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Original Article

Dietary Oxidized Frying Oil Induces Hyperglycemia and Decreases Activities of Energy Metabolism Related Enzymes in Mice

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Abstract

The aim of this study was to examine the effect of high dietary oxidized frying oil (OFO) on insulin and energy metabolism related enzymes in leukocytes of mice. Three groups of mice were fed basal diet containing fresh sunflower oil (Control), OFO fried for 12 and 24 hours. Plasma glucose level was significantly increased after OFO feeding, whereas plasma immuno-reactive insulin (IRI) and insulin staining in islets cells showed significant decrease. Furthermore cholesterol and triglycerides levels were decreased. The activities of malate dehydrogenase (MDH) were significantly reduced in cytosolic and mitochondrial fractions of leukocytes in OFO compared to the control group. Also cytosolic MDH/lactic dehydrogenase (LDH) activity ratio was significantly decreased as LDH showed no significant changes between groups. The results of this study demonstrated that OFO feeding significantly decreased IRI, insulin secretion from pancreas and the activity of MDH in leukocytes of mice, therefore hyperglycemia and glucose intolerance occurred.

Keywords: Oxidized frying oil, malate dehydrogenase, glucose intolerance, insulin immunostaining

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Introduction

Impaired glucose tolerance (IGT) is a pre-diabetic state of dysglycemia that is associated with insulin resistance and increased risk of cardiovascular pathology. IGT may precede type 2 diabetes mellitus by many years and is defined as two -hours' high blood glucose levels on the glucose tolerance test. There are numerous cases associated with IGT such as obesity (Despres, 2006) and high dietary oxidized frying oil (OFO) (Chao *et al*., 2007). Dietary oxidized lipid presents a potential health hazard for both companion animals and humans.

Thermally OFO is usually ingested with the fried foods. (Sulzle *et al.,* 2004) have shown that ingestion of OFO influenced lipid metabolism through reduction of triacylglycerol (TG) levels in the liver, plasma, and very low-density lipoprotein. Additionally, animals fed such oils usually exhibit a markedly lower growth rate and decreased adiposity (Eder *et al*., 2003). The antiadiposity effect of OFO is accompanied with impairment of glucose tolerance. The glucose intolerance induced by OFO has been proposed to be mediated by impairment of insulin secretion, higher oxidative stress, or increased prostaglandin E2 expression in the pancreatic islets (Liao *et al*., 2008).

The activities of certain enzymes related to energy metabolism (especially malate dehydrogenase, MDH and lactic dehydrogenase, LDH) in peripheral leukocytes are considered to reflect the metabolic state in the whole body tissues (Arai *et al*., 2003a). Moreover, changes in MDH activities may induce hyperglycemia (Ferrannini *et al*., 1985).

Therefore, the aim of the present study was to examine the effect of OFO on blood glucose, insulin (in plasma and β cells) and energy metabolism related enzymes (MDH and LDH) in peripheral blood leukocytes of mice.

Materials and Methods

Preparation of OFO

To prepare OFO, deep-frying procedures were done as described previously (Huang *et al*., 1988). Briefly, 3 kg of sunflower oil was poured into a cast iron wok and heated on an adjusted gas stove to maintain the oil temperature at 200 ºC. Wheat flour dough sheets (10 X 4.5 X 0.15 cm) were fried in the oil separately for 3 minutes. About 1.5 kg dough per day was used. The total frying time was 12 hours for half of the oil (OFO-12) and 24 hours for the other half (OFO-24). The final oil yield was about 45 % after removing the dregs. The oxidized oil was stored at -20 °C till used in the diet preparation. Peroxide value of the fresh and oxidized oil mixture before inclusion into the diet was 4.8, 450.4 (OFO-12) and 670.2 (OFO-24) meq O_2/kg respectively. It was measured iodometrically according to (Eder and Kirchgessner, 1998) to indicate the oxidation level.

Animals and diets

Thirty male mice weighing 26–30 g and 7-week-old were purchased from the National research center (Egypt). Animals were placed in stainless steel cages and maintained on a 12 hrs light–dark cycle, suitable room temperature (21 ± 2°C) and hygienic conditions. Drinking water was provided ad-libitum. Mice were observed for 7 days prior to experimentation. Control mice were fed on a basal diet containing 15 g/100 g fresh sunflower oil, based on 1g equal 8.5 kcal (Subcommittee *et al*., 1995). The Second and third groups received diet containing a similar amount of OFO-12 and OFO-24 respectively. The composition and calculated analysis of the three test diets are shown in Table 1.

Body weight changes

Individual live body weight in grams was carried out for mice in all experimental groups at the beginning of the experiment and also at weekly intervals.

Blood sampling

Blood from the animals was collected after 8 weeks of feeding the test diets under anesthesia (sodium pentobarbital, Hypnol 3%, 46 mg/kg), by cardiac puncture after a 12-h fast into heparinized tubes. The plasma was recovered by centrifuging at 4 °C. Plasma was separated with a pipette, and stored in polypropylene microtubes at -20ºC until analysis. Leukocytes were isolated by gradient

centrifugation with a lymphocyte-isolating solution, Ficoll and washed with cold phosphate buffered saline (PBS) by the method described previously (Washizu *et al*., 2002).

Fractionation of leukocytes cellular components

Cytosolic and mitochondrial fractions of leukocytes were prepared by the method described by (Washizu *et al*., 1998). Briefly, the washed WBCs were re-suspended in 2 ml ice-cold STE solution, followed by homogenization with an ultrasonic processor for 5 seconds. The homogenates were centrifuged at 100g for 1 min. The collected supernatant was used as the cytosol fraction. An aliquot (0.5 ml) of the pellets was re-suspended in 1.0 ml of STE solution, dispersed in 8 ml of iso-osmotic Percoll solution, and centrifuged for 20 min at 10,000g. Two membranous bands were collected separately; one immediately below the surface (plasma membrane) and the other close to the bottom (mitochondria).

Table 1: composition and calculated analysis of the experimental diets

Feed ingredients	**Control diet (%)**	**OFO-12 diet (%)**	**OFO-24 diet (%)**
Fresh sunflower oil[1]	15.00	0.00	0.00
OFO-12	0.00	15.00	0.00
OFO-24	00.00	0.00	15.00
Concentrate mixture (52%)[2]	10.00	10.00	10.00
Yellow corn	36.57	36.57	36.57
Soybean meal (44%)	22.43	22.43	22.43
Wheat bran	10.00	10.00	10.00
Mollasses	3.00	3.00	3.00
Common salt	0.50	0.50	0.50
Ground limestone	0.30	0.30	0.30
Dicalcium phosphate	0.50	0.50	0.50
DL-Methionine	0.20	0.20	0.20
Mineral-vitamin premix [2]	1.50	1.50	1.50
Total	**100.0**	**100.0**	**100.0**
Calculated analysis		%	
Crude protein %		20.00	
ME (kcal/kg diet)[a]		3938.98	
Calcium		0.551	
Available phosphorus		0.42	
Crude fiber		3.99	
Arginine		1.05	
Histidine		0.43	
Isoleucine		0.83	
Leucine		1.43	
Lysine		1.099	
Methionine		0.585	
Phenylalanine		0.785	
Threonine		0.616	
Tryptophan		0.219	
Valine		0.824	

[1]Based on 1g = 8.5 kcal (Small Animal Nutrition, 2001. Sandi Agar. Elsevier Limited. British Library Cataloguing in Publication Data. Butterworth Heinemann, Oxford).
[2]Concentrate mixture composed of corn gluten 60%, meat and bone meal 50%, sunflower meal 44%, fish meal 45%, dry yeast, limestone, broiler premix, L-lysine HCl, Dicalcium phosphate, bone meal, Choline chloride, DL-methionine, and common salt (Royal Food, Sharqia, Egypt).

Biochemical assays

Glucose concentrations were measured by the glucose oxidase method (Glick et al. 1986). Immuno-Reactive Insulin (IRI) concentrations were measured by the ELISA as described previously (Miyake et al. 2002). For the analysis of the effect of OFO on plasma lipids, Triglycerides, High density lipoprotein (HDL), low density lipoprotein (LDL) and total cholesterol were quantified using commercial kits (Stanbio, Texas, USA) according to (Wahlefeld, 1974). The activities of the enzymes in the cytosol and mitochondrial fractions were measured by previously reported methods: LDH with lactate as substrate (Kachmar and Moss, 1976); MDH (Zeikus *et al*., 1977). All enzymatic activities were determined at 24-26 °C and expressed as mU/mg of protein (in leukocytes fractions). MDH/ LDH ratio (M/L ratio) was calculated as MDH activity divided by LDH activity.

RT-PCR analysis of leukocyte MDH

The total RNA was extracted from leukocytes using QIAamp RNA Kit for purification of RNA from fresh whole blood (QIAGEN, Germany). RT-PCR was used to detect the conserved region of cytosolic MDH mRNA positioned at 283-715 base pairs of the cDNA sequence of mouse liver MDH (Joh *et al.,* 1987). The sense and antisense primers were 5′-GGCATGGAGAGGAAGGAC-3′ and 5'-TCCGAGCCTTGATGACAG-3′, respectively. PCR consisted of an initial denaturation at 94 °C for 5 min and 35 cycles at 94 °C for 60 seconds, annealing at 60 °C for 60 seconds and extension at 72 °C for 60 seconds, using a DNA thermal cycler (PTC 100, MJ research, USA). The mice β- actin mRNA was used as an internal standard in RT-PCR analysis (Gene Link - Hawthorne, New York). The PCR products were separated on 2% agarose gels and stained with Ethidium Bromide. Relative intensities of bands were analyzed using BIO- RAD Quantity One.

Immunohistochemical staining for insulin

It was performed according to the protocol described before (Miko *et al.,* 2001; Fararh *et al*., 2002). Briefly, Pancreases from all groups of animals were collected and fixed in 10% buffered formalin and then embedded in paraffin. Five µm sections were made and deparaffinized in xylene and alcohol. Horse plasma was used to block nonspecific antigens. Sections were incubated with (antimouse insulin) primary antibody diluted 1 to 250 in buffered saline. After rinsing in PBS, specimens were incubated with secondary antibody (horse anti-mouse IgG). Specimens were stained using the avidin-biotin peroxidase complex method (ABC Kit, Vector Laboratories, Burlingame, USA), then incubated with DAB (3,3-diamino benzidine tetrahydrochloride) in Tris–HCl. Sections were stained with hematoxylin for few seconds, dehydrated in alcohol and xylene, finally, mounted and covered with Canada balsam. Evaluation of immunohistochemical preparations was made by examination of 8–10 islets for each mouse. Analysis of granularity and staining of images was performed using the CellProfiler (cell image analysis software) designed to quantitatively measure cell phenotypes. This software was developed as an open source project in a non-profit institution and is found at http://www.cellprofiler.org (Carpenter *et al.,* 2006).

Statistical Analysis

Results are expressed as the mean ± SD for the ten mice in each group. One-way ANOVA was used to analyze the significance of differences between groups. A *P*- value of <0.05 was considered significant (Sigma Stat 3.1 for Windows).

Results and Discussion

Effect of OFO on body weight changes

Results of body weight revealed no significant changes in body weight between different experimental groups.

Effect of OFO on plasma glucose level and Leukocyte enzymes Activities

The average gain in body weight in mice fed OFO was significantly (P <0.01) lower than that of the control group (control, 64 ± 23.5; OFO-12, 39 ± 23.51; OFO-24, 44.24 ± 12.58 gram respectively).

Determination of plasma glucose level (Fig. 1) revealed that OFO induced an impairment of glucose metabolism in mice. Impairment of glucose induced by the OFO diet was observed as hyperglycemia. Glucose was highest in mice fed

OFO-24 followed by OFO-12 compared with the fresh sunflower oil group.

The activities of cytosolic LDH in peripheral leukocytes were not significantly different in mice fed OFO-12 and OFO-24 compared to those fed a diet containing fresh sunflower oil. On the other hand the activities of cytosolic and mitochondrial MDH in leukocytes of OFO-fed groups were significantly decreased. Also M/L ratio was decreased (18% and 33% in OFO-12 and OFO-24 respectively) compared to those in the control mice (Table 2).

Table 2: Peripheral leukocyte enzyme activities; malate dehydrogenase (MDH) and Lactic dehydrogenase (LDH) (mU/mg protein) and ratio between cytosolic MDH/LDH (M/L ratio) in mice fed a diet containing fresh sunflower oil (control), OFO-12 and OFO-24

	Control	OFO-12	OFO-24
Cytosol			
LDH	571.66 ±16.94	551.50 ± 15.81	566.50 ± 21.29
MDH	259 ± 6.86	206.42 ± 4.74[b]	201.85 ± 8.75[b]
M/L ratio	0.45 ±0.01	0.37 ± 0.02[b]	0.35 ± 0.01[b]
Mitochondria			
MDH	96 ± 3.54	76.42 ± 3.57[b]	71.14 ± 3.94[b]

The significance of differences between the three groups was analyzed by one-way ANOVA test. Values are expressed as means ± SD, n = 10 for each group. Mean values within a row with different superscript letters are significantly different (P< 0·05).

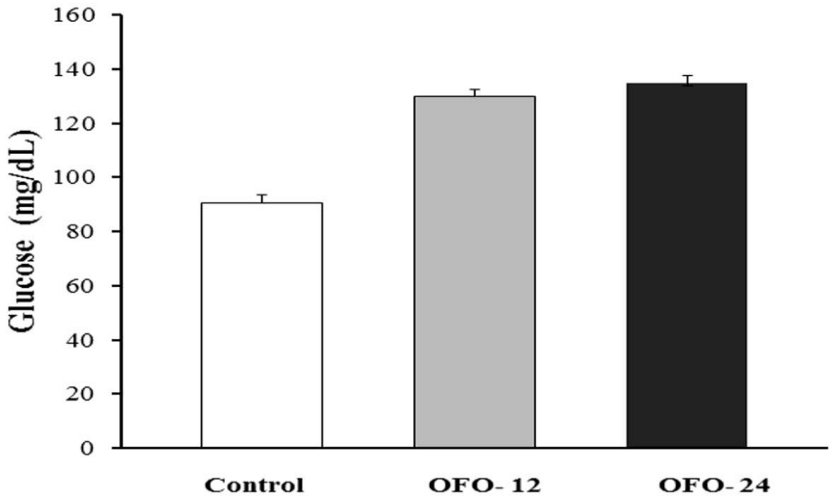

Fig. 1: Plasma glucose level increased in mice fed diet containing OFO. Glucose was highest in mice fed OFO-24 followed by OFO-12 compared to the fresh sunflower oil (control) group. One-way ANOVA test, P < 0.05.

RNA isolation and mRNA detection (RT – PCR) of MDH

The amplification of the cytosolic MDH complementary DNA (cDNA) by RT-PCR (predicted size 360 bp) showed that the cytosolic MDH fragments were clearly detected in the PCR products of leukocytes from mice fed a diet containing fresh sunflower oil and OFO. Relative intensities of bands were 2078.91, 2184.43, 2207.54 int x mm^2 in OFO-24, OFO-12, and control group respectively. The fragments of β- actin mRNA (predicted size 289 bp) as an internal control for quality and quantity of RNA were clearly detected in all groups (Fig. 2).

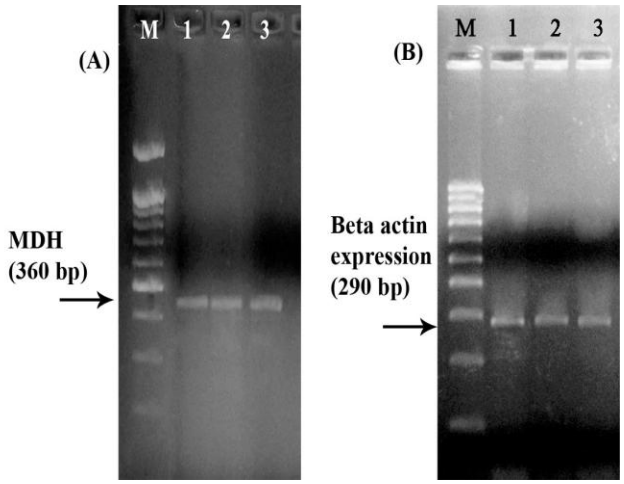

Fig. 2: (A) Analysis of cytosolic MDH mRNA with RT-PCR in leukocytes of mice fed OFO-24 (Lane 1), OFO-12 (Lane 2), and fresh sunflower oil (control) (Lane 3). Relative intensities of bands were 2078.91, 2184.43, 2207.54 int x mm2 respectively. PCR products were stained with ethidium bromide. (B) β-actin mRNA was determined as a control for the PCR technique.

Changes in plasma lipids

The OFO-24 diet resulted in a significant reduction in plasma lipids, including cholesterol, LDL, HDL and triglycerides levels (P <0.01) compared to the control mice (Table 3). In contrast, no significant change in triglyceride level was seen in mice fed OFO-12.

Responses of plasma IRI and Immunohistochemical staining of the Pancreas to OFO

IRI in mice fed a diet containing OFO-12 and OFO-24 were significantly decreased compared to the fresh sunflower oil group. The hypoinsulinemia induced by the OFO diet were more marked after OFO-24 feeding than OFO-12 (Table 3).

Fig. 3 shows insulin immunostaining of β cells in islets of pancreas from mice fed fresh sunflower oil, OFO-12 and OFO-24 groups respectively. Positive staining appeared as dark insulin granules distributed in the β cells in the islets of Langerhans. Immuno-reactivity for the presence of insulin was significantly lower in OFO-fed groups (approximately 19% and 20% in OFO-12 and OFO-24 decreased staining compared with the control group).

Table 3: Immuno-reactive insulin (IRI) and lipid profile in mice fed a diet containing fresh sunflower oil (control), OFO-12, and OFO-24.

	Control	OFO-12	OFO-24
IRI (μU/L)	6.04 ± 0.43[a]	4.86 ± 0.51[b]	4.04 ± 0.33[b]
Cholesterol (mg/dl)	148.69 ± 21.76[a]	47.05 ± 16.58[b]	52.57 ± 13.80[b]
LDL (mg/dl)	57.26 ± 3.51[a]	13.14 ± 1.24[b]	17.91 ± 2.10[b]
HDL (mg/dl)	70.49 ± 4.56[a]	15.24 ± 1.25[b]	18.17 ± 1.94[b]
Triglycerides (mg/dl)	106.90 ± 18.52[a]	96.44 ± 15.81[a]	80.34 ± 7.98[b]

[a,b]Means within a row without common superscripts are significantly different (P < 0.05).

Deep-fat frying is one of the most commonly used procedures for the preparation and manufacture of foods in the world. During deep-fat frying, oxidative and thermal decompositions may take place with the formation of volatile and nonvolatile decomposition products, some of which in excessive amounts are harmful to animals and human health including glucose intolerance induction (Ichinose *et al.*, 2004). In this study, mice fed OFO showed a significantly increased blood glucose level. These results showed that dietary OFO has a greater hyperglycemic effect than similar levels of dietary fresh sunflower oil.

Hyperglycemia and compromised glucose tolerance was shown in OFO-fed rodents (Chao *et al*., 2007). Several factors have been proposed that could potentially increase blood glucose level in OFO-fed mice. These factors includes, decreased peripheral glucose uptake, decreased insulin level and destructive effect on the pancreas.

To clarify the relationship between glucose utilization by blood leukocytes and hyperglycemia as a consequence of OFO feeding, this study compared energy metabolism related enzymes in OFO and control mice. The activity of enzymes related to energy metabolism in peripheral leukocytes is considered to be an indicator of ATP production in the whole body (Hosoya *et al.*, 2004). In particular, changes in activities of MDH and LDH in cytosolic and mitochondrial fractions of leukocytes reflect the redox state and NAD/NADH ratio in oxidative ATP production (Eto *et al*., 1999).

In this study the activities of cytosolic and mitochondrial MDH in leukocytes of OFO-fed mice were significantly decreased compared to those in the control ones. Cytosolic LDH activities showed no significant changes between groups. Therefore in OFO-fed mice the M/L ratio significantly decreased compared to the control value. Decreased M/L ratio is also considered to reflect reduction of energy metabolism including ATP production in mitochondria of leukocytes (Washizu *et al.*, 2001; Fararh *et al*., 2010). Leukocytes produce less ATP due to reduced activity of MDH utilize lower glucose as energy source, which resulted in hyperglycemia (Kimura *et al.*, 2007). Minor reduction in the expression (1%, 5.8% decrease of band intensity in OFO-12, OFO-24 respectively) of mRNA of cytosolic MDH was confirmed by the RT-PCR analysis in mice fed OFO compared to the control ones. Therefore hyperglycemia and decreased ATP production in leukocytes of OFO-fed mice may occur as consequence of decreased MDH activities (Mainly) and decreased MDH mRNA expression (partially). The mechanism underlying reduced activities of MDH in mice fed OFO may be due to general disturbances of energy production. Ingestion of autoxidized oils has been shown to cause profound alterations in membrane composition and fluidity associated with alterations of some membrane-bound enzymes involved in energy metabolism (Lee and Csallany 1994; Hochgraf *et al*., 1997).

Considering the effect of OFO on plasma lipids it was noted that plasma TG, LDL, HDL and cholesterol concentrations decreased significantly in mice fed OFO-24 compared to the control group fed fresh sunflower oil. In contrast triglyceride showed no significant decrease in OFO-12 group. These finding are supported by a previous study provided by Chao et al. (2005) who found that dietary OFO was a more potent anti-adiposity agent than fish oil.

The anti-adiposity effect of OFO could be partly attributed to the reduced fat absorption as a consequence of decreased digestion and absorption of the oxidized lipid, especially for the polymeric materials formed during the deep-frying process (Gonza´lez-Mun˜oz *et al*., 1998; Nwanguma *et al*., 1999).

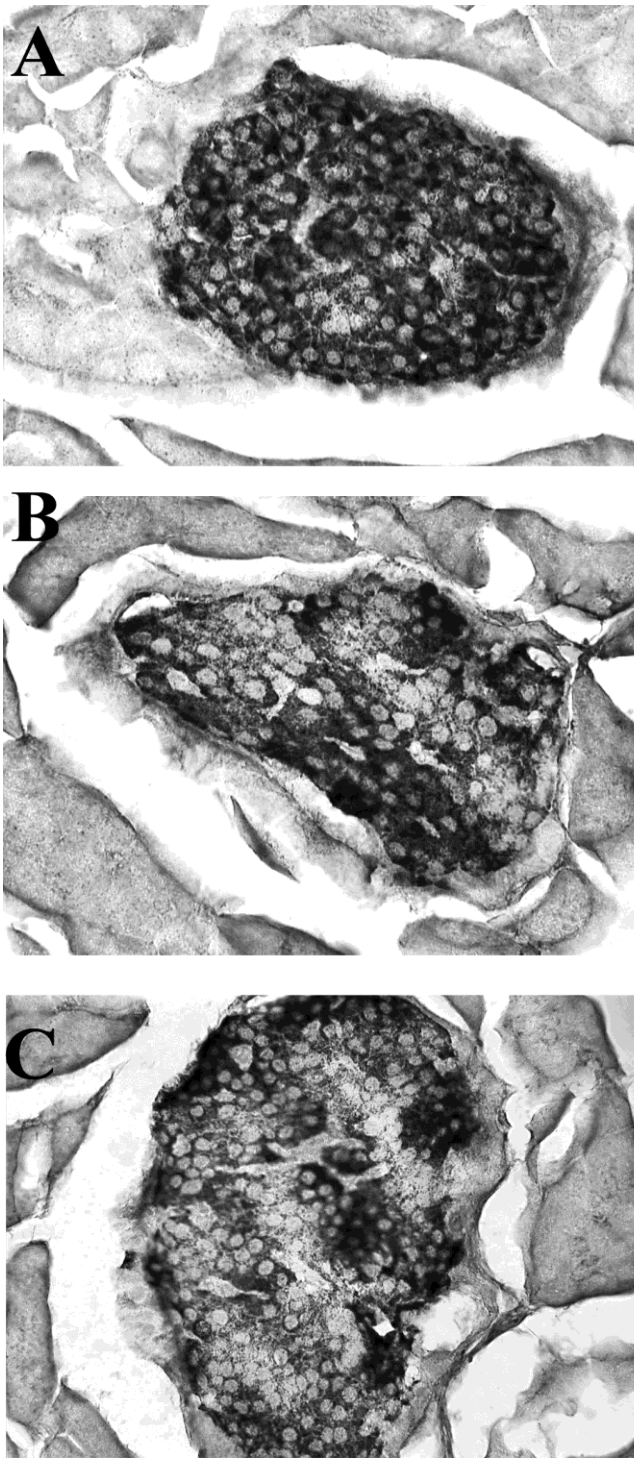

Fig. 3: Insulin immunohistochemistry of the islets of Langerhans in pancreas. A, immunostaining of section from mice fed fresh sunflower oil (control), OFO-12 (B) and OFO-24 (C). Staining was concentrated in cytoplasm of β cells and appeared as dark insulin granules. Amount of staining was lower in OFO-fed groups, although occasional strongly stained areas were visible. Original magnification 400x.

The anti-adiposity effect of OFO might also be related to activation of the peroxisome proliferator-activated receptor α (PPARα) signaling pathway. PPARα activation up-regulate the expression of genes involved in fatty acid oxidation, which cause reduction in liver and serum lipids (Schoonjans *et al*., 1996).

It is generally accepted that a reduction in adipose mass can improve insulin sensitivity due to decreased circulating levels of adipose tissue-released mediators of insulin resistance. In this study, OFO reduced plasma cholesterol and triglycerides levels; however, hypoinsulinaemia was seen in mice fed OFO-12 and OFO-24 diets, suggesting that the hyperglycemia is mediated partly by insulin deficiency. The decreased circulating insulin levels decrease glucose uptake by skeletal muscle and adipose tissue and impair glucose tolerance. The mechanism of the hypoinsulinaemia caused by high OFO consumption may involve impaired insulin secretion due to impaired function of pancreatic β cells (Tsujinaka *et al*., 2005). In this study, the inactivation of β cells was demonstrated by immunohistochemical staining of pancreas using anti-insulin antibodies. Significantly decreased immuno-reactivity to anti-insulin antibodies was observed in the pancreases from OFO-fed mice. Insulin staining in the cytoplasm of β cells in OFO was approximately 20% less intensely than the control mice. These results indicate that β cells activities in synthesis and secretion of insulin were diminished. Several factors have been described that could potentially diminish β cells activity. It is known that OFO feeding induced higher oxidative stress (Izaki *et al*., 1984; Brandsch and Eder, 2004), which may result in oxidative damage to the pancreas. Reactive oxygen species were proved to play a role in insulin resistance induced by tumor necrosis factor-α or dexamethasone (Houstis *et al*., 2006). Furthermore Oshima *et al*. (2006) showed that the increased prostaglandin E2 expression in the islets resulted in the destruction of pancreatic beta cells. Significantly higher prostaglandin E2 metabolites in plasma and urine of OFO-fed rats have been observed (Huang, 2003). In addition, the decreased insulin immuno-reactivity in the pancreas of OFO-fed mice might partially be related to decreased vitamin E, since most of the vitamin E in the oil was destroyed during the frying process (Liu and Huang, 1995). Vitamin E-deficient high OFO diet might be a cause of development of impaired glucose tolerance in rats, because of both insulin resistance and impaired insulin secretion (Koch *et al*., 2007).

Impaired glucose tolerance is believed to be caused not only by impaired insulin secretion but also persistent hyperglycemia. The progression of impaired glucose tolerance may lead to type II diabetes mellitus (Fujita *et al*., 1998). Therefore, the quality and oxidative level of frying oil should be well controlled, especially in developing countries.

This study may clarify the causes of hyperglycemia occurred due to the effect of OFO feeding in mice. Our findings support the relationship between hyperglycemia and decreased activities of MDH (an energy metabolism related enzyme in peripheral blood leukocytes), hypoinsulinemia and reduced activities of β cells in synthesis and secretion of insulin as indicated by decreased insulin immunostaining in OFO-fed mice.

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