Increased cerebral expressions of MMPs, CLDN5, OCLN, ZO1 and AQP5s are associated with brain edema following fatal heat stroke

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Human brain samples were collected from 46 autopsy cases, including 23 fatal heat stroke cases and 23 age-matched controls. Nine candidate reference genes (PES1, POLR2A, IPO8, HMB5, SDHA, GAPDH, UBC, B2M, ACTB) were evaluated in the cerebral cortex of 10 forensic autopsy cases (5 heat stroke and 5 controls), using the geNorm module in qBaseplus software. SDHA, POLR2A, IPO8 and HMB5 were identified as the most stable reference genes. Using these validated reference genes, mRNA expressions of Matrix metalloproteinases (MMPs, MMP2 and MMP9), Claudin5 (CLDN5), Occludin (OCLN), Zona occludens protein-1 (ZO1) and Aquaporins (AQP5, AQP1 and AQP4) in the cerebral cortex were examined. Relative mRNA quantification using Taqman real-time PCR assay demonstrated increased calibrated normalized relative quantity (CNRQ) values of MMP9, CLDN5, OCLN, ZO1 and AQP4 in heat stroke cases. Heat stroke cases showed an increase in brain water content, which was found to be positively correlated with MMP9, CLDN5, ZO1 and CLDN5 mRNA. When using one conventional reference gene (GAPDH or ACTB) for normalization, no difference was detected between heat stroke and controls. In immunostaining, only AQP4 showed more intense staining in most heat stroke cases. The present study, for the first time, reports increased cerebral MMP9, CLDN5, OCLN, ZO1 and AQP4 in heat stroke and suggest a crucial role of reference gene selection when using postmortem human tissues.

Heat stroke is defined as a form of hyperthermia associated with a systemic inflammatory response leading to a syndrome of multi organ dysfunction in which encephalopathy predominates1. The mortality is as high as 10–15% in patients with heat stroke. Nearly 30% of patients with heat stroke are accompanied by central nervous system (CNS) dysfunction that results in delirium, convulsions, or coma1. Brain edema is an important factor associated with brain damage causing long-term disability and death in patients with heat-related illness. In fact, forensic autopsy data showed profound brain edema in heat stroke cases1. However, this phenomenon has not been fully emphasized in clinical treatment. The potential mechanism of brain edema formation following heat stroke has not been fully clarified.

Matrix metalloproteinases (MMPs) belong to a family of calcium-dependent zinc-containing endopeptidases, which are involved in the tissue remodeling and degradation of the extracellular matrix (ECM)5. Considerable research has been conducted on the role of two secreted MMPs, MMP2, and MMP9. Both of them have positive and negative roles in the healthy and diseased CNS5. MMP9 is responsible for blood-brain barrier (BBB) opening in several pathological conditions and the marked increase of MMP9 causes severe BBB disruption.

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**Results**

**Brain water content.** There was a significant increase in brain water content in the heat stroke group as compared to the control group (Table 1, heat stroke: 82.3% ± 2.9%; control: 79.3% ± 2.6%, p < 0.05, Student’s t test).

**RNA concentration, purity and integrity.** RNA concentrations ranged from 25.9 to 348.7 ng/μL (mean 194.1 ng/μL). There were no age or postmortem interval dependences on Pearson correlation analysis (p > 0.05). RNA concentrations showed no significant differences between the heat stroke group and control group.

**Amplification efficiency.** The amplification efficiencies of targets and reference genes ranged from 86.2% (ACTB) to 104.6% (UBC), showing small inter-individual variations (standard deviation, SD < 5%). Details are shown in Table 2.

**Reference genes validation.** The geNorm module in qBase® software ranked the 9 reference genes. The most stable one was SDHA followed by POLR2A, IPO8 and HMBS. The least stable one was ACTB (Fig. 1). Pairwise variation (V) was calculated based on normalization factor values (NFn and NFn + 1) after the inclusion of the least stable reference gene and indicated if the extra reference gene added to the stability of the normalization factor. The V-value was the lowest when the fifth most stable gene (PES1) was added (Fig. 2). However, when the fourth most stable gene (HMBS) was added, V3/4 showed a V-value of 0.153, near the threshold of 0.15. Therefore, to save on cost and time, four reference genes SDHA, POLR2A, IPO8 and HMBS, were selected for normalization.

**Data analysis**

**Normalization against validated reference genes.** Raw Ct values and amplification efficiencies of targets and 4 validated reference genes, SDHA, POLR2A, IPO8 and HMBS, were imported into the qBase® software. CNRQ values were exported and statistically investigated.

There were no gender-related differences, or age or postmortem interval dependence in CNRQ values of target genes on Pearson correlation (p > 0.05).

CNRQ values of AQP4, CLDN5, OCLN, ZO1 and MMP9 were significantly higher in the heat stroke group as compared to the control group (Fig. 3).

CNRQ values of CLDN5 and MMP9 were found to be positively correlated with brain water contents ($r^2 = 0.1225$ and 0.1486, p < 0.05).

**Table 1.** Case profiles n = 46. Significantly lower RIN values were detected in heat stroke group as compared to the control group (*p < 0.05). There was a significant increase in brain water content in the heat stroke group as compared to the control group (*p < 0.05).
| Gene Symbol | Gene Name                  | TaqMan Assay ID    | Amplicon Length (bp) | Amplification efficiency (mean ± SD) |
|-------------|---------------------------|--------------------|----------------------|--------------------------------------|
| **Gene of interest**                           |                                          |                    |                                     |
| AQP1        | aquaporin 1                | Hs00166067_m1      | 86                   | 0.896 ± 0.021                        |
| AQP4        | aquaporin 4                | Hs00242342_m1      | 92                   | 0.955 ± 0.014                        |
| CLDN5       | claudin 5                  | Hs01561351_m1      | 55                   | 0.903 ± 0.016                        |
| MMP2        | matrix metallopeptidase 2  | Hs01548727_m1      | 65                   | 0.953 ± 0.031                        |
| MMP9        | matrix metallopeptidase 9  | Hs0234579_m1       | 54                   | 0.939 ± 0.028                        |
| OCLN        | occludin                   | Hs0170162_m1       | 68                   | 1.0387 ± 0.039                       |
| ZO1         | zona occludens protein-1   | Hs01551861_m1      | 148                  | 0.974 ± 0.028                        |
| **Reference gene**                             |                                          |                    |                                     |
| PES1        | pescadillo homolog 1       | Hs00362795_g1      | 56                   | 0.985 ± 0.029                        |
| POLR2A      | polymerase (RNA II) (DNA directed) polypeptide A | Hs00172187_m1    | 54                   | 1.010 ± 0.017                        |
| IPO8        | importin 8                 | Hs00183533_m1      | 71                   | 1.025 ± 0.033                        |
| HMBS3       | hydroxymethylbilane synthase| Hs00699297_m1      | 64                   | 1.019 ± 0.025                        |
| SDHA4       | succinate dehydrogenase complex | Hs0188166_m1   | 70                   | 1.018 ± 0.037                        |
| GAPDH       | glyceraldehyde-3-phosphate dehydrogenase | Hs99999905_m1 | 122                  | 0.927 ± 0.016                        |
| UBC         | ubiquitin C                | Hs0024723_m1       | 71                   | 1.046 ± 0.027                        |
| B2M         | beta-2-microglobulin       | Hs99999907_m1      | 75                   | 1.041 ± 0.037                        |
| ACTB        | beta-actin                 | Hs99999903_m1      | 171                  | 0.862 ± 0.019                        |

Table 2. Introduction of the PCR primers and probes. "_m" indicates the assay’s probe spans an exon junction and will not detect genomic DNA. "_g" indicates the assay may detect genomic DNA. Detailed information for each TaqMan Assay is available from Applied Biosystems.

![Figure 1](image1.png)

**Figure 1.** Average expression stability values (M). Expression stability values of genes from the least stable (left) to most stable (right).

![Figure 2](image2.png)

**Figure 2.** Pairwise variation of candidate reference genes using geNorm analysis. Pairwise variation analysis to determine the optimal number of reference genes for normalization.
Normalization against ACTB or GAPDH. When ACTB or GAPDH alone was used for normalization, there was no significant difference in the expression of any target gene (Figs 4 and 5).

Immunostaining. Immunostaining showed substantial interindividual variations in each group. AQP1 (Fig. 6a and b) and AQP4 (Fig. 6c and d, Fig. 7) were mainly detected in glial cells which were morphologically identified as astrocytes, and only AQP4 showed more intense staining in most heat stroke cases. CLDN5 was strongly positive in capillary endothelia, and no significant differences in distribution or intensity were detected between heat stroke and control group (Fig. 6e and f, Fig. 7). MMP2 was detected clearly in the neurons, showing no significant differences in distribution or intensity between heat stroke and control group (Fig. 6g and h, Fig. 7). MMP9 was located in glial cells, neurons and capillary endothelia, and no significant differences in distribution or intensity were detected between heat stroke and control group (Fig. 6i and j, Fig. 7). OCLN was positive in capillary endothelia, sporadically in neurons and glial cells, and no significant differences in distribution or intensity were detected between heat stroke and control group (Fig. 6k and l, Fig. 7). ZO1 was located in capillary endothelia, and no significant differences in distribution or intensity were detected between heat stroke and control group (Fig. 6m and n, Fig. 7).
Discussion

RT-qPCR is increasingly applied to determine changes in gene expressions due to the high sensitivity and accuracy of the technique. The most common procedures in RT-qPCR are relative measurements of genes of interest after normalization with the endogenous reference gene(s). Accurate and reliable relative RT-qPCR requires ideal reference gene(s). However, expressions of several conventional reference genes were shown to vary due to

Figure 5. AQP1, AQP4, CLDN5, OCLN, ZO1, MMP2 and MMP9 mRNA expression levels after normalization against GAPDH. There was no significant difference in the expression of any target gene.

Figure 6. Immunostaining of AQP1 (a and b), AQP4 (c and d), CLDN5 (e and f), MMP2 (g and h), MMP9 (i and j), OCLN (k and l) and ZO1 (m and n) in the brain. Peracute death due to blunt injury (a, c, e, g, i, k and m), a 52-year-old male, 27 h postmortem. Death due to heat stroke (b, d, f, h, j, l and n), a 64-year-old male, 30 h postmortem.
In the present study, with the help of geNorm module in qBaseplus software, nine reference genes were evaluated. The V-value was the lowest when the fifth most stable gene (PES1) was added. Further addition of genes increased V-values, indicating a negative influence on the normalization process. Ideally, a threshold V-value of 0.15 is recommended as a cut-off value by geNorm to determine the optimal number of reference genes. However, when the fourth most stable gene (HMBS) was added, V3/4 showed a V-value of 0.153, near the threshold of 0.15. Therefore, to save on cost and time, four reference genes SDHA, POLR2A, IPO8 and HMBS, were selected for normalization. In the present study, CLW/H ratios were higher in the heat stroke group as compared to the control group, indicating brain edema in heat stroke cases. However, using mRNA measurements of intracerebral MMPs, CLDN5, OCLN, ZO1, and AQPs as markers of brain edema, inconsistent results were detected by different normalization methods. When those four validated reference genes, SDHA, POLR2A, IPO8 and HMBS, were used for normalization, increased cerebral expressions of AQPs, CLDN5, OCLN, ZO1 and MMP9 were detected in heat stroke group. However, these findings cannot be detected when GAPDH or 18S was used alone for normalization. Expression stability values of these five reference genes calculated by geNorm showed V-values, indicating a negative influence on the normalization process. Ideally, a threshold V-value of 0.15 is recommended as a cut-off value by geNorm to determine the optimal number of reference genes.

Another considerable factor influencing the accuracy of gene expression analysis using RT-qPCR is the integrity of RNA. However, unlike animal experimentation, RNA degradation is inevitable and unpredictable for human tissues collected at autopsy. In the present study of the human brain tissues, RIN values showed no postmortem interval-dependent changes but were significantly lower in the heat stroke group as compared to the control group, indicating that RNA quality was more seriously affected in cases of hyperthermia. The up-regulations of MMP2 and MMP9 in brain are associated with an increase of BBB permeability by degrading the endothelial basal lamina of the BBB which results in vasogenic edema. Despite the well-documented effects of systemic inflammatory response, the impact of hyperthermia on the BBB has been overlooked and the probable mechanism has not been fully addressed. In the present study, brain tissues in heat stroke group showed evidently higher CNRQ values of MMP9, but not MMP2. These findings suggest independent contributions of MMP2 and MMP9 in the brain tissues of heat stroke group, which require further investigation. MMP9 was regarded as a key player in the alteration of BBB permeability. Several studies in animal models have shown that increased MMP9 is closely related to the breakdown of BBB, by digesting ECM. Both inhibiting MMP9 and deleting MMP9 gene can attenuate the BBB disruption.

In addition, intercellular junctions between endothelial cells are essential for vascular integrity and function. Breaching of endothelial barriers is a key event in the development of brain edema. The loss of CLDN5, OCLN and ZO1 in TJs can open the BBB, lead to brain edema and neuronal cell death. Decreased expression of CLDN5, OCLN and ZO1 is closely associated with BBB damage. However, in the present study, increased CLDN5, OCLN and ZO1 mRNA expression was detected in the heat stroke group. These findings might be considered a compensatory mechanism to mend junctional complexes and restore barrier function.

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AQPs, the principal AQPs in mammalian brain, highly correlate with a variety of pathophysiological processes of brain edema. Increased expression of AQPs in the brain indicated that AQPs participated in the formation of brain edema. In the vasogenic edema resolution phase, an increase of AQPs was observed in several studies.

Figure 7. Quantification of immunostaining data. Ratio for fold change of AQP4 was significantly higher in the heat stroke group as compared to the control group. Significantly lower RIN values were detected in heat stroke group as compared to the control group (*p < 0.05).
Therefore, in heat stroke cases, AQP4 might play a beneficial role in eliminating accumulating water from the extracellular space of the CNS, suggesting an activation of the self-protective system.

In the present study, the immunostaining did not detect any evident differences in distribution or intensity among all the causes of death except for AQP4. These findings may be caused by a lower sensitivity of immunostaining in detecting changes in gene products than that with quantitative analyses of gene expressions using RT-qPCR. The major limitation of the present study is that the protein levels of all targets have not been examined because of the limitation of the postmortem materials, which need further investigation.

In conclusion, the present study shows that brain edema is evident in heat stroke, and human brain retains a self-protective response capacity in victims who died due to heat stroke. Systematic analysis of gene expressions using RT-qPCR is a useful procedure and validation of reference genes is crucial.

Materials and Methods

Sample collection. A total of 46 human forensic autopsy cases were selected from autopsy documents. The demographics of study subjects are described in Table 1. In each case, the cause of death was carefully diagnosed on the basis of autopsy examination, including macromorphological, histological, toxicological and biochemical analyses. Cases were divided into two groups as follows: 23 fatal heat stroke cases and 23 age, gender and post-mortem interval (PMI)-matched control subjects, including three hanging cases, three strangulation cases, five blunt injury cases, six fire fatality cases, and six acute cardiac death cases. A thorough neuropathological analysis was performed as part of our routine investigation, and cases with any preexisting neurological pathologies were excluded in the present study.

In the present study, clearly accountable cases without any other complications that may have contributed to the death, supported by well-established circumstantial evidence, were collected. Postmortem interval was defined as the estimated time from death to autopsy which was estimated on the basis of autopsy findings and circumstantial evidence recorded in autopsy documents. The definition and criteria of deaths due to heat stroke are described in our previous report32; drug abusers and chronic alcoholics were excluded from the heat stroke group. This work was approved by our institutional Ethics Committee of Southern Medical University. All sampling methods were followed anatomical practices and carried out in accordance with regulations of Methods of extraction, fixation, packing and inspection of forensic pathology of The PRC Public Safety Industry Standard (GA/T 148–1996) and Forensic pathology materials extraction, fixed operating instructions of Southern Medical University (NYSJ-IS-BL04). As one of our routine work, the informed consent paper was obtained from the immediate family members of deceased before starting autopsy.

Brain water content. Brain water content was measured by a halogen moisture analyzer (model HB43, METTLER TOLEDO, Switzerland) automatically according to the manufacturer’s instructions33. Briefly, brain tissue samples were taken from consistent sites in the parietal lobe of left cerebral hemispheres at autopsy. About one gram of brain tissue was weighted first to obtain a wet weight (WW), then placed in the analyzer at 150°C for about 30 min and weighted again to obtain a dry weight (DW). The water content was calculated using the equation: (WW – DW)/WW × 100%.

Toxicological analyses. The procedures of drug testing and analysis, including chemicals and reagents, sample preparation and conditions of the instrument were performed by gas chromatography/mass spectrometry33.

Extraction of total RNA and cDNA synthesis. Tissue specimens were taken from consistent sites in the central anterior of left cerebral hemispheres (precentral gyrus) at autopsy, then immediately submerged in 1 ml of RNA stabilization solution (RNAlaterTM, Ambion, Austin). Total RNA was isolated from 100 mg of sample using RNAiso Plus (Takara Bio, Inc., Shiga, Japan) according to the manufacturer’s instructions. After extraction, the RNA concentration was estimated by spectrophotometric analysis using a NanoDrop 1000 (Thermo Scientific, Wilmington, USA). cDNA copies of total RNA were obtained using a High Capacity RNA-to-cDNA kit (Applied Biosystems Japan, Ltd.), then were adjusted to a concentration equivalent to 5 ng/μl of total RNA using nuclease-free water.

Evaluation of the quality and integrity of RNA samples. RNA purity was determined using 260/280 absorbance (A260/A280) ratios. The RNA integrity number (RIN) was determined using a RNA 6000 Nano Labchip, METTLER TOLEDO, Switzerland) automatically according to the manufacturer’s instructions33. Briefly, brain tissue samples were taken from consistent sites in the parietal lobe of left cerebral hemispheres at autopsy. About one gram of brain tissue was weighted first to obtain a wet weight (WW), then placed in the analyzer at 150°C for about 30 min and weighted again to obtain a dry weight (DW). The water content was calculated using the equation: (WW – DW)/WW × 100%.

Reference genes selection. Nine candidate reference genes were evaluated in the brain tissue of 10 forensic autopsy cases (Heat stroke, n = 5 and control, n = 5); pescadillo homolog 1 (PES1), polymerase (RNA) II (DNA directed) polypeptide A (POLR2A), importin 8 (IPO8), hydroxymethylbilane synthase (HMBS), succinate dehydrogenase complex (SDHA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ubiquitin C (UBC), beta-2-microglobulin (B2M), beta-actin (ACTB), PES1, POLR2A, IPO8, HMBS, SDHA were chosen from the relevant literature and have been validated in postmortem brain tissues34, 35. GAPDH, B2M and ACTB were conventional reference genes. Details are shown in Table 2.

Quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR). The PCR primers and probes (TaqMan Gene Expression Assay) were purchased from Applied Biosystems, Inc. (Carlsbad, CA, USA). Details are shown in Table 2. RT-qPCR reactions were run in 96-well reaction plates with an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). RT-qPCR was performed with 10 μl cDNA (corresponding to the cDNA reverse transcribed from approximately 50ng RNA) in 20 μl reaction mix containing 10 μl TaqMan Gene Expression Master Mix (2 × ) and the above-mentioned TaqMan
Gene Expression Assays (lyophilized powder). Thermal cycling conditions included 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 15 sec, and 60 °C for 1 min. The threshold cycle (Ct) was calculated by the instrument software automatically (threshold value at 0.2). Raw fluorescent data (normalized reporter values, Rn values) were also exported.

**Amplification efficiency calculation.** Amplification efficiencies were calculated from raw fluorescent data (Rn values), using a completely objective and noise-resistant algorithm, the Real-time PCR Miner program (http://ewindup.info/miner/).

**Data analysis**

**Normalization against validated reference genes.** Raw Ct values and calculated amplification efficiencies of these 9 reference genes were imported into the qBaseplus software. In qBaseplus software, geNorm module was used to identify the most stable reference genes and determine the minimum number of reference genes.

After determining the minimum number of reference genes, raw Ct values and amplification efficiencies of targets and validated reference genes were imported into the qBaseplus software again. Using a calibrator case (peracute death due to blunt injury, 52-year-old male; 27 h postmortem), calibrated normalized relative quantity (CNRQ) values were exported from the qBaseplus software, and statistically investigated.

**Normalization against conventional reference gene (ACTB or GAPDH).** The expression levels of mRNA transcripts are described as the ratios of the targets normalized to the endogenous reference (GAPDH or B2M), using the 2−ΔΔCt method, as the ratios for fold change relative to the above mentioned calibrator. The 2−ΔΔCt method assumes that the amplification efficiency of the reaction is ideal (100%) and constant for each sample.

**Immunostaining.** The brains were fixed in buffered 4% formaldehyde for 2 weeks and the cerebral hemispheres were cut coronally into 1-cm-thick sections, and the sections of cerebellum and brain stem were also prepared. Paraffin–embedded brain tissue specimens were taken from the standardized anatomical regions. Serial sections (5 μm thick) were cut and stained with hematoxylin–eosin (HE) as part of routine laboratory investigation. In the present study, parietal lobes of left cerebral hemispheres were used for immunostaining.

Mouse monoclonal anti-AQP1 antibody (Abcam, Cambridge, code ab9566, diluted 500-fold), rabbit polyclonal anti-AQP4 antibody (Santa Cruz Biotechnology, Santa Cruz, code sc-20812, diluted 500-fold), rabbit polyclonal anti-CLDN5 antibody (Abcam, Cambridge, code ab53765, diluted 500-fold), rabbit polyclonal anti-MMP2 antibody (Abcam, Cambridge, code ab79781, diluted 100-fold), rabbit polyclonal anti-OCLN antibody (Abcam, Cambridge, code ab168986, diluted 200-fold), rabbit polyclonal anti-ZO1 antibody (Santa Cruz Biotechnology, Santa Cruz, code sc-33725, diluted 500-fold) and rabbit polyclonal anti-MMP9 antibody (Abcam, Cambridge, code ab38898, diluted 800-fold) were used. Following overnight incubation with the primary antibodies described above at room temperature, immunoreactions were visualized by the polymer method (ChemMate Envision, Dako, Tokyo, code k5027) and color was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB liquid system, DAB, Tokyo, code K3466), according to the manufacturer’s instructions (counterstaining with hematoxylin).

Image J 1.46 R (NIH, USA) was used to calculate the intensity and extent of staining for the detected molecules. The per-area density of staining was calculated in this manner to reflect the percentage of the positive staining, resulting in a semi-quantitative analysis. A total of 5 microscopic fields were randomly selected, and their images were cropped. The integral optical density (IOD) levels of the stained cells in the tissue samples were then calculated by image analysis. Results were expressed as the mean ± standard deviation (SD) per tissue examined. Using a calibrator case (peracute death due to blunt injury, 52-year-old male; 27 h postmortem), ratios for fold change relative to the calibrator were used for statistical analysis.

**Statistic.** All the RT-qPCR experiments were performed in triplicate, and results are reported as the mean ± SD. Correlation analyses between pairs of parameters were performed using linear regression (Pearson correlation analysis). The Student’s t test (two-tailed) was used to compare groups. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, USA). Values of p < 0.05 were considered as statistically significant.

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Author Contributions

Y.D., J.-T.X. and H.-N.J. collected the samples, carried out RT-qPCR experiments. D.Z. and R.Z. performed the brain water content and toxicological analyses. S.-H.D., J.-T.X. and Y.X. carried out the immunostaining, statistical analysis and helped to draft the manuscript. Q.W. and X.-L.X. designed the study and wrote the manuscript. All authors read and approved the final manuscript.
Additional Information

Competing Interests: The authors declare that they have no competing interests.

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