ISSN: 2347-467X, Vol. 09, No. (1) 2021, Pg. 139-146

Current Research in Nutrition and Food Science

www.foodandnutritionjournal.org

Trans Fatty Acid Intake Modulates the Expression of Uncoupling Proteins 2 and 3 (UCP2 & UCP3) mRNA in Children

WALAA M SARHAN[1]*, SAFAA EL-HUSSIENTAWFIK[2], AMAL S EL-SHAL[1], VISHRUTIMAKANI[3] and HANIM M. ABDEL-NOUR[1]

[1]Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt.
[2]National Nutrition Institute, Cairo, Egypt.
[3]Wake Forest Institute for Regenerative Medicine, Wake Forest University Health Sciences, Winston-Salem, North Carolina, United States of America.

Abstract

Increased consumption of Trans Fats is associated with increased risk of Coronary Heart Disease. Uncoupling proteins (UCPs) are mitochondrial proteins that disperse the inter-membrane electrochemical potential as heat. We aimed todetect the relation of high Trans-fat intake in diet on the expression of UCP 2 & 3 m-RNA in children. A specific questionnaire to parents of sixty-eight children (4-15 years) was conducted. Accordingly, the subjects were sub-grouped into; High Trans-fat consuming group (37 subjects) and a Medium Trans-fat consuming one (31 subjects). Samples collected from Peripheral blood to analyze UCP 2 & 3 mRNA expression by Real Time Polymerase Chain Reaction (RT-PCR). Levels of UCP2 expression was reduced in children consuming High Trans-fat (2.5 ±0.7) in comparison with Medium Trans-fat consuming ones (1.5± 0.2) with ($p<0.001$). However, not much significance was showed in UCP3 expression with values (2.1±0.5) in the High consuming group and (1.9±0.2) in Medium consuming group with ($p=0.08$). In Delta relationship the diet-induced changes in UCP2 ($r=0.66$, $P=0.002$) and UCP3 ($r=0.61$, $P=0.06$) mRNA expression was negatively correlated with percentage of Trans-fat in diet. The correlation of UCP 2 & 3 mRNA expression and high Trans-fat intake suggests a mechanism by which high Trans-fat diet plays a role in childhood obesity.

Article History

Received: 10 November 2020
Accepted: 16 February 2021

Keywords

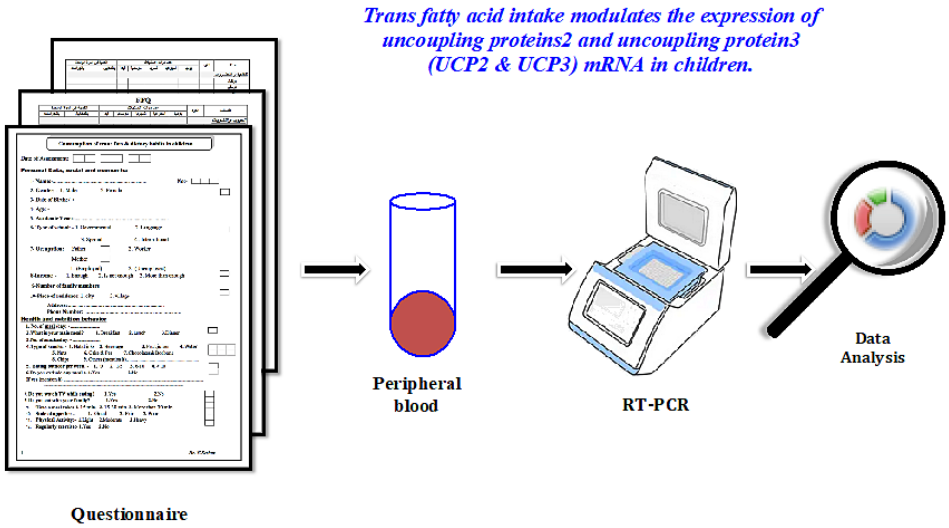
mRNA Expression; Mitochondrial Uncoupling; Questionnaire; Real Time Polymerase Chain Reaction (RTPCR); Trans Fatty Acids; Uncoupling Proteins (UCPs).

CONTACT Walaa M Sarhan walaasarhan@hotmail.com Medical Biochemistry and Molecular Biology Department, Faculty of Medicine, Zagazig University, Zagazig, Egypt.

Doi: 10.12944/CRNFSJ.9.1.14

Highlights

- Children in Egypt showed high consumption of Trans-fat.
- Levels of UCP2 and UCP3 mRNA expression are negatively correlated with the amount of Trans-fat intake.



Graphical Abstract

Introduction

Trans fatty acids (TFA) are generally geometrical isomers of unsaturated fatty acids and have at minimum one non-conjugated carbon-carbon double bond in the form of trans-configuration.[1] Dietary TFA could be of artificial or organic sources. The artificial TFA are generated by partial hydrogenation of unsaturated vegetable oils.[2] The major sources of artificial TFA in food are margarines, deeply fried fast foods, commercially baked products and packaged snack foods.[3]

Consuming high amounts of TFAs (>1% of total daily energy intake) is associated with high risk of Coronary Heart Disease (CHD).[4,5] That could be linked to increase low-density lipoprotein cholesterol (LDL), decrease high-density lipoprotein cholesterol (HDL), release of pro-inflammatory cytokines and endothelial dysfunction.[3,6] Moreover, high TFA has been identified as a major risk factor for obesity.[6,7] Lesions in the big arteries can begin to appear in childhood and are majorly linked to dyslipidemia.[8] The negative health effects of TFA were attributed mainly to TFA coming from artificial origin and some studies have suggested that natural TFA might have beneficial effects.[9]

Non-communicable disease (NCDs) is accused of about 68% of deaths in the Eastern Mediterranean Region (EMR).[10] Elevated numbers of children (6.9% of children under five years) higher than the global average of (6.2%) showed overweight. In most EMR countries, more than 50% of the adolescents are overweight or obese.[11] In 2016 The total mortalities of cardiovascular diseases (CVDs) in the EMR was around 1.3 million, representing 31.9% of total mortality rate.[12]

Uncoupling proteins (UCPs) biochemically are proteins disperse the mitochondrial inter-membrane electrochemical potential as heat . UCPs are classified UCP-1, 2 and 3. UCP-1 is overexpressed when exposing to cold and overeating, and its half-life ranges from hours to days.[13]

The second isoform, UCP-2, has shown to mediate cyto-protection, immune cell modulation and enhance glucose sensitivity in tissues as the brain and the pancreas, having a half-life of only 1h. Lastly, UCP-3 is markedly translated in skeletal tissue, with a half-life of 1 to 4h.[14] UCP3 could be of high thermo-genic importance under specific situations and it is an attractive drug target for the management

of obesity. A major function of UCP 2 & 3 is to attenuate mitochondrial production of free radicals in the mitochondria, in cells fighting against oxidative damage.[15]

Activation of proton transport of UCP2 by Reactive oxygen species (ROS) reduces insulin production in pancreatic β cells. This may be pathologically negative effect of the preservative mechanism that restricts production of ROS and damage of pancreatic islet during high fat feeding or hyperglycemia and causes type II diabetes.[16] This action could be considered a physiological mechanism to control the use of fatty acids and glucose as sources of energy.[17]

Subjects and Methods

Subjects

The subjects comprised 68 child (31 males and 37 females, mean age 9 ± 6yr) visiting Zagazig University hospital clinics - Egypt.Approval for the study was obtained from the Institutional Review Board (IRB). Dietary Trans-fat intake was assessed using a 162-item semi quantitative food frequency questionnaire (SFFQ). The questionnaire was designed to evaluate socio-demographic data, nutritional habits and the frequency of consuming selected foods. There were 5 options for respondents to choose from, ranging from never to daily with determination of the sizes. We asked their parents about the type of fat ordinarily used for baking and frying food and at the table. All procedures performed in our study were in accordance with the ethical standards of the institution and an informed consent approval from the parents. Composition values for dietary fats and other nutrients were obtained from National Nutrition Institute Food Composition Tables for Egypt[18] with the help of other reports to determine Trans-fat contents.[19,20, 21]

After analysis of the food frequency questionnaire data, the amount of Trans fat in diet were estimated and accordingly the subjects were sub-grouped into: High Trans-fat consuming group included 37 individuals with a mean ± SD (2.2 ± 0.36) g per day, which represented 11.2% of dietary fat, Medium Trans-fat consuming group comprised 31 subjects with a mean ± SD (1.2 ± 0.22) g per day, which represented 6.1% of dietary fat which comprised 31 children.

Collection of Blood Samples

Three ml of whole blood was collected from each participant under complete aseptic condition in sterile EDTA (solute form) containing tubes and kept frozen at -20°C till analysis.

RNA Extraction

The RNA extraction was done using The IQeasy TM plus Blood RNA Extraction Mini Kit purchased from iNtRON Biotechnology. Evaluation of purity and concentration of each RNA sample was done by determination of the A260/ A280 ratio using Milton Roy Spectronic 3000 Array.

Reverse Transcription

The cDNA was prepared by reverse transcription of total RNA template using Maxime RT PreMix Kit purchased from iNtRON Biotechnology.

Table 1: Primers for PCR

Gene	Sense Primer (5'-3')	Antisense Primer (5'-3')
UCP-2	GACCTATGACCTCATCAAGG	ATAGGTGACGAACATCACCACG
UCP-3	ATGGACGCCTACAGAACCAT	TACGAACATCACCACGTTCC
β-actin	AGAGCTACGAGCTGCCTGAC	AGCACTGTGTTGGCGTACAG

Real-Time qPCR

2µl of first-strand cDNA was used for real time PCR (RT-PCR) in 20µl reactions containing 10 ul of qPCR Green Master, 1ul of each primer and 4ul PCR grade water. To avoid co-amplification of the gtenomic DNA, that may cause contamination of the RNA preparation, we designed All primer combinations to span at least one intron. The primers used for amplifying cDNA fragments are shown in Table 1. PCR conditions were as follows for 40 cycles: 1 min at 94°C, 45 sec at 57°C and 2 min at 72°C proceeded by initial denaturation at 95 °C for 10 min to fully activate the chemically modified PCR DNA polymerase and were quantified by Agilent

Mx3005P Real-Time PCR Systems. Levels of mRNA were cleared up as the ratio of signal intensity for the target gene relative to β-actin. all the determinations were performed in duplicate.

Statistical Analysis

Statistical analysis was conducted using the statistical package for Social Science (SPSS) version 23. All results are presented as the means ± SD (standard deviation) and $p < 0.05$ was considered statistically significant.

Results

For both genders, the age ranged from 4 to 15 years. More than half of the parents felt they had sufficient money. Thirty eight percent of the sample was overweight (BMI > 25 kg/m2). Daily food consumption was similar for both genders Table 2.

Table 2: Socio demographic and lifestyle characteristics and food intake by gender

Variable	Females		Males	
	N	%	N	%
	37	54%	31	46%
Age Group				
4 – <10	15	22%	18	27%
10 – 15	22	32%	13	19%
Economic situation (income)				
Mostly sufficient	22	32.50%	20	29.50%
Mostly insufficient	14	21%	11	16.50%
More than enough	1	0.50%	0	0%
BMI				
Below normal (<18.5kg/m^2)	10	15%	8	12.50%
Normal (18.5–25 kg/m^2)	12	17%	12	17%
Overweight (>25 kg/m^2)	15	22%	11	16.50%
Food frequency questionnaire (FFQ)				
Sweets	36	53%	30	44.50%
Cake, cookies	33	48.50%	29	43%
Snacks	34	50%	20	29%
Fresh fruits	34	50%	20	29%
Salad, raw vegetables	30	44.50%	18	26.50%
Cooked vegetables	29	43%	17	25%
Fast food, canned food	31	46%	30	44.50%
Lemonade, soft drinks	20	29%	13	19%
Meat, sausages	29	43%	27	40%
Fish, sea food	20	29%	20	29%
Milk and milk products	37	54%	30	44.50%
Cereals and their products				

Saturated fat intake was correlated with both monounsaturated fat (r = 0.73) and trans-fat (r = 0.5) intake, but not with polyunsaturated fat (r = 0.01). Monounsaturated fat intake was correlated with both trans-fat (r = 0.57) and polyunsaturated fat (r = 0.5) intake. Polyunsaturated fat intake was correlated with trans-fat (r = 0.57) intake only. Common daily dietary sources could be the cause of this high correlation between monounsaturated fatty acids and saturated fatty acids.

On average, Total fat consumption was about 18.5 ± 0.7% of the total calories. Mean reported

energy with trans-fat contributes to ≥ 2.3± 0.9% of the total calories and fat intake were similar for males and females, although the range of intake was more among the females.

Table 3: mRNA expression in relation to Trans Fat Consumption

	High Trans fat consuming	Medium Trans fat consuming	p
UCP2 mRNA expression	2.5 ± 0.7	1.5 ± 0.2	< 0.001
UCP3 mRNA expression	2.1 ± 0.5	1.9 ± 0.2	0.08

Effect of the Trans-Fat Content of Diet on UCP2 and UCP3 mRNA Expression

UCP2 mRNA expression was lower in the High Trans-fat consuming group compared to Medium Trans-fat consuming one (2.5 ± 0.7 vs. 1.5 ± 0.2, p < 0.001). However, UCP3 mRNA expression was not significantly different between both groups (2.1 ± 0.5 vs. 1.9 ± 0.2, P=0.08) Table 3.

In relationship between Delta (= mRNA expression on Medium Trans-fat consuming group minus High Trans-fat consuming one) we found that the diet-induced changes (delta) in mRNA expression of UCP2 (r = 0.66, P = 0.002) and UCP3 (r = 0.61, P = 0.06) mRNA expression (ratio with β-actin) were negatively correlated with percentage of Trans fat in diet.

Discussion

The major sources of trans fats are meat or dairy products if we considered natural TFA. On the other hand, margarines, deeply fried fast foods, commercially baked products, packaged snack foods and other prepared foods are rich with artificial TFA.[2,3] Increased consumption of trans fats has been associated with increased risk of Coronary Heart Disease (CHD) morbidity and mortality.[4,5] Moreover, high TFA has been identified as a risk factor for obesity.[6,7] In most countries, large numbers of children and adolescents suffer from overweight or obesity.[11]

Uncoupling proteins (UCPs) are mitochondrial proteins, which act by increasing the permeability of the inner mitochondrial membrane to protons returning them back into the mitochondrial matrix without energy production from the Electron Transport Chain.[13] There are three isoforms discovered: UCP-1, 2 and 3. UCP-1 expression is increased following cold exposure and overeating.[13] Uncoupling proteins types 2 and 3 are suggested to be involved in energy metabolism and obesity development.[22] UCP2 is expressed in most of human tissues. Initially, a lot of studies had reported the role of UCP2 in the regulation of energy homeostasis and metabolism.[23] Moreover, the UCP2 expression has been linked to obesity, metabolic syndrome and basal metabolic rate alterations.[24,25] Vidal-Puig, A. J. concluded that; UCP2 and UCP3 are not thermogenic to UCP1 degree.[26] Moreover, UCP2 and UCP3 genes expression was detected to be as 1000-fold less than UCP1 expression.[27] Activation of all UCP types requires fatty acids.[28] Schrauwen P and Hesselink MK suggested that UCP3 transport fatty acids out of mitochondria thus protecting the mitochondria from the toxic effects of fatty acid peroxidation and trans fats.[29]

Previous study had investigated the relation between UCP2 and UCP3 expression and fasting.[30] Based on the central role of the UCP2 and UCP3 in metabolism, in the present study we examined the effect of the Trans-fat food consumption on the expression of UCP2 and UCP3 mRNA in children.

The levels of UCP2 mRNA expression was lower in the High Trans-fat consuming children (2.5 ± 0.7) compared to Medium Trans-fat consuming ones (1.5 ± 0.2) with p value (< 0.001). However, UCP3 mRNA expression levels didn't show that much significance as the values were (2.1 ± 0.5) in the High Trans-fat consuming group. While showed (1.9 ± 0.2) Medium Trans-fat consuming one with p value (0.08). In relationship between Delta we found that the diet-induced changes in mRNA expression of UCP2 (r = 0.66, P = 0.002) and UCP3 (r = 0.61, P = 0.06) mRNA expression (ratio with β-actin) were negatively correlated with percentage of Trans fat in diet.

Rodríguez E *et al* showed similar findings and reported that Trans fatty acids inhibited uncoupling protein (UCP) 1 induction by norepinephrine (NE), decreased the leptin mRNA levels and caused defective brown adipose tissue (BAT) thermogenesis.[31] Machado RM *et al* research results showed the role of trans fatty acid consumption in the development of the key features of metabolic syndrome.[32] Jeyakumar SM *et al* concluded that the chronic consumption of a TFA-rich diet significantly impaired insulin sensitivity, which is a main characteristic for metabolic syndrome.[33] Zhao X *et al* research results support our findings as they concluded that; consumption of high trans-fatty acids diet induces higher rates of obesity and insulin resistance.[34]

In the same line of our data, Siagian CM results showed that high TFA intake without concurrent increase in calories caused an increase in intra-abdominal fat deposit and defective insulin sensitivity.[35] In cooperation with Tjahjono DK, Siagian CM has emphasized his results in another study.[36] Also, in agreement with our results, West DB *et al* reported that administration of conjugated linoleic acids (CLA) down regulates UCP2 expression.[37] However, other studies have observed that a high fat diet influence UCP3 mRNA expression especially in skeletal muscle.[38, 39] Conversely, substitution with a low-fat diet was found to reduce UCP2 expression and increase ROS production.[40] This could be attributed to correlating with total fat in diet including saturated, monounsaturated and polyunsaturated fats and not only with trans-fat consumption. And the effects on metabolism and gene expression depend on the type and source of lipids present in the diet and could be attributed to energy expenditure and adipose tissue metabolism, such as lipoprotein lipase, lipogenesis, and lipolysis activities.

Putting these findings together, indicate that the increased levels of trans fat consumption in diet affects the levels of UCP2 and UCP3 mRNA expression which in turn affects their levels. These effects are expected to have direct relation with obesity and its comorbidities. Our findings should have implications for additional clinical and laboratory research.

Conclusion

The levels of UCP2 and UCP3 mRNA expressions are reduced by high Trans-fat content in diet. This down regulation is more significant on UCP2. This gives a possible mechanism by which Trans-fat leads to obesity and metabolic syndrome in children.

Recommendations

Presenting the trans-fat content in the food composition tables for Egypt is required. And further studies with higher subject number with more demographic correlation is recommended.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Conflict of Interest

The authors declare that they have no competing interests.

References

1. Nishida, C., & Uauy, R. (2009). WHO Scientific Update on health consequences of trans fatty acids: introduction. *European journal of clinical nutrition,* 63(S2), S1.
2. Bhardwaj, S., Passi, S. J., &Misra, A. (2011). Overview of trans fatty acids: biochemistry and health effects. Diabetes & Metabolic Syndrome: *Clinical Research & Reviews,* 5(3), 161-164.
3. Mozaffarian, D., Katan, M. B., Ascherio, A., Stampfer, M. J., & Willett, W. C. (2006). Trans fatty acids and cardiovascular disease. *New England Journal of Medicine*, 354(15), 1601-1613.
4. World Health Organization. (2003) Diet, Nutrition and the Prevention of Chronic Diseases: Report of a Joint WHO/FAO Expert Consultation; World Health Organization: Geneva, Switzerland. Available online: http://whqlibdoc. who.int/trs/WHO_TRS_916.pdf (accessed on 1 February 2018).
5. Wang, Q., Afshin, A., Yakoob, M. Y., Singh, G. M., Rehm, C. D., Khatibzadeh, S., ... &Mozaffarian, D. (2016). Impact of nonoptimal

intakes of saturated, polyunsaturated, and trans-fat on global burdens of coronary heart disease. *Journal of the American Heart Association*, 5 (1), e002891.

6. Mozaffarian, D., Aro, A., & Willett, W. C. (2009). Health effects of trans-fatty acids: experimental and observational evidence. *European journal of clinical nutrition,* 63(2), S5-S21.
7. Thompson, A. K., Minihane, A. M., & Williams, C. M. (2011). Trans fatty acids and weight gain. *International journal of obesity,* 35(3), 315-324.
8. World Health Organization. (2009). Global Health Risks: Mortality and Burden of Disease Attributable to Selected Major Risks; World Health Organization: Geneva, Switzerland.
9. Kleber, M. E., Delgado, G. E., Lorkowski, S., März, W., & von Schacky, C. (2016). Trans-fatty acids and mortality in patients referred for coronary angiography: the Ludwigshafen Risk and Cardiovascular Health Study. *European heart journal,* 37(13), 1072-1078.
10. World Health Organization. (2014). Global Status Report on Noncommunicable Diseases 2014; World Health Organization: Geneva, Switzerland.
11. Alwan, A., McColl, K., Al-Jawaldeh, A., & World Health Organization. (2017). Proposed policy priorities for preventing obesity and diabetes in the Eastern Mediterranean Region.
12. World Health Organization. Global Observatory (GHO) Data. Available online: http://www.who.int/gho/ en/ (accessed on 10 May 2018).
13. Busiello, R. A., Savarese, S., & Lombardi, A. (2015). Mitochondrial uncoupling proteins and energy metabolism. *Frontiers in physiology,* 6, 36.
14. Azzu, V., Jastroch, M., Divakaruni, A. S., & Brand, M. D. (2010). The regulation and turnover of mitochondrial uncoupling proteins. *Biochimicaet Biophysica Acta (BBA)- Bioenergetics,* 1797(6-7), 785-791.
15. Brand, M. D., & Esteves, T. C. (2005). Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. *Cell metabolism,* 2(2), 85-93.
16. Joseph, J. W., Koshkin, V., Saleh, M. C., Sivitz, W. I., Zhang, C. Y., Lowell, B. B., ... & Wheeler, M. B. (2004). Free fatty acid-induced β-cell defects are dependent on uncoupling protein 2 expression. *Journal of Biological Chemistry,* 279(49), 51049- 51056.
17. Brand, M. D., Affourtit, C., Esteves, T. C., Green, K., Lambert, A. J., Miwa, S., ... & Parker, N. (2004). Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radical Biology and Medicine,* 37(6), 755-767.
18. National Nutrition Institute (2006) Food Composition Tables for Egypt. 2nd Edition, ARE, Cairo, 119.
19. Roe, M., Church, S., Pinchen, H., &Finglas, P. (2013). Nutrient Analysis of a Range of Processed Foods with Particular Reference to Trans Fatty Acids. *Technical report.*
20. Costa, N., Cruz, R., Graça, P., Breda, J., &Casal, S. (2016). Trans fatty acids in the Portuguese food market. *Food control,* 64, 128-134.
21. Sadek, M. A. S., Daoud, J. R., Ahmed, H. Y., &Mosaad, G. M. (2018). Nutritive value and trans fatty acid content of fast foods in Qena city, Egypt. *Nutrition & Food Science.*
22. Jabůrek, M., Vařecha, M., Gimeno, R. E., Dembski, M., Ježek, P., Zhang, M., ... &Garlid, K. D. (1999). Transport function and regulation of mitochondrial uncoupling proteins 2 and 3. *Journal of Biological Chemistry*, 274(37), 26003-26007.
23. Myara, I., Alamowitch, C., Michel, O., Heudes, D., Bariety, J., Guy-Grand, B., & Chevalier, J. (2003). Lipoprotein oxidation and plasma vitamin E in nondiabetic normotensive obese patients. *Obesity research,* 11(1), 112-120.
24. Shen, H., Qi, L., Tai, E. S., Chew, S. K., Tan, C. E., & Ordovas, J. M. (2006). Uncoupling protein 2 promoter polymorphism− 866G/A, central adiposity, and metabolic syndrome in Asians. *Obesity,* 14(4), 656-661.
25. Bulotta A., Ludovico O., Coco A., Di Paola R., Quattrone A., Carella M., Pellegrini F., Prudente S. &Trischitta V. (2005). The common K866G/A polymorphism in the promoter region of the UCP-2 gene is associated with reduced risk of type 2 diabetes in Caucasians from Italy. *Journal of Clinical Endocrinology and Metabolism* 90 1176– 1180. (doi:10.1210/ jc.2004-1072)
26. Vidal-Puig A.J. (2000) Uncoupling expectations *naturegenetics,*

26(4), 387-388.

27. Pecqueur C., Alves-Guerra M. C., Gelly C., Levi-Meyrueis C., Couplan E., Collins S., Ricquier D., Bouillaud F. & Miroux B. (2001). Uncoupling protein 2, *in vivo* distribution, induction upon oxidative stress, and evidence for translational regulation. *J Biol Chem.* 276 (12): 8705-8712. 10.1074/jbc.M006938200.
28. Esteves T. C. & Brand M. D. (2005). The reactions catalysed by the mitochondrial uncoupling proteins UCP2 and UCP3. *Biochim Biophys Acta.* 1709 (1): 35-44. 10.1016/j.bbabio.2005.06.002.
29. Schrauwen P. &Hesselink M. K. (2004). The role of uncoupling protein 3 in fatty acid metabolism: protection against lipotoxicity?. *Proc Nutr Soc.* 63 (2): 287-292. 10.1079/PNS2003336.
30. Samec S., Seydoux J. &Dulloo A. G. (1999). Post-starvation gene expression of skeletal muscle uncoupling protein 2 and uncoupling protein 3 in response to dietary fat levels and fatty acid composition: a link with insulin resistance. *Diabetes.* 48 (2): 436-441.
31. Rodríguez, E., Ribot, J., & Palou, A. (2002). Trans-10, cis-12, but not cis-9, trans-11 CLA isomer, inhibits brown adipocyte thermogenic capacity. *American Journal of Physiology-Regulatory*, Integrative and Comparative Physiology, 282(6), R1789-R1797.
32. Machado, R. M., Stefano, J. T., Oliveira, C. P., Mello, E. S., Ferreira, F. D., Nunes, V. S., ... & Lottenberg, A. M. P. (2010). Intake of trans fatty acids causes nonalcoholic steatohepatitis and reduces adipose tissue fat content. *The Journal of nutrition*, 140(6), 1127-1132.
33. Jeyakumar, S. M., Prashant, A., Rani, K. S., Laxmi, R., Vani, A., Kumar, P. U., & Vajreswari, A. (2011). Chronic consumption of trans-fat-rich diet increases hepatic cholesterol levels and impairs muscle insulin sensitivity without leading to hepatic steatosis and hypertriglyceridemia in female Fischer rats. *Annals of Nutrition and Metabolism*, 58(4), 272-280.
34. Zhao, X., Shen, C., Zhu, H., Wang, C., Liu, X., Sun, X., ... & Hu, K. (2016). Trans-fatty acids aggravate obesity, insulin resistance and hepatic steatosis in C57BL/6 mice, possibly by suppressing the IRS1 dependent pathway. *Molecules,* 21(6), 705.
35. Siagian, C. M. (2015). Trans Fatty Acid: TFA Implication: Does it helpful or harmful?. *International Journal of Sciences: Basic and Applied Research* (IJSBAR), 23(1), 69-79.
36. Siagian, C. M., & Tjahjono, D. K. (2015). Weight Gain and Blood Glucose Level in Granting Trans Fatty Acids (Study at Sprague Dawley). *International Journal of Sciences: Basic and Applied Research* (IJSBAR), 23(1), 294-307.
37. West, D. B., Blohm, F. Y., Truett, A. A., & DeLany, J. P. (2000). Conjugated linoleic acid persistently increases total energy expenditure in AKR/J mice without increasing uncoupling protein gene expression. *The Journal of nutrition*, 130(10), 2471-2477.
38. Gong D. W., He Y. & Reitman M. L. (1999). Genomic organization and regulation by dietary fat of the uncoupling protein 3 and 2 genes. *Biochem Biophys Res Commun*. 256 (1): 27-32. 10.1006/bbrc.1999.0239.
39. Hesselink M. K., Greenhaff P. L, Constantin-Teodosiu D., Hultman E., Saris W. H., Nieuwlaat R., Schaart G., Kornips E. &Schrauwen P. (2003). Increased uncoupling protein 3 content does not affect mitochondrial function in human skeletal muscle *in vivo*. *J Clin Invest.* 111 (4): 479-486. 10.1172/JCI200316653.
40. Sullivan P. G., Dube C., Dorenbos K., Steward O. & Baram T. Z. (2003). Mitochondrial uncoupling protein-2 protects the immature brain from excitotoxic neuronal death. *Ann Neurol.* 53 (6): 711-717. 10.1002/ana.10543.