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doi:10.3748/wjg.14.3891

World J Gastroenterol 2008 June 28; 14(24): 3891-3896
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Change of choline compounds in sodium selenite-induced apoptosis of rats used as quantitative analysis by *in vitro* 9.4T MR spectroscopy

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Supported by The National Natural Science Foundation of China, No. 30570480
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Received: February 2, 2008 Revised: April 30, 2008
Accepted: May 7, 2008
Published online: June 28, 2008

Abstract

AIM: To study liver cell apoptosis caused by the toxicity of selenium and observe the alteration of choline compounds using *in vitro* 9.4T high resolution magnetic resonance spectroscopy.

METHODS: Twenty male Wistar rats were randomly divided into two groups. The rats in the treatment group were intraperitoneally injected with sodium selenite and the control group with distilled water. All rats were sacrificed and the livers were dissected. ^{1}H-MRS data were collected using *in vitro* 9.4T high resolution magnetic resonance spectrometer. Spectra were processed using XWINNMR and MestRe-c 4.3. HE and TUNEL staining was employed to detect and confirm the change of liver cells.

RESULTS: Good ^{1}H-MR spectra of perchloric acid extract from liver tissue of rats were obtained. The conventional metabolites were detected and assigned. Concentrations of different ingredient choline compounds in treatment group *vs* control group were as follows: total choline compounds, 5.08 ± 0.97 mmol/L *vs* 3.81 ± 1.16 mmol/L (P = 0.05); and free choline, 1.07 ± 0.23 mmol/L *vs* 0.65 ± 0.20 mmol/L (P = 0.00). However, there was no statistical significance between the two groups. The hepatic sinus and cellular structure of hepatic cells in treatment group were abnormal. Apoptosis of hepatic cells was confirmed by TUNEL assay.

CONCLUSION: High dose selenium compounds can cause the rat liver lesion and induce cell apoptosis *in vivo*. High resolution ^{1}H-MRS *in vitro* can detect diversified metabolism. The changing trend for different ingredient of choline compounds is not completely the same at early period of apoptosis.

Key words: Apoptosis of liver cell; Choline compounds; Sodium selenite; *In vitro* ^{1}H-MRS; Quantitative analysis

Peer reviewers: Eva Herrmann, Professor, Saarland University, Kirrberger Street, Homburg/Saar 66421, Germany; Natalia A Osna, Liver Study Unit, Research Service (151), VA Medical Center, 4101 Woolworth Avenue, Omaha NE 68105, United States

Cao Z, Wu LP, Li YX, Guo YB, Chen YW, Wu RH. Change of choline compounds in sodium selenite–induced apoptosis of rats used as quantitative analysis by *in vitro* 9.4T MR spectroscopy. *World J Gastroenterol* 2008; 14(24): 3891-3896 Available from: URL: http://www.wjgnet.com/1007-9327/14/3891.asp DOI: http://dx.doi.org/10.3748/wjg.14.3891

INTRODUCTION

Apoptosis is a programmed, active, highly selective mechanism of cell death. Multicellular organisms' apoptosis is an essential component of cellular regulation. Abnormal regulation of apoptosis can lead to disorders such as cancer[1,2].

The field of cell death research has undergone an explosion of new knowledge over the past decade. The methods to evaluate death of cells, especially in intact tissues, have led to the development of several techniques. However, the properties revealed in these assays are not always applicable to study of diversified metabolite of apoptosis at one time[3].

Nuclear magnetic resonance spectroscopy is a non-destructive and non-invasive technique that can provide complete structural analysis of a wide range of organic molecules in complex mixtures. It generates

quantitative information, as the peak intensities can be proportional to analyze concentrations[4]. Because of their low sensitivity and small magnet gaps, the early spectrometers had limited applications, primarily to synthetic chemistry[5]. Over the past three decades, however, sensitivity has increased by orders of magnitude so that this technique can be used to detect the metabolite alteration of apoptosis[6-8].

Choline compounds are one kind of biologically interesting metabolites that can be detected by ^{1}H-MRS. Declining of choline compounds is considered as a ^{1}H-NMR metabolite marker of advanced stage of apoptosis[9]. Lehtimaki *et al*[10] consider that choline compounds stay unchanged despite reduced cell density. However, how the intensity choline compounds change when apoptosis occurs is still confused.

Our hypothesis was that there is no reason for choline compounds to stay unchanged when apoptosis of liver cell occurs. The alteration of choline compounds could be observed through detailed quantitative analysis by high-resolution ^{1}H-MR spectroscopy. Therefore, the purpose of this study was to observe the liver cell apoptosis caused by the toxicity of selenium and the alteration of choline compounds using *in vitro* 9.4T high resolution magnetic resonance spectroscopy.

MATERIALS AND METHODS

Animal

Twenty male Wistar rats, weighing 280-320 g, were randomly divided into two groups (n = 10). The rats in the treatment group were intraperitoneally injected with sodium selenite liquor (Na_2SeO_3) at a dose of 20 μmol/kg and the control group with distilled water at a dose of 1 ml/kg. The rats of both groups were fasted for 12 h but with free access to water. All the rats were sacrificed after 24 h. The livers of all rats were immediately dissected. Parts of livers were frozen in liquid nitrogen and then stored at -70℃ until measured. The rest parts of livers were fixed in formalin. All animal experiments were performed according to the guidelines approved by the Ethical Committee of the Medical College of Shantou University.

In vitro ^{1}H-MR spectroscopy

Frozen liver tissue was pulverized with a pestle and mixed with a volume of 2 mmol/mL ice-cold perchloric acid. The mixture was transferred to a homogenizer and homogenized for 20 min at 4℃. The mixture was neutralized with ice-cold 3mmol/mL and 2 mmol/mL KOH and then centrifuged at 10 000 g for 30 min in order to eliminate perchlorate salts. The resulting supernatant was lyophilized and the precipitate discarded. The powder of extracts was transferred into a 5-mm NMR tube and redissolved in 500 μL D_2O containing 1 mmol/L 2,2'-3,3'-tetradeutero-trimethyl-sylilpropionate (TMSP). D_2O was added for locking signal. TMSP was used as an internal chemical shift reference at 0.00 ppm. Each sample was collected using *in vitro* 9.4T high resolution magnetic resonance spectrometer (Bruker Avance 400 MHz). Spectra of extracts were acquired with a pulse sequence with water suppression from the Bruker zgpr pulse program. Data were obtained over a 5000 Hz sweep width and digitized with 4096 data points. The number of scans was 128. Total acquisition time was 6 min. Spectra were primarily processed in the frequency domain using XWINNMR (Bruker GmBH), including fourier-transformation, phased correction and baseline correction. The chemical shift was assigned according to the internal standard TMSP and advanced analysis was then performed using MestRe-c 4.3.

Statistical analysis

The peak areas which were assigned as metabolites containing choline and TMSP and integral values were calculated separately by MestRe-c 4.3. The concentration of choline was calculated following the amended formula[11].

$$(\text{metabolite}) = \frac{\text{square(metabolite)}}{\text{square(TMSP)}} \times \frac{\text{number of protons of metabolite}}{9} \times (\text{TMSP})$$

Square (metabolite) and square (TMSP) stand for the area of peaks of choline compounds and TMSP; 9 correspond to the number of protons giving rise to the TMSP peak; (TMSP) and (metabolite) represent the concentration of TMSP and metabolite.

The concentration data were put into computer and analyzed using SPSS 13.0 software. A two-sample t test was used for comparison of choline concentration of the samples in both groups. Significance level was set at $P < 0.05$.

Histopathology

Parts of livers presenting no necrosis by visual inspection underwent histopathological examination during follow-up. Formalin fixed samples were embedded in paraffin and 4-mm sections were cut. Samples were stained with hematoxylin and eosin and examined under light microscopy.

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using the *in situ* cell death detection kit according to Schrum LW[12]. Briefly, DNA ends were tagged with fluorescein-labeled dUTP using terminal deoxynucleotidyl transferase by incubating the samples at 37℃ in a humidified chamber. Liver sections were then incubated with anti-fluorescein-alkaline phosphatase conjugate for 30 min in a humidified chamber. Slides were incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium for 20 min at room temperature and were counterstained with hematoxylin. The sections were observed under light microscopy.

RESULTS

^{1}H-MRS spectra of perchloric acid extract from rat liver tissues are shown in Figure 1. The conventional metabolites were detected and assigned. The assignments of spectra were performed following the

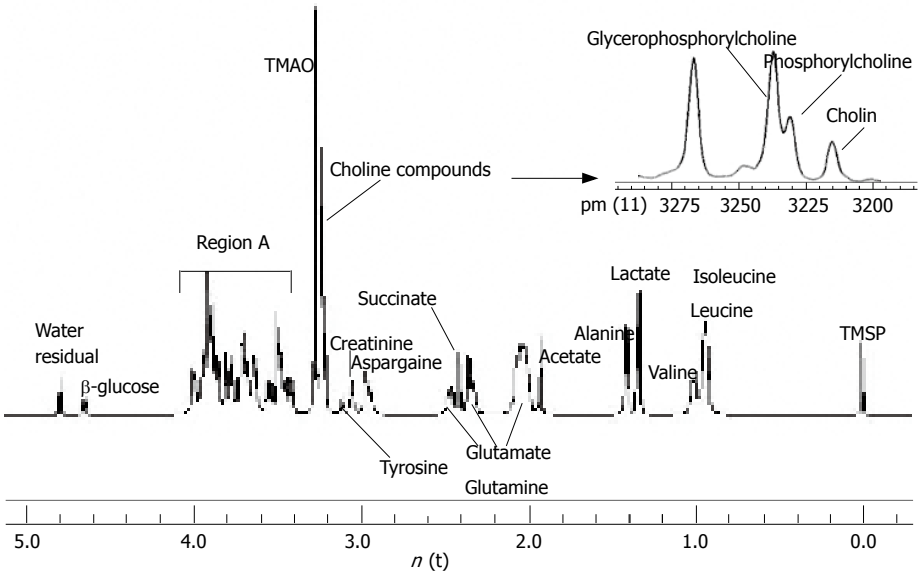

Figure 1 High-resolution proton spectra of liver tissue of a rat in control group and internal standard (TMSP). The field 0.0-5.0 ppm is shown.

Table 1 Assignments of liver metabolites from male Wistar rats

Metabolite	Chemical shift (ppm)
TMSP	0
Isoleucine and Leucine	0.87
Valine	0.96
Lactate	1.32
Lysine	1.47
Alanine	1.48
Acetate	1.92
Glutamate	2.07-2.34
Succinate	2.41
Glutamine	2.13-2.45
Aspargaine	2.95
Creatinine	3.05
Tyrosine	3.11
Choline compounds	3.20-3.23
TMAO	3.27
Region A	3.41-4.0
β-glucose	4.67
Water (residual)	4.75

TMSP: 2,2′-3,3′-tetradeutero-trimethyl-sylilpropionate; TMAO: Trimethylamine-N-oxide methyl; Region A: Glucose and amino acid CH resonances.

previous studies[13-16] and presented in Table 1. The resonances of partially megascopic region 3.20-3.27 ppm were well resolved, where $N(CH_3)_3$ signals from the compounds choline, phosphorylcholine and glycerophosphorylcholine can be separated. Following the formula, the total choline compounds and free choline concentrations were calculated. The mean concentration of total choline compounds was 5.08 ± 0.97 mmol/L in control group and 3.81 ± 1.16 in treatment group and the mean concentration of free choline was 1.07 ± 0.23 mmol/L in control group and 0.65 ± 0.20 in treatment group. The differences of the two groups were statistically significant (P = 0.05 and P = 0.00, respectively). However, there were no statistical significances if we compared the concentrations of synthetical choline, including phosphorylcholine and glycerophosphorylcholin (3.71 ± 0.74 mmol/L *vs* 3.01 ± 0.94 mmol/L, P = 0.46) between the two groups.

Although the structure of liver lobules was normal in both groups in the view of 100 times magnification, the hepatic sinus was wider in treatment group than in the control group. Cell shape was then inspected in 400 times magnification under light microscopy. The normal hepatic cells were like short shuttle while hepatic cells in treatment group were irregular and verge was not clear. Some of the cells were slightly bigger than the normal ones, but smaller ones were more often observed. We found that the cytoplasm was dyed redder than normal ones. Simultaneously, the condensed or diffuse chromatin remained randomly distributed, and the nuclear pores disappeared (Figure 2). No obviously inflamed cells could be detected under light microscope. Apoptosis of hepatic cells in both groups was confirmed by TUNEL assay. The brown ones in TUNEL assay were the apoptosis positive (Figure 3).

Although positive cells were detected in both groups, the number of positive cells was conspicuously more in treatment group than in control group. The positive cells were spread around in the control group but relatively assembled in the treatment group. Some of the apoptosis positive hepatocytes were vacuolated in treatment group. We found a very interesting phenomenon in our results: Few of apoptosis positive cells were detected in a rat of the treatment group. The ^{1}H-MRS data showed that choline concentrations were also much higher than the mean concentration of the treatment group, the concentration of total choline, free choline and synthetical choline of this rat was 5.67, 0.87 and 4.04 mmol/L, respectively.

DISCUSSION

Selenium is an essential trace element for human health. The cellular effects of selenite appear quite complex and are concentration-dependent. It is demonstrated that the serum level of selenite affects cell proliferation[17]. At intermediate concentrations, selenite appears to exert its chemopreventive activity. At higher concentrations,

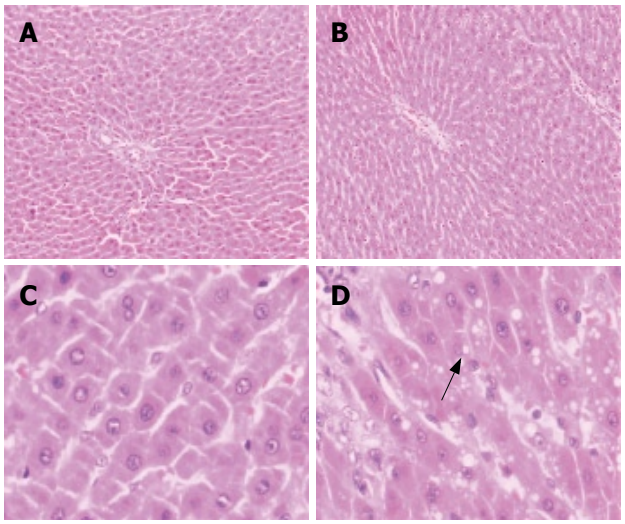

Figure 2 Light microscopy of HE stained livers. **A**: Specimen from control group (x 100); **B**: Specimen from treatment group (x 100); **C:** Specimen from control group (x 400); **D**: Specimen from treatment group (x 400). Arrowhead indicates vacuolated hepatocytes.

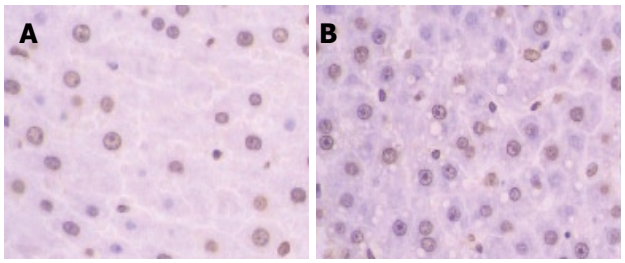

Figure 3 The brown hepatocytes showing the cell apoptosis (TUNEL staining). **A**: Specimen from control group (x 400); **B:** Specimen from treatment group (x 400).

selenite induces oxidative stress and may become toxic[18]. Many researchers focused on the induction of apoptosis by toxic concentrations of selenite. Selenocompounds have been reported to induce apoptosis in non-malignant cell lines, such as the Chang liver cells[19]. The results of our experiments also intensively suggested that high-concentration selenium was able to cause lesions in rat livers and induce apoptosis *in vivo*. However, we also observed that one rat liver in treatment group did not present obvious cell apoptosis. We think that this phenomenon might be related to the individual diversity. Some of the rats are not sensitive towards this remedy. Bollard *et al* pointed out that various intrinsic physiological factors were known to affect the metabolic composition of biological samples from healthy experimental animals. These included well-being, genetic drift, strain, hormonal differences, rate of metabolism, age, and gender[20]. It is still unclear how selenocompounds might induce apoptosis. Many potential mechanisms have been proposed, including protection against oxidative damage, modulation of metabolism of carcinogens, cytotoxicity of selenium metabolites, induction of apoptosis secondary to production of ROS, regulation of the thioredoxin redox system, regulation of the cell cycle, and inhibition of angiogenesis[21].

It has been reported that, with Se supplementation, the liver Se concentration increases disproportionately[22]. How to predict the liver lesion and inspect the efficiency and toxicity of selenocompounds is an important issue. ^{1}H-MRS is one of suitable, low-cost, and accurate methods for this study.

MR spectroscopy possesses the sensitivity required to measure subtle biochemical changes[23]. Although MR spectroscopy detects only a fairly small number of metabolites, it can still be used to monitor the activity of many cellular activities, because so many metabolic pathways are connected[24]. Choline compounds are one group of metabolites that can be detected by ^{1}H-MRS.

Choline is a nutrient essential for normal function of all cells[25]. It is a precursor not only for acetylcholine but also for phospholipids that are found in intracellular membranes and in the cell membrane[26]. The total choline peaks consist of glycerophosphorylcholin, phosphorylcholineand free choline, but the low resolution of *in vivo* spectroscopy can not identify the individual peaks from these compounds[27]. Because of the high resolution of the spectrometer which we used in this experiment, free choline is detached among the choline compounds so that we can further find out how free choline changes are when apoptosis of cells takes place separately. In our research, we found that the total choline declined when apoptosis occurred in the liver cells. This result is quite conformable with the result of Blankenberg FG and the conventional idea, but different from the results of Lehtimaki[10]. In our opinion, there are three reasons for this phenomenon: firstly, it is related to the inspected organ, which is liver but not brain. As it is known, all ingested choline and free choline generated by phospholipid metabolism enter the hepatic circulation, making the liver, where there are very active biochemical pathways for choline metabolism, a significant "sink" for choline[28]. But when a lesion arises in the liver, the liver loses the function of absorbing choline and causes choline declining. Secondly, the method used to induce liver cell apoptosis could cause this difference. One of the reasons why the selenocompounds cause cell apoptosis is that it is capable of inducing rapid superoxide generation and *p53* phosphorylation[29]. This activation can initiate mitochondrial dysfunction and result in energy insufficiency. Ultimately, it may affect the exchange of the cell containing substance, including choline compounds. This is testified by Luck *et al*[30]. Finally, cell apoptosis period is also one of the influencing factors towards the result. In our study, the apoptosis was in its early phase. At the beginning of cell apoptosis, the total choline compounds declined because free choline decreased. This was supported by our spectroscopic and light microscopic data. It is also very interesting that the total concentration of synthetical choline, including phosphorylcholine and glycerophosphorylcholin did not have statistical difference between the two groups. Energy insufficiency and activity decline may originally cause the concentration decrease of synthetical choline when apoptosis takes place in liver cells. Because of the synthetical choline supplement by membrane dilapidation

and release when the samples were mashed, the total concentration of synthetical choline finally remained fairly constant. This process of membrane perturbations is mainly the function of phospholipase A_2 activity *in vivo*[31].

In summary, high dose selenium compounds can cause lesion of rat liver and induce cell apoptosis *in vivo*. *In vitro* high resolution ^{1}H-MRS can detect diversified metabolism that can resolve in water. The data of the spectroscopy include quantitative and qualitative information. Moreover, it can repetitively and accurately evaluate the lesion of the organ at early stage. Thus, this method has a potential role in oncology, including detection of malignancy, grading of tumor, predicting and monitoring the treatment response, and identifying persistent or recurrent diseases[32,33]. In our study, we found that the changing trend for different ingredient of choline compounds is not completely the same at early period of apoptosis. Further studies are needed to know how choline compounds change at the advanced and final stage of apoptosis.

ACKNOWLEDGMENTS

We thank Shi-Lin Cao and Xu Zhang for their help in preparing the work, and Dr. Ming-Xia Fan and Zhi-Wei Shen for their discussions.

COMMENTS

Background

The field of cell death research has undergone an explosion of new knowledge over the past decade. The methods to evaluate death of cells have led to the development of several techniques. However, the properties revealed in these assays are not always applicable to study of diversified metabolite of apoptosis at one time. Magnetic resonance spectroscopy is a non-destructive and non-invasive technique that can provide complete structural analysis of a wide range of organic molecules in complex mixtures. This technique used to detect the metabolite alteration of apoptosis has been reported. Choline compounds are one kind of biologically interesting metabolites that can be detected by ^{1}H-MRS. However, how the intensity of choline compounds changes when apoptosis occurs is still confused.

Research frontiers

The alteration of different ingredient of choline compounds could be quantitatively analyzed by *in vitro* 9.4T high-resolution ^{1}H-MR spectroscopy when apoptosis of liver cell takes place because of the toxicity of selenium. The results of present article will be helpful for further studies concerning metabolism of apoptosis.

Innovations and breakthroughs

When apoptosis of liver cell takes place, the concentrations of total choline and free choline decline, whereas the total concentration of synthetical choline, including phosphorylcholine and glycerophosphorylcholine, stays unchanged.

Applications

This study is useful to explain how apoptosis of liver cell occurs. It may also play an important role in guiding the clinical diagnosis and treatment of tumors.

Peer review

This is an interesting study, where the advanced technique of ^{1}H-NMR to detect the effect of choline compounds is paralleled to TUNEL staining of liver tissues of rats injected with selenium.

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