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Original Article

Effects on high cholesterol-fed to liver, retina, hippocampus, and Harderian gland in Goto-Kakizaki rat

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Abstract: To understand the relationship among cholesterolemia, hyperglycemic stage in non obese type 2 diabetes mellitus, and histological perturbations on liver, retina, hippocampus, and Harderian gland, we maintained rat on a diet high in cholesterol for fourteen weeks, then analyzed blood lipid profiles, blood glucose, hepatic enzymes, and microscopic lesion of those tissues. We observed that high cholesterol-treated rat elevated in cholesterol and low density lipoprotein with not correlated to hyperglycemia. Histopathological changing in Goto-Kakizaki rat on liver (microvesicular steatosis) and Harderain gland (tubular lesions) were related to hyperglycemic effect rather than cholesterolemic effect. These may be related to hypoinsulinemic characteristic of this diabetic model. However increasing pyknotic nuclei on hippocampus and reducing of retinal ganglionic cell were related to the high level of cholesterol loaded with synergized effect due to diabetic stage.

Keywords: High cholesterol, liver, retina, hippocampus, Harderian gland, Goto-Kakizaki rat

Introduction

The Goto-Kakizaki (GK) rat is a spontaneously diabetic rat model of non-insulin-dependent or type 2 diabetes mellitus (T2DM) [1, 2] which shows progressive loss of β cells in the pancreatic islets, fibrosis, and consequence to hyperglycemia or metabolic alterations [3] with aging progression [4]. This model is used to investigate of human T2DM whose absence obesity to allow the dissociation of confounding obesity factors [5]. It is characterized by non obesity, moderate but stable fasting hyperglycemia, hypoinsulinemia, normolipidaemia, insulin resistance, impaired glucose tolerance which appears at 2 wk of age in all animals, and an early onset of diabetic complications [1, 2, 6-8]. There have been reported the association between high cholesterol diet effect and obese T2DM rodent models [9-11]. The effect of diabetes on cholesterol metabolism is associated with a decrease turnover of cholesterol in the liver tissues or a decrease ability to catabolize cholesterol [12]. However, reports related to non-obese T2DM and cholesterol effect are limited [6].

T2DM is a heterogeneous group of diseases that is progressive and involves multiple tissues especially pancreas [3, 4, 13], kidney [14, 15], liver [16], retina [17], intestine [18], heart [19], and skeletal muscle [20]. Interestingly, the Harderian gland of the rodent, sand rat (*Psammomys obesus*), known as a model for obese diabetes mellitus is histological affected by the diabetic syndrome [21-23]. Specific binding of 125I-insulin has been observed by autoradiography in the Harderian gland of mouse [24], suggesting that the Harderian gland is also be the target organ for hormone action. Moreover, although the brain is not included as a classical target organ of insulin, it has recently been shown that dysregulation of insulin receptor signaling in various mental illnesses [25] and an alteration of expression pattern in the hippocampus and pre-frontal cortex in T2DM model [26].

For these reasons we carried out to this present study, examined the 12 weeks effects of high cholesterol feeding on the liver, hippocampus, retina, and Harderian gland pathology in GK rat, non-obese T2DM. Four-week old GK rat were fed with 3% cholesterol diet for 12 weeks to induce hypercholesterolemia, and their condition was compared with that of non treated rat. Age matched normal Wistar rat were also given cholesterol for the same periods and used for comparison. These were purposed that what were the effects on high cholesterol diet to those organs that related to T2DM in association with blood clinical chemistry of lipid profiles and hepatic enzymes.

Materials and methods

Animals

All of the animal studies (30 rat, 4 weeks of age) were conducted on age-matched female GK (20 rat) and Wistar (control) rat (10 rat) those imported from Center for Laboratory Experimental Animals (CLEA), Japan, in accordance with the Mahidol University, Thailand, policy for the care and use of animals for scientific purposes and approved by the institutional ethics committee. All rat were group (2 rat/cage) housed and maintained in low barrier with heating, ventilating, and air conditioning system. They were provided 12:12h light and dark cycle and fed *ad libitum* 3% cholesterol coated diet (N=10 of each strain) or standard diet (N=5 of each strain) with 7-10 ppm of chlorinated reverse osmosis water. Daily observation and weighing were routinely conducted by the well-trained animal caretaker.

High cholesterol diet

Three percentage of pure cholesterol was coated to the standard pellet diet number 082 (Perfect Companion Ltd., Thailand) which composes of 26.7% total protein, 2.15% fat, 2.91% fiber, 5.54% ash, 0.597% sodium chloride, 0.939% calcium, 0.809% phosphorus. The diet must be free from aflatoxin and *salmonella spp*.

Blood clinical chemistry

Fasting blood glucose was measured by stripped glucometer (AccuCheck Performa, F. Hoffmann-La Roche Ltd., Switzerland) at 5, 8, 12, and 16 weeks of age. The blood samples were collected via needle puncture of tail vein. At the end point, all of the rat were anesthetized with carbon-dioxide inhalation, the blood samples were collected via heart puncture. LDL (low density lipoprotein), HDL (high density lipoprotein), cholesterol, triglyceride, albumin, total bilirubin, AST (aspartate aminotransferase), ALT (alanine transaminase), and ALP (alkaline phosphatase) were measured by a Hitachi 902 automated blood analyzer (Hitachi Science Systems Ltd., Ibaraki, Japan).

Histopathology

At the end of the study, all groups of rat were euthanized by over dose inhalation of carbon-dioxide. They were subjected to gross necropsy. The liver, brain, eye ball, and Harderian gland were removed and fixed in 10% neutral buffer formalin for 48 hours. Fixed specimens were processed in standard tissue processing and embedded in paraffin and sectioned (4 μm thick) then stained with haematoxylin and eosin (H & E).

All tissues were examined histopathological finding as following, (1) liver, microvesicular steatosis was measured as percent area fraction of fat vacuole surrounding periportal area/ field (N=10 each group), an area measurement was all done by using image analysis program (ImageJ®, NIH, version 1.36, by Wayne Rassband) [27], (2) retina, number of retinal ganglion cell (at the same areas) and (3) hippocampus, percentage of pyknotic nuclei on C2&3 of the amnion horn were manually single blind counted as cell/high power field (hpf) ×400 (N=10 each group), (4) Harderian gland was evaluated by severity scoring of tubular lesion (dilated tubule with squamous cell metaplasia, flattened and vacuolated tubular cells, tubular tissue debris), prophyrin pigment accumulation and inflammation. The severity scores were classified as absent: 0, focal: 1, mild: 2, moderate: 3, and severe: 4.

Statistical analysis

Quantitative results were expressed as mean ± standard error of mean. Data were statistically analyzed with IBM® SPSS® Statistic software version 20, using a one way analysis of variance (ANOVA) followed by Levene's test. To differentiate the difference between groups, the multiple comparison Bonferroni test and

Table 1. Mean body weight gains and blood clinical chemistry in GK rat and Wistar rat fed normal diet (ND) and high cholesterol diet (HCD)

Group		BWG (g)	LDL (mg/dl)	HDL (mg/dl)	CHOL (mg/dl)	TG (mg/dl)	ALB (g/dl)	Bili-T (U/L)	AST (U/L)	ALT (U/L)	ALP (U/L)
GK rat											
ND	Mean	98.2	30.1	130.0	174.4	101.3	6.8	0.25	184.2	61.3	116.5
	SD	42	9.9	33.3	45.9	24.2	1.5	0.07	119.4	21.1	29.0
HCD	Mean	94.1	149.8^{ns}	97.0^{c}	270.3^{ns}	107.2^{c}	7.1^{b}	0.19^{c}	226.2^{ns}	58.1^{c}	137.7^{c}
	SD	39	72.7	12.3	71.9	17.1	0.3	0.01	24.1	5.8	14.2
p-value		ns	***	*	**	ns	ns	ns	ns	ns	ns
Wistar rat											
ND	Mean	112.8	1.8	40.0	54.8	134.6	5.8	0.13	152.9	44.7	59.1
	SD	48	1.5	8.8	11.8	57.0	1.0	0.0	49.4	11.4	14.2
HCD	Mean	108.4	174.1^{ns}	43.0^{c}	227.8^{ns}	75.2^{c}	6.1^{b}	0.12^{c}	226.2^{ns}	155.8^{c}	78.0^{c}
	SD	45	90.5	8.0	94.2	7.8	0.5	0.0	30.1	50.6	13.4
p-value		ns	***	ns	***	*	ns	ns	**	***	*

Significantly different in each group of rat: *: p<0.05, **: p<0.01, ***: p<0.001, ns: non significant. Significantly different between group of match pair: ^{a}p<0.05, ^{b}p<0.01, ^{c}p<0.001, ^{ns}non significant. BWG: Body weight gains, LDL: low density lipoprotein, HDL: high density lipoprotein, CHOL: cholesterol, TG: triglyceride, ALB: albumin, Bili-T: total bilirubin, AST: aspartate aminotransferase, ALT: alanine transaminase, ALP: alkaline phosphatase.

Table 2. Mean fasting blood glucose (mg/dl) in GK rat and Wistar rat fed normal diet (ND) and high cholesterol diet (HCD) during 12 weeks

Group		5th week	8th week	12th week	16th week
GK rat					
ND	Mean	104.0	129.0	142.0	147.3
	SD	7.61	9.89	15.64	17.24
HCD	Mean	112.0	133.6	138.2	131.9
	SD	8.0	16.57	13.66	23.51
p-value		ns	ns	ns	ns
Wistar rat					
ND	Mean	81.0	89.3	92.6	109.6
	SD	12.16	4.27	5.64	11.94
HCD	Mean	86.4	90.6	91.7	93.0
	SD	10.94	6.49	5.57	10.04
p-value		ns	ns	ns	**

Significantly different in each group of rat: *: p<0.05, **: p<0.01, ***: p<0.001, ns: non significant.

Dunnett test were performed for equal and non-equal variance assumption respectively.

Results

Body weight gains

For body weight gains, there were not significantly different between cholesterol-treated and non-treated groups both in GK and Wistar rat as shown in **Table 1**.

Blood glucose, lipid, and hepatic enzymes level

Mean fasting blood glucose of both cholesterol-treated GK rat and non-treated GK rat were gradually increased, while there was no observation in both cholesterol-treated WR and non-treated WR. Cholesterol-treated GK rat were significantly higher LDL and cholesterol than non treated GK rat, while HDL was reduced. It was also similar to cholesterol-treated WR, but HDL was not reduced. Triglyceride, albumin, and total bilirubin in all groups had no significantly different. Interestingly higher level of AST, ALT, and ALP were demonstrated in cholesterol-treated WR than non-treated WR while there was no observation in both cholesterol-treated GK rat and non-treated GK rat as shown in **Table 2**.

Liver histopathology

Percentage of hepatic steatosis area fraction in hypercholesterolemic rat was significantly higher than those in the control groups (**Figure 1**). Interestingly in treated GK rat were significantly lower than treated WR. Calculate values among experimental groups were shown as follows, GK-HC: 9.25±0.9%, GK-N: 0±0%, WR-HC: 46.22±2.8%, WR-N: 2.11±0.7%.

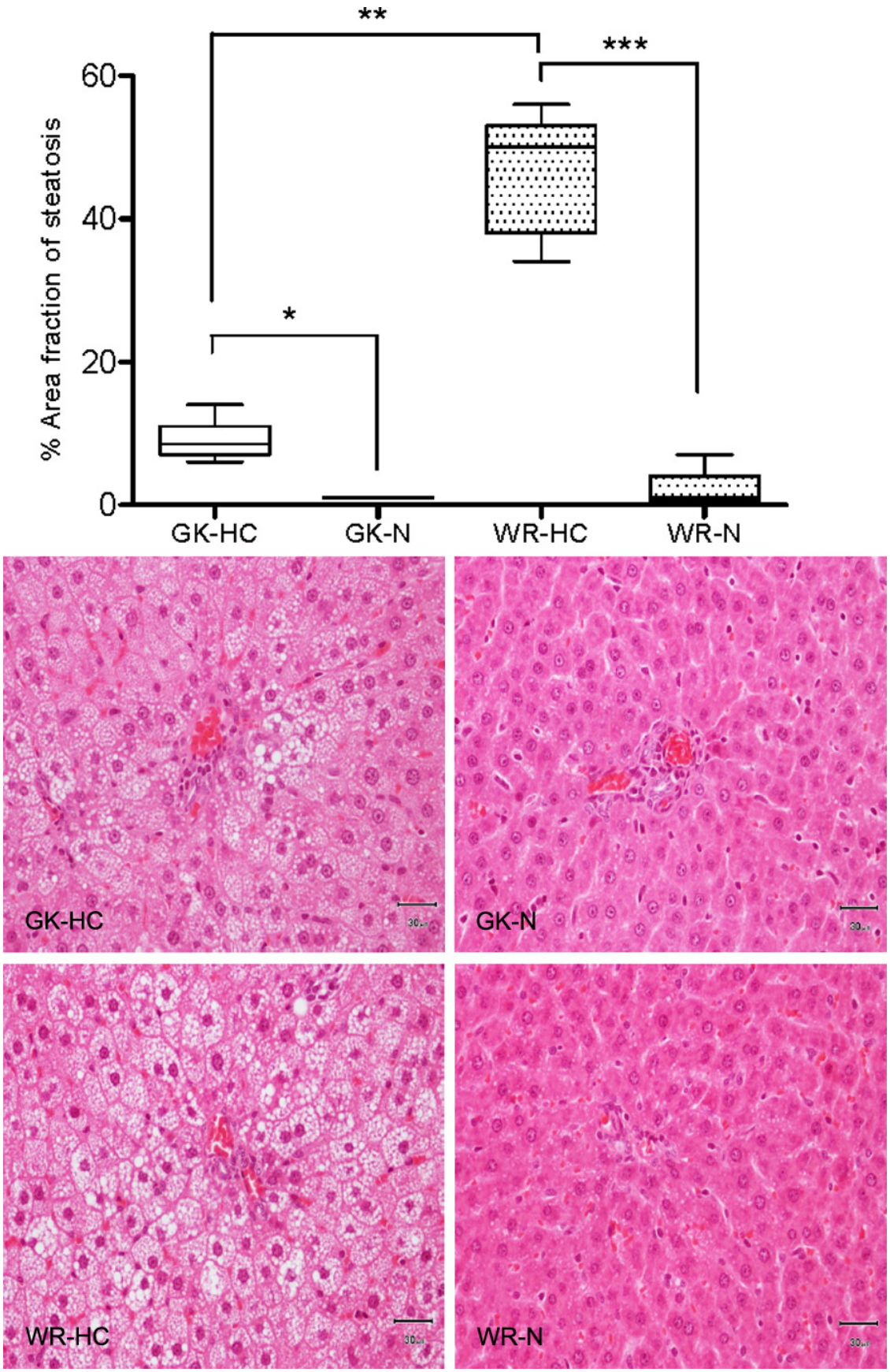

**
***
*
60
40
20
0
% Area fraction of steatosis
GK-HC
GK-N
WR-HC
WR-N
GK-HC
GK-N
WR-HC
WR-N

Figure 1. The comparison of percentage of steatosis area among Goto Kakizaki (GK) rat and Wistar rat (WR) those fed high cholesterol (-HC) diet and Normal (-N) diet. Light microscopic micrograph of periportal microvesicular steatosis (GK-HC and WR-HC), Hematoxylin & Eosin staining, ×400.

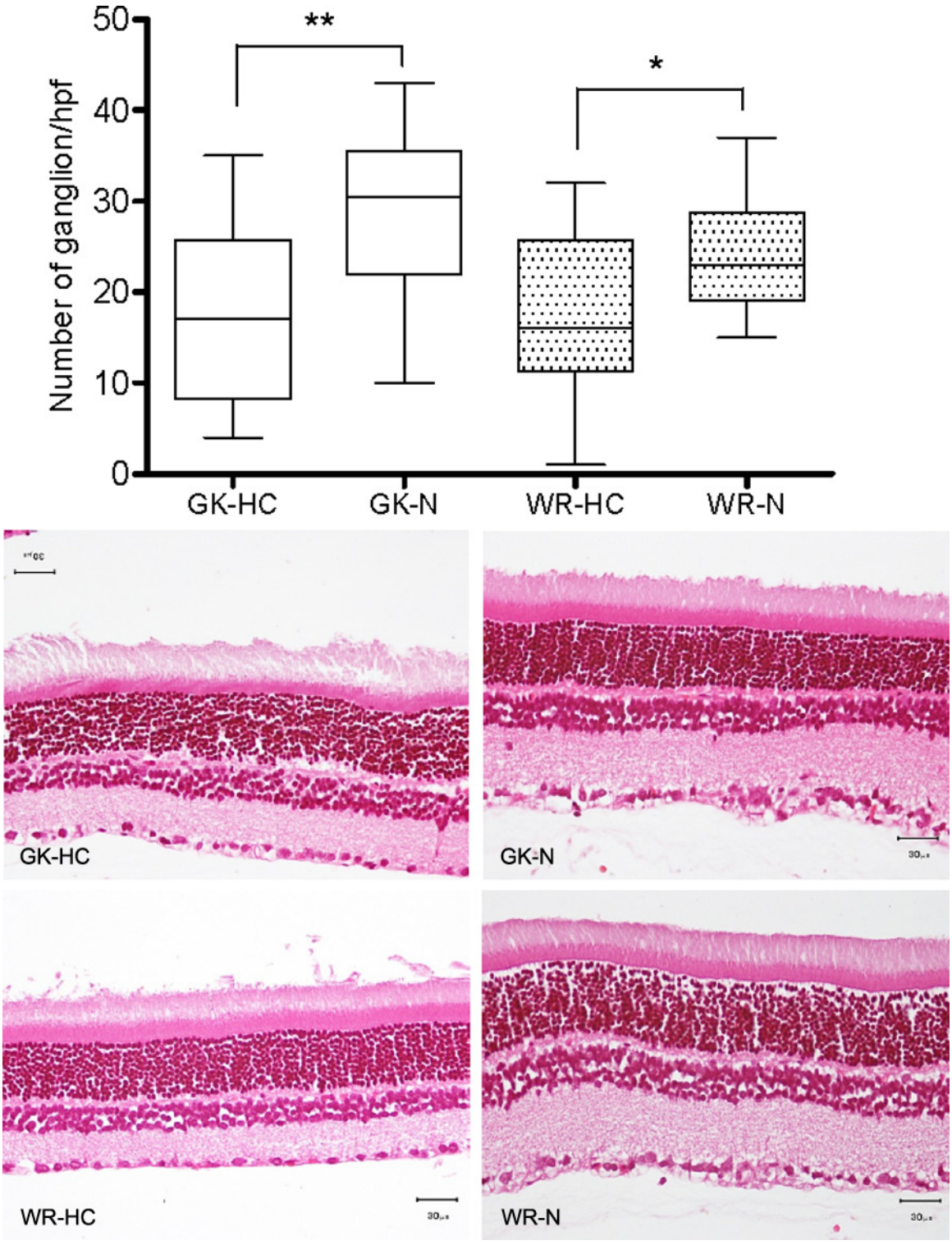


Figure 2. The comparison of number of ganglion cell on retina among GK rat and WR those fed HC diet and N diet. Light microscopic micrograph of retina, Hematoxylin & Eosin staining, ×400.

Retinal ganglion cell

The number of retinal ganglion cell in hypercholesterolemic rat was significantly lower than those of each control group (**Figure 2**). Calculate values among experimental groups were shown as follows, GK-HC: 17.32±9.4 cells, GK-N: 28.78±9.3 cells, WR-HC: 16.92±9.2 cells, WR-N: 24.0±6.4 cells.

Pyknotic nuclei in hippocampus

Percentage of pyknotic nuclei of amnion horn on C2&3 areas demonstrated that cholesterol-treated GK rat had significantly higher than presented in normal diet. Contrast to cholesterol-treated and non treated WR were not different (**Figure 3**). Calculate values among experimental groups were shown as follows, GK-HC: 26.75±9.4%, GK-N: 10.56±0.49%, WR-HC: 16.88±10.9%, WR-N: 20.36±3.64%.

Harderian gland

The tubular histopathology of Harderian gland comparing between GK rat and WR those fed both HC and N diet exhibited that the severity score in diabetic rat was much higher than non-diabetic rat (**Figure 4**) which was not related to high cholesterol content. In Diabetic stage of GK rat, some of the tubules are dilated and their epithelium flattened from columnar to cuboidal and squamous (or squamous cell metaplasia) with some of porphyrin pigment accumulations. The interlobular connective tissue is well developed. Focal leukocyte infiltrations, tubular degeneration, and necrosis are also observed.

Discussion

Short-term high-cholesterol feeding induced minimal changes in body weight gains in both normal and diabetic rat in this study. In previous studies, longer-term high-fat feeding clearly induced body weight gain in various animals [28]. Cholesterol-treated rat relative to untreated rat had higher cholesterol and LDL levels after 12 weeks on cholesterol load. However cholesterol-treated GK rat had lower severity of lipid storage, microvesicular steatosis, than cholesterol-treated WR with no significantly increased hepatic enzymes. These suggesting the presence of stronger hypercholesterolemic stress in cholesterol-treated GK rat are lowered than cholesterol-treated WR. It was noted that plasma glucose levels of GK and Wistar rat treated with high cholesterol diet were almost the same levels of each untreated group. In this study cholesterol feeding thus only forced lipid storage in liver rather than enhanced hyperglycemic effect especially in Wistar rat those have normal insulin level, while GK rat, non obese T2DM, tended to be hypoinsulinemia [1, 2, 8, 10, 17], then they had lower occurrence of fatty liver. In contrast to obese T2DM, such as sand rat [29], Zucker fatty rat [30], and *JCR/LA-cp* rat [31], that tend to be hyperinsulinemia, then lipid infiltration had been noted in some stage of diabetic syndromes in the liver. In animal models, chronic hyperinsulinemia is found to predispose the liver to relative resistance to insulin, leading to increased lipogenesis and promoting fatty liver [32, 33].

From the 16 weeks of age histopathological changing of the Harderian gland indicated that, cholesterol-treated GK rat are similar presented the diabetic syndrome to untreated GK rat characterized by several glandular lesions as described in Djeridane et al., while cholesterol-treated WR had indeed significantly lower severity. This exhibited that high cholesterol diet might not be affected to the Harderian gland than the hyperglycemic effect would be, particularly in non-obese T2DM model. In contrast to the obese T2DM rat models such as sand rat, in the present study there was not found free lipid vacuoles in the interstitial tissue of the Harderian gland. Therefore as in the liver, low severity of hepatic steatosis, we can hypothesize that hypoinsulinemia during non-obese T2DM of GK rat [7, 8, 10] may have the same effect on the Harderian gland.

Furthermore, there was a relationship between hyperinsulinaemia and cholesterol synthesis, whereas there was no relationship between blood sugar and cholesterol synthesis after the meal [34]. In animal models, chronic hyperinsulinemia is found to predispose the liver to relative resistance to insulin, leading to increased lipogenesis [35]. The effect of high dietary fat on insulin secretion in GK rat was reported by Shang et al., glucose metabolism in diabetic rat after high fat diet deteriorated partly because of insufficient insulin secretion caused by genetic defects and lipotoxicity due to chronically high FFA levels [36]. Several articles [37-40] demonstrated the cholesterol metabolism

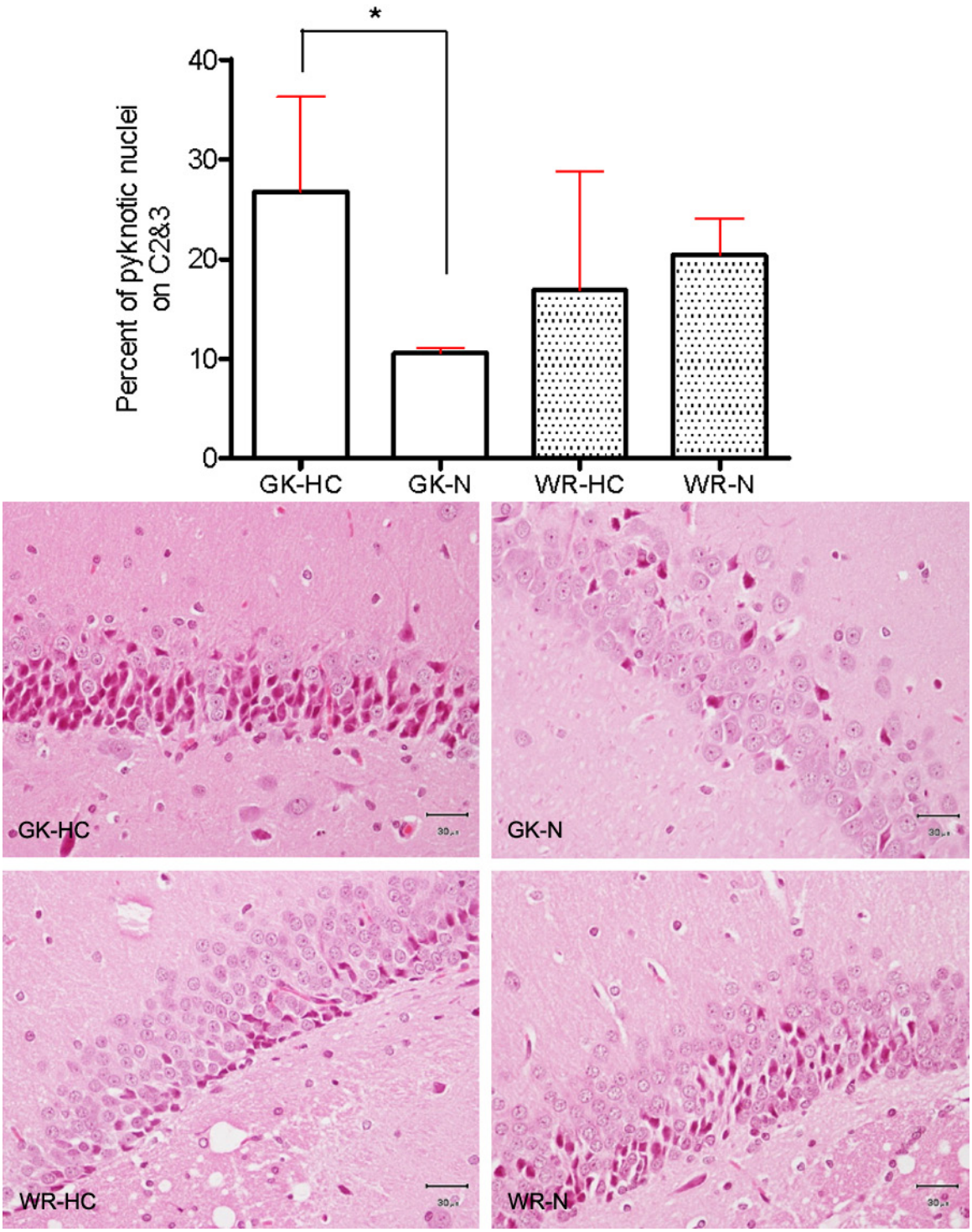
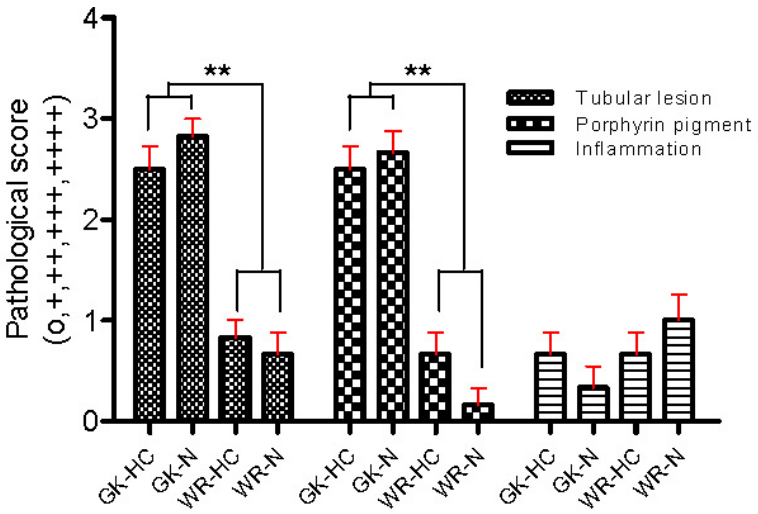


Figure 3. The comparison of percentage of pyknotic nuclei on C2&3 of amnion horn on among GK rat and WR those fed HC diet and N diet. Light microscopic micrograph of hippocampus, Hematoxylin & Eosin staining, ×400.

in obese patients with T2DM, a possible mechanism suggested by the "succinate hypothesis". The pathway occurs in the liver, the increased glucose of diabetes would be metabolized to pyruvate that can then enter to the mitochondria. Conversion to acetyl-CoA and oxaloacetate then could result in increased production of HMG-CoA, mevalonate, and, presumably, cholesterol. All contrast to non obese with diabetes type 2.


Pathological score
(o,+,++,+++,++++)
4
3
2
1
0
**
**
Tubular lesion
Porphyrin pigment
Inflammation
GK-HC
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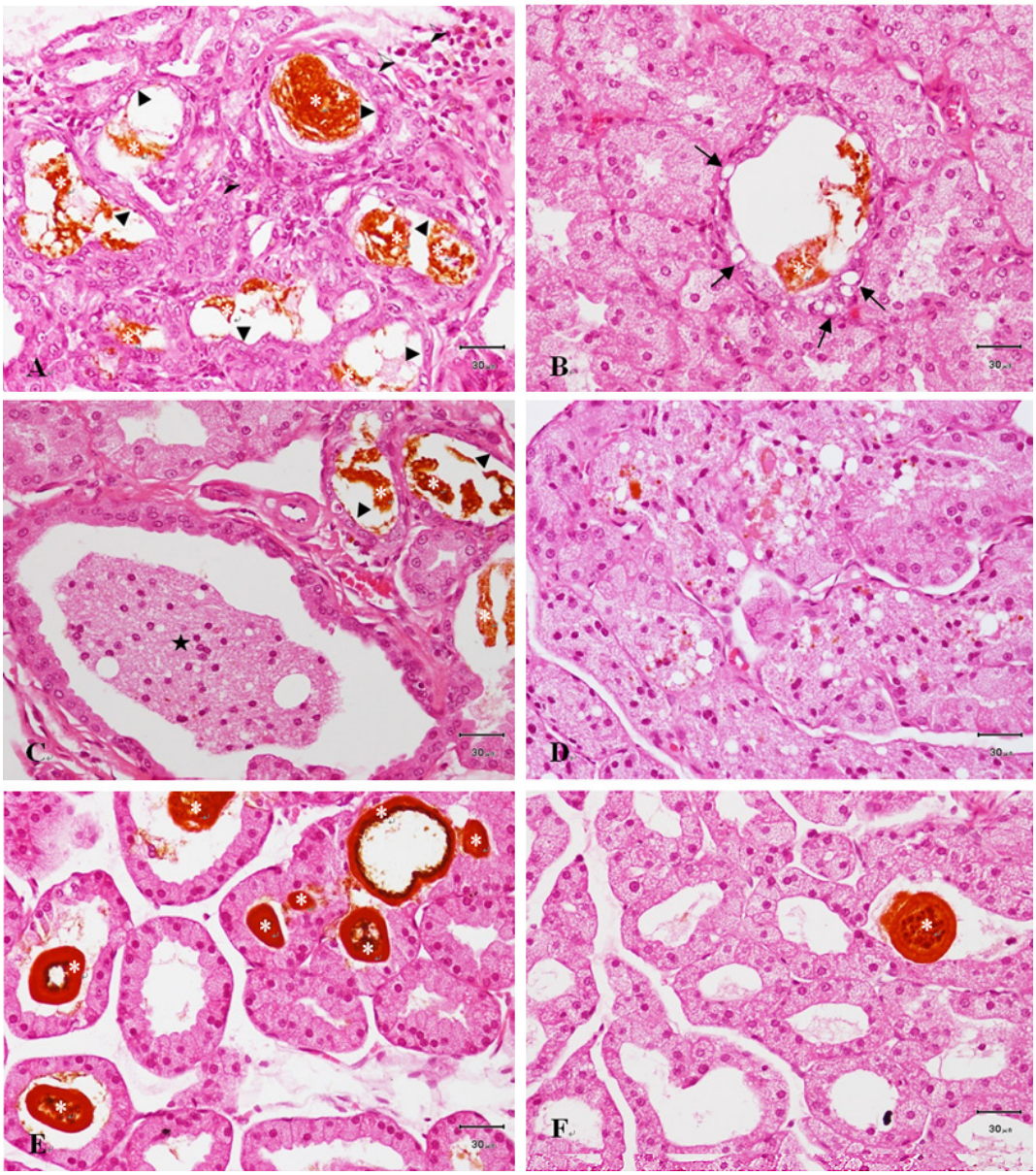
A
B
C
D
E
F

Figure 4. The comparison of the Harderian gland, tubular pathological score among GK rat and WR those fed HC diet and N diet. Light microscopic micrograph of tubular lesions showing early stage of diabetic onset of GK-HC and GK-N (A-D), dilated tubule with cuboidal and squamous epithelial cell (►), a luminal porphyrin pigment (*), focal interstitial connective tissue is infiltrated with inflammatory cells (A: ➢), tubular cells are flattened and vacuolated (B: →), extraglandular duct is filled with tissue debris (C: ★), focal tubular degeneration with white cells infiltration (D), porphyrin pigment between GK rat (E) and WR (F), Hematoxylin & Eosin staining, ×400.

In this study, clinical chemistry of the hepatic function demonstrated that all groups are normal albumin and total bilirubin levels, these referred to normal liver synthetic function and biliary function with no cholestasis. Individuals with T2DM have a higher incidence of liver function test abnormalities than individuals who do not have diabetes [32]. Mild chronic elevations of transaminases often reflect underlying insulin resistance [32]. The present study exhibited that ALT in non-treated GK rat was higher than non-treated WR, this should be probably claimed stage of insulin resistance in GK rat. Moreover hepatic enzymes in cholesterol-treated WR were higher than non-treated one, while they were normal in GK rat. These increasing may be related to the effect of high cholesterol load rather than insulin effect which reflected to the occurrence of hepatic steatosis.

Chronic hyperglycemia in GK rat causes several alterations of retina such as low grade inflammation, macular edema, subretinal accumulation of activated microglia/macrophages, and visual cell loss [41], microvascular complication such as decreased retinal blood flow, prolongation in retinal microcirculation time, microvascular occlusion, and ischemia [41-43]. Cholesterol loaded may be related to decrease retinal blood flow and leading to the reduction of retinal ganglionic cell.

They have recently been shown that the brain is another target organ of T2DM leading to mental illnesses [25, 26]. A high cholesterol diet elevates hippocampal cytokine expression in female rat [44]. The structure and function of the hippocampus, a brain region critical for learning and memory, is impaired by obesity and hyperlipidemia [45, 46]. The high fat diet was associated with increased serum and liver cholesterol and triglyceride levels, and also promoted cholesterol accumulation in the hippocampus and increased oxidative stress [46]. Cholesterol-treated GK rat exhibited higher pyknotic nuclei than non treated one, while cholesterol-treated and non treated WR rat were the same. These may be referred that diabetes stage was predisposed to hippocampal damage by cholesterol loaded inducing oxidative stress.

The conclusion of this study is that, short term effects of high cholesterol feeding in GK rat on histopathological changing of liver and the Harderain gland are related to hyperglycemic effect rather than cholesterolemic effect that may be associated with hypoinsulinemic stage of non-obese T2DM model. However on hippocampus and retina, themselves, are related to the high level of cholesterol loaded with synergized effect due to diabetic stage.

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