDH (Fig. 5.2). Also The housekeeping mRNA results is considered for housekeeping genes PGK2 and TUBB in Table 5.4 and mean Cp is comparing for PGK2 (Fig. 5.3) and TUBB (Fig. 5.4). .The GAPDH was most constant with variance 1.43 related to other housekeeping genes, so it used for normalisation of target mRNA expression.

Table 5.3: Housekeeping Cp and absolute quantity results for different test groups for B2M and GAPDH housekeeping Gene:

Name	B2M Cp1	Cp2	Cp3	Average	Abs Quantity
Control	22.46	22.31	22.31	22.34667	1.87492E-07
FB	22.9	22.6	22.6	22.74	1.42751E-07
FG	22.7	22.34	22.34	22.49	1.6976E-07
LB	21.65	21.93	21.93	21.88	2.59098E-07
LG	23.13	22.43	22.43	22.63333	1.53705E-07
SB	23.7	23.11	23.11	23.44333	8.76705E-08
SG	22.5	22.41	22.41	22.41667	1.78612E-07
Name	GAPDH Cp1	Cp2	Cp3	Average	Abs Quantity
Control	27.48	27.48	27.29	27.41667	5.58E-09
FB	27.6	27.37	27.45	27.47333	5.37E-09
FG	28.05	27.67	27.42	27.71333	4.54E-09
LB	27.41	27.13	26.68	27.07333	7.08E-09
LG	27.68	27.59	27.4	27.55667	5.07E-09
SB	28.6	28.27	28.09	28.32	2.98E-09
SG	27.97	27.46	27.4	27.61	4.88E-09

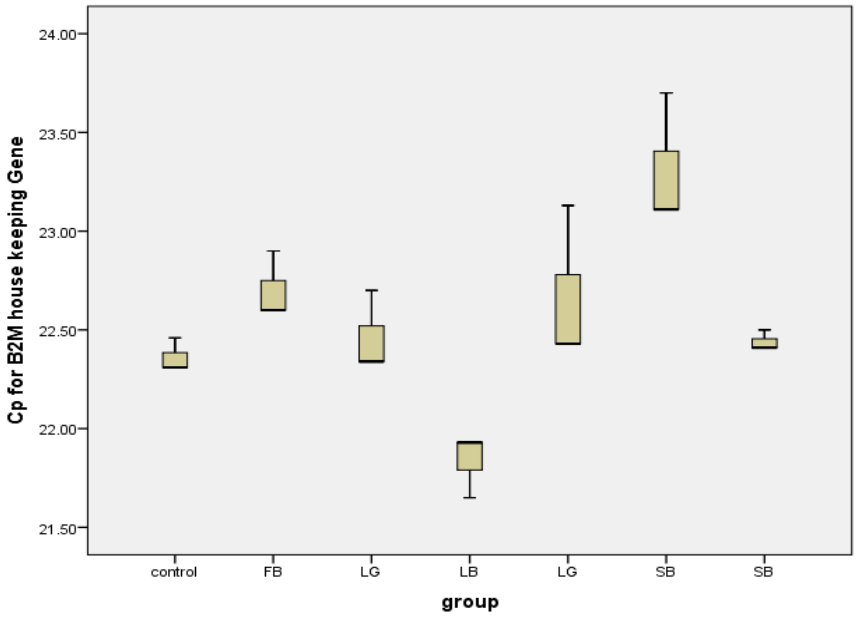


Figure 5.1: Housekeeping Cp mean comparison between different sample groups for B2M

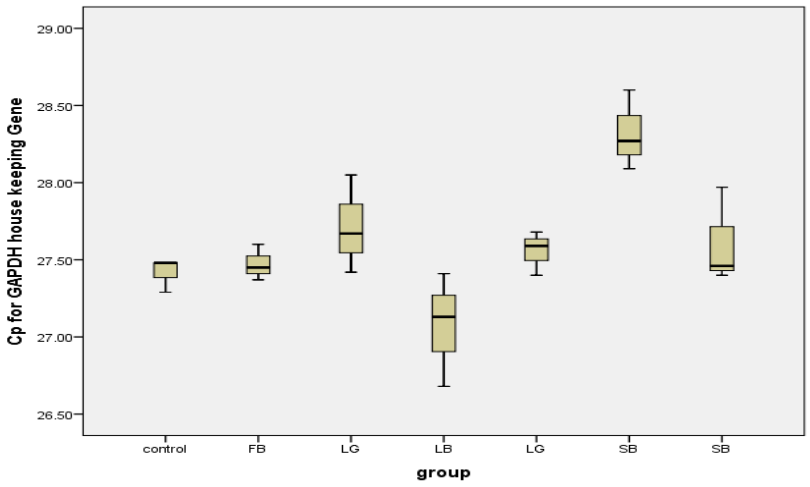


Figure 5.2: Housekeeping Cp mean comparison between different sample groups for GAPDH

Table 5.4: Housekeeping Cp and absolute Quantity results for different test group for PGK1 and TUBB housekeeping Gene:

	PGK1				
Name	Cp1	Cp2	Cp3	Average	Abs Quantity
Control	23.58	23.54	23.62	23.58	7.97E-08
FB	24.27	24.09	24.1	24.15333	5.36E-08
FG	23.9	23.84	23.84	23.86	6.57E-08
LB	23.25	23.27	22.92	23.14667	1.08E-07
LG	23.81	23.87	24.21	23.96333	6.11E-08
SB	24.78	24.74	24.85	24.79	3.45E-08
SG	23.85	23.64	23.82	23.77	6.99E-08
	TUBB				
Name	Cp1	Cp2	Cp3	Average	Abs Quantity
Control	28.35	28.86	28.51	28.57333	2.50363E-09
FB	27.75	28.06	27.69	27.83333	4.1815E-09
FG	28.41	28.1	28.3	28.27	3.08946E-09
LB	27.99	27.54	27.84	27.79	4.309E-09
LG	28.37	28.52	28.33	28.40667	2.81023E-09
SB	29.18	29.1	28.96	29.08	1.76217E-09
SG	28.41	28.28	28.56	28.41667	2.79081E-09

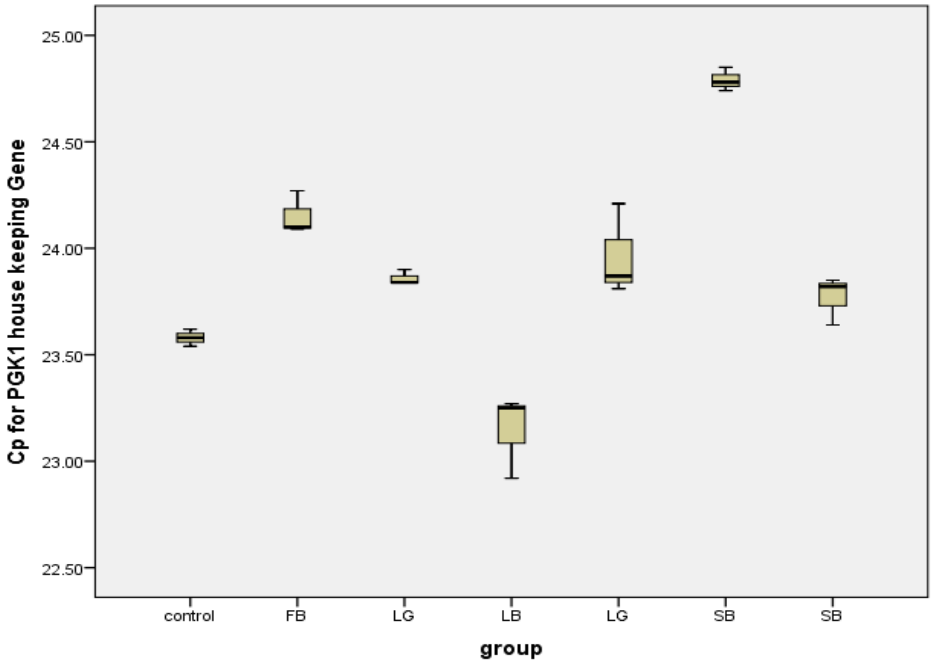


Figure 5.3: Housekeeping Cp mean comparison between different sample groups for PGK1

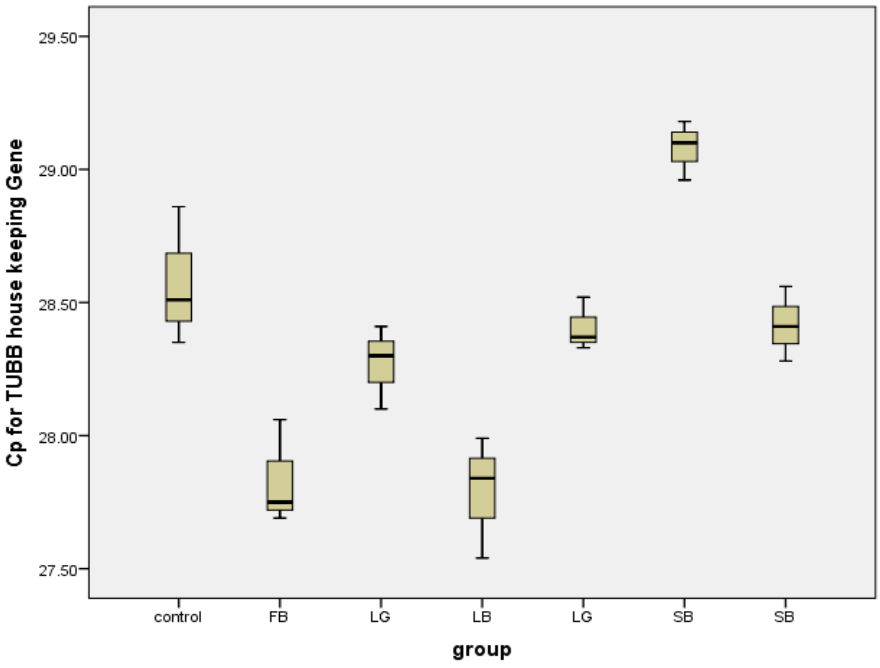


Figure 5.4: Housekeeping Cp mean comparison between different sample groups for TUBB

The qPCR results of specific target mRNA are shown in Table 5.5 for osteonectin and Table 5.6 for osteocalcin. The Cp mean comparison is cited un Fig. 5.5 for Osteonectin and Fig. 5.6 for Osteocalcin.

Table 5-5: Osteoinductive specific mRNA Cp and absolute quantity results for different test groups for osteonectin mRNA:

Osteonectin					
	Cp1	Cp2	Cp3	Average	Abs Quantity
Control	21.21	21.03	21.03	21.09	4.48E-07
FB	21.2	21.11	21.18	21.16333	4.26E-07
FG	21.36	21.28	21.15	21.26333	3.97E-07
LB	20.83	20.63	20.76	20.74	5.71E-07
LG	20.85	20.69	20.86	20.8	5.48E-07
SB	21.77	21.62	21.92	21.77	2.8E-07
SG	21.01	20.94	21.01	20.98667	4.81E-07

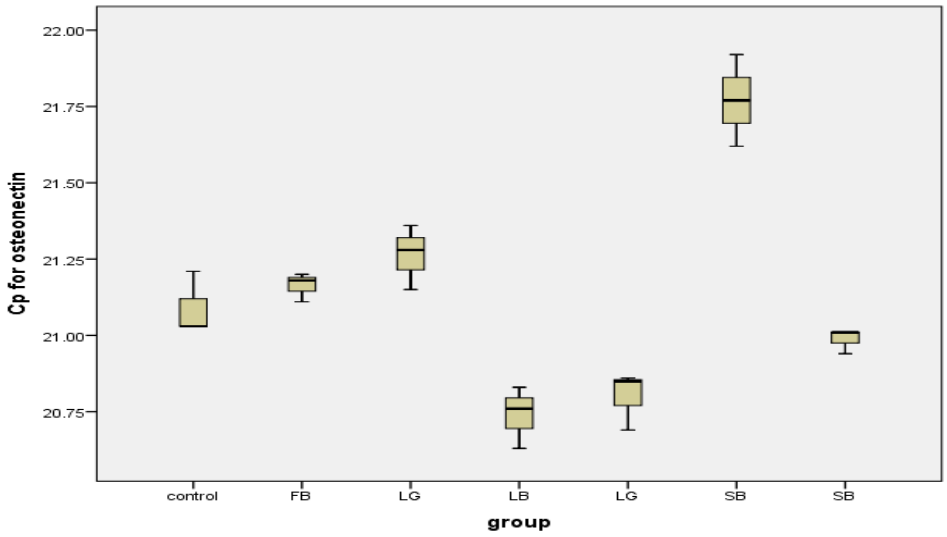


Figure 5.5: Osteoinductive specific mRNA Cp mean comparison between different sample groups for Osteonectin

Table 5-6: Osteoinductive specific mRNA Cp and absolute quantity results for different test groups for osteocalcin mRNA:

Osteocalcin					
	Cp1	Cp2	Cp3	Average	Abs Quantity
Control	27.79	27.76	27.77	27.77333	4.36E-09
FB	27.63	27.6	27.66	27.63	4.81E-09
FG	27.93	27.78	27.94	27.88333	4.04E-09
LB	27.3	27.46	27.34	27.36667	5.78E-09
LG	27.73	27.8	27.77	27.76667	4.38E-09
SB	27.86	28.66	28.48	28.33333	2.96E-09
SG	27.87	28.57	27.98	28.14	3.38E-09

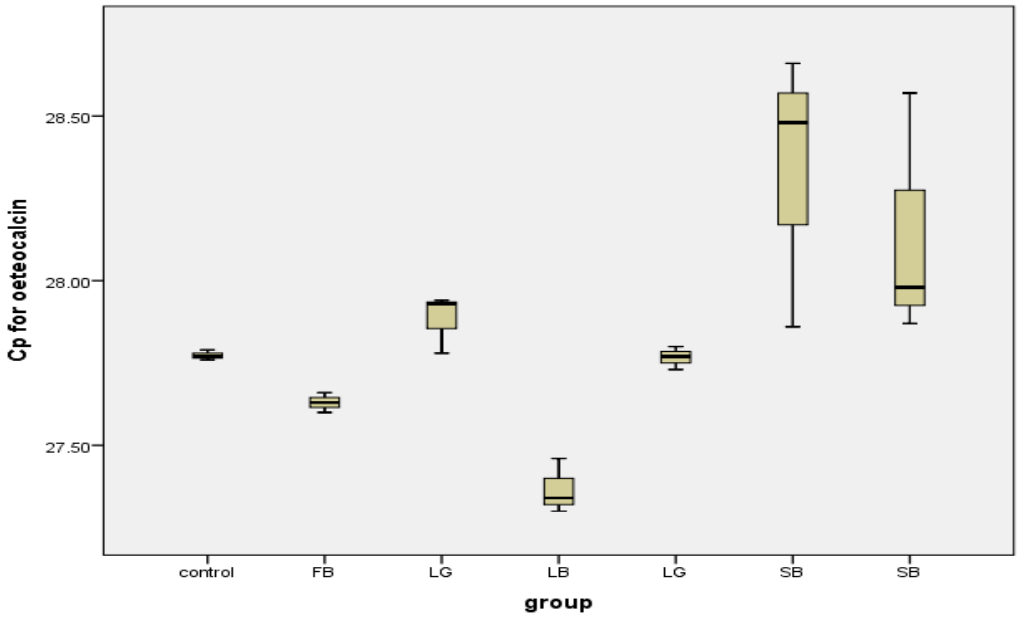


Figure 5.6: Osteoinductive specific mRNA Cp mean comparison between different sample groups for Osteocalcin

Quantitative RT-PCR was done for Osteopontin too but due to it reach to Cp limit (due to primary mRNA low concentration in target cells so the test was repeated so there is two series of test in Table 5-7 for osteopontin series 1 that the mean comparison in different group is shown in Fig. 5.7. As osteopontin was detected in low quantities so the test was repeated with 5 samples for each group for better evaluation. The results can be found in Table 5-8 series 2 and Fig. 5.7 .

Table 5-7: Osteoinductive-specific mRNA Cp and absolute quantity results for different test groups for osteopontin (SPP1) mRNA series 1:

	osteopontin SPP1 (test series1)				
	Cp1	Cp2	Cp3	Average	Abs Quantity
Control	32.11	32.05	32.26	32.14	2.11E-10
FB	32.48	32.3	33.63	32.80333	1.33E-10
FG	32.79	31.62	~~24.6~~	32.205	2.02E-10
LB	32.59	32.32	32.3	32.40333	1.76E-10
LG	31.77	32.17	31.67	31.87	2.55E-10
SB	32.98	32.61	32.26	32.61667	1.52E-10
SG	32.77	32.49	31.69	32.31667	1.87E-10

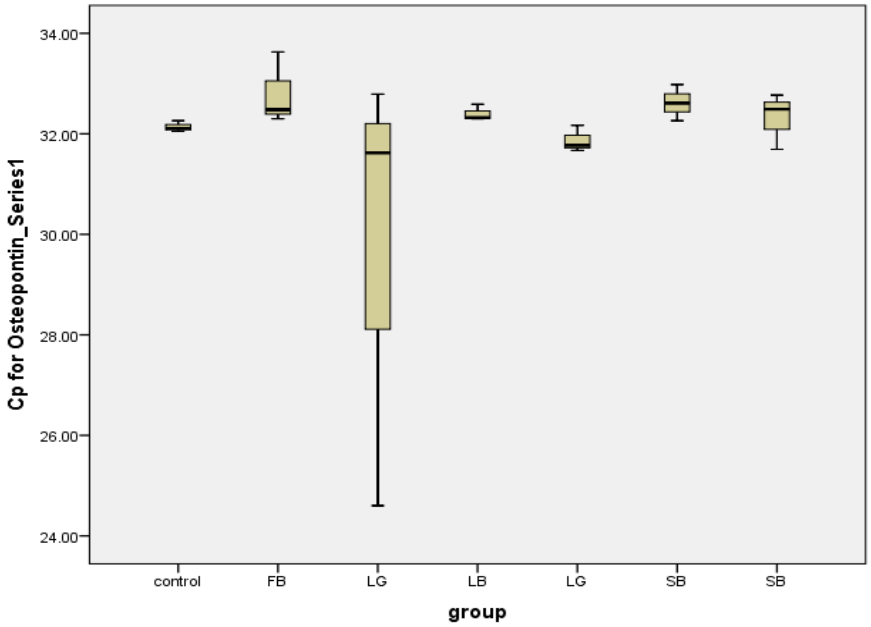


Figure 5.7: Osteoinductive specific mRNA Cp mean comparison between different sample groups for Osteopontin series1

Table 5-8: Osteoinductive-specific mRNA Cp and absolute quantity results for different test groups for osteopontin (SPP1) mRNA series2:

Osteopontin SPP1(test seris2)							
	Cp1	Cp2	Cp3	Cp4	Cp5	Average	Abs Quantity
Control	31.15	31.11	31.65	32.03	30.87	31.362	3.62324E-10
FB	32.72	31.68	32.08	32.74	31.63	32.17	2.0695E-10
FG	31.36	31.44	30.53	31.15	31.56	31.208	4.0314E-10
LB	31.12	31.52	32.17	31.41	31.67	31.578	3.11943E-10
LG	31.44	32.5	31.61	31.33	31.09	31.594	3.08502E-10
SB	31.58	32.21	32.44	32.04	31.67	31.988	2.34775E-10
SG	31.79	31.98	31.76	31.23	31.88	31.728	2.81138E-10

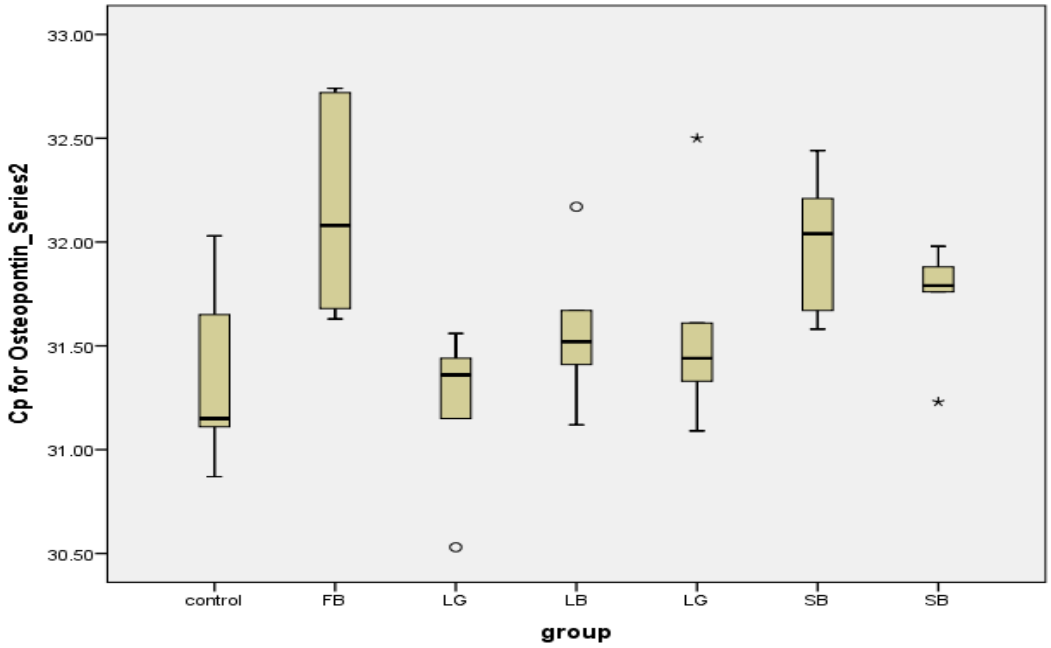
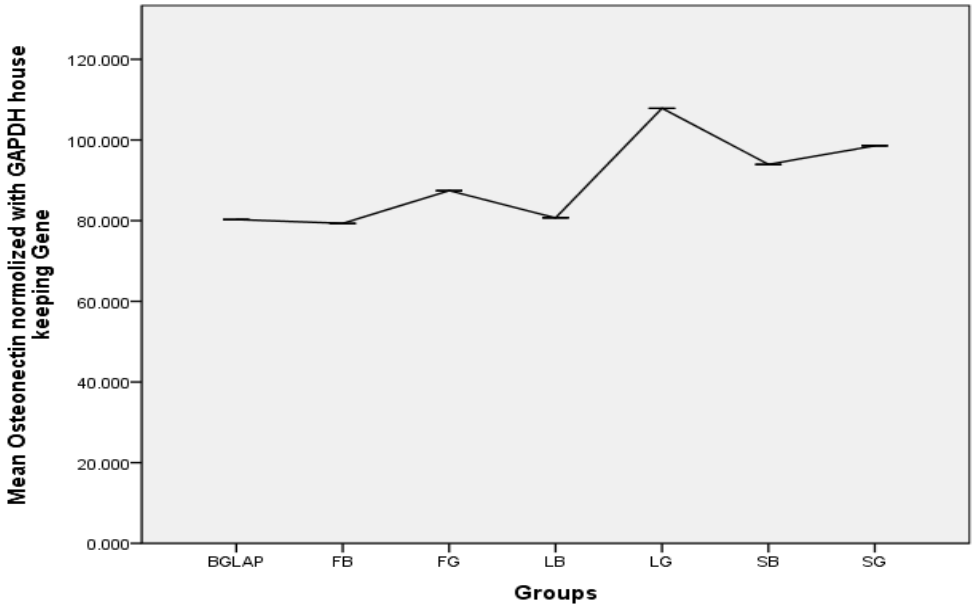


Figure 5.8: Osteoinductive specific mRNA Cp mean comparison between different sample groups for Osteopontin series2

5-12 Osteoinductive specific mRNA comparison:

Normalised results to GAPDH were compared using non parametric analysis. There is a significant up- regulation in different target mRNA include osteonectin mRNA ($p<0.003$ see Fig. 5.8), osteocalcin ($p<0.004$ see Fig. 5.9), osteopontin ($p<0.003$ see Fig. 5.10). As series 2 test was done on more samples it was also used for osteopontin mRNA expression analysis.

Osteonectin by GAPDH



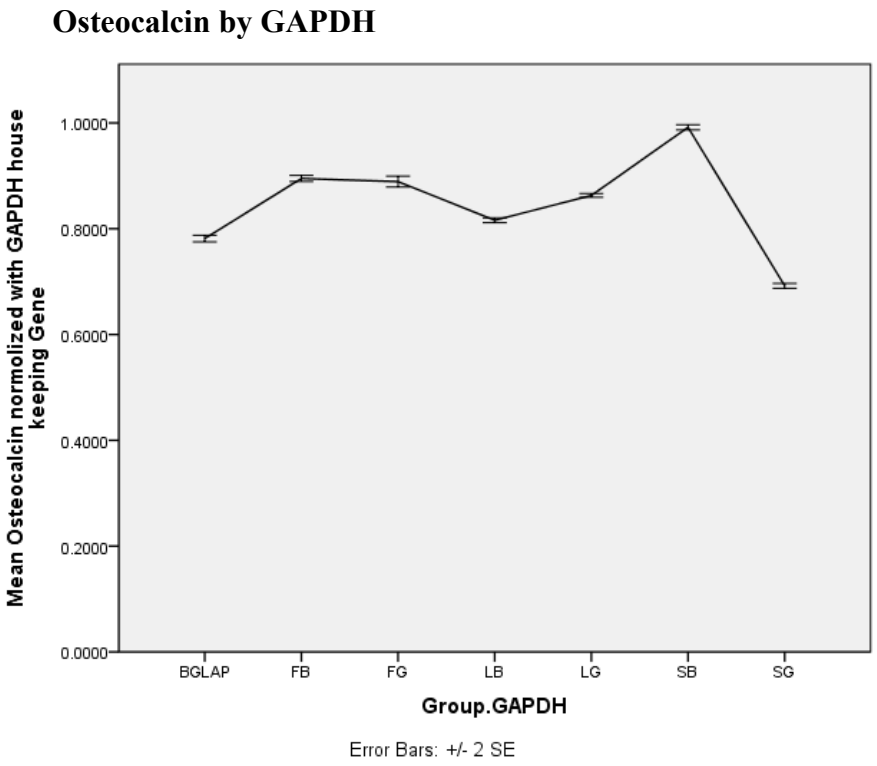
Test Statistics[a,b]

	Osteonectin
Chi-Square	19.662
df	6
Asymp. Sig.	**0.003**

a. Kruskal Wallis Test

b. Grouping Variable: Group.GAPDH

Figure 5.9: Nonparametric analysis of results between different DBM/Carrier groups and control for Osteonectin, there is a significant ($p<0.000$) higher mRNA expression in FG, LG, SB and SG group in compare to control.



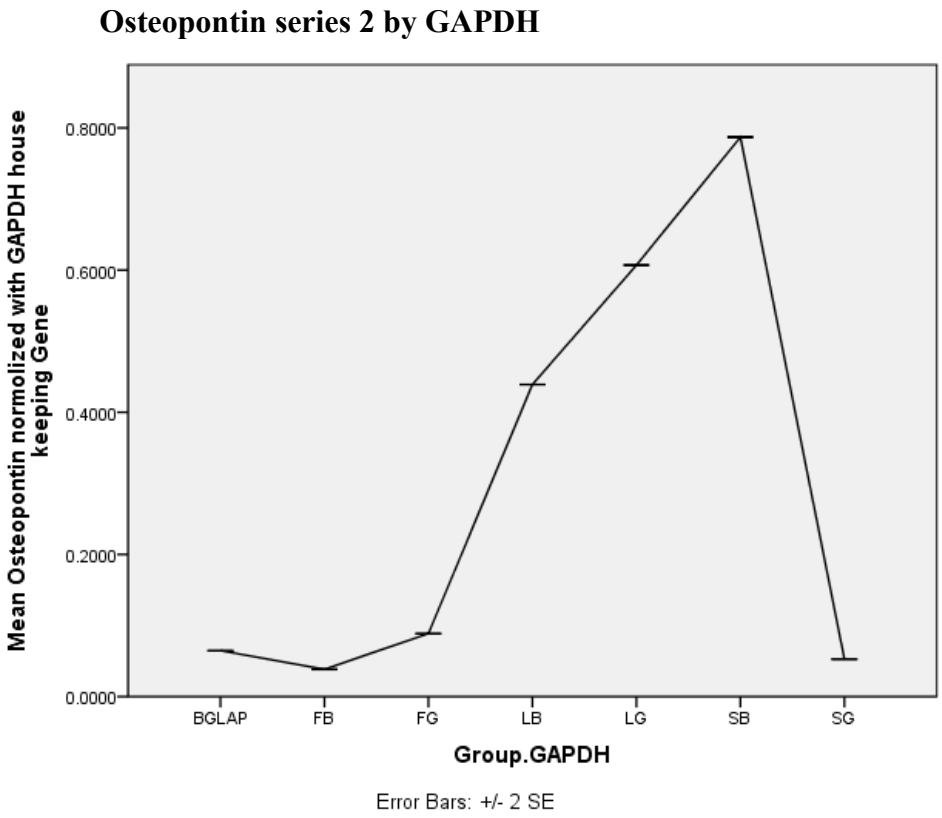
Test Statistics[a,b]

	Osteocalcin
Chi-Square	19.394
df	6
Asymp. Sig.	**.004**

a. Kruskal Wallis Test

b. Grouping Variable: Group.GAPDH

Figure 5.10: Nonparametric analysis of results between different DBM/Carrier encountered groups and control for Osteocalcin**.** There is a significant ($p<0.000$) higher mRNA expression in FB, FG, LG and SB group in compare to control.



Test Statistics[a,b]

	Osteopontin2
Chi-Square	19.636
df	6
Asymp. Sig.	**.003**

a. Kruskal Wallis Test

b. Grouping Variable: Group.GAPDH

Figure 5.11: Nonparametric analysis of results between different DBM/Carrier encountered groups and control for Osteopontin. There is a significant ($p<0.000$) higher mRNA expression in LB, LG and SB group when compared to control.

Chapter6

Discussion

The end product of the bone demineralization process is a DBM powder or other shap that may be difficult to manage clinically. Consequently, several carriers have been used to incorporate high mass fractions of DBM powder, facilitate handling, formulation and reliable delivery of DBM products clinically. Diverse types of commercial DBM-carrier products are known and available. The current most popular clinical DBM format is a moldable putty that can be packed into bone defects and resists dispersion from irrigation and blood during surgery. Conversion of DBM powder to putty involves formulation with a biocompatible viscous carrier that provides a stable suspension of DBM powder particles. The viscous carriers can be classified as water-soluble polymers such as sodium hyaluronate or carboxymethylcellulose, or anhydrous water-miscible solvents such as glycerol. In some cases, the carrier selection has implications for other processing steps, compatibilities, applications, and even sterilization. Further, DBM can be mixed with these carriers to produceflexible sheets that may contain both DBM and cortical bone chips as a composite biomaterial. Additionally, the polymer carrier Pluronic (BASF product, synonymous with poloxamer), is a temperature-sensitive biomedical copolymer carrier used with DBM. The composition becomes firmer as it warms to body temperature. Another carrier is a thermoplastic, porcine collagen-based hydrogel that is non-water-soluble. The DBM-porcine collagen can be extruded through a syringe after it is heated to 46–50°C. In situ at body temperature the composition becomes firm. [59]

Regards to Biocompability two type of carrier was tested . alginate base carrier have a potential to become firm. Both carrier compounds show good biocompatibility even in concentrated dose. The viability is above 70% in both experiments (2 days and 6 days contact with the gel carrier). Also cell count was generally unaffected by dose. Although the results are slightly better for the CMC base carrier, but alginate also shows acceptable biocompatibility.

Also the qPCR results confirmed that the putty and DBM has effect on cell differentiation and osteoinduction in gene translation on Saos2 cells. It can up regulate and also in some

instances also down regulate the target genes. The in vitro osteoblastic differentiation and RT-PCR phenotyping was compared in different groups (Table 6.1) to compare effect of DBM size and shape and also the potential effect of carrier polymer.

Table 6.1: Sample codes in RT-PCR test

Sample code	**Sample description**	**Carrier**
Control	Control –cell culture without any compound	No
SG	small particle powder(<500 micron) DBM with CMC base carrier	Yes- CMC base
LG	Large particle powder (<1000 micron) DBM with CMC base carrier	Yes- CMC base
FG	Fibre DBM with Alginate base carrier	Yes- Alginate base
SB	small particle powder (<500 micron) DBM	No
LB	Large particle powder (<1000 micron) DBM	No
FB	Fibre DBM	No

If the extent of bone demineralization is constant, then DBM particle size remaining after morselization in the powder, or DBMfiber geometry produced by processing the bone-derived collagen proteins then defines DBM surface area as a clinical variable. Different surface geometries may impact host cellular interactions as well as diffusion rates of DBM-resident biological molecules and endogenous agents such as BMPs or growth factors in and out of DBM. Consequently, some discussion regarding the optimal size and size range of particle for DBM preparations suggests that particles less than 250μm are not as osteoinductive as larger-sized (420–840μm) particles [33-35]. Generally, preclinical data are inconsistent regarding DBM compositions as particles and their size ranges, as well as for fibres, and DBM formulations in sheets and gels. Inconsistency is due to different animal models and outcome measures, many of which are highly subjective and are unique to one laboratory and not universally accepted. Moreover, human data on DBM are weak due in part to the emphasis on DBM as a bone graft extender rather than as a stand-alone therapy. Consequently, the efficacy for different formulations for DBM has not been clearly duplicidated. Formulation design features are largely empirically ascertained in arbitrary test bed[59] .

There is no any previous research to evaluate the carrier effect in cell model osteoinduction gene regulation . According to our results of this study there is different finding regards to DBM particle size and shape in presence of carrier or without it that is summerize in follow. it could be related to cell attachment property alteration due to carrier so the results shown different in DBM and DBM+carrier but further study need to be done with more controlling the other parmeter.

The mRNA expression is significantly higher in SB, LG and FG treated culture in comparison to control group for all three genes OC, OP, ON, but other groups show some up-regulation as well. Also there is a significant gene expression between similar group of DBM shape and size with or without carrier. For example osteonectin absolute quantification (normalised with GAPDH) are significantly expressed in samples containing the carrier, independently of the size or shape of the DBM present (Fig. 6.2). For osteocalcin and osteopontin the small particle powder DBM leads to lower expression when carrier is present (SG) (see Fig. 6.1, 6.3). It could be due to effect of carrier in cell attachment and surface characteristic and its effect on DBM and interconnection to cells. Wohlfart et al., [60] has shown that apart from architectural organisation, the surface characteristics such as micro and macro pore size, an interconnecting system, the in vitro solubility, the chemical composition of the materials seems to have a significant impact on the target cells. Although this study compares well to the one performed by Wohlfart, in that cells were cultivated for 5 days on target compound, future studies where the cells are cultured for longer periods (14 to 30 days) could give more information about trends and effects of carrier on DBM presentation and osteoblastic differentiation. Sampath and Reddi stated 'the functional collaboration between the soluble extract and insoluble collagenous substratum' (i.e., the demineralised matrix) were necessary for 'optimal ostegenic activity'. Reddi and his colleagues identified factors in the 'soluble extract' crucial to ectopic ossification. These BMP molecules are part of the 'soluble extract' Reddi first identified in his original demineralised bone matrix (DBM) (reviewed in [24]). As carrier encapsulates the DBM (BMP containing part of putty) so it could affect the cells encountering in contact with soluble extracts.

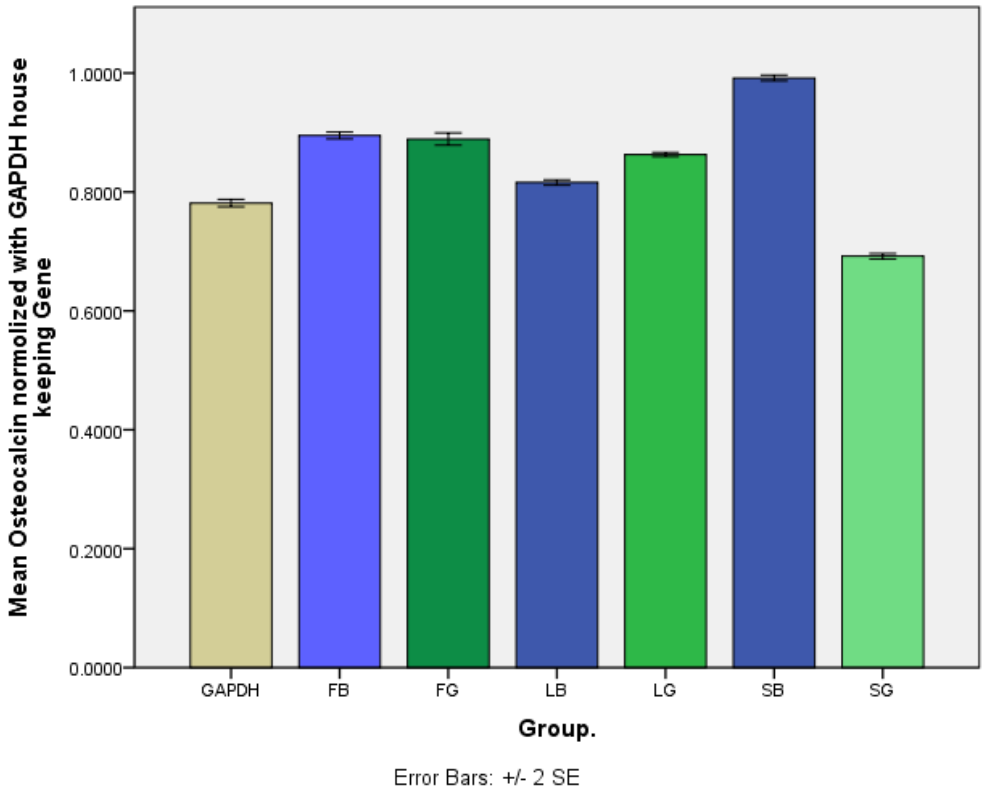


Figure 6.1: Comparison of Osteocalcin mRNA expression in different groups with carrier (Green) and without carrier (blue). With exception of the carrier with small particle size DBM, all others show higher expression when compared to samples without carrier.

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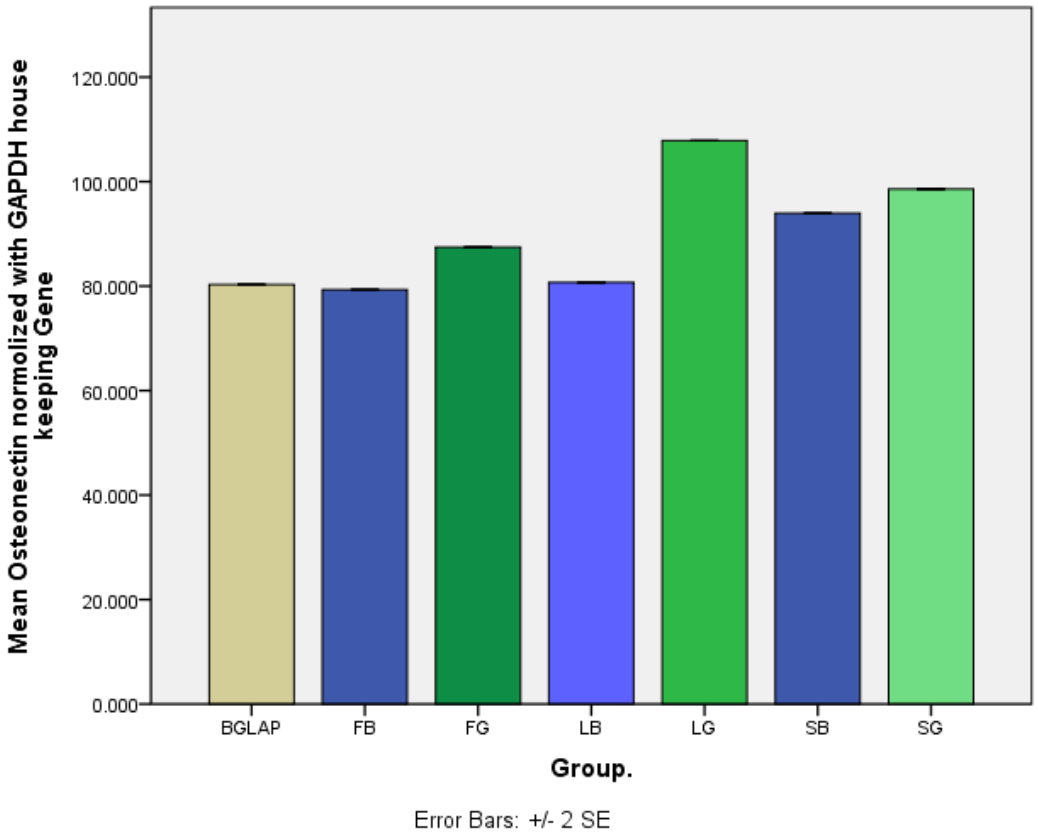

Figure 6.2: Comparison of Osteonectin mRNA expression in different groups with carrier (Green) and without carrier (blue) it shows higher expression when compared to samples without carrier

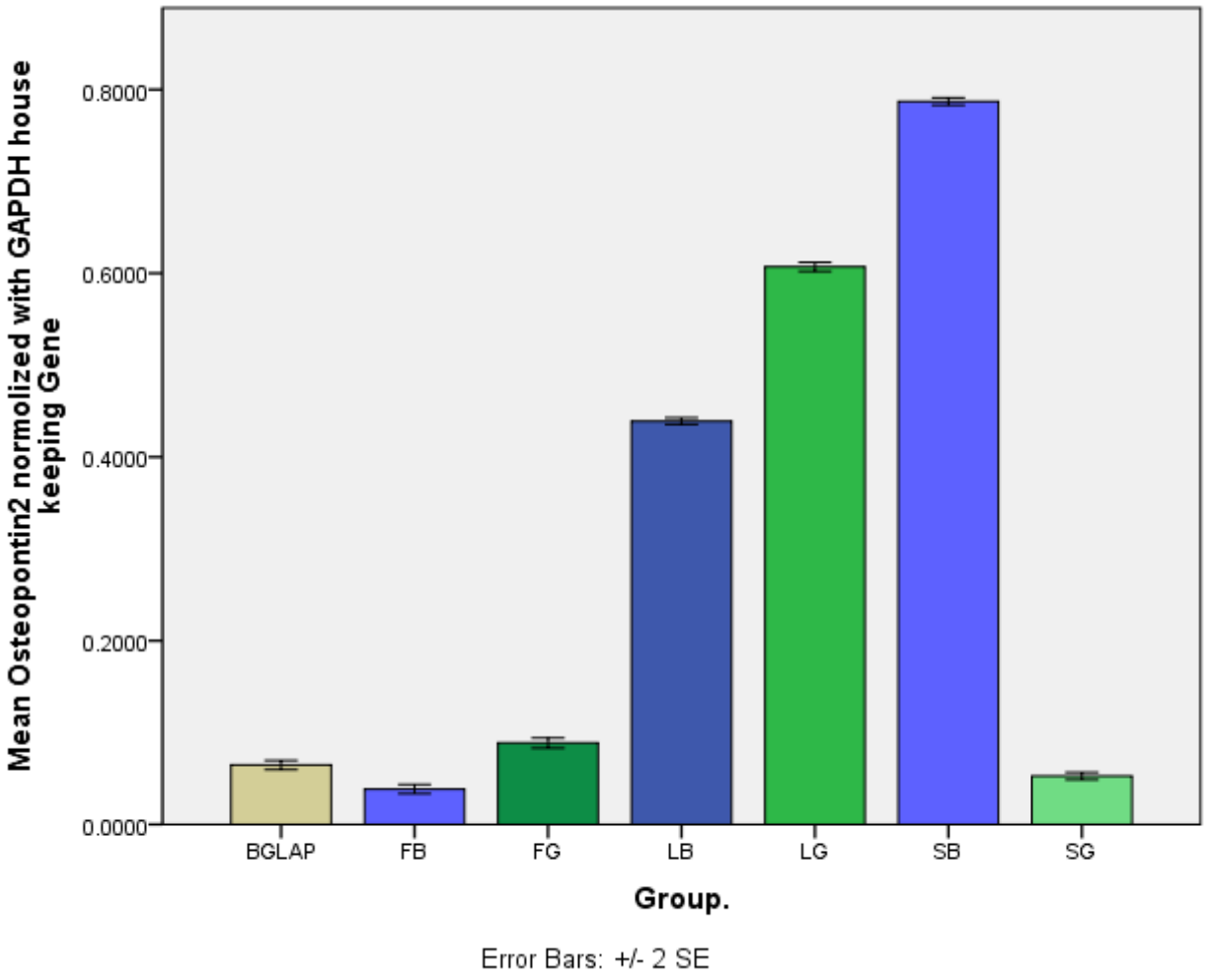

Figure 6.3: Comparison of Osteopontin mRNA expression in different groups with carrier (Green) and without carrier (blue). With exception of the carrier with small particle size DBM, all others show higher expression when compared to samples without carrier.

Studies designed to discriminate the effectiveness of various carriers on DBM (pre)clinical efficacy are limited. Wang implanted athymic rats with commercially available DBMs Dynagraft putty, Grafton putty, or Osteofil allograft bone paste [61]for spinal fusion. Most of the segments implanted with Grafton and Osteofil fused and none of the segments implanted with Dynagraft fused. Sassard and colleagues reported on a retrospective review

of patients who had undergone instrumented posterolateral lumbar spinal fusion with an autogenous bone graft and Grafton gel [9]. Age-, gender-, and procedure-matched group of patients were involved. There were no differences between treatment groups (autograft vs autograft plus Grafton) based on radiographs up to 24months after surgery. The fusion rates in the autograft with Grafton group and the autograft-only groups were only 60% and 56%, respectively. The most important predictor of 24-month bone mineralization was a correlation between the type of instrumentation and fusion success. Bostrom and co-workers implanted Grafton subcutaneously into athymic rats and reported acute tubular necrosis[62] .It was speculated the glycerol component in Grafton led to the nephrotxoicity.

However, the dose was 10 times the human clinical dose. Nevertheless, glycerol-containing products should be used with caution in pediatric patients and in those at risk of renal disease[63].

Acarturk and Hollinger determined in a pre-clinical model in an orthopic critical-sized defect site that treatment with either Grafton or DBX promoted significantly more bone regeneration than other DBM-carrier products [64]They concluded that differences in osteogenic activity among commercial DBM products may be related to differences in the carrier, the amount of DBM in the carrier and ability of the carrier to localize the DBM particulate to the bone defect site for a sufficient period to promote bone regeneration.

An important variable inherent to these products is that the DBM content among different commercial composites (i.e., DBM plus carrier) is non-standardized and inconsistent. Therefore, different DBM doses will be delivered to tissue sites by different products. This could explain the variability in responses reported by Acarturk and Hollinger[64]. Moreover, individual‘DBM lots’ processed by the same tissue bank may possess different osteoinductive capacities, and these capacities may vary among different donors. While biological activity is measured by osteoinduction and is reported as theosteoinductive index,OI,no DBM“potency”or“compositional”regulatory standards are enacted across DBM products to understand or control these differences. It is also noteworthy to emphasize the significance of shelf life and carrier stability for DBM composite products.

If precise control of conditions is not maintained, then endogenous osteogenic proteins in the DBM, most importantly, BMPs, might be susceptible to chemical and physical degradation[65]. Variations in the shelf life of a specific carrier may or may not affect the overall activity of the product. This is an important clinical and manufacturing issue that has not been sufficiently described or controlled. Further study about stability of DBM with carrier need to be done about the CenoBiologic putty formulation.

In this study we tried to control extraneous variables that could have an effect on the osteoinductive properties of DBM apart from particle size and shape. For example all DBM was processed from same donor, similar gamma sterilisation dose, similar freeze drying and water content.

Also, calcium content of all groups was in the recommended range for this production of osteobiologic material (less than 8 %) with exception of fibre DBM which had 5 times more residual calcium in comparison to powder groups, and it could have affected the results. Honsaek and co-workers suggested that BMPs in DBM maybe more readily extracted as calcium content in the DBM decreases [66]. However, BMP content of the DBM is also suggested to likely be less important than its extractability and release into the host implant site.

6.1 Technical considerations

There is a considerable increase in the volume of DBM fibre after rehydration for the osteoinductivity tests. In the experiments, a similar weight of fibre was used, in comparison with the powdered DBM, though these have shown different swelling ratios. This can lead to differences in the space available for cell growth, and so might also affect the final results. In future experiments, it might be more appropriate to use volume to surface area index of DBM in order to normalise the results, instead of just weight.

Chapter 7
Conclusions

The importance of carriers is to deliver and retain in place osteoinductive DBM to a site requiring bone regeneration while not inhibiting bone formation. The ultimate goal is to design a system that provides ostegenic, osteoinductive, and osteoconductive properties resulting in bone fusion rates comparable to auto graft without the associated risks and limitations of autologous harvest. Also, bio compatibility of carrier is important for clinical licensing. According to results obtained here, the biocompatibility of CMC base and alginate base carrier is at an acceptable level and also that all groups are capable of supporting the in vitro growth and maturation of osteoblasts-like cells. Although the osteoblastic cell differentiation is demonstrated by qPCR with up-regulation of osteopontin and osteocalcin, there is a significant difference when different DBM size and shape were used, favouring larger particle sizes. In order to obtain more definitive answers, tests should be repeated in a longer time frame (10-20 days), also the influence of the carrier polymer on the qPCR results should be quantified in more detail in the future.

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