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Choline reduces the hepatocyte nuclear factor-4α (HNF-4α) in HepG2 cells

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Choline is a semi-essential nutrient that is required to make main membrane phospholipids including phosphatidylcholine and sphingomyeline. The liver is the most important organ for metabolism dietary choline and phosphatidylcholine. The liver is enriched transcription factors of genes that are preferentially expressed in this tissue. Hepatocyte nuclear factor-4α (HNF-4α) is known as a master regulator of liver-specific gene expression. The activity of HNF-4α may be regulated by dietary components. In this study, to investigate the effects of choline and lecithin on HNF-4α expression, the HepG2 cells were treated with several concentrations of these compounds, including concentration of 11.25μM, 22.5μM, 35μM for choline and 0.23 mg/ml and 0.46 mg/ml for lecithin, at three time intervals 6, 12 and 24 hours. Then expression of HNF-4α at mRNA level was assessed by real-time PCR. The results showed a 50% decrease in HNF-4α mRNA levels when HepG2 cells were treated with choline chloride at concentration 35μM for 24 hours.

Keywords: Phosphatidylcholine, Real-time PCR, gene expression, DNA methylation, lecithin

INTRODUCTION

Choline is a semi-essential nutrient that is found in dietary sources such as egg yolk, meat, liver, nuts and soybean. It functions as a methyl group donor [1-2] and is involved in many physiological processes, including metabolism and transport of lipids, methylation reactions, and neurotransmitter synthesis [1]. The majority of choline in our body is found in phospholipids such as phosphatidylcholine and sphingomyelin. Phosphatidylcholine (lecithin) is the most abundant choline species in mammalian tissues that accounts for 95% of the total choline pool [2-3]. It is a major structural component of membrane bilayers and lipoproteins, and thereby it participates in signaling and transport across membranes [3-5]. Phoaphatidylcholine is the important choline metabolite that is necessary for packaging and export of triglycerides in VLDL (very low density lipoprotein) and solubilization of bile salts for secretion [6-7]. Liver is an important site for choline metabolism and storage and also, it requires choline for proper function, so that choline deficiency causes abnormal deposition of fat in the liver and results in fatty liver disease [6]. In human, choline deprivation causes liver cell death and Hepatosteatosis. The spectrum of effects choline in the liver and several mechanisms for these effects has been identified [8-10].

The liver is enriched transcription factors of genes that are preferentially expressed in this tissue. Hepatocyte nuclear factor-4α (HNF-4α) is a highly conserved member of the nuclear receptor superfamily that regulates the expression of genes involved in fatty acid, cholesterol and glucose metabolism [11-13]. The study with liver-specific HNF-4α knockout (KO) has shown that inactivation of HNF-4α leads to abnormal deposition glycogen and of lipid in the liver. Lipid accumulation and fatty liver phenotype in this animal are indicated the role of HNF-4α in the regulation of genes involved in fatty acid metabolism [11, 14]. It can influence the levels and activity of HNF-4α but it is not known if HNF-4α may be affected by choline, as an endogenous factor and also a component of dietary. Thus, the objective of this study was an investigation of the effects of choline and also lecithin on HNF-4α expression.

MATERIALS AND METHODS

Cell culture

HepG2 cells were prepared from the Stem cell Research Center of Kerman University of Medical Sciences. After thawing and cell recovery in Dulbecco's Modified Eagle Medium (DMED) containing fetal bovine serum (at ratio 1:10 as 1ml from thawed cells and 9ml medium), the cells were cultured in DMEM supplemented with 10% of FBS and 1.5% of penicillin/streptomycin and were maintained at 5% CO2 and temperature of 37°C. The medium was changed every 3-4 days. Each T-25 flask of Cultures was split (1:4) when it reached to 80–90% Confluency using 1ml trypsin/EDTA (0.05% trypsin, 0.5mM EDTA). For treatment, cells were plated at a seeding density of 1.5-2×10^6 cells

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per each 60 mm petri dish in medium and after 24 hours, the medium was replaced with medium containing choline (11.25µM, 22.5µM, 35µM) or lecithin (0.23 mg/ml and 0.46 mg/ml) and the cells were incubated at 37°C and 5% of CO2 for 6, 12 or 24 hours. At the end of treatment period, the cells were washed twice with phosphate buffered saline (PBS) and then were detached by scraping in Tripure reagent (Roche applied science, Switzerland) for RNA extraction. The samples were frozen at -80 °C until RNA isolation could be carried out.

RNA extraction

Total RNA was extracted from HepG2 cells using Trizol reagent (Roche applied science, Switzerland) according to the manufacturer's instructions. The quality and integrity of RNA were checked by electrophoresis on 1% agarose gel. The concentration of total RNA was quantified spectrophotometrically at 260 nm using a ND-1000 nanodrop (Thermo Scientific).

Reverse Transcription and Real-time PCR

First-strand cDNA was synthesized from 1µg of total RNA using Revert Aid First Strand cDNA synthesis kit (Thermo Scientific, USA) according to the manufacturer's protocol. In brief, the reverse transcription reaction mixture was prepared in a final volume of 20µl containing 4µl reaction buffer (5X), 2µl dNTP mix (10mM), 1µl random Hexamer (100µM), total RNA (1µg) and nuclease-free water. Thermal program for cDNA synthesis was as follows: first step incubation at 25 °C for 5 min, then one cycle incubation at 42 °C for 60 min that terminated by heating at 70 °C for 5min. The cDNAs were stored at -20 °C until use. Real time PCR were performed with 3µl cDNA and specific primers (Table 1) for HNF-4α(hepatocyte nuclear factor-4α), ChDH (Choline dehydrogenase), BHMT1 (Betaine-homocysteine methyltransferase1) genes and HPRT (Hypoxanthine phosphoribosyl transferase) gene as a reference gene using 2µl from RealQ Plus 2x Master Mix Green, high ROX (Ampliqon, Denmark), 1µl forward and 1µl revers primers (500nM), 3µl cDNA and water to final volume of 20µl in StepOne Real-time PCR System (Applied Biosystems). The thermal conditions were used as follows: initial incubation at 95 °C for 5min, followed by 40 cycles at 95 °C for 10 sec, annealing temperature (55-58 °C) for 30 sec and 72°C for 30sec. The relative levels of mRNAs were calculated by Delta-Delta CT ($2^{\Delta\Delta Ct}$) method as $\Delta\Delta Ct = (CT\ target\ gene - Ct_{HPRT})_{treated} - (Cttarget_{gene} - Ct_{HPRT})_{control}$ [15] that target genes are HNF-4α, ChDH and BHMT1 and HPRT is an internal reference gene.

Table 1. The primer sequences used in real-time PCR experiments

Gene symbol	Forward Primer	Reverse Primer	Product size (bp)	Accession numbers	Reference
HNF-4α[a]	5' TGCCTACCTCAAAGCCATC 3'	5'ATGTAGTCCTCCAAGCTCA C 3'	111	NM_000457.4	Designed
BHMT1[b]	5' CGTGGACTTCTTGATTGCAG 3'	5'AATCTCCTTCTGGGCCAAT G 3'	122	NM_001713.2	[21]
CHDH[c]	5' GCAAGGAGGTGATTCTGAGTG G 3'	5'GGATGCCCAGTTTCTTGAG GTC 3'	99	XM_017006799	[21]
HPRT[d]	5' GACCAGTCAACAGGGGACAT 3'	5'GTCCTTTTCACCAGCAAGC T 3'	182	XM_011531328	[23]

[a] Hepatocyte nuclear factor-4α, [b] Betaine-Homocysteine methyltransferase1, [c] Choline dehydrogenase, [d]Hypoxanthine phosphoribosyl transferase.

Statistical analysis

Statistical analysis was performed using SPSS 22 software. To investigate of changes of HNF-4α mRNA levels in treatment groups (choline chloride, lecithin and control) we used one way ANOVA test that followed by post hoc Scheffe test. Comparison of ChDH and BHMT1 mRNA levels between treated cells with choline chloride and control group was done with an unpaired t-test. The statistical significance was considered at $P < 0.05$.

RESULTS AND DISCUSSION

Nutritious diet has been effective to possess various biological activities such as antimutagenic, hypolipidaemic, antioxidant, antitumour, anti-fungal and hepatoprotective [16]. To investigate the effects of choline and lecithin on HNF-4α expression, the human hepatoma cell line, HepG2, was used. The HepG2 cell line is similar to hepatocytes in term of biological responsiveness. In addition, these cells are relatively easier than hepatocytes to maintain in

culture so that they are used widely for in vitro studies [12, 17]. In this study, HepG2 cells were treated with choline chloride and L-α-lecithin and then relative levels of HNF-4α mRNA were measured by real-time PCR. The results were shown that choline chloride exposure decreases mRNA levels of HNF-4α. Different concentrations of choline chloride reduced the expression of mRNA in a dose-dependent manner.

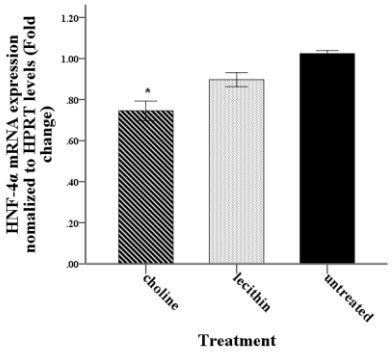

Fig. 1. The expression of HNF-4α mRNA in HepG2 cells after treatment with choline chloride and lecithin. The relative mRNA levels of HNF-4α were measured using SYBR Green quantitative real-time PCR and according to ΔΔCt method using HPRT as an endogenous control. Data analysis was performed by one-way ANOVA followed by post hoc Scheffe test. n=4; error bars represent mean ± SEM. * P <0.002.

Figure1 shows the expression of HNF-4α mRNA in HepG2 cells after treatment with choline chloride and lecithin, and concentration effects of choline on mRNA levels of HNF-4α were shown in Figure 2. As it can be seen, the concentrations of 11.25 µM, 22.5 µM and 35 µM from choline had reduced HNF-4α mRNA to 10, 20, and 50%, respectively that it was only statistically significant at concentration 35 µM. The change of HNF-4α mRNA levels in 35 µM concentration from choline chloride was investigated at three time intervals of 6, 12, and 24 hour treatment. The expression of HNF-4α mRNA decreased after 12 and 24 hours of exposure to choline chloride that it was only significant after 24 hours It was shown in Figure 3.

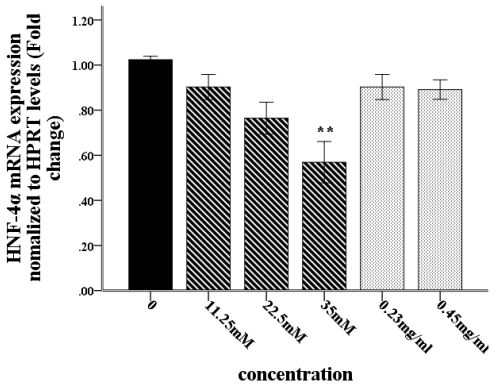

Fig. 2. The concentration effects of choline chloride and L-α-lecithin on mRNA levels of HNF-4α. The HepG2 cells were treated with Choline chloride at three concentrations (11.25, 22.5 and 35 µM) and L-α-lecithin at two concentrations (0.23 and 0.46 mg/ml). The relative mRNA levels HNF-4α were quantified using of real-time PCR. Changes of HNF-4α mRNA expression were only significant at 35µM of choline chloride. Data represents mean ± SEM from four independent cultures **P<0.0001.

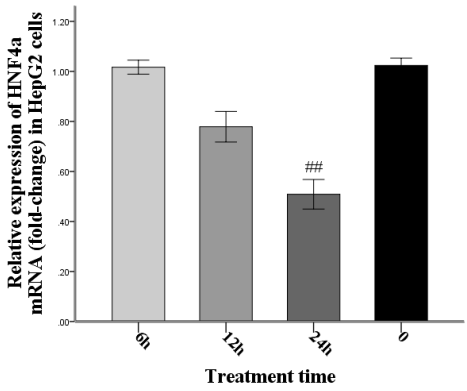

Fig. 3. The change of mRNA levels of HNF-4α after exposure to 35µM concentration of choline chloride at three interval times 6, 12, and 24h. A decrease of HNF-4α expression was approximately 20% at 12 hour treatment and that was 50% at 24 hours and it was only significant at the letter. Data represents mean ± SEM from four independent cultures, ## P <0.05.

Previous studies have been shown that diet can influence the levels and hence the activity of HNF-4α. For example, despite of that HNF-4α is known as a nuclear receptor that does not require the addition ligand in order to activation [18-19], it is found that in livers of mice, linoleic acid (C18:2 ω6) is bound to HNF-4α [19]. Choline is an essential nutrient for proper function of nearly every cell. It contributes to epigenetic modification via its role as a source of methyl-groups [20]. For this purpose, choline is first oxidized to betaine by choline dehydrogenase (ChDH) enzyme and then betaine denoted its methyl group to homocysteine for

remethylation to methionine, that this reaction is catalyzed by betaine-homocysteine methyltransferase1 (BHMT1).

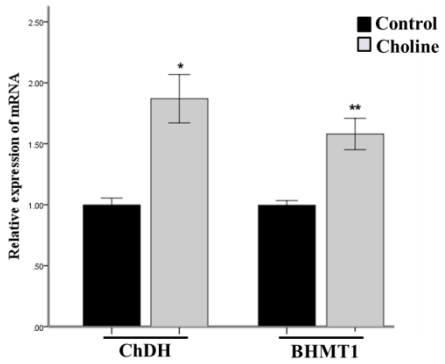

Fig. 4. The expression of ChDH (choline dehydrogenase) and BHMT1 (Betaine-Homocysteine methyltransferase1) mRNA in HepG2 cells after treatment with choline chloride at 35µM concentration and 24 hours exposure. An unpaired t-test analysis was used to compare mean differences between treatment and control groups. Data represents mean±SEM from four independent cultures. * P <0.0001 and ** P <0.003.

Finally, methionine is converted to methyl donor S-adenosylmethionine and it is ready for methylation reactions [21-22]. The study of Jiang et al. (2016) has been shown that in the HepG2 cells, choline supplementation increases global DNA methylation. They also observed overexpression of ChDH and BHMT1 enzymes at mRNA level after choline exposure [21]. In the present study, we assessed mRNA expression of enzymes ChDH and BHMT1 by real-time PCR. Figure 4 indicates expression of ChDH and BHMT1 mRNA after utilization of choline chloride at 35 µM concentration for 24 hours. As it can be seen, choline exposure increased mRNA levels of both enzymes. This observation is consistent with findings of Jiang X et al. (2016). It is possible that choline treatment caused DNA methylation in HepG2 cell and hence it changed HNF-4α expression [23]. However, we did not investigate that whether the reduction in HNF-4α is resulted from up-regulation of DNA methylation specific genes (such as ChDH and BHMT1 genes that increased) in HepG2 cells. The decrease of HNF-4α expression may influence on cell function and expression a large number of genes, because HNF-4α is the nuclear receptor that exerts its function via both direct transcriptional effects on its target genes and indirectly with positive or negative regulation of other transcription factors, each of which regulates numerous downstream targets [12]. The study by Wang et al (2011) has indicated that when HNF-4α expression is suppressed in HepG2 cells by the technique of RNA interference, a large number of genes (approximately 3088 of genes) are up-regulated. Although, this study did not explain about the underlying causes, but, their observations suggest that HNF-4α directly or indirectly regulates many liver specific genes [12].

Another study on mice with disrupted HNF-4α gene in liver, has indicated that HNF4a is central to the maintenance of hepatic function and it is a major in vivo regulator of genes involved in the control of lipid homeostasis, so that the mice lacking hepatic HNF-4α display decreased expression of essential gene for VLDL (very low density lipoprotein) secretion and they have accumulation of lipid in hepatocytes [11]. In the present study, the decrease of HNF-4α mRNA level was observed after treatment of HepG2 cells with choline. Considering the above study [11], it can be concluded that choline may cause lipid accumulation in liver, but this effect has not been observed so far. Further studies are required for understanding the mechanisms by which choline influence on liver function, and also to know how decreased HNF-4α expression affects the liver.

CONCLUSION

The conclusion that choline as a semi-essential nutrient, is able to change gene expression. Choline is mainly metabolized in the liver. The present study showed that choline affects the expression of hepatic specific transcription factor, HNF-4α. The possible mechanism by which choline reduces HNF-4α expression may involve increased DNA methylation that it should be investigated by further experiments. Up-regulation of genes involved in formation of active methyl group, ChDH and BHMT1, can suggest the possibility of DNA methylation.

REFERENCES

1. J. W. E. Jr, I. A. Macdonald, S. H. Zeisel, Present Knowledge in Nutrition. Choline, John Wiley & Sons, Inc, (2012).
2. S. V. Konstantinova, G. S. Tell, S. E. Vollset, O. Nygard, O. Bleie, P. M. Ueland, *J. Nutr.*, **138**, 914 (2008).
3. P. M. Ueland, *J. Inherit. Metabo. Dis.*, **34**, 3 (2011).
4. C. Kent, P. Gee, S. Y. Lee, X. Bian, J. C. Fenno, *Mol. Microbiol.*, **51**, 471 (2004).
5. S. Robertson, Choline chloride, SIDS Initial Assessment Report For SIAM 19. UNEP PUBLICATIONS, (2004) .
6. K. D. Corbin, S. H. Zeisel, *Curr. Opin. Gastroen.*, **28**, 159 (2012).
7. Z. Li, L. B. Agellon, D. E. Vance, *J. Biol. Chem.*, **280**, 37798 (2005).

8. A. R. Johnson, C. N. Craciunescu, Z. G. Teng, R. J. Thresher, J. K. Blusztajn, *FASEB J.*, **24**, 2752 (2010).
9. A. A. Noga, Y. Zhao, D. E. Vance, *J. Biol. Chem.*, **277**, 42358 (2002).
10. K. A. Waite, N. R. Cabilio, D. E. Vance, *J. Nutr.*, **132**, 68 (2002).
11. G. P. Hayhurst, Y. -H. Lee, G. Lambert, J. M. Ward, F. J. Gonzalez, *Mol. Cell Biol.*, **21**, 1393 (2001).
12. Z. Wang, E. P. Bishop, P. A. Burke, *BMC Genomics.*, **12**, 1 (2011).
13. C. A. Wiwi, M. Gupte, D. J. Waxman, *Mol. Endocrinol.*, **18**, 1975 (2004).
14. C. P. Martinez-Jimenez, I. Kyrmizi, P. Cardot, F. J. Gonzalez, I. Talianidis, *Mol. Cell Biol.*, **30**, 565 (2010).
15. A. Mohammadi, M. R. Baneshi, Z. Vahabzadeh, T. Khalili, *Biosci. Biotechnol. Res. Asia*, **13**, 1797 (2016).
16. C. Ge, H. Radnezhad, M. F. Abari, M. Sadeghi, G. Kashi, *Appl. Ecol. Env. Res.*,**14**, 547 (2016).
17. L. Guo, S. Dial, L. Shi, W. Branham, J. Liu, J. L. Fang, *Drug Metab. Dispos.*, **39**, 528 (2011).
18. W. W. Hwang-Verslues, F. M. Sladek, *Curr. Opin. Pharmacol.*, **10**, 698 (2010).
19. X. Yuan, T. C. Ta, M. Lin, J. R. Evans, Y. Dong, E. Bolotin, *PloS one*, **4**, e5609 (2009).
20. S. Lao, The University Of North Carolina At Chapel Hill, p. 42 (2013).
21. X. Jiang, E. Greenwald, C. Jack-Roberts, *Nutr. metab. insights*, **9**, 11 (2016).
22. Y. W. Teng, M. G. Mehedint, T. A. Garrow, S. H. Zeisel, *J. Biol. Chem.*, **286**, 36258 (2011).
23. J. Zhang, J. R. Cashman, *Drug Metabl. Dispos.*, **34**, 19 (2006).