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REDUCED GRAPHENE OXIDE NANOPARTICLES AND PLATELET RICH PLASMA IN REGENERATING BONE DEFECTS OF IRRITATED BONE IN RATS: RANDOMIZED CONTROLLED STUDY

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ABSTRACT

The ability of reduced graphene oxide (rGo) in regenerating bone tissue has recently gained significant attention in biomedicine. **Aim**: we investigated the effects of rGo nanoparticles and platelet rich plasma (PRP) in regenerating induced defects in irradiated 2 types of rat bones. **Materials and Methods**: We had 4 experimental rat groups, each was 6 in number, all of them were irradiated then a bone defect was surgically induced at the condyle representing endochondral bone and ramus representing intramembranous bone in each rat. The bone defect was then grafted with rGo alone in (**GpI**), PRP alone in **GpII**, combination of PRP and rGO in **GPIII** or left ungrafted as a control in **GpIV**. 8 weeks later samples were explanted for histological studies. **Results:** All groups showed bone formation with highest organized bone formation in **GPIII**. **Conclusion:** regeneration of irradiated bony defects using the combination of rGO nanoparticles and PRP can provide the highest active healthy bone formation.

INTRODUCTION

Malignancy of the oral and maxillofacial area whether in early stage or in the form of locally advanced disease is treated by surgical resection and reconstruction with adjuvant radiotherapy as a standard approach[(1)]. Unfortunately, radiotherapy has a detrimental effects on tissues through a radiation induced fibro-atrophic mechanism [(2)]. The cellular and molecular responses to tissue irradiation are immediate, dose and technique dependent and can induce early and late consequences. These consequences reduce the soft and hard tissue capacity for regeneration and repair, diminish the patient's quality of life, damage their physical appearance, and ultimately necessitate invasive corrective surgery to repair and rebuild the affected area [(3,4)].

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Current options for substituting bone defects include autograft, xenograft and alloplastic materials and they can be used alone or in combinations [5] Also, lots of growth factors such as bone morphogenic proteins (BMPs) and platelet derived growth factors (PDGF) alone or in combination with different kinds of bone substitutes were examined. Platelet rich plasma (PRP) contains many growth factors including platelet derived growth factors (PDGF), vascular endothelial growth factors (VEGF), and transforming growth factors (TGF-b)[6,7,8,9,10]. PRP works by providing a concentrated growth factors necessary for initiation and completion of bone augmentation [11,12].

Graphene family is one of the newly investigated alloplastic nanomaterials and it proved to have osteoinductive and osteoconductive properties in non-irradiated bone defects [13]. In this research we designated a randomized controlled study aimed to investigate the effect of reduced Graphene nanoparticles (rGo) alone, PRP alone or in combination to regenerate a cortical defect in irradiated rat condyle and ramus.

MATERIALS AND METHODS

Rats will be irradiated on their mandibles. Three weeks later, surgery will be executed to create condylar head and Mandibular body bone defect and will be kept empty (control) Gp IV or filled with either reduced graphene oxide alone group GpI, PRP alone Gp II or PRP with reduced nanoparticles of graphene oxide Gp III. 8 weeks after implantation, samples will be explanted for histological analysis (Fig.1).

Animals

Eight-week-old inbred Albino Wistar rats (n = 24 females), provided by a certified breeding centre (Laboratory Animals Farm, Veterinary Serum and Vaccine Research Institute , Helwan province),weighting about 225 g, were used for this study. Animal care was provided by the Department of Experimental Therapeutic (Faculty of Veterinary, South Valley University). Rats were kept for acclimatization two weeks prior to experiment, during which animals were handled regularly to allow acclimatization. Animals were given a standard diet with balanced contents during the whole period of the experiment.

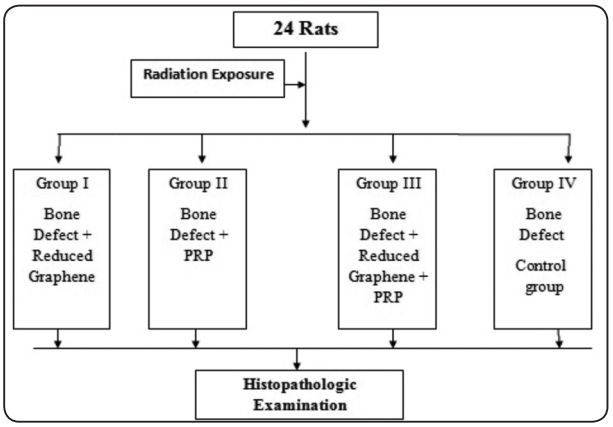

Fig. (1): Scheme demonstrates the study design.

The Ethics Committee of the South Valley University reviewed and approved the study design.

All rats were exposed to radiation using the fractionated regimen. 3 weeks later, bony defects were done in all rats. After that rats were randomly divided into 4 treatment groups (n=6 pergroup): Gp I: rGO-nanoparticles alone; Gp II: PRP alone; Gp III: rGO-nanoparticles + PRP as grafting materials and Gp IV: control. Animals were followed up daily till 8 wks postoperative. Then, the animals were sacrificed and mandibular ramal bones and condyles were harvested.

Radiation Exposure Technique

Rats were irradiated on linear accelerator using photon X-ray 6Mev energy.

Technique: Teletherapy, SSD = 100 cm. During radiotherapy of the animals, fixation was needed

so general anesthesia was applied. Irradiation targeted the left side of the face at the mandibular condyle and ramus area and the rest of the body was protected by a lead bridge. We used 2D technique, square field were arranged treating multiple mice every session. Irradiation done using the hypo-fractionated external radiotherapy protocol total dose of 75Gy(5*15Gy) every other week for 5 weeks with a total period of 9 weeks [14,15].

Reduced Graphene Oxide fabrication

Graphene oxide (GO) preparation Graphene oxide was synthesized from graphite powder using a modified method as described by Marcano et al.,[16] Briefly, 1 g of graphite powder was slowly added under stirring conditions to H2SO4/H3PO4 mixture (9:1 v/v) to form a viscous solution. 6 g of KMnO4 was added to this mixture and stirred overnight at 50 °C in an incubator. The mixture was then poured on to 200 ml of ice with gentle stirring to cool down the reaction temperature. To stop the reaction, 30% H2O2 was added in drops and the solution colour turned into bright yellow indicating the formation of graphene oxide. The mixture was centrifuged to collect GO, washed multiple times with water, HCL and absolute ethanol to obtain purified GO dispersion. The final GO product was collected by centrifugation and lyophilized to obtain GO powder.

Defect Creation and Grafting

Surgical procedures started 6 weeks after last radiation session. All interventional procedures were performed under generala nesthesia. Surgical sites at the condyles and rami were shaved and prepped with povidone iodine as antiseptic agent. To create a unicortical bone defect, an incision approximately 2.5 cm in length was made over the condyles and ramus area. After surgical exposure a bone defect in the condyle of a diameter 3mm by a round bur no. 4 of head size diameter 1.4mm for a depth of 2mm while in the ramus we used the same bur and depth but for a length of 5mm.(Fig.2) Defect drilling was done under copious irrigation of sterile normal saline to avoid heat generation and bone necrosis. Also defect wash was done using the same solution before the application of the graft material. Then all tissue layers were repaired using polygalactin 910 suture material (VICRYL – ETHICON). rGO was prepared in 300 mgm weight increments and mixed with sterile normal saline before application.

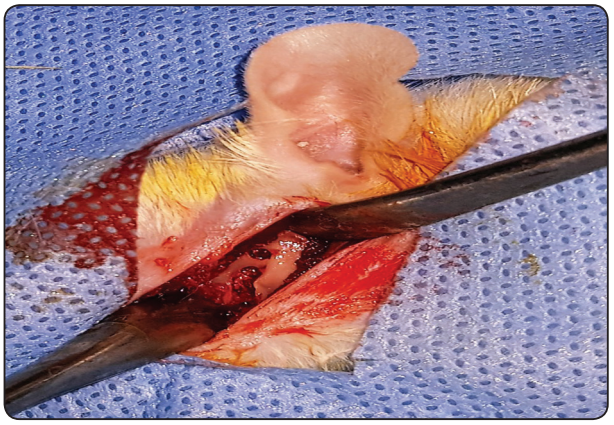

Fig. (2) Surgical exposure and defect drilling at the condyle and ramus.

Preparation of Platelets Rich Plasma (PRP)

Five samples, 8 ml blood for each, were collected form 5 rats using tubes containing K2-EDTA as anticoagulant. Afterwards, samples were centrifuged at 200g for 15 minutes leading to separation of concentrated red blood cells in the bottom and PRP in supernatant. At least, one ml of supernatant fluid was obtained and used freshly just before treatment. Platelets were allowed to be activated through direct contact with collagen of exposed matrix protein[17].

Histological analysis

For histological analysis, mandibular ramal bones of all groups will be harvested and fixed immediately in 10% formalin for 48- 72 hours, washed overnight with tap water, decalcification with 10% EDTA for 4-5weeks was done and then embedded in paraffin blocks. The paraffin embedded

specimens were serially cut in bucco-lingual plane into sections of 5 microns thickness. Sections were then mounted on slides and staining procedure was performed.

The Staining Technique Maisson's Trichrome Stain

Mordant in Bouin's solution were added to the cut sections, which were microwaved for 1 minute and allowed to stand for 15 minutes, washed in running tap water, then Weigert's working hematoxylin was added for 10 minutes, the slides were rinsed in distilled water and Biebrich scarlet was introduced for 5 minutes, slides were rinsed again in distilled water, after that Phosphotungstic/phosphomolybdic acid was applied for 10 minutes, slides were transferred directly to Aniline Blue and rinsed with distilled water, finally 1% Acetic acid was added for 1 minute, the solution then was discarded, rinsed in distilled water finally rehydration and covering the slide was achieved. By this procedure collagen fiber will retain the blue stain. The sections were examined and evaluated by experienced two pathologists according to the criteria presented in (table 1). [18]

TABLE (1): Scoring system for the bone formation.

Grade	Characterization
scoring system for Bone formation	
0	Absent
1	Mild bone formation
2	Moderate bone formation
3	Massive bone formation

Statistical test:

The Mann-Whitney test is used for comparing the efficacy of two treatments in clinical trials. It is often presented as an alternative to a *t* test when the data are not normally distributed. Whereas a *t* test is a test of population means, the Mann-Whitney test is commonly regarded as a test of population medians.

RESULTS

During radiation No acute complications were noticed by any degree.

Histological Findings: On examination of sections stained by Ttrichrome stain for GpI , showing intramembranous ossification; osteoid tissue and new bone formation Fig.(3) as well Gp III showed mild bone formation more fibrosis and tendency for collagen fibers to organize and for new bone was noted Fig (4,6). Gp III (using the combination for PRP and Graphene) showed the highest organization for bone formation either endochondral or intra-membranous bone formation Fig. (5,7,8).

Statistical Finding:

The Mann-Whitney test is sometimes used for comparing the efficacy of two treatments in clinical trials. Using PRP and graphene oxide having positive effect on bone formation table (2-5), as well the most effective one was the combination between PRP and graphene is statistically significant having the greatest amount of bone formation table (6,7)

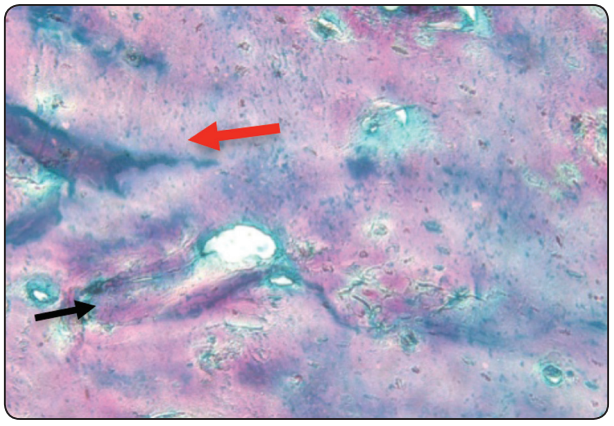

Fig (3) Photomicrograph for defect filled with graphene group I showing intramembranous ossification; osteoid tissue (black arrow) and new bone formation (red arrow) (Trichrome staining X400))

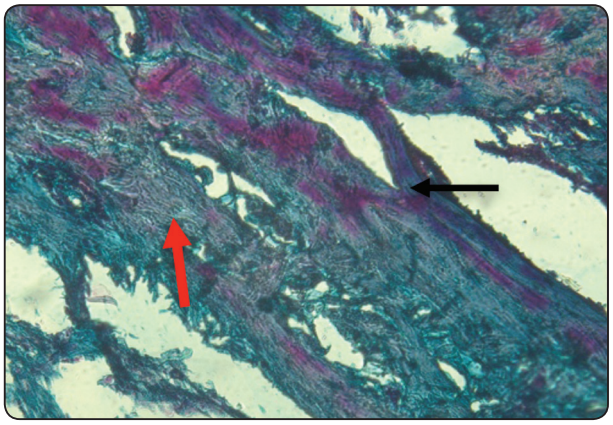

Fig (4) Photomicrograph for defect filled with PRP group II- showing intramembranous ossification; osteoid tissue (black arrow) and new bone formation (red arrow) (Trichrome staining X400)

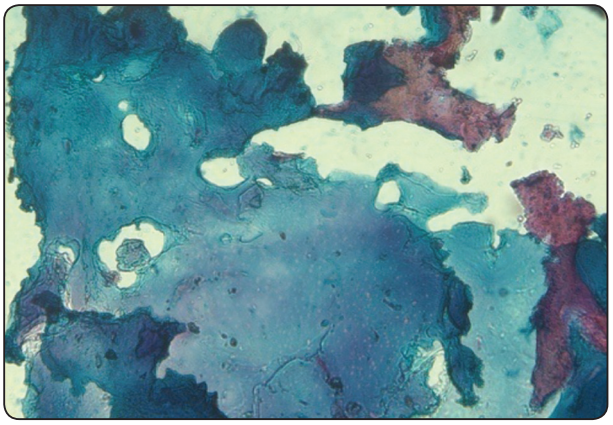

Fig. (5) Photomicrograph for defect filled with Graphene and PRPgroup III showing intramembranous ossification; new bone formation (Trichrome staining X400)

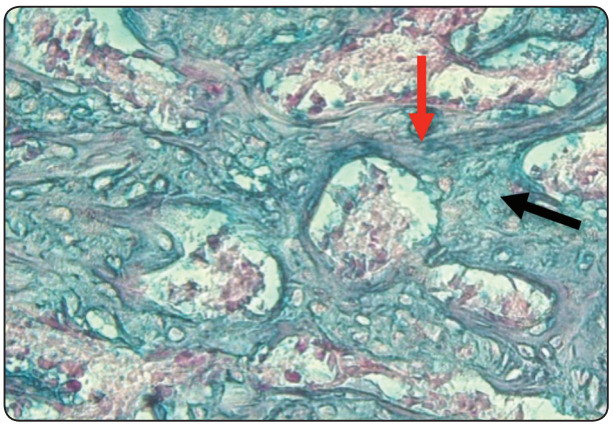

Fig. (6) Photomicrograph for defect filled with Graphene group I showing zone of calcified cartilage trabeculae (black arrow) and bone marrow (red arrow) (Trichrome 400x)

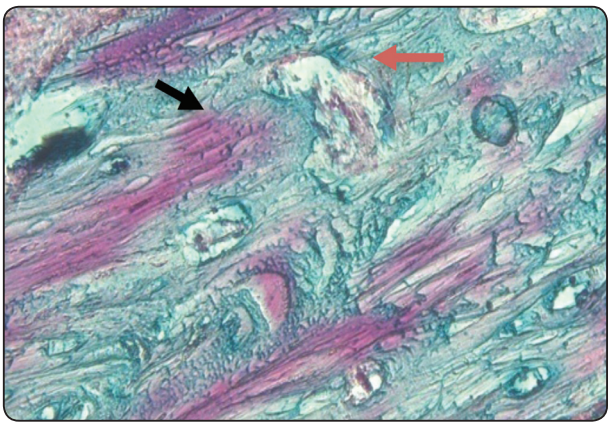

Fig. (7) Photomicrograph for defect filled with graphene and PRP group III showing growth plate of bone (black arrow) within basophilic calcifying cartilage (red arrow) (Trichrome x400)

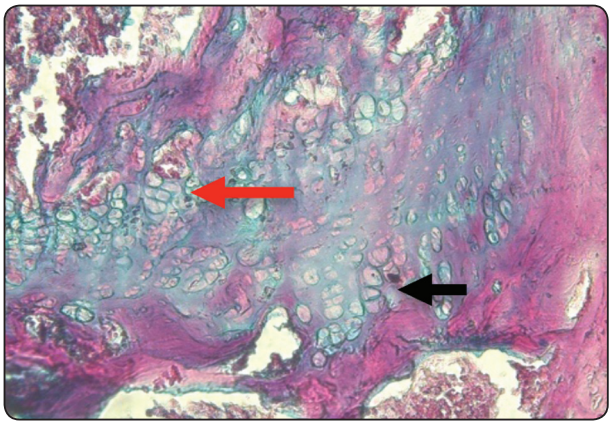

Fig. (8) Photomicrograph for defect filled with graphene and PRP group III showing steps of endochondral ossification: proliferative cartilage zone with cells lined up to form little disc (black arrow) and hypertrophy cartilage zone with large cell and big lacunae (red arrow) (Trichrome staining X400)

TABLE (2): Bone formation using PRP and no material (Ranks)

Bone Formation		N	Mean Rank	Sum of Ranks
Scores	Control	10	7.50	75.00
	PRP	10	13.50	135.00
	Total	20		

Table (3): Bone formation using PRP and no-material (Test Statistics)

Bone formation	Scores
Mann-Whitney U	20.000
Wilcoxon W	75.000
Asymp. Sig. (2-tailed)	.018

a. Grouping Variable: BoneFormation

TABLE (4): Bone Formation using graphene oxide and no material (Ranks)

BoneFormation		N	Mean Rank	Sum of Ranks
Scores	Control	10	7.50	75.00
	Graphene Oxide	10	13.50	135.00
	Total	20		

TABLE (5): Bone Formation using graphene oxide and no material (Test Statistics)

Bone formation	Scores
Mann-Whitney U	50.000
Wilcoxon W	105.000
Z	.000
Asymp. Sig. (2-tailed)	1.000

Grouping Variable: BoneFormation

TABLE (6): Bone formation using combination of PRP+Graphene and no material (Ranks)

BoneFormation		N	Mean Rank	Sum of Ranks
Scores	Control	10	6.00	60.00
	PRP+Graphene	10	15.00	150.00
	Total	20		

TABLE (7) Bone formation using PRP+Graphene and no material (Ranks)Test Statistics

	Scores
Mann-Whitney U	5.000
Wilcoxon W	60.000
Z	-3.489-
Asymp. Sig. (2-tailed)	.000
Exact Sig. [2*(1-tailed Sig.)]	.000[b]

Grouping Variable: BoneFormation

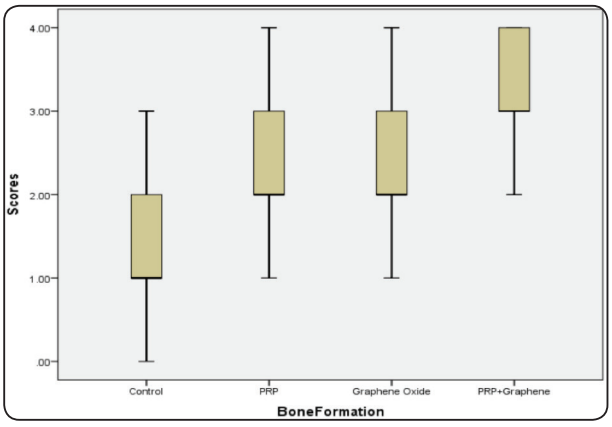

Fig. (9): showing the greatest amount of bone formation related to the combination between PRP and graphene

DISCUSSION

One of the biggest clinical challenges in Oral and Maxillofacial specialty is managing patients with big defects and poor tissues healing capacity as in patients experienced bony tumor resection followed by irradiation. Beside the problem of lost bony skeleton, neighboring soft tissue became hypocellular, hypoxic and hypovascular[19,20]. These consequences reduce the targeted tissue capacity for regeneration and repair as well as necessitate invasive corrective surgery to rebuild the affected area[3,4].

To achieve adequate repair of the irradiated osteogenic tissues we must provide a scaffold for new bone growth (osteoconduction), MSCs and osteoblasts (osteogenesis), and some growth factors to stimulate new bone growth (osteoinduction)[21].

Contemporary choices for bone regeneration consist of autograft, xenograft, and allograft[5] . While autogenous bone is by far the gold standard in restoring bone defects due to its inherent osteoinductive and osteoconductive properties, it may show donor site morbidity plus limited quantity. Xenograft is another choice which provide unlimited amounts of graft and avoid both donor site morbidity as well extended hospital stay but unfortunately it lacks the osteoinductive property which limit its use to small well vascularized defects plus it carries the risk of disease transmission[24].

In this study we used tissue engineering in irradiated bone defects by introducing rGO nanoparticles and PRP as graft materials. rGo is one of the recently applied alloplastic nanomaterials in bone tissue regeneration field. Graphene family members include graphene oxide, carboxyl graphene, rGO, and graphene quantum dots. Graphene possesses an inherent phenomenal conductive and mechanical properties which stimulate cellular activity and bone tissue repair[25]. rGO is synthesized by the use of GO with controlled reducing agents to render it improved chemical and physical properties[26].

Graphene oxide has received extensive invitro and invivo attention exploring its biological and biomedical properties. In medicine, it has a wide range of applications like cancer diagnosis and/or therapy, detection of diseases like Influenza virus RNA, potential MRI contrast agent in addition to its antibacterial ability. Moreover, in the field of tissue engineering and regenerative medicine, it exhibited a proved potential for the differentiation of stem cells to mature osteoblasts and eventually the ability for repairing bone defects[21,22].

rGO was studied with stem cells in the form of 2D coatings and 3D scaffolds for bony tissue engineering. It could significantly promote the proliferation and osteogenic differentiation of stem cells from different sources to osteoblasts and chondroblasts who show bone deposition by intramembranous and endochondral ossification[29]. Also, Kem JW et al.,[23] in 2017 studied the combination of rGO and calcium phosphate as a graft material with different concentrations in critically sized calvarial non-irradiated bone defect and the combination proved to be more effective in osteogenesis. Moreover, it improved the mechanical properties and new bone formation when added to both resorbable and titanium membranes during the process of guided bone regeneration(24). Elkhenany H et al., in 2016 published a paper studying the osteoinductive and osteoconductive properties of rGO nanoparticles with stem cells against stem cells alone in non-irradiated bone defects and they found that both the MSCs alone or in combination with nanoparticles had the potential to heal bone[13]. Also, the combination of mesenchymal stem cells and rGO nanoparticles resulted in improved active bone formation and increased mineralization. In our model we made a bone defect in irradiated bone which is more difficult to heal and regenerate compared to previous models. We mixed the rGO predetermined dose with saline before its application in bony defects and this method showed bone formation and defect regeneration comparable to that obtained with Kem JW et al., and Elkhenany H et al[13,23].

PRP is the portion of blood containing the concentrate of platelets which are rich in platelet derived growth factors (PDGFs), transforming growth factor beta TGF-β, epidermal growth factor EGF, insulin like growth factor IGF and vascular endothelial growth factor VEGF. PRP is the natural source of mitogenic and chemotactic factors necessary for different tissues healing. It showed both osteoconductive and inductive capabilities in different experimental studies. Moreover, it was reported to help in regeneration of critical size bone defect in human with osteoradionecrosis [26]. In our model PRP could achieve bone regeneration in irradiated bone defects comparable to that gained with rGo group.

In our experimental model we could gain new bone formation in irradiated bone defects in groupI, group II and group III with highest bone formation reported in group III which was grafted by a combination of PRP + rGO. This can be explained by the role of rGO which could successively recruited cells from nearby tissue, act as source of calcium and as a mesh framework upon which cells can spread and lay down bone, moreover PRP which supplied concentrated growth factors needed for cells survival and function in an area deprived from vascularity, nutrients, oxygenation and native cells. The role of rGO and PRP was clear when compared with results of group IV which received radiation and defect formation with no grafting. Also, the incorporation of rGO could improve the properties of collagen matrix to affect cell behaviors.

Previous reports showed that the cell adhesion of MSCs on substrates was promoted by the incorporation of rGO through the modulation of integrins and the formation of ECMs. The cell adhesion and the rearrangement of F-actin filament were promoted by the incorporation of rGO. Importantly, the osteogenesis from MSCs was promoted by the incorporation of rGO in time-course and dose-dependent manner (Graphene Oxide-Collagen Matrix Accelerated Osteogenic Differentiation of Rat Bone Marrow Derived Mesenchymal Stem Cell). This has been emphasized in our histological results that displayed the role of rGO to gain highest amounts of bone formation in GII and GIII.

In formulating our experimental model we planned to do 2 bony defects to check the healing in 2 embryologically and histologically different bones, the condyle demonstrating the endochondral type and the ramus representing intramembranous bone. We didn't find a difference in bone gain within the same group.

Radiation was one of our study model corner stones. We needed to mimic a clinical problem in patients exposed to radiation after bony tumor mass resection. The commonly followed practice in postoperative irradiation in humans is a fractionated protocol in the form of 2Gy daily for 5 days a week, over a 5- to 7-week period with a total dose of 50 to 70 Gy[30] . In our rat model we used a regimen of 15 Gy every other week till reaching a total dose of 75 Gy, which is a documented radiation model in rats with a predicted effects on bone[14].

We conclude that regenerating irradiated bony defects using the combination of rGO nanoparticles and PRP can provide the highest active healthy bone formation.

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