Control of Blood Glucose Levels in the Streptozotocin Diabetic Rat Using a Long-Acting Heat-Treated Insulin

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Summary. Diurnal plasma glucose levels have been studied two hourly in streptozotocin diabetic rats during insulin treatment. Protamine Zinc Insulin induced a very steep fall in plasma glucose level from 359 ± 100 (SD) mg/100 ml to 91 ± 49 mg/100 ml within two hours. Plasma glucose was then low ($13\text{--}60$ mg/100 ml) until after 18 hours when an equally steep rise in glucose concentration occurred. Six other insulin preparations and several insulin treatment regimens were tested with the aim of normalising the 24 hour plasma glucose profile of streptozotocin diabetic rats. One preparation, a non-commercial, very long acting Ultralente NOVO (Mc, ox pH 5.5) yielded a diurnal plasma glucose profile reasonably close to normal when it was administered once a day and when the dose was based on daily blood glucose measurements. Mean plasma glucose was 122 ± 55 mg/100 ml with a nadir of 55 ± 15 and a maximal of 187 ± 99 mg/100 ml.

Key words: Streptozotocin diabetes, blood glucose control, rat, insulin treatment, diurnal plasma glucose, long acting insulin.

Animal models such as the pancreatectomised rat, the alloxanised or streptozotocin diabetic rat have been widely used in studies related to clinical problems in human diabetes mellitus. In many kinds of studies, it is of importance to be able to produce conditions simulating different degrees of diabetic control in human subjects [3]. This is notoriously difficult, especially if normalisation of plasma glucose profiles by insulin administration is required.

Because detailed information about plasma glucose levels in streptozotocin diabetic rats undergoing

insulin treatment is not available this study was performed to assess diurnal plasma glucose profiles in such animals.

A series of commercially available insulin preparations together with other preparations have been tested with the aim of finding a suitable preparation that could normalise the 24 h plasma glucose profile of streptozotocin diabetic rats.

Materials and Methods

Female Wistar rats weighing 200–300 g were used. Two days after intravenous injection of streptozotocin (65 mg/kg) insulin treatment was started in the animals that had developed gross glucosuria and ketonuria. Thereafter they were kept in metabolic cages with free access to commercial chow pellets and water (except in group 4, see text).

Diabetic animals were studied in two different metabolic conditions: 1) 'Well-controlled', termed type A control and 2) 'poorly-controlled', termed type B control. Weight matched non-diabetic control animals, type C, were included in all experiments. Diabetic state was assessed by the 24 h urine volume which was brought as close to normal as possible in the animals in type A control by giving increasing doses of insulin [14]. The animals in type B control were given small doses of insulin to keep them free from ketosis, assessed with Ketostix (Ames Company, Inc.).

The following insulin preparations were studied: Protamine Zinc Insulin NOVO, Protamine Zinc Insulin Wellcome, Ultralente NOVO, Depot-Insulin Hoechst, Protamine Zinc Insulin with 3 times the ordinary protamine content, long acting porcine Ultralente, MC, pH 5.5 (2V52-S) and very long acting bovine Ultralente MC, pH 5.5 (1V52-O). The latter is a bovine insulin crystal suspension stored at 25°C for 3 months buffered in 0.01 mol/l sodium acetate ($2 \mu\text{g Zn}^{++}$ /unit). The prolongation of action by storing at 25°C (or at higher temperatures) is not obtained at pH 7.4 as in conventional Ultralente for clinical use. It is described by Schlichtkrull in 1958 as the thermic effect on insulin crystal suspensions [11].

The insulins were given as one or more subcutaneous injections per day, in one case combined with food restriction.

The protocol comprised 2 parts.

Table 1

Group	n	Basis for regulation	Insulin preparation	Injections /day	Type of control	Amount of insulin (U/kg)	24 h urine volume (ml)	Urine glucose/24 h (g)	Mean plasma glucose (mg/100 ml)	Plasma glucose, mean range (mg/100 ml)
1	6	D	No insulin	1	C	0	16 ± 6	0.03 ± 0.008	92 ± 11	82 ± 12 109 ± 14
2	6	D	Protamine Zinc Insulin NOVO	1	A	8.5 ± 1.3	25 ± 2	1.4 ± 0.2	149 ± 57	28 ± 10 410 ± 20
3	6	D	Protamine Zinc Insulin NOVO	1	B	2.7 ± 0.4	75 ± 23	6.7 ± 2.0	356 ± 68	167 ± 116 457 ± 52
4 ^a	6	D	Protamine Zinc Insulin NOVO	1	A	8.6 ± 1.6	26 ± 5	1.4 ± 0.8	154 ± 57	21 ± 4 396 ± 36
5	6	D	Protamine Zinc Insulin NOVO	2	A	3.2 ± 1.1	19 ± 5	1.1 ± 0.6	140 ± 26	30 ± 7 387 ± 69
6	6	D	Protamine Zinc Insulin NOVO	3	A	1.8 ± 0.3	22 ± 6	1.8 ± 1.0	186 ± 63	51 ± 21 383 ± 93
7	6	D	Ultralente NOVO	1	A	8.5 ± 2.9	27 ± 5	1.5 ± 0.6	144 ± 51	32 ± 14 399 ± 41
8	6	D	Depot-Insulin Hoechst	1	A	12 ± 3.2	23 ± 7	1.5 ± 0.5	189 ± 40	36 ± 13 395 ± 47
9	6	D	Protamine Zinc Insulin Wellcome	1	A	6.6 ± 1.3	16 ± 6	0.6 ± 0.3	131 ± 45	31 ± 18 383 ± 117
10	6	D	Protamine Zinc Insulin NOVO + 3 × protamine 1	1	A	31.2 ± 12	33 ± 6	1.8 ± 0.7	138 ± 39	30 ± 8 517 ± 41
11	6	D	Long acting Ultralente NOVO	1	A	45.6 ± 17	25 ± 9	1.2 ± 0.6	198 ± 82	28 ± 15 414 ± 37
12	6	D	Very long acting Ultralente NOVO	1	A	11.7 ± 1.9	20 ± 4	1.1 ± 0.6	189 ± 83	68 ± 32 307 ± 77
13	6	D	Very long acting Ultralente NOVO	1	B	1.6 ± 0	99 ± 42	9.7 ± 3.1	375 ± 35	212 ± 88 487 ± 57
14	7	PG	Very long acting Ultralente NOVO	1	A	13.2 ± 1.8	19 ± 5	0.6 ± 0.4	122 ± 55	55 ± 15 187 ± 99
15	6	PG	Protamine Zinc Insulin NOVO	1	A	7.0 ± 1.4	16 ± 5	^b	101 ± 42	27 ± 5 236 ± 102

In the columns are shown: The basis for the regulation of plasma glucose, which was either diuresis or plasma glucose, insulin treatment, injection schedule, type of control, amount of insulin administered, diuresis, urine glucose excretion, mean plasma glucose and range of means. Results are given as mean ± SD. D = diuresis; PG = plasma glucose; C = nondiabetic animals; A = animals in which optimal control was attempted; B animals kept free of ketonuria but poorly controlled. ^a In group 4 the animals were left without food for 8 h prior to insulin injection. ^b missing

1. Three Week Preparatory Period. During this period the individual diabetic animals were treated with various doses of insulin in order to produce either a mild 'well-controlled' (A) or a 'badly controlled' (B) diabetes. Different insulins and injection schedules were used as shown in Table 1. The adjustment of the insulin dose during the preparatory period was performed on the basis of daily determinations of urine volume [12] combined with measurements with Clinistix (Ames Company, Inc.). In practice it was found that a certain small degree of glucosuria had to be tolerated to avoid loss of animals in hypoglycaemia. In two groups however, (group 14 and 15) see Table 1, the adjustment, during the 3 week period, was based on plasma glucose measured just prior to the daily insulin injection using Dextrostix and a Reflectance Meter (Ames Company, Inc.) [8].

2. Five Day Test Period. During this period the type and amount of insulin as well as the injection schedule were the same as at the end of the preparatory period. Urine was collected on the second, third and fifth day. The results are expressed as a mean of the 24 h urine volumes and glucose excretion from the three days. Twenty four h plasma glucose estimations were made on the fourth day starting at 4 p. m. and followed by 2 hourly blood sampling until 4 p. m. the following day. The insulin injection was given at the start of the 24 h period just after the first plasma glucose measurement, unless otherwise specified.

Plasma glucose was measured on 20 µl samples of tail vein blood collected in heparinised microcapillary tubes using a glucose oxidase method [2]. Urine glucose was also analysed with a glucose oxidase method (Glox KABI).

Each diurnal experiment was performed on 6–7 rats.

Results are expressed as mean ± SD.

Results

The correlation coefficient between urine volume and urine glucose excretion calculated from the urine specimens of the experiments was 0.95 ($p < 0.001$). Only urines with a positive reaction to Clinistix were used in the calculation.

The results obtained in the testperiod of the 15 experiments are given in Table 1, which shows the average insulin dose, the 24 h urine volume and glucose excretion, 24 h mean plasma glucose and mean plasma glucose range.

The 24 h plasma glucose profiles from a number of the groups are shown in Fig. 1–5.

Fig. 1 shows the 24 h plasma glucose profiles from normal animals (group 1) and animals in whom good control was attempted with Protamine Zinc Insulin (group 2) as well as animals who were maintained in a poor state of control but also treated with Protamine Zinc Insulin (group 3). In group 2 plasma glucose concentration showed a very steep fall from 359 ± 100 (SD) mg/100 ml to 91 ± 49 mg/100 ml within two hours after the insulin injection. Thereafter the plasma glucose concentration was below normal values, from 13–60 mg/100 ml for several hours. High values, however, were found during the last 6 of the 24 h. In group 3 a steep fall in plasma

Fig. 1. Effect of once-daily Protamine Zinc Insulin on plasma glucose profile in diabetic rats with diabetic control based on diuresis. Protamine Zinc Insulin NOVO was injected just after the first plasma measurement at 4 p. m. Results are shown as mean $\pm$ SD. ($n = 6$ in each group). *Upper curve:* Group 3, diabetic rats in type B control. Amount of insulin: 2.7 ± 0.4 U/kg (●—●). *Lower curve:* Group 2, diabetic rats in type A control. Amount of insulin: 8.5 ± 1.3 U/kg (●—●). (---): Group 1, non diabetic control rats. No insulin

glucose concentration was also found within 2 hours, followed by high values during the rest of the 24 h period.

Group 4 was a type A controlled group. Food was withdrawn from the animals 8 hours before the insulin injection and food and insulin were given at the same time. This feeding pattern was repeated daily during the preparatory period and the test period. The plasma glucose curves obtained were undistinguishable from those obtained from group 2 animals on unrestricted food intake. Protamine Zinc Insulin from another source was subsequently tested and results were no better (group 9).

In two further experiments Protamine Zinc Insulin was administered 2 or 3 times per day (group 5 and 6, Figs. 2 and 3). It can be clearly seen that acute changes in plasma glucose levels were still found.

Two other commercially available insulin preparations were tested: Ultralente® (group 7) and Depot-Insulin Hoechst® (group 8). Neither of these insulin preparations gave a 24 h plasma glucose different from that previously found with Protamine Zinc Insulins.

Several Insulin preparations not commercially available were then tested. Protamine Zinc Insulin with 3 times the normal protamine content was tested (group 10) but no difference in 24 h plasma glucose pattern was found compared to the normal Protamine Zinc Insulin.

Heat treated, extra long acting Ultralente preparations were then assessed. The long acting Ultralente

Fig. 2. Effect of twice-daily Protamine Zinc Insulin on plasma glucose profile in diabetic rats with diabetic control based on diuresis. Protamine Zinc Insulin NOVO was injected at 8 p. m. and 8 a. m. just after the plasma glucose measurements. Results are given as mean $\pm$ SD ($n = 6$). (●—●): Group 5, diabetic rats in type A control. Amount of insulin: 3.2 ± 1.1 twice for day. (---): Group 1, non diabetic control rats. No insulin

Fig. 3. Effect of three times-daily Protamine Zinc Insulin on plasma glucose profile in diabetic rats with diabetic control based on diuresis. Protamine Zinc Insulin NOVO was injected at 4 p. m., at 12 p. m. and at 8 a. m. just after the plasma glucose measurements. Results are given as mean $\pm$ SD ($n = 6$). (●—●): Group 6, diabetic rats in type A control. Amount of insulin: 1.8 ± 0.3 U/kg three times per day. (---): Group 1, non diabetic control rats, no insulin

preparation (group 11) had no advantage over any of the above described insulins, but the very long acting Ultralente (group 12–14) was superior to all the other insulins tested.

In group 12 adjustment of insulin dose was based on urine volume. The plasma glucose falls were less steep than with Protamine Zinc Insulin and the lowest plasma glucose values in the groups in type A control were close to normal. However, plasma glu-

Fig. 4. Effect of once-daily very long acting Ultralente NOVO on plasma glucose profile in diabetic rats with diabetic control based on diuresis. Very long acting Ultralente was injected just after the first plasma glucose measurement at 4 p. m. Results are given as mean $\pm$ SD ($n = 6$). (●—●): Group 12, diabetic rats in type A control. Amount of insulin 11.7 ± 1.9 U/kg. (---): Group 1, non diabetic controls. No insulin

Fig. 5. Effect of once-daily injection of very long acting Ultralente where diabetic control was based on plasma glucose measurements just prior to the insulin injection. Results are given as mean $\pm$ SD ($n = 6$). Upper curve: Group 13, diabetic rats in type B control. Amount of insulin: 1.6 ± 0 U/kg (●—●). Lower curve: Group 14, diabetic rats in type A control. Amount of insulin 13.2 ± 1.8 U/kg (●—●). Dotted line: Non diabetic controls. No insulin

glucose concentration prior to the next insulin injection was still high (Fig. 4).

In the next series (group 14) plasma glucose concentration just prior to the next insulin injection was measured and the adjustment of dose was based on plasma glucose measurement instead of diuresis as hitherto. As the plasma glucose concentration 24 hours after the last injection nearly always is the highest value in the diurnal plasma glucose pattern in the rat a value of 150 mg/100 ml was intentionally

Fig. 6. Initial plasma glucose fall after injection of 2 different insulin preparations. Plasma glucose measurements were done every 20 min for 4 hours. Results are given as mean $\pm$ SD ($n = 4$). One animal was given 8 U/kg, two animals were given 16 U/kg and one animal was given 22.4 U/kg of insulin. Left: Protamine Zinc Insulin NOVO. Right: Very long acting Ultralente NOVO

chosen. If plasma glucose was below 50 mg/100 ml, no insulin was given; if between 50 and 150 mg/100 ml the same dose was administered as on the previous day, if more than 150 mg/100 ml on more than two consecutive days a higher dose was administered.

The same insulin preparation was also used in a group in type B control (group 13). Only a small dose of insulin was administered to prevent ketonuria. Fig. 5 shows that in this way two distinctly different groups of rats were obtained. The group in type A control had nearly normalised plasma glucose throughout the day, the group in type B control had consistently high plasma glucose. Similar results were obtained when this experiment was repeated with other batches of this particular insulin.

In order to follow the initial plasma glucose fall more closely, plasma glucose was measured every 20 minutes for the first 4 h after injection of the 7 different insulin preparations from group 2 and groups 7–12. These animals were treated for a few days before the experiment with 6.4 U/kg. On the experimental day 8, 16 or 22.4 U/kg was then given subcutaneously. In the short interval of 4 hours all the insulins except one showed the same steep initial fall. Fig. 6 shows the results obtained in this type of experiment with two of the insulins, the Protamine Zinc Insulin NOVO and the very long acting Ultralente preparation. The figure clearly shows a less steep fall in plasma glucose using the very long acting Ultralente as compared to Protamine Zinc Insulin.

Discussion

The availability of methods for obtaining complete control of plasma glucose concentration in experimental diabetes is obviously of considerable interest, especially in the studies of diabetic angiopathy and neuropathy using the insulin-deprived animal as a model [3, 7, 9, 10].

The control state of diabetic rats in insulin treatment can be judged in different ways.

Steiner et al. [13] compared the growth rate of groups of diabetic rats on different insulin dosages to normals. He thereby found a suitable insulin dose. In the present study all groups in type A control had the same weight gain as the controls. This means that a wide range of plasma glucose can be tolerated in animals preserving normal growth rate.

Blackshear et al. [1] has shown that normal plasma glucose can be found 24 h after the last Protamine Zinc Insulin injection. The insulin dose was however larger than that used in the present study. With this larger dose it is possible that they were measuring at a point of time when the plasma glucose was just beginning to rise, as it was in the present experiments 18 to 20 hours after the last dose.

In other studies with diabetic rats in insulin treatment the problem has been that good diabetic control results in hypoglycaemia and loss of animals [4, 5, 6, 12].

In the study of Greene et al. [5] one group of diabetic rats an insulin treatment was intentionally kept at plasma glucose levels below the highest measured normal value. In this group plasma glucose was sometimes normal but hypoglycaemic values often occurred causing a loss of 7 out of 24 animals in 14 days. In other groups where hyperglycaemia was sometimes found, no loss of animals was seen. These observations are in agreement with the present study where it was found that either slight glucosuria or elevated pre-injectional plasma glucose had to be present nearly every day to prevent loss of animals when the commercial insulin preparations were used. However, with the very long acting Ultralente used in the present study it is possible to select levels even lower than the one selected for type A control without hypoglycaemia. Jacobsen [7] has used a level of 100 mg/100 ml without loss of animals during one month. In this study the daily individual insulin dose was adjusted according to plasma glucose measurements, as in the present study.

The present investigations demonstrate that it is possible to achieve considerably improved plasma glucose control in streptozotocin diabetic rats when a heat-treated long acting insulin is used. An acceptable result was not obtained with any of the other

insulin preparations tested whether given once a day or in divided doses several times a day.

The experimental design originally included adjustment of the control based on 24 h urine output [15]. This is justified by the well-known relationship between urine volume and glucose excretion, also appearing in these experiments [14]. However, urine volume and the glucose content gives no information about normoglycaemia versus hypoglycaemia.

In the present series of experiments the use of urine volume as an index of glucose control pointed to the very long acting Ultralente as the most promising candidate. Afterwards when the control state was adjusted on the basis of plasma glucose values it could be shown that nearly normal plasma glucose levels could be obtained with this insulin. The pre-injection value was slightly elevated while the values were close to normal the rest of the day. The reason for choosing a level a little above normal was that the animal model was going to be used for long term experiments (6–9 months) and loss of animals due to hypoglycaemia was to be avoided.

The model described here has been used in our laboratory for periods of many months and has been found to be reliable. Its effectiveness in plasma glucose control in rats has been proved in experiments lasting 6 and 9 months where the weight of the animals in type A control was the same as that of the non-diabetic control group. Urine volume was a little higher than that of the controls, mean urine glucose excretion was 0.19 and 0.29 mg/ml. Mean morning plasma glucose was 182 mg/100 ml and no animals were lost due to hypoglycaemia [9, 10].

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