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An oligonucleotide microarray study on gene expression profile in mouse testis of experimental cryptorchidism

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1. ABSTRACT

To investigate the germ cell apoptosis under body temperature in testis, we analyzed the gene expression patterns on day 1, day 4, day 7, day 14, day 28 and normal control adult mouse testis after experimental cryptorchidism (EC) using Affymetrix MOE430A microarray. Our data showed that EC led to the oxidative stress and gene expression fluctuation in the first 28 days, both of which were highly coincident in timing. Cryptorchid testis showed more effective antioxidative capability in the first 4 days, and suddenly lowered the capability from day 5 on, then gradually restored the antioxidation from day 10 to day 14, and turned to worse on day 28 again. The extensive high gene expression on day 4 after EC and the up-rising of oxidative stress level on day 5 and the abrupt down-regulation of the gene on day 7 were closely related. From the chip data, we have found that the high level of reactive oxidative species (ROS) was not only related to the dysfunction or abnormality of the direct origin of ROS generation, but also related to the abnormality of the more upstream physiological events in energy metabolism, lipid metabolism. The selective regulation of metabolic substrate transporter in different cell population implied the existence of various regulation of the selective signal pathways among different cell populations by EC.

2. INTRODUCTION

Germ cells consume a lot of carbohydrates, lipids and sterol hormones to proliferate and differentiate, and at the same time, the cells generate quantitative metabolic wastes among many of which are harmful oxidative wastes. Reproduction as a whole is a process that is exposed to severe oxidative stress at various stages (1). Owing to the blood-testis barrier, the germ cells at later stage, such as spermatocytes and spermatids, meet their most demands of energy and building materials and the metabolic waste cleanup through Sertoli cells (2). In this context, spermatogensis and spermiogensis physiologically lack the capability of “paddle one's own canoe”. Germ cells are being put under the control of Sertoli cells and other somatic cells (3, 4). Therefore, when testis is afflicted with stress, germ cells will be the prime victim. Even at body temperature, testis can only withstand for several days in abdomen operated by EC. Concomitantly germ cells perform a large wave apoptosis (5).

We propose that body temperature itself does not directly injure the genes in rodent testis since the spermatogenesis is normal in birds, hyraxes and cane mouse (Zygodontomys brevicauda) at the body temperature or near body temperature (6-8). Deep-body temperature induces only a partial suppression of function in the

inguinal testis of natural cryptorchidism as compared to experimental cryptorchidism. Sensitivity to body temperature of testis also varies among different species.

There is no absolute distinction between the scrotal and ascrotal states among mammals with respect to the temperature sensitivity of the testis (9). However, temperature might exert its influence through other factors such as oxidative stress indirectly on germ cells as previously reviewed (1, 10, 11). The energy metabolism is also another possible factor that affects the apoptosis of germ cells (12). But why germ cell apoptosis takes place only within a few days after experimental cryptorchidism? Which kind of physiological process would be affected by the heat stress? How do different genes response to hyperthermia? For such questions, there is no comprehensive analysis and answer available in the literatures owing to the complexity of the pathological changes in cryptorchid testis.

Study on the whole testis in vivo means to deal with various populations of germ cells and somatic cells simultaneously. It brings many technical problems to analyze the data. In vivo analysis, however, can bring the more actual physiological changes than that in vitro. In this report, we used the whole testis as the subject combining with oligo DNA arrays to investigate gene expression profile in the testis of experimental cryptorchidism. Our results show that EC changes the oxidative status and energy metabolism in the cryptorchid testis. Our data provide the preliminary and valuable information for further understanding the mechanism of germ cell apoptosis induced by heat stress.

3. MATERIALS AND METHODS

3.1. Animals and Unilateral EC

Male CD-1 mice, 10 weeks old, were housed singly in polycarbonate cages with a bedding of pine shavings, and they had free access to food and water in a controlled environment. The mice were randomly divided into 10 groups (group n = 8; including control group). The unilateral cryptorchidism was experimentally induced in the mice under pentobarbital anesthesia by dividing the distal gubernaculum on the right side and displacing the testis inside the abdominal cavity, followed by suture of the inguinal canal. The right testis remained in the scrotum as the control which underwent a sham operation. The mice were deeply anesthetized by an overdose of pentobarbital anesthesia and killed by cervical dislocation, both testes were removed at intervals of 1, 2, 4, 5, 6, 7, 10, 14, and 28 days post operation. After testicular weights were recorded, the same treated tissues were pooled and cut into small parts for storage in nitro liquid or for fixation. The protocol for animal experimentation was approved by the Animal Research Center Committee of Beijing. All experiments adhered to Guidelines for Animal Experimentation of Institute of Zoology, Chinese Academy of Sciences.

3.2. Affymetrix Microarrays

8 unilateral cryptorchid testes and 8 normal contol testes were pooled respectively for RNA isolation. Total RNA was prepared with TRIzol Reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. DNase-I treated RNAs were purified further using the RNeasy Mini Kit (QIAGEN). Total RNA was used to synthesize double-stranded cDNA using Superscript Choice System incorporating a T7 RNA polymerase promoter. Biotin-labeled antisense cRNA was prepared using the Enzo RNA Transcript Labeling Kit (Affymetrix). cRNAs were purified with the RNeasy columns. The fragmented samples were hybridized with the Affymetrix Test3 Arrays and the mouse MOE430A GeneChips and scanned with G2500A GeneArray Scanner (Affymetrix). This array contained probe sets (oligos) designed to interrogate mainly full-length genes with previously identified functions representing approximately 12,000 characterized full-length genes as well as thousands expressed sequence tags (ESTs).

3.3. Data Analysis

The data of untreated versus treated samples were analyzed using the Microarray Suite Version 5.0 with the default parameters. Algorithms in the microarray analysis suite (Affymetrix) were used to determine whether the expression of each transcript was present, absent or marginal in the sample. Transcripts designated as absent in all samples, both treated and control, were excluded from further analysis. Mean signal values were computed by experimental group, and the differences between the mean signal value of each treated group and the control group was calculated. Fold change values were calculated for each treated sample signal against the control mean signal. Genes displaying either increase or decrease signal were selected for further analysis only if they meet the following filtration criteria: (1) Treated or control detection should be present ($p < 0.05$). (2) Fold change should be equal to or greater than 2 (signal $\log_2$ ratio mean value$\geq$1 or $\leq$-1) in either group. (3) Signal value in either treatment or control should be equal to or greater than 100. All gene annotations and symbols can be referred to web site: http://www.affymetrix.com/ and http://nciarray.nci.nih.gov/cards/. All the significant changed genes from each category of genes were subjectively chosen to draw dendrogram in GEPAS server (http://gepas.bioinfo.cnio.es) (13).

3.4. Total antioxidative capacity (TAC), malondialdehyde (MDA) and tissue sulfhydral assay

Testis tissue was homogenized in 0.9% NaCl water and centrifuged at 10,000 g for 5min to save the supernatant for assay. Total antioxidative capacity was performed using a total antioxidative capacity (T-AOC) detection kit (Nanjing Jiancheng Bioengineering Institute, China). The method was based on the reduction of the Fe3+-TPTZ complex to the ferrous form at low pH. This reaction was monitored by measuring light absorption at 520 nm. Malondialdehyde (MDA) was a stable product of lipid peroxidation and therefore was used as a measure of cumulative lipid peroxidation. MDA assay was performed with thiobarbituric acid test in the supernatant using a Malonaldehyde Detection Kit (Nanjing Jiancheng Bioengineering Institute, China), according to previous method (14). MDA reacted with thiobarbituric acid to give

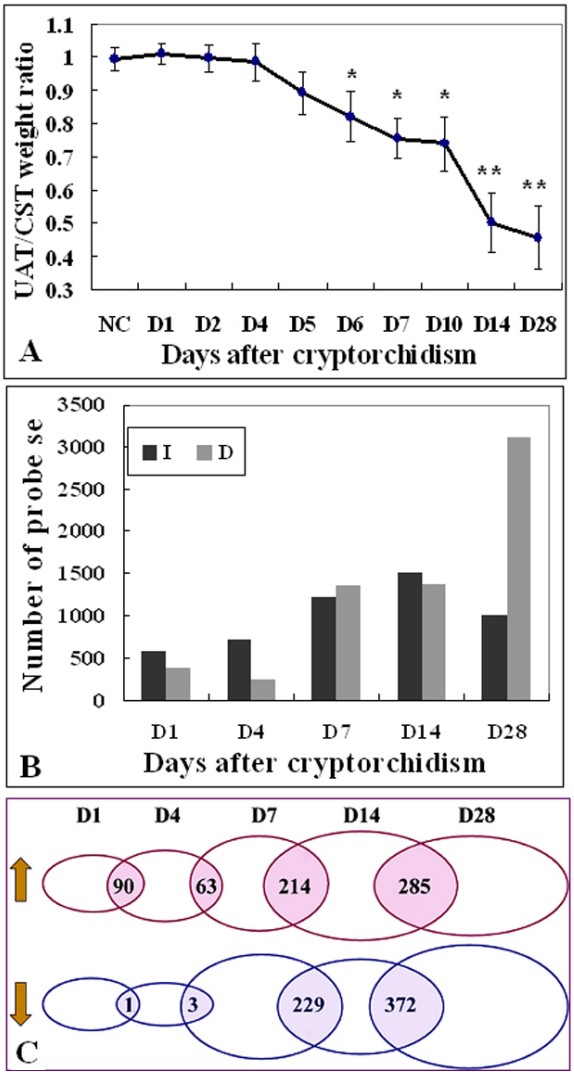

Figure 1. (A) The unilateral abdomen testis (UAT) weight to contralateral scrotum testis (CST) weight ratio on day 1, 2, 4, 5, 6, 7, 10, 14, 28 and NC (normal control) after EC operation on CD-1 mice. Results are shown as the mean±SD. Values significantly different from the normal control group were labeled with asterisk (*, $p<0.05$ **, $p<0.01$) (one-way ANOVA). (B) Number of genes significantly up-regulated (signal log ratio (SLR) ≥1) and significantly down-regulated genes (SLR ≤-1) on day 1, 4, 7, 14, 28 after EC. (C) The numbers in the space between overlapping elliptical circles represent the number of probe sets that were all significantly changed in the adjacent treatments. Number of probe sets in each treatment was represented by the size of respective circle. Up arrow denoted up-regulated probe sets, down arrow denoted down-regulated probe sets. IC, increase; DC, decrease.

a red compound absorbing at 532 nm. The results were expressed per unit weight of tissue. One unit was defined as the capacity of per microgram homogenized tissue gains 0.01 OD (optical density) with light absorbance in one minute at 37^{o} C.

Thiols were determined according to the standard "Ellman's Test" (15) using a hydrosulfuryl (-SH) detection kit (Nanjing Jiancheng Bioengineering Institute, China). Absorbance at 412 nm was recorded and calculated according to manufacture's guidelines.

3.5. Semi-quantitative RT-PCR

Total RNA (2 μg) was reverse-transcribed in a final volume of 20 μl containing 100 units of reverse transcriptase Superscript III (Invitrogen), 30 units of RNasin, and oligo-dT primer. To increase the sensitivity and reproducibility of semi-quantitative RT-PCR, we prolonged the cycles in the exponential phase by diluting the cDNA template to 0.5μl, 0.1μl and 0.02μl of the reverse product using gene specific primer pairs in 25μl amplification system. All results showed were generated from the system with 0.1μl cDNA templates. Primer pairs were shown in table 1.

3.6. Criterion for estimating changes in gene expression by EC

To study the effects of EC on germ cell apoptosis, the most difficult problem we faced was to define the changes in the gene expression since the down-regulated and the up-regulated genes in apoptotic cells might be a result of cell loss in certain cell populations in the testis. Two extreme criteria (high and low) were used to define the gene increasing and decreasing level. The high criterion (criterion for increasing, C-I =-log2 (UAT/CST)) was used to define the change of genes for those exclusively expressed in unaffected cell populations, especially in somatic cells and spermatogonia. The change in the value which was higher than or lower than C-I value in each treatment could be regarded as the up-regulated or the down-regulated genes.

The low criterion (criterion for decreasing, C-D) was another uttermost standard for estimating the changes in down-regulated genes. The criteria above or below the mean value of C-D was regarded as up- or down-regulated genes**.** The genes exclusively expressed in late stages of spermiogenesis would be an uttermost criterion for gene down-regulation as observed from Figure 3. Therefore, we utilized the values of the average change in the levels of germ cell-specific gene expression at the late stage on each time point as the criteria as shown in table 2. The more genes we used, the more precise could be obtained.

However, as to the more extensively expressed genes as those related to antioxidation, we have to utilize the data of NC as the standard to evaluate the profile of the gene expression.

4. RESULTS

4.1. The changes in the weight of the unilateral cryptorchid testis and the gene expression pattern

Figure 1 showed the testis weight ratio (TWR) of the experimental mice at various time points after unilateral cryptorchid operation. The initial obvious drop in TWR started from day 5 and continued to decline abruptly in the

Table 1. Oligonucleotide primers used for RT-PCR

Gene symbol	5' primer	3' primer
Txn1	5′-GCTTGTCGTGGTGGACTTCTC-3′	5′-GCTTTTCCTTGTTAGCACCG-3′
Gsta4	5′-AACCCCCAAGGAAAAAGAGG-3	5′-CACCTTAAAGCACGCTGCAC-3′
Gstp2	5′-TGGTGAATGATGGGGTGGAG-3′	5′-GGGAAAACGGGGACAAGAAG-3′
Gpx3	5′-GAGCCCAAAGGAAACACAAGT-3′	5′-CCTGGGAGCCTAAGCCTGA-3′
Cbr2	5′-GTGCCAGGGTCCATTGTCA-3′	5′-ATGAGGGCGGAGTCACGAG-3′
Gclm	5′-GGCTTCGCCTCCGATTGA-3′	5′-ACCGAGTACCTCAGCAGCCA-3′
Gss	5′-TGACCAGCGTGCCGTAGA-3′	5′-CTCTGGGGCTTCAGCACAA-3'
Bcdo1	5′-GTTTGCTGTGCCCCTCCA-3′	5′-TCAGTTCTGGTGCTGTCGGA-3′
Rbp1	5′-CCTTACGCAAAATCGCCAA-3′	5′-GGTGGGTATGCGTTTCGGT-3′
Ttpa	5′-AAACACCTCTGGCTTATCAACC-3′	5′-GCAGCACGGTGAGCACCTAT-3′
Cygb	5′-CCGAGGAATTTGCCAATGA-3′	5′-GAAGAGTGTGCCCCTGCTGT-3′
Slc2a1	5′-GAGACCTCTTCCGAACCGAC-3′	5′-AGAGACAGGAATGGGCGAAT-3′
Slc2a3	5′-AACTTTCTGGTCGGAATGCTC-3′	5′-TGGAGTGTGTGGGGTGAGAAT-3′
Slc16a7	5′-GCCATCTCTTATGCCCCCT-3′	5′-GGGAGGATTGTGTGCGTTTG-3′
Hk1	5′-GACCCCCCTCTTGGACTGT-3′	5′-AGATTCCAGTGATGGCGTTG-3′
Pgd	5′-GTGTTGGCTGTGATTGCTGAA-3′	5′-GTGCCAGTAAGAAATACCCGA-3′
Idh1	5′-TGATGGGCGTTTCAAAGACA-3′	5′-AGCCAGCCTCAATGGTCTCA-3′
Abca1	5′-GGTGTCTGTACCGCAAGCAT-3′	5′-TAGGGCAGGAGGGAAAAGAA-3′
Scd2	5′-GGCGAGGGCTTCCACAACT-3′	5′-ACTTCCGATTTTTGTCCGTTTT-3′
Fads2	5′-CTCCTGTCCCACATCATCGTC-3′	5′-GCTCCCAAGATGCCGTAGA-3′
Fasn	5′-CTATGGCGAGGACTTGGGTG-3′	5′-GTAGAAAAGGCTCAGTTTGGCT-3′
Hmgcs1	5′-CACAGAAGGACTTACGCCCG-3′	5′-CCAGACCACAACAGGAAGCAT-3′
Dhcr7	5′-GGATTGTAGCCTGGACCCTC-3′	5′-CCAGAAGCCTGAGACCAACA-3′
Fdxr	5′-GCTGCTGGAGGACCTGAAG-3′	5′-GCACGGTTCGGCGTCTAA-3′
Cyp11a1	5′-GTGAATGACCTGGTGCTTCG-3′	5′-AGGATGAAGGAGAGGAGAGCC-3′
Star	5′-GCAGGAACAAAATCTTGGGAGT-3′	5′-TAAAGTCGTGTGCCACCGTC-3′
Hsd17b3	5′-TCCCAAGCCATTTCCTGAG-3′	5′-GGCTTCCCCCACTATCTGTT-3′
Shbg	5′-AAGCGTGGTTCTGTCTTCCG-3′	5′-CCCGATTCTCCCAACTTCAC-3′
Ptgis	5′-CACACGGTCTGGCAAGCA-3′	5′-CAGCCGTTTCCCATCTTTG-3′

Table 2. The expression profiling of selected genes expressed specifically in later stages of spermatogenesis

Gene Title	Gene Symbol	D1	D4	D7	D14	D28	Accession NO.
preproacrosin	Acr	-0.3	-0.1	0.3	-0.7	-1.4	M85170
proacrosin binding protein	*Acrbp*	-0.3	0.1	-0.1	-0.3	-1.7	D17574
cAMP responsive element modulator	*Crem*	-0.2	0.2	-0.2	-0.6	-0.9	AI467599
histone H1-like protein in spermatids 1	*Hils1*	-0.1	0.1	-0.2	-0.3	-1.5	NM_018792
histone 1, H1t	*Hist1h1t*	0	0	-0.2	-0.3	-2.3	NM_010377
mitochondrial capsule selenoprotein	*Mcsp*	0	0.5	0.2	-0.4	-2.5	NM_008574
outer dense fiber of sperm tails 1	*Odf1*	0	0.5	0	-0.7	-2.2	NM_008757
outer dense fiber of sperm tails 2	*Odf2*	0	0.4	-0.4	-0.2	-1.6	AF000968
outer dense fiber of sperm tails 3	*Odf3*	-0.3	0	-1.1	-1	-2	NM_027019
protamine 1	*Prm1*	0.3	0.7	0.8	0.4	-1.5	AV209063
protamine 2	*Prm2*	-0.1	0.5	0.1	0	-1.9	NM_008933
protamine 3	*Prm3*	-0.2	-0.1	-1.1	-0.8	-3.1	NM_013638
sperm motility kinase 2	*Smok2*	0.5	1.2	1	0.8	-1.7	AJ245454
sperm autoantigenic protein 17	*Spa17*	-0.1	0.1	-0.2	-0.4	-1.4	NM_011449
sperm associated antigen 1	*Spag1*	0.2	0.2	-1.1	-0.2	-1	NM_012031
sperm associated antigen 5	*Spag5*	-0.1	-0.2	-0.5	-0.3	-0.9	BM208112
sperm associated antigen 6	*Spag6*	0.1	0.2	-0.2	0.1	-0.9	NM_015773
sperm associated antigen 7	*Spag7*	-0.2	0.2	-1.1	-0.5	-1.6	BB239907
spermatid perinuclear RNA binding protein	*Spnr*	-0.2	-0.1	-2	-0.4	-0.1	AK006314
sperm specific antigen 1	*Ssfa1*	0	-0.1	0.3	-0.4	0.4	NM_011482
sperm specific antigen 2	*Ssfa2*	0.1	0.1	-0.8	0.8	0.4	BB033597
testicular haploid expressed gene	*Theg*	-0.3	0.3	-0.8	-1.3	-2.5	AB033128
transition protein 1	*Tnp1*	-0.1	0.4	-0.3	-0.1	-1.5	NM_009407
transition protein 2	*Tnp2*	0	0.4	0.1	0.1	-2.4	NM_013694
zonadhesin	*Zan*	-0.1	0.4	0.3	-0.4	-1.9	NM_011741
zona pellucida glycoprotein 2	*Zp2*	-0.4	0.1	-0.1	0.4	-1.6	NM_011775
zona pellucida glycoprotein 3	*Zp3*	0.1	0.3	-2.3	-0.5	-3.4	NM_011776
zona pellucida 3 receptor	*Zp3r*	-0.4	-0.2	-1.7	-1.4	-1.8	NM_009581
Mean value		-0.07	0.21	-0.40	-0.29	-1.59	

Data were shown with mean signal $\log_2$ ratio value, the mean change value was used as criterion (C-D) for determining gene expression levels at the later time points after EC.

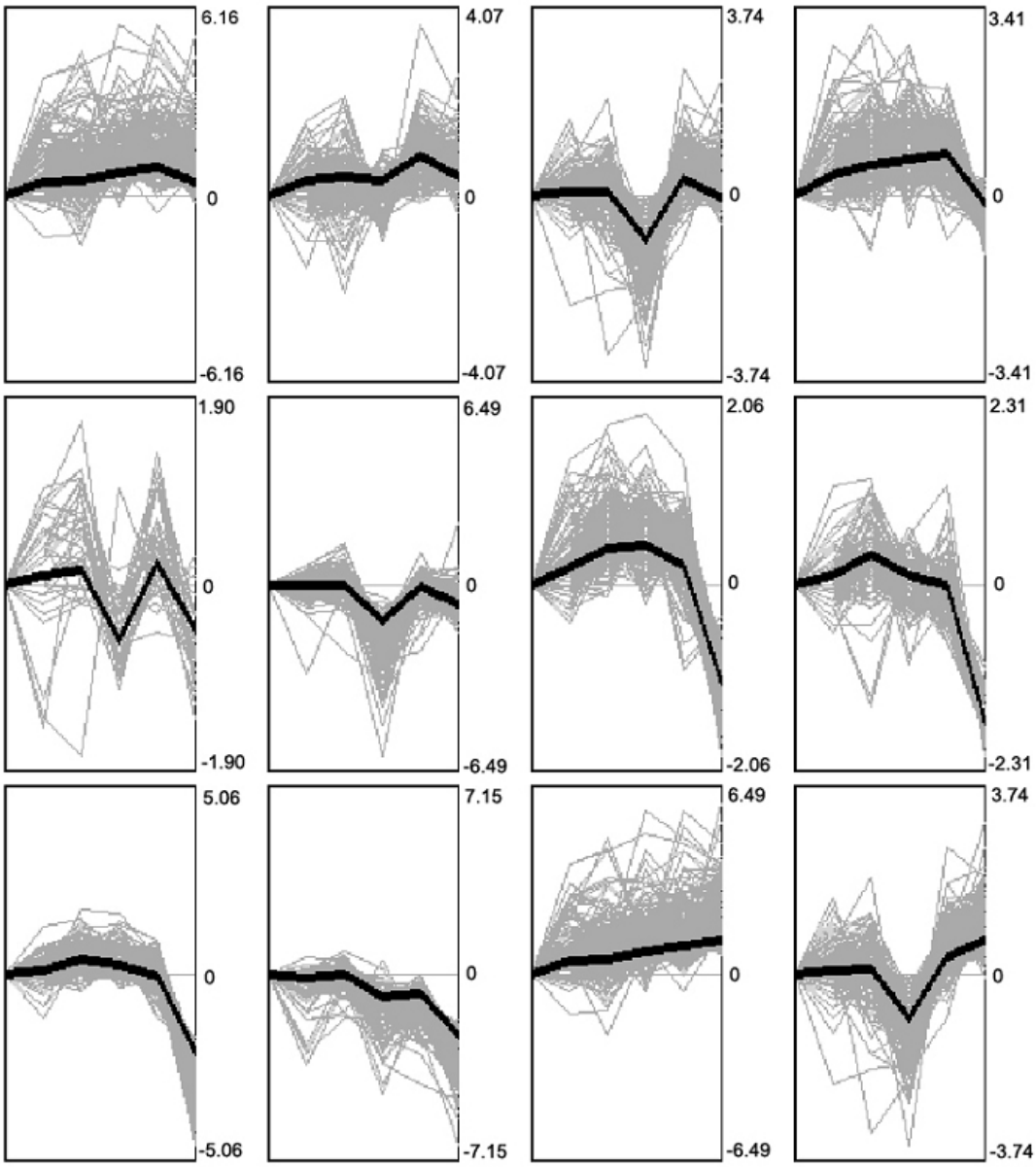

Figure 2. The gene expression patterns in testis after EC. The clustering was performed by using program SOM-Tree. 12 clusters can be divided from the significantly regulated genes according to the program.

first 14 days, then reached a platform with a slight drop from day 14 to day 28. TWR on day 7 was significant different from that on day 14 ($p<0.01$). According to the previous reports, the decline of testis weight was closely associated with germ cell apoptosis (16). The germ cell apoptosis was accompanied by a great change in the gene expression patterns as shown in Figure 1B, 1C. On Day 4, the numbers of significantly up-regulated genes surpassed that of being down-regulated. The number of the up-regulated genes increased stably after the induction of cryptorchidism in the first 14 days, while that of down-regulated genes presented two abrupt peaks on day 7 and day 28 respectively. Significantly regulated genes in the all treatments amounted to 5299 probe sets. It occupied about 24% of the total probe sets on MOE430A chip.

12 clusters could be divided from the significantly regulated genes according to the SOM-Tree program on GEPAS server (Figure 2). But the distinct clusters did not represent distinct categories of genes which showed the extensive and intricate effects of EC on testis.

4.2. Germ cells from late steps were affected mostly by EC

As shown in Figure 3, elongating and elongated spermatids were hardly observed in the lumen of the seminiferous tubules from the histological sections on day 7 to day 14, indicating that the spermiogenesis was almost stopped at the late stages. Germ cell differentiation was almost stagnated at the round spermatid steps and seldom reached the elongating and elongated spermatid steps

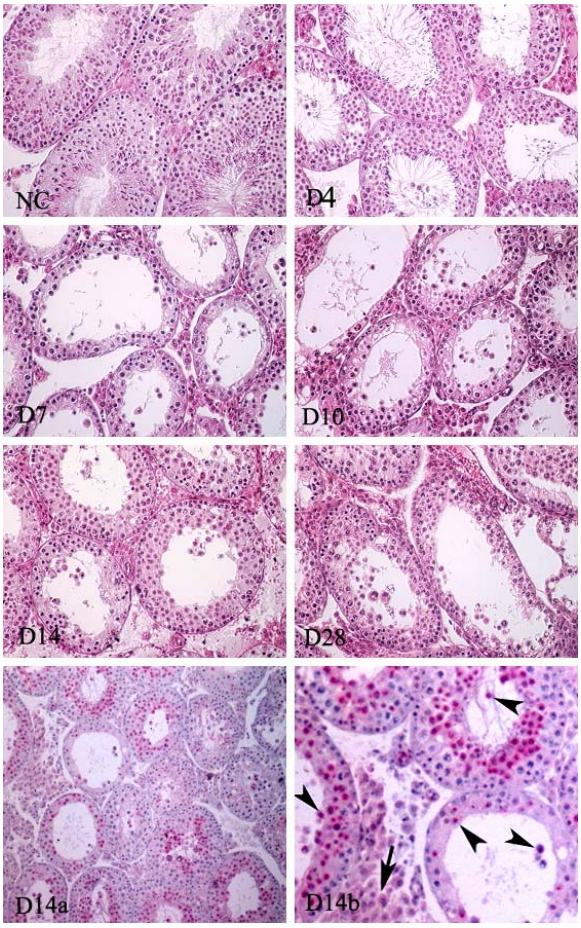

Figure 3. Hematoxylin-eosin (HE) staining and acrosome staining the testis tissue slides on day 4, 7, 10, 14, 28 and NC (normal control) after EC operation on CD-1 mice. D14a and D14b were stained with acrosomes (red) to visualize the round and elongating spermatids by anti-Afaf antibody, which was localized predominantly in the acrosome in mouse testis (data will be published in another paper). The slides were then incubated with biotin-coupled secondary antibody and alkaline phosphatase-conjugated streptavidin and color were developed with alkaline phosphate red substrate (Vector Laboratories). In the inner side of the lumen, round spermatids were seen in most seminiferous tubules with some easily being detached to the lumen (arrow head). More interstitial cells were seen (arrow). All slides were Bouin's fixative fixed and paraffin-embeded. D4, D7, D10, D14, D28, D14b and NC, 100×amplification; D14a, 40×amplification. D14b was partial amplification of D14a.

during spermiogenesis. From day 7 on, more germ cells could be observed with detachment from the seminiferous epithelium. The pathological process, therefore, could be utilized in this study to make a criterion below for estimating the changes in gene expression after EC.

4.3. Oxido-redox-associated gene expression were affected significantly and extensively by EC

The genes including DNA transcription, cytoskeleton, metabolism, transport, stress response, immune defense, and signal transduction were all affected extensively in cryptorchid testis in the first 28 days (data were not shown). Among the various categories of the genes, we found that antioxidant and redox-related genes, such as thioredoxin 1 (Txn1) and glutathione peroxidase 3 (Gpx3) (Figure 4A, Figure 5A) responded to the treatment more rapidly and persistently. We chose all the significantly changed redox-related genes and analysed on the GEPAS server to get the cluster dendrogram as well as their expression profiles as shown in Figure 4 B. Thioredoxin (Trx) and glutaredoxin (Grx) systems in the cell cytoplasm and mitochondrion were affected significantly. Most of the genes involved in GSH metabolism increased steadily on day 7, day 14, day 28, indicating that the testis was afflicted under severe oxidative stress at the corresponding time points. Gclm (glutamate-cysteine ligase, modifier subunit) was the key enzymes participating in the synthesis of GSH, the actual protecting enzymes against oxidative stress (Figure 4 A, Figure 5B). Its reduced expression also meant the severe affection of cryptorchidism on the de novo synthesis of GSH. However, Gss was up-regulated in our RT-PCR results contrary to DNA array data. Bcdo1 (beta-carotene 15, 15'-dioxygenase 1), the limiting enzyme in the synthesis of retinol, was also reduced, while at the same time the expression of retinol binding protein 1 (cellular, RBP1) and tocopherol (alpha) transfer protein (Ttpa) were enhanced, indicating shortage and/or larger consumption of non-enzymatic antioxidants (Figure 4A, Figure 5B).

4.4. Biochemical assays verified the existence of high oxidative stress in cryptorchid testis

A normal redox status maintained in testis was crucial for spermatogenesis. DNA array data analysis suggested that the cryptorchid testis was shocked by the oxidative stress. To get direct evidence, we detected TAS, MDA and thiol contents in the mouse cryptorchid testis as shown in Figure 6. The anti-oxidative system of the organism was made up of enzymatic components and non-enzymatic components, such as Vc, Ve, GSH (reduced glutathione), glucose, β-carotene etc. They guaranteed the normal TAS of tissue and cells to scavenge free radicals. The TAS in the cryptorchid testis reached the lowest level on day 5 ($p<0.01$) and lasted in the low levels on day 6 and day 7 ($p<0.05$), increased slightly during day 10 and day 14, then reduced again on day 28 ($p<0.05$) (Figure 6A).

MDA was one of the final products of polyunsaturated fatty acids (PUFA) peroxidation in the cells. An increase in free radicals caused overproduction of MDA. MDA level was commonly known as a marker of oxidative stress and the antioxidant status in organism. MDA level changed slightly in the first 4 days and rapidly reached its highest peak on day 6 in the testis, at the same time the testis weight dropped dramatically (Figure 1A). The MDA peak appeared on day 28, the patterns of TAS and MDA levels were coincident with the down-regulated gene peak and the peak level of germ cell apoptosis (Figure 1B). However, thiol contents were reduced continuously from day 2 on and reached the lowest level on day 28 (Figure 6C). Fluctuation of TAS on day 10 and day 14 did not directly influence the thiol contents.

Antioxidants, rather than thiols, may lead to the rising of TAS level in the testis, implying that the

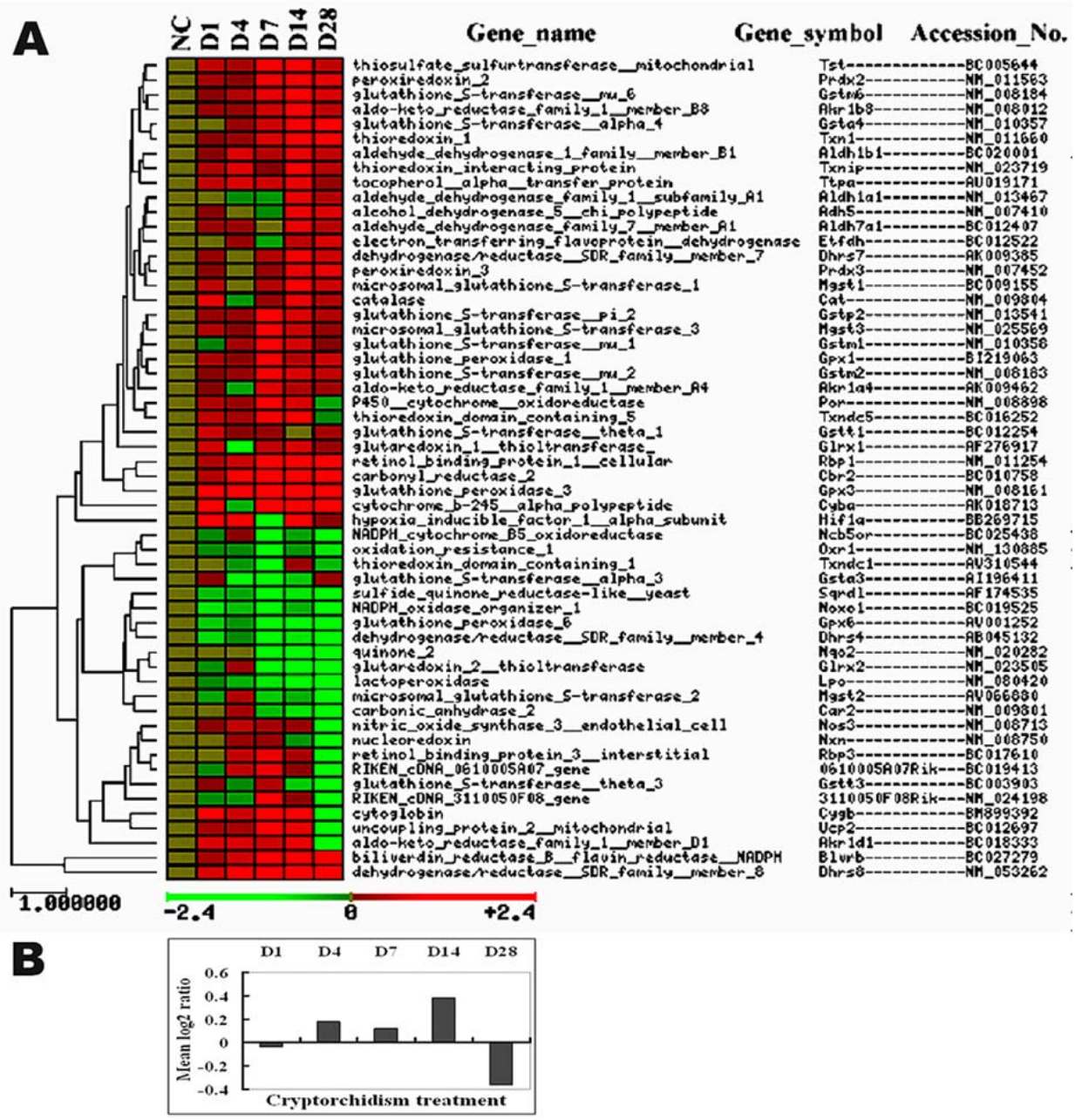

Figure 4. A EC altered expression profiling of antioxidant and redox-associated gene. Significant changed genes in either treatment were chosen to draw the dendrogram. The metric used in the expression profiling was Euclidean distance, and clustering was done by the SOM-Tree method as implemented in the GEPAS server. Significant changed genes in either treatment were chosen to draw the dendrogram. B. The average representative log2 ratio of each treatment. Green and red symbols indicate different patterns in which expression levels of transcripts are decreased (Green) or increased (red) in the indicated scale.

cryptorchid testis may be greatly short of NADPH, the most powerful reducing agent for the regeneration of GSH.

4.5. The direct origins of ROS in cryptorchid testis

Since mitochondrion was the main producer of ROS and RNS (reactive nitride species) (17), the ROS generated by oxygenase was only the partial reason to induce apoptosis in the crytorchid testis. The electron transport chain of oxidative phosphorylation in mitochondrion, therefore was investigated in the DNA chip. We demonstrated that Ndufa1, Ndufa4, Ndufs7, Ndufs8 and Ndufb9 in complex I, Cox6b, Cox6a, Cox6a2, Cox7a1, Cox7a2, Cox7c, Cox8a, Cycs in complex IV (cytochrome oxidase complex) changed significantly (Figure 7, Figure 5C). Body temperature directly or indirectly affected energy

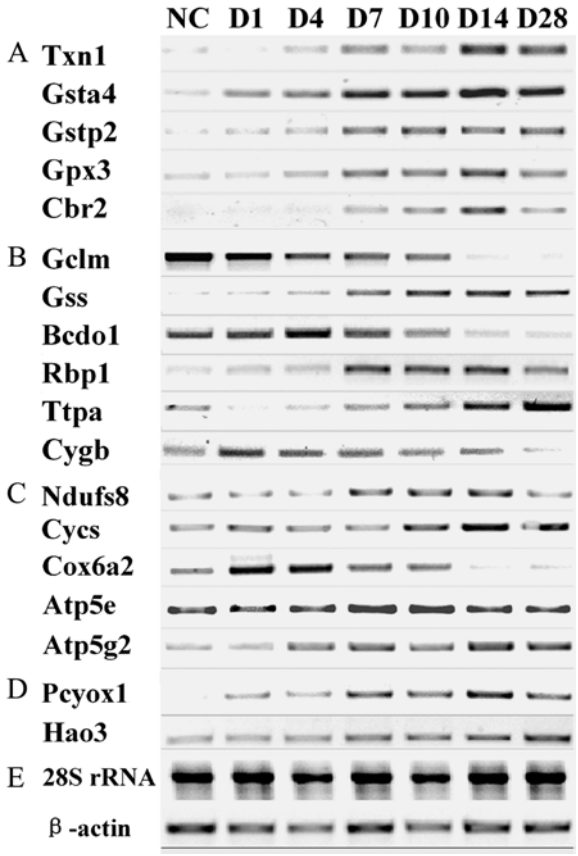

Figure 5. RT-PCR detected the oxido-redox associated gene expression upon EC. (A) RT-PCR verification of thioredoxin and glutathione redox system expression change in cryptorchid testis. Txn1, thioredoxin; Gsta4, GSH metabolism; Gstp2, GSH metabolism; Gpx3, GSH metabolism; Cbr2, carbonyl reductase 2, carbonyl compounds metabolism. (B) GSH, vitamin and oxygen mobilization upon stress in cryptorchid testis. Gclm, GSH biosynthesis, first step; Gss, GSH biosynthesis, second step; Bcdo1, vitamin A biosynthesis; Rbp1, cellular, intracellular transport of retinol; Ttpa, vitamin E binding and transport; Cygb, cytoglobin, intracellular oxygen storage or transfer. (C) Genes selected in oxidative phosphorylation in mitochondrion. Ndufs8, mitochondrial electron transport chain complex I; Cycs, mitochondrial electron transport chain; Cox6a2, mitochondrial electron transport chain complex IV; Atp5e, mitochondrial ATPase complex; Atp5g2, mitochondrial ATPase complex (D) Pcyox1 with catalytic activity: an S-prenyl-L-cysteine + O(2) + H(2)O = a prenal + L-cysteine + H(2)O(2); Hao3 with catalytic activity: (S)-2-hydroxy acid + O(2) = 2-oxo acid + H(2)O(2). (E) Internal controls, all the following RT-PCR results have the same internal controls.

transformation through ROS and the superoxide generation might be via the two sites in mitochondrion.

F0 and F1 complex of ATP synthetase were also affected by the cryptorchidism. The direct evidence related to why testis upon stress of body temperature increased the formation of superoxide in mitochondrion needs to be investigated further.

Another origin of ROS was the various oxygenases and oxidases. They mainly catalyzed phospholipids, sterols and toxicants in cells. Such enzymes used O(2) to detoxify or degrade their substrates at the expense of consuming plenty of reduced flavoprotein such as NADPH, NADH. Pcyox1 and Hao3 have been reported to generate reactive wastes that also could bring injury to the cells (Figure 5D).

4.6. Energy metabolism: another possible origin of ROS production in the upstream

Reductive status was the balance process of oxidation and anti-oxidation. Both were determined by the physiological activity and metabolism direction. Therefore it is suggested that metabolism may be a more important upstream event of ROS stress.

According to DNA microarray data and RT-PCR results, the glucose transport and metabolic enzymes were changed significantly upon EC. Owning to the germ cell loss, gene down-regulation was hard to determine at the later time points from day 7 on after EC. The mean change values below the criteria (C-I or C-D) were regarded as the gene down-regulation. Hk1 in glycolysis path increased initially and then declined, that affected the ATP generation and lactate production at late time points of EC. We demonstrated that the expression of enzymes in glycolysis path changed significantly by the cryptorchidism (data not shown). Uptake of glucose in certain cell types was influenced significantly. Slc2a3/Glut3, which was initially found to be expressed in Sertoli cells, peritubular myoid cells, macrophage-like interstitial cells, testicular endothelial cells and early spermatocytes (18, 19), increased obviously in the first 4 days and declined significantly on day 7, day 28 (Figure 8A). Slc2a1/Glut1 which was initially found in rat testicular peritubular myoid cells, macrophage-like interstitial cells, endothelial cells and spermatocytes (18), our data showed an increased expression at the later time points of the mouse cryptorchid testis that was contrary to the expression profiling of Slc2a3/Glut3. Slc16a7/MCT2, a lactate transporter exclusively to elongated spermatids (20), was also decreased significantly on day 7 and day 28 (Figure 8B).

We found the expression of enzymes in glycolysis path changed significantly by EC. Hk1 (hexokinase 1, rate-limiting enzyme), which was expressed high in late stage germ cells (21, 22), was also declined. Cryptorchid testis still kept high level of phosphogluconate dehydrogenase (Pgd) in pentose phosphate shunt and isocitrate dehydrogenase I (NADP+) (Idh1) in cytoplasm and peroxisome on day 14 and day 28 (Figure 8C).

4.7. Perturbation on lipid synthesis by EC

Sterol, hormone and cytokines are important factors for spermatogensis. Our data demonstrated that lipid synthesis was perturbed extensively by cryptorchidism. Since many sterol related genes were supposed to be expressed in the testosterone secreting cells (Leydig) and

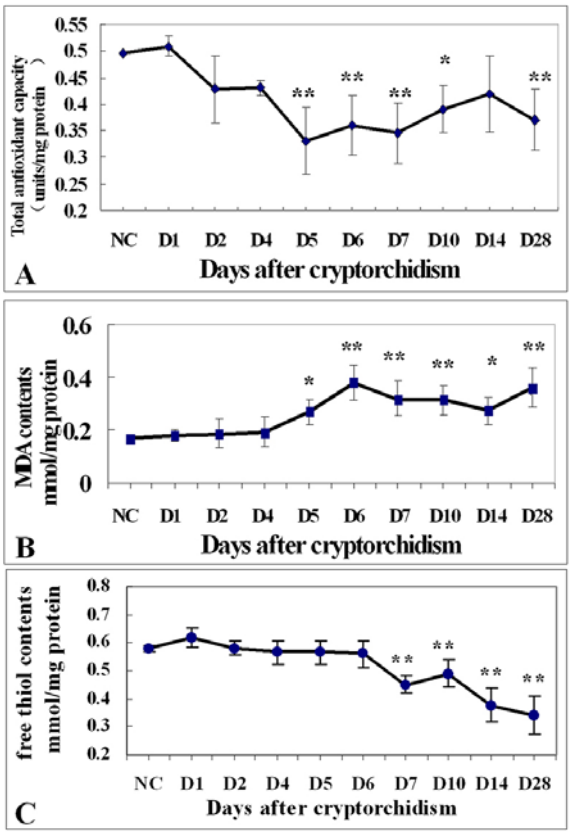

Figure 6. Biochemical assay of the oxidative stress in cryptorchid testis. (A, B, C) Changes in total antioxidant capacity (TAC), malondialdehyde (MDA) contents and thiol contents in testis. Results were shown as the mean±SD. Values significantly different from the normal control group were labeled with asterisk (*, $p<0.05$ □ **, $p<0.01$) (one-way ANOVA).

many desaturated fatty acids and lipid transport mainly in Sertoli cells (23-25), the evaluation of expression change can utilize the C-I criterion. Genes of lipid synthesis especially sterol and sterol hormone synthesis in testis were also reinforced especially on day 14 (Figure 9 and 10). Unsaturated fatty acids peroxidation led to the desaturases such as Scd2 and Fads2 to enhance the gene expression. Lipid transporter (Abca1, Apoe and Hdlbp) and de novo synthesis (Fasn, Hmagcs1 and Lss) increased at the later time points of the cryptorchidism. The genes responsible for steroid hormone synthesis and transport increased extensively especially on day 14. Up-regulation of lipid synthesis reflected the high destroy of lipid by peroxidation. It might be another factor that exacerbated the shortage of reductants and increased the oxidative stress in certain cells of the cryptorchid testis.

5. DISCUSSIONS

Spermatogenesis normally occurs in scrotum below the body temperature in most mammals (26). If being put into abdomen cavity, the testis must extensively adjusted the scale of physiological activity especially energy metabolism to keep a normal level as that in scrotum. On studying the oligo DNA microarray data of cryptorchid testis, we did not find obvious extensive down-regulation of gene expression levels after EC. However, in the initial 4 days, the cryptorchid testis displayed higher gene expression levels concerning the up-regulation patterns especially on day 4. This, however, was related to the concomitant testis weight decline and the rising of oxidative stress. Biochemical assays verified the existence of oxidative stress in cryptorchid testis. TAS declined slowly in the first 4 days and dramatically on day 5 and MDA concomitantly reached its highest level on day 6, which was coincident in timing with the drop of testis weight from day 5 and the large wave of gene down-regulation on day 7 where a large wave apoptosis took place as previous reported (5, 27).

Among the various categories of genes that were changed by EC, genes from redox system were more distinct. Glutethioredoxin (Gr) system and thioredoxin (Tr) system was the prime antioxidative components using GSH as the reductant (28). Many members in the system changed significantly (see figure 4). Gclm was the rate-limiting enzyme in GSH de novo synthesis path and was reduced gradually in the early time points and dramatically on day 14 and day 28. Its expression reduction together with the higher oxidative stress accounted for thiol contents decline after cryptorchidism. GSH must be recycled through de novo synthesis and glutathione redox system and thioredoxin redox system using NADPH as the reductant (28). The reduction of free thiols largely reflected the decline of reduced GSH level and of the methionine residues with reduced thiols. Shifting the GSH toward the oxidizing state GSSG activated several signaling pathways thereby reducing cell proliferation and increasing apoptosis (29). Previous reports also indicated cryptorchid testis had reduced GSH contents and GSH-dependent enzyme activities (11). GSH-dependent enzymes had more power to antagonize the ROS than GSH (30). The decline of GSH-dependent enzyme activity and reduced GSH contents in testis had dual impacts on total antioxidative capability (TAS) in germ cells. Therefore, thioredoxin 1 (TRX1) overexpression can suppress apoptosis in testicular germ cells induced by EC (31). Thiol contents and GSH-dependent enzyme activity decline was an important factor for the milieu deterioration of cryptorchid testis in later time points which also reflected the deficiency of NADPH production and/or NADPH overconsumption in cryptorchid testis discussed further below.

With the more and more consumption of antioxidants such as reduced thiols, vitamins, unsaturated fatty acids, and NADPH, testis had to curb the more and more ROS production. But in mouse testis, there seemed to have not enough capability or efficient means other than lowering the germ cell number by apoptosis to reduce the metabolic scale upon body temperature stress. The similar apoptosis also happened during the first round of spermatogenesis as reported (32), since a Sertoli cell can only support a certain number of germ cells to undergo continuous proliferation and differentiation (33). Testis as a whole was famous for its continuous proliferation and

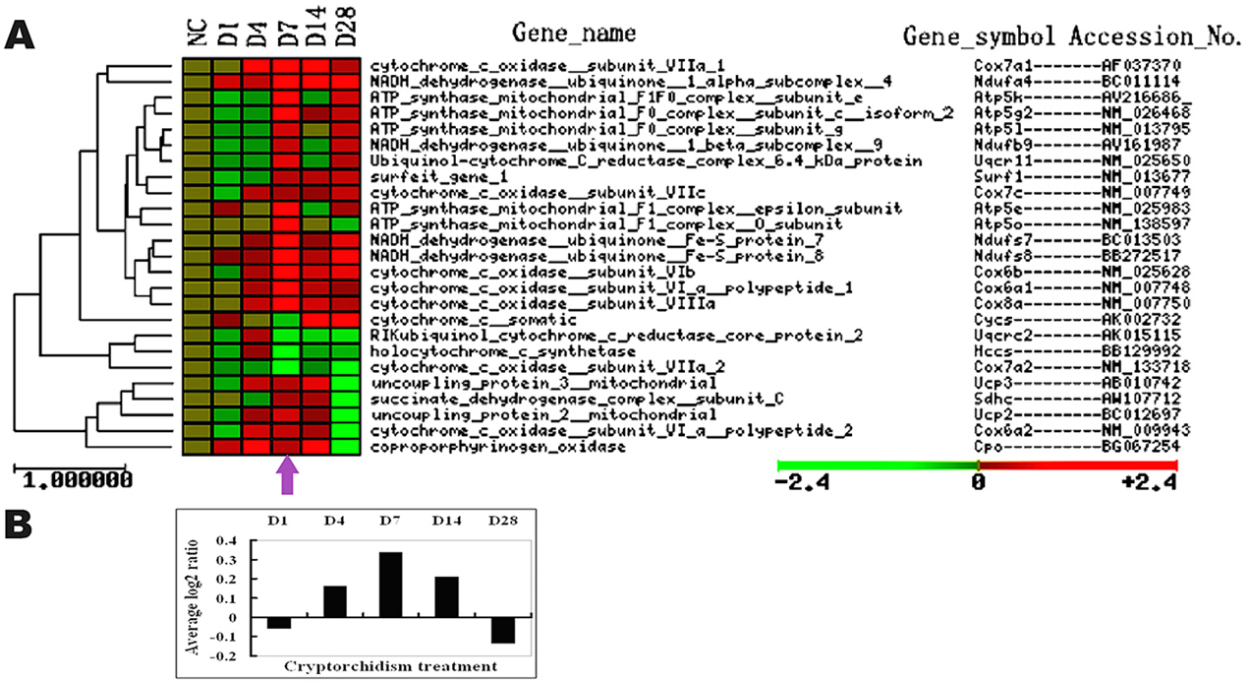

Figure 7. (A) Effects of Cryptorchidism on mitochondrion oxidative phosphorylation in testis mitochondrion by EC. Significant changed genes in either treatment were chosen to draw the dendrogram. Electrons are transported through the electron-transport chain by the enzyme complexes, cytochrome *c* and the electron carrier ubiquinone (Q). Ndufa1, Ndufa4, Ndufs7, Ndufs8, and Ndufb9 in complex I, Cox6b, Cox6a, Cox6a2, Cox7a1, Cox7a2, Cox7c, Cox8a, Cycs in complex IV changed significantly. Body temperature or ROS affected energy metabolism perhaps through the two sites in mitochondrion. F0 and F1 complex of ATP synthetase were also affected by cryptorchidism. Genes in D7 were up-regulated more highly (arrow). (B) The average representative log2 ratio of each treatment. Green and red symbols indicate different patterns in which expression levels of transcripts are decreased (Green) or increased (red) in the indicated scale.

differentiation (34). No evidences showed germ cells could enter quiescent phase during this preprogrammed process to shunt bad impacts under adverse environments as many somatic cells did. Reduced number of germ cells can objectively curb the metabolism scale on day 7 by apoptosis. This regulation together with the up-regulation of redox-associated genes and germ cell number decline can restore antioxidative capability on day 10 and 14, but not completely.

That the oxidative stress was one reason account for the germ cell degeneration being addressed more before, but the origin of ROS was still intangible. Oxidative phosphorylation in mitochondrion was the largest suspicion, since electron transport chain consumed about 95% oxygen and about 1-5% of which were being transformed into superoxide anion in cell (35). Many genes in the electron transport chain in mitochondrion were affected by cryptorchidism and raised their expression on day 7. Respiratory complex I, complex IV seemed to be influenced greatly. Apoptotic cells demanded on the ATP pool to ensure the repair of DNA, the completion of the effector phase of apoptosis and the maintenance of ion pumps to prevent cell swelling and necrosis (36). During apoptosis, more glucose would be preferentially directed to the utilization of apoptosis and this would further enhance the deficiency of glucose. Oxidative stress exerted stress on mitochondrion (37). More importantly, in many pathological conditions, electron transport chain can produce more superoxide. Complex I was the primary source of ROS in a variety of pathological scenarios ranging from ageing to Parkinson's disease (38). Higher ROS can reversibly impact the normal function of complex I which initiated more superoxide production (39). Superoxide anion was immediately dismuted into hydrogen peroxide (H_2O_2) and then was transformed into hydroxyl radicals (40). High contents of H_2O_2 destroyed the enzymes especially those with low pKa values of Cys-SH residues in testis like thioredoxin (41). ROS disrupted cholesterol transport in the mitochondria in MA-10 tumor Leydig cells (42).

In cells, another resource of ROS was the various oxygenases and oxidases especially members of cytochrome P450 family. In cryptorchid testis, both Pcyox1 and Hao3 could generate H2O2 and expressed with higher level at later time points. Therefore, inhibiting oxygenases and oxidases activity, such as xanthine oxidase, can suppress germ cell apoptosis (43). To reduce the production of reactive substrate and save NADPH, testis had to selectively reduce many oxygenase expressions. But that may severely affect the normal process of spermatogenesis and spermiogenesis.

In cryptorchid testis, both the antioxidative system and the ROS production system were all largely dependent

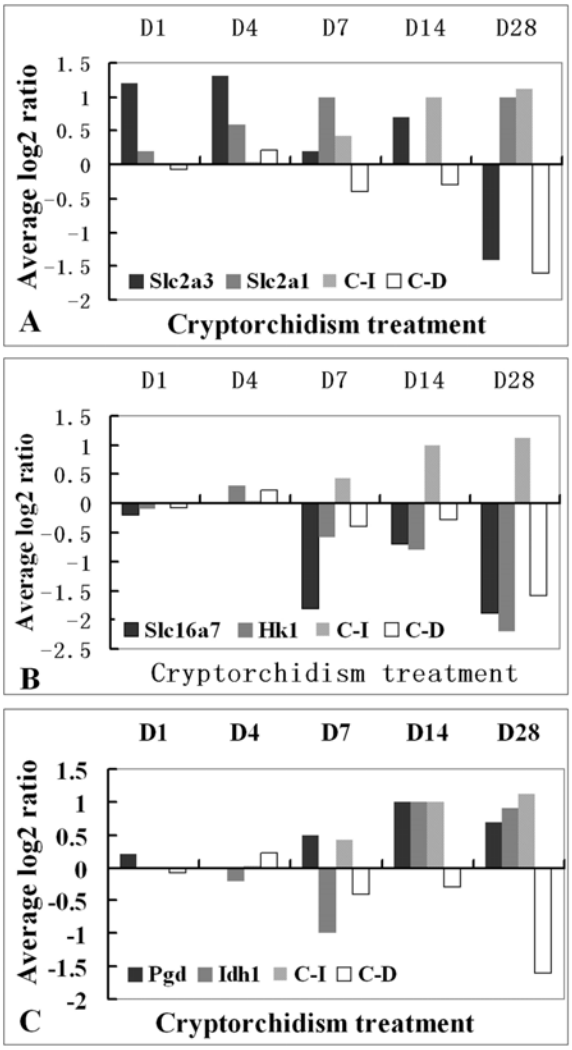

Figure 8. EC altered the key enzymes and transporters in glucose, lactate and NADPH metabolism (data were derived from mean change value of the chip data and verified by RT-PCR, RT-PCR data not shown). (A)Slc2a3/Glut3 (solute carrier family 2, member 3), glucose transporter; Slc2a1/Glut1 (solute carrier family 2, member 1), glucose transporter. (B)Slc16a7/MCT2 (solute carrier family 16, member 7), monocarboxylate transporter; Hk1 (hexokinase 1), first step of several metabolic pathways of carbohydrate (rate limiting step). (C)Pgd (phosphogluconate dehydrogenase) with catalytic activity: 6-phospho-D-gluconate + NADP(+) = D-ribulose 5-phosphate + CO(2) + NADPH, pentose phosphate pathway (rate-limiting step); Idh1 (isocitrate dehydrogenase 1 (NADP+), soluble) with catalytic activity: isocitrate + NADP(+) = 2-oxoglutarate + CO(2) + NADPH in citrate cycle. C-I was the high criterion used to counteract the decrease of cell number due to apoptosis after EC. C-D was low criterion that represented the average expression level of the most impacted cell populations as elongating and elongated spermatids.

on the energy metabolic system to supply the essential substrate, glucose, NADH and NADPH. Our results showed the genes from carbohydrate metabolism seemed to play a key role in anti-oxidative stress and apoptosis in the testis. Glucose served not only as the raw materials for building the fast proliferating and differentiating germ cells, but also the sources of ATP as well as the sources of NADPH. Glucose was first transported into testis and oxidized by glycolysis path to supply pyruvate to tricarboxylic acid cycle (TCA) and then the oxidative phosphorylation in mitochondrion. Pentose phosphate path utilized glucose for the supply of ribose for nucleic acid synthesis and the supply of NADPH for redox system and lipid synthesis. More differentiated germ cells such as spermatocytes, round spermatids, as well as spermatozoa, were more dependent on glycolysis and lactate (44-46). Most lactate was generated by Sertoli cells and was transported to germ cells (reviewed in 47). In this regards, glucose metabolism and lactate generation and transport played a key role in controlling the redox status in testis. The apoptosis in different cells had different mechanism by glucose deficiency. Please refer to the detailed description by Moley *et al* (48).

Slc2a3/Glut3 was the main glucose supplier to Sertoli cells and germ cells and was associated with the degeneration of cryptorchid testis (18, 19). Slc2a3/Glut3 elevated expression on day 1 day 4 and day 14 and declined on day 7 and day 28. It implied low glucose supply to those cells was associated with cell apoptosis. Slc2a1/Glut1 expressed in a pattern contrary to Slc2a3/Glut3, and elevated more obviously on day 7 and day 28 when the testis performed two peaks of apoptosis. It reflected the differential regulation of glucose supply to different cell populations in the cryptorchid testis. The cryptorchid testis may selectively regulate the glucose transport benefiting the somatic cells, but may be unfavorable to the more differentiated germ cells on day 7 and day 28 when antioxidation was most urgent. This was also reinforced by the pattern of glycolysis with an initial uprising on day 4 and day 7 and then reduction on day 14 and day 28 which in testis might affect the more need of lactate production especially at later time points. The lactate and succinate dehydrogenase activity decline (49) made the energy supply even worse in cryptorchid testis. Since glucose and lactate were the preferred energy source, to supply the cryptorchid testis with essential energy substrates such as lactate, can significantly prolong the germ cell survival upon EC (50, 51).

Upon oxidative stress, cryptorchid testis elevated NADPH production to scavenge ROS. The key enzyme of pentose phosphate path, Pgd, raised its expression (figure 7B). Pentose phosphate path had more activities in cryptorchid testis than the normal one (11). Epididymal spermatozoa during spontaneous lipid peroxidation also increased the activities of pentose phosphate path (52). To generate more NADPH, other metabolic pathways that can produce NADPH were also enhanced with the gene up-regulation of Idh1, Fdxr, Hsd17b3 and Blvrb (data were not shown).

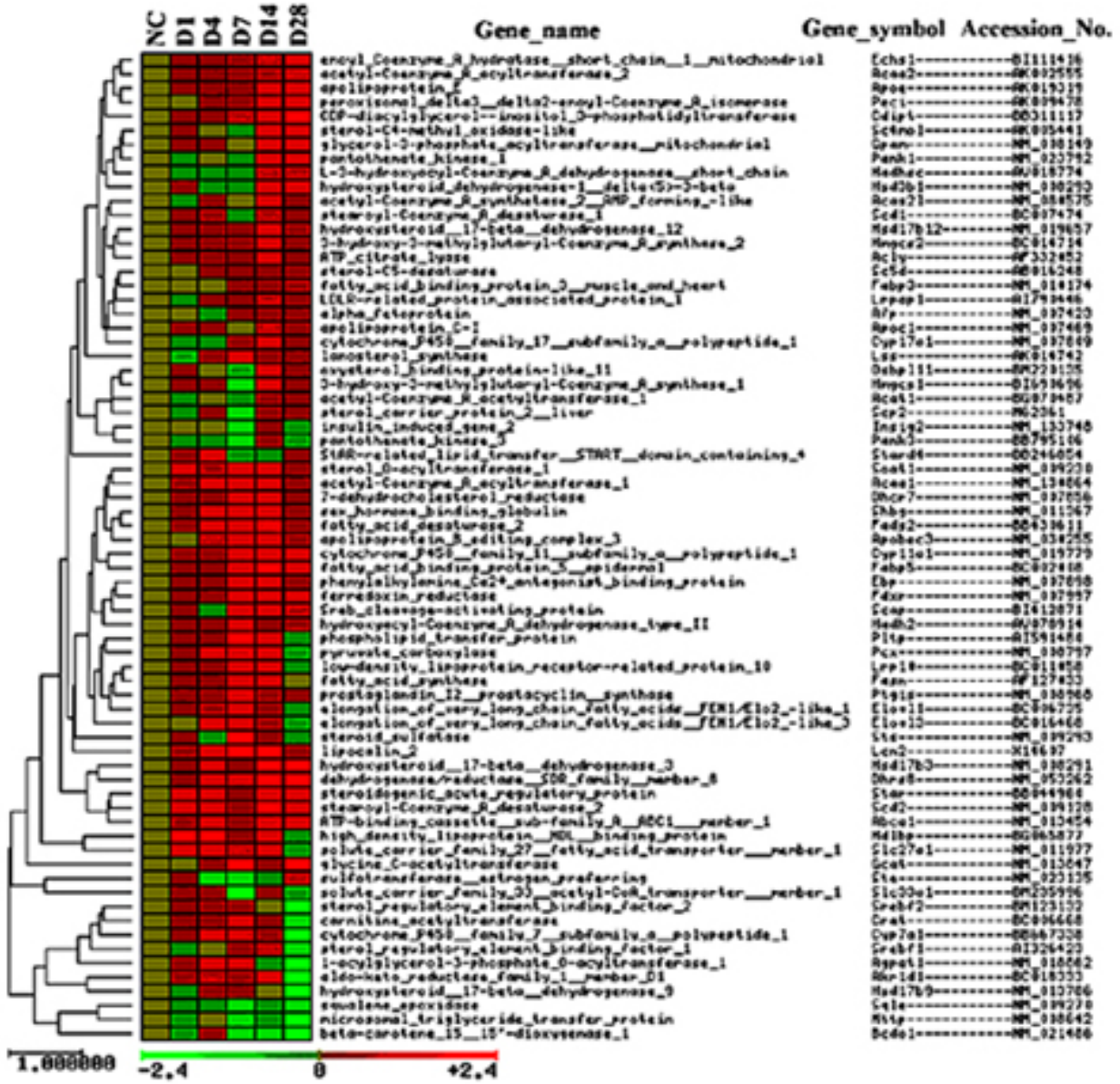

Figure 9. EC altered the lipid synthesis associated genes expression. Significant changed genes in either treatment were chosen to draw the dendrogram. Green and red symbols indicate different patterns in which expression levels of transcripts are decreased (Green) or increased (red) in the indicated scale.

From Figure 3, we also found that the round spermatids can easily be detached from the seminiferous tubules, which implied the junction between Sertoli cells and the germ cells was also disrupted by EC. This suggestion has been confirmed by our previous studies. Heat stress could induce Sertoli cell dedifferentiation by disrupted expression of intermediate filament and activation of extracellular signal-regulated kinase 1 and 2 in the cryptorchid testis (61) and in vitro heat-treated Sertoli cells (62).

The substrate delivery from Sertoli to spermatids might be impacted which can account for the partial inefficiency of energy supply in these cells.

In addition to the glucose and lactate metabolism, lipid metabolism was also an important factor contributing to the high ROS by EC. According to the criterion C-I, in late time points as day 14 and day 28, most genes in the lipid synthesis displayed a slight decreasing. It implied the fact that the somatic cells were also impacted in late time points. But comparing to sperm production and other categories of genes in our chip, lipid synthesis as a whole was high in expression profile.

Since lipid synthesis can consume a large amount of NADPH, why did testis at all costs to maintain the extravagant activity of lipid anabolism when being highly short of NADPH under oxidative stress? First, fatty acids

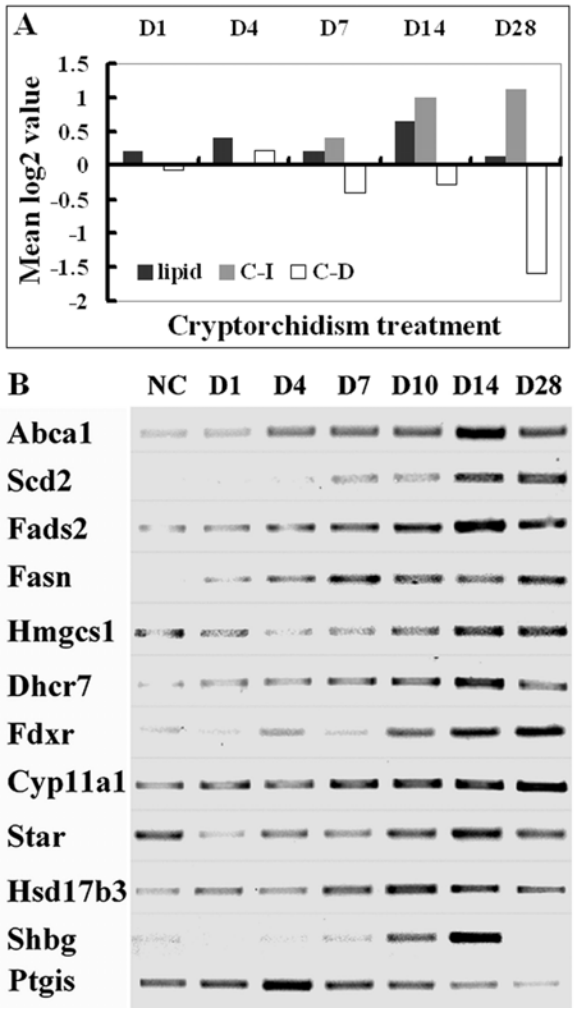

Figure 10. Lipid synthesis associated genes expression. (A) The mean representative log2 ratio of each treatment in figure 9. (B) RT-PCR verification of the up-regulation of genes in lipid synthesis and transport. Abca1, cholesterol transporter; Scd2, fatty acid desaturase; Fads2, fatty acid desaturase; Fasn, fatty acid synthetase (key enzyme); Hmgcs1, cholesterol synthesis; Dhcr7, cholesterol synthesis; Fdxr, mitochondrial p450 system and cholesterol synthesis (rate-limiting step); Cyp11a1, steroid hormone synthesis (first step, rate-limiting); Star, steroidogenesis (rate-limiting step); Hsd17b3, testosterone biosynthesis (last step); Shbg, androgen transport. Ptgis prostaglandin I_2 synthesis.

especially unsaturated fatty acids had peculiar meanings for the spermatogenesis. In the progression of germ cell differentiation from spermatogonia to condensing spermatids in mouse, the total amount of 22:5(n-6) increased from 2% to 20% of total fatty acids (23). High ROS and high MDA contents in cryptorchid testis meant high destroy of unsaturated double bonds in lipid molecules. Membrane fluidity and the ratio of unsaturated to saturated fatty acids decreased on the 3rd day of intraabdominal testis while the ratio of cholesterol to phospholipids increased significantly (53). Therefore, renewal of the destroyed lipid by peroxidation and change the lipid components were the preferred mission for cryptorchid testis. We propose that the large volume of cytoplasmic membrane and the high content of unsaturated fatty acids were the attack targets of ROS. And that high dependence on other cells and highly differentiation of the elongating and elongated spermatids were why they degenerated more severely than other type of testis cells. Significant increase in activities of anabolic enzymes and decrease in activities of catabolic enzymes from the meiotic germ cells to post-meiotic germ cells indicated a shift in lipid metabolism towards fatty acid synthesis during meiosis (46). Hormone-sensitive lipase (HSL) increased in Sertoli cells and Leydig cells in cryptorchid testes (54). Secondly, sterol hormones, especially testosterone, were extremely important for spermatogenesis and spermiogenesis. Oestradiol-17 beta (E2) was three-fold increased in unilateral cryptorchid testes (55), steroidogenesis-stimulating activity □ SSA □ from cryptorchid rats was significantly greater (2- to 3-fold) than normal at all time points, the major increase in SSA occurred within the first 4 weeks after treatment (56), which was also coincident with the more previous reported (57). It also suggested that cryptorchidism badly impacted the endocrine homeostasis in testis and enhanced the activities of anabolic enzymes of sterols. But we cannot rule out the compensation from contralateral scrotum testis in secreting testosterone, though there were still many questions. Different stages in cryptorchid testis might have different effects on the testosterone secretion.

Cholesterol transport activity was also changed in cryptorchid testis such as Abca1, an important protein regulating intracellular cholesterol transport (58, 59). The ratio of cholesterol to phospholipids increased significantly in cryptorchid testis (53). The bioactivity of the cryptorchid testis lipoprotein fraction was 8-fold higher than the lipoprotein fraction from untreated testes (56). The increased volumes of lipid droplets in Sertoli cells and multi-vacuolated Leydig cells in the cryptorchid testis (16, 60), might be evidence of continuous up-regulation of lipid synthesis and transporting dysfunction in the cryptorchid testis.

Overall, body temperature changed the energy metabolism, antioxidation and redox system, and thus generated more ROS in cryptorchid testis. The unknown regulation process between day 5 and day 7 after EC was a key event for germ cell apoptosis.

In our previous studies we have demonstrated that heat stress induced germ cell apoptosis mainly occurred at the early stage in rhesus monkey (63-65), cynomolgus monkeys (66,67), the profile of germ cell apoptosis is consistent with the changes in testicular weight and the gene expression pattern at the early stage of the heat stress observed in this study. The expression of the germ cell apoptosis-related genes, such as Fas/FasL, Bax, BcL-2, changed dramatically in correlation with the extent of the germ cell apoptosis induced by the heat stress (66)

The sudden up-rising of oxidation level on day 5 was apparently a turning point in the pathological process after EC. The extensive up-regulation of genes on day 4 might lay the curse for germ cell apoptosis. From the gene expression profile, the ROS level fluctuation and histological tests on the pathological tissue, we proposed that cryptorchid testis tried to protect all the cells initially, and between day 5 and day 7 after EC, when the high ROS exceeded the limitation of testis, cryptorchid testis then selectively regulated different cell populations and prompted apoptosis of late stage germ cell and preserved the somatic cells and the early stage germ cells. The alteration in the main ROS production system, the antioxidative system and the energy metabolism system might contribute to the high ROS level by EC. The massive germ cell apoptosis on day 7 can objectively decrease the metabolic scale in testis and thus effectively curb the ROS level as seen on day 10 and day 14, but not completely. Our data suggested that experimental cryptorchid testis of adult mouse had no capability to curb the oxidative levels in body temperature and thus cannot change the fate of cell apoptosis.

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