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# Bone Engineering by Biomimetic Injectable Hydrogel

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*Osteoporosis is a multifactorial bone disease characterized by low bone mineral density (BMD) and deterioration of micro-architecture of cancellous bone leading to bone fragility and risk of fractures. In the current work, a novel tissue engineering strategy was experimented to enhance bone architecture in the risk areas via local injection of a biomimetic/osteoinductive injectable hyaluronan based hydrogel loaded with nano-hydroxyapatite crystals (Hya/HA) with/without bone morphogenetic protein (BMP-2), in distal femur of normal and ovariectomized New Zealand white rabbits. Our results revealed the osteoinductive effect of the Hya/HA composite that enhanced bone density and architecture of the rabbit distal femur.*

**Keywords** Bone engineering; hyaluronic acid; hydroxyapatite; injectable hydrogels; osteoinduction; osteoporosis

## Introduction

Osteoporosis is a multifactorial, age-related metabolic bone disease characterized by low bone mineral density (BMD) and deterioration of its physical and histological micro-architcture, leading to enhanced bone fragility and a consequent increased pain, disability and risk of fractures, with an enormous costs of treatment for senior patients [1,2].

Recent evidence reveals that femoral neck fracture occurs more than a quarter of million per year [3,4]. Interestingly, through the years a number of therapeutic agents have demonstrated effectiveness in reducing the risk of fracture either by preventing bone loss or stimulating bone formation. However, drugs do have side effects together with its cost which significantly curtailed its continuous use which consequently leads to threat of fracture [5,6].

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Developing an animal model has been done to induce osteoporosis and explore various treatment modalities in relation to enhanced bone micro-architectures [7–9]. The latter should include other parameters such as the number and thickness of individual bone trabecule together with their interconnectivity which affects bone mechanical strength [10,11]. Also, clinical trials have shown that bone turnover, damage accumulation and mineralization are important determinants of bone strength [12,13].

Since the introduction of tissue engineering by Langer and Vacanti [14] in the last two decades, different strategies have been carried out through different approaches including the use of bioinert, bioactive [15] or bioresorbable, or the more recently introduced biomimetic composite biomaterials [16]. Noteworthy, the last approach seems to be promising for bone regeneration to restore, maintain and improve its structure and function; thus enhancing its biomechanical and biochemical properties.

Polymer-ceramic composites which combine organic and inorganic components have been shown recently to mimic the specifically organized nanoscale structure of the bone ECM [17,18]. Hyaluronic acid (Hya) is a glycosaminoglycan, synthesized inside the cell and present with high molecular weight in the ECM of most animal tissues [19]. Hya promotes angiogenesis, interacts with other macromolecules and plays a dominant role in tissue morphogenesis, cell migration, differentiation, and adhesion [20–22]. Native hyaluronic acid was shown to have a poor effect on bone repair even in the presence of BMP-2 [23], whereas pre-formed hydrogels based on cross linked hyaluronic acid were demonstrated to enhance bone formation in-vivo [24–26]. On the other hand, calcium phosphate-based biomaterials have frequently been used as bone substitute scaffolds due to their chemical similarity to the inorganic phase of bone. Calcium phosphate-based biomaterials have frequently been used as bone osteoconductive scaffolds. In 1990, modified forms of calcium phosphate biomaterials have shown osteoinductivity when tested in ectopic sites, together with long-term sustainability and size stability of the neo-bone [27–33]. These results were supposed to be due to implantation of specifically architectured scaffolds with pores big enough for soft tissues and blood vessels to grow in, which is also believed to act as a solid-state matrix for adsorption, storage and controlled release of endogenous BMPs which locally initiate bone formation [29,34]. Nano HA particles in particular have been used in different composites with polymers to promote osteoblast adhesion, migration and differentiation for potential applications in bone engineering [35,36] with good biocompatibility and osteogenesis [37].

Since the pioneering work of Urist using demineralized bone matrix [38], recombinant BMPs have been genetically produced and associated with natural or synthetic carriers to induce ectopic bone formation in vivo [39,40].

In the current study, we used a rapid in situ cross-linked Injectable Hyaluronan based hydrogel loaded with 25% Nano particles HA with and without rhBMP-2 to assess the osteogenic effect of this novel scaffold system for bone engineering in rabbit models. Also, we developed an OVX rabbit model to inject our innovated material into the rabbit femur metaphysis with the assumption that the hydrogel will penetrate through the bone trabeculea and lay down neo-bone which will increase both trabecular thickness and interconnectivity in a minimal invasive approach.

## Materials and Methods

### *Preparation and Characterization of the OVX Rabbit Model*

A controlled preliminary study was performed on 12 mature female rabbits to establish an osteoporotic rabbit model prior to the commencement of the current work. Results were confirmed via hormonal analysis (estrogen and progesterone), histological evidence

(thinning and loss of bone-trabeculae, etc.), and BMD (digital radiographs for the neck of the femur). Also, the success of ovariectomy was confirmed after the rabbits were euthanatized, at different intervals, through macroscopic absence of both right and left ovaries. Our results revealed that all female rabbits developed osteoporosis in femur and spine two months post ovariectomy. Thus, it is confirmed that, in rabbits fed on regular calcium free diet, 8 weeks post ovariectomy (OVX) were sufficient for induction of bone loss and osteopenia.

### *Preparation of Hya/HA Injectable Hydrogel with/without BMP-2*

*Synthesis and characterization of polymer derivatives.* Aldehyde-modified hyaluronan (HAA) was prepared according to a previously described protocol [24] with minor modifications. In brief, protonated hyaluronan (HA) was prepared by stirring a solution of 2 g HA (1.78 MDa, Shiseido Co Ltd., Japan) in 400 mL deionized water with 8 mL Dowex $H^+$ during 30 min, followed by filtration and lyophilization. The product (1.52 g) was then dissolved in 100mL deionized water and 30 mL acetonitrile mixed with 450 $\mu$L N-methylmorpholine. The solution was mixed with 282 mg 2-chloro-4,6-dimethoxy-1,3,5-triazine and 440 $\mu$L aminoacetaldehyde dimethylacetal, and stirred at room temperature overnight. Dowex $H^+$ (5 mL) was then added followed by agitation during 20 min and filtration. The step was repeated using Dowex saturated with sodium and the solution dialyzed (3500 $M_w$ cutoff) and lyophilized. The product was dissolved to a concentration of 10 mg/mL in 0.5M HCl and stirred at room temperature for 4 h. Neutralization with NaOH, dialysis and lyophilization yielded 1.33 g HAA with 4% aldehyde functionality and a molecular weight of 180 kDa, as determined by performing an aldehyde-assay and by static light scattering measurements respectively, according to previously described procedures [41]. Using polyvinyl alcohol (PVA) with a molecular weight of 16kDa, a derivative with 7.5% hydrazide functionality (PVAH) was synthesized following previously described methods [42].

*Preparation of gels.* HAA was dissolved in PBS (Sigma) to a concentration of 27.3 mg/mL and filtered through a sterile 0.45 $\mu$m syringe filter. Recombinant human BMP-2 (rhBMP-2, InductOs$^{TM}$, Wyeth Europe Ltd.), delivered as a lyophilized powder in a formulation buffer containing 2.5% glycine, 0.5% sucrose, 0.01% Polysorbate 80.5 mM sodium chloride and 5 mM L-glutamic acid, was reconstituted by the addition of deionized water to obtain a concentration of 0.3 mg/mL. The concentration of BMP-2 was determined by performing a Quantakine colorimetric sandwich ELISA assay (R&D Systems, UK). PVAH was dissolved in the BMP-2 solution to a concentration of 2.7 mg/mL and filtered through a sterile 0.22 $\mu$m syringe filter. PVAH was dissolved in the formulation buffer alone for gels without BMP-2.

Hydroxyapatite powder "nano particle size" (HAP, Plasma Biotal Ltd., UK) was sterilized by heating to 200°C for a minimum of 2 h, then added to each polymer solution and mixed thoroughly to form 25% w/v suspensions. Gels were formed by mixing equal volumes of the two polymer/HAP suspensions using dual barrelled syringes equipped with static mixing tips (TAH Industries Inc., US) and 21 gauge needles.

### *Injection Technique Feasibility*

To determine the feasibility of our injection technique, another study was performed prior to the main experiment. In this study we utilized 3 mature female rabbits which were anaesthetized and prepared for aseptic surgery. Each rabbit was injected with Xylazin HCl 2% in a dose of 5 mg/kg I.M. as a sedative pre-anaesthetic followed by shaving of the surgical area. Anaesthesia was induced by intramuscular administration of ketamine-HCl

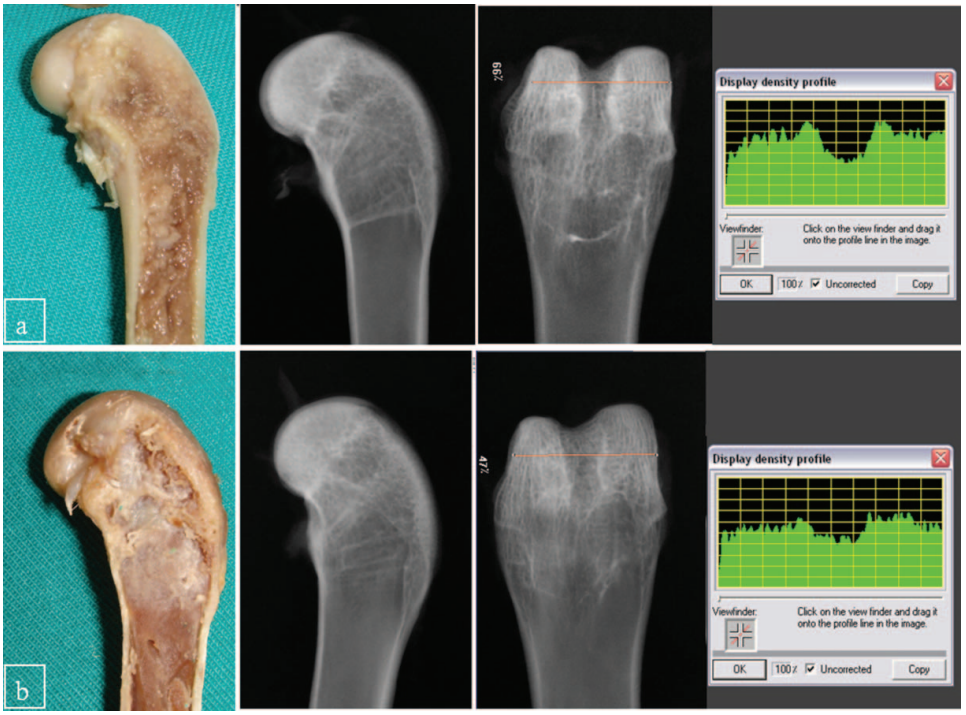


**Figure 1.** Lateral macroscopic photograph of the distal femur of female New Zealand White rabbit together with lateral and frontal digital radiographs and the measurement for the bone density profile for (a) normal (after skeletal maturity at the age of 8 months) and (b) OVX rabbit (12 weeks post ovariectomy). (digital X-rays using XIOS Intraoral Sensor System, Sirona Dental Systems, LLC and SIDEXIS XG Imaging Software, Sirona - Germany).

in a dose of 50 mg/kg then draping of the surgical area for aseptic surgery. The right thigh was exposed through 3–4 cm skin incision made at the level of the lower third of the thigh. The subcutaneous tissue above the stifle joint was opened together with the joint capsule itself with a lateral deviation of the patella. A 1.2 mm hole was drilled in the side of the most distal part of the femur (medial epicondoyl) of its articulating surface (using a 1.2 mm diameter drill mounted on contra-angle low speed hand-piece at 10,000RPM with copious irrigation) (Fig. 2).

### *Injection of Hya/Ha Hydrogel Scaffold with/without BMP-2 in Distal Femur Metaphysis of Normal and OVX Rabbits*

The main objective of this procedure was to compare the efficacy of cross-linked Hya/HA hydrogel as a biomimetic injectable scaffold for in situ bone tissue engineering compared with and without BMP-2. Thus we could utilize this technique in repairing trabecular architecture and to increase BMD in a very challenging condition (the distal femur of an OVX-rabbit).

The material was injected into two animal groups. GpA are normal female New Zealand white rabbits at age 30 weeks (average weight 3.4 kg) (n = 5). GpB are OVX female New Zealand white rabbits with similar weight and age (8 weeks post ovariectomy) (n = 5).

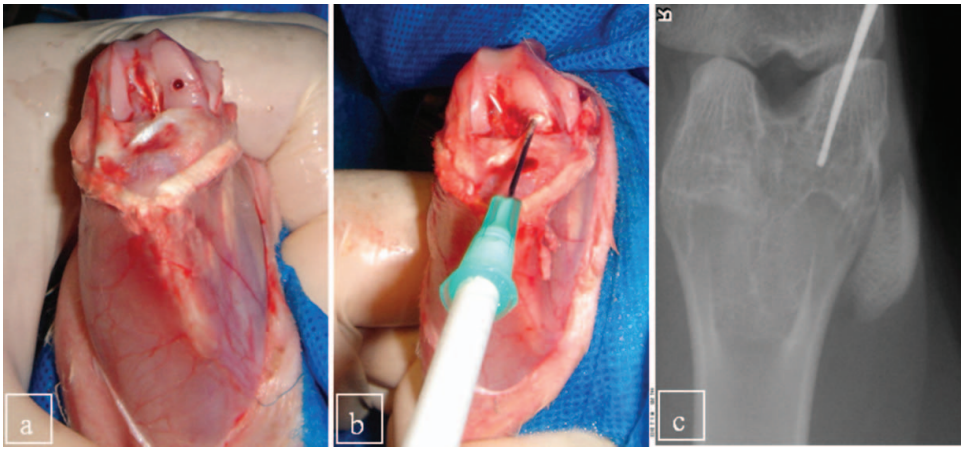


**Figure 2.** Photographs showing the minimally invasive surgical injection of the Hya/HA gel in the medial epicondoyl of the distal femur in New Zealand white female rabbit. (a) Exposing the stifle joint with lateral deviation of the patella and a hole was drilled parallel to the long axis of the distal femur. (b) Injecting the hydrogel system through the hole. (c) Digital radiograph shows the site of injection with the needle inside.

Each group was divided into 2 subgroups. Subgroup 1 was injected with Hya/HA (n = 3), subgroup 2 was injected with hya/HA/BMP-2 (n = 2).

Hya hydrogel (0.5 ml) was injected slowly with adequate pressure in the drilled hole of the distal femur of the right limb (Fig. 2). After setting of the hydrogel (1–3 min later), the patella was returned again to its place. The joint capsule was then sutured and the skin incision was closed. As a control, the same surgical procedures were performed on the left limb without the injection of any material.

The rabbits were recovered from anesthesia, monitored in the recovery units for the next 6 hours until they were resting comfortably then returned back to their cages. Also, they were injected with Enrofloxacin (0.1mg/kg I.M) twice daily to relief pain, left to feed on standard commercial rabbit chow and to move freely in their cages. The rabbits were then monitored regularly and weighed weekly.

Rabbits were euthanatized 5 weeks post operation, samples were retrieved, digital photographs and regular radiographs were taken, then samples were processed for undecalcified poly(methyl methacrylate) PMMA embedding [43], sectioned and grinded up to 150 $\mu$m thickness then stained with Stevenel's blue and Van Geison's picro-fuschin for light microscope examination and histomorphometric analysis of bone volume/total volume and trabecular bone thickness (BioPix iQ - software, Göteborg, Sweden).

## Results

### *Characterization and Confirmation of the OVX Rabbit Model*

Macroscopic bone loss in OVX group was supported positively by digital radiographs. Sidexes software measurements emphasized the difference between bone density profiles in normal and OVX rabbits (Fig. 1). Samples of the examined bone demonstrated most of the histological features for osteoporosis. The distal head of both right and left femur depicted disrupted, isolated and free-end thin bone trabeculae with almost complete absence

of connecting trabeculae. Also, bone trabeculae were aligned parallel to the long axis of the bone with an apparent osteoprotic marrow spaces that had minimal cellularity. In addition, the cortical bone demonstrated scalloped bone surface with many Hawship's lacunae and few underlying spongy bone trabeculae with an apparent osteoclastic activity.

### *Injection Technique Feasibility*

After drilling, in the distal femur of the rabbit, the injected hydrogels (Hya/HA) spread through the metaphyseal trabuclae (Fig. 3) and the over-pushed material was seen to internally invaginate into the marrow space.

### *Injection of Hya/HA Hydrogel Scaffold with/without BMP-2 in Distal Femur Metaphysis of Normal and Ovx Rabbits*

Photomicrograph of the undecalcified histological sections of the distal right femur injected with Hya/HA with/without BMP-2 showed enhancement for cancellous bone architecture and interconnectivity in both normal and OVX rabbits when compared to the uninjected (left control side) (Figs. 4 and 5). The histomorphometric analysis of the histological sections revealed increase in both trabecular thickness and bone area/total area in the injected right side, compared to the uninjected left control side (Fig. 6).

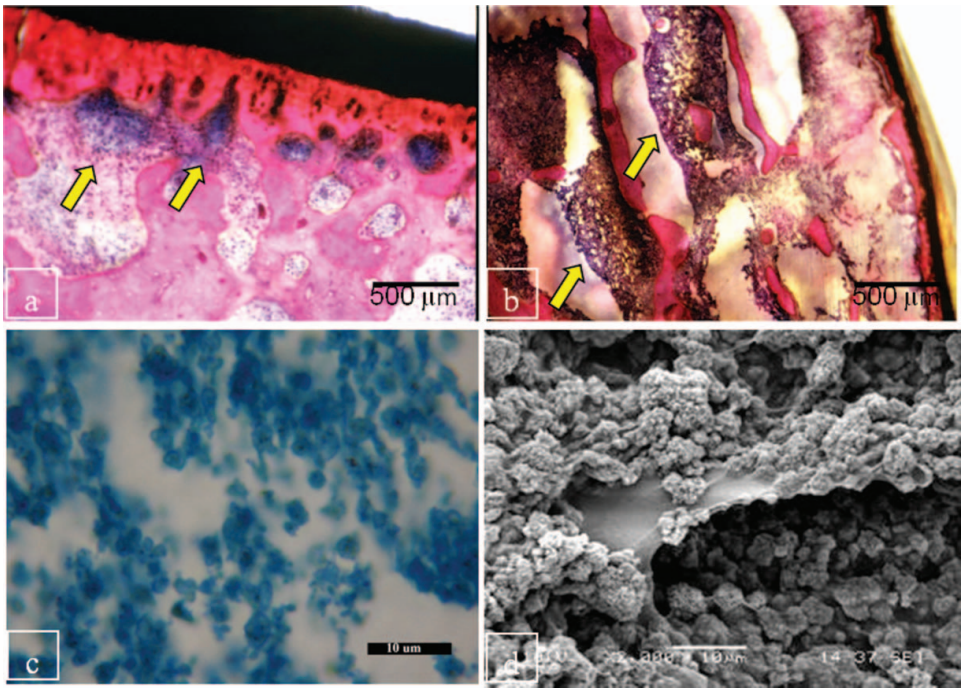


**Figure 3.** Undecalcified histological micrographs of the distal femur of New Zealand White rabbit 1-day post injection allocate the hydrogel through bone trabeculae; material (bluish color) distributed among bone trabeculae (yellow arrows) (a and b). Higher magnification for the gel distributed among bone showing the HA particles stained in blue. (c) Sections stained with Stevenel's blue &Van Geison's picro-fuschin. (d) SEM for the hydrogel material.

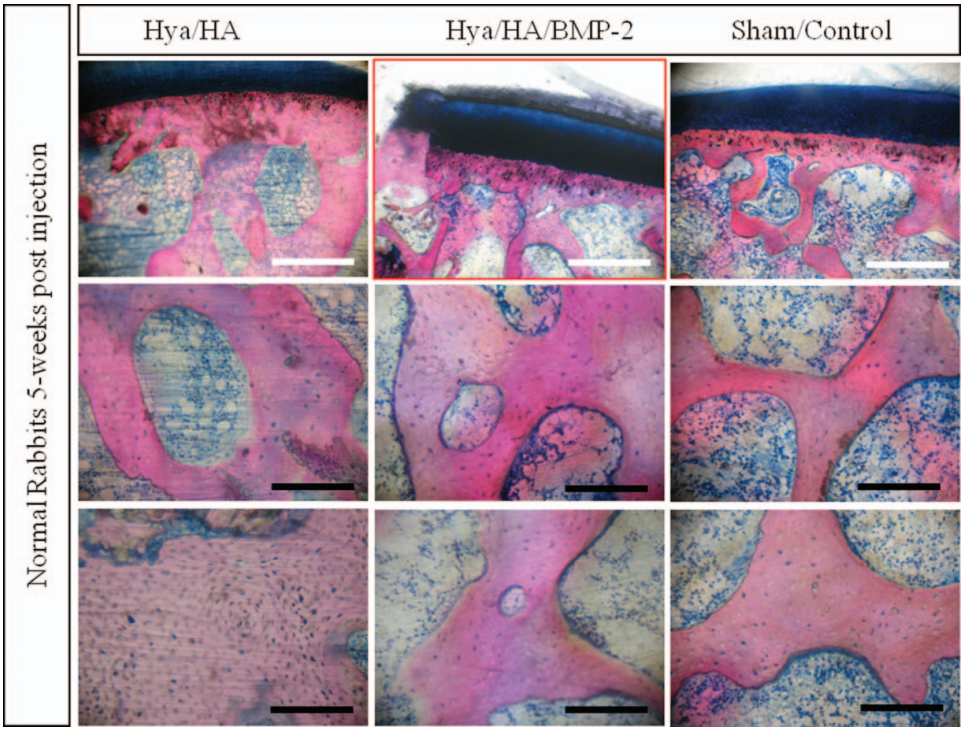


**Figure 4.** Undecalcified histological micrographs stained with Stevenel's blue and Van Geison's picro-fuschin comparing the distal femurs of uninjected normal New Zealand White rabbit with those injected with Hya/HA with and without BMP-2. Note the increase in trabecular bone thickness in cases injected with the hydrogel without BMP-2 than those given BMP-2 loaded hydrogel. White scale bar = 500 $\mu$m, black scale bar = 200 $\mu$m.

There seems to be no difference in the rate of new bone formation between the OVX and normal group. However, the architecture and rate of new bone were clearly better in case of injecting Hya/HA without BMP-2 (Fig. 6). For most cases in the injected groups (OVX and normal) (with and without BMP-2), the over-pushed material seen to internally invaginate into the marrow space was converted to a mass of woven bone with marrow like tissue (Fig. 7).

## Discussion

The key message of this pilot study includes three main ideas. First; among the various ways tissue engineering could deal with osteoporosis might be through injecting hydrogels that are loaded with cells, as done by previous studies [9] though it could be inconvenient clinically. Hydrogels could also act as a vehicle for growth factors that might have some adverse effects. However, it is profitable for hydrogels to be a biomimetic osteoconductive scaffold which is considered an easy, cheap, clinically convenient and on-the-shelf product. The second idea is whether a biomimetic osteoconductive scaffold is sufficient to induce bone when exposed to bone microenvironment and suspended in an in vivo bioreactor [44]. Thirdly, the significance of adding BMP-2 and other growth factors delivered to induce bone in orthotopic sites and claimed to have a reverse action most of the time [23].

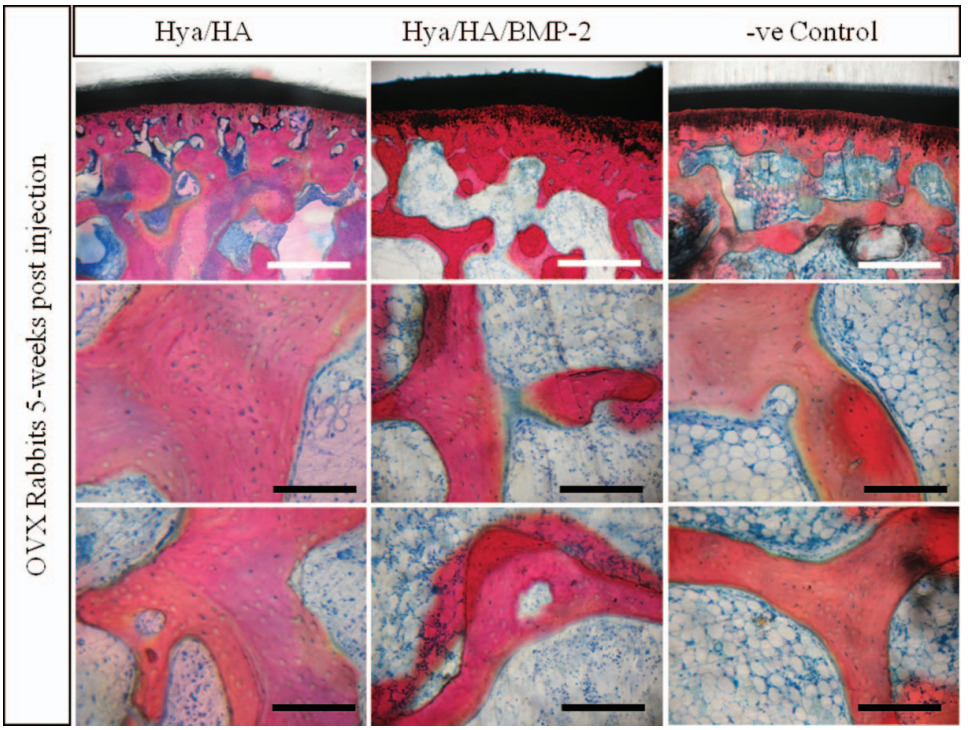


**Figure 5.** Undecalcified histological micrographs stained with Stevenel's blue and Van Geison's picro-fuschin compare the distal femurs of uninjected OVX New Zealand White rabbit with those injected with Hya/HA with/without BMP-2. Note the increase in trabecular bone thickness in cases injected with the hydrogel without BMP-2 than those given BMP-2 loaded hydrogel. White scale bar = 500 $\mu$m, black scale bar = 200 $\mu$m.

Thus to target the ultimate goal of repairing trabecular architecture and to increase BMD in specific area, we determined the efficacy of cross-linked Hya and 25% w/v HA as a biomimetic injectable scaffold for bone tissue engineering compared with the same system carrying BMP-2. We also developed and utilized a feasible surgical technique to study the effect of injecting this Hya/Nano-HA biomimetic composite with or without BMP-2 in bone engineering in a very challenging condition; distal femur of OVX rabbit animal model.

It is a rule of thumb that the process of bone remodelling occurs once the regenerative processes are well under way. Significantly, the surface characteristics and geometric configurations of the delivery system are critical for bone induction to occur with and without the exogenous application of BMPs [34].

Although other studies on rats, lacked ectopic bone formation with injectable Hya/HA hydrogel unless combined with exogenous BMP-2 [45], our study on the rabbit model revealed orthotopic bone formation using the same injectable Hya/HA hydrogel with and without incorporating exogenous BMP-2 (Fig. 7). The latter could be related to our chosen site of injection; contiguous to the bone marrow.

Notably, native high molecular weight Hya is anti-angiogenic, whereas Hya degradation products stimulate endothelial cell (EC) proliferation, migration and tube formation

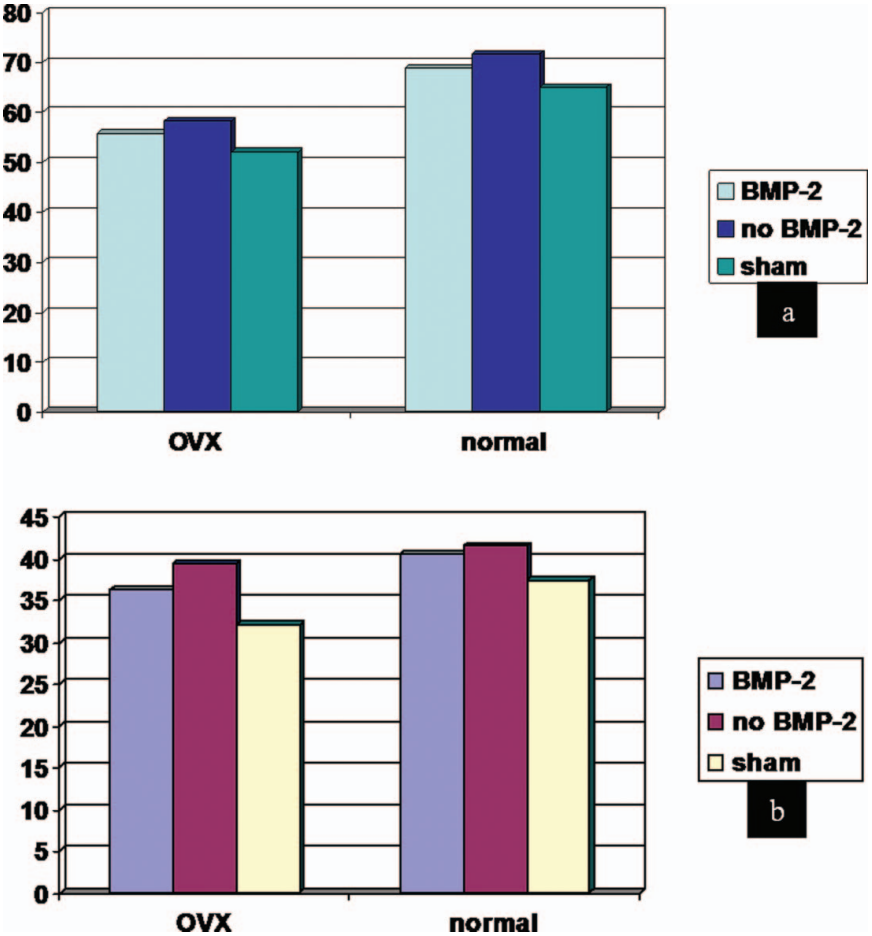


**Figure 6.** Histo-morphometrical diagram compare between (a) Bone area/total area and (b) Trabecular thickness of cancellous bone of normal and OVX rabbits. Note the increase in total bone area and trabecular bone thickness in cases injected with hydrogel without BMP-2 than those given BMP-2 loaded hydrogel in both normal and OVX rabbit groups.

following activation of specific Hya receptors in particular, CD44 and Receptor for Hya-Mediated Motility (RHAMM, CD168) [22].

Interestingly, studies on the material factors related to osteoinductivity of calcium phosphate biomaterials revealed that its chemistry, geometry and porous structures are essential for bone induction [46]. Also, they strongly suggested that calcium phosphate biomaterials could be endowed with osteoinductivity just by optimizing the materials themselves [47,48] rather than through tissue engineering in which biomaterials were made to be osteoinductive by adding BMPs or osteogenic cells. Controlled delivery of exogenous BMPs was considered by numerous researchers to be crucial for bone engineering strategies [49], to guarantee proper vasculature and osteogenic mechanism.

The use of injectable bulking agent to augment tissues has become a common practice since it facilitates minimally invasive delivery, has low cost, low patient morbidity, performed in an outpatient clinic and under local anesthesia [50].

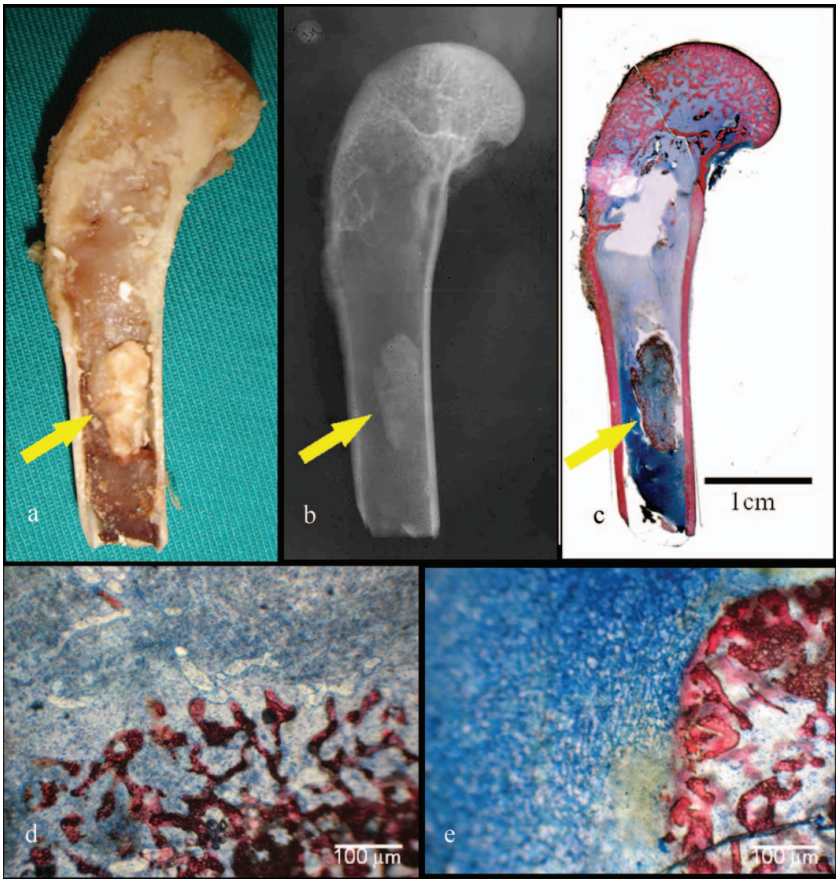


**Figure 7.** An in-situ orthotopic woven bone formation within the marrow space of normal and OVX New Zealand white rabbits injected with Hya/HA without BMP-2 (5-weeks) (a) macroscopic (b) radiographic and (c) light microscopic undecalcified section Stained with Stevenel's blue and Van Geison's picro-fuschin of the distal femur sectioned vertically. (d & e) Light microscopy micrographs of the orthotopic woven bone formed inside the marrow space of the distal femur.

Furthermore, using two geometrical forms of HA (beads and discs) with/without BMP exposed that the geometry of the delivery system is critical for optimal bone induction. The discs were consistently osteoinductive with BMPs in rats [34]. In certain species, HA alone was proved to be "osteoinductive" [29]. In subhuman primates, the HA induces bone albeit at a much slower rate. It is possible that the circulating BMPs could progressively bind to the implanted HA disc and after achieving its optimal threshold concentration, HA becomes osteoinductive. Thus, in certain species, the osteoconductive HA substrata could progressively function as an osteoinductive substance by binding to endogenous BMPs [51]. It is possible that modification of Hya/HA scaffold composition or ratio alters its biological action that makes BMP-2 induces more bone formation in rabbits.

In the current study, it is reasonable to report that the Hya/HA composite in the gel form induced a 3D matrix (Fig. 3(c) and (d)) with the HA nano-particles in a specified geometry to induce bone formation without the need of exogenous BMPs which could be

considered as a smart way to enhance intra-bone trabeculations. Adding exogenous BMP-2 or other growth factors delivered to induce orthotopic bone could also have a reverse action.

## Conclusion

Nano HA crystals are osteoinductive. They act as a matrix for adsorption, storage and controlled release of endogenous BMPs. Combined with Hya hydrogel, nano HA crystals would have the required geometry to induce bone, in addition to promoting angiogenesis, cell adhesion and migration. Being injectable, the nano HA crystals/Hya hydrogel composite has an added advantage of being minimally invasive, leading to less patient trauma and easier targeting for infeasible clinical sites of osteoporosis.



## Acknowledgement

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