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***In vitro/In vivo* evaluation of sustained release of glucagon-like peptide-1 (GLP-1) from nanocomposite hydrogel to induce angiogenesis**

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Abstract

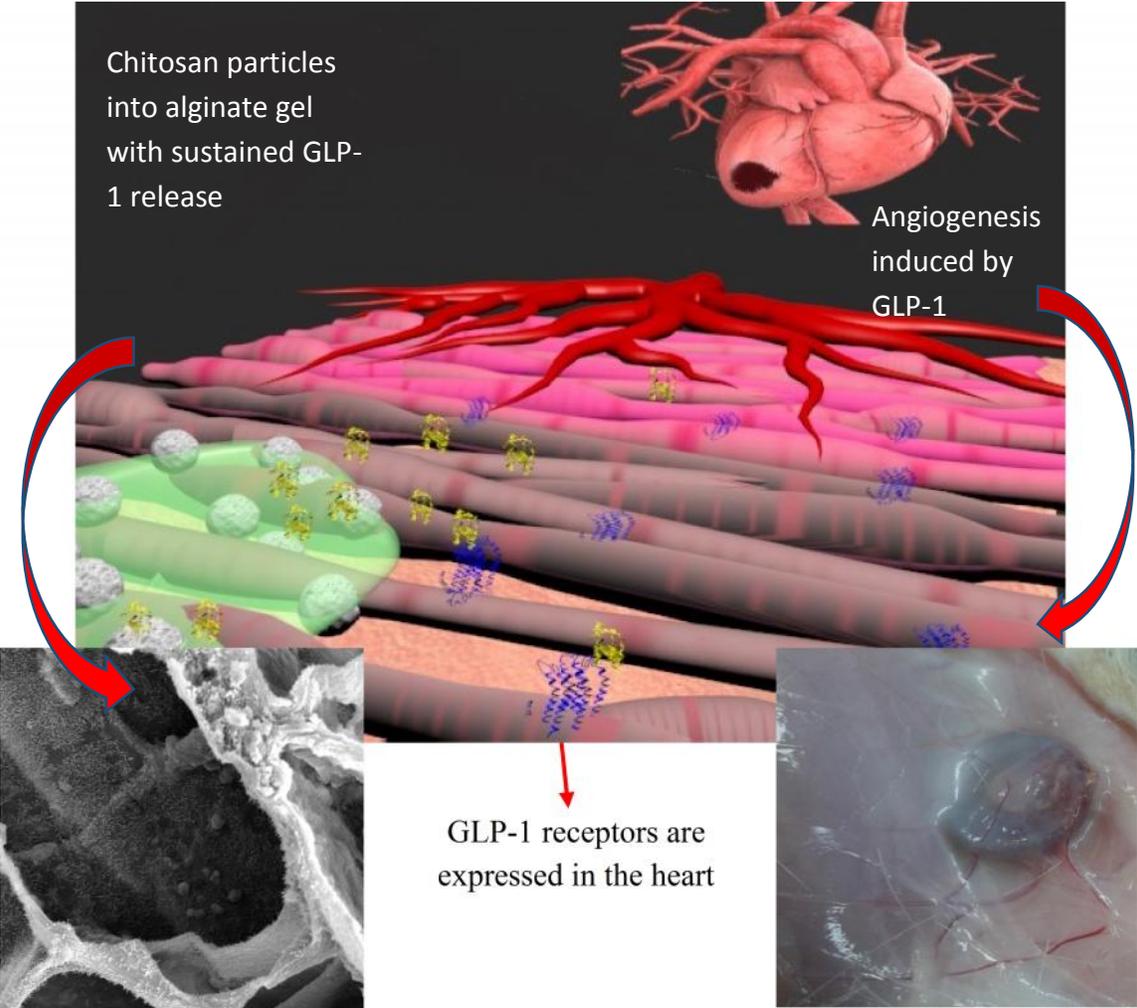
Background: Glucagon-like peptide-1 (GLP-1) is a proglucagon hormone with cardioprotective effects and angiogenic ability.

Results: Chitosan nanoparticles (65nm) possess the ability to encapsulate $65\pm 4.1\%$ of the GLP-1 used for the formulation of nanoparticles. Freeze-dried gels containing these particles released 40% of the total loaded GLP-1 during 192 hours. Field emission scanning electron microscopy was done to observe the morphology of particles both alone or when embedded into a gel. Results of *in vivo* studies and histological images showed that following the gradual release of GLP-1, new vessels formed into and around gels implanted into the back of rats. Through Drabkin assay, it was observed that the concentration of hemoglobin in the samples containing GLP-1 loading were similar to samples with VEGF loading, while a significant difference exists between hemoglobin concentration in sponges with or without GLP-1 release ($P < 0.05$).

Conclusion: Sustained release of GLP-1 induced new vessel formation in a subcutaneous *in vivo* model of angiogenesis. This system has the potential to induce angiogenesis in myocardial infarction situations.

Keywords: glucagon-like peptide-1; Angiogenesis, Myocardial Infarction; Alginate; Nanoparticles

Graphical Abstract



1- Background

Therapeutic angiogenesis is an effective tool for the treatment of certain types of diseases that deal with a shortage of angiogenesis such as hypertension, diabetes and cardiovascular diseases such as atherosclerosis and myocardial infarction (MI). MI is a significant disease dealing with angiogenesis, being a condition in which blockage of a coronary artery occurs and consequently limits collateral blood circulation to the heart muscle, potentially leading to necrosis in the myocardial tissue. The balance between oxygen requirement and its delivery determines the fate of myocardial regions at risk for infarction [1-3]. Induction of angiogenesis in the ischemic tissue of the heart is a valid strategy to overcome infarction, especially when vessel grafting and bypass surgery are not applicable [4, 5]. Many different approaches have been investigated for angiogenesis induction, including angiogenic growth factors administered to the site of ischemia with or without a carrier through multiple injections or through secretion of these factors from encapsulated genetically engineered cells [6]. Gene therapy is another approach, which aims to increase capillary density in ischemic areas through the transfer of genes encoding angiogenic growth factors by means of viral and non-viral vectors [7]. Although encapsulation of genetically engineered cell and gene therapy seem to be useful approaches to address the problem with short half-lives of growth factors seen in the simple injection methods, several significant concerns such as toxicity, inflammation, and cancer still exist for the clinical development of these methods [7, 8].

GLP-1 is considered as an effective material for heart tissue angiogenesis. GLP-1 is one of the proglucagon hormones affects appetite and satiety and is regarded as a suitable treatment for diabetic patients [9]. GLP-1 receptors are expressed in the heart. The cardioprotective effects of GLP-1 have been proven previously [10, 11]. Moreover, researchers have proved the angiogenic effects of GLP-1 through a 3D culture of cells in Matrigel [12]. Despite the importance of angiogenic potential of GLP-1 for heart tissue regeneration, which could be combined with its cardioprotective effects to bring about promising results, little studies to date investigated the angiogenicity of GLP-1 in vivo [13]. In this study we designed a carrier to release GLP-1 slowly to induce angiogenesis in vivo.

Generally, in terms of developing protein pharmaceuticals, the most demanding task is to avert physical and chemical instabilities of the protein. This instability is the reason why continuous injections are required for a favorable therapeutic response. Like other proteins, GLP-1 has the limitations of short half-life in serum and its rapid inactivation by the enzyme dipeptidyl peptidase-4 (DPP-4). [9]. Therefore, a system for sustained delivery of protein drug over a period of time that would be able to protect GLP-1 from enzymatic attacks would be the most effective delivery method. In this respect, hydrogels are a good candidate. Selecting among many different gel materials and considering their affinity to the drug molecule, controlling diffusion of the drug from gel structure, and tailoring degradation rate of the gel enable us to have a precise control over the delivery rate and the drug release behavior. Along with these advantages, hydrogels can also protect sensitive structures, such as protein, from being exposed to serum and degradation [14]. Their hydrophilic properties and high water content makes them a suitable choice not only for cell encapsulation applications or as tissue scaffolds [15, 16] but also non-invasive in vivo drug delivery [17, 18]. swelling of the gel network provides an environment for the free movement of water molecules and the release of the embedded drug [19].

In this article, chitosan and alginate combined were chosen as a protein carrier. These two polysaccharides are both naturally occurring biopolymers that could be applied as pH-sensitive hydrogels applicable for the delivery of proteins [20-22]. Both of these materials were selected due to high biocompatibility, biodegradability, and non-toxicity [22, 23]. Carboxylic acid groups are a proper site of amine binding. Therefore, the binding of proteins to alginate in order to enhance cell attachment of this material when it is used as a cell scaffold is favored by forming amide bonds [24, 25]. Also, the ability of alginate to form a physical gel due to binding with divalent cations, such as calcium, makes it favorable in tissue engineering applications as an irreversible in situ formation of a gel [26]. In further steps, the system proposed in this study could be used as an injectable alginate gel which could be injected into heart muscle and release GLP-1 locally to increase drug bioavailability and effectiveness. Localized delivery

directly improves angiogenesis in the infarcted area of the diseased heart, and consequently, reconstruction of this region will occur.

2-Results

3-1 Physicochemical characteristics of nanoparticles and selection of optimal formulation

Results of release and encapsulation efficiency (EE) of nanoparticles with different concentrations of GLP-1 in the formulation are presented in Fig 1. It is obvious that with increasing GLP-1 concentration, release rate decreased and encapsulation efficiency increased. After 192 hours, the released amount was not different in samples with 1.2 and 1.5 mg/ml GLP-1. The released GLP-1 from the sample with 0.9 mg/ml GLP-1 in the initial formulation was significantly different from other samples (1.2 and 1.5 mg/ml, and 0.2 mg/ml) as it is shown in Fig 1. Samples with a low concentration of GLP-1 had higher release rate (40-60% of the loaded GLP-1) and among them, a sample with 0.9 mg/ml GLP-1 had the highest value of EE. Therefore, this sample was selected for further experiments.

3-2 Characteristics of alginate gel incorporating nanoparticles

The porous structure of freeze-dried alginate gel (1.5 % w/w) is obvious in Fig 2. The presence of intact nanoparticles of chitosan over gel structure is shown in higher magnification in this figure as well, and it seems nanoparticles are able to preserve their spherical shape into a gel. The mean particle diameter as calculated by Image J software was 65 nm. The pores of alginate gel are evidently greater than the radius of nanoparticles, which will impact the mechanism of protein release from this template. In Fig 3 it is shown that the amount of released GLP-1 from particles, whether incorporated into alginate gel or not, is about 40% of the loaded GLP-1 after 192 hours.

3-3 Results of in vivo test

The sponge implant model of angiogenesis used in this study represented the angiogenic potential of this system designed. In Fig 4, many small vessels were obvious within and around the implant as a result of the sustained release of GLP-1. Compared to VEGF-releasing sponges, while the dose of VEGF and GLP-1 are the same (0.9 mg/ml), it appears better integration with nearby tissues was achieved in the case of GLP-1 releasing sponges. Furthermore, hemoglobin concentration in sponges was measured to augment angiogenesis results of in vivo test with some quantitative data. Hemoglobin content of VEGF-releasing sponges was comparable to its amount in sponges with the GLP-1 release, which was significantly higher than sponges without loading. This remark was indicative of the potential angiogenic ability of GLP-1.

3-Discussion

As suggested before, localized delivery of GLP-1 from chitosan particles is to be achieved via embedding these particles into alginate gel. Protein entrapment in alginate beads is routine, especially in oral formulations. Some properties of alginate make it suitable for protein delivery systems such as biocompatibility, non-toxicity, biodegradability, pH sensitivity, extremely mild gelation conditions with nontoxic reagents and its ability to form gels by reaction with divalent cations, such as Ca²⁺, via association of the G-block regions by calcium [20]. Apart from these features, the most important reason for selecting alginate in this study is the history of using an intracoronary injection of this biomaterial to the infarcted area, which had been helpful in the prevention of left ventricular remodeling and dysfunction. Alternatively, in this study, alginate is the best choice among other biomaterials because it is non-thrombogenic when it enters inadvertently into blood circulation [29-33]. Gelation of alginate results in forming a solid hydrogel in a determined shape with the ability to keep high water content (99–

99.5% of the structure), resist mechanical stresses and protect the loaded protein structure owing to not having inorganic solvents and crosslinkers. These gels could be temporary when lower concentrations of calcium are used or permanent with higher levels of calcium. In addition to calcium concentration, the chemical structure and molecular size of alginate, and kinetics of alginate gel formation are parameters determining the final characteristics of the gel, such as porosity, swelling behavior, stability or biodegradability, strength and biocompatibility [20].

There are two problems regarding using alginate gel alone. Firstly, negatively charged protein structures remain active in the alginate gel network, as in the case of this study, while positively charged proteins, instead of calcium ions, might interact with carboxylic acid sites of alginate resulting in protein inactivation. Secondly, alginate has the problem of rapid dissolution.

To address this issue, protein would be inserted into chitosan particles and then dispersed into alginate gel [14]. Chitosan itself can protect the protein structure due to the fact that polymers having a carboxylic group are able to prevent protein inactivation by interacting with divalent extracellular cations [34]. Such composite drug delivery systems in the form of nanoparticle-loaded gels have gained the acceptance of researchers for the purpose of localized delivery of the drugs from these particles through lengthening the retention time of these particles in the desired site, and consequently, increasing bioavailability [35, 36] or to alter release rate and kinetic rate to prevent burst release [37-39]. This concept could be useful in releasing a hydrophobic drug embedded into a particle from a hydrophilic gel structure [40-43] or for the release of more than one growth factor from gel scaffolds in tissue engineering applications [44]. Furthermore, inserting particles into gel has improved the release of drugs from the gel matrix in a number of studies [45]. Regardless of gel/particle mixture systems, binding of alginate and chitosan is interesting in drug delivery applications. In these platforms, polyelectrolyte complex of chitosan and alginate solutions will be provided, and then the particle will either form in CaCl_2 solution through coacervation/ionotropic gelation method or chitosan will be coated on the alginate beads in order to promote swelling ratio, cross-linking density, and drug loading capacity of the resulted system [34, 46-49]. Williams et al. provided a blended chitosan-alginate gel to achieve sustained release of an angiogenic factor [50].

In this study, chitosan particle synthesis was performed by ionic gelation method. The basis for this method is electrostatic interactions between two oppositely charged ions. Therefore, gelation of chitosan chains occurs as a result of the interaction of amino groups of chitosan with negatively charged phosphates of TPP in an immediate manner. Moreover, due to the lower isoelectric point of GLP-1 (~5.5) than pH value of the solution (=6), GLP-1 will be negatively charged, and chitosan, as a cationic material, is able to entrap this protein via electrostatic interactions. Regarding the release of GLP-1 from this platform, it is known that absorption of water by the hydrogel causes swelling and protein release to take place simultaneously. Since the degradation of polymer networks (both chitosan and alginate) is longer than the duration of drug release measurement in this study, diffusion and capillary action are the prevailing mechanisms [51, 52]. At the first step, the protein diffuses out from nanoparticles' pores and then passes through micropores of alginate network via capillary force [53, 54]. Fig 3 shows that the release of protein from alginate incorporating chitosan particles had an insignificant difference from the particles alone. There are two possible reasons for this observation. First, as seen in Fig 3, the average pore size of this network is about 20 micrometers. Hence, the alginate network could not restrict protein release significantly because the protein molecule is smaller than pore size. Second, the negative charge of the protein allows the anionic alginate network to repel it rapidly after being released from chitosan particles. The diffusion of several proteins from alginate beads has been reported including IgG, fibrinogen, and insulin [24].

Not only histological staining but also the appearance of sponges showed the effective role of GLP-1 in angiogenesis induction. As observed in Fig 4, sponges with VEGF release were completely red and full of blood vessels. These sponges are like a hank of tiny vessels while in the case of sponges with GLP-1 release large and apparent vessels are generated and sponges without GLP-1 release had an insignificant change in their color and vessel ingrowths. Histological examination of the sponges reveals the presence of blood cells, further confirming the observation of vessels into sponges. The various amounts of hemoglobin in the Drabkin assay also confirm the

formation of blood vessels into sponges. As depicted in Fig 4, the concentration of hemoglobin in the samples with GLP-1 loading is similar to samples with VEGF loading suggesting the potential angiogenic ability of GLP-1 is comparable to VEGF angiogenesis induction. Interestingly, there exists a significant difference between the number of red blood cells into sponges with or without GLP-1 release. It is suggested that the angiogenic effect of GLP-1 is mediated by the Akt, PKC and Src pathways [12]. Additionally, some angiogenic effects of GLP-1 are receptor-dependent [55]. Results of in vitro studies indicate that GLP-1 could promote proliferation of endothelial cells via a GLP-1 receptor-dependent mechanism because endothelial cells express GLP-1 receptors on their surfaces. In fact, with increasing GLP-1 level, levels of circulating endothelial progenitor cells will increase, leading to upregulation for tissue expression of endothelial nitric oxide synthase (eNOS), and ultimately, enhanced angiogenesis [12, 56, 57]. The angiogenic ability of GLP-1 has been combined to other cardioprotective effects which makes it a better choice than VEGF for angiogenesis in the heart. GLP-1 infusion has been shown to reduce infarct size and lead to myocardial recovery post-infarction through the mechanism of increasing glucose uptake, enhancement of resistance to ischemia and increasing contractile strength of the heart muscle [58-61].

4-Conclusions

In summary, we achieved a system composed of nanoparticles incorporated into alginate gel to release GLP-1 locally and in a sustained manner. Favorably, sustained release of GLP-1 from this platform induced angiogenesis in the sponges implanted in the back of rats as confirmed by an in vivo sponge implant model of angiogenesis. In conclusion, the system introduced in this investigation has the potential to be served as a useful tool for the treatment and regeneration of damaged heart in MI situations. The designed system could be easily injectable to a determined area of the heart muscle with the gelation procedure being in place immediately, release GLP-1 and provide angiogenesis beside other cardioprotective effects.

5-Methods

5-1 Synthesis of particles and gels loaded with GLP-1

Ionic gelation method was used to prepare chitosan nanoparticles according to our previous work [23]. After preparing clear solutions of chitosan (0.2 % w/v) in acetic acid, 0.5 % (v/v), with dissolved GLP-1 (0.2, 0.5, 0.9, 1.2, 1.5 mg/ml), tripolyphosphate (TPP) (1mg/ml) was added dropwise to chitosan and stirred for 15 min at 60°C. The formed nanoparticles were then ultra-sonicated, separated, and re-dispersed in fresh medium. Alginate Sodium (1.5% w/v) solution in phosphate buffer saline (PBS) was prepared. 10 mg of calcium sulfate powder was added to each milliliter of the solution and ultrasonicated. GLP-1 loaded chitosan particles were dispersed into this pre-gel.

5-2 Encapsulation Efficiency and drug release measurement

Encapsulation efficiency and release of GLP-1 was calculated via the Bradford assay [27]. To begin this assay, separation of supernatant from suspensions of nanoparticles was completed by ultracentrifugation (20000 rpm, 20 minutes, 10°C). The amount of free GLP-1 in supernatants of samples was determined using the Bradford assay. In this assay, the addition of Coomassie brilliant blue, a dye, to a protein solution leads to the formation of a complex with optical absorbance at 595 nm. In fact, the absorbance of different supernatant solutions at 595 nm was measured by spectrophotometry. Absorbance is proportional to the concentration of protein. As a result, with the help of a standard curve produced by samples with known concentrations, the prediction of the unknown concentrations is possible. Thus, a standard curve was produced and GLP-1 concentration was measured. The linear range of the standard curve in this study was 1-100 micrograms per milliliter.

In order to measure protein release from nanoparticles, all samples were centrifuged for 20 min (RPM= 20000g). Accordingly, 150 microliters of their supernatant were transferred to 96 wells plate using a sampler and mixed with 150 microliters of Bradford reagent. The absorbance of this mixture in 595 nm was measured and compared to a standard curve. The taken volume was replaced quickly with 150 microliters of fresh medium. The sampling procedure was repeated at certain times (1-3-6-12-24-48-96-192 hours). During the test, all samples were inserted into a shaking incubator at a temperature of 37 ° C simulating physiological conditions of the human body.

5-3 Morphological characterization

The shape, morphology and surface characteristics of the nanoparticles were observed by field emission scanning electron microscopy (FE-SEM) (Philips XL30–Netherlands). With this method, ten microliters of nanoparticle suspension were inserted on a glass slide, and after complete dryness, sputter coated with gold in an argon atmosphere. Drying step may result in agglomeration of nanoparticles owing to high surface tension during drying. Then, the aforementioned pre-gel was cross-linked with acetic acid and then freeze-dried until complete removal of solvents. Then, these sponges were frozen, fractured and placed onto a glass slide, followed by mounting on a Fe-SEM stub and sputter-coated with gold in an argon atmosphere. The coated samples were examined by Fe-SEM.

5-4 The procedure of in vivo sponge implant model of angiogenesis

Sodium alginate, calcium sulfate, and chitosan powders were UV irradiated for 2 hours. Solutions of acetic acid, sodium tripolyphosphate, PBS, sodium hydroxide and their containers were autoclaved for one hour, then immediately transferred underneath the hood. Synthesis of nanoparticles and gel was done under aseptic conditions. Polyurethane foams were prepared with specified dimensions (circular surface, the diameter of 1cm and thickness of 3mm), sterilized through immersion in ethanol, and re-dried under UV exposure for two hours, three times. Test samples were freeze-dried and placed at the center of this foam. The internal radius of some sponges filled with freeze-dried gel/ particle with or without GLP-1 loading. To deliver VEGF, a cannula was inserted at the center of the foam and fixed in place with sutures. VEGF injection into the cannula was done via an insulin syringe.

Male Wistar rats (n=15) weighing approximately 250 ± 20g were selected and prepared for the test. Animals were transferred to standard separate cages and were kept at standard conditions of 12/12 hour light-dark cycle, 25°C temperature and 60% humidity. First, the animals received an intraperitoneal injection of the anesthetics, Ketamine (50 mg/kg) and Xylazine (5 mg/kg). Their backs were shaved, incisions were made on their backs and then the sponges were inserted carefully, then sutured according to protocol. The rats were kept in standard cages for three weeks after recovery. Last, the animals were sacrificed after 8 days (192 h) and sponges were removed for further tests.

5-5 Histological examination

Eventually, the animals were euthanized 8 days post-implantation of samples and the sponges were fixed in the 10% neutral buffered formalin (NBF, PH. 7.26) for 48 h, then processed and embedded in paraffin. Sponges were then cut into 6-micron sections and stained with Hematoxylin-Eosin (H&E). The histological slides were evaluated by the independent reviewer, using light microscopy (Olympus BX51; Olympus, Tokyo, Japan).

5-6 Measurement of hemoglobin concentration in sponges as an indication of angiogenesis

In order to measure hemoglobin concentration in the sponges, Drabkin assay was utilized [28]. This is a quantitative and precise test to indicate the degree of angiogenesis present in the samples. First, the sponges were weighted and their homogenates prepared with 2ml of Drabkin reagent, consisting of sodium bicarbonate, potassium cyanide, and potassium ferricyanide. This reagent was used to dilute the blood into samples (20µl blood/5 µl Drabkin). The sponge material was removed from the homogenate with centrifugation (12000g, 20 min) and filtration (0.22 µm filter paper). Red blood cells lyse by detergents present in Drabkin agent, and consequently, their hemoglobin is

released. Afterward, under the effect of potassium ferricyanide, the hemoglobin will be converted into methemoglobin and have an optical absorbance of 540 nm. Therefore, the value of absorbance of samples in this wavelength is proportional to hemoglobin concentration, and therefore, blood cells. The calibration curve was drawn using hemoglobin calibrators.

5-7 Statistical analysis

Release data and quantitative results of Drabkin assay were analyzed through multiple t-tests of repeated measures one-way ANOVA. All values are presented as a mean \pm standard error of the mean (SEM) with a level of significance set at $P < 0.05$ (*) and $P < 0.001$ (**). All analyses were performed using Graph Pad Prism version 6 for Windows (Graph Pad Software, San Diego, CA).

- **Ethics approval and consent to participate:** All in vivo procedures approved by the ethics committee of Tehran University of Medical Sciences and the investigation conformed to the “Guide for the Care and Use of Laboratory Animals” published by the United States National Institutes of health.
- **Consent for publication :** all authors consent to the publication of the manuscript in Biomedical Engineering Online
- **Competing interests :** The authors declare that there is no conflict of interest
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- **Availability of data and material:** The material required to reproduce these findings are available. The processed data required to reproduce these findings are available.

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Figures

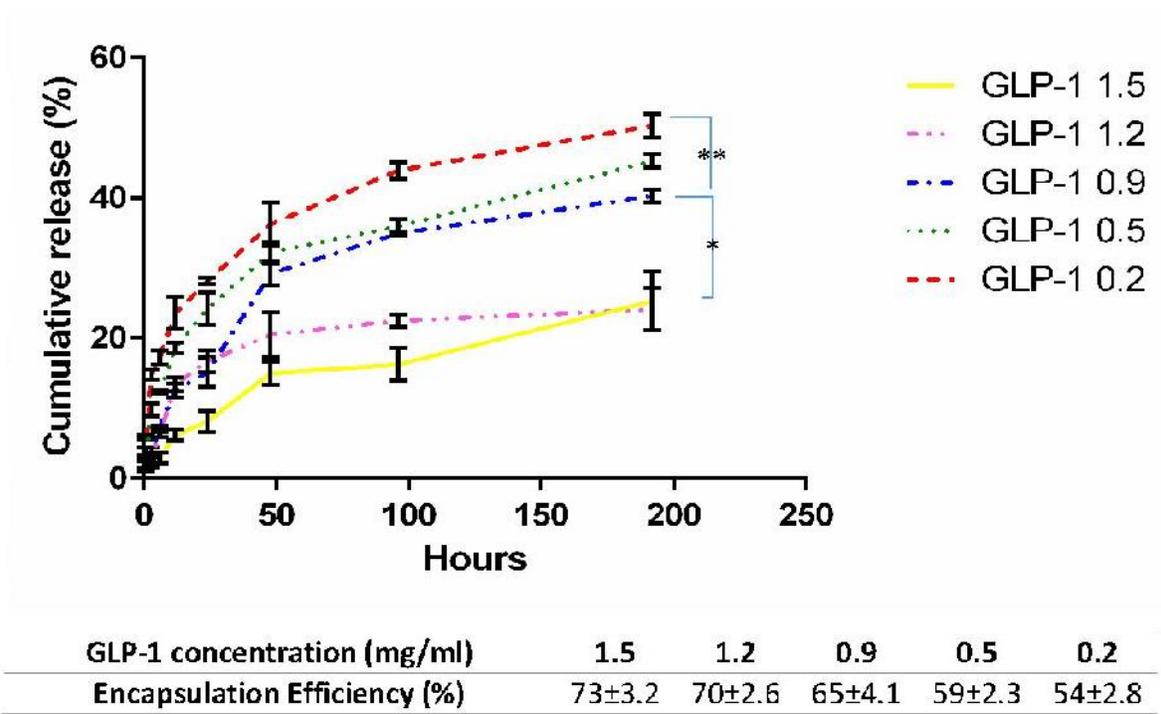


Fig 1. The cumulative release profile of nanoparticles with the different loaded GLP-1 amount. Chitosan concentration of 0.2% (w/v), Chitosan to TPP volume ratio of 5, and pH=6. n=3,* P<0.05 and **P<0.001 for the time point of 192h.

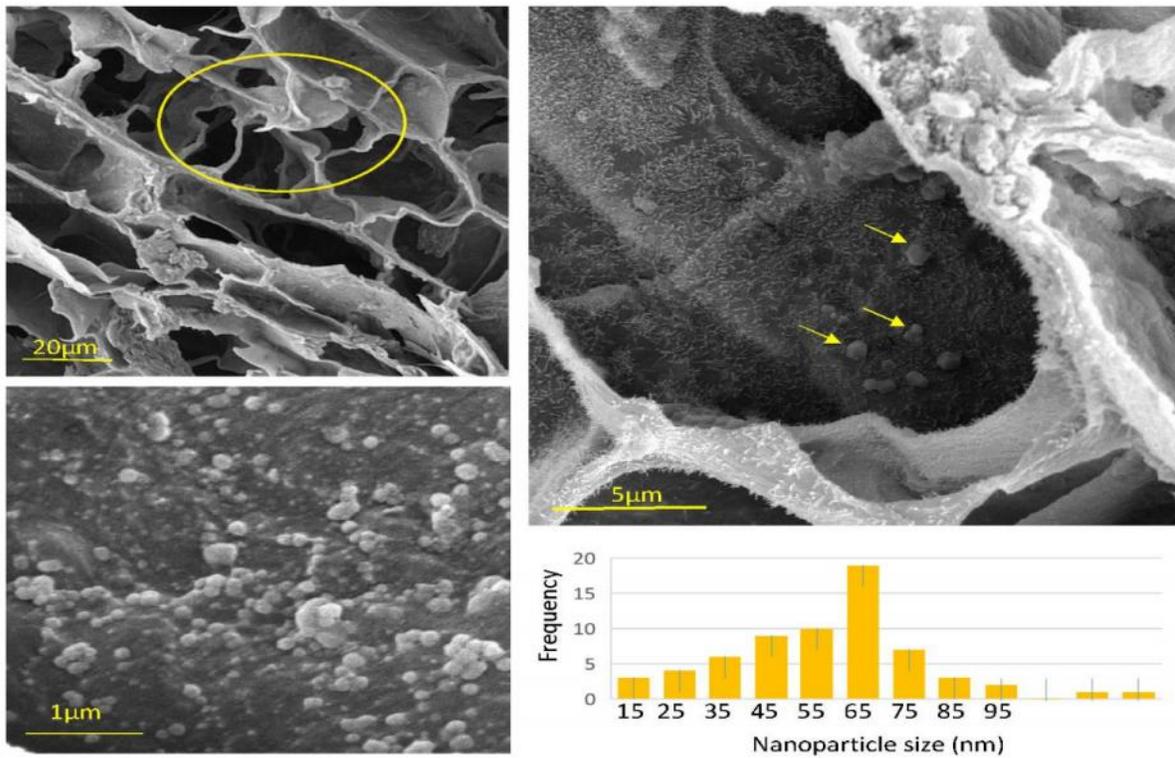


Fig 2. Scanning Electron Microscopy images of the porous structure of freeze-dried alginate gel (1.5 % w/w) containing chitosan nanoparticles at magnifications of a) 2kX, b) 10kX, c) 50 kX, and d) chitosan particle's size distribution.

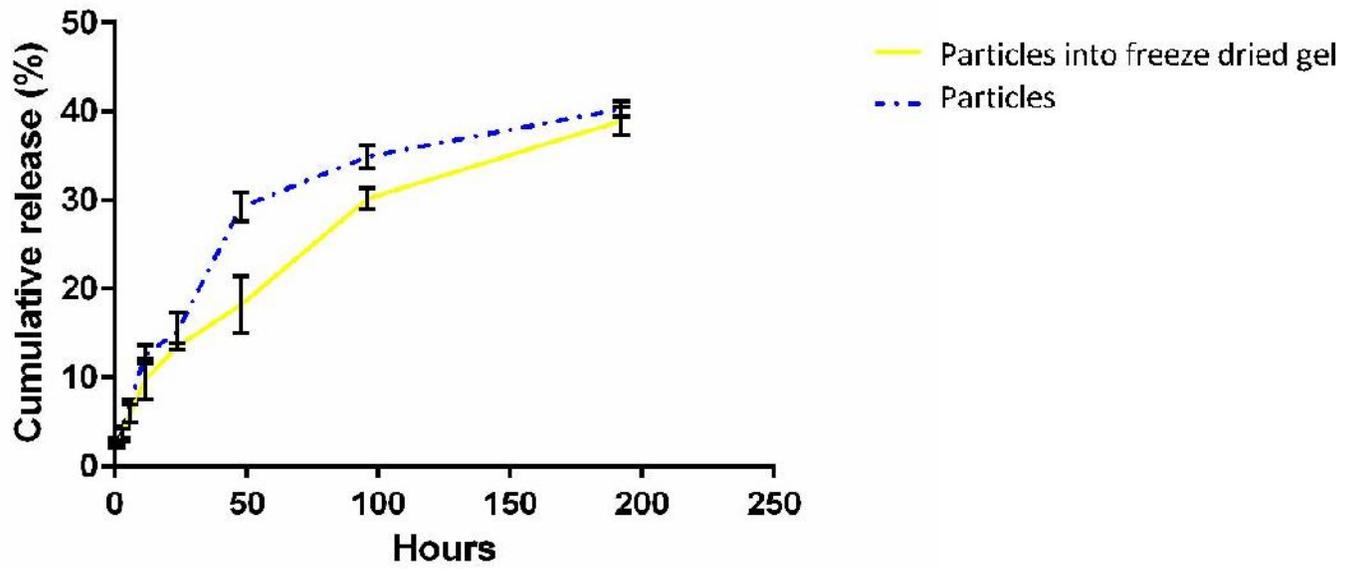


Fig 3. Comparison of release profiles of particles before and after incorporation into alginate freeze died gel (n=3).

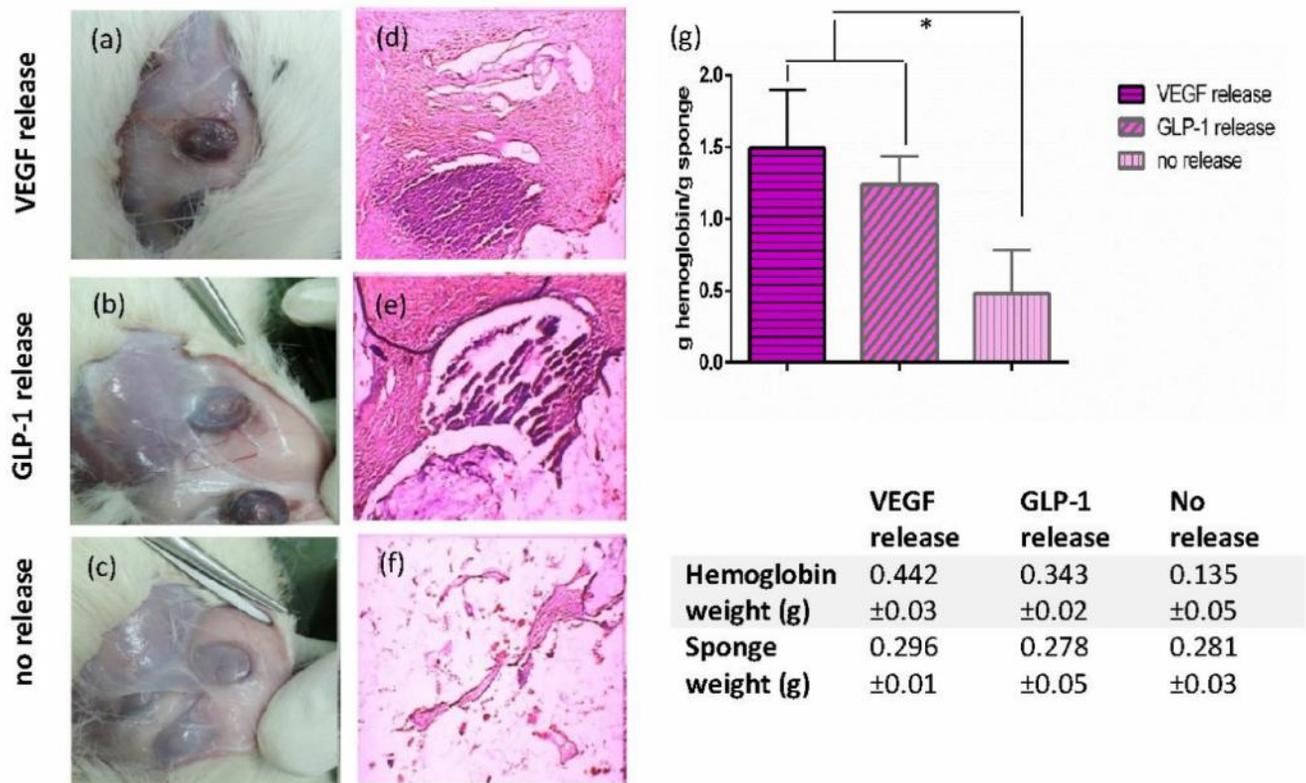
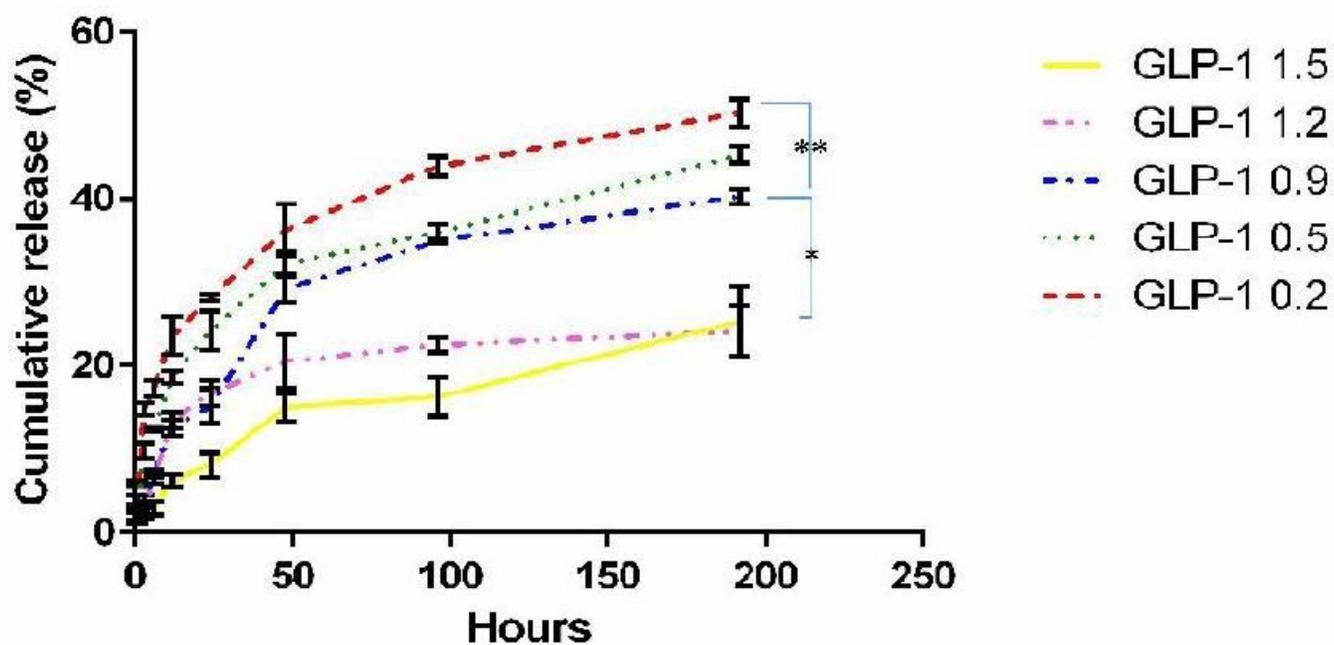


Fig 4. Results of in vivo test of sponge implant model of angiogenesis. (a,b,c) The appearance of samples after 192 h implantation into the back of mice; (d,e,f) histopathologic sections of alginate sponges after H&E staining in different experimental groups, (a and d: alginate sponges with VEGF delivery, b and e:alginate sponges containing chitosan nanoparticles with GLP-1 delivery , c and f: alginate sponges with no release), (g) hemoglobin content of three sponges. n=5 and P<0.05.

Figures



GLP-1 concentration (mg/ml)	1.5	1.2	0.9	0.5	0.2
Encapsulation Efficiency (%)	73±3.2	70±2.6	65±4.1	59±2.3	54±2.8

Figure 1

The cumulative release profile of nanoparticles with the different loaded GLP-1 amount. Chitosan concentration of 0.2% (w/v), Chitosan to TPP volume ratio of 5, and pH=6. n=3,* P<0.05 and **P<0.001 for the time point of 192h.

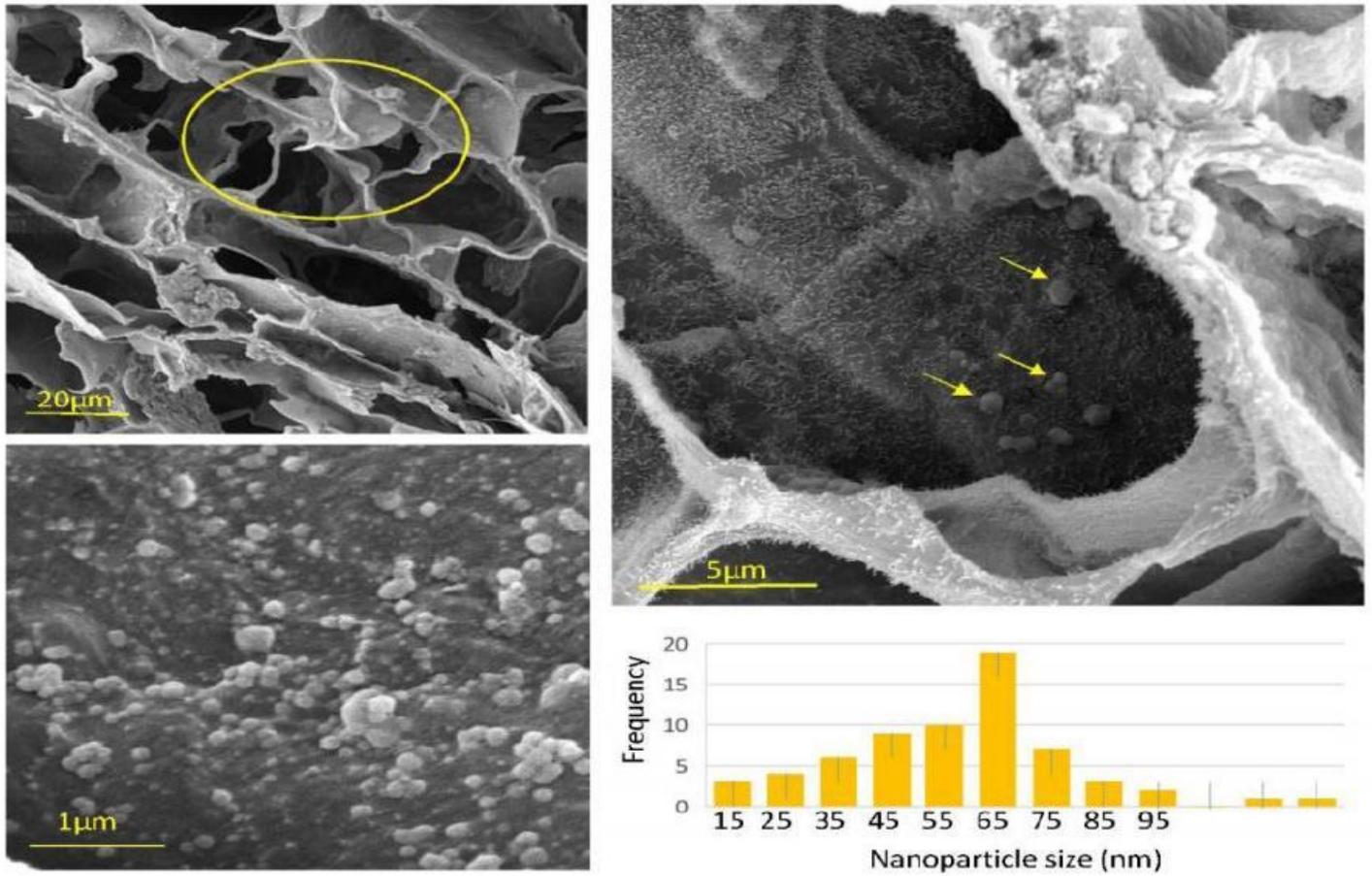


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Scanning Electron Microscopy images of the porous structure of freeze-dried alginate gel (1.5 % w/w) containing chitosan nanoparticles at magnifications of a) 2kX, b) 10kX, c) 50 kX, and d) chitosan particle's size distribution.

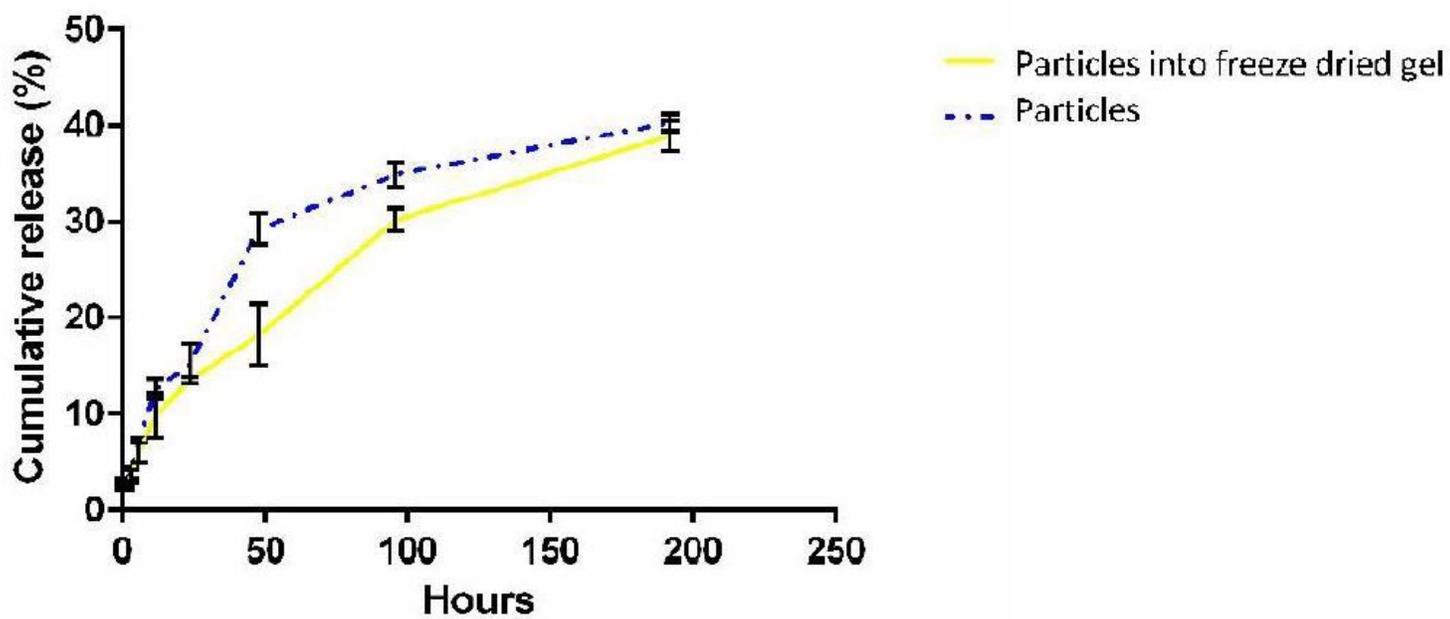


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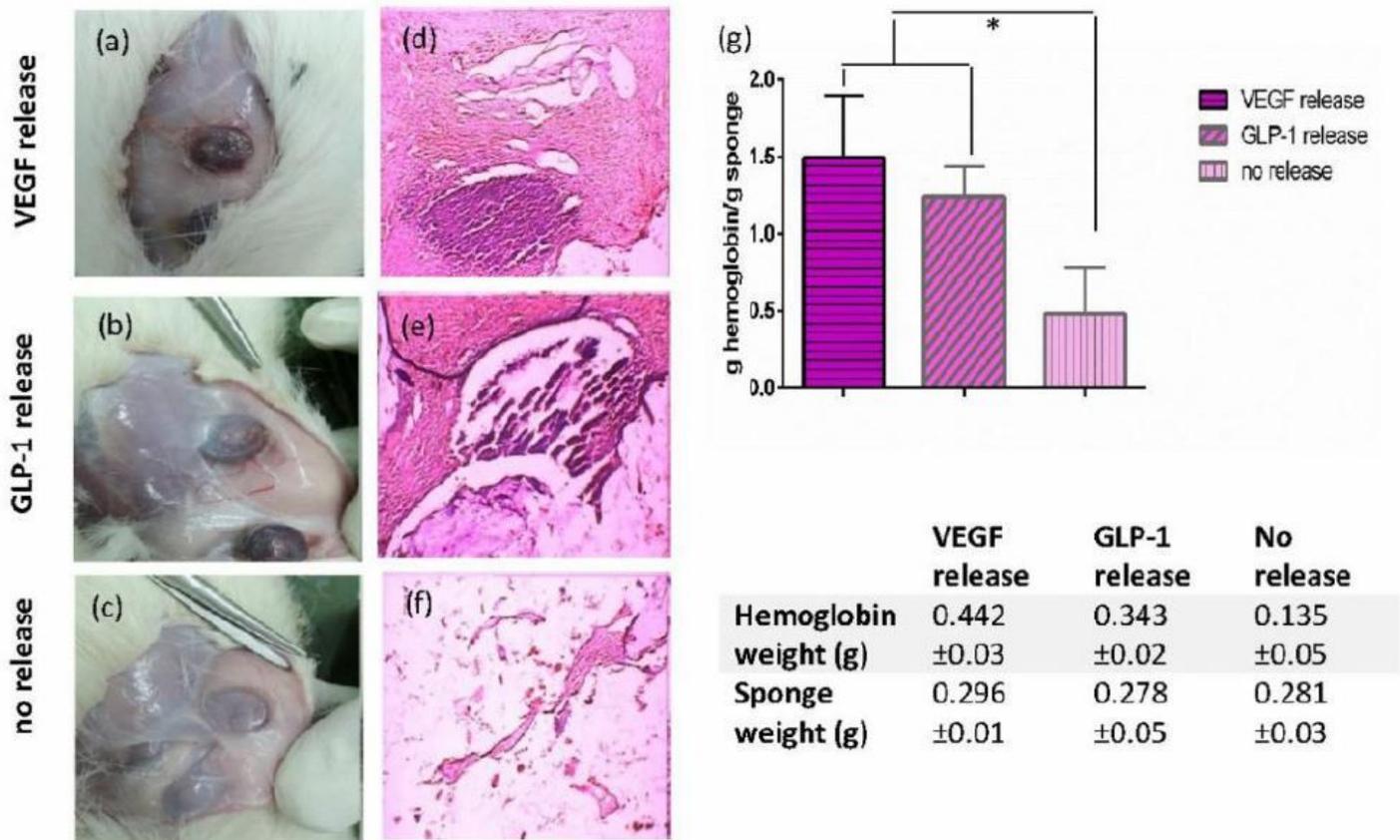


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