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RESEARCH ARTICLE

COMPARATIVE STUDY FOR THE ASSESSMENT OF TISSUE ENGINEERED OSTEOCHONDRAL GRAFT VERSUS AUTOGENOUS GRAFT IN CRITICAL SIZE DEFECT: AN EXPERIMENTAL STUDY ON THE TEMPOROMANDIBULAR JOINT OF DOGS.

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Tissue engineering, osteochondral graft, Biphasic scaffold, autogenous graft, TMJ reconstruction.

Abstract

This study was performed to demonstrate the ability of mesenchymal stem cells using biphasic scaffolds, to produce osteochondral graft, assess its function, and compare it with autogenous grafting of condylar defects. Eighteen male adult mongrel dogs ranging from 12-15 kg in weight were selected for this study and were randomly divided into twoequal groups: Group A: Tissue engineering procedure was performed in 2 stages, Stage 1: Aspiration of bone marrow samples from the canine subjects was performed, Stage 2: In vitro isolation, expansion, differentiation and characterization of Mesenchyme Stem Cells was performed, followed by implantation of the tissue engineered graft. Group B: autogenous bone grafting was performed. After 16 weeks from implantation of the grafts in the created condylar defects, the dogs were sacrificed, and bone samples were prepared for histological assessment and follow up. Microscopic and histochemical analysis revealedwell-formed tissue- engineered osteochondral graft in the test group(Group A), versus very limited cartilage regeneration in the control group (Group B).Moreover, Radiographic evaluation was performed immediate post-operatively, 6 weeks, 12 weeks, and 16 weeks postoperatively. Based on our finding, we concluded that the replacement of TMJ bone and cartilage with tissue-engineered osteochondral graft was superior to autogenous bone graft at the same step.

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Introduction:-

Temporomandibular joint (TMJ) is a complex system that is regularly subjected to trauma, metabolic and inflammatory processes (Scheller, et.al. 2009). It is one of the most difficult tissues to treat due to the limited blood

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supply and hence limited capacity for self- repair. The biological basis of TMJ problems is the deterioration of articular cartilage, which covers the bone at the joint surface and performs many complex functions (Sherwooda, et.al. 2002).The articular cartilage of TMJ has a surface layer of fibro-cartilaginous and deep layer of hyaline-like hypertrophic zone with a thin intermediate proliferative zone (Atkinson and Haut 2001). Thus it has unique properties, such as viscoelastic deformation, that allow it to absorb shock, distribute loads, and facilitate stable motion (Mao, et.al. 1992). Therefore, Osteochondral defect repair is a challenging problem in the field of oral and maxillofacial surgery (Hunziker 1999, Grande, et.al. 1999).

Tissue engineering provides a new era for therapeutic medicine; it is progressing very rapidly and extends to involve all tissues in our body. Engineering the osteochondral interface with its complex structure and its cartilaginous component with its zones of different structures and organization is very challenging (Wojciech, et.al. 2014).
Tissue engineering approaches have the potential to overcome the lack of donor tissue and create TMJ replacement that is both biologically and mechanically functional (Schek, et.al. 2005, Hu, et.al. 2003, Tanaka, et.al. 2006). The standard approach of tissue engineering is to seed cells on a three-dimensional (3D) biomaterial scaffold. The scaffold is designed to create a 3D environment that promotes tissue development of cells that are placed on or within the scaffold. Gene vectors, soluble factors, and chemical signals may be incorporated into the scaffold to help promote tissue development during in vitro incubation or in vivo implantation (Elisseeff, et.al. 2005).

Since bone and cartilage require different competing conditions for their regeneration, growing a biphasic osteochondral construct in vitrocontaining a cartilage region and a bone-appropriate region to guide the growth of these two different tissues into a single implant is therefore very difficult (Scheafer, et.al. 2000, Jill, et.al. 2002). The Biphasic scaffolds have been studied for osteochondral regeneration, and it was reported that they could support cell growth and differentiation into bone and cartilage (Kreklau, et.al. 1999, Schaefer, et.al. 2002, Gao, et.al. 2001). Furthermore, numerous studies reported the successful use of tissue engineered osteochondral implants using biphasic scaffolds in the repair of TMJ defects (Weng, et.al. 2001, Chen, et.al. 2001, Schek, et.al. 2005).

However, to date few studies have attempted to produce a tissue engineeredosteochondral graft, and assess its function compared to autogenous grafting. Therefore, the objective of this study was to demonstrate the ability of mesenchymal stem cells seeded in biphasic scaffolds, to produce osteochondral graft, assess its function, and compare it with autogenous grafting of condylar defects.

Materials &Methods:-

Ethics statement. This study was approved by CU-IACUC, (approval number CU II S 17 16). Eighteen male adult mongrel dogs ranging from 12-15 kg in weight were selected for this study. All animals to be included were examined to be sure that they were free from any disease. The animals were hosted and quarantined in separate cages for one week prior to the surgery to become acclimatized to housing and diet. Then the animals were randomly divided into twoequal groups: Group A: for tissue engineering procedure and group B: for autogenous bone grafting.

Group A (Tissue engineering):-

Preparation of the engineered tissue involved 2 stages. Stage 1: in which aspiration of bone marrow samples from the canine subjects was performed, Stage 2: in which in vitro isolation, expansion, differentiation and characterization of MSCs from canine bone marrow samples was performed.

Stage one: Bone marrow collection:-

Under general anesthesia and strict aseptic conditions the head of each canine's tibia was palpated carefully for the detection of the tibia tuberosity followed by introduction of special biopsy needle (aspirating needle) for about 1.5 cm depth. Bone marrow samples were aspirated in 20-ml syringe containing 2 ml heparin (preservative free, 400 units per ml).

Stage two: Tissue collection and engineering:-

According to the departmental standard operating procedures (SOP), strict aseptic conditions were followed throughout this study. The bone-marrow samples were flushed by means of an 18-gauge needle and 10-mL syringe loaded with Dulbecco's Modified Eagle's Medium-Low Glucose (DMEMLG; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Biocell, Rancho Dominguez, CA, USA) and 1% antibiotic-antimitotic (Gibco, Carlsbad, CA, USA). Marrow samples were mechanically disrupted by passage through 16-, 18-,

and 20-gauge needles. Marrow cells were centrifuged, suspended in serum-supplemented medium, counted, plated at 5 x 107 cells/100-mm culture dish, and incubated for two weeks in 95% air/5% CO2 at 37°C, with fresh medium change every 3-4 days. Upon reaching 80-90% confluence, primary MSCs were trypsinized, counted, and passaged at a density 5-7 x 105 cells/100- mm culture plate.

Treatment of MSCs with Chondrogenic and Osteogenic Differentiation Factors:
The same population of first-passage MSCs was treated separately with chondrogenic or osteogenic specially formulated medium. The chondrogenic medium was supplemented with DMEM-LG, 10%FBS, BMP-7, insulin like growth factor-1, antibiotic, dexamethasone, ascorbic acid , whereas the osteogenic medium contained 100 nM dexamethasone, 10 mM ß -glycerophosphate, and 0.05 mM ascorbic acid-2-phosphate. Cultures were incubated for 1 week in 95% air/5% CO2 at 37°C, with fresh medium change every 3-4 days.

Histochemical staining of subcultures
Histochemical staining was performed for subculture in osteogenic mediumat days 7, 14, 21 and 30 to detect expression of alkaline phosphatase enzyme. Moreover, Von kossa staining was performed at 7, 14, 21 and 30 days of subculture, to detect extra cellular matrix mineralization (ECM) in osteogenic medium. Furthermore, Safranin-O staining was performed at day 30 of subculture to detect proteoglycans and glycosaminoglycan found in chondrocytes (in chondrogenic medium). AllStained cultures were inspected by the naked eye and under the inverted phase contrast microscope.

Cells seeding onto the Biphasic-scaffold:-
The Biphasic-scaffold (composed of porous silica calcium phosphate (SCPC) color coded discs) was sterilized, and seeded bymost of the trypsinized cells after four weeks of osteogenic and chondrogenic differentiation.

Group B (autogenous bone grafting):-
After subjection of each canine to general anesthesia,an extra-oral incision and flap reflection was performed. Autogenous graft was harvested from the buccal cortical plate of the body of the mandible using trephine bur (0.5 cm diameter) at a low speed under copious irrigation with saline.

Implantation of the engineered osteochondral tissue/ autogenous graft:-
After subjection of each canine to general anesthesia, Under copious irrigation with saline and using trephine bur (0.5 cm diameter) at a low speed, a cylindrical bony defect was done at the condylar head having the same length and width of the engineered osteochondral tissue at the scaffold in group A, and the harvested autogenous graft in group B. The biphasic scaffolds seeded with differentiated cells and the autogenous bone grafts were implanted inside the surgical defects. The wound was closed in layers (Fig 1A and 1B).

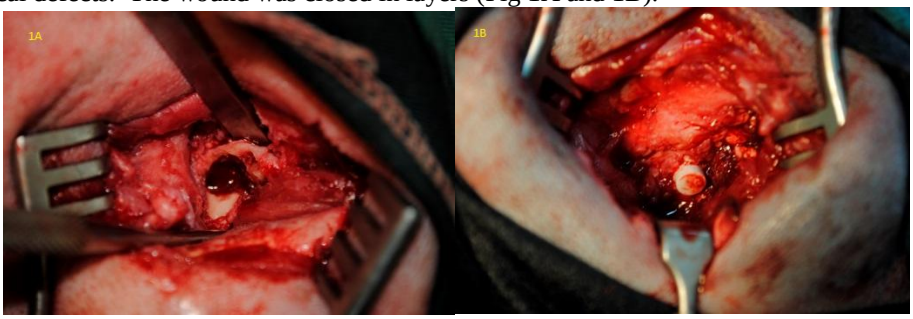

Fig 1: (A) Clinical picture of the surgically created defect at the canine's condylar head. (B) Biphasic scaffold seeded with BM-MSCs after its placement at the created condylar defect. (group A)

The dogs were euthanized (sacrificed) after 16 weeks from implantation using hyper dosage of Thiopental Sodium 10% injected directly through the cannulated cephalic vein. The bilateral condylar bones with overlying capsules were freed in each dog by surgical dissection, examined grossly and bisected vertically by electrical saw. The bones were de-mineralized for one week in a mixture of formic acid and hydrochloric acid solution. Samples were then fixed for 24 hours in 10% neutral buffered formalin,then processed and paraffin-embedded in a standard manner.

Assessment of the implanted osteochondral tissue/autogenous graft:-
Radiographic follow up was performed using lateral oblique contact film immediate post-operatively, 6 weeks, 12 weeks, and 16 weeks postoperatively.

While Histological assessment and follow up were performed using Hematoxylin and Eosin (H&E) stain, Tartrate resistant acid-phosphatase (TRAP) special stain, Goldner's Masson Trichome special stain, and Toluidine blue staining.

Results:-

Histologic Evaluation:-
Bone marrow-derived MSCs in monolayer culture incubated for 4 weeks in osteogenic and chondrogenic medium reacted positively to alkaline phosphatase and Von Kossa silver stain indicating their osteogenic phenotype and mineral deposition respectively (Fig. 2A and 2B). Also they exhibited positive reaction to safranin O indicating their chondrogenic phenotype (Fig.2C).

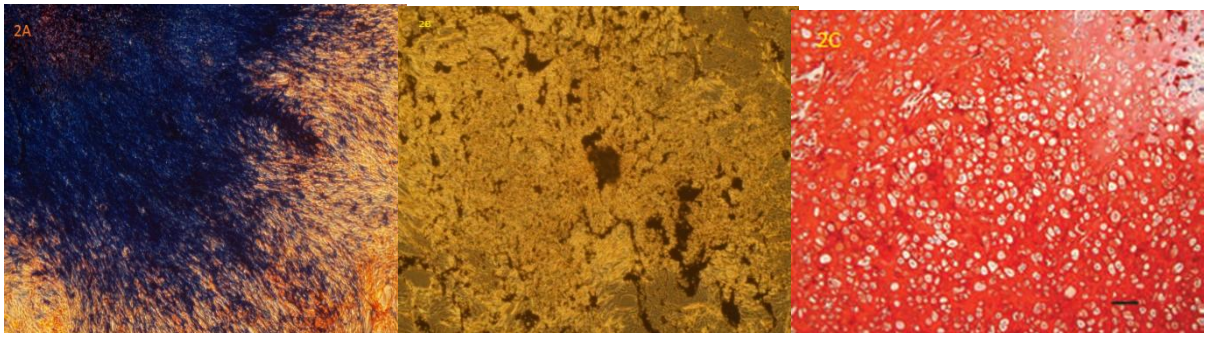

Fig. (2): (A) BM-MSCs subjected to osteogenic differentiation showing +ve reaction to alkaline phosphatase histochemical staining (blue spots). (B) BM-MSCs subjected to osteogenic differentiation showing +ve reaction to Von Kossahistochemical staining (black spots). (C) BM-MSCs subjected to chondrogenic differentiation, showing positive reaction of their extra cellular matrix to Safranin O histochemical stain (red-orange stain)

Regarding the histological evaluation of theosteochondral defect repair, in group (A) the H&E stained sections from de-mineralized engineered condylar bones revealed repair of the defect by formation of an upper layer of well-formed cartilage in 100% of cases overlying mature osteogenic layer in 100% of cases (Fig.3A).While in group (B) H&E stained sections from de-mineralized grafted condylar bones revealed repair of the defect by formation of an upper layer of well-formed cartilage in 10% of cases overlying mature osteogenic layer in 100% of cases (Fig.3B).

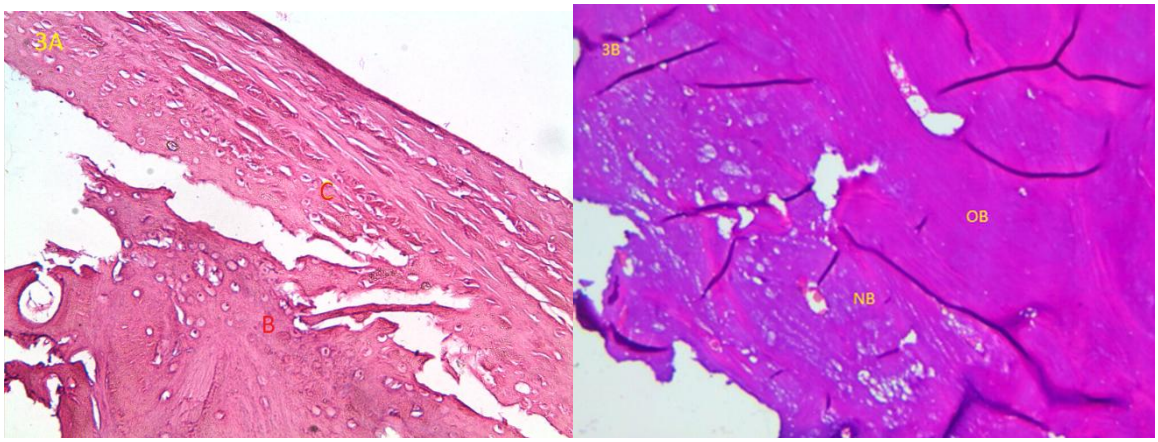

Fig. (3): (A) H&E stained section at the biphasic/cell composite site showing the formation of well-formed cartilage layer overlying mature osteogenic layer. (B) H&E stained section at the autogenous graft site showing borders of the bony defect old bone (OB), trabeculea of new bone (NB)

TRAP special stain was used for the detection of osteoclasts and osteoblasts in the osteogenic layer of the engineered and grafted condylar bones. In both groups (A&B) Osteoblasts and osteoclasts were strongly positive to TRAP special stain at the osteogenic layer. This was illustrated by the presence of small orange-brown spots denoting the osteoblasts and large orange-brown spots denoting the osteoclasts in their Hawships lacunae (Fig. 4A and 4B).

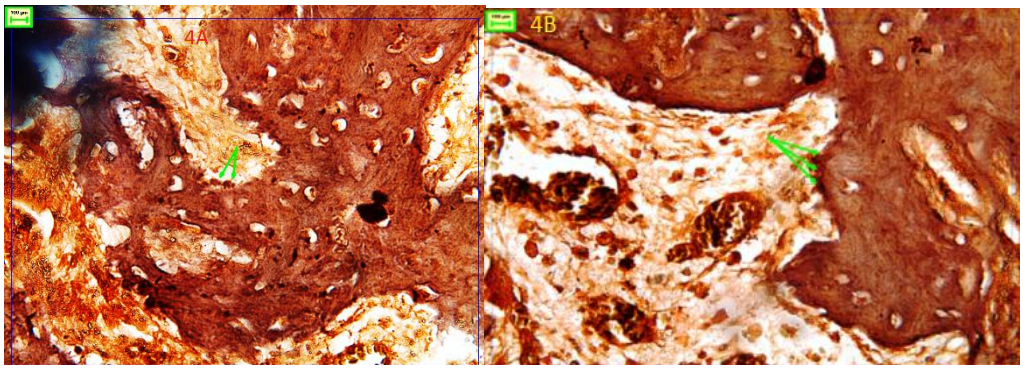

Fig. (4) :(A) strong TRAP positive expression by osteoclasts present in their Hawships lacunae(arrow)in group A.(B) strong TRAP positive expression by osteoclasts present in their Hawships lacunae(arrow) in group B.

The use of Goldner's Masson Trichrome stain in both groups (A&B) demonstrated clear differentiation between mineralized mature bone that stained blue or green and un-mineralized new immature bone that stained red. It also provided excellent highlighting of bone-forming cells or osteoblasts (red-orange) lining trabeculae of immature bone (Fig.5A and 5B).

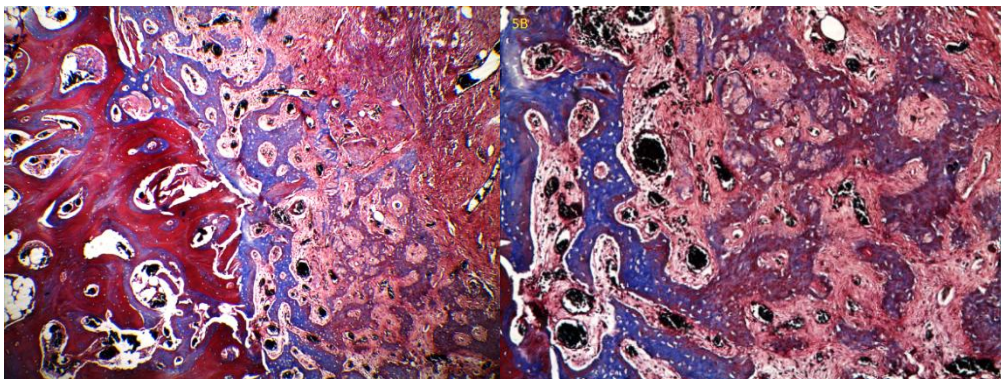

Fig. (5) :(A) Goldner's Masson Trichrome staining section exhibiting the presence of newly formed woven bone taking the light red stain, underlined with old mature bone taking a blue stain in group A. (B) Goldner's Masson Trichrome staining section exhibiting the presence of newly formed woven bone taking the light red stain, underlined with old mature bone taking a blue stain in group B.

In group (A) the Presence of chondrocytes, which reacted positively with the Toluidine blue stain by taking the blue color, was detected in all of the experiments, as shown in figure (Fig.6A). While in group (B) Presence of chondrocytes, was detected only in 10% of the experiments (Fig. 6B).

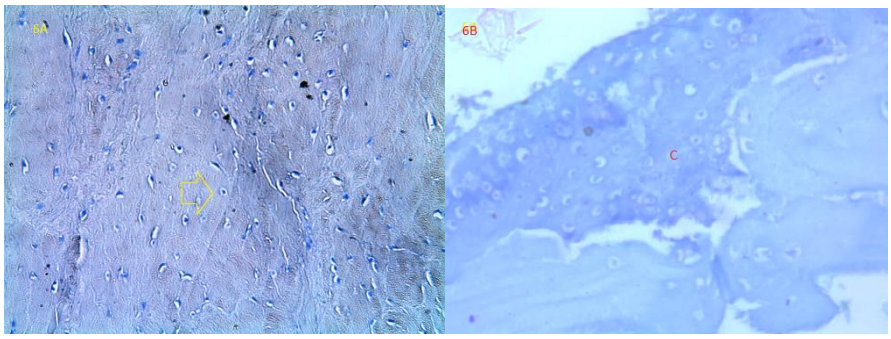

Fig. (6): (A) Toluidine blue special stain sections in the chondrogenic layer of the engineered osteochondral graft, showing the presence of chondrocytes (arrow) in the engineered cartilaginous layer.(B)Toluidine blue stained sections of the autogenous graft condyle showing areas of cartilage (C)

Radiographic evaluation:-

The radiographic follow up of (group A) showed increase in the radio-opacity at the implanted defect site packed with the seeded biphasic scaffold, accompanied with disappearance of the radiolucent rim separating between the seeded biphasic scaffold and the normal surrounding bone denoting de novo bone and cartilage formation at the defect site (Fig.7). As for (group B) radiographs showed complete integration between the autogenous graft and the normal condylar bone (Fig. 8)

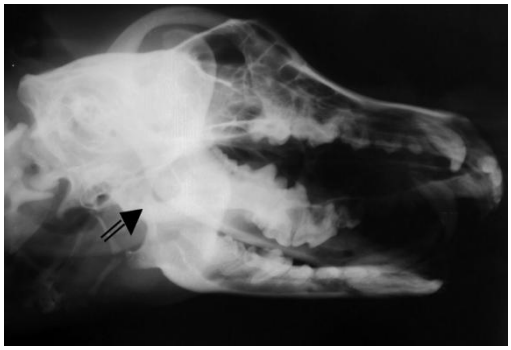

Fig. (7): A lateral oblique radiographic picture showing total remodeling of the condyle (arrow), with difficulty to distinguish between the newly regenerated bone and the rest of the condyle.

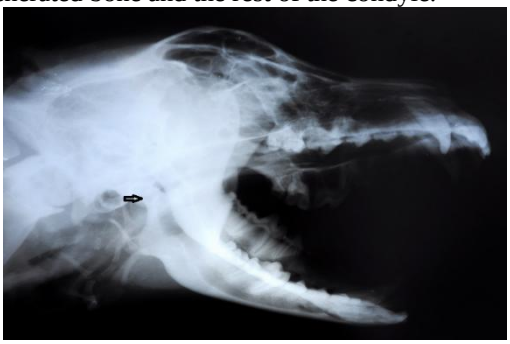

Fig. (8): A lateral oblique radiographic picture showing total integration between the autogenous graft and the normal condylar bone.

Discussion:-

Temporomandibular joint (TMJ) is one of the most difficult tissues to treat due to the limited blood supply and hence limited capacity for self-repair. Since bone and cartilage require different competing conditions for their regeneration, growing a biphasic osteo-chondral construct in vitro is therefore very challenging.

The canine model was selected in this study according to the similarities between human and canine TMJ morphology and composition as proved in some studies(Aerssens, et.al. 1998, Reichert, et.al. 2009). Adult bone marrow was selected as the source of MSCs as these cells possess a multilineage differentiation capability into bone,

cartilage, adipose, tendon and muscle tissue (Ferrari, et.al. 1998, Jones, et.al. 2002).The differentiation of BM-MSCs into chondrogenic cells and osteogenic cells and active syntheses of chondral and osseous matrix in vitro is consistent with someprevious work(Goldberg and Caplan 1994,Schaefer, et.al. 2000, Gao, et.al.2001).

The data of the present study demonstrated that BM-MSCs derived chondrogenic and osteogenic cells continue their phenotypic differentiations in vivo. BM-MSC-derived chondrogenic and osteogenic cells were encapsulated into the SCPC biphasic scaffold with a dimension of 5mm diameter x5mm height. This result is consistent with the result of (Alhadlaq and Mao 2003)who used aPoly-Ethylene Glycol hydrogel scaffold and encapsulated the BM-MSCs-derived chondrogenic and osteogenic cells into the shape of a human mandibular condyle with a dimension of 11 x 4 x 7 mm. Moreover, (Weng, et.al. 2001) have successfully used mature bovine chondrocytes and osteoblasts seeded on a polymeric scaffold molded into the shape of human mandibular condyle and yielded discrete layers of cartilage and bone. Furthermore, this result is consistent with other studies (Elisseeff, et.al. 2000, Poshusta and Anseth 2001, Burdick and Anseth 2002, Halestenberg, et.al. 2002, Martens and Bryant 2003).

In the present study, the improvement in bone and cartilage repair found by the use of rh-BMP7 with the biphasic SCPC scaffold coincide with those of (Herford, et.al. 2002, Krebsbach, et.al. 2000) who used recombinant OP-1/BMP-7 delivered on bone-derived type I collagen scaffolds press-fitted into large focal defects in rabbit models. When implanted in TMJ, the silica calcium phosphate (SCPC) biphasic scaffold with its high porosity enhanced cell colonization and osteochondral formation within the graft material. Thus it may have a strong stimulatory effect on bone and cartilage cell function, and resorbs in harmony with the rate of new bone and cartilage formation. It also showed no signs of inflammation or immunologic rejection into the surrounding tissues.

Regarding the control group, the histologic characteristics of the newly formed bone in defects received autogenous bone grafts was similar to the normal bone formation, and mineralization. This finding agrees with those of (Zhu,et.al. 2006) who reported in a study on goats that the histological characteristics of the neocondyle from autogenous coronoid process were similar to the normal one with fibrous connective tissue covering the head of the neocondyle.

In the current study a mature osteogenic layer in 100% of cases was found due to its cortico-cancellous nature, which causes the new bone to be stiff, and can resist heavier forces. This finding agrees with the finding of several studies (Zhu, et.al. 2006, Liu, et.al. 2010, Zhu, et.al. 2008). Moreover, our study found the formation of an upper layer of well-formed cartilage in 10% of the control group cases overlying mature osteogenic layer which could be interpreted as a kind of tissue response to local mechanical stimulation and/or the periosteum that have exerted its chondrogenic and osteogenic potential. This finding agrees with several studies that reported the occurrence of the fibrocartilage proliferation and the sufficiently organized cartilage at the resected condylar surface after condylectomy (Murnane and Doku 1971, Sprinz 1963, Glanelly, et.al. 1965).

Comparing the 2 groups, this study showed very limited cartilage regeneration in the control group, and well-formed tissue- engineered osteochondral graft in the test group. Thus tissue-engineered osteochondral graft from BM-MSC represents another step toward therapeutic applications of TMJ reconstruction.

Conclusion:-

Based on our finding, we can conclude that the tissue-engineered osteochondral graft was superior in the replacement of the bony and the cartilaginous part of the TMJ at the same step compared to the autogenous bone graft.

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