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Design a lymphatic specific delivery system of rHuKGF in rat and assessment of intestinal lymphatic uptake

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Abstract: The intestinal lymphatic (IL) delivery of polypeptides is a useful formulation technology to suppress chemical decomposition in the stomach and improve oral absorption. Moreover, the possibility of the absorption through the gut-associated lymphoid system could prevent the loss by presystemic hepatic first-pass metabolism. The aim of this work is to develop a method to deliver rHuKGF to the IL in rat and determine its uptake by the mesenteric lymph nodes (MLNs). The ionic gelation method was utilized for the preparation of rHuKGF-loaded chitosan nanoparticles (rHuKGF-CNPs), followed by freeze-drying and filled in Kollicoat MAE 100 P-coated porcine hard gelatin (KM100-PHG) capsules. rHuKGF-CNPs were administered intravenously (IV) and orally to rats. Finally, the pharmacokinetic parameters and IL uptake were determined by KGF (FGF7) Human SimpleStep ELISA Kit. The absolute bioavailability (%F) for the oral doses of rHuKGF, rHuKGF-CNPs, and KM100-PHG capsule filled with rHuKGF-CNPs were calculated to be 17%, 22%, and 74%, respectively. The results showed that oral administration of rHuKGF-CNPs in KM100-PHG capsules is a practical alternative to the IV administration since the capsule has successfully passed the stomach and released its contents in the small intestine. Moreover, a significantly higher extent of rHuKGF uptake by rat MLNs was detected with the oral administration of KM100-PHG capsule.

Keywords: lymphatic specific delivery system, rHuKGF, chitosan nanoparticles, pharmacokinetics, intestinal lymphatic uptake, cell regeneration.

1. Introduction

Fibroblast growth factor-7 (FGF7), also known as keratinocyte growth factor (KGF) is a signaling protein molecule that acts exclusively through the fibroblast growth factor receptor 2b (FGFR2b) and stimulates cell regeneration and wound healing by discrete expression manners [1].

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For the majority of epithelial derived cells, KGF is an essential mitogen and is a crucial mediator for their morphogenesis, growth, and differentiation. The expression of KGF is usually linked to wound repair and tissue expansion [2], while it is extremely up regulated in cutaneous wounds where it can stimulate gastrointestinal tract (GIT) wall thickening and speed the repair process [3]. Angiogenesis is important to support the nutritional demands of tissues that are being repaired or expanding. It occurs transiently in cycling reproductive organs and during wound healing by facilitating the growth of new capillaries from preexisting blood vessels. Angiogenesis is essential for several pathological conditions ranging from tumor growth to psoriasis [4,5]. Researchers observations revealed that KGF and five other members of the FGF family are also angiogenesis-dependent as one of their activities is the ability to stimulate epithelial cell regeneration by inducing the growth of new blood vessels and recruiting neutrophils [6,7]. Recently, researchers have reported that after the incidence of lung injury induced by influenza virus, epithelial cell regeneration is critically dependent on FGFR2b signaling. Quantius et al. [8] suggested that in the uninfected stem cell niche, inducing FGFR2b signaling by the therapeutic intrapulmonary application of FGF10 was found to counteract the failure of lung injury repair induced by influenza virus and to restore barrier function. Wang et al. [9] studies have shown that the administration of recombinant FGF2 resulted in recruitment of neutrophils and protected mice against influenza virus infection. Palifermin is a recombinant human KGF (rHuKGF) used to improve the functioning of patients with hematologic malignancies and treated with myeloablative chemotherapy and/or radiotherapy. It acts by reducing the duration and severity of oral mucositis and related clinical sequences [10].

Nanoparticles (NPs) represent a suitable platform to deliver polypeptides into the lymphatic system. However, NPs are required to efficiently reach the lymphatic vessels and remain inside for a significant period in order to achieve a selective lymphatic uptake [11]. There are various essential aspects for lymphatic delivering of conventional therapeutics by NPs; a smooth texture, size <200 nm, and spherical shape. Particles >500 nm are known to be eliminated rapidly from the circulation [12]. Nevertheless, it is still a challenge for therapeutic agents to reach the lymphatic targeted site (such as lymph nodes) due to the distinctive anatomy of the lymphatic system. Thus, an effective drug delivery system is urgently needed for efficient delivery of these therapeutics to the lymphatic system [13]. Recent studies have reported the potential of NPs delivery of drugs into the lymphatic tissues, which consequently reduces their side effects on healthy organs. Cai et al. [14] reported a successful lymphatic metastasis delivery of pH-sensitive hyaluronic acid doxorubicin complexes after peritumoral subcutaneous injection, resulting in improved anti-tumor activity compared with the free doxorubicin treatment. Fan et al. [15] reported that NPs conjugated to the follicle-stimulating hormone polypeptide have efficiently targeted the lymphatic metastases of ovarian cancer. Tan et al. [16] studies showed that poly-butyl cyanoacrylate NPs significantly improved the lymphatic targeting of vincristine sulfate. He et al. [17] have reported that calcium carbonate NPs were capable of delivering vascular endothelial growth factor into targeted cells and efficiently suppresses tumor growth, regional lymph-node metastasis, and tumor lymphangiogenesis of gastric cancer. In addition, Luo et al. [18] have reported that LyP-1-conjugated NPs can specifically target lymphatic metastases of pancreatic cancer.

Chitosan-based NPs have attracted increasing interest for their wide therapeutic applications including genes, proteins, and antineoplastic agents, administered through various routes including intravenous (IV), oral, ocular, and nasal delivery [19]. In 2015, Tummala et al. [20] developed 5-Fluorouracil-loaded chitosan NPs (CNPs) for colon-specific targeting to minimize the toxic effects of this drug on healthy cells. Due to the mucoadhesive properties of chitosan, these NPs improved the localization of the drug at the colon region.

In this work, a method for oral delivery of rHuKGF to the intestinal lymphatic (IL) system in the rat was developed to prevent the loss of this growth factor by chemical decomposition in the stomach and presystemic hepatic first-pass metabolism to increase its blood concentration and improve oral absorption.

2. Materials and Methods

2.1. Materials

Chitosan (Aldrich, Iceland), rHuKGF (Sigma, USA), trehalose (Merck, Germany), sodium tripolyphosphate (Sigma Aldrich, USA), Kollicoat MAE 100 P, molecular weight 135,000 (Sigma, Germany), KGF (FGF7) ELISA kit (abcam, UK), and veterinary porcine hard gelatin (PHG) capsules size M (Torpac Inc., USA) were used for this study.

2.2. Animals

Sixty-three Sprague-Dawley male rats, 250–300 g, 8-10 weeks old, were received humane care in accordance with the regulations of OECD guidelines. They were maintained in an air-conditioned room, subjected to 12 hours light and dark cycles, and given standard laboratory rat chow and free access to drinking water. During the experiments, animals were fasted for 12 hours before dosages administration and until 4 hours post-administration [21].

2.3. Methods

2.3.1. Dose calculation

In the dose calculation, bodyweight is not the single factor, although it is equally related and influences the scaling of the dose. Therefore, the correction factor (K_m) is usually used for the dose calculation and it is estimated by dividing the average body weight in kg of species to its body surface area in m^2 [22]. Typically, the human K_m factor is calculated by dividing the average human body weight (60 kg) by the human body surface area (1.62 m^2) and the resulted human K_m is 37. The rat K_m factor is calculated by dividing 0.15 by 0.025, and the resulted rat K_m is 6. The rat equivalent dose for rHuKGF was estimated from the Human Equivalent Dose (HED) of palifermin as:

$$\text{Animal equivalent dose (mg/kg)} = \text{HED mg/kg} \times (\text{Human } K_m / \text{Animal } K_m) \quad (1)$$

2.3.2. Nanoparticles preparation and capsules filling

Ionic gelation between chitosan and tripolyphosphate was utilized to prepare rHuKGF-loaded CNPs (rHuKGF-CNPs) as described in our previous report [23]. Following the preparation of rHuKGF-CNPs, 17.5 µL of trehalose (40 mg/mL) was added as a cryoprotectant. The mixture was frozen at -80°C for 24 hours, then lyophilized in a freeze dryer. Veterinary PHG capsules size M (Figure 1) were uniformly filled by the appropriate capsule-filling funnel (Torpac, X-M, USA) with 2 mg of the lyophilized rHuKGF-CNPs, to obtain the required rat dose of rHuKGF, combined with lactose as a diluent. To avoid capsule disintegration in the rat stomach, the filled capsules were coated with Kollicoat MAE 100 P (KM100) by dip-coating method as described in our previous report [24].

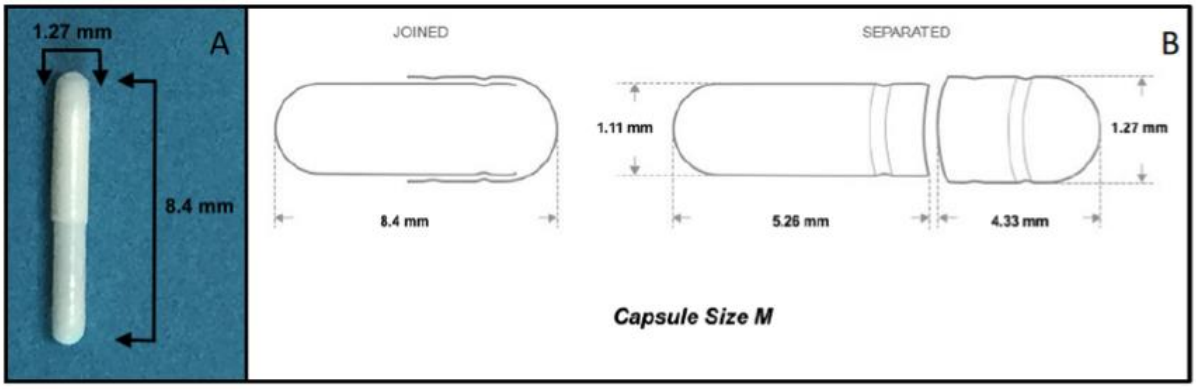

Figure 1. Empty capsule size M (A), and schematic diagram showing dimensions of capsule (B).

2.3.3. *In vivo* study design

This experiment was complied with the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines [25] and was performed according to the EU Directive 2010/63/EU for animal experiments. This protocol was approved by the University Kebangsaan Malaysia Animal Ethical Committee (UKMAEC), UKMAEC approval

number (UCSI/2018/PALANIRAJAN/25-JULY/936-DEC.-2018-DEC.-2019).

The animals were divided into six groups. The control group with eight rats given a placebo, a drug group with eight rats given 0.372 mg/kg/day recombinant growth factor as an oral solution, a drug group of eight rats given 0.372 mg/kg/day rHuKGF as an IV injection, a treatment group with eight rats given 0.372 mg/kg/day rHuKGF-CNPs as an oral solution, a treatment group with eight rats given 0.372 mg/kg/day rHuKGF-CNPs as an IV injection, and a treatment group with eight rats given KM100-coated PHG capsules filled with 0.372 mg/kg/day rHuKGF-CNPs. After treatment, anesthesia was administered to rats by intraperitoneal injection of ketamine (90 mg/kg), xylazine (4 mg/kg), and zoletil (20 mg/kg). Blood samples were immediately collected by venipuncture from the jugular vein in needles with no anticoagulant (for serum samples collection) or with ethylene diamine tetraacetic acid (EDTA).

Rats were sacrificed by euthanasia under anesthesia in accordance with the regulations of AVMA guidelines [26], by using a lethal dose (5 times anesthesia dose) of ketamine/xylazine/zoletil mixture and their intestines and mesenteric lymph nodes (MLNs) were removed immediately and placed in formaldehyde for further studies.

The prepared rHuKGF-CNPs formulation was given to the rats orally for 24 hours treatment, followed by anesthesia injected intraperitoneally to the examined rats.

The MLNs were removed after locating the duodenum by small incision to the rat abdomen. The collected MLNs were washed and homogenized in PBS by a homogenizer. 200 µL of the homogenate was added in a test tube containing 1.5 mL methanol. The resulted suspension was stirred for 3 hours followed by incubation at 65°C for 30 minutes, then centrifugation for 10 minutes [27]. The supernatant was collected into a new test tube and dried. To reconstitute the residues, 100 µL of methanol was added and centrifuged for 10 minutes. Finally, KGF (FGF7) Human Simple Step ELISA® Kit was used to determine the amount of rHuKGF present in the serum.

2.3.4. Data analysis

Each experiment was performed independently in triplicate at least. Statistical analysis was performed by GraphPad Prism Software 7.0c (La Jolla, CA). Animals' grouping was performed in a randomized manner. The statistical analysis of the difference between each group was tested by one-way ANOVA, followed by the Post hoc test for between-group comparisons. All results are presented as mean ± SD. Significance was accepted at $P < 0.05$.

3. Results and Discussion

3.1. Dose Calculation

The rat equivalent dose was calculated based on the human dose using formula (1). Since the human dose of palifermin is 0.06 mg/kg, the human K_m is 37 and the rat K_m is 6, the rat equivalent dose = 0.06 x (37/6) = 0.372 mg/kg.

3.2. Nanoparticles preparation and capsules filling

According to our previous report [23], the prepared rHuKGF-CNPs were in a monodisperse system with 0.217 PdI, + 20.3 ± 6.46 mV surface charge, 119 ± 74.62 nm particles size, and with a loading capacity of 93.3 ± 2.02 %. PHG capsules sizes M were filled with 2 mg of the rHuKGF-CNPs, to obtain the required rat dose of rHuKGF, combined with lactose as a diluent. To avoid capsule disintegration in the rat stomach, the filled capsules were coated with Kollicoat MAE 100 P (KM100) by dip-coating method.

3.3. *In vivo* study

3.3.1. Pharmacokinetics parameters

Veterinary KM-coated PHG capsules filled with rHuKGF-CNPs were developed and optimized for IL-specific delivery of rHuKGF. The summary of the obtained pharmacokinetic parameters for the developed rHuKGF formulations is presented in Table 1.

Table 1. Pharmacokinetic parameters of rHuKGF formulations. (mean ± SD, n=8)*.

Parameter	IV administration of rHuKGF	IV administration of rHuKGF-CNPs	Oral administration of rHuKGF	Oral administration of rHuKGF-CNPs	Oral administration of capsule filled with rHuKGF-CNPs
C_{Max} (pg/µL)	262.43±4.17	197.12±3.58	36.35±1.08	45.44±3.13	151.47±6.07
$t_{1/2}$ (hour)	1.86±0.12	2.93±0.14	0.89±0.07	1.07±0.12	3.58±0.29
$AUC_{(0-\infty)}$ (pg/h/µL)	1162.06±4.98	2202.59±13.80	105.51±3.76	131.88±2.91	439.69±12.36
$AUMC_{(0-\infty)}$ (pg/h/µL)	4065.816± 20.15	18587.32±19.64	178.72±8.03	223.40±5.74	744.68±10.12
Cl (L/hour)	0.1073±0.004	0.0809±0.005	1.27±0.07	0.8112±0.004	0.0730±0.006
Vd (L)	0.3861±0.0007	0.9631±0.001	0.0216±0.003	0.0271±0.006	0.0934±0.009
%F	100%	98%	17%	22%	74%

*$p < 0.05$

The blood concentration of palifermin is known to decrease after 30 minutes of a single IV dose and followed by a slight increase, then a terminal decline phase that occurs within 1 to 4 hours [28]. The extravascular distribution of palifermin normally shows a linear pharmacokinetics. Whereas, its volume of distribution in diseased subjects is 2-fold higher and its clearance is 2- to 4-fold higher than in healthy subjects. In both groups of healthy and diseased subjects, palifermin elimination half-life ($t_{1/2}$) was found to be 4.5 hours [28]. The IL delivery of rHuKGF could be a useful formulation technology to increase its blood concentration and improve oral absorption. The purpose of our study is to develop a method to deliver rHuKGF-CNPs to the rat IL system by utilizing the lower gastrointestinal localization of KM-100-coated PHG capsule since it only releases its contents at pH ≥5 (Figure 3).

The intestinal absorption of rHuKGF was promoted by utilizing the properties of both chitosan (mucoadhesive and absorption enhancement) and KM-100 (protective effect against the release of rHuKGF into the stomach). The pH-dependent solubility of KM100-coated PHG capsule was increased at pH ≥5, as the lowest bloom strength of porcine skin gelatin was found to be at pH 5 [29], and KM100 only dissolves at pH >6. Therefore, this capsule on oral administration would release its content only into rat intestine, suggesting a potential intestinal-targeted drug delivery system for rHuKGF.

In this study, the pharmacokinetic parameters and the IL uptake of the developed rHuKGF formulations were determined in rats based on the serum rHuKGF levels. After a single-dose administration, rHuKGF exhibited a one-compartment linear pharmacokinetics model with first-order kinetics in the dose of 0.372 mg/kg. Table 1 is presenting the summary of the obtained pharmacokinetic parameters for the developed rHuKGF formulations. Following IV administration, the serum concentration of rHuKGF was rapidly increased up to 262.43 ± 4.17 pg/µL over 3 hours of treatment. Whereas slower absorption and sustained elimination were observed after the IV administration of rHuKGF-CNPs, reaching rHuKGF C_{max} of 197.12 ± 3.58 pg/µL after 6 hours of treatment and that was mainly because of the sustained-release of rHuKGF from CNPs. On the other hand, lower levels of serum rHuKGF (36.35 ± 1.08 pg/µL) were detected after the oral administration of rHuKGF due to the denaturation of rHuKGF in the gastric environment. But, a slight increase in rHuKGF C_{max} (45.44 ± 3.13 pg/µL) was detected, $P < 0.05$, after the oral administration of rHuKGF-CNPs, suggesting that CNPs provided little protection from the gastric environment.

The oral administration of KM100-coated PHG capsule filled with rHuKGF-CNPs resulted in a rHuKGF C_{max} of 151.47 ± 6.07 pg/µL (after 6 hours from the administration) and accompanied by delayed absorption and sustained elimination. This delay can be explained as the capsule remained intact in the gastric conditions, and released its content only at the intestinal pH since this condition is optimal for the capsule to be disintegrated. The elimination of the IV doses of rHuKGF and rHuKGF-CNPs was rapid in comparison to the oral capsule dose, with $t_{1/2}$ of 1.86 ± 0.12, 2.93 ± 0.14 hours, and 3.58±0.29 hours, respectively.

Furthermore, rHuKGF serum concentration of the oral capsule group was significantly (P <0.05) different from that of the oral rHuKGF and rHuKGF-CNPs groups.

The AUC of the orally administered capsules was 439.69 ± 12.36 pg/h/µL and for oral rHuKGF and rHuKGF-CNPs groups were 105.51 ± 3.76 pg/h/µL and 131.88 ± 2.91 pg/h/µL, respectively. The %F for the oral doses of rHuKGF, rHuKGF-CNPs, and KM100-coated PHG capsule filled with rHuKGF-CNPs was calculated to be 17%, 22%, and 74%, respectively. Following the oral administration of rHuKGF-CNPs, the initially detected serum levels of rHuKGF indicated that the absorption of rHuKGF from this formulation is significantly higher compared to pure rHuKGF, orally-administered, formulations, P <0.05. The subsequent continuous absorption of rHuKGF from CNPs-formulation, over 6 hours, is mainly due to the ongoing sustained-release of rHuKGF during the adhesion of CNPs to the intestinal mucosa. It also could be due to the NPs passage to the posterior small intestine, which contributed to this absorption pattern. Despite the mucoadhesive properties, CNPs could slide along the intestine while maintaining their physical structure integrity. In fact, CNPs exhibit slow transit at the upper small intestinal *in vivo* and mucoadhesive capacities *in vitro*. However, this intestinal retention was only limited to a short period. The CNPs arrival to the posterior small intestine occurs in a few hours and due to the maintained mucoadhesive properties, the intestinal-uptake is promoted [30,31].

3.3.2. Intestinal lymphatic uptake study in rats

As shown in Figure 2, the uncoated PHG capsule has released its contents in both the small intestine (Figure 2A) and stomach (Figure 2B) after 1 hour of administration. On the other hand, the KM100-coated PHG capsule has successfully passed the stomach and released its contents only in the small intestine (Figure 3A) and there was no release detected in the rat stomach (Figure 3B). Thereafter, the lymphatic uptake of rHuKGF was determined after each administration as presented in Figure 4. A significantly higher extent of lymphatic uptake was only detected after the oral administration of KM100-coated PHG capsule filled with rHuKGF-CNPs formulation, P <0.05.

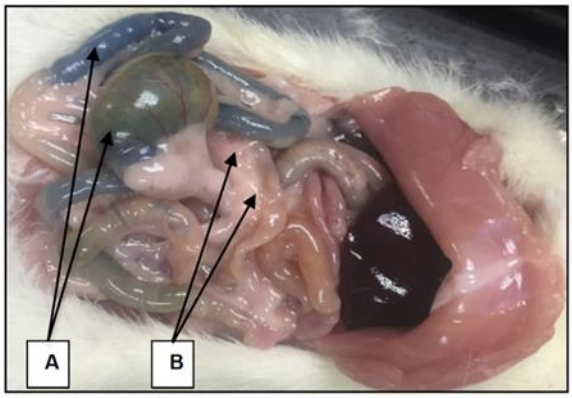

Figure 2. The release of PHG capsule (uncoated) filled with Coomassie Brilliant Blue in rat stomach and small intestine after 1 hour of administration (A). The location of MLNs (B).

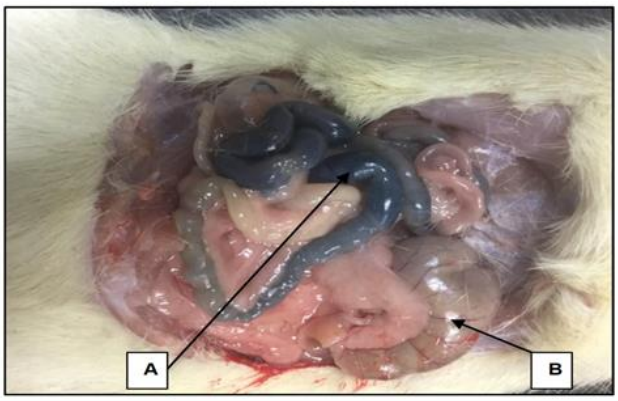

Figure 3. The release of KM100-coated PHG capsule filled with Coomassie Brilliant Blue in rat small intestine after 1 hour of administration (A). There was no release detected in the rat stomach (B).

IL-targeted drug delivery system is currently attracting more attention as it offers improvement of the oral absorption of macromolecular drugs. To achieve selective intestinal delivery, the developed capsule needs to successfully pass the stomach and only release its content in the intestine. Recently, Gomez-Lado et al. [32] have reported that in non-anaesthetized rats, the majority of the size 9 Eudragit L100-coated capsules did not empty from the stomach, but most of the smaller size 9h Eudragit L100-coated capsules were successfully emptied from the stomach to the intestine. Therefore, even smaller capsule size (size M) was used in this study to ensure successful gastric

emptying. Therefore, the developed delivery method for rHuKGF appeared to stabilize and protect it from the degradation, in the stomach, as rHuKGF was detected in high concentration in serum, and able to deliver rHuKGF to rat MLNs at higher extent following the KM100-coated PHG capsule administration.

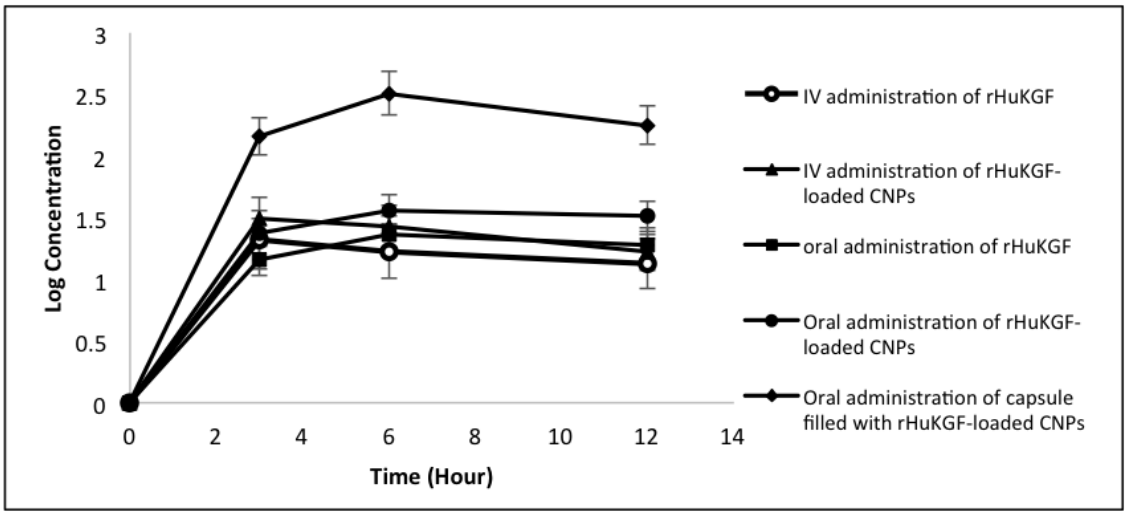

Figure 4. The concentration of rHuKGF present in rat MLNs fluid after each administration determined by ELISA test-based quantitative analysis (n = 3).

4. Conclusions

This study revealed that intestinal-lymphatic delivery of rHuKGF in rats through oral administration of rHuKGF-loaded CNPs filled in Kollicoat MAE 100 P-coated PHG capsules is a practical alternative to the IV administration since the capsule has successfully passed the stomach and released its contents in the small intestine and resulted in 74% absolute bioavailability. Moreover, a significantly higher extent of rHuKGF uptake by rat MLNs was detected with the oral administration of KM100-PHG capsule.

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Conflicts of Interest

The authors declare no conflict of interest.

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