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Research Article

DIABETOGENIC INFLUENCE OF HYPERDIET AND CYCLOPHOSPHAMIDE ON THE NON OBESE DIABETIC (NOD) MOUSE.

M.NAJMA HABEEB, PRAKASH R. NAIK*

Department of Zoology, University of Mysore, Mysore-06. Email: prakashrnaik@yahoo.co.in

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ABSTRACT

Objective: The objective of the study is to evaluate whether the hyper diet and cyclophosphamide accelerates diabetes related metabolic changes in the male NOD mice stock.

Materials and Methods: Different groups of NOD male mice were fed with hyperdiet and injected intraperitoneally with cyclophosphamide single dose and multiple split doses. Hepatic Hexokinase Glucose 6 phosphatase, Glucose-6-phosphate dehydrogenase, Glutathione reductase and Glutathione S Transferase activities were determined.

Results: Significancant differences showed in hyperglycemia in hyperdiet, cyclophosphamide single dose and cyclophosphamide multiple split dose treated animals. Glycosylated hemoglobin showed that there was a significant difference in all the three groups when compared with that of the control group. Whereas, hyperdiet treated and the cyclophosphamide multiple split dose treated animals showed significant similarity. The altered activities of hexokinase, glucose 6 phosphatase, and glucose 6 phosphate dehydrogenase in the liver of NOD male mice resulted in the hyperglycemic state of the treated animals.

Conclusion: Hyperdiet and cyclophosphamide treated animals showed significant hyperglycemia and also altered few enzyme activity of carbohydrate metabolism. Hence, the non diabetic NOD male mice treated with hyper diet and cyclophosphamide may be a useful model to test therapeutic approaches for amelioration of chronic diabetic complications in humans.

Keywords: NOD mice, Diabetes, Hyperdiet, Cyclophosphamide

INTRODUCTION

Diabetes mellitus is a chronic metabolic disease as old as mankind and its incidence is considered to be high 4-5% all over the world [1]. The world health organization [2] estimates that more than 180 million people worldwide have diabetes. Ethanopharmacological survey indicates that more than 1200 plants are used worldwide in traditional medicine for their potential hypoglycemic activity [3, 4, 5].

Different animal models have been used extensively in diabetes research. Early studies used pancreatectomised dogs to confirm the central role of the pancreas in glucose homeostasis, culminating in the discovery and purification of insulin [6]. Today, animal experimentation is contentious and subject to legal and ethical restrictions that vary throughout the world [6]. Most experiments are carried out on rodents, although some studies are still performed on larger animals. Several toxins, including streptozotocin and alloxan [7, 8] induce hyperglycemia in rats and mice. Selective inbreeding has produced several strains of animal that are considered reasonable models of Type 1 diabetes, Type 2 diabetes and related phenotypes such as obesity and insulin resistance. In recent years, molecular biological techniques have produced a large number of new animal models for the study of diabetes, including knock-in, generalized knock-out and tissue-specific knockout mice. The non-obese diabetic mouse [NOD] is a well studied model of spontaneous type 1 diabetes in which predominantly the female develops an insulitis between 5 and 8 weeks of age and most of them will become clinically diabetic by 20-30 weeks [9].The NOD mouse and bio breeding [BB] rat are the two most commonly used animals that spontaneously develop diseases with similarities to human Type 1 diabetes [10]. Diabetes NOD/ShiLtJ mouse is characterized by insulinities of leukocytic infiltration of pancreatic islets. The occurrence of overt diabetes in this strain is sex related [11]. Type 1 diabetes mellitus in human and NOD mouse is the consequence of selective, autoimmune-mediated destruction of pancreatic islet beta cells [12]. Marked decrease in pancreatic insulin content occurs in females at about 12 weeks of age and several weeks later in the males. Onset of diabetes is marked by moderate glycosuria and non- fasting plasma glucose higher than 250mg/dl. [Female mice are more prone to the disease]. In NOD/ShiLtJ mice, females are more widely used than males because of the onset of IDDM which occurs earlier and with a higher incidence [90-100%] by 30 weeks, whereas in males diabetes occurs approximately 10% within 30 weeks from birth with the cumulative incidence. However histological examinations have revealed that insulities occurs in all most all the mice of both sexes after 5 weeks of age and is followed by islet atrophy [11]. NOD/ShiLtJ males develop IDDM at a frequency of 40-60% by 30-40 weeks of the age. Diet is a critical factor influencing the penetrance of the diabetogenic genotype of NOD strain [13]. Nutritional sufficiency in early life can influence the incidence and the time of onset of auto immune diabetes in animal models [14,15]. Cyclophosphamide is an alkylating agent with many immunological properties and has been used in the treatment of human diseases [16].Cyclophosphamide is used in the treatment of chronic lymphocytic leukemia, lymphomas and solid tumors as well as an immunomodulatory agent. Cyclophosphamide has been shown to accelerate the diabetic process in NOD mice and induce diabetes in diabetes-resistant BB rats [17]. Diabetes development can be accelerated and synchronized by cyclophosphamide in young NOD mice. According to Harda, [18] cyclophosphamide promoted the onset of overt diabetes in non-obese diabetes-prone mice of both sexes.

There was no consistency in diabetes after breeding them in our laboratory for nearly one year. Hence in order to induce diabetes in NOD male mice and to obtain consistently diabetic status, present experiment was undertaken. The aim of present investigation was to find out whether the hyper diet, cyclophosphamide accelerated the diabetes related metabolic changes in the male NOD mice stock.

MATERIALS AND METHODS

Animals

NOD/ShiLtJ mice were purchased from Center for Cellular and Molecular Biology [CCMB] Hyderabad. The animals were maintained

in a room with temperature 22 ±2^{0}C and 50-70% Relative humidity [R.H] and 12:12 h L:D cycle. The animals were fed with standard diet supplied by Ambruth feeds Pvt. Ltd Bangalore and water *ad libitum* throughout the experiment. 30 weeks old non- diabetic male NOD mice whose serum glucose level didn't show hyperglycemia were used. The institutional animal ethics committee [IAEC] approved the experimental protocols of the present study.

Experimental design

The non diabetic NOD male mice weighing in the range of 22 - 25 grams were selected for the experiment and divided as follows, each group consisting of ten animals, the experiment was conducted for thirty days. Serum glucose was measured using glucometer before the commencement of the experiment.

Group 1: Control group- Non treated NOD mice which did not show hyperglycemia [42.48-51.25 mg/dl].

Group 2: Animals were fed with hyper diet. The diet was made according to the concentration as prescribed by American Institute of Nutrition [19].

Group 3: Animals were treated with single dose cyclophosphamide: 150 mg/kg body weight dissolved in 250µl PBS was injected intraperitoneally once, Harada [18].

Group 4: Animals were treated with multiple split doses cyclophosphamide: 40 mg/kg body weight of cyclophosphamide dissolved in 250µl PBS and injected consecutively for 5 days [total 200mg/kg body weight]. The diabetic state in animals was assessed by measuring body weight and serum glucose levels.

Biochemical analysis

Serum glucose levels were estimated using glucometer [EZ Omnitest] every week to ascertain the status of diabetes in different groups, simultaneously body weight also recorded. The experiment was terminated after thirty days. The animals were deprived of food before autopsy and sacrificed. The blood sample was collected from carotid artery at the time of autopsy and centrifuged at 4^{o}C at 10,000 rpm for 10 minutes; the separated serum was used for various biochemical analyses.

Serum glucose was estimated by Trinder's method using GOD-POD enzymatic kit [20]. Blood urea was estimated by urea- glutamate dehydrogenase [GLDH] method [21]. Triglycerides [22], HDL-cholesterol [23], Glycosylated hemoglobin [24] all the parameters were estimated by using semi bioauto analyzer from Swemed diagnostics with appropriate kits supplied by the manufacturer of the instrument. Liver glycogen was estimated by Vies, [25] method. For the determination of VLDL and LDL- cholesterol Friedwald's [26] formula was used which states that, VLDL cholesterol = TG /5 and LDL = total cholesterol – [VLDL + HDL-cholesterol].

Determination of carbohydrate metabolizing enzymes

Hepatic Hexokinase activity was assayed by the method of Brandstrup et al.,[27]. Glucose 6 phosphatase was assayed by the method of Koida and Oda [28].The phosphorous content of the supernatant of the tissue homogenate was estimated by the method of Fiske and Subbarow [29]. The protein precipitate was removed by centrifugation and the residual glucose in the supernatant of tissue homogenate was estimated by the method of Trinder as described previously. Glucose-6-phosphate dehydrogenase was estimated based on the Worthing manual method [30]. Glutathione reductase was assayed based on the method of Corlberg and Manniervik, [31].Glutathione S Transferase was estimated by using Habig et al, [32].

Statistical analysis: The statistical analyses were carried out by using SPSS version 11.5. Results were expressed as mean ± S.E.M. The comparison of means between the groups was done by using analysis of variance [ANOVA] followed by Duncan's multiple range tests.

Results: The characteristic symptoms of diabetes viz hyperphagia, polydipsia leading to hyperglycemia, uremia and loss of body weight were recorded in mice during the study period. The effect of two different dosage of cyclophosphamide on the development of diabetes in NOD male mice was studied, in the single dose experiment. i.p injection of cyclophosphamide caused death of the more number of animals having high mortality rate [60 %] whereas, the multiple split dose injection showed comparatively less mortality rate [40%], and in the hyperdiet there was no mortality. The development of diabetes was significantly slower in cyclophosphamide single dose treated animals compared with the multiple split dose and hyperdiet treated animals.

Body weight

Table 1: Shows initial and final body weight of different experimental animals. There was a significant body weight gain in control animals and the hyperdiet fed animals. Whereas, in cyclop hosphamide treated animal's there was loss of body weight compared with that of initial body weight (P<0.05).

Table1: Body weight of different experimental groups.

Parameters → / Groups ↓	Initial body weight (g)	Final body weight (g)	Body weight(gain/loss) (%)
NOD Control	23.37^a ±1.54	28.31 ^{b}±1.24	(+)17.41
Hyper diet	22.66^a ±1.47	36.73 ^{c}±1.85	(+)38.31
Cyclophosphamide (Single dose)	23.93^a ±1.34	19.50^a ±0.73	(-)18.51
Cyclophosphamide (multiple split dose)	25.77^a ±1.32	17.58^a ±0.96	(-)31.78

The values are mean ±S.E. Superscripts (a,b,c) are obtained from Duncan's post hoc test.

Serum Glucose: Table 2 shows the mean serum glucose level in the experimental animals. There was no significant variation in the serum glucose in the NOD control animals whereas hyperglycemia was achieved in the hyperdiet fed animals and cyclophosphamide multiple split dose treated animals. Cyclophosphamide single dose also showed significant elevation in the serum glucose level, however it did not match with that of the hyperdiet and cyclophosphamide multiple split dose treated animals.

Table2: Serum glucose level of different experimental groups

Parameters → / Groups ↓	Initial blood glucose(mg/dl)	Final blood glucose (mg/dl)
NOD control	42.48^a±0.63	51.25^a ±0.63
Hyperdiet	43.77^a ±1.07	150.06^c±0.55
Cyclophosphamide (Single dose)	43.99^a ±1.17	77.57^b±0.94
Cyclophosphamide (Multiple split dose)	43.67^a ±0.99	189.94^d±0.31

Values are mean ± S.E. All values are significant at P< 0.05 vs. NOD mice.

Blood urea: Table 3 shows the mean blood urea level in the experimental animals. There was a significant elevation in the Urea level in hyperdiet and cyclophosphamide multiple split dose treated animals compared to that of control groups. Whereas, cyclophosphamide single dose did not show significant changes in blood urea when compared to that of control.

Effect on Lipid Profile: Triglycerides level was elevated significantly in cyclophosphamide multiple split dose and hyper diet treated animals when compared with that of the control. **Cholesterol:** there was significant elevation of cholesterol in all the three experimental groups.**HDL**: There was no significant variation between different experimental groups. However there was a significant reduction of HDL compared with that of the control group.**LDL**: There was no significant difference between the two

groups-hyper diet and cyclophosphamide multiple split dose, whereas the cyclophosphamide single dose treated animals showed significant difference when compared to that of the control group.

Table 3: Comparison of biochemical parameters of different experimental groups

Parameters → / Groups ↓	NOD control MEAN± S.E	NOD WITH DIET MEAN± S.E	CY Single DOSE MEAN± S.E	CY multiple split dose MEAN± S.E	F Value	P Value
UREA(mg/dl)	31.27 ^{a} ±0.86	50.17 ^{b}±0.69	40.10 ^{a}±1.74	56.36 ^{c}±8.21	6.84	0.004**
TG(mg/dl)	99.81 ^{a} ± 0.82	137.05 ^{c} ±1.6	116.43 ^{b} ± 3.03	136.5 ^{c} ±0.65	100.29	0.000***
CHOLESTROL (mg/dl)	71.22^{a} ± 0.74	113.51^{d}±3.23	95.17 ^{b} ±1.12	102.2^{c}±0.89	98.02	0.000***
HDL(mg/dl)	34.85 ^{b} ± 1.53	28.17 ^{a} ± 0.87	28.7^{a} ± 0.82	28.06 ^{a} ± 0.79	9.81	0.001**
LDL(mg/dl)	17.34^{a} ±0.92	48.71^{c} ±1.21	43.32^{b}±0.98	46.14^{c}±0.93	203.72	0.000***
Gly Hb(%)	6.17^{a}±2.9	9.91^{c}±0.35	8.05^{b}±0.2	9.15^{c}±0.45	25.36	0.000***
Glycogen (mg/g)	7.99 ^{b} ±0.83	14.18 ^{c} ±0.95	3.56 ^{a} ±0.52	4.25 ^{a} ±0.61	42.14	0.000***
HK (U/mg protein)	2.90^{a}±0.05	2.19^{a}±0.06`	2.64^{a}±0.04	2.63^{a}±0.06	1.08	0.384^{NS}
G6Pase (µmol/mg/min protein)	2.66^{a}±0.24	3.73^{b}±0.09	2.68^{a}±0.03	3.68^{b}±0.58	3.54	0.039*
G6PDH (U/mg protein)	4.84^{b}±0.3	3.28^{a}±0.2	4.03^{b}±0.05	3.04^{a}±0.85	3.18	0.053*
GST (µmole/mg /min protein)	18.72^{a}±0.51	22.99^{b}±0.02	18.12^{a}±0.21	21.85^{b}±0.77	23.8	0.000***
GR (nmol/mg/min protein)	0.8^{b}±0.03	0.6^{a}±0.01	0.6^{a}±0.03	0.6^{a}±0.05	7.62	0.002**

n=10, Values are mean± S.E. The superscripts are obtained by Duncan's Test. CY=Cyclophosphamide, HDL= High density lipoprotein, TG=Triglycerides, LDL=low density lipoprotein, Gly Hb= Glycosylated haemoglobin, G6PDH= Glucose 6 phosphate dehydrogenase, G6Pase= Glucose 6 Phosphatase, HK= Hexokinase, GST= Glutathione S Transfarease, GR= Glutathione Reductase.

Effect on Glycosylated hemoglobin: From the results [table 3] it is clear that all the three groups showed significant difference from the control group. Whereas, hyperdiet treated and the cyclophosphamide multiple split dose treated animals showed significant similarity.

Effect on Glycogen level in the tissue: Glycogen content in liver was significantly less in the cyclophosphamide single dose and multiple split doses treated animals, whereas there was significantly higher glycogen level in the hyperdiet treated animals compared with that of the control animals [table 3].

Effect on carbohydrate metabolizing enzymes: Table 3 shows the activities of hexokinase, Glucose 6 Phosphatase, glucose 6 phosphate dehydrogenase, Glutathione S Transferase and glutathione reductase. There was no significant change in the activity of hepatic hexokinase in all the groups. The activity of the hepatic gluconeogenic enzyme glucose 6 phosphatase was significantly increased in the hyperdiet treated and cyclophosphamide multiple split dose treated animals whereas, cyclophosphamide single dose showed no significant change when compared with the control. Glucose 6 phosphate dehydrogenase activities significantly decreased in hyperdiet and cyclophosphamide multiple split dose treated animals compared with the cyclophosphamide single dose treated and control animals. GST activity was significantly increased in both the hyperdiet and cyclophosphamide multiple split dose treated animals. Whereas, the GR activity decreased significantly in all the three groups when compared with the control group.

DISCUSSION

A NOD mouse provides a condition of insulities and has been described as a useful experimental model to evaluate the activity of hypoglycemic agents. The result showed that hyperdiet and cyclophosphamide can induce diabetes in male NOD mice of 30 weeks and also demonstrated that it significantly altered the biochemical parameters as that of type 1diabetes. Dietary components act as catalysts determing the rate at which diabetogenesis proceeds. The type of dietary protein has a major impact on the incidence of diabetes in the NOD mouse with meat meal or casein resulting in a high rate of onset while casein hydrolysate a denatured form or lactalbumin based diets being relatively protective [13]. Elliote et al, [33] stated that the presence of cow's milk trigger's the diabetes in the NOD mouse if it is introduced at weaning. In concurrence with the above postulates, the present experiment showed that there was a significant hyperglycemia in the hyperdiet group. Cyclophosphamide can enhance immune response to various imunogens including proteinous antigens and foreign RBC by depleting suppressor T lymphocytes or their precursors. It has been shown that cyclophosphamide converts resistant mouse strains into susceptible ones to some auto immune diseases such as experimental allergic encephalomyelitis. As suggested by Sobel et al., [34] treatment with cyclophosphamide decreases the development of diabetes in the diabetes- prone BB rat by inhibiting the development of insulities. Hence, this could be the reason for less response in the single dose cyclophosphamide injection. Cyclophosphamide has been shown to accelerate the onset of diabetes in NOD mice which seems to be due to a decrease in the suppressor like cell activity [35]. Whereas, the frequency of diabetes is less in males and thus cyclophosphamide is used to accelerate the diabetes [18]. Thereby, providing a platform to make use of non diabetic NOD male mice for further evaluation. Therefore, in the present investigation cyclophosphamide was used to induce diabetes in the male mice. It is notable that, both Hyperdiet and cyclophosphamide treated NOD male mouse has not been reported for its effect on biochemical pathways, as these parameters plays a major role in inducing diabetes. Accordingly the study revealed that the altered biochemical pathway may be due to the effect of hyperdiet and cyclophosphamide. In earlier reports hyper diet and Cyclophosphamides were used to make NOD mice diabetes. They had almost the same characterisitics of type 1 diabetic patients such as higher FBG and lipid levels. The most common lipid abnormalities in diabetes are hypertriglyceridemia and hypercholesterolemia [36, 37].

In the present study, body weight loss was observed in the treated animals which could be due to acquired diabetics in NOD male mice. The result is in agreement with Furuse et al., [38], their study suggested that, the body weight is reduced in the diabetic state.

As results show, the significant elevation in the blood urea levels in the hyperdiet and cyclophosphamide treated animals. This may be due to increased lipid and protein metabolism for gaining energy in

diabetic animals. Mulec et al [39] reported almost same result stating that rise in blood urea levels has been reported in patients with diabetes.

In the present study, hyperdiet and cyclophosphamide treated animals had an elevation in the serum lipids. Diabetic conditions are associated with the elevated lipid levels along with the hyperglycemia.

According to Ravi et al., [40] abnormalities in lipid profile are one of the most common complications in diabetes mellitus found in 40% of the diabetic cases. Diabetics cause an increase in the triglycerides, cholesterol and LDL [41].

The result clearly showed that, the activity of the hepatic gluconeogenic enzyme glucose 6 phosphatase was significantly increased in the hyperdiet treated and cyclophosphamide multiple split dose treated animals. Whereas, cyclophosphamide showed no significant change when compared with that of the control. Hexokinase and glucose 6 phosphate dehydrogenase were significantly decreased in cyclophosphamide treated animals compared with the control, hyperdiet and cyclophosphamide high dose treated. Increased hepatic glucose production plus decreased hepatic glycogen synthesis and glycolysis are the major symptoms of diabetes that results in hyperglycemia [42]. The gluconeogenic enzyme glucose-6-phosphatase is a crucial enzyme of glucose homeostasis. The results of few earlier studies are also in agreement with the present investigation. For example Mithievre et al [43] stated that because it catalyzes the ultimate biochemical reaction of both glycogenolysis and glyconeogenesis. Increased glucose-6-phosphatase activity in diabetic rats provides hydrogen, which binds with $NADP^+$ to form NADPH and enhances the synthesis of fats from carbohydrates i.e. lipogenesis [44], and finally contributes to increased levels of glucose in blood. Increased hepatic glucose production in diabetes is associated with impaired suppression of the gluconeogenic enzyme fructose-1, 6-bisphosphatase. Hepatic hexokinase is the most sensitive indicator of the glycolytic pathway in diabetes and its increase can increase the utilization of blood glucose for glycogen storage in the liver [45]. In the present study there was a marked decrease in the hexokinase, thereby increasing the blood glucose level and generating high glycemic levels and indicating the level of diabetes.

In uncontrolled or poorly controlled diabetes, there is an increase in glycosylation of a number of proteins including hemoglobin and beta crystalline of lens [46]. Glycosylated hemoglobin was found to increases in diabetes, and the amount of increase is directly proportional to the fasting blood glucose level [47]. Glycosylated hemoglobin is considered to be a good measure to indicate the average blood glucose concentration over the preceding weeks while a single glucose determination gives a value which is true only at time the blood sample is drawn [48, 49, 50]. Glycosylated hemoglobin has found to increase in patients with diabetic mellitus [51, 52] and the magnitude of this is directly proportional to the fasting blood glucose level [53]. In the present study there was an increase in glycosylated haemoglobin in the hyperdiet and cyclophosphamide multiple split dose treated animals, thereby indicating diabetic status in the mice.

CONCLUSION

The present study reveals that the Hyperdiet apart from altering the beta cell morphology and function, it brings about alteration in metabolic enzymes. Cyclophosphamide, apart from enhancing autoimmune disorder and causing intra islet nitric oxide (NO) production to beta cell destruction, disturb the biological pathway which is involved in glucose homeostasis by altering the rate of carbohydrate metabolizing enzymes and also it will increase the production of free radicals thereby indicating that it causes toxic effect to the cells and thus enhancing diabetic state in non diabetic NOD mice.

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