Impaired Retrograde Axonal Transport from a Nerve Crush in Streptozotocin Diabetic Rats

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Summary. The axonal transport of proteins in crushed nerves of streptozotocin (40 mg/kg) diabetic rats was investigated 4 weeks after induction of diabetes. ^{35}S -methionine was used as a marker for protein and ^3H -fucose as a marker for glycoprotein. The precursors were injected into the fifth lumbar spinal ganglion and the accumulation of TCA-insoluble activity proximal and distal to a sciatic nerve ligature was measured at different time intervals after application of a crush. The start of accumulation distal to the ligature was delayed by 1 hour for proteins as well as for glycoproteins. Furthermore, the total amount of accumulated protein after 19 h was decreased by 18% while the decrease was 21% for glycoprotein. By insulin treatment the differences could both be prevented and reversed after 3 days of normoglycaemia. These findings demonstrate an impaired response to a nerve crush and might be the explanation for the regenerative abnormalities of peripheral nerves in diabetes.

Key words: Diabetic neuropathy, axonal transport, streptozotocin diabetes, nerve crush, nerve regeneration, insulin treatment.

The functional changes of the nerves in human diabetes are well known [15, 33]. Similar changes have been demonstrated in streptozotocin diabetic rats, namely a reduction of conduction velocity and a prolonged preservation of nerve conduction during ischaemia [18, 19].

Diminished axon calibre in the common peroneal nerve has been demonstrated in these rats [16, 17] and reduced amounts of smooth endoplasmic reticulum have also been observed suggesting an abnormality of axonal transport [18]. Recently we have found a reduction of the perikaryal volume of

the lower motor and primary sensory neurons, indicating that the entire neuron is involved [31]. Finally, axonal transport studies have shown decreased retrograde flux of transported glycoproteins in the diabetic model [20, 30].

Axonal transport of endogenous synthesized proteins and glycoproteins, various enzymes and organelles has been demonstrated by various techniques [8, 11, 12, 23]. Forward transport from the nerve cell body has been demonstrated consisting of membrane material [1], molecules involved in transmitter function [14, 24] and structural proteins of the axon [10]. Retrograde axonal transport has been demonstrated for exogenous molecules taken up by axon terminals, for endogenous enzyme molecules [21, 24] and for the return of labelled proteins synthesized in the perikaryon of the cell [5, 32].

Recently Bisby has proposed a method for analysing the flux of retrogradely transported protein in intact and in crushed nerves [2, 3]. A premature reversal of axonal transport at a nerve crush was demonstrated [3, 6]. He proposed this as the signal for the chromatolytic changes of the nerve cell following a nerve crush.

It is known from human studies that the regenerative capacity of peripheral nerves is reduced in diabetic patients [27, 28]. Therefore, the present study was undertaken to investigate whether a derangement of the retrograde axonal transport from a nerve crush occurs in streptozotocin diabetic rats. We have estimated retrograde axonal transport as proposed by Bisby [2, 3]. The experiments were performed with and without insulin treatment of the animals.

Materials and Methods

Male Wistar rats aged 24 weeks and weighing between 325 and 455 g were injected intravenously with streptozotocin in citrate buffer pH 4.5 (40 mg/kg) to induce diabetes. Blood glucose was

Fig. 1. Distribution of TCA insoluble activity after a 2 h collection period proximal and distal to two ligatures placed 7 h after dorsal root ganglion injection of ^{35}S -methionine and crushing of the nerve. The bars are 3 mm wide except for the three 2 mm bars between the ligatures. The activity is given relatively to activity in the interligature segment (mean $\pm$ SEM). The full drawn line represents the control group ($n = 7$) and the dashed line the diabetic group ($n = 8$). The symbols in the figure designates the amount of activity in the nerve segments used for calculation of accumulations. A_p , activity in proximal collection segment; A_d , activity in distal collection segment; A_{cr} , activity in the crush segment; S_p , activity in 6 mm segment proximal to A_p ; S_i , activity in 6 mm interligature segment; S_d , activity in 6 mm segment between A_d and A_{cr} .

measured approximately with an reflectance meter (Ames) in whole blood from a tail vein. Blood glucose values above 13.9 mmol/l 24 h after injection of streptozotocin and on the day of the experiment were the criteria for inclusion in the diabetic group. The axonal transport experiments were carried out at the age of 28 weeks in a group of age and weight matched controls and in two groups of insulin treated rats.

The precursors used were L-(6- ^3H)fucose (Amersham) 26 Ci/mmol, and L-(^{35}S)methionine (Amersham) 720–1100 Ci/mmol. They were concentrated by freeze drying and dissolved in a buffered salt solution to give a final concentration of 10 mCi/ml ^3H -fucose and 5 mCi/ml ^{35}S -methionine.

Sodium pentobarbitone (50 mg/kg) was used for anaesthesia and body temperature was maintained by placing the rats on an electrically heated pad [30]. One microlitre of the precursor solution was injected into the fifth lumbar ganglia at various times before sacrifice. The nerve was crushed at a fixed site in the distal thigh by a ligature tightened against a glass rod. This was done either 5–10 min after injection or on the day before. The crush was left without a ligature. Two hours before sacrifice two ligatures separated by a 6 mm interval were applied 15 mm proximal to the crush [3]. The nerves were dissected out immediately after sacrifice and axonal transport was stopped by cooling the nerve on a cold copperblock (-70°C).

The nerve was cut into 3 mm segments which were placed in numbered vials containing 1 ml icecold trichloroacetic acid, w/v 10% (TCA). On the following day the TCA solution was aspirated and the nerve segment washed in an additional 1 ml 10% TCA for 1 h at room temperature. The TCA treated nerve segments were

dissolved in 150 μl Lumasolve (Lumac) and counted for 10 min in 4 ml Lipoluma (Lumac). The counts were corrected for quenching and expressed as dpm.

The axonal flux of material which designates the amount moving through a cross section of the nerve per unit time was measured as the accumulation proximal and distal to ligatures [20]. As units for activity the pre-existing level in the collection segment was chosen [2, 20]. This level was calculated as the mean activity of equal length of nerve proximal and distal to the collection segments. The formulas used for calculation of proximal and distal accumulation were

$$\text{PA} = \frac{\frac{A_p}{S_p + S_i} - 1}{t} \times 3 \text{ and } \text{DA} = \frac{\frac{A_d}{S_d + S_i} - 1}{t} \times 3$$

respectively (Fig. 1). PA, proximal accumulation; DA, distal accumulation; A_p , activity in proximal collection segment (3 mm); A_d , activity in distal collection segment (3 mm); S_p , activity in 6 mm segment proximal to A_p ; S_i , activity in 6 mm segment between the ligatures; S_d , activity in 6 mm segment distal to A_d ; and t , time interval in h between placing of the ligatures and stopping of axonal transport. The quantity measured in this way is the flux in the axons of activity anterogradely and retrogradely respectively, assuming that the accumulations are caused only by axonal transport [20]. Activity is measured as amount of radioactivity per mm nerve of the neighbouring segments, (pre-existing activity)/mm. Thus the unit of transport is (pre-existing activity) $\text{mm}^{-1} \text{h}^{-1}$.

The accumulation at the crush was calculated relative to the 6 mm segment proximal to the crush region (S_d) using the formula

$$\text{CA} = \left(\frac{A_{cr}}{\frac{3}{2} S_d} - 1 \right) \times 9. \text{ CA, accumulation at the crush; } A_{cr},$$

activity in 9 mm segment containing the crush; and S_d , activity in 6 mm segment proximal to A_{cr} . The units are (pre-existing activity) mm^{-1} . The study was carried out in three parts.

Study 1

Following spinal ganglion injection and subsequent application of the crush axonal flux was estimated at the intervals 5–7, 7–9, 9–11, 11–13 and 17–19 h. Each diabetic animal was paired with an age and weight matched control. If the same time interval was used for both nerves of an animal the average value was taken as a single determination.

Study 2

The axonal flux was estimated in the interval 7–9 h with the crush applied 24 h before injection of the precursors in a diabetic group and in age and weight matched controls.

Study 3

After induction of diabetes two groups were treated with different insulin regimes. A long-acting insulin preparation (modified Ultralente, pH 5.5), kindly provided by Dr. J. Schlichtkrull, Novo Research Institute, was used in this study. Insulin was given daily at 1530 h and the dose was adjusted according to blood glucose determined just before injection [18, 26]. In the first group the first insulin dose was given 24 h after injection of streptozotocin and the last on the day of the experiment. The insulin dose given was reduced during the first week from 8–6 U/day to about 4 U/day. Blood glucose concentration was kept as close to normal values as possible. Values between 3.3 and 6.7 mmol/l were accepted without change of insulin dose.

In the second group no insulin was given for 19 days. The first insulin dose was given 20 days after injection of streptozotocin and

Fig. 2. The distal accumulation (DA) in the upper half of the figure is given as a function of time for proteins and glycoproteins. The full drawn line represents the control group ($n = 5-7$) and the dashed line the diabetic group ($n = 6-8$). The measured values are given in units of (pre-existing activity) $\text{mm}^{-1} \text{h}^{-1}$ (mean $\pm$ SEM). The horizontal bars show the collection period for each estimate. In the lower half the DA of the various intervals is summated as a function of time the unit being (pre-existing activity) mm^{-1} to give the total amount of material returned by retrograde transport. The abscissa shows the hours post injection (h p. i.)

the last on the day of the experiment 28 days after induction of diabetes. The initial dose was 14 U/day which was gradually reduced to about 8 U/day. The same criteria for acceptable blood glucose values was used as in the first group.

The axonal flux in both groups were estimated at the interval 7-9 h after injection with the crush applied at the time of the injection.

Statistics

Estimates of population parameters are given as mean $\pm$ SD. Student's unpaired *t* test was employed for statistical analysis using a 5% limit of significance.

Results

During the four week experimental period all diabetic rats showed weight loss (mean 93 g, range 5-175 g). Mean body weight of controls increased

36 g (range 0-70 g). The blood glucose concentration of diabetic rats was 21.4 mmol/l (range 15.0-27.2 mmol/l) on the day of the experiment.

Axonal Transport in Crushed Nerves of Diabetic Rats and Controls Studied in 5 Time Intervals

The accumulation of ³⁵S-methionine labelled proteins at a crush and at a double ligature applied 7 h after injection of the precursor is shown in Figure 1. Labelled material accumulated at the crush and the two ligatures. Much less activity accumulated in the distal segment (A_d) in the diabetic group than by the controls. In Figure 2 distal accumulation (DA) is shown as a function of time after injection for methionine and fucose labelled material, respectively. A delay of approximately 1 h before accumulation commenced and a smaller maximum was seen for the diabetic group as compared to the control group. The total

amount of material returned from the crush to the distal collection segment for the period 5 to 19 h was decreased by 19% for methionine labelled protein and by 21% for fucose labelled glycoprotein in the diabetic group. The difference was most pronounced in the 7–9 h interval which therefore was chosen for the following two series of experiments (Table 1).

Table 2 shows proximal accumulation (PA) for the different time intervals. Significantly increased

accumulation was only reached for glycoprotein in the late intervals in the diabetic group.

The accumulation of protein and glycoprotein at the crush (CA) is shown in Table 3. Accumulation in the diabetic group was significantly increased compared to the controls for protein while accumulation of glycoprotein was only significantly increased in the late time interval. For both precursors the maximal accumulation seems to be earlier in the control group than in the diabetic group.

Table 1. Accumulation of protein and glycoprotein distal to a ligature (DA) 7–9 h after precursor injection for 2 control groups, 2 diabetic groups and 2 insulin-treated diabetic groups. The accumulation of material was measured in units of (pre-existing activity) $\text{mm}^{-1} \text{h}^{-1}$. Thus, the accumulation was used for estimating the axonal flux of material relative to the level in nerve segments with unrestricted transport. Results are given in mean $\pm$ SD (n)

Group	^{35}S -methionine (pre-existing activity) $\text{mm}^{-1} \text{h}^{-1}$	^3H -fucose $\text{mm}^{-1} \text{h}^{-1}$
Control, day 0	3.6 ± 0.5 (7)	2.1 ± 0.7 (6)
Control, day 1	3.4 ± 0.7 (8)	2.3 ± 0.7 (8)
Diabetes, day 0	1.4 ± 0.8 (8)	0.3 ± 0.8 (7)
Diabetes, day 1	2.2 ± 1.3 (8)	1.2 ± 1.1 (8)
Insulin-treated 28 d	3.3 ± 0.4 (9)	2.6 ± 0.6 (9)
Insulin-treated 8 d	3.1 ± 0.8 (5)	2.4 ± 0.5 (5)

The following differences are statistically significant. For ^{35}S -methionine labelled protein: $\text{D}_0\text{-C}_0$, $2p < 0.001$; $\text{D}_1\text{-C}_1$, $2p < 0.05$; $\text{I}_{28}\text{-D}_0$, $2p < 0.001$; $\text{I}_8\text{-D}_0$, $2p < 0.01$ and for ^3H -fucose labelled glycoprotein: $\text{D}_0\text{-C}_0$, $2p < 0.01$; $\text{D}_1\text{-C}_1$, $2p < 0.05$; $\text{I}_{28}\text{-D}_0$, $2p < 0.001$; $\text{I}_8\text{-D}_0$, $2p < 0.001$

Axonal Transport 7–9 h after Precursor Injection in Diabetic Rats and Controls 24 h after Application of a Crush

In Table 1 distal accumulation (DA) for both precursors is given. Again distal accumulation was decreased in the diabetic group and no statistical differences were obtained between day 0 and day 1 after the crush.

Proximal accumulation (PA) was similar in both groups for both precursors to the values found on day 0 (Table 2).

However, an increase in accumulation at the crush (CA) was seen at day 1 after the crush. As shown in Table 3 the relative accumulation at the crush was increased on day 1 for both precursors in the control group. In the diabetic group the increase was only statistically significant for the fucose labelled glycoprotein.

Table 2. Accumulation of protein and glycoprotein proximal to a ligature (PA) in various time intervals after injection of precursor. PA is given for control, diabetic and insulin treated diabetic groups in mean $\pm$ SD (n). The accumulation of material was measured in units of (pre-existing activity) $\text{mm}^{-1} \text{h}^{-1}$. Thus, the accumulation was used for estimating the axonal flux of material relative to the level in nerve segments with unrestricted transport

Precursor	Group	Hours post injection				
		5–7 (pre-existing activity) $\text{mm}^{-1} \text{h}^{-1}$	7–9 $\text{mm}^{-1} \text{h}^{-1}$	9–11	11–13	17–19
^{35}S -methionine	Control, day 0	5.0 ± 1.1 (7)	3.9 ± 0.8 (7)	2.5 ± 0.3 (5)	2.5 ± 0.3 (7)	1.8 ± 0.3 (7)
	Control, day 1		4.0 ± 0.7 (8)			
	Diabetes, day 0	6.0 ± 1.2 (7)	4.4 ± 0.6 (8)	3.1 ± 0.8 (6)	2.6 ± 0.5 (7)	2.0 ± 0.4 (6)
	Diabetes, day 1		4.1 ± 0.6 (8)			
	Insulin-treated 28 d		3.2 ± 0.4 (9)			
	Insulin-treated 8 d		3.3 ± 0.3 (5)			
^3H -fucose	Control, day 0	6.7 ± 1.6 (7)	5.7 ± 1.6 (6)	3.6 ± 0.0 (5)	3.1 ± 0.5 (7)	2.2 ± 0.2 (7)
	Control, day 1		6.6 ± 0.8 (8)			
	Diabetes, day 0	7.7 ± 1.7 (6)	6.3 ± 0.7 (7)	5.1 ± 1.2 (6)	3.9 ± 0.7 (7)	3.3 ± 0.9 (6)
	Diabetes, day 1		6.7 ± 1.0 (8)			
	Insulin-treated 28 d		4.4 ± 0.8 (9)			
	Insulin-treated 8 d		4.9 ± 0.8 (5)			

The following differences are statistically significant. For ^{35}S -methionine labelled protein $\text{I}_{28}\text{-C}_0$ (7–9), $2p < 0.05$; $\text{I}_{28}\text{-D}_0$ (7–9), $2p < 0.001$; $\text{I}_8\text{-D}_0$ (7–9), $2p < 0.01$ and for ^3H -fucose labelled glycoprotein $\text{D}_0\text{-C}_0$ (9–11, 11–13, 17–19), $2p < 0.05$; $\text{I}_{28}\text{-D}_0$ (7–9), $2p < 0.001$; $\text{I}_8\text{-D}_0$ (7–9), $2p < 0.01$

Table 3. Accumulation of protein and glycoprotein at a crush (CA) at various time intervals after injection of precursor. CA is given for control, diabetic and insulin-treated diabetic groups in mean $\pm$ SD (n). The accumulation is measured relative to the level of activity in an unrestricted segment of the nerve proximal to the nerve crush. The units for accumulation are (pre-existing activity) mm^{-1}

Precursor	Group	Hours post injection			
		9 (pre-existing activity)	11 mm^{-1}	13	19
^{35}S -methionine	Control, day 0	21.6 $\pm$ 1.9 (7)	25.2 $\pm$ 5.2 (6)	24.1 $\pm$ 4.2 (7)	22.5 $\pm$ 4.9 (7)
	Control, day 1	29.8 $\pm$ 6.6 (8)			
	Diabetes, day 0	28.2 $\pm$ 3.6 (8)	26.4 $\pm$ 3.2 (6)	30.7 $\pm$ 3.3 (6)	27.7 $\pm$ 2.8 (6)
	Diabetes, day 1	30.6 $\pm$ 4.1 (8)			
	Insulin-treated 28 d	23.1 $\pm$ 3.8 (9)			
	Insulin-treated 8 d	21.2 $\pm$ 5.1 (5)			
^3H -fucose	Control, day 0	22.1 $\pm$ 3.5 (8)	23.6 $\pm$ 4.1 (6)	25.4 $\pm$ 6.1 (8)	23.4 $\pm$ 4.2 (8)
	Control, day 1	30.5 $\pm$ 8.4 (8)			
	Diabetes, day 0	24.6 $\pm$ 5.2 (7)	26.4 $\pm$ 2.9 (6)	28.3 $\pm$ 2.2 (6)	29.3 $\pm$ 2.6 (6)
	Diabetes, day 1	32.6 $\pm$ 4.6 (8)			
	Insulin-treated 28 d	23.5 $\pm$ 3.3 (9)			
	Insulin-treated 8 d	21.8 $\pm$ 2.5 (5)			

The following differences are statistically significant. For ^{35}S -methionine labelled protein $\text{D}_0\text{-C}_0$ (9 h), $2p < 0.001$; $\text{D}_0\text{-C}_0$ (13 h), $2p < 0.01$; $\text{D}_0\text{-C}_0$ (19 h), $2p < 0.05$; $\text{C}_1\text{-C}_0$, $2p < 0.01$; $\text{I}_{28}\text{-D}_0$, $\text{I}_8\text{-D}_0$, $2p < 0.05$ and for ^3H -fucose labelled glycoprotein $\text{D}_0\text{-C}_0$ (19 h), $\text{C}_1\text{-C}_0$, $2p < 0.05$; $\text{D}_1\text{-D}_0$, $2p < 0.01$

Axonal Transport in Insulin-treated Diabetic Rats

Of the insulin treated rats all except two survived.

The rats treated with insulin from the first day of diabetes gained 37 g in weight (range 10–80 g), similar to the weight gain in the control group. The blood glucose values measured 24 h after the daily insulin injection were 3.7, 6.0 and 10.7 mmol/l at the 20th, 50th and 80th percentiles. The mean blood glucose concentration during the day of the experiment was 5.6 ± 1.7 mmol/l.

The rats which were untreated for the first 20 days lost 55 g (range 0–110 g) body weight. During the subsequent 8 days of treatment they regained their starting weight. The blood glucose values 24 h after the daily insulin injection, were normal for the group for 3 days before sacrifice, with a mean blood glucose level on the day of the experiment of 4.3 ± 1.1 mmol/l.

Distal accumulation (DA) values for the individual animals 7–9 h after precursor injection are given for the control, the first insulin treated group (28 days of treatment), the second insulin treated group (8 days of treatment) and the diabetic group in Figures 3 and 4 for methionine labelled proteins and fucose labelled glycoproteins, respectively. Normalization occurred for proteins and glycoproteins in both groups of insulin treated rats. The statistical significances for the differences are shown in Table 1.

Fig. 3. Individual values for distal accumulation (DA) of proteins 7–9 h after spinal ganglion injection of ^{35}S -methionine for the control group (C, ●), the group treated with insulin from the first day of diabetes (D + I₁, ○), the group treated with insulin for only 8 days (D + I₂, ○), and the diabetic group (D, ○)

The crush accumulations (CA) were also normalized in the insulin treated groups although there was a decreased proximal accumulation (PA) in the insulin treated groups compared to the normal as well as to the diabetic group (Table 2).

Fig. 4. Individual values for distal accumulation (DA) of glycoproteins 7–9 h after spinal ganglion injection of ^3H -fucose for the control group (C, ●), the group treated with insulin from the first day of diabetes (D + I₁, ○), the group treated with insulin for only 8 days (D + I₂, ○), and the diabetic group (D, ○)

Discussion

This study has demonstrated a delay and a decrease in the early response of the retrograde axonal transport from a nerve crush in diabetic rats. The changes were found a few hours as well as one day after the application of the crush for glycoprotein marked by ^3H -fucose [25] and for protein marked by ^{35}S -methionine.

A maximal estimate of the blood borne labelling is 30% of that accumulated by axonal transport for a crush applied before injection of precursor [4]. However, the level of precursor in the blood after intraperitoneal injection is decreased to less than 10% of maximal concentration after 2 min and to less than 2% after 2 h [9]. The contribution of blood borne activity to the accumulation at the crushes can therefore be neglected as the nerve crush was applied 5 min or 24 h after precursor injection and the ligatures several hours later. An increased local degradation of the accumulated proteins should affect all accumulations equally and consequently cannot explain the decrease in accumulation at the distal collection ligature in the diabetic group. We therefore conclude that the changes observed in accumulation distal to the collection ligatures in this experiment were due to a defect in the reversal of axonal transport at a crush. Besides the delay observed a decrease of the total amount of material returned must also occur, as the retrograde flux in the diabetic nerves is less than or equal to that of the control nerves in all intervals examined.

It is shown that the defect is present on day 1 after the crush.

The increased proximal accumulation (PA) in diabetic rats (Table 2), most pronounced in the late intervals for fucose-labelled glycoproteins, could be due to a differing early response to the nerve crush in the two metabolic states. An alternative explanation is that an increase in the fast axonal transport is present in diabetic nerve. However, in an earlier study we concluded after investigation of the 4–6 and the 19–21 h interval that no difference was present in intact nerve [20]. So the other possibility should be a different time pattern of the anterograde transport.

An increased accumulation at the crush (CA) is seen at all time intervals in the diabetic group when compared to the controls. This can be explained by the decreased retrograde transport.

When comparing the results obtained in the first experiments where the transport study was started just after the crush was applied, to the second series where it began one day after the crush, a difference was found. The accumulation at the crush was more pronounced in nerves crushed one day prior to the measurement. There were no differences in either anterograde or retrograde transport. Consequently the difference must be due to local factors. Proximal to the site of injury in regenerating fibres structural axon changes have been demonstrated [7]. We calculated the accumulation relative to the segment proximal to the crush so the difference could be explained by very early changes in the regenerating stump.

The first insulin experiment demonstrates a complete prevention of the changes in retrograde transport. The changes in retrograde transport are present after two days of diabetes (Sidenius and Jakobsen, unpublished observations). The normal findings in the second insulin experiment, therefore, shows a reversal after only three days of normal blood sugar. This signifies that the axonal changes are reversible at least after a short duration of diabetes. A curious finding is the decreased proximal accumulation in the insulin treated groups compared both to the control and the diabetic group. An increased exploitation of the anterogradely transported material by the axon stimulated by the insulin treatment would result in a decreased accumulation. Further studies are necessary before any conclusions can be made concerning this finding.

The retrograde transport probably conveys information concerning the state of the axons and the nerve endings to the synthetic machinery situated in the nerve cell body [3, 13, 22]. Thus a defect in the retrograde transport from a nerve crush as shown in the present study could give rise to a reduction in the regenerative capacity of the nerve fibres. Sharma and Thomas found no differences in fibre size, fibre

number and axon-myelin ratio 18 weeks after injury to peripheral nerves of diabetic rats [29]. However, a slight lag in myelination was found at 3 weeks. As the peripheral nerve is functionally regenerated in the rat 30 days after a crush [6], abnormal regeneration is most likely to be demonstrated in an earlier time interval after the injury.

Studies by Reske-Nielsen et al. on the terminal motor innervation in muscle biopsies from diabetic patients have shown signs of denervation in recently diagnosed diabetics [28] and defective regeneration in patients with long-term diabetes [27].

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