Expression profiling of microRNAs in lipopolysaccharide-induced acute lung injury after hypothermia treatment

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Abstract We investigated the expression profiles of miRNAs in acute lung injury (ALI) rats after hypothermia treatment. ALI rats were induced with lipopolysaccharide (LPS) and maintained with hypothermia (HT) or normothermia (NT) for 6 hours. HT attenuated inflammatory cell infiltration in the lung and improved biochemical indicators of multi-organ dysfunction. Nineteen miRNAs were significantly differentially expressed in the HT group compared with the NT group. miR-142, miR-98, miR-541, miR-503, miR-653, miR-223, miR-323 and miR-196b exhibited opposite patterns of expression between the two groups. These dysregulated miRNAs were mainly involved in the immune and inflammatory response on functional annotation analyses. This study shows that HT has lung protective effects and influences expression profiles of miRNAs in ALI. And dysregulated miRNAs after HT modulate the immune and inflammation in ALI. These results suggest that dysregulated miRNAs play a role in the mechanism of the lung protective effects of HT in ALI.

Keywords Acute lung injury, MicroRNA, Hypothermia

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Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) commonly develop in patients afflicted with pneumonia, sepsis, multiple trauma, massive blood transfusions, and patients undergoing cardiopulmonary bypass. Though the causative factors may be diverse, ARDS is uniformly characterized by overwhelming lung inflammation, increased microvascular permeability and endothelial and epithelial disruption. Despite the recent advances in the elucidation of the pathophysiology and in supportive therapy, ARDS is still associated with a high mortality rate and there is no effective therapy as yet.

Recently, induced mild hypothermia (HT) has been clinically used in patients with post-resuscitation syndrome to prevent ischemia-reperfusion injury, resulting in a better neurologic outcome and reduced mortality. In addition, several animal studies show that HT can attenuate ischemia-reperfusion lung injury, ventilator-induced lung injury and endotoxin-induced lung injury. Several physiological effects and mechanisms involving therapeutic effects of induced HT have been suggested, including a reduced metabolic rate, slower energy depletion and decreases in inflammatory responses. However, the exact mechanisms by which HT exerts these lung protective effects are still unknown.

MicroRNAs (miRNAs) are known as regulatory molecules involved in numerous biological processes. miRNAs are single-stranded, highly conserved small non-coding RNAs that recognize complementary sequences in the 3′-untranslated region of target mRNAs. The miRNAs control gene expression by binding to their target genes, resulting in either reduction of protein translation or downregulation of mRNA expression. miRNAs are associated with diverse biological processes, such as cell proliferation, differentiation, migration, immune response, inflammation, apoptosis, angiogen-
esis, and metastasis. Therefore, altered miRNA expression levels are correlated with disease occurrence and progression\textsuperscript{11,12}.

The expression profiles of miRNAs have been studied to identify the pathogenesis of various lung diseases, such as in a mouse model of ventilator-induced lung injury\textsuperscript{13}, chronic obstructive pulmonary disease\textsuperscript{14}, idiopathic pulmonary fibrosis\textsuperscript{15}, and rat ARDS\textsuperscript{16}. Sonkoly et al.\textsuperscript{17} reported that miRNAs are novel players in the regulation of immune function and inflammation, which is the first line of defense response. Tili’s study\textsuperscript{18} showed that upregulated miR-155 and downregulated miR-125b were responsible for the pathogenesis of LPS-induced endotoxemia through the TNF-α stimulation pathway.

We put forward the hypotheses that HT affects the expression profiles of miRNAs and the dysregulated miRNAs are correlated with the effects of HT in ALI. We confirmed the effects of HT in ALI by lung pathology studies, inflammatory cell counts in BAL fluid and multi-organ dysfunction studies and analysis of the differentially expressed miRNAs in rat ALI after HT treatment.

**Hypothermia attenuated LPS-induced acute lung injury**

The representative lung tissues from three different groups are shown in Figure 1. Histopathological examination of lung tissues confirmed the presence of moderate pulmonary injuries, congested alveolar capillaries, hemorrhage, inflammatory cell infiltration, and some alveolar wall thickening in LPS (ALI with NT treatment) comparing to LPS + HT (ALI with HT treatment) (Figure 1B, 1C).

To quantify the effects of HT on lung injury, we estimated the LIS. Based on the scoring system, all 4 components for controls had the score 0. Consistent with the histopathological examination, LIS in NT rats (10.6 ± 1.14) was significantly higher than that in the controls (\(P < 0.001\), Figure 1D). In contrast, LIS in HT rats was lower than in NT rats (9.8 ± 0.83 vs. 10.6 ± 1.14), but was not statistically significant.

To investigate the protective effect of HT in LPS-induced ALI, we measured the total cell number and the number of neutrophils in the BAL fluid. We found that HT decreased the number of total inflammatory cells and neutrophils in the BAL fluid compared with NT (Figure 1E, 1F).

**Hypothermia improved multi-organ dysfunction induced by LPS**

Because the lethality of ALI is associated with multi-organ failure, we examined the dysfunction of major organs. Organ dysfunction was determined by measur-

![Figure 1](image-url)
ing biochemical indicators in serum samples collected 6 hours after LPS challenge. LPS challenge significantly increased the levels of each biomarker (Figure 2). The concentrations of the liver enzymes released into the circulation upon injury, ALT and especially AST, were significantly lower in the HT group than in the NT group. In kidney injury, Blood Urea Nitrogen (BUN) and creatinine were also significantly lower in the HT than in the NT group ($P<0.05$). The concentration of lactate, an indicator of tissue hypoperfusion, was lower in the HT group than in the NT group, but the difference between the HT and the NT groups was not statistically significant.

Expression profiles of miRNAs in ALI rats relative to control rats

To identify the altered miRNAs in the lungs of the ALI rats, we performed miRNA profiling using an in-house printed microarray containing 690 rat miRNAs. In total, 126 miRNAs showed differences in expression levels between the LPS-induced ALI rats and the control rats. Out of the 126 miRNAs, 67 miRNAs were upregulated and 59 miRNAs were downregulated. Based on a $P$ value of $<0.05$ and a fold change $>1.5$, 29 miRNAs showed significant alteration after LPS challenge, and are presented in Table 1. Among them, 14 miRNAs were upregulated and 15 miRNAs were downregulated. miR-760 (mean fold change (mFC) = 3.7; $p=0.03$) and miR-541 (mFC = 3.2; $p=0.03$) were the top two most highly expressed miRNAs after LPS challenge. In contrast, miR-100 (mFC = 2.4; $p=0.02$), miR-99 (mFC = 2.3; $p<0.01$), and miR-199a (mFC = 2.3; $p=0.02$) were most significantly downregulated.

**Table 1. Altered miRNAs in rat ALI relative to control rats.**

| miRNA_name   | Fold change | Regulation | $P$-value |
|--------------|-------------|------------|-----------|
| rno-miR-760-3p | 3.7        | up         | 0.032     |
| rno-miR-541-5p | 3.2        | up         | 0.030     |
| rno-miR-223-3p | 2.9        | up         | 0.048     |
| rno-miR-653-3p | 2.3        | up         | 0.040     |
| rno-miR-98-3p  | 2.1        | up         | 0.030     |
| rno-miR-449c-3p | 2.0       | up         | 0.043     |
| rno-miR-503-3p | 2.0        | up         | 0.048     |
| rno-miR-380-5p | 1.8        | up         | 0.041     |
| rno-miR-935    | 1.8        | up         | 0.042     |
| rno-miR-124-5p | 1.7        | up         | 0.048     |
| rno-miR-450a-5p | 1.7       | up         | 0.040     |
| rno-miR-154-3p | 1.6        | up         | 0.048     |
| rno-miR-142-3p | 1.6        | up         | 0.049     |
| rno-miR-496-3p | 1.5        | up         | 0.049     |
| rno-miR-100-5p | 2.4        | down       | 0.023     |
| rno-miR-99a-5p | 2.3        | down       | 0.006     |
| rno-miR-199a-5p | 2.3       | down       | 0.028     |
| rno-miR-199a-3p | 2.2        | down       | 0.029     |
| rno-miR-181a-5p | 2.1        | down       | 0.040     |
| rno-miR-191a-5p | 2.1        | down       | 0.011     |
| rno-miR-497-5p | 2.1        | down       | 0.034     |
| rno-miR-28-5p  | 2.0        | down       | 0.033     |
| rno-miR-196b-3p | 2.0       | down       | 0.040     |
| rno-miR-3065-5p | 2.0       | down       | 0.041     |
| rno-miR-653-5p | 1.9        | down       | 0.029     |
| rno-miR-200c-3p | 1.8       | down       | 0.022     |
| rno-miR-322-5p | 1.8        | down       | 0.045     |
| rno-miR-148b-3p | 1.8       | down       | 0.043     |
| rno-miR-323-3p | 1.8        | down       | 0.049     |

A list of increased and decreased miRNAs in the lung tissues of the ALI rats compared with normal rat lung tissues, with a $P$ value $<0.05$ and a fold change $>1.5$.

Differential miRNAs expression in rat ALI after hypothermia treatment

In total, 117 miRNAs showed differences in expression levels between the HT and the NT group. Out of these 117 miRNAs, 46 miRNAs were upregulated and 71 miRNAs were downregulated. Furthermore, 19 miRNAs were significantly differentially expressed in the HT group compared with the NT, wherein significant values were those that had the $p$-value $<0.05$ and
Table 2. Altered miRNAs in the ALI rats after hypothermia treatment.

| miRNA_name    | Fold change | Regulation | P-value |
|---------------|-------------|------------|---------|
| rno-miR-183-5p| 4.4         | up         | 0.047   |
| rno-miR-182  | 3.2         | up         | 0.019   |
| rno-miR-323-3p| 3.2         | up         | 0.023   |
| rno-miR-196b-3p| 2.0        | up         | 0.004   |
| rno-miR-129-2-3p| 1.9       | up         | 0.043   |
| rno-miR-760-3p| 1.8         | up         | 0.002   |
| rno-miR-328a-3p| 1.6        | up         | 0.045   |
| rno-miR-711  | 1.5         | up         | 0.047   |
| rno-miR-142-3p| 2.7         | down       | 0.013   |
| rno-miR-98-3p | 2.4         | down       | 0.041   |
| rno-miR-541-5p| 2.3         | down       | 0.044   |
| rno-miR-503-3p| 2.1         | down       | 0.035   |
| rno-miR-653-3p| 2.1         | down       | 0.005   |
| rno-miR-144-3p| 1.8         | down       | 0.047   |
| rno-miR-193-3p| 1.7         | down       | 0.042   |
| rno-miR-142-5p| 1.6         | down       | 0.046   |
| rno-miR-301b-5p| 1.6        | down       | 0.044   |
| rno-miR-223-3p| 1.6         | down       | 0.041   |
| rno-miR-19a-3p| 1.6         | down       | 0.046   |

A list of increased and decreased miRNAs in the HT-treated ALI rats compared with the NT-treated ALI rats with a P value < 0.05 and a fold change > 1.5. Out of these 19 miRNAs, 8 miRNAs were upregulated and 11 miRNAs were downregulated. The most significantly upregulated miRNAs were miR-183 (mFC = 4.4; p = 0.04), miR-182, and miR-323 (mFC = 3.2; p = 0.02). In contrast, the most significantly downregulated miRNAs were miR-142 (mFC = 2.7; p = 0.01), miR-98 (mFC = 2.4; p = 0.04), and miR-541 (mFC = 2.3; p = 0.03).

To identify the effects of hypothermia in ALI, we analyzed the miRNAs inversely expressed in the HT group compared to the NT group, at a significant manner. We found that 8 miRNAs were inversely expressed between the two groups. Among them, 6 miRNAs (miR-142, miR-98, miR-541, miR-503, miR-653, and miR-223) were upregulated in the NT group and downregulated in the HT group. Two miRNAs (miR-323 and miR-196b) were downregulated in the NT group and upregulated in the HT group (Table 1, Table 2).

Potential functions of the differentially expressed miRNAs after hypothermia

We hypothesized that the 8 miRNAs that are differentially expressed between the HT and the NT group may be involved in the pathways related to the effects of HT treatment. Since each miRNA potentially regulates a large number of targets, two miRNA target databases, the miRanda and Targetscan (version 7.0), were used to improve the accuracy of target prediction. Gene ontology (GO) term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation analyses were then performed using the DAVID version 6.7. DAVID provides a tool for annotating biological meaning for input genes regulated by specific miRNAs and functional annotation clustering, and it uses an algorithm to explore relationships among the annotation terms via co-associated genes. The 8 lists of predicted targets (upregulated and downregulated miRNAs) were separately submitted to the functional annotation tool. The similar annotation contents were clustered into annotation clusters due to their similar biological meaning. The results showed that dysregulated target genes could be categorized into several major categories. (1) immune response: defense response, response to external stimuli, (2) inflammation: inflammatory cell activation, (3) cell survival: regulation of apoptosis, (4) cellular growth: epidermal growth factor activation, (5) cell-cell interaction: cell-cell communication, and (6) cell migration and adhesion. The main biological process in which the differentially expressed 8 miRNAs in HT were involved was the immune response (cluster 1), inflammation (cluster 2) and cellular apoptosis (cluster 3) (Table 3).

Pathway analysis of the differentially expressed miRNAs in hypothermia

The sorted gene lists of 8 miRNAs were overlapped with KEGG pathway database to identify signaling pathways regulated by those miRNAs. After removing redundant terms, we identified 12 annotated KEGG pathways for the differentially expressed miRNAs (Figure 3). Cytokine-cytokine receptor interaction, chemokine signaling pathway had high scores and were likely to be controlled by the differentially expressed miRNAs in HT. The pathways for these miRNAs were associated with the immune response: Cytokine-cytokine receptor interaction, chemokine signaling pathway via JAK-STAT signaling pathway; the cell migration and adhesion (leukocyte trans endothelial migration and adherens junction). Individually, miR-142 was mainly associated with the immunity and the cell growth via JAK-STAT signaling pathway via cytokine-cytokine receptor interaction; and apoptosis via the MAPK signaling pathway. miR-223 was also involved in cytokine-cytokine receptor interaction, and miR-653 participated in the cell adhesion and migration molecules through T cell receptor signaling pathway, tight junction, and leukocyte transendothelial migration. miR-98 was related to the hematopoietic cell lineage through NK cells, neutrophils, mast cells and platelets.

Construction of an altered miRNA interaction network

To perform static topological analysis, a network of

### Table 3. Functional annotation clustering of differently expressed miRNAs after hypothermia.

| Annotation Cluster 1 | Term                                                                 | Count | P-value  |
|----------------------|----------------------------------------------------------------------|-------|----------|
| G0TERM_BP FAT        | GO:0006952~defense response                                           | 19    | 0.0117   |
| G0TERM_BP FAT        | GO:0006935~chemotaxis                                                | 8     | 0.0157   |
| G0TERM_BP FAT        | GO:0032103~positive regulation of response to external stimulus      | 5     | 0.0202   |
| G0TERM_BP FAT        | GO:0009611~response to wounding                                       | 16    | 0.0261   |
| G0TERM_BP FAT        | GO:0031349~positive regulation of defense response                   | 5     | 0.0310   |
| G0TERM_BP FAT        | GO:0032101~regulation of response to external stimulus               | 7     | 0.0455   |
| G0TERM_BP FAT        | GO:0002712~regulation of B cell mediated immunity                     | 3     | 0.0475   |
| G0TERM_BP FAT        | GO:0002889~regulation of immunoglobulin mediated immune response     | 4     | 0.0488   |
| G0TERM_BP FAT        | GO:0002520~immune system development                                 | 9     | 0.0489   |

Cluster 2  Enrichment Score: 1.67

| G0TERM_BP FAT        | GO:0046649~lymphocyte activation                                     | 12    | 0.0004   |
| G0TERM_BP FAT        | GO:0045321~leukocyte activation                                      | 12    | 0.0020   |
| G0TERM_BP FAT        | GO:0042110~T cell activation                                         | 8     | 0.0045   |
| G0TERM_BP FAT        | GO:0002521~leukocyte differentiation                                 | 8     | 0.0056   |
| G0TERM_BP FAT        | GO:0030098~lymphocyte differentiation                                | 7     | 0.0067   |
| G0TERM_BP FAT        | GO:0030217~T cell differentiation                                    | 5     | 0.0213   |
| G0TERM_BP FAT        | GO:0042113~B cell activation                                         | 5     | 0.0352   |
| G0TERM_BP FAT        | GO:0030097~hemopoiesis                                              | 9     | 0.0394   |
| G0TERM_BP FAT        | GO:0006954~inflammatory response                                     | 11    | 0.0398   |

Cluster 3  Enrichment Score: 1.61

| G0TERM_BP FAT        | GO:0006915~apoptosis                                                | 18    | 0.0193   |
| G0TERM_BP FAT        | GO:0012501~programmed cell death                                    | 18    | 0.0219   |
| G0TERM_BP FAT        | GO:0008219~cell death                                               | 20    | 0.0255   |
| G0TERM_BP FAT        | GO:0016265~death                                                    | 20    | 0.0271   |
| G0TERM_BP FAT        | GO:0006916~anti-apoptosis                                           | 8     | 0.0415   |
| G0TERM_BP FAT        | GO:0043066~negative regulation of apoptosis                         | 11    | 0.0439   |
| G0TERM_BP FAT        | GO:0043069~negative regulation of programmed cell death             | 11    | 0.0485   |

The functional annotation of differentially expressed 8 miRNAs in HT-treated ALI rats compared with the NT-treated ALI rats was conducted by DAVID software (http://david.abcc.ncifcrf.gov). Annotation Cluster: a group of terms having similar biological functions; Enrichment Score: The geometric mean (in -log scale) of member’s P-values in a corresponding annotation cluster is the rank of their biological significance. Count: Genes involved in the terms.

**Figure 3.** Functional annotation of the differently expressed 8 miRNAs in HT-treated rat ALI compared with NT-treated rat ALI. The DAVID informatics resources tool was used for the analysis. The sorted gene list was input to KEGG pathway analysis to reveal the potential biological functions and pathways, which were ranked by significance level.
altered miRNAs after HT was established with their putative target molecules. After the predicted target genes associated with the HT-dysregulated miRNAs were imported into the network, the Cytoscape plug-in NetworkAnalyzer was applied. As a result, we obtained a network of 8 miRNAs and 769 target genes (Figure 4), and found that miR-503-3p and miR-142 correlate with targets GZMB, C5orf51, and STAM, whereas miR-503-3p and miR-541-3p correlate with target SH3BGR, DCDC1, and LRRC19 (Table 4).

**Table 4.** Genes related to the altered miRNAs in ALI after hypothermia.

| miRNAs                        | Genes                      |
|-------------------------------|-----------------------------|
| miR-503-3p & miR-142-3p       | GZMB, C5orf51, STAM         |
| miR-503-3p & miR-541-5p       | SH3BGR, DCDC1, LRRC19      |
| miR-503-3p & miR-653-3p       | POUSF1B, CNTD1              |
| miR-503-3p & miR-98-3p        | FLG, POLR2K                |
| miR-503-3p & miR-323-3p       | DRAM1                      |
| miR-503-3p & miR-196b-3p      | SAMD12, F3                 |
| miR-142-3p & miR-223-3p       | DCUN1D4, MORF4L2, IL7      |
| miR-142-3p & miR-323-3p       | IL17D, TWF1                |
| miR-142-3p & miR-196b-3p      | NXX2-3                     |
| miR-223-3p & miR-541-5p       | AC002451.1                 |
| miR-223-3p & miR-653-3p       | PPP3R2                     |
| miR-223-3p & miR323-3p        | FBXO8, HMGCS1              |
| miR-223-3p & miR-196b-3p      | RDIH10                     |
| miR-541-5p & miR-98-3p        | CBLN4                      |
| miR-541-5p & miR-323-3p       | MCTP1, SH3BGRL             |
| miR-541-5p & miR-196b-3p      | SPRED2, RP4-559A3.7        |
| miR-653-3p & miR-98-3p        | AC107021.1                 |
| miR-653-3p & miR-196b-3p      | RPI-170019.20, HOXA9       |
| miR-98-3p & miR-323-3p        | MDM1, CFHR3                |
| miR-98-3p & miR-196b-3p       | LYSMD3, ELOVL2             |
| miR-323-3p & miR-196b-3p      | COMMD8                     |

**Discussion**

There have been recent reports showing that induced mild hypothermia can improve clinical outcome in several severe clinical situations. In refractory ARDS patients’ study, it was found that HT reduced metabolic rate and allowed successful ventilation with a very low tidal volume without an increase in PaCO₂ and respiratory acidosis. However, the therapeutic effect of HT is still under debate. Some studies have reported that HT can have beneficial effects in endotoxemic rats via
modulation of the inflammatory response and attenuation of lung injury. Kira et al. also demonstrated that HT inhibits the adhesion, activation, and accumulation of neutrophils during the acute phase of ALI in rats and may have the potential to reduce ongoing inflammation of ALI. In contrast, Torossian et al. reported that HT increased mortality and impaired immune response in a septic rat model. Stewart et al. also reported that mild HT increased the levels of IL-6 and IL-10 in endotoxemic mice. In our current study, we studied the therapeutic effects of HT in LPS-induced ALI rat model compared with NT treatment. ALT/AST (hepatic dysfunction), BUN/Cr (renal dysfunction), and Lactate (tissue hypoperfusion) have previously been investigated as predictive markers of organ dysfunction. Our results show that mild HT ameliorates liver and kidney injury and improves tissue perfusion. Upon histological examination, we observed that lung injuries in the HT group were less prominent than in the NT group. These results led us to investigate the mechanisms that are involved in these therapeutic effects of HT in ALI.

Functional genomics approaches provide new promising insights into understanding gene-mechanism interactions. Actually, the expression profiles of miRNAs have been studied for several diseases, including different types of malignancies, acute myocardial infarction, congestive heart failure, type 1 diabetes mellitus, infectious diseases. In present study, the differential expression profiles of miRNAs in ALI rats with HT compared to that in the NT group permitted us to find that up-regulated 6 miRNAs (miR-142, miR-98, miR-541, miR-503, miR-653, miR-223) in NT were inversely downregulated in the HT group. In contrast, 2 miRNAs (miR-323, miR-196b) that were downregulated in the NT were upregulated in the HT group. We searched for biological processes related with significantly differently expressed 8 miRNAs in HT using with DAVID. We also found that the dysregulated miRNAs in the HT rats were involved in the immunity and the inflammation pathways, as determined by the KEGG pathway analysis. Previous studies have shown that miRNAs may have a role in the immune response and inflammation in sepsis, or ARDS. One such example is that upregulated miR-155 and downregulated miR-125b is one of the pathogenesis in LPS-induced endotoxemic rats, and acts through tumor necrosis factor (TNF)-α stimulation. Dysregulation of miR-146, miR-155, miR-181b, miR-21, and miR-301a were related to NF-κB activation, which is an essential mediator in the immune system. miR-146 inhibited Toll-like receptor and cytokine signaling through downregulation of IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6 (TRAF 6). In this study, dysregulated miR-142 and miR-223 are involved in the inflammatory response by modulating interleukin 2/3 (IL-2/3), interleukin 6 signal (Transducer (IL6ST), IL-7, TNF-α/TNFR (TNF receptor) interaction, and CCL4 (chemokine ligand 4) in JAK-STAT signaling pathway and cytokine-cytokine receptor interaction. The interaction of the inflammatory mediators, TNF-α/ TNFR is very important due to its position at the apex of the proinflammatory cytokine cascade and its dominance in the pathogenesis of various diseases. Moreover, TNF-α is one of the main cytokines involved in the response to LPS. Thus, we hypothesize that the alteration of the expression of many genes involved in immune response and inflammation contributes to the therapeutic effects of HT in ALI.

In this study, analysis of the differentially expressed miRNAs in HT by KEGG pathway analysis showed that they may be involved in the regulation of apoptosis. Apoptosis of epithelial and endothelial cells is known for the major metabolic pathway that activated in the lung of ARDS patients. Lee et al. also reported that the levels of apoptosis mediators were increased in the BAL fluid of ARDS patients, and, a delayed apoptosis of intra-alveolar neutrophils and increased apoptosis of alveolar epithelium increased the severity of lung injury. Some studies showed that miRNAs are related with the regulation of apoptosis. Upregulated miR-26a increased apoptosis in hypoxic rat cardiomyocytes via the caspase-3 pathway and miR-let-7 regulated apoptosis in tumors. In this study, the upregulated miR-142 expression profile in NT was downregulated after HT treatment. We confirmed that miR-142 is participated in the regulation of apoptosis through ASK2 (apoptosis signal-regulating kinase), MLK3 (mixed-lineage protein kinase 3), and TAB2 (TAK1 binding protein 2) in MAPK signaling pathway in Gene ontology and functional annotation analysis.

There have been a few studies about miR-223 that is differentially expressed in different situations. Serum miR-223 expression levels are significantly reduced in septic patients compared with healthy controls, but lung miR-223 levels are increased in influenza virus infected mice. Another study suggested that the protective effect of 5, 14-HEDGE was related to decreased serum miR-223 level through down-regulation of MyD88/TAk/I/IKKb/IKBα/NF-kB pathway in the rat model of septic shock. In our study, lung miR-223 expression levels were increased at 6 h after LPS challenge but decreased in HT-treated rat ALI. The discrepancy among previous studies as well as our results could be attributed to factors such as differences in between humans and animals, type of infection, type of sample, and time points for measurement of miRNA expression levels.
In a review of miR-223, miR-223 was described to affect multiple targets simultaneously for key processes. Such processes include hematopoietic cell differentiation, particularly towards the granulocyte lineage (where miR-223 is abundant) and the myeloid lineage (where miR-223 expression decreases). NF-kB is an important immunological mechanism that is dampened by miR-223. Anca et al. concluded that miR-223 regulates leukocyte chemotaxis by directly targeting the chemokines CXCL2, CCL3, and IL-6 in myeloid cells in TB. Similarly, downregulated miR-223 in HT was involved in the cytokine-cytokine receptor interaction via CCL4, CCL3, and CCL8, and, the hematopoiesis via IL13.

Despite a few studies on miRNAs expression profiling in ARDS or sepsis, there have been no previous attempts to identify the expression profiles in rat ALI after HT treatment. In conclusion, this study showed that mild hypothermia restored indicators of multi-organ dysfunction and attenuated lung injury. Moreover, we systematically analyzed the expression of miRNAs in ALI after HT treatment compared with those after NT. The expression levels of lung miR-98, miR-142, miR-223, miR-503, and miR-541, miR-653, miR-323 and miR-196b were significantly altered in HT treatment compared to NT. These differently expressed 8 miRNAs may play certain roles in the therapeutic effects of HT in ALI by targeting genes that regulate the immunity, inflammation, and apoptosis. This is the first report of miRNAs expression profile in hypothermia treated rat ALI. Further studies should be encouraged because mild hypothermia may provide an approach to the modulation of ongoing inflammatory response in ALI.

Materials & Methods

Rat model of ALI

Male Sprague Dawley rats (180-200 g) purchased from Samtaco Bio Korea (Cheonan, South Korea) were used for this study. The rats were housed under virtually identical conditions for at least 3 days for adaptation. They had free access to standard food and filtered water, were under a 12:12 h light-dark cycle, and were kept at a room temperature maintained at 23-26°C. They were grouped as two or three animals per cage before any procedure and singly after the procedures. All procedures were performed by the same investigator in order to minimize variability.

To induce ALI, bacterial lipopolysaccharide (LPS, serotype 055:B5, 20 mg/kg, Sigma Chemical, St Louis, MO), which is the outer membrane of Escherichia Coli, diluted in saline was used. Bacterial LPS is an effective trigger of the inflammatory response during infection with gram-negative bacilli. ALI rats were intraperitoneally infiltrated with LPS (5 mg/kg) at time 0, control rats received the same amount of saline intraperitoneally. At 16 h after LPS or saline challenge, the rats were anesthetized with an intraperitoneal injection of 1% ketamine (80 mg/kg) and xylazine (5 mg/kg). Rats were placed in a supine position at a 60° angle. Rats were endotracheally intubated with a sterile polyethylene catheter (PE-190 BD, NJ, USA). LPS (10 mg/kg, 100 μL) or saline (100 μL) was infiltrated via the endotracheal catheter slowly, and 4 mL of air was infused to expand LPS diffusely into both the lungs.

Induction of hypothermia

All rats were randomly assigned to three groups: saline-treated controls (Cont, n = 5), LPS-induced ALI with Normothermia (LPS, n = 5), and LPS-induced ALI with Hypothermia (LPS + HT, n = 5). Body temperature was monitored using a rectal probe (Physitemp, Physitemp Instrument Inc, NJ). After intra-tracheal infiltration of LPS, rectal temperature was maintained at 32-34°C for 6 h by external cooling with ice cube and spray in LPS + HT group. The target temperature was reached within 10 minutes. The LPS group kept in normothermia, 37±0.5°C, was placed on a heated pad (FHC, Bowdoinham, ME, USA) and a blanket for 6 h. Anesthesia was maintained with an intermittent infusion of 1% ketamine (80 mg/kg) and xylazine (5 mg/kg) for 6 h.

Bronchoalveolar lavage (BAL) and sample collection

Sepsis-related lung injury rat model induced by LPS study showed that at 6 hours after LPS administration, LPS developed the severe state, thrombocytopenia, elevated lactate levels, hypoxemia, and liver and renal injury. Hypoxemia at 6 hours was severe and slightly improved at 24 hours. So we collected the BAL and the sample at 6 hours after LPS infiltration.

The rats were sacrificed at 6 h after the NT or HT treatments. Blood was collected through cardiac puncture and centrifuged at 3,000 rpm for 10 mins to obtain plasma. Plasma samples were frozen at −70°C before measurements were made using a specific kinetic enzymatic analyzer, IDEXX VetTest® Chemistry Analyzer (IDEXX Laboratories, Inc., ME). The trachea was incised, and a 14-G tube was placed into the trachea. Left (Lt.) main bronchus was ligated with 3-0 silk. After 5 mL of PBS was flushed back and forth three times through the 14-G tube placed in trachea, BAL fluid was collected from the Right (Rt.) lung. The BAL fluid was centrifuged (3,000 rpm, 4°C, 10 mins), the pellet was diluted with 500 μL PBS, and the total cells were
counted using LUNA automated cell counter (Logos-Biosystems, VA) according to the manufacturer’s instructions. Of the 500 μL diluted pellet, 200 μL was cytopspinned, prepared on a slide, and then Wright-Giemsa-stained. Differential cell counts were performed by counting 100 cells under a microscope (Olympus, Tokyo, Japan) in four representative slide sections, and the number of neutrophils was calculated as the percentage of neutrophils multiplied by the total number of cells in the BAL fluid.

**Lung histology**

Lt. upper lobe was fixed with 10% formalin for hema-toxylin and eosin (HE) stain, and Lt. lower lobe was stored at −70°C for miRNA study. After 24 h, the lung tissue was dehydrated and embedded in paraffin. Sections were stained with HE for evaluation of the severity of lung injury. Each lung section was blindly assigned by a clinical pathologist to obtain the lung injury score (LIS). LIS comprising of 4 components (alveolar capillary congestion, hemorrhage, inflammatory cells infiltrating the airspace or interstitium, and thickness of the alveolar wall) scored on a 5-point scale (0 = minimal damage, 1 = mild damage, 2 = moderate damage, 3 = severe damage, 4 = maximal damage) each and summed.[20]

**RNA isolation**

Total RNA was extracted from the rat lung tissue using the TRI REAGENT (MRC, OH) according to the manufacturer’s instructions. Following homogenization, 1 mL of solution was transferred to a 1.5 mL Eppendorf tube and centrifuged at 12,000 rpm for 10 min at 4°C to remove insoluble material. The supernatant containing RNA was collected, mixed with 0.2 mL of chloroform, and centrifuged at 12,000 rpm for 15 mins at 4°C. After RNA in the aqueous phase was transferred into a new tube, the RNA was precipitated by mixing 0.5 mL of isopropyl alcohol and recovered by centrifuging the tube at 12,000 rpm for 10 mins at 4°C. The RNA pellet was washed briefly in 1 mL of 75% ethanol and centrifuged at 7,500 rpm for 5 mins at 4°C. Finally, the total RNA pellet was dissolved in Nuclease-free water, and its quality and quantity was assessed by an Agilent Bioanalyzer 2100.

**miRNA microarray**

Rat microRNA expression was analyzed using miCURY LNA™ microRNA Array (7th gen-has, mmu & rno array; Exiqon, Vedbaek, Denmark), covering 690 well-characterized rat microRNA among 3,100 capture probes for rat miRNAs. In this procedure, 5′-phosphates from 800 ng of total RNA were removed by treating with Calf Intestinal Alkaline Phosphatase (CIP) followed by labeling with Hy3 green fluorescent dye. Labeled samples were subsequently hybridized by loading onto a microarray slide using Hybridization Chamber Kit part # G2534A (Agilent Technologies, Santa Clara, CA, USA) and Hybridization Gasket Slide Kit part # G2534-60003 (Agilent Technologies). Hybridization was performed over 16 h at 56°C followed by washing the microarray slide as recommended by the manufacturer. Processed microarray slides were then scanned with Agilent G2565CA Microarray Scanner System (Agilent Technologies). Scanned images were imported by Agilent Feature Extraction software version 10.7.3.1 (Agilent Technologies), and fluorescence intensities of each image were quantified using the modified Exiqon protocol and corresponding GAL files.

**Functional annotation analysis of the predicted targets**

miRNA target prediction was performed by the miRanda and Targetscan, version 7.0. To identify the functions of the differentially expressed miRNAs, the lists of predicted targets of dysregulated miRNAs were separately submitted to the functional annotation tool provided by the Database for Annotation, Visualization, and Integrated Discovery (DAVID), version 6.7.[21,22] The predicted targets were annotated by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. A pathway was considered to be significant only if it passed the count threshold of three genes per annotation term and presented EASE score, with Benjamin-Hochberg correction set to <0.05.[23,24]

**Statistical analysis**

The results are expressed as the mean ± standard deviation. Differences between the two groups were determined using Student’s t-test. A p value <0.05 was considered to be statistically significant. Statistical analysis was performed by using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA).

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**Ethical statements for animal experiments** All animal experiments were approved by Institutional Animal Care and Use Committee in Daejeon St. Mary’s Hospital, Catholic University.

**Conflict of Interest** The authors declare no conflict of interest.
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