[原著] Increased Susceptibility of B-cells in Protein-Calorie Malnutrition to Long Term Low Dose Streptozotocin and Its Effect on Re-feeding

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Increased Susceptibility of B-cells in Protein-Calorie Malnutrition to Long Term Low Dose Streptozotocin and Its Effect on Re-feeding

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Abstract

The susceptibility of pancreatic B-cells of protein-calorie malnourished(PCM) rats to long term low dose streptozotocin(STZ) and the effect of this low dose STZ on re-feeding were studied. Male weanling Wistar rats were fed either by 20%(30=normal) or 4%(30=PCM) protein diet. Twenty of each two groups were treated with STZ (5 mg/kg/day i.m.) and the rest with buffer for one month. Ten of each STZ treated normal and PCM rats were re-fed with 20% protein diet for another one month called as "RF-normal" and "RF-PCM" respectively. Wet wt. of liver and pancreas, plasma lipids and insulin were measured. IPGTT, morphometric and histologic study of islets were done. PCM+STZ showed significantly high fasting plasma glucose (FPG) 175.9±46.6 compared to 88.8±12.9 mg/dl (mean±SD) in normal+STZ rats. Both STZ and vehicle treated PCM rats showed impaired IPGTT with peak plasma glucose 663.4±87.8 and 465.8±97.2 mg/dl (mean±SD) respectively. Areas under curves in case of IPGTT were significantly high in PCM with and without STZ than in normal with and without STZ. These were also significantly high in normal and PCM with STZ, compared to normal and PCM without STZ. No./field and size of islet, % of B-cell area/islet and insulin levels were significantly reduced ($p<0.01$ vs. normal) in both STZ and vehicle treated PCM rats. Degree of reduction was more prominent in PCM+STZ. FPG remained high with 221.0±98.8 mg/dl despite some nutritional recovery in RF-PCM rats. Areas under curves in case of IPGTT were significantly high in RF-PCM than RF-normal in both before and after re-feeding. The area under curve reduced significantly in RF-normal after re-feeding but it was unchanged in RF-PCM rats. RF-PCM rats also showed significant reduction in morphometry and insulin level ($p<0.01$ vs. RF-nomal) and degree of reduction was more in RE-PCM after re-feeding. Histologic findings showed close correlationship with morphometry. We concluded that B-cells of morphologically altered islets of PCM rats were more susceptible to STZ and the effect of STZ was persistent after re-feeding. These suggest the possibility of increased susceptibility of pancreatic B-cells to other B-cytotoxic influences as STZ in PCM in childhood and exposure to these agents might produce an irreversible change to the endocrine pancreas.

Introduction

Exocrine pancreatic function is extremely vulnerable to protein depletion[1] but the position regarding endocrine function is controversial. There are some contradictory reports as decreased granulation and atrophied[2], hypertrophied[3] and normal islets of Langerhans[4] in protein-calorie malnutrition (PCM). Malnutrition has been regarded by some as a possible cause of diabetes mellitus (DM)[5]. WHO expert committee in 1985 has given the hypothesis that protein malnutrition with chronic intake of some cyanide containing food may cause diabetes known as malnutrition related diabetes mellitus (MRDM)[6]. R. Harsha Rao has also hypothesized that the endocrine pancreas of protein-calorie malnourished subjects may be more susceptible to toxic substances (chemical, viral or environmental)[7]. But confusion arises when we see some contradictory reports about these hypotheses[8,9] in both morphometric data and in susceptibility of endocrine pancreas of PCM to different B-cytotoxic agents. And as there is lack of sufficient direct experimental evidence, specially about susceptibility and no report about the persistency of the effects of B-cytotoxic agents after re-feeding the PCM rats with adequate protein diet, we performed this study to evaluate the morphom etric change of the islet more precisely and its susceptibility to long term low dose B-cytotoxic stimulation by low dose Streptozotoc in (STZ) in PCM rats, and to determine whether the effects of increased susceptibility of toxic substances are persistent or not after re-feeding of the PCM rats with adequate protein diet.

Materials and Methods

Sixty male weanling Wistar rats were randomly divided into two groups of 30. Thirty rats were fed ad libitum for one month with 20% protein diet and another 30 rats were fed with 4% protein diet for the same duration. The composition of diets in Table 1. indicates that reduction of protein from 20% to 4% was compensated for by increasing the carbohydrate ingredient and further supplementation of 0.2% DL-methionine, the first limiting amino acid in casein[10], to obviate an acute deficiency of this amino acid. 20% protein fed rats were called as "normal rats" and 4% protein fed rats were called as "PCM rats". 20 rats from each normal and PCM groups were treated with STZ (5 mg/kg/day i.m.) and the other 10 rats from each two groups were treated with buffered vehicle in the same manner. After one month 10 STZ treated rats from each normal and PCM groups and all buffered treated rats were used in investigation. The other 10 STZ treated rats from each normal and PCM groups were re-fed with 20% protein diet for another one month without STZ injection. These re-fed normal and PCM rats were called as "RF-normal" and "RF-PCM" respectively. All animals were housed in air-conditioned room (24±1℃) with 12 hour alternately light and dark. Tap water was supplied freely and every alternate day body weight and weight of the consumed food were taken. At the end of the experiment intraperitoneal glucose tolerance test (IPGTT) was done in the morning at about 8:00 am after overnight fasting and about three hours later rats were anesthetized and blood was collected from inferior vena-cave for determining serum lipids and plasma insulin levels.

Table 1. Composition of experimental diets

Composition	4% protein diet	20% protein diet
Casein[1]	4%	20%
DL-Methionine[2]	0.20%	—
Corn starch[1]	55.20%	44.67%
Sucrose[3]	27.60%	22.33%
Corn oil[4]	5%	5%
Mineral mixture[1]	5%	5%
Vitamin mixture[1]	1%	1%
Cellulose Fiber[1]	2%	2%
Calories/100 g[5]	393	393

[1]Casein, corn starch, mineral mixture, vitamin mixture and cellulose fiber were obtained from Oriental Kobo Co. Tokyo, Japan. Mineral mixture consisted of (mg/kg diet) $CaHPO_4.2H_2O$:7,280; KH_2PO_4:12,860; NaH_2PO_4:4,680; NaCl:2,330; Ca-lactate:17,550; Fe-citrate:1,590; $MgSO_4$:3,590; $ZnCO_3$:55; $MnSO_4.4\text{-}6H_2O$:60; $CuSO_4.5H_2O$:15; KI:5. Vitamin mixture consisted of (expressed in units or milligrams of vitamin per kg diet) thiamine HCL:12; riboflavin:40; pyridoxine HCL:8; vitamin B- 12:0.005; ascorbic acid:300; D-biotin:0.2; folic acid:2; calcium pantothenate:50; p-aminobenzoic acid:50; niacin:60; inositol:60; choline chloride:2,000; retinyl acetate:5,000; ergocalciferol:1000 IU; tocopheryl acetate:50; menadione:52;

[2]DL-Methionine was obtained from Wako pure chemical industries Ltd. Tokyo, Japan.

[3]Granular sugar was used.

[4]Corn oil was obtained from Yoshihara oil Co. Osaka, Japan.

[5]Computation based on 4 kcal per g protein and carbohydrate and 9 kcal per g fat.

IPGTT

IPGTT was carried out according to the methods of Wexler and Fisher[11] and Cole and Harned[12]. Blood samples were collected by heparinized capillary tube from retro-orbital sinus of fasted rats and 30,60,120 and 180 minutes after i.p. administration fo 35 ml/kg of 10% w/v glucose solution. After collecting blood it was centrifuged immediately and serum was separated and stored in deep

freeze under -20℃ until measurement. Glucose was determined by glucose oxidase method by using kit "Shinotest for glucose" (Shinotest Shoji Co. Ltd. Tokyo, Japan).

Serum lipids

Serum total cholesterol (TCHO) and serum triglyceride (TG) were measured by enzyme assay method by using kit "Shinotest for TCHO" and "Shinotest for TG" (Shinotest Shoji Co. Ltd. Tokyo, Japan) respectively.

Serum albumin

Serum albumin was measured by "aca discrete clinical analyzer" (Du Pont Company, Delaware 19898, U.S.A.)

Plasma insulin

Plasma insulin was measured by enzyme immunoassay (EIA) method using EIA kit "Isulotec Mochida" (Mochida Pharmacutical Co. Tokyo, Japan). Rat insulin (Novo Research Institute, Denmark) was used as standard.

Histological study

By opening the abdomen of anesthetized rats liver and whole pancreas were dissected out carefully, wet weight of liver and pancreas was measured. Pancreas was fixed in 2.5% glutaraldehyde solution and processed for routine paraffin embedding. About 5 μm thick pancreatic sections were stained with hematox ylin-eosin (H & E) and immunoperoxidase staining for insulin. Immunoperoxidase staining was carried out by avidin-biotin-complex (ABC) method using commercial kit "Histoscan for insulin" (Biomeda Corp. U.S.A.). Primary antibody was produced in guineapig and secondary antibody was anti-mouse antibody. Morphometric analysis of islets was performed. The number of islets was counted from three random sections, one from each head, body and tail of each pancreas under light microscope by counting all islets in 20 fields per section of 200 magnification and the date presented as the mean of three random sections of each pancreas. Similarly the size of whole islets and immunoperoxidase positive areas were measured from three random sections of each pancreas by computer controlled digitizer under light microscope of 200 magnification with the help of 3 dimension image analysis system "Nikon Cosmozone 98" (Nippon Kogaku K.K.Japan) and expressed as mm^2. Beta-cell area was expressed as percentage of whole islet size.

Statistical analysis

For statistical analysis, analysis of variance was used for multiple comparison of IPGTT data and Student's t-test was used for comparison between two groups. the results were expressed as mean±SD.

Results

Nutritional status of the experimental animals is given in Table 2. and in Table 3. Both with and without STZ treated PCM rats showed failure to gain adequate body weight ($p<0.05$), coarseness of hair, reduced pancreatic weight ($p<0.05$), increased liver weight ($p<0.05$), reduced serum albumin ($p<0.05$), reduced serum lipids (TG and TCHO, $p<0.05$). Food intake (mg/kg/day) was reduced significantly in both with and without STZ treated PCM rats ($p<0.05$). The statistical analyses in normal or PCM between with and without STZ treated rats in all parameters is given in Table 2. RF-PCM rats have got some nutritional recovery relative to PCM rats when they were returned to 20% protein diet

Table 2. Nutritional status of Normal and PGM rats

	Normal rats		PCM rats	
	With STZ	Without STZ	With STZ	Without STZ
Total Body wt. gain (g/month/rat)	142.2±10.8†	145.3±14.4	4.3±3.3	6.0±4.1*
Absolute Body wt. (g)	176.8±11.2†	182.9±17.0	41.4±5.9	48.2±6.5*†
Food consumed (g/kg body wt./day)	142.9±45.3†	146.4±29.7	126.4±31.4	129.2±19.8*
Wet pancreatic wt. (% of body wt.)	0.450±0.054*†	0.386±0.035	0.340±0.038	0.290±0.059*†
Wet liver wt. (% of body wt.)	3.796±0.158*†	3.390±0.246	5.043±0.248	4.896±0.641*
Serum albumin (g/dl)	2.8±0.2†	3.0±0.3	1.2±0.1	1.3±0.3*
Serum lipids:				
Triglyceride (mg/dl)	186.8±29.5*†	138.6±31.5	69.3±18.5	47.2±10.1*†
Total cholesterol (mg/dl)	83.0±10.9*†	67.8±8.8	64.0±21.1	56.9±24.9

Mean±SD *p<0.05 vs. normal without STZ

†p<0.05 vs. PCM with STZ

Table 3. Nutritional status of re-feeding rats

	RF-normal rats	RF-PCM rats
Total body wt. gain(g)		
a) First month	146.3±8.6	4.8±3.2**
b) Second month	91.5±12.2	91.8±18.9
Absolute body wt.(g)		
a) First month	183.3±8.5	49.8±8.1**
b) Second month	274.8±19.4	141.6±24.1**
Food consumed(g/kg body wt./day))		
a) First month	142.9±45.3	126.4±31.5*
b) Second month	152.3±21.4	150.8±23.5
Wet pancreatic wt.(% of body wt.)	0.463±0.154	0.352±0.040
Wet liver wt. (% of body wt.)	3.323±0.296	4.999±0.468**
Serum albumin (g/dl)	2.8±0.3	2.4±0.5
Serum lipids:		
Triglyceride (mg/dl)	140.6±67.7	175.5±62.9
Total cholesterol(mg/dl)	77.0±12.0	98.0±32.2

Mean±SD * p<0.05, ** p<0.01 vs. RF-normal

as indicated by table 3. There was no significant difference between RF-normal and RF-PCM rats in body weight gain and food intake in the second month. Though the wet liver weight remained significantly increased in RF-PCM rats, wet pancreatic weight, serum lipids and serum albumin level were not significantly different between RF-normal and RF-PCM rats.

Figure 1 is representing the results of IPGTT in PCM, normal, PCM+STZ and normal+STZ. PCM+STZ showed significant high fasting plasma glucose (FPG) of 175.9±46.6 mg/dl compared to 88.8±12.9 mg/dl in normal +STZ rats ($p<0.05$). Plasma glucose levels increased at all the intervals after glycemic stimulation with significance at 30 and 120 minutes in PCM +STZ rats compared to normal+STZ. Though FPG was not increased plasma glucose levels after glycemic stimulation was increased significantly at all intervals in PCM rats. Plasma glucose level increased at all interval in both normal+STZ and PCM+STZ compared to normal and PCM without STZ ($p<0.05$). The areas under curves of these groups are represented in Table 4. The area under curve was significantly increased in both with or without STZ treated PCM compared to both with or without STZ treated normal rats ($p<0.01$). It was also significantly increased in STZ treated normal and PCM rats compared to normal and PCM without STZ respectively ($p<0.01$). Figure 2. is representing the results of IPGTT in re-feeding rats. FPG level was significantly high in RF-PCM rats than RF-normal rats in both before and after re-feeding ($p<0.05$). Plasma glucose level in RF-PCM rats after re-feeding increased significantly at all the intervals after glycemic stimula-

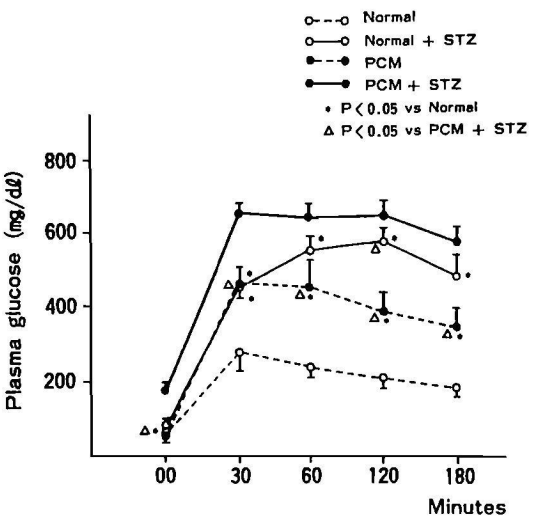

Fig. 1 The changes of plasma glucose levels during intraperitoneal glucose tolerance test (IPGTT) in normal, PCM and STZ (5 mg/kg/day i.m.) treated normal and PCM rats (mean±SD).

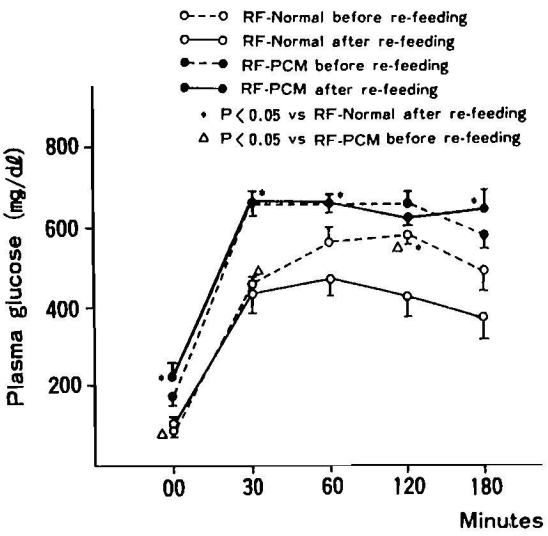

Fig. 2 The changes of plasma glucose levels during intraperitoneal glucose tolerance test (IPGTT) in RF-normal and RF-PCM rats in both before and after re-feeding. RF-normal and RF-PCM rats were treated with STZ (5mg/kg/day i.m.) for first one month (mean±SD).

tion ($p<0.05$). After re-feeding there was some recovery in glucose intolerance in RF-normal but no change in RF-PCM rats. The areas under curves of these groups are given in Table 5. The area under curve was significantly high in RF-PCM in both before and after re-feeding compared to RF-normal rats ($p<0.01$). It was significantly decreased in RF-normal after re-feeding ($p<0.01$) but no change in RF-PCM rats.

Plasma insulin level (Table 6). was significantly reduced ($p<0.01$) in both STZ and vehicle treated PCM rats than STZ and vehicle treated normal rats. In RF-PCM rats it was also significantly reduced than RF-normal rats ($p<0.01$).

Results of the morphometric analysis of islets of normal and PCM rats are expressed in Figure 3. Average number of pancreatic islets per field, mean size of islet and percentage of B-cell area per islet were significantly reduced in both STZ and vehicle treated rats PCM rats ($p<0.01$) compared to vehicle and STZ treated normal rats. All the

Table 4. Results of IPGTT expressed as areas under curve in normal and PCM rats

	Normal rats	PCM rats
Without STZ	995.0±193.1	1772.8±481.6*†
With STZ	2227.2±249.7*†	2712.7±146.0

Mean±SD *$p<0.01$ vs. normal without STZ
†$p<0.01$ vs. PCM with STZ

Table 5. Results of IPGTT expressed as area under curve in re-feeding rats

	RF-normal rats	RF-PCM rats
Before re-feeding	2227.2±264.9	2712.7±146.0*
After re-feeding	1845.1±441.0*†	2770.7±78.4

Mean±SD *$p<0.05$ vs. RF-normal before re-feeding
†$p<0.01$ vs. RF-PCM after re-feeding

morphometric parameters of islet were significantly reduced in STZ treated normal and PCM rats compared to vehicle treated normal and PCM rats ($p<0.01$, $p<0.05$). The degree of change (percent of normal) of these parameters in PCM rats when treated with STZ in given in Table 7. The degree of change in case of mean islet size and B-cell area was significantly prominent in STZ treated PCM rats compared to vehicle treated PCM rats. Morphometric analysis of islets of re-feeding rats is expressed in Figure 4. Average number of islets per field, mean size of islet and percentage of B-cell area per islet were reduced significantly in RF-PCM compared to RF-normal rats ($p<0.01$). The degree of change (percent od RF-normal) of islet morphology in RF-PCM rats after re-feeding is presented

Table 6. Plasma insulin concentration

	Plasma insulin (μU/ml) Vehicle	Plasma insulin (μU/ml) STZ	Plasma insulin (μU/ml) Re-feeding rats
Normal rats	122.1±76.7	90.6±45.3	112.2±54.4
PCM rats	44.0±4.0**	34.7±15.4**	35.6±19.3**

Mean±SD **p<0.01 vs. Normal rats

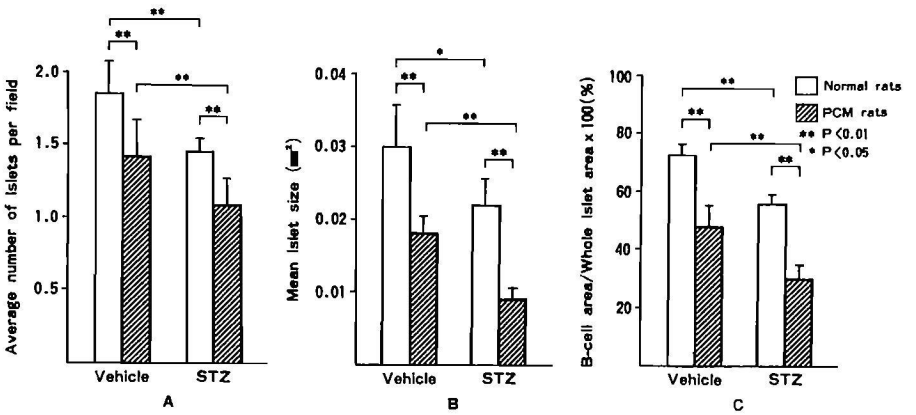

Fig. 3 Average number of pancreatic islets per field (A), mean size of islet (B) and percentage of B-cell area per islet (C) of normal and PCM rats. Islets were examined from 20 fields/section with 200 magnification of three random sections, one from each head, body and tail of each pancreas. The data expressed was the mean from these three random sections. Mean size of islet and percentage of B-cell were measured by computer controlled digitizer under light microscope arter immunostaining for B-cells (mean±SD).

in Table 8. The degree of change in case of number of islet and B-cell area was significantly prominent in RF-PCM rats after re-feeding ($p<0.05$).

Figure 5 is representing the histological findings of pancreatic islets of normal and PCM rats, which is showing close correlationship with morphometric data, reduction of the islet size and density of islet cells at the central part of islet in both vehicle and STZ treated PCM rats, but the reduction was more in STZ treated rats.

Table 7. Degree of change (Percent of normal) of islet morphology in PCM rats, when treated with STZ.

	No. of islet/field (percent of normal)	Mean islet size (percent of normal)	% of Beta cell area/islet (percent of normal)
Vehicle treated PCM	76.1±13.5	61.01±9.6	66.7±11.7
STZ treated PCM	74.5±12.7	38.9±10.9**	53.2±13.1*

Mean±SD *p<0.05, **p<0.01 vs. vehicle treated PCM

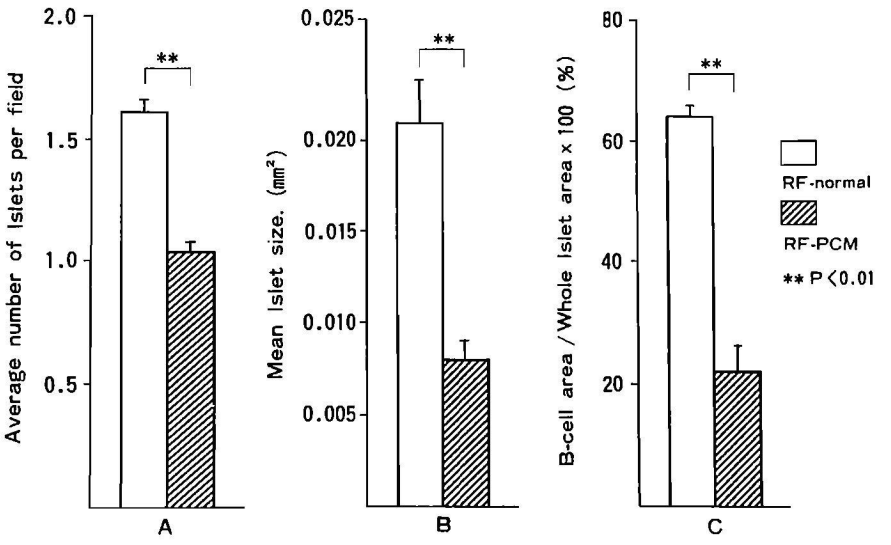

Fig. 4 Average number of pancreatic islets per field (A), mean size of islet (B) and percentage of B-cell area per islet (C) in re-feeding normal and PCM rats, which were treated with STZ (5mg/kg/day i.m.) for first one month (mean±SD).

Table 8. Degree of change (percent of RF-normal) of islet morphology in RF-PCM rat after re-feeding.

	No. of islet/field (percent of RF-normal)	Mean islet size (percent of RF-normal)	% of Beta cell area/islet (percent of RF-normal)
Before re-feeding	74.5±12.7	38.9±10.9	53.2±13.1
After re-feeding	64.1±7.3*	34.7±9.6	34.6±15.9*

Mean±SD *p<0.05 vs. before re-feeding

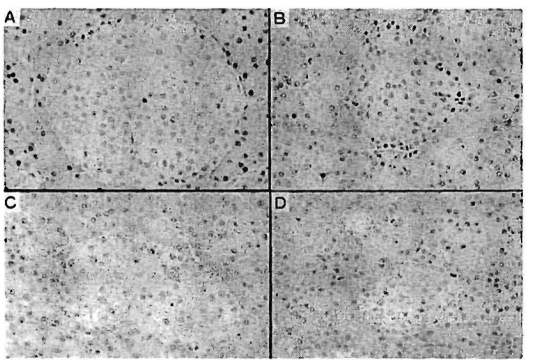

Fig. 5 Pancreatic sections showing size of islets and density of islet cells at the central portion of islet and B-cell area per islet. (A) Normal rat + vehicle, (B) PCM rat + vehicle, (C) Normal rat + STZ, (D) PCM rat + STZ. Glutaraldehyde fixation. Hydroxymethyl-methacrylate embedding. Hematoxylin-eohin stain. X100.

Discussion and Conclusion

The PCM rats in our experiment showed more or less all the features seen in human PCM, such as failure to gain adequate body weight, coarseness of the hair, reduced pancreatic weight, increased liver weight, reduced serum albumin, reduced serum lipids both TG and TCHO, impaired glucose tolerance. Though the two diets were of same energy value per gram, malnourished rats were reduced their food intake. As with malnourished child these animals suffered from protein-calorie deficiency, not only protein deficiency, and which were regarded as similar to human PCM.

The usual dose of STZ as diabetogenic agent is 45-60 ㎎/㎏ i.v. or i.m. but the dose, we used in this experiment of STZ (5 ㎎/㎏/day i.m.), was thought to have no hyperglycemic effect on endocrine pancreas by single injection. It has been known that STZ has 15 minutes of serum half life[13], rapid onset of action, rapid renal clearance and 6-24 hours duration of action. By the whole body autoradiographic study of rats injected with radiolabeled STZ also showed rapid clearance with 70-80% excretion via kidney and 8-9% via feces within 6 hours of administration[14]. Thus there is less chance of cumulative effect of STZ after one month administration. We used 5 ㎎/㎏ of STZ, which is lower than the minimum effective dose (20 ㎎/㎏) for hyperglycemia by single injection[15], therefore suggesting no direct destructive effect on pancreatic B-cells by this dose of STZ. Despite of this low dose, by repeated injection, a

significant toxic effect on the endocrine pancreas of PCM rats was observed. The STZ treated PCM rats showed significantly high FPG of 175.9±46.6 mg/dl compared to 88.8±12.9 mg/dl in STZ treated normal rats. Pancreas has the highest turnover of protein, it could be easily affected by the change of protein metabolism[16]. So it can be suggested that protein deficiency might cause these animals vulnerable to this low dose STZ. It has been reported that continued protein deprivation could result in irreparable damage to pancreatic B-cells or would increase their vulnerability to harmful environmental influences.[17,18] On the contrary it has been proved experimentally that previous adaptation to a high-protein diet protects against streptozotocin induced inhibition of insulin release from isolated rat islets[19]. Furthermore STZ is thought to produce its B-cytotoxic effects by depleting NAD in B-cells. Nicotinami de administration before or immediately after exposure to STZ in rats prevents diabetes. Thus it may be suggested that a subject with deficiency of the vitamin could be more susceptible to the B-cytotoxic effect of the drug[7]. We observed in our experiment that vehicle treated PCM rats compared to vehicle treated normal rats were intolerant to glycemic stimulation which correlates with other reports[5,20-22].

In morphometric analysis of the islets, PCM rats showed significant reduction in average size of islets, total number of islets per field and percentege of B-cells in islets, and the reduction was more prominent in PCM when treated with STZ. Though our results contradict with Camain et al.[3], who reported an increase in number and hypertrophy of islet tissue in PCM, our results correlate with Heard CRC et al.[23,24] and Weinkove C et al.,[25] the later report shows that malnourished rats have significantly reduced islet volume, with a loss of large diameter islets. One of the reasons of this discrepancy might be the difference of method for morphometric analysis. We used computer controlled digitizer for direct plotting of microscopic image and calculated the size with the help of 3 dimension image analysis system. The use of this method suggests more accuracy. It has been shown that the essential amino acids are potent stimulators of B-cell growth. All forms of PCM are characterized by a moderate to severe reduction in serum and tissue levels of essential amino acids[7], which might be applicable in our experimental rats. Our results are in accordance with some reports which suggest that glucose intolerance in PCM is due to impaired insulin secretion.[26-28] Morphometric changes in islets, we observed, might be one of the backgrounds of impaired insulin secretion.

RF-PCM rats remained with significantly high FPG of 221.0±98.8mg/dl and retained impaired glucose tolerance with abnormality in B-cell morphology despite partial nutritional recovery by 20% protein diet. It has been suggested that by the treatment of protein-calorie malnutrition, cell replication and longitudinal growth is rapidly resumed at normal or even increased rates[27]. However, persisting reduction of protein/DNA ratio in several tissues observed in human[29] and rats[27], indicates that cellular protein accretion and growth in size is impaired despite adequate protein diet. It may be derived from reduced insulin secretion. Morphometric change in the islets of RF-PCM rats also indicates some reduction of insulin secretion. These RF-PCM rats were fed with 4% protein diet and treated with STZ in first one month, when they showed increased susceptibility to STZ. Thus the reduced insulin

secretion together with initial low dose STZ challenge might cause these rats to be persistent intolerant to glucose despite re-feeding.

We have not observed any evidence of insulitis in our STZ treated normal or PCM rats as found in mice by multiple injection of sub-diabetogenic dose of STZ[30], probably due to change of species or duration and dose of STZ injections.

In conclusion we have seen in our experiment that PCM produced a definite functional and structural lesion to endocrine pancreas. Furthermore PCM rats were more susceptible to STZ and the effects of STZ were persistent after one month of re-feeding with 20% protein diet. These suggest the possibility of increased susceptibility of pancreatic B-cells to other B-cytotoxic viral, chemical or environmental agents in protein-calorie malnutrition in childhood, and exposure to these diabetogenic influences might produce an irreversible change to the endocrine pancreas.

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