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Trans fatty acids increase nitric oxide levels and pancreatic beta-cell necrosis in rats

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ABSTRACT

BACKGROUND

The prevalence of diabetes in Indonesia is increasing due to various factors, including life style changes such as trans fatty acid (TFA) intake. High TFA intake is known to be related to blood lipid profile changes resulting in cardiovascular disorders. This study was to identify the effect of TFA on nitric oxide (NO) production and on necrosis of pancreatic beta cells.

METHODS

A study of randomized pre-test post–test design with control group. Thirty Sprague Dawley rats were divided into 3 groups, i.e. group K (control), group P1 receiving a diet with 5% TFA, and P2 receiving 10% TFA. The intervention was performed for 8 weeks. NO level and pancreatic beta-cell necrosis were analyzed using Pearson's chi square test.

RESULTS

After 4 weeks of treatment there was no change in NO levels in group K, but increased NO in P2 (2.6-3.8 ìM). At 8 weeks after treatment, NO levels in groups P1 and P2 increased to 2.6-3.4 ìM and 4.2-14.3 ìM, respectively, while in group K only 2 rats had increased NO levels of 2.8-2.9 ìM. With Pearson's chi-square test, there was a signifant difference in the proportions of necrotic pancreatic beta cells after 4 weeks and 8 weeks (p= 0.000). No necrosis of beta cells was found in group K, mild necrosis in group P1 (1-25%) and moderate necrosis in group P2 (26-50%).

CONCLUSION

TFA consumption significantly increases NO levels in Sprague Dawley rats and also results in moderate grades of necrosis of pancreatic beta cells.

Keywords : Trans fatty acids, nitric oxide levels, necrosis of pancreatic beta cells

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Asam lemak trans meningkatkan kadar oksida nitrit dan neksrosis sel beta pancreas pada tikus

ABSTRAK

LATAR BELAKANG

Prevalensi diabetes melitus di Indonesia semakin tahun semakin meningkat. Asupan tinggi asam lemak trans selama ini dihubungkan dengan memburuknya profil lipid, yang berakibat terjadinya penyakit kardiovaskuler. Tujuan penelitian ini untuk membuktikan asupan tinggi asam lemak trans terhadap stres oksidatif, produksi oksida nitrit (NO), dan nekrosis sel beta pankreas.

METODE

Penelitian ini merupakan penelitian eksperimental laboratorik dengan Randomized Pre-test Post –test control group design dengan Rancangan Acak Lengkap. Tiga puluh tikus Spargue dawley dibagi dalam 3 kelompok kontrol (K), kelompok perlakuan (P1) 5% asam lemak trans, kelompok perlakuan (P2) dengan pakan 10%, perlakuan diberikan selama 8 minggu. Kadar NO dianalisis dengan uji Pearson-Chi Square, *nekrosis sel beta pankreas dengan analisis deskriptif*

HASIL

Pengukuran kadar NO diklasifikasikan menjadi kelompok dengan kadar NO < 2,5 ìM, dan meningkat bila > 2,5 ìM. Kelompok kontrol tidak terdapat perubahan, P1 terdapat peningkatan pada 1 tikus (2,6) ìM, P2 terjadi peningkatan pada semua tikus (2,6-3,8) ìM. Setelah 8 minggu, kelompok K, 2 tikus mengalami peningkatan (2,8-2,9) ìM, P1 terdapat peningkatan pada semua tikus (2,6-3,4) ìM, P2 terjadi peningkatan pada semua tikus (4,2-14,3). Pada P1 dan P2 dengan uji beda Pearson Chi- Square, *setelah 4 minggu dan 8 minggu terdapat perbedaan proporsi yang bermakna (p=0,000). Kelompok K tidak dijumpai nekrosis sel beta pankreas, P1 semua tikus mengalami nekrosis sel beta pankreas dengan derajat ringan (1-25%) dan P2 dengan derajat sedang (26-50%).*

KESIMPULAN

Terjadi peningkatan kadar NO secara bermakna pada P1 dan P2 dan terjadi nekrosis derajat ringan pada P1 dan derajat sedang pada P2.

Kata kunci: *Asam lemak trans, nitric oxide, nekrosis sel beta pankreas*

INTRODUCTION

The prevalence of diabetes mellitus (DM) is steadily increasing worldwide. In 2009 there were 285 million patients with DM and for the year 2030 the prevalence is projected to increase to 438 juta patients, 60% of whom will be Asians. Epidemiologically, type 2 DM may be found in developed as well as developing countries.[1,2] The prevalence of DM in Indonesia is estimated to be up to 5.7% of the total population.[3] One of the risk factors for DM is the changing dietary pattern, from the traditional diet to a Western diet, including increased consumption of fast foods.[3,4] The results of various studies have revealed that currently a large amount of fast foods is consumed by Indonesian communities. Fast foods are not only high in calories and fats but also high in trans fatty acids (TFA).[5]

Trans fatty acids are naturally present in small amounts in products from ruminants, such as beef, milk, cheese and butter.[6] Most of the TFA comes from the processing of cis-unsaturated fatty acids which are abundantly found in nature. In Indonesian communties the major sources of TFA are a variety of hydrogenated plant oil products, such as margarine, shortening, hydrogenated vegetable oils (HVO) and other products processed with industrially hydrogenated fats, such as breads, packaged snacks (chips, cereals and biscuits) and deep fried foods. These ready-made products are in high demand in the communities, because they are tasty, crispy, practical, not easily becoming rancid, and easily transported, since they are in semi-solid form.[5,7] Currently TFA are attracting attention because they have more negative effects on health in comparison with saturated fatty acids, formerly considered to be bad for health.[8] Thus far, studies have shown that high intakes of TFA have a negative effect on the lipid profile, which is a predictor of the development of atherosclerosis [9] and a major cause underlying cardiovascular disease (CVD).[10,11] Theoretically a high TFA intake may cause oxidative stress, resulting in the formation of intracellular reactive oxygen species (ROS),[12,13] thus causing cellular damage and dysfunction.[12-15] One of the ROS compounds formed by oxidative stress is nitric oxide or nitrogen monoxide (NO).[12,16]

NO is an important intracellular signaling and messenger molecule in humans. NO in normal concentrations is important for protecting organs against ischemia. On the other hand chronic expression of NO may kill healthy host cells, particularly in chronic inflammation, and may be associated with the development of degenerative disease. Mitochondria play a role in NO-induced cell death. This occurs through inhibition of respiration and reactive nitrogen species (RNS)-induced mitochondrial permeability transition (MPT), thus resulting in depletion of adenosine triphosphate (ATP), ultimately leading to necrosis. Production of NO in large amounts may also cause DNA damage, have toxic effects, and cause necrosis.[17] Similar processes presumably take place in pancreatic beta cells as a result of high TFA intake.

The present study used high dosages of TFA of 5% and 10% of total calories, with reference ot previous studies, where at these dosages TFA may result in nutritional metabolic disturbances.[18] The limit for TFA intake based on studies up to date is 1% of total calories. The pathophysiology of high TFA intake is still controversial and unclear. The objective of the present study was to evaluate the effect of administration of high dosages of TFA on NO levels, as a result of oxidative stress, and in connection with the hypothesis of an in vitro study that increased NO levels causes necrosis of pancreatic beta cells [17,19] in male Sprague Dawley rats as experimental animals.

METHODS

Research design

This a true experimental laboratory study, with completely randomized pre-test post-test design with control group. Simple randomization was done using a computer. The study was conducted in 2012 at the Integrated Research and Development Laboratory of the Preclinical Research Service for Development of Experimental Animals (LPPTLP3HP), Gajah Mada University, Yogyakarta.

Animals and experimental protocol

This study was conducted on the experimental animals for 8 weeks, with reference to the study conducted by Dorfman[18] on the effect of TFA on nutrient metabolism in liver and adipose tissues using male Sprague Dawley rats with the following inclusion criteria: i) aged 7 weeks, ii) weighing 200-295 grams, iii) being in healthy condition, actively moving, and without anatomical abnormalities, iv) not suffering from weight loss in the adaptation period, and v) having an initial fasting blood glucose of <110 mg/dL. The exclusion criteria

were: i) animals suffering from diarrhea during the intervention period, marked by unformed stools, ii) weight changes of >10% in the adaptation period, and iii) rats dying during the study period. All rats were obtained from the Integrated Research and Development Laboratory of the Preclinical Research Service for Development of Experimental Animals (LPPTLP3HP), Gajah Mada University, Yogyakarta.

This study was conducted on Sprague Dawley rats, comprising 3 groups. Group K was the control group receiving standard feed, group P_1 was the first intervention group receiving rat feed pellets containing TFA corresponding to 5% of the total daily energy requirement, and group P_2 was the second intervention group receiving rat feed pellets TFA corresponding to 10% of the total daily energy requirement.

Calculation of the sample size was based on the WHO guidelines on the use of experimental animals for evaluation of the safety and effectiveness of herbal medications,[20] with a mininum of 5 animals per group and including a control group. The calculation was also based on a previous study conducted by Dorfman,[18] using 8 Sprague Dawley rats per group. The present study used 10 rats per group.

Care of experimental animals

The rats were placed in individual cages in a well-ventilated at a temperature between 28 - 32^{o}C, with cycles of 12 hours light and 12 hours darkness, and were acclimatized to adapt them to the weather and environment. The cages were cleaned daily. The rats were daily given pelleted feed and water *ad libitum*. The pelleted feed was produced by Research Diet, NJ (USA).[18]

Assessment of NO levels

NO levels were assessed indirectly by spectrophotometry, followed by nitrite quantitation using Griess' reagent at an absorbance of 450 nm. NO measurements were classified into a group without increased NO levels (<2.5 ìM), and a group with increased NO levels (>2.5 ìM).

Histopathological examination of pancreatic â-cells

After completion of the interventions, the Sprague Dawley rats were terminated in accordance with the principles set forth in the Medical Ethics Manual 2005.[21] The rats were anesthesized with ether by group and terminated by cervical dislocation. The pancreas was extracted from the abdomen, weighedpan, and processed into paraffin blocks. The blocks were made into hematoxyline eosin (HE) histopathologic preparations. From each slide five fields were examined at 400x magnification for necrotic pancreatic â-cells. The criteria used for determining the grade of necrosis were according to the study by Matveyenk et al.[22] The necrosis was considered to be severe when more than 50% of the â-cells were necrotic. In diabetes a more severe grade of necrosis ($\geq$65%) was expected. The criteria to be used in this case was according to a study conducted at the Bogor Agricultural Institute (Institut Pertanian Bogor, IPB).[23] The necrosis was considered moderate when there was necrosis in 26-50% of â-cells and mild when there was necrosis in 1-25%, of â-cells, while in normal rats there are no necrotic â-cells (0%).

Data analysis

The collected primary data were tabulated for further analysis. The P1 and P2 data were analyzed using statistical difference tests (Pearson's chi square). Data on necrosis in cells are on the categorical (ordinal) scale and therefore the Pearson chi-square test was used to find differences in proportions between groups.

Ethical clearance

Ethical clearance for this study was issued under No. 173/EC/FK/RSDK/2011 by the Commission on Medical Research Ethics,

Table 1. Between-group differences in proportions of NO levels at weeks 0, 4, and 8

NO level(µM)	Group			p value
	Control (n=10)	P1 (n=10)	P2 (n=10)	
= 2.5 at week 0	10 (100.0%)	10 (100%)	10 (100.0%)	0.0001*
> 2.5 at week 0	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.0001*
= 2.5 at week 4	10 (100.0%)	9 (90.0%)	0 (0.0%)	0.0001*
> 2.5 at week 4	0(0.0%)	1(10.0%)	10 (100.0%)	0.0001*
= 2.5 at week 8	8(80.0%)	0 (0.0%)	0 (0.0%)	0.0001*
> 2.5 at week 8	2 (20.0%)	10 (100.0%)	10 (100.0%)	0.0001*

*Pearson chi-square test

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RESULTS

Initially all rats had NO levels of 2.5 ì M, and after a four-week intervention no changes in NO levels were found in the control group. The Pearson chi-square test for differences between proportions found significant differences in NO levels in the intervention groups at the end of the fourth and eighth weeks, with p=0.000.

Necrosis of pancreatic â-cells on histopathologic examination

On histopathologic examination of the pancreas at the end of the intervention period no necrosis was found in the control cells (group K). In group P1 all rats had mild necrosis of the pancreatic â-cells (1-25%), while in group P2 all rats had moderate necrosis of the pancreatic â-cells (26-50%).

The results of Pearson's chi-square test showed a significant difference between P1 and P2 with a p value of 0.000. The proportions of necrotic events by group is presented in Table 2 and the difference in necrosis is shown graphically in Figure 1.

DISCUSSION

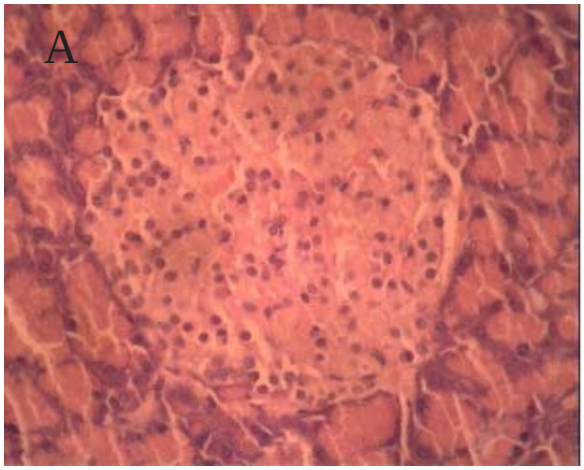
TFA and NO levels

Nitric oxide (NO) or nitrogen monoxide is an important signaling molecule in humans and an important messenger molecule in the cell. NO in normal concentrations is important for protecting the organs against ischemia, On the other hand chronic and excessive NO expression may result in the death of healthy cells,[24] particularly in chronic inflammation and in connection with the development of degenerative diseases such as cancer and diabetes. Chronically raised NO levels induce inhibition of mitochondrial respiration, through RNS-induced mitochondrial permeability transition (MPT), thus causing depletion of ATP and ultimately cell necrosis. Production of large amounts of NO may also lead to DNA damage, toxic effects, and necrosis.[17]

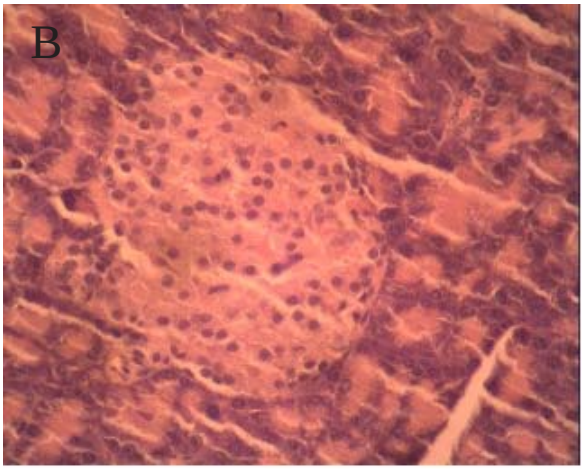
Table 2. Between-group difference in proportions of necrosis in pancreatic â=cells

Degree of necrosis (%)	Group			p value
	Control	P1	P2	
None	10	0	0	0.000
Mild (1-25%)	0	10	0	
Moderate (26-50%)	0	0	10	
> 50%	0	0	0	

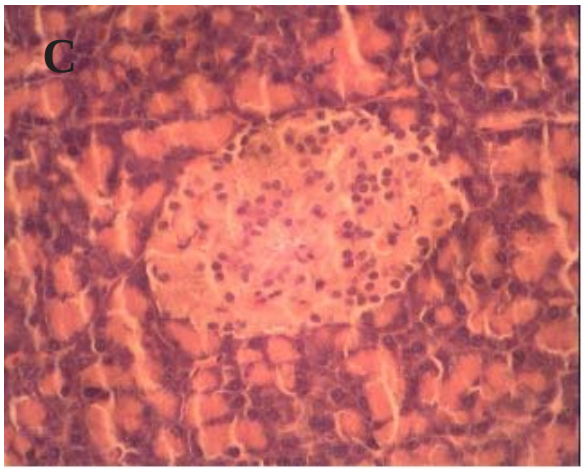
*Pearson's chi square test



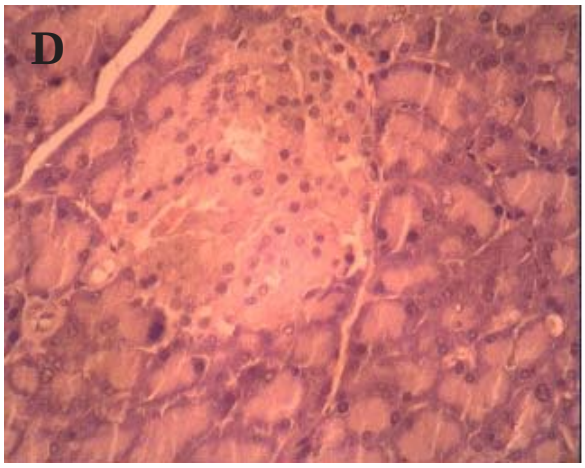
(A) No necrosis in islets of Langerhans (x400)



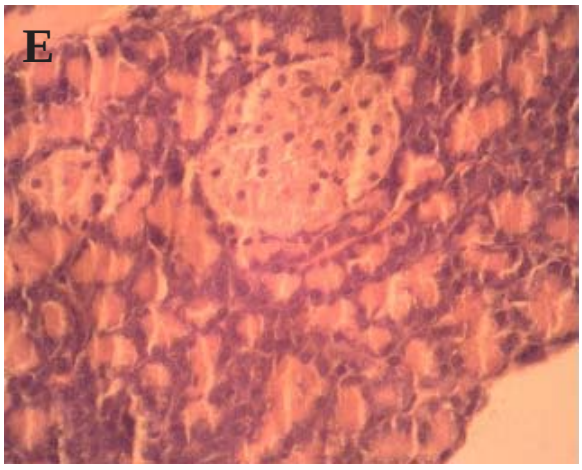
(B) Mild (10%) necrosis in islet of Langerhans as indicated by cells without nuclei (x 400)



(C) Moderate (20%) necrosis in islet of Langerhans as indicated by cells without nuclei (x 400)



(D) Moderate (30%) necrosis in islet of Langerhans as indicated by many cells without nuclei (x 400)



(E) Moderate (40%) necrosis in islet of Langerhans as indicated by scarcity of cells (x 400)

Figure 1. Necrosis of pancreatic â-cells (arrows indicate cell nuclei)

At physiological concentrations (approximately 1-100 nM), NO tends to be non-reactive and most of its physiological functions is mediated by NO bound to Fe^{2+} by guanylate cyclase causing NO activation and cGMP production. NO may be converted into many derivatives, designated reactive nitrogen species (RNS). At high concentrations in the cell or the cell, NO reacts directly with oxygen producing NO_2, which subsequently reacts with NO form N_2O_3.

The results of this study show that NO levels after 4 weeks was ≤2.5 µM in all control rats, identical with baseline values (signifying no change in NO levels). In group P1 only one rat (10%) showed NO levels of ≥2.5 ìM, while in group P2 all rats (100%) had NO levels of ≥2.5 ì M. After 8 weeks 2 rats (20%) in the control group had NO levels of ≥2.5 ìM (these being 2.8 and 2.9 ì M, respectively), in group P1 all rats (100%) had NO levels of ≥2.5 ìM, as did the rats in group P2.

These findings are in agreement with an in vitro study by Downar et al. stating that high TFA intakes may cause oxidative stress, leading to formation of intracellular ROS and may thus cause cellular damage and ultimately cellular dysfunction.[14,15] One of the ROS formed by oxidative stress is NO.[12,25]

The study conducted by Mukhopadhyay [26] in animal experiments showed that the increase in NO due to oxidative stress may cause myocardial cell death. This may presumably also occur in pancreatic â-cells as a result of high TFA intakes.

The study of Rao et al.[27] on the nervous system has reported that free radicals produce lipid peroxides and more prominent is the formation of ROS and NO free radicals resulting in ischemic attacks. High TFA intakes result in the formation of ROS/RNS causing oxidative stress, such that it causes lipid peroxidation which triggers NO formation. Continuous peroxidation of lipids will cause higher increases in NO levels. Cells exposed to NO or cells producing NO show inhibition of respiration in cytochrome oxidase that occurs rapidly but is reversible. After exposure to NO, an irreversible inhibition occurs, made possible by the conversion of NO to RNS, which cause irreversible inhibition of respiration in other enzymes. The resulting peroxynitrites are strong antioxidants and can cause DNA damage, induce lipid peroxidation, produce oxidants and cause death of cells, including pancreatic â-cells. For the abovementioned reasons, high TFA intakes may cause pancreatic â-cell necrosis through NO formation.

Trans fatty acids and pancreatic â-cell necrosis

Necrosis is cell death, with karyolysis and severe damage to the cell membrane (resulting in loss of membrane integrity). In addition, the lysosomes release enzymes that enter the cytoplasm and cause damage the cell's own organelles, resulting in irreversible necrosis. Cell necrosis cause an efflux of cellular contents through the damaged plasma membrane and thus result in an inflammatory reaction. Necrosis of pancreatic â-cells may be caused by a variety of factors, such as genetics, infection, toxic diabetogenic substances, oxidative stress and nutrition. TFA are among the factors capable of causing pancreatic â-cell necrosis.

On histopathological examination of pancreatic tissues in the present study, no pancreatic â-cell necrosis was found at the end of the eighth week in the control group K (0.0% necrosis). However, in group P1 all rats had pancreatic â-cell necrosis in varying degrees, viz. 6 rats had 5% necrosis and 4 had 10% necrosis, both of which are considered to represent mild necrosis. In group P2, 3 rats had mild necrosis (20% necrosis), while 1 rat had 30% necrosis and 6 rats had 40% necrosis, both being categorized as moderate necrosis.

The cause of pancreatic â-cell necrosis in this study as a result of the high TFA intakes presumably occurs through inflammation (leading to CRP production) and oxidative stress. Both induce NO synthesis, thus

increasing NO levels. Oxidative stress in group P1 with administration of 10% TFA causes more severe cell necrosis in comparison with group P2 with administration of 5% TFA.

Previous studies by Szmitko et al.[15] and Malhi [28] have advanced the lipotoxicity theory, stating that cell death may be due to abnormal lipid accumulation. Free fatty acids are hydrophobic and therefore able to pass the cell membrane directly or through fatty acid transport protein (FATP) or fatty acid transporter CD36. Saturated fatty acids may increase apoptosis (programmed cell death) and thus may cause cell damage and ultimately cellular dysfunction. However, in this study no apoptosis was found, possibly because TFA are more toxic than free fatty acids and thus did not cause programmed cell death, which is a physiological process to kill unwanted cells.

The study of Stumfold et al.[29] focussed on an explanation of â-cell necrosis by glucotoxicity, as glucose metabolism in pancreatic â-cells is oxidative and thus causes ROS formation which in turn damages the â-cells. Pancreatic â-cells have limited amounts of catalase and superoxide dismutase for neutralizing ROS. ROS induces the production of NF-êB which causes the proapoptotic effect of hyperglycemia in the pancreas and decreases the expression of the duodenal homeobox-1 gene. This is a regulator of insulin transcription and causes abnormalities in the final stages of insulin exostosis.

The pancreatic â-cell necrosis occurring in the present study may presumably be produced by an inflammatory process through the activation of cytokines such as IL-6 and TNF-á or through induction of CRP production. These processes occur continuously because the administration of high TFA feed was conducted daily, and thus continuously inducing NO production. Another mechanism of â-cell necrosis in this study is through increased ROS/RNS free radical production, resulting in oxidative stress which in turn triggers lipid peroxidation and thus increasing NO production and pancreatic â-cell necrosis.

Although this study did not perform an in-depth analysis of the relationship between severity of TFA intake by the experimental animals on the one hand, and NO production and pancreatic cell necrosis on the other hand, it may still be concluded that TFA cause increased NO production and necrosis of pancreatic â-cells.

CONCLUSIONS

Administration of TFA at the higher dosage of 10% within the longer intervention period of 8 weeks reults in increased NO levels and more severe necrosis of pancreatic â-cells.

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