Knockdown of the Cysteine Dioxygenase Gene in Damaged Mitochondrial Cardiomyocytes May Be Protected by the Effective Range of Taurine Level

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Abstract: Cardiomyopathy is a disease caused by various reasons, including myocardial hypertrophy and autologous gene defects. Taurine has been used in cardiomyopathy treatment for many years. It is a cytoprotective agent that can protect the damaged mitochondria of the cardiomyocytes. Previous studies suggest that the high level of taurine is primarily controlled by the cysteine dioxygenase (CDO) gene. This paper investigates the optimum value of taurine concentration into CDO knockdown cardiomyocytes in vitro and vivo. In vitro, taurine solution (ranges from 0μL to 1000μL) was added into the CDO knockdown cell culture medium daily for every six groups. Then, we use ATP detection and Mitochondria membrane potential (MMP) detection to investigate the effect of the taurine supplement. In vivo, 50 male mice are divided into ten groups fed with taurine ranging from 1.0g to 3.0g as 0.2g increments. There is no result since all experiments are conducted in virtual due to COVID-19. However, this paper is the first that provides the protocol to detect the right taurine concentration in the case of CDO knockdown in cardiomyocytes. Thus, it may provide some ideas in treating cardiomyopathy using a suitable amount of taurine in clinical.

1 INTRODUCTION

1.1 Background

Cardiomyopathy is characterized by a collection of abnormal myocardial conditions including myocardial mechanical and/or electrical dysfunction, ventricular hypertrophy, and dilation, which can eventually lead to cardiac death or progressive heart failure (Zhuge, Ruiqi et al, 2017, Holmgren, D et al, 2003, Debray, François-Guillaume et al. 2007). And this disease is often caused by genetic inheritance or autoimmune disease (like lupus) (Cardiomyopathy 2017). A possible cause of cardiomyopathy is mitochondria failure (Suzuki, Takeo, et al, 2002). Cardiomyopathy can cause damage to the mitochondria of myocardial cells, leading to a greater possibility of heart failure (Chen, Yu-Han et al., 2019, Pion et al., 1987, Moise, N S et al., 1991, Marcinkiewicz, Janusz, Ewa Kontny. 2014).

Taurine (2-aminoethanesulfonic acid) is the most abundant free amino acid in the human body. It plays an important role in many important biological processes, such as bile acid-binding, calcium maintenance. Homeostasis, osmotic adjustment, and membrane stability. In addition, the reduction of apoptosis and its antioxidants. Activity seems to be essential for cell protection taurine (Marcinkiewicz, Janusz, Ewa Kontny, 2014, Lambert et al., 2015). In 1985, taurine (2-aminoethanesulphonic acid) was first used in the treatment of congestive heart failure in Japan (Azuma, et al., 1985, Azuma, et al., 1983), and it is now established by numerous contemporary works of literature that a decrease in the cellular level of taurine considerably increases the possibility of mitochondrial diseases, especially cardiomyopathy (Suzuki, Takeo et al, 2002, Chen, Yu-Han et al., 2019, Pion et al., 1987, Marcinkiewicz, Janusz, Ewa Kontny., 2014). Some studies have shown that taurine as a cytoprotective agent can protect damaged cardiomyocyte mitochondria (Marcinkiewicz,

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Janusz, Ewa Kontny, 2014, Lambert et al., 2015). As the most abundant free amino acid in excitable tissue, taurine plays an essential role in several biological functions including central nervous system development and membrane stabilization. Studies have also shown that mammals need to supplement taurine by eating foods rich in taurine (Lambert, et al., 2015).

In humans, taurine can be synthesized from other sulfur-containing amino acids (Polakof, Sergio et al. 2018, Sampath, et al. 2020), one of which is cysteine. It was long discovered that an enzyme, cysteine dioxygenase (CDO), primarily controls the high level of taurine in the human body (Wl, et al., 2019). Regulating through the oxidation pathway of cysteine, CDO expression level contributes to the taurine biosynthesis in multiple human organs, including the two major contributing organs: liver and mammal glands (Ueki, Iori, Martha H Stipanuk. 2007). Through the CDO gene synthesis pathway, the taurine content of taurine-containing plants can be increased. Controlling the CDO gene can also help control the content of taurine in the body. (Tevatia, Rahul, et al. 2019)

Gene knockdown is considered better since it achieves the same purpose and attains the same results without directly regulating the genes, like deletion in gene knockout or addition in gene knock-in. It only affects the transcription and mostly translation of a specific gene of interest, with high accuracy and specificity. Plus, it will be far easier to conduct knockdown than knockout.

Adenosine triphosphate (ATP) is formed by connecting adenine, ribose, and 3 phosphate groups. It releases more energy during hydrolysis and is the most direct source of energy in the body. ATP release and autocrine signals through purinergic receptors promote T cell activation to form the immune synapse formed by T cells and APC. (Ledderose, Carola et al. 2018) ATP can help human cells to carry out immunity, and cardiomyopathy can reduce the ATP produced by autogenous movement in the heart. (Bloemink, Marieke et al., 2014, Ichihara, Sahoko, et al., 2017)

1.2 Hypothesis

Therefore, this review will outline the important role of taurine in mitochondrial cardiomyopathy. We believe that increasing the content of taurine in the body to a certain extent can help protect the damaged mitochondria of cardiomyocytes. Change the original CDO in the body to control the initial content of taurine in the body. By changing the content of taurine in the food used to help the experimenter to supplement taurine, at the same time can detect the content of ATP to select the most appropriate taurine supplement. We believe that there should be a suitable range for supplementing taurine content, which should not be too high or too low.

2 EXPERIMENT DESIGN

2.1 Cardiomyocyte Cell Culture

Cardiomyocyte cell culture. Two groups of neonatal cardiomyocytes are isolated from three-day-old murine hearts, one from wild-type, the other from mice with cardiomyopathy. The cells are resuspended in DMEM supplemented with 10% fetal bovine serum, 100units ml−1 penicillin, 100μg ml−1 streptomycin. After another 24 h with a regular culture medium, 20μg ml−1 cytosine β-D-arabinofuranoside will be added into the medium to suppress non-interest cells. (Ladeira, et al, 2010)

2.2 CDO Knockdown in Cardiomyocyte Cell Culture

Short interfering RNA (siRNA) Oligonucleotides. In order to obtain CDO sequence siRNA oligonucleotides, the experiment requires siRNA manufacturer companies to design the required complementary sequences, select potential target sites, and then search with NCBI Blast to confirm the specificity of each CDO exon expression. Since there is no commercially available or known siRNA that specifically downregulates the CDO gene in murine cardiomyocytes, a positive control group cannot be carried out. For the negative control group, this work design non-targeting siRNAs that lack the RNA sequences of interest in the targeting genome to eliminate the possible experimental material interference. (Han 2018) Aliquot the resuspended or annealed siRNA into new tubes and store at −20 °C.

Single-Wall Carbon nanotubes (SWCNTs). Ladeira *et al.* (Ladeira, et al, 2010) have already validified that the covalent conjugation of siRNA to SWCNTs for RNA interference and gene knockdown is of high efficiency, especially in cell lines that are poorly transfected, such as cardiomyocytes. SWCNT is added into the cell medium with a concentration of 0.0250mg ml−1 for 48-h incubation of cardiomyocytes. To guarantee the presence of SWCNTs in the cells, the work use Raman spectroscopy. The sample cell is excited by a He-Ne laser (632.8 nm), and an oil objective lens with a

magnification of 60 times and NA = 1.4 is used to focus on the sample surface.

Transfection. Prepare a stable short SWCNT (~200 nm length) aqueous solution using high-purity short COOH-SWCNT dissolved in MilliQ water. After centrifugation, 50nM CDO-specific siRNA was added to 50μL of CNT aqueous solution, sonicated for 30 minutes, and added to the cell culture medium. For RNAifect preparation, wash the cells and provide 1 ml of fresh tissue culture medium. 50nM siRNA is added to 3μL RNAifect, QIAGEN transfection reagent, and then add 100μL tissue culture medium. Following a 15-minute incubation at 37°C to allow for complex formation, the mixture was reconstituted with 900 mL of tissue culture media and subsequently poured over the cells dropwise. The cells then are plated into fibronectin-coated culture dishes at 37°C in a 5% CO_2 incubator for two days. (Ladeira, et al, 2010)

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Gel Electrophoresis. RT-PCR is used for the evaluation of CDO knockdown efficacy and the after-treatment reactions in the negative control group. To purify the CDO mRNA of interest, add 0.75mL of Trizol-LS® Reagent to 0.25ml of freshly isolated cardiomyocytes. To allow the full dissociation of nucleoprotein complexes, mix the cells multiple times with a pipette and then incubate the lysates for 5 min at room temperature. Then, centrifuge the lysates at 12,000 g for 10 min at 4°C, and discard the suspended liquid. To conduct RT-PCR, using the High-Capacity cDNA Reverse Transcription Kit, follow the instructions, and use NCBI BLAST to create primers of interest. Then place the tubes in a thermal-cycler and run RT-PCR with 10 mins at 25°C, 2 hrs at 37°C, and 5 secs at 85°C. (Guan, Yang 2008) For the ethidium bromide-stained gel analysis, 10μL each RT-PCR product is loaded on a 7% polyacrylamide (19:1) gel and run for 3 hours at 120 V. The two groups of results are compared and analyzed.

Western Blot. For a more detailed and comprehensive investigation, the protein expression of CDO is tested through Western Blot. Antibodies are targeted towards CDO protein, and the protocol is available online, See reference for more (“ Antibodies, Proteins, Kits and Reagents for Life Science.” Abcam).

In vivo *siRNA delivery.* Purchase C57BL/6 6–8-week male mice from Beijing University. They are kept in a 12:12 light/dark condition at 25°C on a chow diet. Mice are anesthetized with pentobarbital sodium (50mg/kg) by intraperitoneal injection. As shown in Figure 1, 3mM of 20μL CDO-specific siRNAs are administered intramyocardially into the left ventricle using a 32G needle at roughly five regions around the beginning section of the left anterior descending coronary artery.

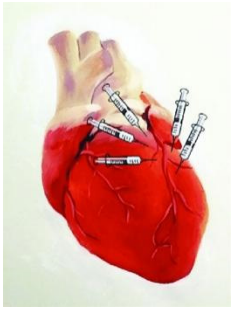

Figure 1: The injected locations are shown by the injectors. Intramyocardial injection into the left ventricle (LV) at roughly five places surrounding the beginning region of the LAD coronary artery was performed. (The figure is adapted from (Huang, Kun et al 2016)) .

2.3 Taurine Supplement into Cardiomyocytes with CDO Knockdown

a) In Vitro

Taurine Supplement. Inject taurine solution onto the cell culture medium daily with 200μL as an increment for each group, ranging from 0μL to 1000μL.

ATP Detection. The Detection Kit named ab113849- Luminescent ATP Detection Assay Kit is used to test the concentration of ATP. To extract ATP in selected cardiomyocytes, PBS is first employed to wash cells, and then add the cell lysis buffer on the culture plate, which is then vibrated on the micro-oscillator for 5 ~ 10min. To obtain the cell suspension for further tests, first use a cell scraper to scrape the cells on the culture plate, transfer the cell suspension into a 1.5ml centrifuge tube, and fully vibrate on a vortex oscillator for 30 seconds. Next, l dilute luciferin with ATP assay buffer in the proportion of 1/20 and then add 1/10 volume of luciferase and ddH_2O to prepare the required volume of an assay reagent. After all, the preparation adds the reagent into the measuring tube and record the luminous unite of firefly luciferase.

Mitochondria Membrane Potential (MMP) Detection. The Detection Kit named ab112134-JC-10 Mitochondrial Membrane Potential Assay Kit is used to test the membrane potential of cardiomyocytes’ mitochondria. For preparation, it is needed to place cardiomyocytes overnight in a 90μL growth medium at a 96-well plate. Then mix 50 μL of 100X JC-10 and 5 mL Assay Buffer A, treat cells by adding 10μL of 10X test compounds into PBS. Next incubate the cell

plate in a 37°C, 5% CO2 incubator for 4-6 hours to induce apoptosis, and then add JC-10 dye-loading solution into the cell plate. After the addition, another 30-min no-light incubation a 37°C, 5% CO2 incubation is needed. Then add the Assay Buffer B into the dye loading plate, read the fluorescence intensity, and monitor the fluorescence intensities at E_x/E_m = 490/525 nm (cut off at 515 nm) and 540/590 nm (cut off at 570 nm) for ratio analysis.

b) In Vivo

Experimental Period Estimation. In each of the cell culture experiments, when the ATP and MMP detection data from experimental groups deviate significantly from the control group, this work will make a note and regard it due to the ineffectiveness of siRNAs. These statistics will be comprehensively considered as an estimation of the efficient period of our siRNA and be considered during in vivo experiment conduction.

Taurine Supplement. Mice are chosen to do an experiment on. Use 12 groups of male mice of the same species, and each group has five mice. The first group is all healthy mice without any treatment. The second group is all mice with cardiomyopathy but without any other treatment. The third group to the twelfth group is mice with cardiomyopathy and with CDO knockdown. These ten groups of mice are fed with taurine, which is fed with 1.0g, 1.2g, 1.4g, 1.6g, 1.8g, 2.0g, 2.2g, 2.4g, 2.6g, 2.8g, and 3.0g.

Magnetic Resonance Imaging (MRI). The mouse is under anesthesia and allowed to breathe freely. First inhaled isoflurane into mice. Isoflurane inhalation is currently the preferred method for mice because anesthesia induction and wake-up are fast, hemodynamic inhibition is minimal, and the depth of anesthesia is easy to adjust. Then, the mice will receive 1.0-2.0% isoflurane, 30%-50% oxygen, 50-70% air. The location of the animals is also a critical step, and they must be reproducible. Location affects the quality of data and the degree to which motion artifacts affect imaging. The mouse should be in the prone position (fixed to an animal sled or other fixture with tape or plastic pins). Then, an anesthesia cone is provided through the nose, a breathing sensor is usually connected to the abdomen, and the temperature is measured through a fixed rectum. After completing the previous step, the expected gating synchronized with the ECG must be performed. Finally, obtain cine cardiac imaging based on multiphase gradient echocardiography.

High-Performance Liquid Chromatography. After the mice are fed for 12 hours, blood samples from their tail vein are collected, and each sample is 100μL. Add 5μL 1% heparin solution into each blood sample and then centrifuge. Then, plasma samples form. The pre-colum derivation is prepared, which mixes 100μL blood sample with 50μL derivatization. Add samples on y-reversed-phase column chromatography, (125*3 mm, ODS Hypersil 3 m) (VDS Optilab, Chromatographie Technik, GmbH), the mobile phase is 27% methanol+73% 0.1 mol/L Na2HPO4, 0.13 mmol/L EDTA water solution, speed is 0.8 mL/min, detected volumetrically (ESA Coulochem II; Bedford, Mass. USA) using three electrodes, a guard (0.4 V), peroxidation (0.4 V) and working (0.6 V) electrodes (analytical cell ESA5011). The concentrations of taurine in mice's blood are tested out.

Computerized tomography (CT) and anatomical observation. Mice euthanasia was performed and left ventricular myocardial tissue was extracted and utilized for detection.

3 RESULTS

3.1 Investigating CDO Knockdown in Vitro

Expect experiment results as in Figure 2.

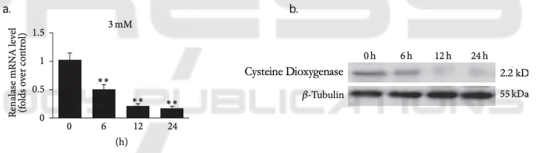

Figure 2: CDO has been knocked down using RT-PCR (a) and Western Blot (b) (Mock trials).

3.2 Investigating Effect of Taurine Levels in Vitro and in Vivo

a) ATP level in vitro using ATP Detection Assay Kit.

From Figure 3, we expect a positive correlation between the taurine supplement diet and ATP level.

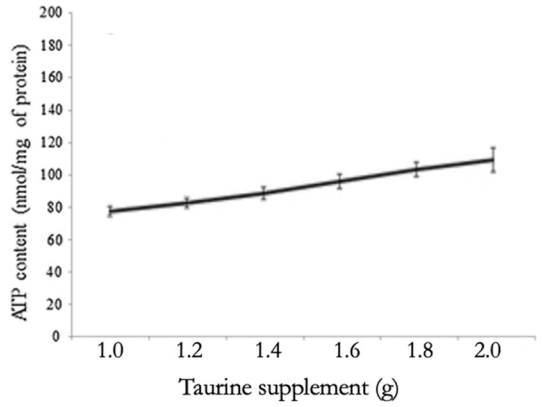

Figure 3: A positive correlation between taurine levels and ATP levels is expected (Mock trials).

b) Mitochondrial Membrane Potential in vivo.

We expect a proper change in cell membrane potential (Figure 4).

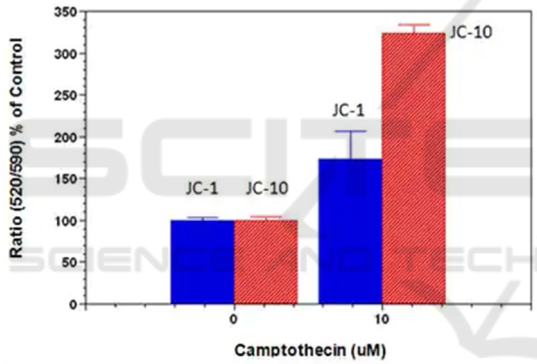

Figure 4: A proper change is expected in mock trails.

c) Taurine level in mice using MMR.

A typical mouse will contain 2.4 mg/g taurine or (for better comparison with the other values: 100g would contain 240mg) 4 ounces of a mouse would equal over 2400 mg taurine (However, 2500mg is minimum for a 10-lb cat). Compared with humans and cats, the mouse exhibits a considerable biosynthetic capacity for taurine.

d) Computerized tomography (CT) and anatomical observation

Two symptoms of cardiomyopathy are thinner myocardial wall and cardiac hypertrophy. CT is used to supervise the change of these two symptoms of each mouse (Figure 5). At last, dissect the hearts of mice to see the condition of hearts (Figure 6). Since it is a virtual experiment, there is no exact data. The ideal situation is to find out mice of which taurine level has a greater condition of the heart through comparing these ill mice's hearts with the healthy ones. From this, the taurine range that can help cure cardiomyopathy can be found out.

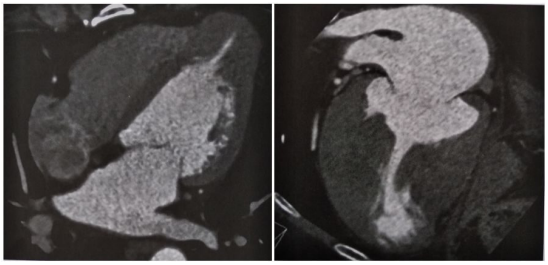

Figure 5: CT shows cardiomyopathy will cause thinner myocardial wall and cardiac hypertrophy.

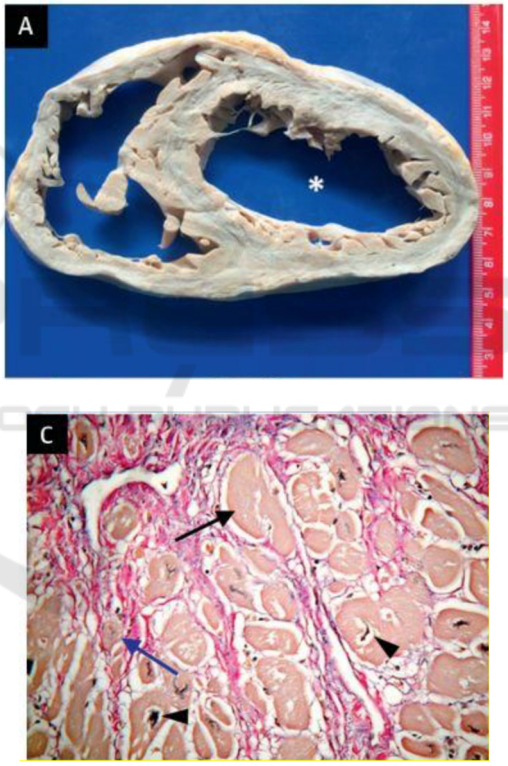

Figure 6: Thinner myocardial wall (a), cardiomyocyte hypertrophy (c).

4 CONCLUSION

Due to the restricted conditions, the results are all predicted. So, if all results meet the hypothesis, we can propose that the protection of damaged mitochondrial of cardiomyocytes with CDO

knockdown by a taurine supplement. However, there will be still have some concerns about methods.

In the beginning of the experiment, this work considered the knockdown CDO gene instead of knockout it. This has also arisen a problem, which is knockdown may have some uncertainty and inaccuracy as we cannot be able to detect whether the CDO gene was still knockdown during the entire experiment. Also, further studies can be investigated by using the knockout CDO gene method if this did happen in a laboratory. Also, due to Han Haiyong (Han 2018) mentioned in his paper that the efficient effect of knockdown should be examined and to determine the optimal time point for assessing cellular effects of siRNA knockdown. Therefore, this work will have some further studies on the monitor the efficiency of knockdown and thus, used in mice model. Moreover, in this report cannot ensure taurine can be absorbed 100% by the human body whether due to taurine consumption. Therefore, although there were several repetitions of the experiment, we cannot be 100% sure that the experiment will not have the same absorption problems. This report also considered the detection by high-pressure liquid chromatography to detect the level of ATP in vitro, but considering its stability is not as good as the kit, we chose to use the kit after careful consideration. If it is not possible to use the kit for an accurate surface in real experiments, we can also choose to use high-pressure liquid chromatography.

Apart from that, we are proud of an experiment is the first to figure out that the range of taurine levels that can affect cardiomyopathy under the condition of CDO knockdown. However, more studies can be taken to understand the underlying molecular mechanisms. For example, test the mitochondrial apoptosis pathway by Western blotting.

If the results of this experiment are true as expected to prove that the content of taurine in the body can have a positive and positive effect on myocarditis, then this technology can be achieved by consuming more taurine-rich foods. It helps patients with cardiomyopathy relieve their symptoms, and at the same time help patients achieve a greater quality of life.

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