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Inducible HSP70 May Play a Protective Role in CUMS Mouse Embryos *In Vitro*

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Abstract

Background: To explore the expression of HSP70 in chronic unpredictable mild stress (CUMS) mouse embryos cultivated *in vitro*.

Methods: We obtained 452 two-cell embryos from 39 CUMS mice and 137 ones from 7control mice, and cultivated them *in vitro* in M2 medium at 37°C and 5% CO_2. Forty-eight hours later, blastocysts were collected and HSP70 expression was analyzed with Immuno Fluorescence (IF) staining and Real-Time Polymerase Chain Reaction (RT-PCR). Twenty-four hours later, morulae were collected for transfecting HSP70-si RNA by electroporation to down-regulation HSP70 and blastocystim plantation rate was assayed.

Results: The blastocyst formation rate in experimental group (38.2%) was significantly lower than in the control group (52.6%, $P< 0.05$). Compared with the control group (5.01±2.11), the level of HSP70 mRNA in experimental group blastocysts (3.35±1.23) also decreased significantly ($P< 0.05$). After transfectingHSP70-siRNA to a morula of experimental group by electroporation, the level of HSP70 mRNA in the HSP70-siRNA group (1.01±0.58) was significantly lower than that in the no electroporation group (3.71±1.29) ($P < 0.05$). And the embryo implantation rate in experimental group (18.8%) was significantly lower than in control group (50%, $P< 0.05$).

Conclusions: Inducible HSP70 may play a protective role in the CUMS embryos *in vitro*.

Keywords: Heat shock protein 70; Chronic Unpredictable Mild Stress; *in vitro*; siRNA; Electroporation

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Background

Embryo culture *in vitro* is an important procedure in human-assisted reproductive technology, and high quality embryos and blastocysts are key to a successful pregnancy. Of course, the environment of the *in vitro* culture system is not the same as the female reproductive tract; gene expression in the embryos *in vitro* may be influenced by media composition, number of embryos present in the culture drop, serum supplementation, and gas atmospheric conditions [1,2]. Oxidative stress *in vitro* can also lead to differences in gene expression [3,4]. The Endoplasmic Reticulum (ER) stress pathway constituents are present at all stages of preimplantation and development *in vitro*, and activation of the ER stress pathways can be induced at the eight-cell, morula, and blastocyst stages [5-7]. Heat Shock Proteins (HSPs) are highly conserved cellular stress proteins present in every organism from bacteria to human beings [8]. HSP70 is a member of the HSP super family. It may act as a molecular chaperone, assist in the cell stress response by resisting oxidative stress and apoptosis, and regulate immune responses [9]. The HSP70 level increases as bovine embryos develop *in vitro* [10]. When subjected to thermal and oxidative stressors, the body may protect its reproductive function by increasing the expression of HSP70 [11]. However, immune sensitization to HSP70 may associate with unsuccessful embryo development and implantation failure in *in vitro* fertilization and embryo transfer (IVF-ET) patients [12].

Chronic Unpredictable Mild Stress (CUMS) is a behavioral animal model used to simulate certain aspects of human depression, such as loss of normal aggressiveness [13]. This model is consistent with ethical principles for scientific experiments on animals and has validity [14]. Our research group established the CUMS animal model with Kunming mice [15]. We found that HSP70 was highly expressed in two-cell embryos and Day 4 embryos from CUMS mice *in vivo*. We also showed that HSP70 mRNA levels in the embryos were positively associated with parameters of

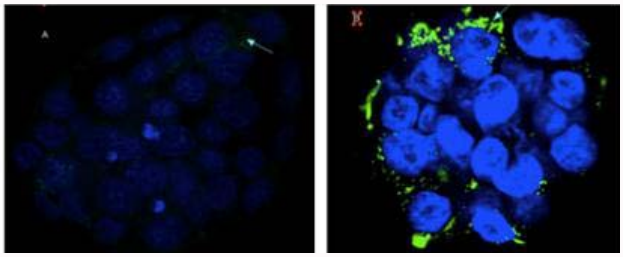

Figure 1: Expression of HSP70 in CUMS mouse' embryos cultured in vitro for 48h demonstrated by immuno fluorescent staining. A. Control embryo(x600). B. Embryo of experimental group (x600). The HSP70 was banded distribution in the control embryos, and punctate distribution in the embryos of experimental group.

oocyte and embryo development potential.

This study continued to explore the expression and role of HSP70 in the CUMS mouse embryos cultivated *in vitro*.

Materials and Methods

Animals

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by our Committee on the Ethics of Animal Experiments of Sichuan University (Permit Number: AE2014034). We performed all surgeries under sodium pentobarbital anesthesia and made all efforts to minimize suffering. We obtained female Kunming mice (4–5 weeks old) from the Animal Service Center of Sichuan University and assigned them randomly to one of two groups: an unhandled control group (n=7) and the experimental CUMS group (n= 39). Mice were housed as our previous study, and the experimental group underwent a 4-week CUMS procedure [15].

CUMS procedure

The experimental mice were stimulated for 4 weeks with one of the nine stressors in the same order each day [15]. The nine stressors were as follows: (1) 24-h damp bedding and cage tilting (cages were tilted to 45°C from the horizontal); (2) 4-h restraint; (3) 5-min swimming in an ice bath; (4) 24-h food deprivation; (5) 24-h water deprivation with empty drinking bottles; (6) 24-h social isolation (one mouse per cage); (7) 5-min heat stress in an oven at 40°C; (8) 1-min tail clamping; (9) 24-h exposure to strange objects such as plastic cups, spoons, or pieces of cloth. Each stressor was carried out in each experimental mouse 3 or 4 times in the 4-week CUMS.

Collection and cultivation of mouse embryos

The mice were injected intraperitoneally with 10 IU Pregnant Mare Serum Gonadotropin (PMSG, Animal Center of Tianjin, P. R. China)at 4 pm, followed by an injection of 10 IU Human Chorionic Gonadotropin (HCG, Biochemical Pharmaceutical Factory of Shanghai, P. R. China) 46-48 h later. After the second injection, female mice were placed two per cage with fertile males. The day of sighting of a vaginal plug was designated as Day 1 (D1) *Post Coitum* (pc). The mated females were separated from males on D1 pc. Two-cell embryos were obtained from these females at 2 pm on D2 and then cultivated *in vitro* in M2 medium at 37°C and 5% CO2. Morulae were collected 24 hours later, and blastocysts were evaluated 48 hours later. The embryos were classified according to the published criteria for human embryos [16].

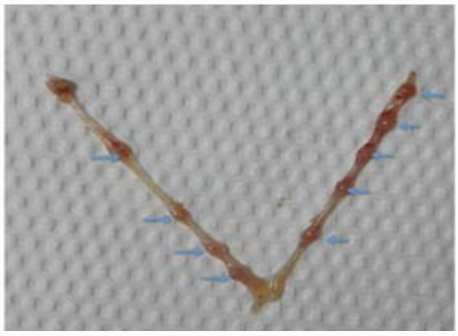

Figure 2: The implantation embryos of transferred blastocysts on pseudopregnant day 7. Arrows show the implantation sites in the uterine horns of a mouse on pseudopregnant day 7. There are 4 implantation sites on the left side transferred with 14 blastocysts of HSP70-siRNA group and 5 implantation sites on the other side with 10 blastocysts of the control group.

Table 1: The blastocyst formation rate of 2- cell embryos of CUMS mouse when cultured *in vitro* for 48 hours.

Groups	No. mice	No.2-cell embryos	No. blastocysts in vitro(%)
Control group	4	76	40 (52.6%)
Experimental group	9	102	39 (38.2%)
P value			< 0.05

Data are shown as rates and were analyzed by χ^2 test.

Table 2: HSP70 mRNA level in CUMS mouse' blastocysts *in vitro*.

Groups	No. blastocysts	$2^{-\Delta\Delta CT}$
Control group	30	5.01±2.11
Experimental group	30	3.35±1.23
P value		< 0.05

Data are shown as the mean ± SD and were analyzed by t-test.

Real-time polymerase chain reaction (RT-PCR)

The RT-PCR protocol was as described in our previous study [15]. The RNA was extracted from 30 embryos per group that had been cultivated 48 h *in vitro*, using RNeasy Micro Kits (Qiagen Inc., Germany) according to the manufacturer's instructions. β-actin was used as the housekeeping gene for data normalization. The RT-PCR reactions were conducted in a final volume of 20 μL, containing 10 μL of SYBR Green Master Mix (Roche Diagnostics, Indianapolis, IN, USA), 3 μL of H_2O, 1 μL each of the forward primer (5′-GAGGAGTTCAAGAGGAAG-3′) and reverse primer (5′-TGATGGATGTGTAGAAGTC-3′), and 5μL of cDNA template or water (non-template negative control). An ABI 7500 thermal cycler (Applied Biosystems, Foster City, CA, USA) was employed for RT-PCR amplification, which was performed under the following conditions: one cycle of 95°C for 10 min; 45 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for 30 s; and a final cycle of 95°C for 15 s, 60°C for 15 s, and then a gradual increase to 95°C over 30 min at a ramp rate of 2% for melting curve analysis.

Immunofluorescence (IF) staining

The IF staining protocol was also described in our previous study [15]. The embryos cultivated for 48 h *in vitro* were fixed in freshly prepared 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) in Phosphate-Buffered Saline (PBS) for 1 h at Room Temperature (RT). The fixed embryos were washed in PBS supplemented with 2% (w/v) bovine serum albumin (PBS-BSA; Sigma-Aldrich) by pipetting through three sequential 50 μL drops, which were then transferred into 0.1 M glycine (Sigma-Aldrich) in PBS-BSA for 5

min at RT to neutralize aldehydes. After washing again in PBS-BSA (as above), embryos were permeabilized in 0.1% Triton X-100 in PBS for 10 min at RT, then washed a third time in PBS-BSA. The embryos were incubated overnight at 4°C with a 1:100 dilution of mouse monoclonal anti-HSP70 antibody (ab5439; Abcam). Negative controls were treated with PBS-BSA alone. Embryos were then washed and incubated with a 1:100 dilution of goat anti-mouse IgG (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, P. R. China). Embryos were washed and counterstained with Hoechst 33258 (0.5 μg/mL; Santa Cruz Biochemical Co., Santa Cruz CA, USA) to stain the cell nuclei.

Embryo Electroporation With siRNA

After confirmed the effectiveness of embryo electroporation for small interfering RNA (siRNA) transfection, we followed the method reported by Zhang SM et al. [17]. During embryo electroporation, mouse morulae from experimental group were transferred to prewarmed droplets of M2 medium covered with paraffin oil and were electroporated to deliver HSP70-siRNA(100 nmol/L; Ribo Biology Co, Guangzhou, China) or control-siRNA(100 nmol/L; Ribo Biology Co, Guangzhou, China) into their cells in an electrode chamber in 50 ml of HEPES-buffered saline (150 mM NaCl, 20 mM HEPES) by a series of direct electrical square pulses (ie, 40 DC voltage/1 ms pulse length/6 pulses) using a 600 Electro Square Porator (BTX Inc, New York, USA).Following electroporation the embryos were washed and cultured in fresh M2 media for about 2 hours and the survival rate of electroporated embryos was calculated. After 24 hours cultured *in vitro*, the formation rate of blastocysts was calculated.

Embryo Transplantation Assay

After transfectedHSP70-siRNA by electroporation, morulae were cultured *in vitro* for 24 hours. And then the blastocysts were transferred into the bilateral uterine horns of 3 recipient mice on pseudopregnant day 4. The implantation embryos were observed after 72 hours.

Statistical analyses

The blastocyst formation rate and blastocyst adhesion rate were evaluated with the χ^2 test. The HSP70 mRNA level was calculated as the mean ± Standard Deviation (SD) and differences were analyzed by t-test and u-test. All statistical analyses were performed using SPSS version 17.0 (IBM Corp., Armonk, NY, USA) and statistical significance was defined as $P< 0.05$.

Results

The blastocyst formation rate of two-cell embryos of CUMS mice cultured *in vitro* for 48 hours

We obtained 76 two-cell embryos from 4 mice of the control group, and 94 two-cell embryos from 9 mice of the experiment groups (CUMS mice). Data on the development of two-cell embryos cultured *in vitro* for 48 hours are shown in Table 1. Compared with the control group (52.6%), the blastocyst formation rate in experimental group (38.2%) decreased significantly ($P< 0.05$).

Expression of HSP70 in CUMS mouse embryos cultured in vitro for 48 hours

When the embryos were cultured *in vitro* for 48 hours, we selected 6 blastocysts of the control group and6 of the experimental group for immuno fluorescence staining. We found that HSP70 was distributed mainly in the embryo cytoplasm *in vitro* (Figure 1). Meanwhile we detected 30 blastocysts of the control group and 30 of the experimental group for RT-PCR. The results showed that compared with the control group (5.01±2.11), the level of HSP70 mRNA in experimental group (3.35±1.23) decreased significantly ($P< 0.05$); see Table 2.

Table 3: HSP70 mRNA level in CUMS mouse' morulae which were transfected HSP70-siRNA by electroporation.

Groups	No. morula	$2^{-\Delta\Delta CT}$
No electroporation group	36	3.71±1.29
HSP70-siRNA group	38	1.01±0.58
Control-siRNA group	36	3.27±1.56
P value		< 0.05

Data are shown as the mean ± SD and were analyzed by u-test.

Expression of HSP70 in CUMS mouse' morulae which were transfected HSP70-siRNA by electroporation

In this section, we cultivated 235 2-cell embryos from 20 mice of the experiment group *in vitro*. Twenty-four hour later, we obtained 110 morula and divided them into HSP70-siRNA group (n=38), control-siRNA group (n=36), and no electroporation group (n=36). After transfected siRNA to cytoplasm by electroporation, we cultivated these embryos in M2 medium 2 hours and then detected HSP70 mRNA by RT-PCR. The RT-PCR results showed that the level of HSP70 mRNA in the HSP70-siRNA group (1.01±0.58) was significantly lower than that in the no electroporation group (3.71±1.29) ($P< 0.05$), which indicated that the method of transfectingHSP70-siRNA into cytoplasm by electroporation was effective.

Embryo Transplantation Assay

Thirty-two blastocysts were obtained from 55 morula which were transfected HSP70-si RNA by electroporation. These blastocysts were transfected into the bilateral uterine horns of 3 recipient mice on pseudopregnant day 4. Statistical analysis showed that the embryo implantation rate in experimental group (18.8%) was significantly lower than in control group (50%, $P< 0.05$); see Table 4 and Figure 2.

Discussion

Embryos produced *in vitro* are generally inferior in quality with lower developmental competence compared with their *in vivo* counterparts [18,19], despite the tremendous advances in culture media and conditions in recent years [20-22]. In our previous study, we established the CUMS animal model with Kunming female mice and found that these mice had decreased ovarian response and oocyte development potential *in vivo* [15]. In this study, we similarly observed that the two-cell embryos from CUMS mice exhibited decreased blastocyst formation *in vitro*.

HSP70 is the most important and well-studied protein of all HSP families. A variety of HSP70s are expressed from the point of zygotic gene activation in cleavage-stage embryos, through blastulation, implantation, gastrulation, neurulation, organogenesis, and on throughout fetal maturation [23]. HSP70 induction in the culture system is known to improve embryo quality and efficiency [24]. In this study, we cultivated the CUMS two-cell embryos *in vitro* for 48hours and found that the HSP70 level in these embryos was lower than that in the control group. We inferred that the stressed embryos had decreased induction of HSP70 *in vitro*.

Previous studies had been demonstrated that the method of transfection siRNA into cells by electroporation was effective [25,26]. In this study, we transfected HSP70-siRNA into morulae by

Table 4: Implantation rate of the blastocyst that was transfected HSP70-siRNA by electroporation.

Groups	No. mice	No. 2-cell embryos	No. morula	No. blastocysts	No. implantation (%)
Control group	3	61	48	34	17 (50%)
HSP70-siRNA group	10	115	55	32	6 (18.8%)
P value					<0.05

Data are shown as rates and were analyzed by χ^2 test.

electroporation to down-regulate HSP70. Our results found a low HSP70 mRNA in morulae after the electroporation, so we believe that the method was successful. Functional experiment showed that the blastocysts that were transfected HSP70-siRNA by electroporation had a lower implantation rate.

A limitation of our study is that we only explored one factor' (HSP70) potential protection on embryos from the stress. But as we know, the relationship between the Hypothalamic-Pituitary-Adrenal (HPA) and the Hypothalamic-Pituitary-Gonadal (HPG) axes was very complex [27]. In our next study, we will explore whether HSP70 plays its protective role in embryos according to interact with carboxyl terminal interacting protein (CHIP) [28], and whether cortisol response happens in this condition. Above all the embryos of CUMS mice exhibited decreased development potential *in vitro* and a low expression of HSP70 mRNA. When transfected HSP70-siRNA by electroporation, the embryos of CUMS had lower level of HSP70 mRNA and implantation rate. So we concluded that inducible HSP70 may play a protective role in the CUMS embryos *in vitro*.

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Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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