Nasal Delivery of Recombinant Human Growth Hormone: *In Vivo* Evaluation with Pheroid™ Technology and N-Trimethyl Chitosan Chloride

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ABSTRACT - Purpose. It was the aim of this study to investigate the possible enhancement of the absorption of recombinant human growth hormone (rhGH) in the nasal cavity, in the presence of a polymeric absorption enhancer, *N*-trimethyl chitosan chloride (TMC) and a patented fatty acid-based delivery system, Pheroid. **Methods**. Two types of Pheroid formulations, Pheroid vesicles and Pheroid microsponges were characterized and evaluated with regard to particle size and morphology. *In vivo* bioavailability studies in rats were performed and the nasal bioavailability of Pheroid vesicles and Pheroid microsponges were compared relative to subcutaneous administration. The results were also compared with different *N*-trimethyl chitosan chloride (TMC) formulations, TMC H-L and TMC H-H, well studied absorption enhancers. **Results.** Pheroid vesicles and Pheroid microsponges showed a size distribution of approximately 2-3 µm and 3-4 µm for Pheroid vesicles and Pheroid microsponges respectively. Using specific RIA, the relative bioavailability of rhGH after comparison with subcutaneous injection was determined to be 38.9, 128.5, 39.9, 136.3, and 8.3 % for Pheroid microsponges, Pheroid vesicles, TMC H-H, TMC H-L and control group (intranasal rhGH alone), respectively. All the enhancers showed significant absorption enhancement ($P < 0.05$) with the highest effect observed with TMC H-L. **Conclusion.** All the enhancers may have promising potential as safe and effective nasal absorption enhancers of rhGH.

The Pheroid delivery system is a novel patented system consisting of a unique submicron emulsion type formulation. The system, which consist mainly of essential fatty acids can entrap and transport pharmacological active compounds. In this study it is shown that two types of Pheroid formulations, Pheroid vesicles and Pheroid microsponges, have the ability to entrap recombinant human growth hormone and improve the nasal absorption and thus bioavailability of rhGH. Intranasal administration of rhGH entrapped in Pheroid technology is an attractive alternative to subcutaneous of intravenous administration.

INTRODUCTION

Recombinant human growth hormone (rhGH) is a long chain amino acid molecule with a molecular weight of ± 22 kDa. Short stature in children due to growth hormone deficiency and chronic renal failure or Turner's syndrome is most often treated with human growth hormone. GH is one of the most widely used hormones in supplementation. Years of administration of this agent have proved its safety and efficacy in the therapy of various conditions associated with short stature [1]. GH plays an important role by increasing serum levels of Insulin growth factor (IGF-I) and IGF-binding proteins, among other metabolic functions. Long term GH exposure in humans exerts similar impacts on IGF-I levels, IGF-binding proteins, bone metabolism, body composition and insulin sensitivity than daily GH injections [2, 3]. While daily injections are still the most accepted route of administration, the bioavailability of GH remains low. The use of a delivery system for nasal administration is a promising alternative to both daily injections and long term continuous subcutaneous infusion of GH. Convenience and compliance in patients will improve by avoiding daily injections. It is also an attractive treatment option for patients who receive GH for other indications rather than replacement therapy [1].

The nasal cavity is a well known route of drug administration for the treatment of various diseases. Despite the extensive blood supply and the relative large surface area in the nasal cavity, the permeability of the nasal mucosa is normally low for polar molecules, including low molecular weight drugs and especially for large molecular weight peptides and proteins [4].

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One approach to counter the limitations of nasal drug administration is to implement alternative delivery systems. Delivery system must have the ability to successfully deliver a molecule into the systemic blood circulation [4, 5, 6]. A delivery system can be designed to make use of some of the various existing epithelial transport mechanisms. These mechanisms include various transcellular transport processes and paracellular diffusion. A drug carrier can for example be employed to target transport carrier proteins or to open tight junctions between the epithelial cells [7, 8, 9]. Several experimental delivery systems for the improved nasal delivery of rhGH have been described. These include dry polymer particles such as starch microspheres [10], chitosan [11] and a thiomer/glutathione microparticulate delivery system [12].

Lipid based colloidal delivery systems are stable isotropic dispersions that enhances the bioavailability of various drugs, including peptides. Pheroid is a novel patented colloidal drug delivery system comprised of submicron lipid based structures. Pheroids can be manipulated to suite various therapeutic applications and are able to entrap virtually any size of drug molecule. Furthermore, Pheroids can be formulated in different forms to manipulate its release characteristics. A further advantage of the Pheroid delivery system is the fact that it only consists of essential fatty acids and other ingredients essential to the body. Pheroid are proposed to improve the transcellular transport of molecules [13, 14, 15]. Polymeric delivery systems, including chitosan and its derivatives, have mucoadhesive properties, good water solubility and tight junction modulatory effects. N-trimethyl chitosan chloride (TMC) is a well described and studied cationic polymer that opens tight junctions and improves paracellular diffusion of hydrophilic drug molecules [16, 17]. The absorption enhancing effect of TMC can be attributed to interactions of the fixed positively charged quaternary amino groups on the C-2 position of TMC directly and indirectly with the cell membranes [18, 19]. It is easy to incorporate TMC into drug formulations and absorption enhancement of peptide molecules can be achieved [20, 21, 22].

The aim of this study was to evaluate the potential application of Pheroid technology as drug carrier system for the nasal delivery of rhGH. The obtained results were related to the absorption enhancement obtained with TMC, a well-known absorption enhancer for macromolecular compounds across mucosal surfaces. Recombinant hGH was administered intranasally to rats in a Pheroid vesicle, a Pheroid microsponge and two different TMC formulations, TMC H-H (high molecular weight with high degree of quaternisation) and TMC H-L (high molecular weight with low degree of quaternisation).

MATERIALS AND METHODS

Materials

Recombinant human growth hormone (rhGH, 1 mg = 3 IU) was supplied by Warren Chem Chemical Specialities (Midrand, RSA) after the necessary authorization on the method was obtained from the Department of Health, South Africa. N-trimethyl chitosan chloride (TMC) was previously synthesised and characterized [23] from Primex chitosan raw material with a DA of 97% and a molecular weight of 263 700 g/mole. Two different TMC polymers were used in this study namely, TMC H-H with a 73.33% degree of quaternisation and a molecular weight of 262 400 g/mole and TMC H-L with a 18.20% degree of quaternisation and a molecular weight of 288 500 g/mole. Heparin sodium (5 000 IU/ml) was supplied by Intramed in South Africa. The Immunoradiometric assay (IRMA) kit for the quantitative measurement of rhGH in plasma was supplied by Biosource (South Africa). Vitamin F ethyl ester was obtained from Kurt Richter Pharma, Germany, Cremaphor EL from BASF, Germany, D-α-tocopherol from Chempure, South Africa, Incromega 7010 and Incromega 3322 (used 1:1) from Croda Chemicals, South Africa.Nile red was supplied by Sigma Aldrich and FluoReporter FITC protein labelling kit by Molecular Probes (Invitrogen).

Flourescent Labelling of rhGH

FITC was covalently bound to rhGH using the FluoReporter FITC protein labelling kit, according to the protocol provided by the manufacturer. In brief, 10 mg/ml of FITC in DMSO was slowly added to 1 mg/ml rhGH prepared in 1M bicarbonate solution. The reaction was stirred at room temperature for one hour. Excess FITC was separated on spin column provided with the kit. The FITC-rhGH conjugate was stored at 4°C until use. The degree of labelling was calculated to be 5.8 FITC dye molecules per protein molecule.

Preparation of the control and TMC formulations

In this study a subcutaneous dose of 0.6 IU/kg bodyweight of rhGH was administered as a reference standard. This formulation was prepared immediately before injection by dissolving the freeze-dried rhGH powder in physiological saline. A dose of 3.6 IU/kg bodyweight of rhGH was administered via the nasal cavity for the control formulation and experimental formulations. The control formulation was prepared by dissolving rhGH powder in physiological saline. The TMC formulations were prepared by dissolving rhGH powder in a 0.5% w/v TMC H-H solution (high molecular weight with high degree of quaternisation) and a 0.5% w/v TMC H-L solution (high molecular weight with low degree of quaternisation). These formulations were shaken for 30 minutes to ensure even distribution and complete dissolution.

Preparation of the Pheroid formulations

Two different Pheroid formulations were used and manufactured as previously described [15]. Pheroid vesicles were prepared by melting vitamin F ethyl ester (2.8 % w/v), Cremaphor EL (1 % w/v) and *D*-α-tocoferol (0.2 % w/v). This mixture constitutes the oil phase of the Pheroid vesicles. Purified water saturated with N_2O was heated and maintained at 75° C. The previously heated oil mixture was then added to the heated N_2O saturated water to form the vesicles. The water-oil mixture was homogenised with a Heidolph Diax 600 homogeniser (Labotec, Johannesburg, South Africa) at 8000 rpm for 2 minutes. This homogenous mixture was then placed in amber glass bottles and shaken continuously and slowly until the mixture reached room temperature after which the bottles, now containing Pheroid vesicles, was placed in a refrigerator and kept at a temperature of 5° C. Pheroid microsponges were prepared in exactly the same way as described above for the Pheroid vesicles, with the addition of Incromega 7010 and Incromega 3322 (0.25:0.25 % w/v) to the oil phase before heating. Formulations for nasal administration were was prepared by warming a sufficient volume of either freshly prepared Pheroid vesicles and Pheroid microsponges to room temperature. The rhGH powder was weighed and dissolved in Pheroid vesicle or Pheroid microsponge formulations and shaken for 30 minutes. Two different formulations for both Pheroid vesicle and Pheroid microsponges were prepared, one as described above (rhGH entrapped for 30 minutes) and another formulation that was prepared in the same way but was left to stand in a fridge at 5° C for a further 24 h before administration. The purpose of this was to allow further entrapment of rhGH (ca 22 kDa) into the Pheroids.

Characterization of Pheroids

The particle size of the Pheroid vesicle and Pheroid microsponge formulations, prior and after entrapment of rhGH, was measured with a Malvern Mastersizer (Malvern Instruments, UK). The morphological features of Pheroid vesicles and Pheroid microsponges were assessed with confocal laser scanning microscopy as previously described [15]. The images were taken with a Nikon D-Eclipse C1 confocal laser scanning microscope with a DXM 1200 digital camera. The entrapment of FITC-rhGH was visulaised with a green krypton laser (excitation 488 nm, emission 515nm) and nile red fluorescence with a red helium neon laser (excitaion 505 nm, emission 563 nm).

Nasal administration

All surgical and experimental procedures were approved by the ethics committee of the North-West University (South Africa). Male Sprague Dawley rats with bodyweights of approximately 300 g were fasted for 18 h, but water was supplied *ad libitum*. Six rats were used per treatment group. Anaesthesia was induced by inhalation of liquid halothane (Fluothane, Zebeca SA (Pty) Ltd, Woodmead, RSA), in medical oxygen. To maintain anaesthesia the rat was attached to a rubber jacket that fits securely over its head. The remaining end of the rubber jacket was connected to a two-way selector that was connected to either one of two 5-litre plastic bags. The bags contained either 2 % v/v or 4 % v/v halothane in oxygen. The *carotis communis* artery was cannulated for blood sampling and fluid replacement.

Between 95 and 105 µl (0.6 IU/kg rhGH) of the subcutaneous formulation was injected directly under the skin in the abdominal region of each rat in the subcutaneous group. The other formulations were administered via the nasal cavity. A single dose ranging between 25-35 µl was administered via the left nostril of each test subject at a dose of 100 µl/kg bodyweight. The final concentration rhGH, administered nasally to each rat was 3.6 IU/kg as determined by a simple calculation based on the bodyweight of the rat. Blood samples with a volume of 800 µl were collected from the cannula in the *carotis*

communis into Eppendorf micro test tubes (Merck, RSA). Samples were collected at different time intervals over a period of 3-5 h. The t = 0 time sample was taken one minute prior to administration and thereafter samples were taken at 5, 10, 15, 30, 60, 120 and 180 minutes after administration. Extended monitoring was done with the Pheroid treatment groups and samples were taken for 300 minutes. Blood samples taken at each time interval were replaced. All samples were centrifuged (Eppendorf centrifuge 5415C, Merck, South Africa) within 20 minutes of collection at 7000 rpm for 7 minutes. The recovered plasma samples were stored at -70° C for not more than 3 weeks before all samples were analyzed.

Quantitative measurement of rhGH in plasma
To determine the plasma rhGH concentrations a commercially available ultra sensitive rhGH-IRMA kit was used (BioSource Europe S.A., Nivelles, Belgium). The instructions provided by the manufacturer were followed exactly and the resulting vials were placed in a Gammatec II gamma counter (Canberra Company, South Africa) to determine the radioactivity and to calculate the plasma rhGH concentrations using a log-linear curve fit.

Pharmacokinetic analysis
Plasma concentrations versus time data obtained for the different formulations in individual rats were analyzed by means of a one-compartment open model analysis method. The area under the rhGH concentration-time curves extrapolated to infinity ($AUC_{0\to\infty}$) following subcutaneous or nasal administration was calculated using the trapezoidal method. The maximum plasma concentrations (C_{max}) and the time to reach the maximum concentration (T_{max}) were determined from the pharmacokinetic profiles generated by plotting the plasma concentrations of rhGH (μIU/ml), obtained from administering the different formulations, against time. The bioavailability for each nasal formulation relative to the subcutaneous injection (s.c) was calculated using the following equation:

$$\text{Bioavailability (\%)} = (AUC_{nasal} \times Dose_{s.c.}) / (AUC_{s.c.} \times Dose_{nasal}) \times 100 \quad (1)$$

Statistical analysis
All values are expressed as the means ± standard deviation (SD). To determine statistical differences the data were analyzed by two-way ANOVA with the Bonferroni post test. All statistical analysis was performed using GraphPad Prism version 4.00 for Windows, (GraphPad Software, San Diego California USA). A *p* value of less than 0.05 was termed significant.

RESULTS

The average particle size of the Pheroid vesicles was 2.19 μm (volume weighted mean). After addition of rhGH (30 minute entrapment period), the particle size increased to 3.01 μm. The particle size of the Pheroid microsponges, before entrapment of rhGH (30 minute entrapment period), was 3.32 μm. After the addition of the rhGH, the particle size increased to 4.33 μm. Similar results were obtained after the 24 hour entrapment period (results not shown). Figure 1 A and C show the porous nature of Pheroid microsponges containing rhGH and Figure 1 B and D the vesicular structure of Pheroid vesicles containg rhGH. Pheroids readily associated with the lipophilic dye and the rhGH which is more hydrophilic can be seen in the aqueous spaces of the Pheroids.

To give an indication of the relative bioavailability of rhGH, a dose of 0.6 IU/kg bodyweight was administered subcutaneously in the lower abdominal region of the rats. Figure 2 shows the resulting rhGH plasma concentration-time profile after subcutaneous injection and the pharmacokinetic data are shown in Table 1. A maximum plasma concentration (C_{max}) of 5.2±2.6 μIU/ml was reached 30 minutes after injection. Negligible absorption was observed for the control (nasal, 3.6 IU/kg bodyweight) formulation at all the time points. There were no adverse systemic events evident in the rats following subcutaneous or nasal administration of rhGH. Relative bioavailability of 8.3±11.9 % compared to subcutaneous injection was calculated for the control formulation (Table 1).

The rhGH plasma concentration-time profiles after administration of the Pheroid microsponge and Pheroid vesicle formulations (rhGH entrapped for 30 minutes), initially measured over 180 minutes, are depicted in Figure 3. In contrast to the control formulation, the nasal administration of rhGH with Pheroid vesicles led to a significant increase in the absorption of rhGH. A plasma concentration of 4.7±1.2 μIU/ml was reached at 180 minutes, which indicated that the Pheroid formulations had to be monitored over an extended time period as the plasma concentration of rhGH was still on the increase.
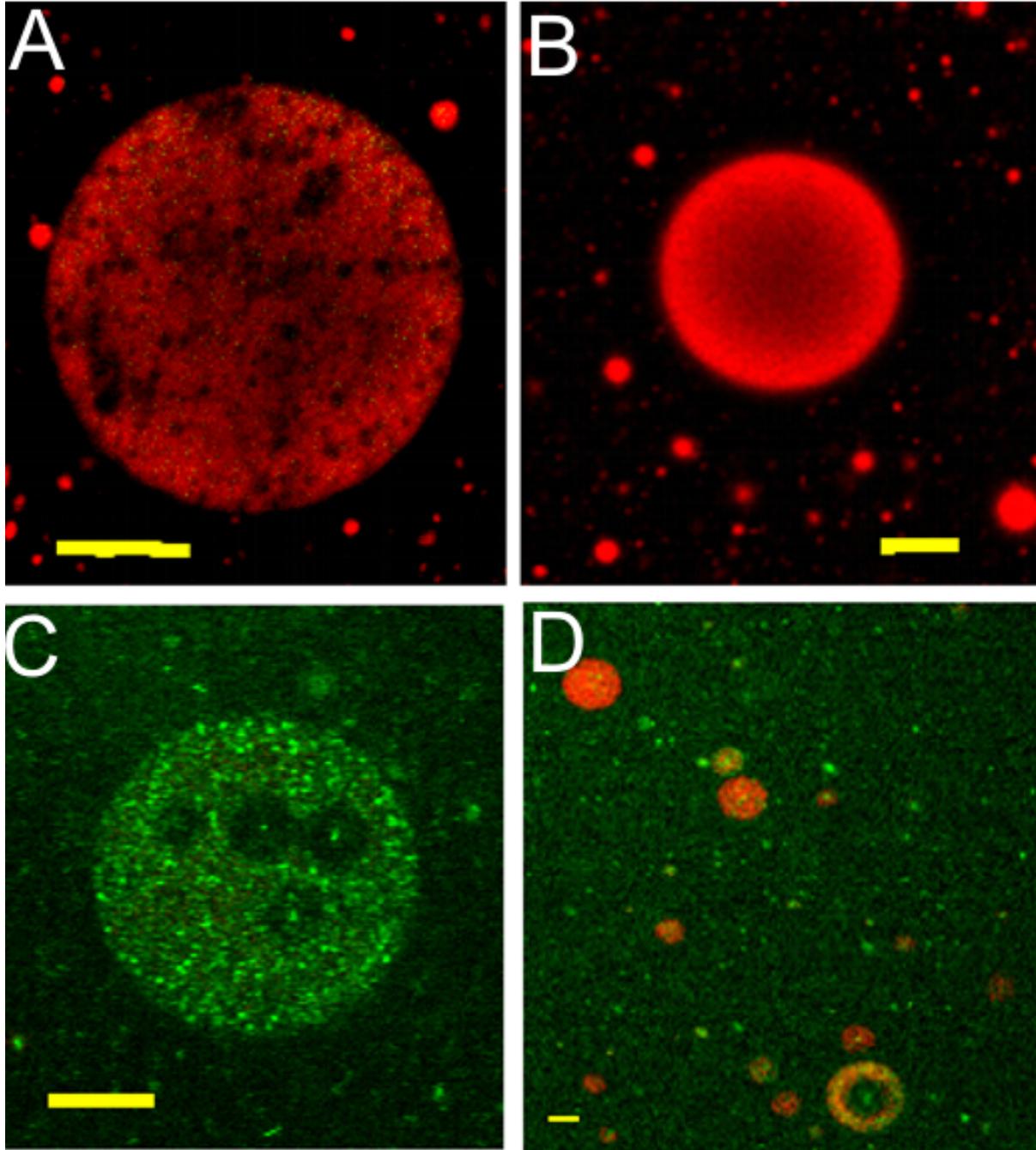
Figure 1. Confocal laser scanning microscopy images of A) a single Pheroid microsponge showing the porous nature, B) Pheroid vesicels, C) Pheroid microsponges entrapped with rhGH and D) Pheroid vesicels entrapped with rhGH. Scale bars represents 1 μm.
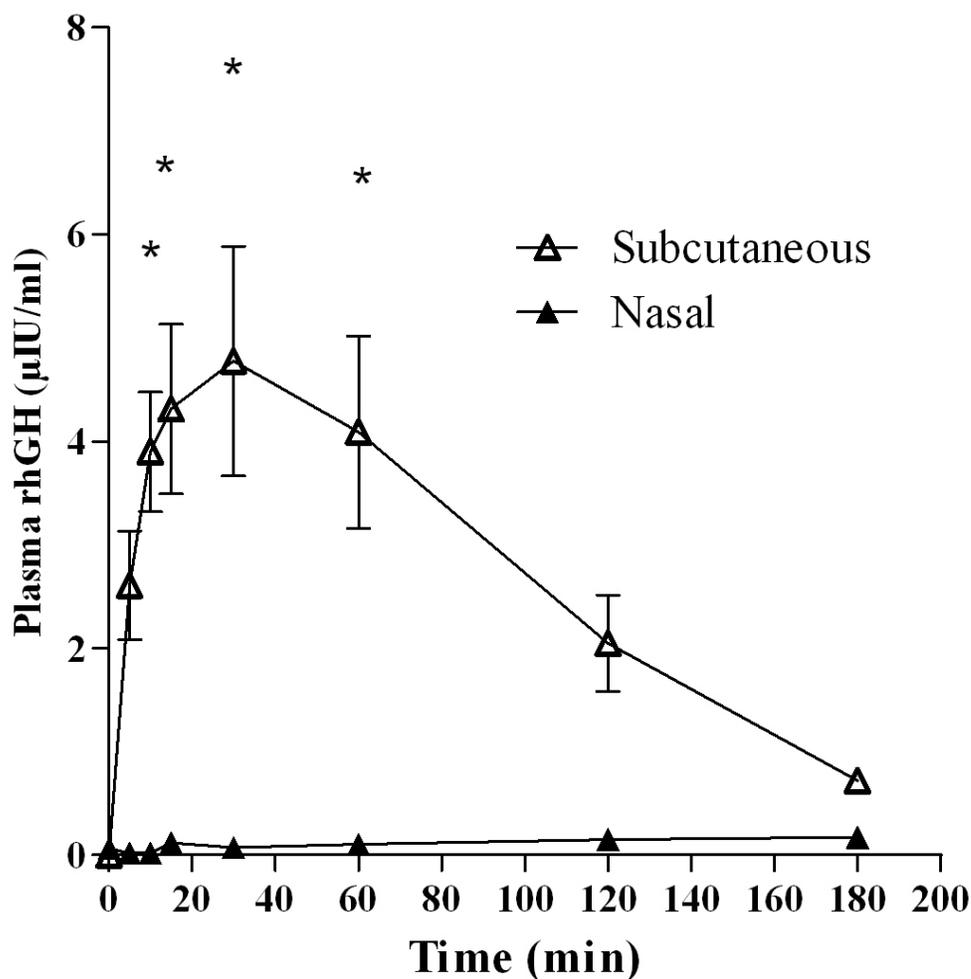
Figure 2. Concentration-time profile of rhGH in rat plasma obtained after subcutaneous injection of 0.6 IU/kg rhGH in physiological saline and after nasal administration of 3.6 IU/kg rhGH in physiological saline (mean ± SD, n=6). *Statistically significant from the control (nasal) $p<0.05$.

Table 1. Main pharmacokinetic parameters after nasal administration of the control, Pheroid microsponges, vesicles, TMC H-L and TMC H-H formulations. The subcutaneous injection of rhGH to rats are also shown (means ± SD, n=6). *Statistically significant from the control $p<0.05$.

| Formulation	Subcutaneous injection	Control	Pheroid microsponges	Pheroid vesicles	TMC H-L	TMC H-H
rhGH dose (IU/kg)	0.6	3.6	3.6	3.6	3.6	3.6
C_{max} (µIU/ml)	4.7±2.6	0.2±0.1	3.9±1.2*	27.9±15.1*	30.4±7.7*	16.0±5.3*
T_{max} (min)	29.2±17.4	69.0±77.6	135.0±57.4	180.0±49.0*	55.0±12.3	80.0±49.0
$AUC_{0\rightarrow\infty}$/rat (µIU/ml $_x$ h)	528.1±255.14	87.9±40.4	1235.3±409.1*	4070.4±1854.2*	4318.5±1419.3*	1263.5±521.2*
Bioavailability (%)	100	8.3±11.9	38.9±26.7*	128.5±121.1*	136.3±9270*	39.9±34.0*
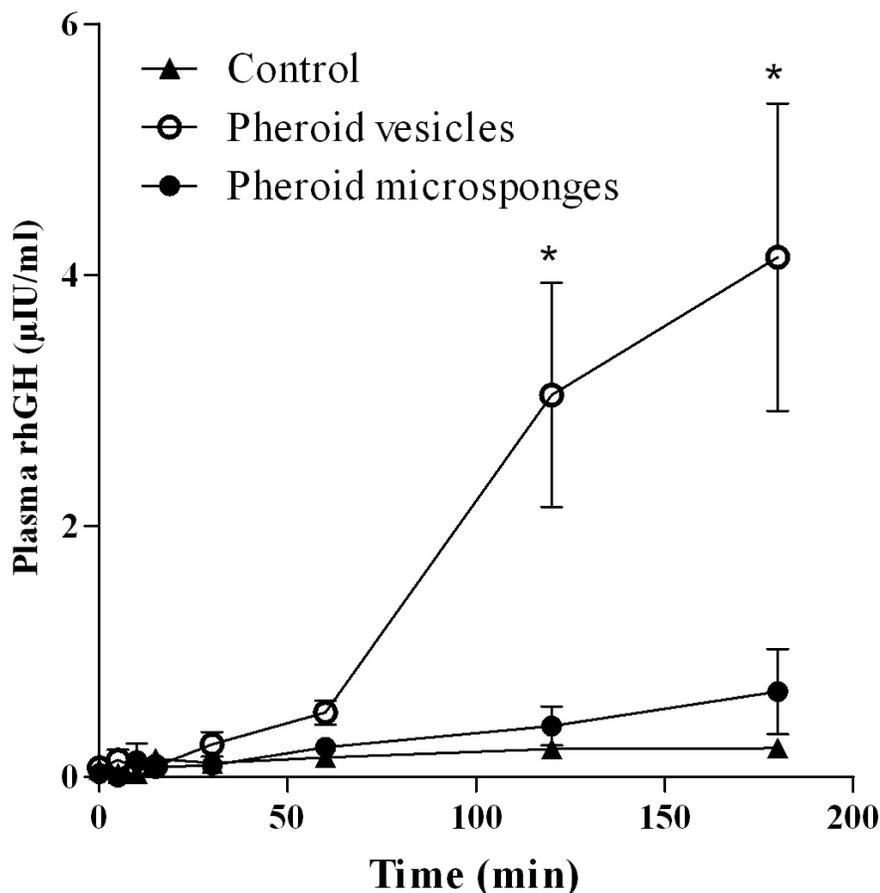
Figure 3. Plasma concentration-time profiles (180 minutes) of rhGH (3.6 IU/kg bodyweight) in a control, Pheroid vesicle and Pheroid microsponge formulations (mean ± SD, n=6). The rhGH was entrapped in the Pheroid formulations for 30 minutes before nasal administration. *Statistically significant from the control and microsponges, $p<0.05$.

Because the plasma concentration-time curve of the Pheroid formulations monitored for 180 minutes did not result in an elimination phase, the data was not used for calculation of pharmacokinetic parameters. The Pheroid vesicle formulation increased the absorption of rhGH significantly ($p < 0.05$) compared to the control formulation. Although the Pheroid microsponge formulation showed slightly higher absorption of rhGH, compared to the control formulation, there was no statistically significant difference between the two formulations. This result also prompted the investigation into a longer entrapment time of rhGH into the Pheroid formulations.

Figure 4 summarise the plasma concentration-time profiles after nasal administration of the Pheroid formulations in which rhGH was entrapped for 24 h. The profiles show that both Pheroid vesicles and Pheroid microsponges display a 60 minute onset time and the maximum rhGH plasma levels were observed at 180 minutes. The increased plasma levels lasted for up to 300 minutes. In contrast to the control formulation, nasal administration of rhGH, entrapped for 24 h in Pheroid vesicles, gave significantly higher plasma concentrations of rhGH and increased T_{max} values (C_{max} 30.8 ± 15.1 μIU/ml reached at 180.0±49.0). Compared to the Pheroid vesicle formulation only slight absorption was obtained in the Pheroid microsponge formulation (4.7±1.2 μIU/ml reached at 135±57.4 minutes). The relative bioavailability of Pheroid vesicles was higher than Pheroid microsponges and both formulations were significantly higher than the control.
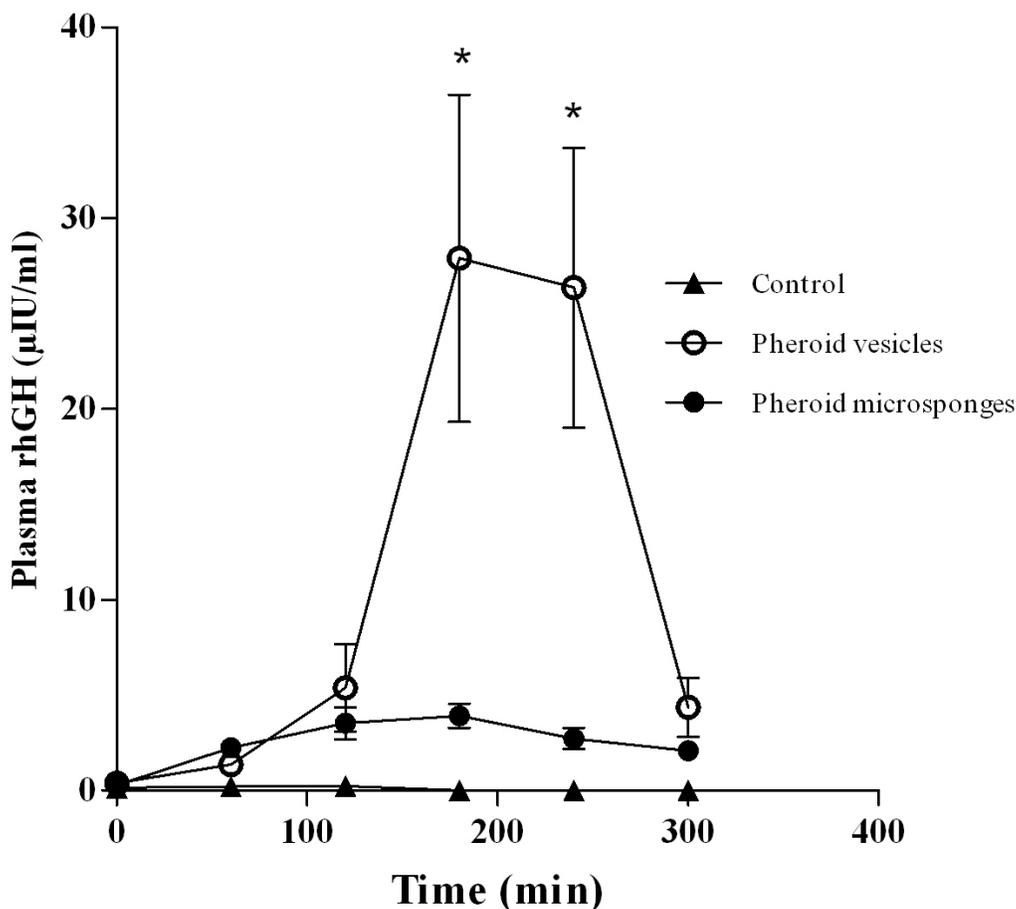
Figure 4. Plasma concentration-time profiles (300 minutes) of rhGH (3.6 IU/kg bodyweight) in a control, Pheroid vesicle and Pheroid microsponge formulations (mean ± SD, n=4). The rhGH was entrapped in the Pheroid formulations for 24 h before nasal administration. *Statistically significant from the control and Pheroid microsponges, $p<0.05$.

Figure 5 summarises the plasma rhGH levels after nasal administration of the TMC formulations. The plasma concentration-time profile shows that both TMC H-H and TMC H-L display a onset time of 10 minutes reaching a peak at 60 minutes and a slow decline towards 180 minutes. The TMC formulations greatly increased the nasal absorption of rhGH. The TMC H-L formulation gave a maximum plasma rhGH concentration (30.8±7.7 µIU/ml) similar to that of Pheroid vesicles, whereas TMC H-H resulted in a lower C_{max} of 17.7±5.3 µIU/ml. It is interesting to note that although the maximum plasma concentration values measured for TMC H-L and TMC H-H differ significantly, the plasma concentration-time profiles are very similar in configuration and reaches similar levels at 180 minutes. The TMC H-L formulation resulted in an

increase in bioavailability of 136.3.3±92.7 % and the TMC H-H in 39.9±34.0 %.

DISCUSSION

Nasal permeability decreases rapidly for hydrophilic molecules larger than 1 kDa and the absorption of large peptides like GH are therefore negligible. To improve the nasal absorption of rhGH, several absorption enhancers were evaluated *in vivo* on male Sprague Dawley rats. The rhGH was entrapped in Pheroid vesicles and Pheroid microsponges to establish their effectiveness as absorption enhancers for large peptide hormones such as rhGH. Pheroid vesicles and microsponges have been recently utilized successfully as absorption enhancers for both oral and nasal calcitonin delivery [15].
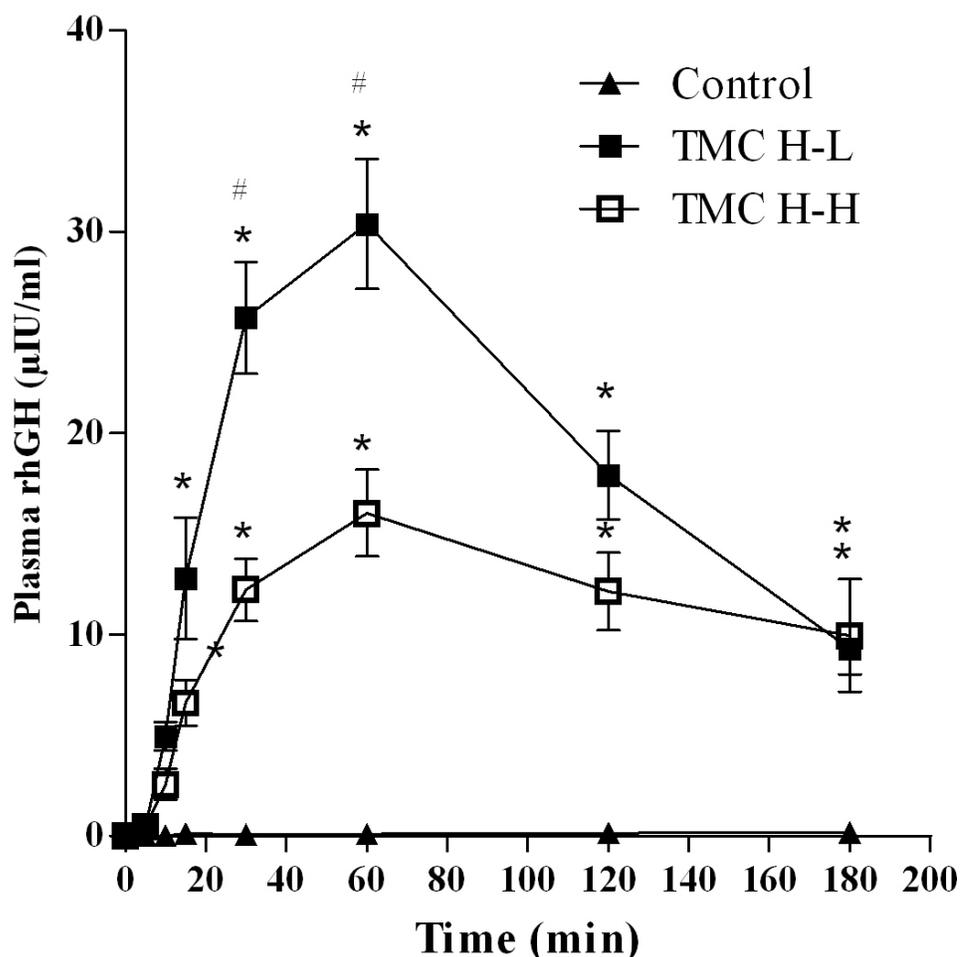
Figure 5. Plasma concentration-time profiles (180 minutes) of rhGH (3.6 IU/kg bodyweight) after nasal administration in the control, TMC H-H and TMC H-L formulations (mean ± SD, n=6). *Statistically significant from the control, # from TMC-HH, *p*<0.05.

The individual plasma concentration-time profiles after nasal administration of Pheroid vesicles and Pheroid microsponges showed that Pheroid vesicles increased the absorption of rhGH to a greater extent than Pheroid microsponges. The particle size of drug delivery systems plays an important role with regard to mucoadhesion and resisdence time in the nasal cavity [12]. Pheroid vesicles are small (~2 μm) liposome-like structures with a bilayer membrane and hydrophylic core. Microsponges are slightly larger than vesicles, with a mean diameter of ~3 μm and have a porous nature. The differences in chemical composition of the membrane can attribute to the difference in the absorption enhancing properties of the Pheroid vesicles and

Pheroid microsponges [13,14, 15]. Lipid based delivery systems have been demonstrated to have good bio-adhesive characteristics when in contact with the nasal mucosa. These drug delivery systems have the ability to control the rate of drug clearance from the nasal cavity as well as protect the drug from enzymatic degradation in nasal secretions [24, 25, 26]. Pheroid vesicles not only increased the bioavailability of rhGH that are administered via the nasal route but a delay in absorption of rhGH was observed. This could possibly be attributed to increased residence time of the formulation in the nasal cavity. When comparing the absorption of rhGH entrapped for 30 minutes in Pheroid vesicles to the absorption of rhGH entrapped for 24 h in Pheroid vesicles it

is interesting to note that a much higher rhGH peak value was reached in the 300 minutes experimental group compared to the 180 minutes experimental group. This result is indicative of the entrapment efficiency of Pheroid vesicles, especially with very large hydrophilic molecules such as rhGH (ca. 22 kDa). Large polar molecules probably needed a longer time period to reach the aqueous inner region of the Pheroid vesicles in contrast to smaller molecules that can enter this region with normal diffusion processes. It is still unclear why the Pheroid microsponges did not have similar absorption enhancement than Pheroid vesicles.

Two other formulations were also prepared for this study, namely TMC H-H and TMC H-L. TMC have been established as an effective absorption enhancer for large hydrophilic molecules including peptide drugs [15, 20, 21]. TMC H-L increased the nasal absorption of rhGH to a greater extent than TMC H-H. The degree of quaternisation of TMC has been shown to play an important role in absorption enhancement with the optimum degree of quaternisation for absorption enhancement of TMC in a neutral environment being 48 % [27]. This could explain the clear differences in absorption enhancement of rhGH seen with TMC H-L and TMC H-H. TMC H-H seems to open the tight junctions to a lesser extent than TMC H-L, which in turn translates to a lower plasma concentration. These results indicate that the degree of quaternisation, 73.3 % for TMC H-H and 18.2 % for TMC H-L, play an important role in the absorption enhancing properties of TMC when used to administer large peptide drug molecules. In comparison with Pheroid vesicles, TMC H-L gave similar C_{max} values, but slightly higher AUC and relative bioavailability. It was possible that the AUC of the Pheroid vesicles was underestimated because the terminal elimination phase could not be calculated accurately due to insufficient time points.

CONCLUSION

The results obtained in this study clearly indicate that TMC H-L, and to a lesser extent TMC H-H, is the most effective absorption enhancers when compared to the other formulations included in this study. Pheroid vesicles and microsponges did improve the absorption of rhGH but to a lesser extent than the other test formulations. It is clear that Pheroid technology has great potential as an absorption enhancer and further studies should be conducted to exploit this system's full potential. Chitosan and TMC polymers are well known polymers and numerous researchers have already confirmed its astounding absorption enhancing capabilities. This study confirmed the absorption enhancing effects of polymeric delivery systems, but also show the potential of a unique lipid based delivery system for the delivery of large hydrophilic molecules like rhGH.

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