N-acetylcysteine (NAC) Inhibits ROS-induced Ferroptosis in CTNS Knockdown β-cells in Vitro

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Abstract: *CTNS* silence or mutation in the human body leads to cystinosis, an autosomal recessive lysosomal storage disease. Ferroptosis is an iron-dependent form of programmed cell death. N-acetylcysteine (NAC), a common antioxidant, is the acetylated precursor of L-cysteine. There are no studies about the relationship between cystinosis and ferroptosis yet. Therefore, in this paper, we aimed to 1) find out the relationship between *CTNS* knockdown and ferroptosis in β-cells and 2) verify that NAC is a potential agent to protect *CTNS* knockdown β-cells and is also a potent ferroptosis inhibitor. Since we do not have the access to do the experiments in a laboratory, all the following results and conclusions are hypothetical or from existing papers: *CTNS*-targeting siRNA inhibit cystinosin expression at mRNA and protein level; *CTNS* knockdown induces ferroptosis in β-cells and NAC attenuates ferroptosis; NAC attenuates oxidative stress in *CTNS* knockdown β-cells; NAC restores energy level and glucose-stimulated insulin secretion in *CTNS* knockdown β-cells. We conclude that, in vitro, the NAC pretreatment can effectively rescue *CTNS* knockdown β-cells from ferroptosis by elevating the GPX4 mRNA and its protein level.

1 INTRODUCTION

Cystinosin is a protein transporter that excretes cystine combined with a proton from the lysosome to the cytosol, which is encoded by the *CTNS* gene. Mutations in the human *CTNS* gene will hinder the efflux of lysosomal cystine and cause lysosomal cystine accumulation, leading to an autosomal recessive inheritance disease, that is, cystinosis (Gahl, Thoene, Schneider 2009). Some researchers discovered that lysosomal cystine accumulation in cystinosin-deprived cells changes the cytosolic redox milieu to higher oxidized status by limitation of glutathione (GSH) synthesis (L. E, de G.-H. A, W. M, van den H. L, M. L, B. H 2005, S. R, M. B, N. P, M. T 2016, B. F et al 2010). Furthermore, abundant research indicated that β-cell is more sensitive to oxidative stress due to low expression of catalase and peroxidase and low GSH level compared to other tissues (N. S, S. H, A. R, T. T, and Y. T 2008, S. K et al 2003, L. S, D. J, and T. M 1996). Former reports showed that intracellular cysteine level, reactive oxygen species (ROS) level, and energy production correlate with insulin secretion (N. S, S. H, A. R, T. T, and Y. T 2008, S. K et al 2003), which is demonstrated in *CTNS*-knockdown β-cells (M. B, S. R, S. C, M. T, and N. P 2015). The elevated cystine and ROS level concomitant with stymied ATP production in cytosol and mitochondria caused attenuated insulin secretion. This conclusion at the cell level keeps aligned with the summary of cystinosis complication (N. G, G. W 2008).

Ferroptosis is an iron-dependent form of regulated cell death. It is related to iron-dependent lipid peroxidation metabolism and regulates cell death through $NADPH/H^+$, polyunsaturated fatty acids, glutamine catabolism, and other signal pathways. Ferroptosis demonstrates cellular atrophy and high-density mitochondria in morphology. The cystine antiporter system mediates the production of GSH, which is an important ferroptosis inhibitor (X. Y et al 2015, S. BR et al. 2017). Cysteine is the rate-limiting metabolite for GSH biosynthesis, so cysteine depletion leads to the lowering of intracellular GSH

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levels. GSH depletion triggers the inactivation of GPX4 which is an enzyme that specifically reduces phospholipid hydroperoxides using GSH as a cofactor (Y. WS et al. 2013). Cystine starvation impairs GPX4 protein expression by inhibiting mTORC1/4E-BP1-mediated protein translation (Z. Y et al. 2021). The inactivation of GPX4 leads to lipid peroxidation which causes the accumulation of ROS (Y. WS et al. 2013). ROS can react with polyunsaturated fatty acids of lipid membranes and induce lipid peroxidation on membranes. Also, intracellular cysteine is used for the biosynthesis of coenzyme A and subsequently CoQ10, a vital metabolite for preventing membrane lipid peroxidation and ferroptosis cell death (D. S et al. 2019, B. K et al. 2019, B. MA et al. 2020). Therefore, the cyst(e)ine level is very essential for regulating redox status and ferroptosis in cells.

N-acetylcysteine (NAC), the acetylated precursor of L-cysteine, is a common antioxidant used for clinical practice and biomedicine research. For example, with its potent antioxidant ability, NAC effectively prevents hemin-induced ferroptosis and ROS/MAPK and p53-mediated ferroptosis and rescues neuron cells having ferroptosis-like phenotype (G. G et al. 2020, L. Y et al. 2020, K. SS et al. 2018). And a clinical trial showed that additional NAC given to nephropathic cystinosis patients with routine cysteamine treatment for three months could reduce oxidative stress and significantly improve renal function without side-effects (P. de F. G. L et al. 2014). However, there is no more cell-level research related to NAC and cystinosis, leaving the relation between NAC and lysosomal cystine accumulation unknown.

Currently, the relationship between cystinosis and ferroptosis has not been studied yet. According to the results from Bernadette's research (M. B, S. R, S. C, M. T, and N. P 2015), the *CTNS*-knockdown β-cells with attenuated insulin secretion bore the characters of high ROS, cyst(e)ine, and oxidized GSH (GSSG) levels. Though the cyst(e)ine level is higher than normal cells, oxidative stress still occurs. What's more, β-cells are more sensitive to oxidative stress than other types of tissues and cells in the human body, which embodies in terms of insulin secretion (N. P, R. E, A. F, K. M, C. A, C. R 2012). Combining the aforementioned information, here we hypothesize that ROS is a potent factor to cause ferroptosis in β-cells regardless of the cyst(e)ine. Therefore, *CTNS*-knockdown β-cells will undergo ROS-induced ferroptosis and NAC could inhibit ferroptosis and reverse the adverse effects caused by lack of cystinosin.

2 MATERIALS AND METHODS

The big picture of our research shows in Figure 1. The Mock means the β-cells underwent all of the siRNA transfection steps without siRNA. β-cells were transfected by non-targeting siRNA (NT) or *CTNS*-targeting siRNA (CT) for 18 h. CT+ cells were cultured with 10 mM NAC for 12 h and transferred to normal culture for the next 12 h after siRNA transfection, while other groups lived in normal culture for 24 h post-transfection. In a word, after 24 h post-transfection, all groups were detected by real-time PCR to justify whether siRNA interference was successful or not. Then the subsequent experiments were practiced for competent groups after 48 h stabilization in normal culture. The specific experiment steps are as follows.

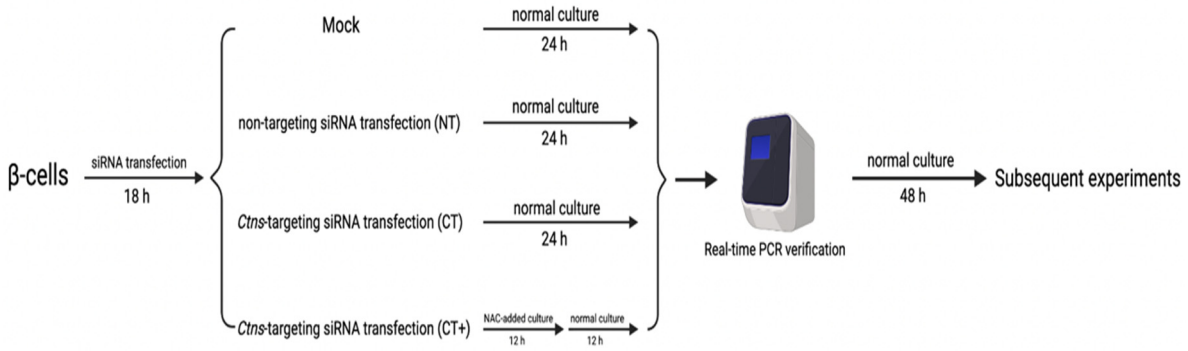

Figure 1: Schematic research design.

The BRIN-BD11 β-cells line was processed and divided into four groups: Mock, non-targeting siRNA transfection (NT), *CTNS*-targeting siRNA transfection (CT), and *CTNS*-targeting siRNA

transfection with NAC treatment (CT+). After transcription-level verification of real-time PCR, all groups were cultured in normal culture for 48 h and then used for subsequent experiments.

2.1 Cell Culture and Process

Here we use the same materials, cell culture protocol, and gene knockdown protocol of Bernadette's research (M. B, S. R, S. C, M. T, and N. P 2015). Briefly, we used ON-TARGETplus SMARTpool technology by GE Dharmacon (Lafayette, CO, USA). BRIN-BD11 cells were seeded and allowed to adhere overnight. *CTNS*-targeting siRNA pool (80 pmol/µl; CT) was transiently transfected into the cells for 18 h using DharmaFECT 1, according to the instructions of the manufacturer. Mock transfection involved the transfection in the absence of siRNA, while the negative control was the non-targeting siRNA pool (NT). Following the NAC addition of Zhang's paper (L. Y et al. 2020), half part of the CT cells is pretreated with NAC (10 mM, CT+) for 12 h and then cultured in normal culture for 12 h before real-time PCR.

2.2 sIRNA Gene Knockdown Verification

The real-time PCR and western blot were practiced demonstrating *CTNS* knockdown at transcriptional and expressional levels. The real-time PCR was practiced 24 h after siRNA transfection and western blot was used at 72 h post-transfection. Experiment steps are strictly repeated according to Bernadette's research (M. B, S. R, S. C, M. T, and N. P 2015).

2.3 Intracellular Iron Level

Intracellular iron levels were assessed using an optimized FerroZine™, an iron-based assay developed by Reimer (R. J, H. HH, C. H, R. SR, and D. R, 2004). Before testing, each sample tube and the standard tube is filled with 6.5 mM FerroZine™, 6.5 mM neocuproine, 2.5 M ammonium acetate, and 1 M ascorbic acid dissolved in water. An iron detection reagent is then being added to each tube. After that, incubation will be performed for half an hour at room temperature, which should allow researchers to observe color development. Then, a microplate reader measures the absorbance at 550 nm when 280 µl from both standard and sample tubes was added in duplicate into wells of a 96-well plate. Finally, the BCA assay will be carried out to determine intracellular iron concentration. The concentration is determined by the amount of dye in the blue ionic from the measurement of the absorbance of the solution.

2.4 GPX4 Level

We use the Glutathione peroxidase 4 (GPX4) kit (Runyu biotechnology co. LTD, Shanghai, China) to detect the GPX4 level in treated cell lines according to Zhou's work (Z. Y 2020). The GPX4 gene level can be detected via Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). RNeasy Mini Kit (Qiagen) was used for the extraction of total RNAs according to the manufacture's protocols. M-MLV Reverse Transcriptase (Promega) was used for DNA synthesis. GAPDH served as the internal reference. The $2^{-\Delta\Delta CT}$ method was used for the calculation of the relative gene transcription level. The GPX4 protein level is detected using western blot following Li's method (L. D et al. 2020).

2.5 MDA Level

The measure of MDA levels is performed by TBARS assay. Glacial acetic acid is used to reconstitute thiobarbituric acid (TBA) since regular acetic acid affects TBA stability by its high-water content. During sample preparation, butylated hydroxytoluene is used in lysis buffer to prevent further peroxidation while processing. After that, TBA solution is added to the sample and then the sample will be incubated at 95°C for 60 minutes. Following that, the samples will cool down to room temperature by using an ice bath for 10 minutes. Finally, a microplate reader is used to measure the output.

2.6 ROS Level

The examination of qualitative ROS level is referring to Li (L. D et al. 2020). The ROS probes solution was diluted to the required concentration in the Phosphate-buffered saline (PBS) buffer and then incubated at room temperature for 45 min. Then the cells were washed with PBS 3 times and excited with green light under a fluorescent microscope to observe and shoot red emission images of the cells, which represent ROS positive cells.

Referring to Bernadette's method (M. B, S. R, S. C, M. T, and N. P 2015), the quantitative ROS level was detected using ROS probe, 5-(and-6)-carboxy-2',7'-difluorodihydro fluorescein diacetate (carboxy-H_2DFFDA). Cells were incubated with 20µM carboxy-H_2DFFDA for 45 min, and the cell

suspension was analyzed immediately using the BD Accuri C6 flow cytometer.

2.7 Intracellular GSH and GSSG Levels

According to Zhou (Z. Y 2020), the levels of GSH and GSSG were detected by GSH/GSSG Ratio Detection Assay II (ab205811, Abcam), which is used for the measurement of GSH, GSSG, and GSH/GSSG. The GSH and GSSG levels were normalized for protein content.

2.8 Cell Viability

Cells incubated in 96-well plates were treated as indicated and cell proliferation was assessed by CCK-8 assay (SAB biotech. College Park, MD, USA) at 24 h after *CTNS* knockdown and at 24 h after NAC treatment following the manufacturer's instruction. Optical density (OD) was recorded at 450 nm.

2.9 Intracellular ATP Level

Following Bernadette's method (M. B, S. R, S. C, M. T, and N. P 2015), cells were detached using 0.05% trypsin–EDTA and washed with ice-cold PBS. The cell pellets were resuspended in 100 µl ice-cold PBS. The cell suspension was then diluted 25-fold and the ATP content was assessed using the ATP Bioluminescence Assay Kit HSII (Roche Diagnostics, Mannheim, Germany), according to the instructions of the manufacturer. The protein concentration of the cell lysates was used to normalize the ATP content.

2.10 Insulin Secretion

The chronic and acute glucose-stimulated insulin secretion was repeated following Bernadette's method(M. B, S. R, S. C, M. T, and N. P 2015). At 72 h post-transfection, the cell supernatant was removed and used to determine chronic insulin release. The cells were then washed with PBS and acute insulin secretion was stimulated after cells were starved for 40 min with Krebs Ringer Buffer (KRB), pH 7.4, containing 1.1 mM D-glucose. The cells were then stimulated with KRB containing 16.7 mM glucose plus 10 mM alanine for 20 min at 37 °C. The KRB was collected and insulin release was determined using the Mercodia ultra-sensitive rat insulin ELISA kit (Uppsala, Sweden), according to the instructions of the manufacturer.

2.11 Statistic Analysis

This research is based on hypotheses and speculations instead of real data in the lab. Therefore, the data presented in the following sections are mock data from existing research and are modified due to our prediction. Data are presented as the means of three independent experiments ± SEM and were analyzed with the GraphPad Prism 9.2 software (GraphPad Software Inc., San Diego, CA, USA). Data were analyzed by Student's *t*-test and differences at a *P*-value of <0.05 were considered significant. One, two, three, four asterisks respectively represent *P*-value of <0.05, <0.01, <0.001, and <0.0001.

3 HYPOTHESIS AND RESULT SPECULATION

Here we hypothesize that *CTNS* knockdown will induce oxidative stress in β-cells BRIN-BD11 and express the phenotype of ferroptosis, while NAC can attenuate ferroptosis by changing redox status in cells. The mock results are listed as follows.

3.1 CTNS-Targeting sIRNA Inhibit Cystinosin Expression at mRNA and Protein Level

Our speculation thoroughly depends on the *CTNS* knockdown in β-cells BRIN-BD11. Therefore, we presume that we successfully silence the *CTNS* gene. The *CTNS* mRNA levels in CT and CT+ are significantly decreased by more than 80 % compared to Mock and NT at 24 h post-transfection (Fig. 2*A*). Combining mRNA level decrease, cystinosin level in CT and CT+ is also decreased by approximately 50 % at 72 h post-transfection (Fig. 2*B*).

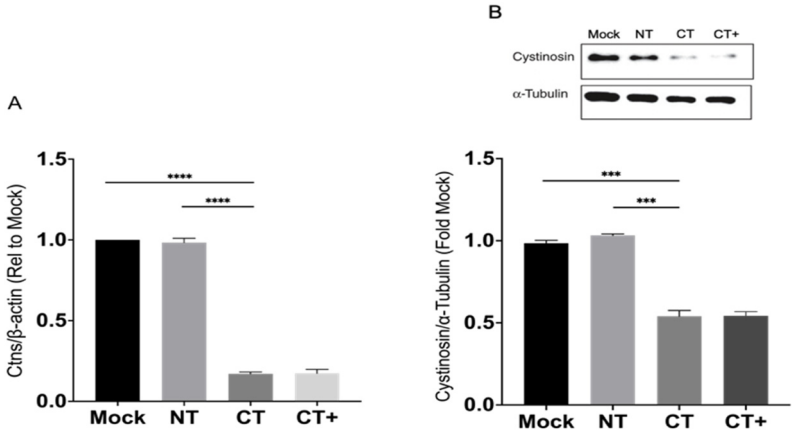

Figure 2: Evaluation of CTNS-targeting siRNA knockdown by real-time PCR and Western blot.

BRIN-BD11 cells were transiently transfected with a *CTNS*-targeting siRNA pool (CT) for 18 h using DharmaFECT 1 transfection reagent, according to the manufacturer's instructions. A part of CT underwent 10 mM NAC pretreatment for 12 h after transfection (CT+). Mock transfection (Mock) was performed in the absence of siRNA, while the negative control contained a scrambled non-targeting siRNA pool (NT). *CTNS* mRNA and cystinosin levels were assessed by real-time PCR (*A*) and Western blot (*B*) at 24 and 72 h post-transfection, respectively. Lanes from 1 to 4 of the Western blot analysis correspond to Mock, NT, CT, and CT+, respectively. Densitometry analysis of Western blots is expressed as a ratio of cystinosin to α-tubulin expression and is presented as mean fold change relative to Mock ± SEM of three or more independent experiments. *** or **** show the CT's statistically significant difference from Mock or NT, as indicated, at P<0.001 and P<0.0001, respectively. Mock, mock transfection; NT, non-targeting siRNA; CT, *CTNS*-targeting siRNA; CT+, *CTNS*-targeting siRNA and NAC treatment.

3.2 CTNS Knockdown Induces Ferroptosis In β-cells and NAC Attenuates Ferroptosis

From artificial Figure 3, we could know that the *CTNS* knockdown β-cells get apparently damaged with strong ferroptosis phenomenons: elevated intracellular iron and MDA level and decreased GPX4 mRNA and protein level. However, NAC could potently attenuate negative effects caused by *CTNS* knockdown.

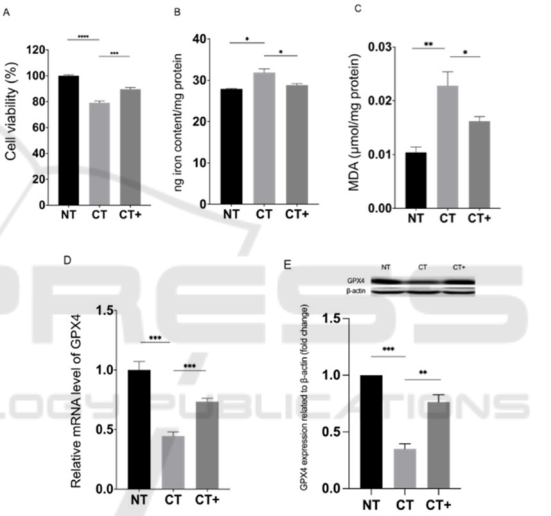

Figure 3: Effect of CTNS knockdown and NAC on ferroptosis in β-cells

At 72 h post-transfection, the BRIN-BD11 cell viability (*A*), iron level (*B*), MDA level (*C*), GPX4 mRNA level (*D*), and GPX4 protein level (*E*) of NT, CT, and CT+ are determined. Data are presented as means ± SEM of at least three independent experiments. *, **, ***, and **** represent significantly difference at P<0.05, 0.01, 0.001, and 0.0001, respectively. NT, non-targeting siRNA treatment; CT, *CTNS*-targeting siRNA treatment; CT+, *CTNS*-targeting siRNA and NAC treatment.

3.3 NAC Attenuates Oxidative Stress in CTNS Knockdown β-cells

We forecast that BRIN-BD11 cells are affected by serious oxidative stress after *CTNS* knockdown (Fig. 4). Compared with NT, the ROS (Fig. 4*B*) and GSSG

(Fig. 4*D*) levels of CT are extremely elevated, while the GSH level has not significantly changed (Fig. 4*C*). The NAC is a competent antioxidant to inhibit oxidative stress (Fig. 4*E*). Interestingly, the total GSH level will not be improved when the cysteine level is normal (B. JM, V. A, and L. BH 1989). Due to the known result that total cysteine in *CTNS* knockdown cells is a bit more than normal cells (M. B, S. R, S. C, M. T, and N. P 2015), we speculate that the total GSH level will not be improved after NAC treatment.

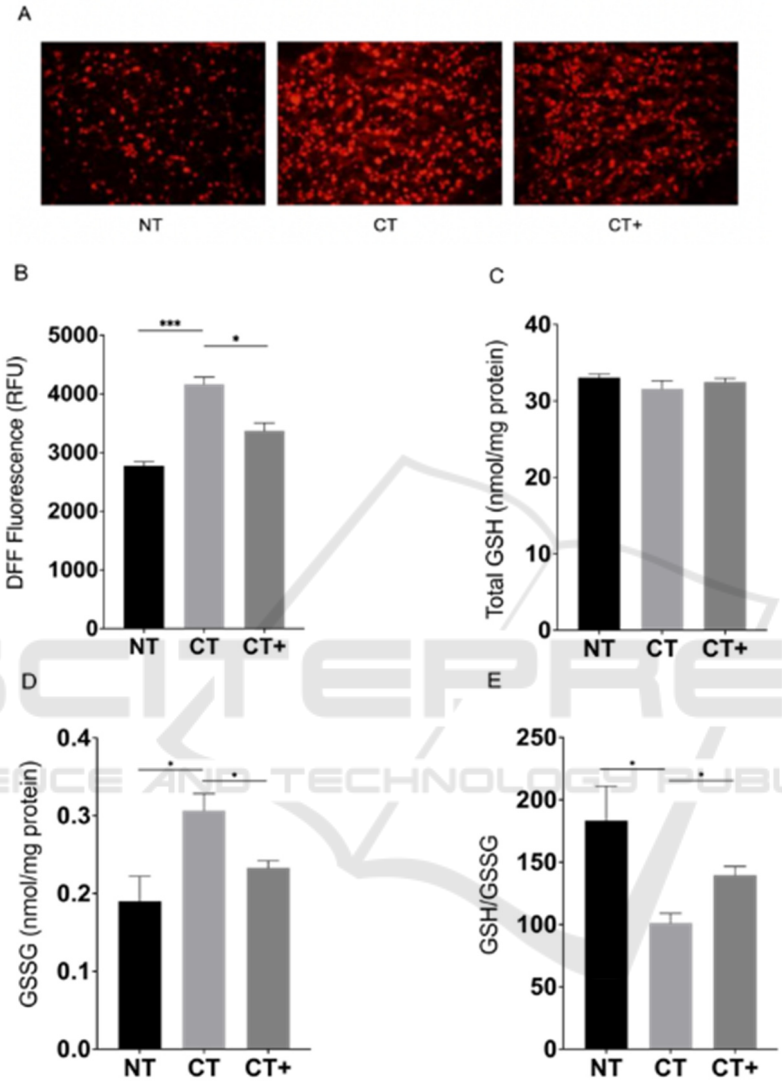

Figure 4: Effect of CTNS knockdown and NAC on redox status in β-cells.

At 72 h post-transfection, the ROS level in BRIN-BD11 cells was showed in qualitative form (*A*) by using Dihydroethidium (DHE) probe and quantitative form (*B*) by using ROS probe carboxy-H_2DFFDA. The total glutathione (GSH) (*C*) and oxidized glutathione (GSSG) (*D*) levels in BRIN-BD11 cells were determined. (E) The ratio of GSH and GSSG is calculated. Data are presented as means ± SEM of at least three independent experiments. * and *** represent the significant difference at $P<0.05$ and <0.001, respectively. NT, non-targeting siRNA treatment; CT, *CTNS*-targeting siRNA treatment; CT+, *CTNS*-targeting siRNA and NAC treatment.

3.4 NAC Restores Energy Level and Glucose-Stimulated Insulin Secretion in CTNS Knockdown β-cells

The decreased total ATP level (Fig. 5*A*) and restricted chronic (Fig. 5*B*) and acute (Fig. 5*C*) insulin secretion

are demonstrated in *CTNS* knockdown β-cells. Based on our hypothesis that β-cells are mainly affected by oxidative stress, 10 mM NAC treatment can significantly increase the intracellular ATP level and improve insulin secretion due to its antioxidant property.

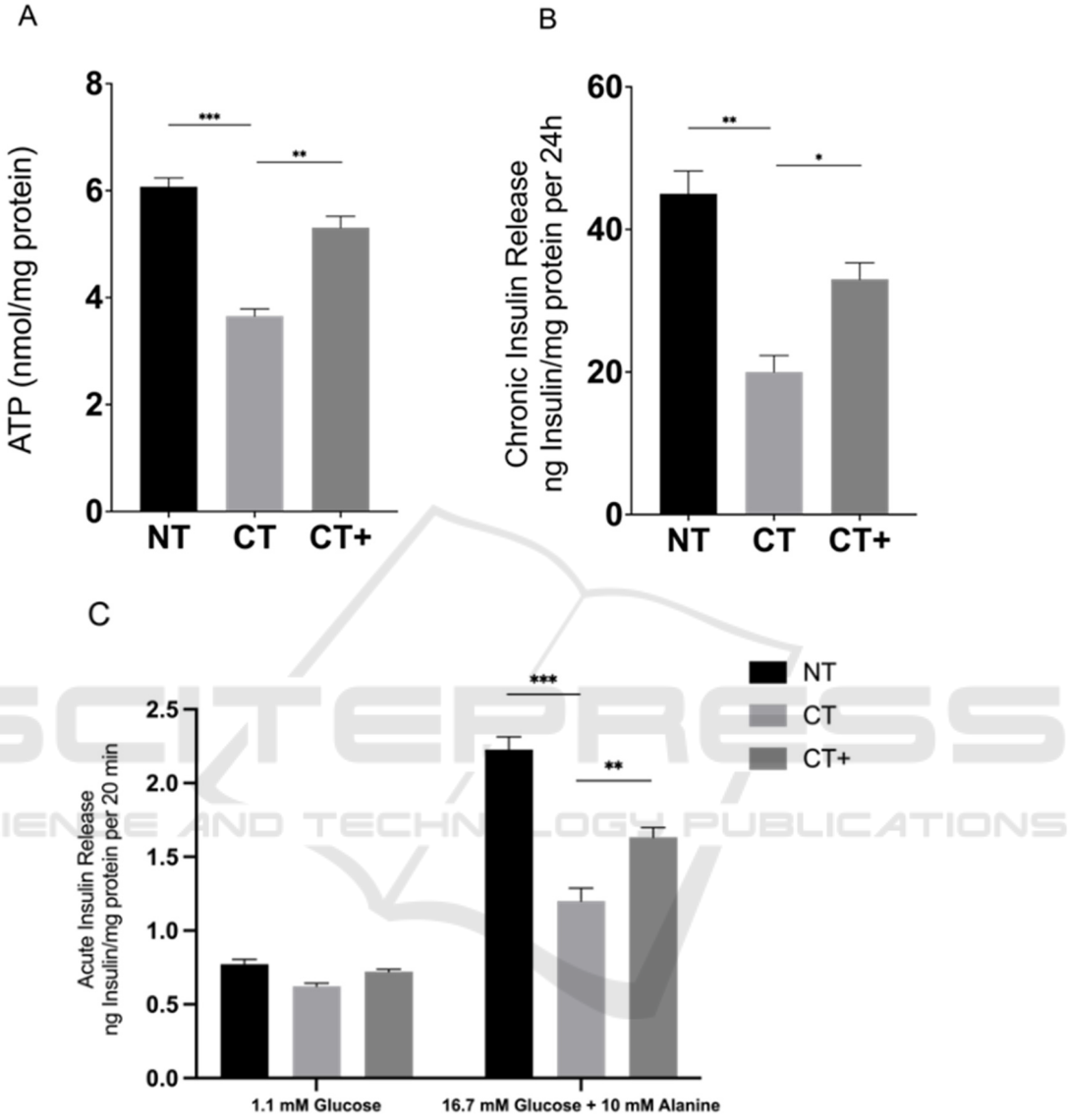

Figure 5. Effect of CTNS knockdown and NAC on ATP level and insulin secretion

A) total ATP concentration is determined in BRIN-BD11 cells at 72 h post-transfection. *B*) the chronic release of insulin was assessed in cells between 24–72 h after transfection. Cells are initially incubated with KRB containing 1.1 mM glucose for 40 min at 37°C before insulin release was stimulated for 20 min using KRB containing 1.1 mM glucose (basal) or 16.7 mM glucose and 10 mM alanine. *C*) acute stimulated insulin secretion was determined in BRIN-BD11 cells at 72 h post-transfection. Data are presented as means ± SEM of at least three independent experiments. *, **, and *** represent significantly difference at $P<0.05$, 0.01 and 0.001, respectively. NT, non-targeting siRNA treatment; CT, *CTNS*-targeting siRNA treatment; CT+, *CTNS*-targeting siRNA and NAC treatment.

4 DISCUSSIONS

During ferroptosis, lipid peroxidation happens, which

is related to the high oxidative level in the cytosol. Currently, researchers think that N-acetylcysteine can inhibit ferroptosis due to its potent antioxidant ability (X. Y et al 2015). However, the specific mechanism of NAC inhibiting ferroptosis has not received much attention. We forecast that NAC may not only scavenge ROS but also restore other substances that act as ferroptosis inhibitors.

According to current research about ferroptosis (X. Y et al 2015), cyst(e)ine starvation can lower the level of GSH, inactivate GPX4, and inhibit the biosynthesis of coenzyme A and subsequent CoQ10 which can greatly prevent lipid peroxidation and ferroptosis. GPX4 can prevent normal oxidative stress and lipid peroxidation by using GSH. No researchers, however, study whether ROS itself can inactivate GPX4 at mRNA and protein level under the condition of normal cyst(e)ine and GSH concentration.

According to Bernadette's research (M. B, S. R, S. C, M. T, and N. P 2015), the level of cystine and cysteine in β-cells both increase after *CTNS* knockdown, but the oxidative stress and the accumulation of ROS still happens, lowering the secretion of insulin and impairing cell viability. The cyst(e)ine-mediated ferroptosis is inactivated because of the sufficient intracellular cyst(e)ine under this situation. Under the hypothesis we raised before, we speculate that the GPX4 level will not be affected by cyst(e)ine but by ROS aggregation. ROS can independently inactivate GPX4 mRNA and protein through a certain unknown mechanism and form positive feedback which leads to more ROS accumulation and inevitable ferroptosis. Following the hypothesis, the mRNA and protein level of GPX4 and cell viability will significantly decrease, the MDA level highly increase. Referring to the discussion above, the NAC treatment is applied to reduce ROS, which can restore the GPX4 transcription and expression. Whether NAC could directly rehabilitate GPX4 in different ways is valuable to study. By rehabilitation from ferroptosis, the insulin secretion and intracellular level of β-cells rise, though we cannot estimate this increase whether is caused by ferroptosis inhibition or oxidative stress inhibition.

5 CONCLUSIONS

After *CTNS* knockdown, the BRIN-BD11 β-cells undergo ROS-induced ferroptosis with impaired insulin secretion and energy production. The NAC pretreatment effectively rescues *CTNS* knockdown β-cells from ferroptosis by increasing GPX4 mRNA and protein level.

Currently, there are no research focusing on the relationship between ferroptosis and cystinosis. The intracellular cyst(e)ine level of cystinosis patients is higher than that of healthy people. Not a few Researchers study the ferroptosis caused by low cyst(e)ine in cells, that is, cyst(e)ine starvation. *CTNS* knockdown β-cells with high oxidative stress and intracellular cyst(e)ine level provide a good model to discover whether ferroptosis occurs with enough cyst(e)ine. We innovatively exclude the influence of cyst(e)ine and study the relationship between ferroptosis and ROS with CTNS knockdown cell model. Our work to some extant offer a new perspective of ferroptosis induction.

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The author's contributions are listed here. Experimental design, H. LIANG; Materials organization, H. LIANG, J. XU; Methodology, H. LIANG, J. YU, R. PENG, Y. CHEN; Hypothesis speculation, H. LIANG, J. YU, J. XU, Y. CHEN, R. PENG; Data visualization, H. LIANG; Paper writing, H. LIANG, J. XU, Y. CHEN; Proofreading, Y. CHEN. All the authors have read and agreed to the final version of the report with no conflict of interest.

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