

Oral delivery of insulin-loaded nanoparticles in diabetic rabbits and in sheep

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ABSTRACT: The objective of this study was to produce Eudragit® RS 100 microparticles (ERS) containing insulin (ILNP), and to evaluate the potential of these nanoparticles as a drug carrier for oral administration in alloxan-induced diabetic rabbits and in sheep. After oral administration of ILNP to diabetic rabbits, a significant hypoglycemic effect was observed and this effect lasted for 2 days. Concentrations of blood glucose were significantly decreased in ILNP-treated sheep compared to those in control and ERS groups on day 5 of the experiment. Orally ILNP-treated sheep exhibited higher concentrations of progesterone compared to control and ERS in all sampling times. Sheep in the ILNP group had lower concentrations of cortisol than the animals in the control group on days 1 and 3. However, other blood hormone parameters were not affected by the treatments. The results show that encapsulation of insulin into nanoparticles allows the preservation of its biological activity when going through digestive system of rabbit and sheep to the blood stream.

Keywords: diabetes; insulin encapsulation; drug delivery; rumen-bypass; glycemia

INTRODUCTION

Diabetes mellitus is a relatively common disease in which the body does not produce enough insulin, produces no insulin, or has cells that do not respond properly to the insulin. Even though, there are several ways the patients can get the necessary insulin their body needs to function properly, needles and syringes remain the most common way to take insulin. However, injection has many disadvantages, including being painful and inconvenient if the patient has to use it in a public place, as well as dosage errors can occur easily. Experts in this area are trying to find other ways of how to deliver insulin (Babu et al., 2008; Chaturvedi et al., 2013). In this regard, the easiest way to control blood sugar for patients would be an effective oral insulin medication. A possible way to achieve this would be protection of insulin from the stomach and elimination of intestines when going

through the digestive system to the blood stream (Cournarie et al., 2002; Rekha and Sharma, 2009; Mundargi et al., 2011a, b).

During late gestation and in early lactation, maternal insulin sensitivity is reduced to ensure that an adequate supply of nutrients is provided to the foetus and to mammary tissues (McDowell et al., 1987; Sano et al., 1993). Therefore, the periparturient period in cattle is characterized by increased concentrations of blood glucagon, growth hormone, non-esterified fatty acid and beta hydroxybutyrate, and decreased concentrations of blood insulin and liver glycogen (Vazquez-Anon et al., 1994). These changes are also observed in induced or spontaneous hepatic lipidosis and ketosis. In addition to elimination of negative energy balance, subcutaneous or intravenous injection of insulin with concomitant infusion of dextrose has been suggested for treating hepatic lipidosis to maintain health and improve performance (Fleming,

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1990). In addition, Hayirli et al. (2002) concluded that a low dose of slow-release insulin (0.14 IU/kg of body weight) could be considered for prophylactic use against hepatic lipidosis and ketosis. However, to our knowledge, no advanced rumen bypass delivery systems for insulin have been investigated until now, based on the physiological factors in the rumen such as pH, microflora, and ruminal fermentation.

Nanomaterials such as nanoparticles, nanocapsules, micelles, micro/nanoemulsions, liposomes, polymersomes, and nanoporous materials are used for drug delivery systems (Fan et al., 2006; Akhlesh et al., 2008). It has been shown that microencapsulation of small peptides in a polymeric system is a useful delivery system for the oral administration in human. It is able to protect the peptide against chemical and/or enzymatic degradation, and may enable the retention of therapeutic properties following oral administration (Trapani et al., 2007). Eudragit® RS 100 (ERS), which is used as a coating material in microencapsulation, is insoluble at physiological pH but undergoes swelling in aqueous medium (Pignatello et al., 2002). This copolymer is commonly used for producing enteric coated tablets and preparation of controlled-release drug forms (Trapani et al., 2007; Mundargi et al., 2011a).

To our knowledge, no advanced rumen bypass delivery systems for insulin have been investigated in ruminants. Therefore, the aim of this study was to encapsulate insulin in ERS microparticles, and to evaluate this insulin delivery system for oral administration in diabetic rabbits and in sheep and its effects on selected blood parameters. In addition, the *in vivo* rumen bypass properties of the encapsulated insulin were evaluated in fistulated ram.

MATERIAL AND METHODS

Materials. Commercially available regular human insulin was obtained from Exir Pharmaceutical Co. (Tehran, Iran). The ERS was kindly provided by Evonik Röhm GmbH (Damstadt, Germany). Polyvinyl alcohol (PVA, 87 hydrolyzed, MW: 31 000) was obtained from Sigma-Aldrich (St. Louis, USA).

Preparation of nanoparticles. The preparation of nanoparticles was carried out by the multiple emulsion technique previously described by Hoffart et al. (2002). Briefly, 1 ml of an aqueous solution of insulin (1000 IU) was first emulsified by sonification (Misonix Sonicator, model XL-2000-010) (Misonix, Inc., Farmingdale, USA) for 30 s in methylene chloride (10 ml) containing 250 mg of

polymer (ERS). The resulting water-in-oil emulsion was thereafter poured into 40 ml of a polyvinyl alcohol aqueous solution (0.1%) and sonicated for 1 min, involving the formation of the second water-in-oil-in-water emulsion. After evaporation of methylene chloride under reduced pressure, the nanoparticles were isolated by centrifugation (45 000 g, 20 min).

Characterization of nanoparticles. The mean diameter of nanoparticles was determined by atomic force microscopy (Bruker Nano GmbH, Berlin, Germany). The amount of insulin entrapped within polymeric nanoparticles was determined by high performance liquid chromatography (HPLC) (Jasco 807-IT; JASCO, Tokyo, Japan) (Moslemi et al., 2003).

Insulin association efficiency. The association efficiency of insulin was determined indirectly. The amount of insulin entrapped into nanoparticles was calculated by the difference between the total amount used to prepare the systems and the amount of insulin that remained in the supernatant. Insulin concentration in the supernatant was determined by HPLC.

$$AE = \frac{\text{Total amount of insulin} - \text{Free insulin in supernatant}}{\text{Total amount of insulin}} \times 100$$

In vivo studies of insulin-loaded nanoparticles (ILNP) in diabetic rabbits. Five adult male white rabbits weighing 2 kg on average were used. Animals were housed in stainless steel caging with slotted floors (80 × 90 × 100 cm), with controlled environmental conditions of temperature (20 ± 2°C), humidity (45–65%), at 12 : 12 light-darkness cycle. Cage pans were cleaned three times per week. The rabbits were fed a standard diet and provided tap water *ad libitum*. In order to exclude effects in the animals by differences of feed intake, amounts of feed were given according to amounts took by animals having lesser intake during one week before the experiment. The animal group having lesser intake was used as control (CTR, *n* = 2) and animals treated with ILNP (*n* = 3) were pair-fed.

Animal protocols were in compliance with the accepted standards of animal care and were approved by the Isfahan University of Technology Animal Care and Use Committee.

Blood glucose levels were measured in overnight-fasted rabbits using a glucometer (Accu-chek Sensor; Roche Diagnostics, Mannheim, Germany) during one week before the experiment. It was established that they had a normal blood glucose level (122.7 ± 13.4 mg/dl) at the start of the experiment.

Diabetes in rabbits was induced by a single injection of alloxan monohydrate into marginal ear vein at a dose of 150 mg/kg body weight. After one week, overnight-fasted rabbits had fasted blood glucose greater than 210 mg/dl and were considered as diabetic.

In order to display the biological efficacy of ILNP after oral administration, a single administration of 20 mg ILNP (i.e. 80 times more than blood insulin levels) was given intragastrically by gavage needle to overnight-fasted (with free access to water) diabetic rabbits. The glycemia of the experimental rabbits was measured in blood sampled from the tail vein prior to and 24 h after administration of nanoparticles.

In vivo studies of ILNP in sheep. Fifteen Iranian Naeini ewes (body weight 28 kg) were used. Animals were allocated in three groups per five ewes. One group without treatment was control (CTR), two treatment groups were fed with one single dose of 1 g ILNP or ERS for 5 days, respectively. During the experimental period, all ewes had free access to water; however, the amounts of feed (alfalfa hay) were given according to amounts took by animals having lesser intake during adaptation period (one week before the experiment). The animal group having lesser intake was used as CTR. Both the CTR and treated animals were pair-fed, in order to rule out the nutrition-related effects on blood parameters, which would allow conclusions to be drawn regarding the efficacy of the ILNP.

Blood glucose levels were measured using a glucometer (Accu-chek Sensor; Roche Diagnostics, Mannheim, Germany) on day 5. In addition, blood was sampled from the jugular vein using vacutainer tubes at the beginning of the experiment (day 1), and on days 3 and 5. All tubes were immediately placed in wet ice and within 1 h centrifuged at 1850 *g* for 20 min. The plasma was obtained and frozen (–20°C) until analyses. Plasma concentrations of estradiol (kit IS-310.400), progesterone (kit IS-310.420), and cortisol (kit SO-313.261) were measured using chemiluminescent immunoassay method by LIAISON analyzer (DiaSorin, Saluggia, Italy). Concentrations of insulin and insulin-like growth factor I (IGFI) were measured using radioimmunoassay with commercial kits (Diagnostic System Laboratories, Webster, USA) and an automatic gamma counter (BioSource International, Camarillo, USA).

Rumen-bypass property of ILNP in sheep. The nylon bag technique was used to evaluate the bypass of ILNP from digestion in ram. The 1.5 g of ILNP was placed into 4 nylon bags. The nylon

bags were incubated in the rumen for 24 h. After the removal of the bags from the rumen, the bags were washed in cold water until rinses were clear and dried at 50°C for 24 h. The residual amount of ILNP retained in the nylon bag was determined by weight changes of the incubated samples. This experiment was repeated two times.

Statistical analysis. The data were analyzed using the MIXED procedure of SAS (Statistical Analysis System, Version 9.1, 2001), using repeated measures with a first-order autoregressive covariance structure in time. The model included treatment, time, and interaction of treatment and time as fixed effects. Animal was used as the repeated subject. Results are presented as the means and the accompanying standard error of the mean. Significance was declared at $P < 0.05$.

RESULTS

Structure and characterization of nanoparticles. The ILNP showed a homogeneous size distribution with a mean diameter of 450 nm (300–600 nm; Figure 1) and high encapsulation efficiency (85%).

Effects of oral administration of ILNP on glycemia in diabetic rabbits. As illustrated in Figure 2, ILNP administered orally by gavage in diabetic overnight fasted rabbits significantly reduced glycemia by 40 units and this effect maintained for 2 days. After oral administration, no differences were in plasma concentrations of glucose between the groups.

Effects of oral administration of ILNP on blood parameters in sheep. As shown in Figure 3, blood glucose was significantly ($P < 0.05$) decreased in ILNP-treated group compared to CTR and

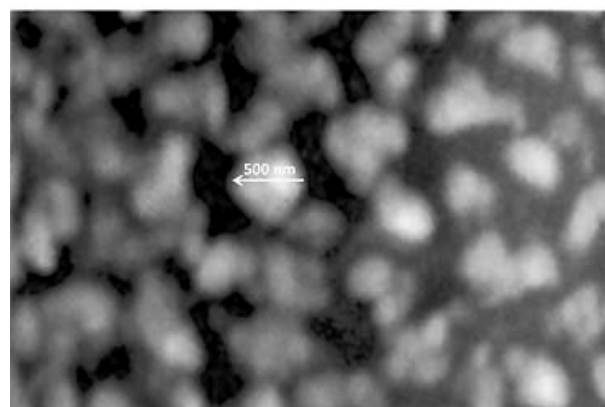


Figure 1. Atomic force microscopy image of insulin-loaded nanoparticles (ILNP)

ILNP show a homogeneous size distribution with a mean diameter of 450 nm (300–600 nm)

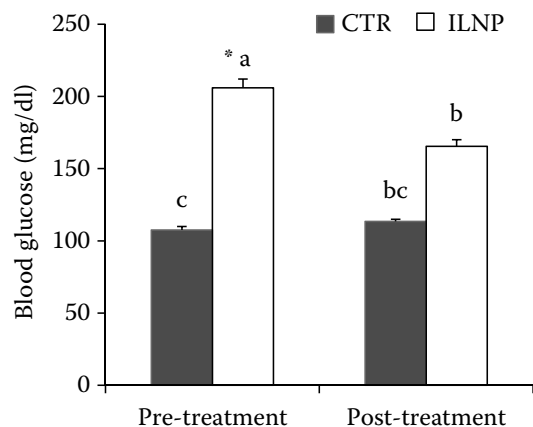


Figure 2. Blood concentrations of glucose (means \pm SEM, mg/dl) after oral administration of insulin-loaded nanoparticles (ILNP) in diabetic overnight fasted rabbits ^{a-c}differences ($P < 0.05$) between pre- and post-treatment (24 h) periods

*significant ($P < 0.05$) difference between control (CTR) and ILNP during pre-treatment period

ERS groups on day 5. In all sampling times, blood concentrations of insulin, IGFI, estradiol, and progesterone were not affected by the treatments (Table 1). However, blood concentrations of progesterone were higher in ILNP group compared with CTR and ERS groups in all sampling times. Animals in the ILNP group had lower ($P < 0.05$) concentrations of cortisol than did animals in the CTR group on days 1 and 3 (Figure 4).

Table 1. Blood concentrations of selected hormones (means \pm SEM) after oral administration of insulin-loaded nanoparticles (ILNP), control (CTR), and Eudragit[®] RS 100 microparticles (ERS) in sheep on days 1, 3, and 5 of the experiment

Trait and group	Sampling time		
	day 1	day 3	day 5
Insulin (μ IU/ml)			
CTR	147.4 \pm 15.1	137.6 \pm 10.8	147.8 \pm 17.1
ERS	113.0 \pm 4.71	160.8 \pm 16.1	142.6 \pm 15.6
ILNP	129.0 \pm 10.3	145.0 \pm 12.9	146.8 \pm 11.3
IGFI (ng/ml)			
CTR	62.6 \pm 9.05	55.6 \pm 8.35	64.6 \pm 8.96
ERS	66.0 \pm 7.12	67.4 \pm 1.96	60.0 \pm 9.49
ILNP	68.0 \pm 4.14	60.0 \pm 11.2	54.0 \pm 4.91
Estradiol (ng/dl)			
CTR	0.24 \pm 0.09	0.36 \pm 0.04	0.16 \pm 0.03
ERS	0.23 \pm 0.008	0.21 \pm 0.04	0.13 \pm 0.03
ILNP	0.19 \pm 0.05	0.28 \pm 0.07	0.11 \pm 0.01
Progesterone (ng/dl)			
CTR	1.90 \pm 0.49	2.66 \pm 1.23	1.84 \pm 0.58
ERS	1.72 \pm 0.50	2.47 \pm 1.17	1.40 \pm 0.69
ILNP	2.82 \pm 0.71	2.93 \pm 0.91	2.52 \pm 0.77

IGFI = insulin-like growth factor I

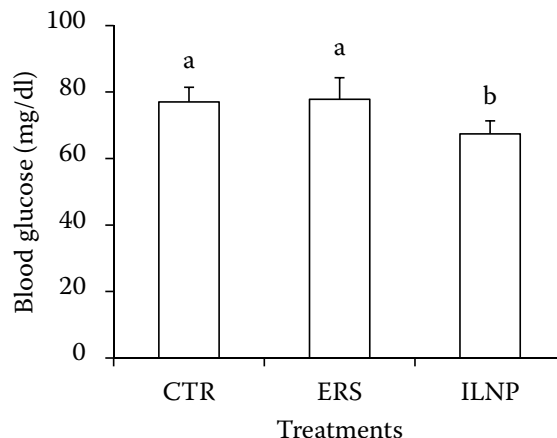


Figure 3. Blood concentrations of glucose (means \pm SEM, mg/dl) after oral administration of insulin-loaded nanoparticles (ILNP), control (CTR), and Eudragit[®] RS 100 microparticles (ERS) in sheep

^{a,b}differences ($P < 0.05$) between treatment groups

In addition, the amount of ILNP placed in the nylon bags remained unchanged after ruminal incubation.

DISCUSSION

Eudragit RS[®] 100 is a copolymer (ethyl acrylate, methyl methacrylate, and a low content of methacrylic acid ester with quaternary ammonium groups). The ammonium groups make the polymers permeable.

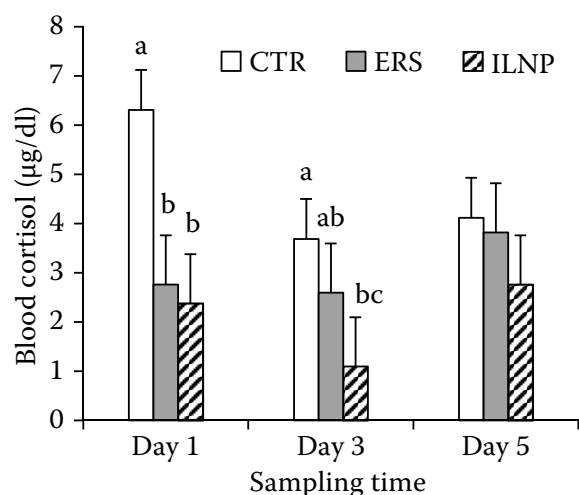


Figure 4. Blood concentrations of cortisol (means \pm SEM, $\mu\text{g}/\text{dl}$) after oral administration of insulin-loaded nanoparticles (ILNP), control (CTR), and Eudragit[®] RS 100 microparticles (ERS) in sheep on days 1, 3, and 5 of the experiment^{a-c} differences ($P < 0.05$) between treatment groups

This polymer, which swells in aqueous medium, is commonly used for the enteric coating of tablets and the preparation of controlled-release drug forms (Trapani et al., 2007; Mundargi et al., 2011a). The mean diameters confirmed that the ILNP produced in the current study were nanoparticles in the range of 300–600 nm.

The first experiment of this study shows that encapsulation of insulin into nanoparticles allows the preservation of its biological activity when going through digestive system to the blood stream, as reflected by 40 units decreases in blood glucose levels in the diabetic rabbit model. Considerable point in this research is that of using insulin associated to nanoparticles at a much lower dosage (almost 2500 times) than that in the previous studies (e.g. Damage et al., 2007).

Taking advantage of this first experiment, it was decided to study whether the ILNP are resistant to the rumen fluid, and the delivery of the ILNP at the low pH of the abomasum to intestinal absorption. The unchanged amount of ILNP in the nylon bags after ruminal incubation alongside with significant decrease in blood glucose using the ILNP clearly show that the polymer has efficiently protected the insulin from proteolytic degradation in the gastroenteric tract of sheep. The decreased levels of blood glucose occurred without changes in blood insulin concentrations among the treatments. It seems that oral administration of the ILNP may have exerted a negative-feedback effect on the

pancreas and reduced endogenous insulin release, resulting in comparable concentrations of insulin in treated (ILNP) and CTR groups.

An interesting finding of the present study was the decreased blood cortisol in the treated animals with the ILNP compared to that in the CTR group. The mechanisms by which ILNP decreased blood cortisol remain unclear. However, it is known that cortisol stimulates gluconeogenesis (formation, in the liver, of glucose from certain amino acids, glycerol, lactate and/or propionate) and thereby increases blood glucose concentrations. Therefore, we assume that decreased blood glucose concentration in animals treated with ILNP compared to the CTR group at least in part has been associated with decreased circulating cortisol levels.

Decreases in steroid clearance by decreasing hepatic monooxygenases cytochrome P450 2C (CYP2C) and cytochrome P450 3A (CYP3A) activities via insulin signalling has been proposed. Lemley et al. (2009) reported a dose-dependent decrease in the activities of CYP2C and CYP3A after challenging hepatocytes with increasing physiological concentrations of insulin. In the present study, blood concentrations of progesterone were higher in ILNP compared to CTR and ERS in all sampling times. It seems thus that numerically higher concentrations of progesterone in the ewes treated with ILNP may be, in part, affected by decreased CYP2C and CYP3A activities. A portion of the early embryonic and fetal loss may be accounted for by inadequate concentrations of progesterone due to increased rates of steroid inactivation, deficiencies in luteal function, or a combination of both (Inskeep and Dailey, 2005). Therefore, the effect of ILNP on hepatic CYP2C and CYP3A activities as well as the resulting biological half-life of progesterone merits more investigation.

CONCLUSION

In conclusion, the present results show that encapsulation of insulin into nanoparticles allows the preservation of its biological activity when going through digestive system of rabbit and sheep to the blood stream. The effects of ILNP to prevent hepatic lipidosis and ketosis especially during the first month postpartum in high producing animals merit more investigation in future studies.

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