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Original Research Article

Insulin-Loaded Prosochit® Nanoparticles Formulation Inhibits Lymphocytosis in Alloxan-Induced Diabetic Rats

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ABSTRACT

Haematological disorder is one of the manifestations in diabetes mellitus. This study aimed to investigate the effect of oral insulin-loaded Prosochit® nanoparticles on blood composition in diabetes. Twenty rats were selected from a community of alloxan-induced diabetic rats, divided into four equal groups and labelled Groups 1 to 4. Another group of 5 rats was kept without induction and without treatment as the healthy control group and labelled HCG. Group 1 animals were administered purified water (untreated), Group 2 was treated with oral insulin-loaded Prosochit® nanoparticles, Group 3 with oral unloaded Prosochit® nanoparticles and Group 4 with subcutaneous insulin - Actrapid® (positive control). The drug administration at the dose of 50 IU/kg was done daily for 28 days. Blood samples were taken and analyzed for glucose level on Days 0, 1 and 28. The samples were also subjected to complete blood count. After 24 h of commencing treatment, insulin-loaded Prosochit® nanoparticles limited the increase in the lymphocytes count to 4.08 x 10^9/L compared to the untreated group with the count of 6.23 x 10^9/L. It also brought forth a slight reduction in haemoglobin level to 11.5 g/L and maintained the number of platelets closest to the normal range. After 28 days, the lymphocytes count only increased to 4.56×10^9/L while that of the untreated diabetic group had increased to 8.92×10^9/L. The red blood cells and platelets counts were similar to those of the healthy rats. Oral insulin-loaded Prosochit® nanoparticles inhibits lymphocytosis in alloxan-induced diabetic rats.

Keywords: Insulin, Lymphocytosis, Nanoparticles, Prosochit®, White blood cells.

Introduction

Oral delivery of insulin has been a subject of extensive research and development for some years now.[1] It is one of the ways of averting the pain, needle-phobia, lipodystrophy and non-compliance associated with the parenteral insulin.[2] Inhaled insulin which was the first approved non-invasive and alternative insulin was withdrawn from the market due to poor acceptance by patients.[3,4] Oral insulin improves the portal level of the drug and curtails peripheral hyperinsulinaemia which is associated with other non-invasive mechanisms such as the nasal and the pulmonary routes.[5,6] It is however inhibited by enzymatic degradation in the stomach and poor absorption in the intestine.[7] Nanotechnology and the use of polymers offer significant advances where insulin for oral delivery can be efficiently protected from degradation and enhanced for absorption.[8,9] Nanoparticles can be prepared with a single or a blend of biodegradable polymers and these have been used as drug carriers for oral administration of insulin.[2] Previous work of Olorunsola *et al.*[10] has demonstrated the effective formulation of insulin nanoparticles using Prosochit®, a coprecipitate of prosopis gum and crab shell chitosan.

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The two polymers used for the development of Prosochit® are versatile drug delivery agents.[11,12] The study on the antidiabetic activity of the orally administered insulin-loaded Prosochit® nanoparticles showed that the formulations are comparable with the subcutaneous insulin and the Type 101 of Prosochit® produced the best result.[10] The net effect of insulin administration is the lowering of blood glucose.[13,14] Blood consists of red blood cells (erythrocytes), white blood cells (leucocytes) and platelets (thrombocytes) which are dispersed in the plasma.[15] The red blood cells are the most common type of blood cells and the human's principal means of delivering oxygen to the body tissues via blood flow.[16] The white blood cells are of various types including neutrophils, monocytes, eosinophils, lymphocytes and basophils; and they protect the body against illness and diseases.[16] Platelets are small, colourless cell fragments that form clots and stop bleeding.[17] Hence, an alteration in the composition of the blood might have a significant effect which may be positive or negative. Chitosan, which is a constituent of Prosochit®, is an absorption enhancer and can promote substance absorption into the blood.[12] Besides, it has the potential of altering blood composition through haemolysis.[18] This research work attempts to assess, in an animal model, if the lowering of blood glucose level by the orally administered insulin-loaded Prosochit® nanoparticles is characterized by alteration of the blood composition. The novelty of this work is in establishing that oral administration of insulin-loaded Prosochit® nanoparticles inhibits diabetes-induced lymphocytosis in addition to lowering the blood glucose level.

Materials and Methods

Materials

Prosochit® 101 (PC 101) described in the work of Olorunsola *et al.*[19], Actrapid® insulin 100 I.U/ml (Novo Nordisk, Germany), soluble insulin powder from bovine pancreas (Lot number. SLB25307, Sigma Aldrich Chemie, Germany), liquid paraffin (BDH Chemicals, England), Span 60 (BDH Chemicals, England), sodium hydroxide (BDH Chemicals, England), and hydrochloric acid (Fisher Scientific International

Company, United States) were used for this work.

Preparation of nanoparticles
A nano-emulsion was formulated as water-in-oil-in-water double emulsion using the method described by Olorunsola *et al*.[10] The inner emulsion was formulated by dissolving 0.175 g (500 IU) insulin in 5 mL purified water and emulsifying with 10 mL liquid paraffin using 0.2 g Span 60 and then homogenizing at 1000 rpm for 10 minutes with the use of a magnetic stirrer (Model 78HW-1, Gallenkamp, United Kingdom). The outer emulsion was formulated using 5 g of Prosochit® 101 as an emulsifier dispersed in 25 mL of 0.2 N hydrochloric acid and the dispersion neutralized with sufficient quantity of 0.1 N sodium hydroxide. The first emulsion was transferred into the dispersion of the second emulsifier and the system homogenized (Magnetic stirrer, 78HW-1, Gallenkamp, United Kingdom) at 1000 rpm for 10 minutes.[10] The process above was repeated to produce the unloaded nanoparticles. In this case, distilled water (without insulin) was used as the inner phase of the first emulsion, Span 60 was used as the emulsifier for the first emulsion and Prosochit® 101 was used as the emulsifier for the second emulsion. Both emulsions were placed in a Clifton freeze dryer for 3 days for the desired nanoparticles to be formed. The final weights were determined after drying to obtain the appropriate nanoparticles as indicated in Table 1.

The degree of drying of formulation was calculated using Equation 1 while the percent drug loading was calculated using Equation 2.

$$\text{Degree of drying} = \frac{weight\ of\ nanoemulsion - final\ weight\ of\ nanoparticles}{\text{weight of nanoemulsion}} \text{ x } 100 \quad \ldots\ldots\ldots (1)$$

$$\%\ \text{drug loading} = \frac{amount\ of\ insulin\ used\ for\ the\ formulation}{final\ weight\ of\ the\ nanoparticles} \text{ x } 100 \quad \ldots (2)$$

Table 1. Formulations administered to the various groups of rats

Groups	Formulations administered
HCG	Nil
1	Purified water: 10 mL per kg weight of rat
2	Insulin-loaded Prosochit® nanoparticles equivalent to 50 I.U. per kg weight of rat
3	Unloaded nanoparticles: 1.82 g per kg weight of the rat
4	Actrapid® insulin equivalent to 50 I.U. per kg weight of rat (positive control)

Ethics approval
Ethical clearance for this work was obtained from the Ethics Committee, Faculty of Pharmacy, University of Uyo (Protocol number UU/Pharm/2019/14).

Experimental animals
Twenty-five albino rats each weighing 100-150 g were housed in a well-ventilated room maintained at 25 ± 2.5 °C for 7 days to acclimatize according to the internationally accepted laboratory animal use before experimentation. Five of the rats were kept normal while the others were subjected to diabetes induction using alloxan.

Induction of diabetes
The twenty rats were made to fast for 12 hours followed by induction of diabetes using intraperitoneal injection of alloxan monohydrate solution in normal saline administered as a single dose of 150 mg/kg body weight of the animal. Fasting blood sugar was taken after 72 hours using a Fine test glucometer (Osang Healthcare, Dongtan, Korea) to validate the induction of diabetes. Manifestation of diabetes was taken as fasting blood sugar > 160mg/dL.[10]

Evaluation of antidiabetic effect of the formulations
No drug formulation was administered to the normal non-diabetic rats (healthy control group). Members of the other groups (diabetic rats) were administered with appropriate formulations as indicated in Table 1. Group 1 animals were given purified water at the dose of 10 mL/kg (untreated), Group 2 members were treated with insulin-loaded nanoparticles with amounts equivalent to 50 I.U. insulin per kg weight of the rat while Group 3 members were treated with unloaded Prosochit® nanoparticles with amounts equal in weight to the loaded nanoparticles using oral canula. Group 4 animals on the other hand were injected with subcutaneous Actrapid® insulin 50 I.U per kg weight of rat (positive control). The formulations were administered daily for 28 days. Blood samples were taken on Days 1 and 28 by drawing blood from the tail vein of the rats. Fasting blood sugar levels were measured using a Fine test glucometer (Osang Healthcare, South Korea).

Complete blood count
After determining the blood glucose level on Day 1, about 5 mL blood sample was drawn from each rat after being sacrificed and the compete blood count was obtained using blood count analyzer (Mindray BC-5380 Auto Haematology Analyzer, China). The average of each parameter was calculated for the test formulations and compared with the various controls and with the reference range. The haematologic evaluation was equally done after 28 days of drug administration and the parameters obtained were compared as on Day 1.

Statistical analysis
Data were analyzed by applying one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test using GraphPad Instat-3 software.

Results and Discussion

Degree of drying and percent drug loading
For the unloaded Prosochit® nanoparticles, the weight of nano-emulsions before drying, final weight of nanoparticles and degree of drying were 59.88 g, 16.20 g, and 72.95% respectively while those of the loaded nanoparticles were 64.80 g, 17.85 g and 72.45% respectively. The percent drug loading was observed to be 0.98%. The percent drug loading of 0.98% when compared with the previous work of Olorunsola *et al*.[10] was found to be within the range obtained then (0.90-0.98%). This shows that the process is reproducible. It is also within the range obtained by Mumuni *et al*.[2] which showed drug loading capacity ranging from 0.70 to 1.70%.

Antidiabetic effect of the formulations
The percent variations in blood glucose level with time for the various groups are shown in Table 2. The blood glucose level at Day 0 (72 h after induction and just before commencing treatment) for each group was taken as 100%. After the first day of drug administration, Actrapid® produced the highest percent reduction in glucose level, followed by insulin-loaded nanoparticles then the unloaded nanoparticles showing the antidiabetic properties of the various formulations.

However, there was no significant difference in the extent of the reduction for Groups 1-4 at Day 28. This shows the wearing out of the induction itself as shown by the effect produced by purified water (in the untreated animals).

Effect of acute administration of formulations on complete blood count
The effects of various formulations on white blood cells, red blood cells and platelets counts after Day 1 of administration are shown in Tables 3, 4 and 5 respectively. From Table 3, it was observed that the total white blood cells (WBC) count of 9.18×10^9/L for Group 1 (the untreated diabetic group) is higher than the counts for the other groups. The group also had the highest lymphocytes count indicating lymphocytosis in the diabetic animals.

Table 2. Percent variation in blood glucose level

Group	Formulation	Percent variation in glucose level		
		Day 0	Day 1	Day 28
HCG	Nil	100.00 ± 2.85	101.23 ± 1.43	101.85 ± 3.57
1	Purified water	100.00 ± 11.55	86.48 ± 4.86	37.47 ± 6.92
2	Insulin-loaded Prosochit® nanoparticles	100.00 ± 12.02	71.76 ± 6.19	29.79 ± 2.61
3	Unloaded Prosochit® nanoparticles	100.00 ± 12.96	73.15 ± 6.91	33.94 ± 2.54
4	Actrapid® insulin (positive control)	100.00 ± 12.34	58.09 ± 13.12	30.14 ± 9.94

Data presented as mean ± S.D, n =5

Table 3. White blood cells count after 1 day of drug administration

Cell type	Reference	HCG	Group 1	Group 2	Group 3	Group 4
Neutrophils	2.00-7.00 x10^9/L	1.00×10^9/L	0.89×10^9/L	0.39×10^9/L	1.68×10^9/L	3.57×10^9/L
Lymphocytes	0.80-4.00 x10^9/L	3.04×10^9 /L	6.23×10^9/L	4.08×10^9/L	4.23×10^9/L	4.89×10^9/L
Monocytes	0.12-1.20 x 10^9/L	0.07×10^9 /L	1.93×10^9/L	0.61×10^9/L	1.44×10^9/L	0.02×10^9/L
Eosinophils	0.02-0.50 x 10^9/L	0.00×10^9/L	0.04×10^9/L	0.00×10^9/L	0.04×10^9/L	0.09×10^9/L
Basophils	0.00-0.10 x 10^9/L	0.00×10^9 /L	0.09×10^9/L	0.00×10^9/L	0.06×10^9/L	0.24×10^9/L
Total WBC	4.00-10.00 x 10^9/L	4.11×10^9/L	9.18×10^9/L	5.08×10^9/L	7.44×10^9/L	8.81×10^9/L

Key: Reference = as indicated by the equipment used (Mindray BC-5380 Auto Haematology Analyzer, China); HCG = healthy control group; Group 1 = diabetic administered with purified water; Group 2 = diabetic treated with Insulin-loaded Prosochit® nanoparticles; Group 3 = diabetic treated with unloaded Prosochit® nanoparticles; Group 4 = diabetic treated with subcutaneous insulin

Table 4. Red blood cells count after 1 day of drug administration

Cell type	Reference	HCG	Group 1	Group 2	Group 3	Group 4
RBCs	3.50-5.50 ×10^{12}/L	6.41x10^{12}/L	7.41×10^{12}/L	6.10×10^{12}/L	7.32×10^{12}/L	6.23×10^{12}/L
Haemoglobin	11.00-16.00 g/dL	12.50 g/dL	13.90 g/dL	11.50 g/dL	13.60 g/dL	12.00 g/dL

Key: Reference = as indicated by the equipment used (Mindray BC-5380 Auto Haematology Analyzer, China); HCG = healthy control group; Group 1 = diabetic administered with purified water; Group 2 = diabetic treated with Insulin-loaded Prosochit® nanoparticles; Group 3 = diabetic treated with unloaded Prosochit® nanoparticles; Group 4 = diabetic treated with subcutaneous insulin

The rats in Group 2 (which received insulin-loaded Prosochit® nanoparticles) had a higher number of total white blood cells compared to the healthy control group, the concentration being 5.08×10^9/L after the first day of drug administration. However, the WBC counts for all the groups still fell within the reference range of 4.00-10.00×10^9/L. Increase in the number of white blood cells is associated with the body fighting an external agent.

From Table 4, Group 1 had a haemoglobin level of 13.90 g/dL, Group 2 had 11.50 g/dL, Group 3 had 13.60 g/dL while Group 4 had 12.00 g/dL as against the reference range of 11.00-16.00 g/dL. Group 2 which received the insulin-loaded Prosochit® nanoparticles had a lower value compared to the healthy control group with 12.50 g/dL. The number of RBC for group 1 was 7.41×10^{12}/L, Group 2 was 6.10×10^{12}/L, Group 3 was 7.32×10^{12} /L while Group 4 was 6.23×10^{12}/L against the reference range of 3.50-5.50×10^{12}/L. The results showed that the number of RBCs exceeded the quoted reference. However, they cannot be considered to be abnormal since they are in line with the results of the normal rats (the untreated non-diabetic group).

From Table 5, the platelet number in Group 3 (Unloaded Prosochit® nanoparticles) with the count of 891×10^9/L exceeded the other test groups. Thrombocytosis (a situation in which a disease or condition causes a high platelet count) could be inferred from the result. However, this is not the situation because, the rats in the healthy control group also had higher values. This is more of specie issue as it has been shown that there are some differences between the body system of human and that of rats.[20]

Effect of sub-chronic administration of formulations on complete blood counts

The effects of the formulations on the white blood cells, red blood cells and platelets counts after 28 days of drug administration are shown in Tables 6, 7 and 8 respectively. From Table 6, there was a remarkable increase in the number of white blood cells of Groups 1 and 3 animals on day 28. The highest count of lymphocytes was observed with Group 1 which received purified water (untreated), the value having increased from 6.23×10^9 /L to 8.92×10^9 /L between Day 1 and Day 28. The count for Group 2 that received the insulin-loaded Prosochit® nanoparticles only increased from 4.08×10^9 /L to 4.56×10^9/L. On Day 28, the lymphocytes count for Group 2 (treated with insulin-loaded nanoparticles) was 51.12% that of the untreated group showing the inhibitory activity of the insulin-loaded Prosochit® nanoparticles on the lymphocytosis observed with alloxan-induced diabetes in rats. However, the count was 128.81% that of the Actrapid®-treated group indicating the superior activity of the subcutaneous insulin against lymphocytosis.

Elevated lymphocyte count is known to be one of the haematological manifestations in diabetes mellitus.[21,22] Therefore, insulin-loaded Prosochit® nanoparticles is capable of controlling diabetes-related lymphocytosis in addition to lowering the blood glucose level.

Table 5. Platelets count after 1 day of drug administration

Cell type	Reference	HCG	Group 1	Group 2	Group 3	Group 4
Number of Platelets	100-300×10^9/L	684×10^9/L	699×10^9/L	338×10^9/L	891×10^9/L	619×10^9/L

Key: Reference = as indicated by the equipment used (Mindray BC-5380 Auto Haematology Analyzer, China); HCG = healthy control group; Group 1 = diabetic administered with purified water; Group 2 = diabetic treated with Insulin-loaded Prosochit® nanoparticles; Group 3 = diabetic treated with unloaded Prosochit® nanoparticles; Group 4 = diabetic treated with subcutaneous insulin

Table 6. White blood cells count after 28 days of drug administration

Cell type	Reference	HCG	Group 1	Group 2	Group 3	Group 4
Neutrophils	2.00-7.00×10^9/L	0.98×10^9/L	5.48×10^9/L	1.44×10^9/L	3.77×10^9 /L	1.45×10^9/L
Lymphocytes	0.80-4.00×10^9/L	3.12×10^9 /L	8.92×10^9 /L	4.56×10^9 /L	4.80×10^9 /L	3.54×10^9 /L
Monocytes	0.12-1.20×10^9/L	0.08×10^9 /L	0.26×10^9 /L	0.18×10^9/L	0.19×10^9 /L	0.09×10^9 /L
Eosinophils	0.02-0.50×10^9/L	0.01×10^9/L	0.16×10^9/L	0.11×10^9/L	0.10×10^9/L	0.03×10^9 /L
Basophils	0.00-0.10×10^9/L	0.00×10^9/L	0.07×10^9 /L	0.04×10^9 /L	0.04×10^9/L	0.01×10^9 /L
Total	4.0-10.00×10^9/L	4.18×10^9/L	14.89×10^9/L	6.33×10^9/L	8.22×10^9/L	5.12×10^9/L

Key: Reference = as indicated by the equipment used (Mindray BC-5380 Auto Haematology Analyzer, China); HCG = healthy control group; Group 1 = diabetic administered with purified water; Group 2 = diabetic treated with Insulin-loaded Prosochit® nanoparticles; Group 3 = diabetic treated with unloaded Prosochit® nanoparticles; Group 4 = diabetic treated with subcutaneous insulin

Table 7. Red blood cells count after 28 days of drug administration

Cell type	Reference	HCG	Group 1	Group 2	Group 3	Group 4
RBCs	3.50-5.50 ×10^{12}/L	6.24×10^{12}/L	7.41×10^{12}/L	6.94×10^{12}/L	7.46×10^{12}/L	6.36×10^{12}/L
Haemoglobin	11.00-16.00 g/dL	12.10 g/dL	13.70 g/dL	12.75 g/dL	13.60 g/dL	12.60 g/dL

Key: Reference = as indicated by the equipment used (Mindray BC-5380 Auto Haematology Analyzer, China); HCG = healthy control group; Group 1 = diabetic administered with purified water; Group 2 = diabetic treated with Insulin-loaded Prosochit® nanoparticles; Group 3 = diabetic treated with unloaded Prosochit® nanoparticles; Group 4 = diabetic treated with subcutaneous insulin

All the values of the concentration of red blood cells were higher than the reference. However, these values are not of concern since the concentration in the healthy control group is equally higher than the reference. This is more of specie difference between humans and rats.[20] The values of haemoglobin concentration were within the reference range for all the groups on Day 1 and on Day 28 (Tables 4 and 7). Assessment of the effect of formulations on platelets reflected Groups 1 and 2 as having remarkable increase in the number of platelets from Day 1 to Day 28 while the healthy control group and Groups 3 and 4 showed slight reduction in the concentration. However, all the values including that of the healthy control group were higher than the reference range as seen in Table 8. Therefore, the difference is more attributable to specie difference between humans and rats.[20]

Successful and safe delivery of oral insulin is a highly desired outcome. This is because it does not only eliminate the pain and poor compliance associated with the parenteral administration but it mimics the physiologic (body) insulin as it goes directly into the portal vein.[23,24] The formulated insulin-loaded nanoparticles and the unloaded nanoparticles showed antidiabetic activity. The limitation of this work is the inability of alloxan administration to sustain diabetes for a long time as observed in the group that was administered with purified water. However, the loaded nanoparticles showed better activity in inhibiting the diabetes-induced lymphocytosis compared to the unloaded nanoparticles. It also reduced the lymphocyte count to 51.12% that of the untreated group (that received purified water).

Table 8. Platelets count after 28 days of drug administration

Identity	Reference	HCG	Group 1	Group 2	Group 3	Group 4
Platelets	100-300×10^9/L	606×10^9/L	837×10^9/L	758×10^9/L	797×10^9/L	541×10^9/L

Key: Reference = as indicated by the equipment used (Mindray BC-5380 Auto Haematology Analyzer, China); HCG = healthy control group; Group 1 = diabetic administered with purified water; Group 2 = diabetic treated with Insulin-loaded Prosochit® nanoparticles; Group 3 = diabetic treated with unloaded Prosochit® nanoparticles; Group 4 = diabetic treated with subcutaneous insulin

Conclusion

Effect of acute administration of insulin-loaded Prosochit® nanoparticles is associated with a slight increase in the total number of white blood cells (due to the slight increase in the lymphocytes count) and a slight reduction in haemoglobin level though still within the normal range while the number of platelets is maintained closest to the normal range. The effect of sub-chronic administration of the formulation manifests as inhibition of the lymphocytosis observed with alloxan-induced diabetes in rats. Further developmental work as toxicological and clinical studies, if carried out on the formulation and it comes out successful, will indicate a landmark in the management of diabetes mellitus.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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