Potential therapeutic strategies aimed at reducing the intensity of mechanical ventilation in ARDS

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Citation for published version (APA):

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Cardiac arrest patients have an impaired immune response, which is not influenced by induced hypothermia

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Submitted
Abstract

Purpose: To study the effect of induced hypothermia on immune response after cardiac arrest.

Methods: Prospective observational cohort study in a mixed surgical-medical intensive care unit (ICU). Patients admitted at the ICU after surviving cardiac arrest were included and during 24 hours body temperature was strictly regulated at 33°C or 36°C. Blood was drawn at three time points: after reaching target temperature, at the end of the target temperature protocol and after rewarming to 37°C. Plasma cytokine levels and response of blood leucocytes to stimulation with Toll-like receptor (TLR) ligands lipopolysaccharide (LPS) from Gram-negative bacteria and lipoteicoic acid (LTA) from Gram-positive bacteria were measured. Also, monocyte HLA-DR expression was determined.

Results: Compared to healthy controls, cardiac arrest patients kept at 36°C had increased plasma cytokines levels, which was not apparent in patients kept at 33°C. Immune response to TLR ligands in patients after cardiac arrest was generally reduced, associated with lower HLA-DR expression. Patients kept at 33°C had preserved ability of immune cells to respond to LPS and LTA compared to patients kept at 36°C. These differences disappeared over time. HLA-DR expression did not differ between 33°C and 36°C.

Conclusions: Patients after cardiac arrest have a modest systemic inflammatory response compared to healthy controls, associated with lower HLA-DR expression and attenuated immune response to Gram-negative and Gram-positive antigens, the latter indicative of an impaired immune response to bacteria. Patients with a body temperature of 33°C did not differ from patients with a body temperature of 36°C, suggesting induced hypothermia does not affect immune response.
Introduction

Induced hypothermia is applied clinically to reduce ischemia-reperfusion injury during operative procedures and following cardiac arrest [1-3]. In cardiopulmonary surgery, hypothermia is associated with improved neurological outcome [4, 5]. Also, avoiding hyperthermia by controlling body temperature is associated with a favourable neurologic function in survivors of cardiac arrest [6], as well as with earlier shock reversal in septic shock patients [7]. The underlying mechanism of mitigating harmful effects of ischemia-reperfusion by controlling body temperature is proposed to be inhibition of an exaggerated systemic inflammatory response syndrome (SIRS). In a pig model of cardiac arrest, hypothermia reduced expression of pro-inflammatory cytokines within the brain [8]. Also in other models of hyper-inflammatory conditions, hypothermia reduced organ failure associated with a decrease of the inflammatory response [9-15]. Thereby, hypothermia may mitigate harm caused by an ‘overshoot’ of a systemic inflammatory response.

On the other hand, an adequate adaptive host immune response to pathogens and infection is crucial [16] and fever is considered an important factor for optimal antimicrobial host defence [17]. Hypothermia inhibits immune response [18], with delayed generation of pro-inflammatory cytokines by monocytes [19] and reduction of neutrophil and monocyte migration [20, 21]. The consequence of inhibiting host immune response by induced hypothermia, may be a higher infection risk. In particular, patients shortly after a cardiac arrest suffer from a dysregulated production of cytokines and may therefore be susceptible to nosocomial infection [22]. Two randomized trials in cardiac arrest patients reported no increased overall infection rates associated with hypothermia [1, 23], although a trend towards more infection could be noted [23]. In addition, prolonged hypothermia (for more than 48 hours) did not increase risk of infection in patients with brain injury, annotated all patients received selective decontamination of the digestive tract [24]. However, a recent systematic review in patients enrolled in randomized controlled clinical trials of therapeutic hypothermia for any indication, showed an association of hypothermia with increased prevalence of pneumonia and sepsis, although overall infection rate was not affected [25].

Patient studies describing the effect of induced hypothermia specifically on the innate immune response are scarce and studies lack an adequate control group [26]. Recently, the Temperature Target Management (TTM) trial was concluded, in which cardiac arrest
Cardiac arrest patients have an impaired immune response, which is not influenced by induced hypothermia [23]. In this predefined sub study of the TTM trial, we investigated the effect of induced hypothermia on the innate immune response to toll like receptors (TLRs) ligands. In humans, TLRs are critical in the first host immune response to pathogens by mediating cytokine secretion [27]. In addition, we measured human leukocyte antigen-DR (HLA-DR) surface expression on monocytes, as low HLA-DR expression is associated with secondary infection and mortality in critically ill patients [28, 29] and hypothermia was shown to reduce HLA-DR expression in vitro [30]. We hypothesized that SIRS is reduced in patients with a target temperature of 33°C compared to patients with a target temperature of 36°C, but that hypothermia does not affect the immune response to pathogens.

**Methods**

*Patient inclusion*

The study was approved by the local medical ethics committee of the Academic Medical Center, University of Amsterdam, the Netherlands and conducted in concordance with the principles of the declaration of Helsinki and good clinical practice. From January 2011 until October 2012, adult patients admitted to the mixed surgical-medical intensive care unit (ICU) of a tertiary referral center in Amsterdam, the Netherlands after out-of-hospital cardiac arrest with a Glasgow Coma Score <8 and treated with therapeutic hypothermia (33°C) for 24 hours, were included in our study after their relatives gave informed consent. From March 2011, our center started enrollment in the TTM trial, patients who enrolled in the TTM trial were only included for our sub study after additional informed consent was obtained from the relatives. Exclusion criteria were pregnancy, out-of-hospital cardiac arrest of presumed non-cardiac cause, in-hospital cardiac arrest, known bleeding diathesis, suspected or confirmed acute intracranial bleeding, suspected or confirmed acute stroke, temperature on admission <30°C, un-witnessed asystole, persistent cardiogenic shock, known limitations in therapy, known disease making 180 day survival unlikely, known pre-arrest cerebral performance category 3 or 4, >240 minutes from return of spontaneous circulation (ROSC) to randomisation [23]. Patients included prior to the TTM trial (n=8) and during the TTM trial (n=12, 3 in the 33°C group and 9 in the 36°C group) did not differ in patients characteristics, Acute Physiology and Chronic Health Evaluation (APACHE) III, Simplified Acute Physiology Score (SAPS) II score, time to ROSC and cause of cardiac arrest (data not shown). Healthy volunteers were recruited and included after informed consent for a single blood donation.
Cardiac arrest patients have an impaired immune response, which is not influenced by induced hypothermia.

**Study procedure**

Patients included in our study received standard post-resuscitation care according to the current best practice or the post-resuscitation protocol of the TTM trial [23], including 24 hours of target temperature management to achieve a core body temperature of either 33°C or 36°C with the use of ice-cold saline (maximum 1 L) and a cooling device (Blanket roll, Cincinatti Sub Zero, Cincinatti). Temperature was measured using a bladder catheter. All patients were sedated with propofol, mechanically ventilated in a pressure controlled mode and selective digestive tract decontamination was administered. Patients received either anticoagulant therapy as deemed appropriate or thrombosis prophylaxis. Patients were not fed. Blood was drawn at three time points: after reaching target temperature (33°C or 36°C; T=1), at the end of the target temperature protocol (T=2) and after reaching 37°C (T=3). Healthy volunteers donated only once.

**Measurements & Data collection**

Data from the patient data monitoring system were collected, including previous medical history, age, gender, weight, length, maximal leukocyte count, APACHE III, SAPS II score, as registered in the Dutch National Intensive Care Evaluation [31]. Serum levels of Interleukin (IL)–1β, IL–1RA, IL–8, IL–10, Macrophage Inflammatory Proteins (MIP)-1, Monocyte Chemotactic Protein (MCP)–1 and soluble CD40 ligand were determined by Luminex, according to instructions of the manufacturer (Merck Millipore Chemicals BV; Amsterdam; the Netherlands). Serum levels of IL–6 and Tumor Necrosis Factor (TNF)-α were determined by ELISA, according to instructions of the manufacturer (R&D Systems; Abingdon, United Kingdom).

**Whole blood stimulation**

The response of blood leucocytes to stimulation with TLR ligands was determined in a whole blood stimulation system. Immediately after drawing, blood was diluted 1:1 with RPMI and stimulated with LPS (100 ng/ml; Sigma Aldrich, Steinheim, Germany) or LTA (10 μg/ml; Invivogen, San Diego, USA) as bacterial antigens of respectively Gram-negative and Gram-positive bacteria. After 2 or 24 hours stimulation in an 37°C incubator with 5% CO₂, whole blood was centrifuged at 600g for 10 minutes at 4°C. Supernatant was stored at -80°C and levels of Interleukin (IL)–6 and Tumor Necrosis Factor (TNF)-α were determined by ELISA, according to instructions of the manufacturer (R&D Systems).
Cardiac arrest patients have an impaired immune response, which is not influenced by induced hypothermia.

**HLA-DR expression**

HLA-DR expression on monocytes was analysed by fluorescence activated cell sorter after labelling by incubation with HLA-DR-FITC and CD14-PE monoclonal antibodies (Becton Dickinson, Erembogem, Belgium). Erythrocytes were lysed with lysis buffer (Becton Dickinson BV, Breda, the Netherlands) and the debris was washed away. The remaining leukocytes were fixated with 1% paraformaldehyde. The final flowcytometric analysis was done, using a flowcytometry (Becton Dickinson BV, Breda, the Netherlands). HLA-DR expression was measured on CD14-bright monocytes which have mainly anti-inflammatory properties [32] and CD14-dull monocytes which are more pro-inflammatory, since they selectively induce production of cytokines in response to viruses and immune complexes containing nucleic acids [33]. To compare patient samples measured at different time points, a standard series of quantum beads which are labelled with a known quantity of FITC fluorescent label (Quantum FITC-5 MESF (premix), Bangs laboratories, Fishers, USA) was analysed simultaneously with each measurement. Therefore, HLA-DR expression could be expressed in Molecules of Equivalent Soluble Fluorochrome (MESF) units.

**Statistical analysis**

Data are expressed by mean ± SD in the table and as median ± range in the figures, depending on distribution of the data. Differences between 33°C and 36°C groups were compared using an unpaired T-test or a Mann-Whitney U test, depending on distribution of the data. The effect of temperature over time was compared using a repeated measurement ANOVA or a Friedman test with either a Bonferroni’s or Dunn’s multiple comparison test correction. Differences between healthy volunteers and patients with a target temperature of either 33°C or 36°C over time were compared using a one-way ANOVA or Kruskal Wallis test, with either a Bonferroni’s or Dunn’s multiple comparison test, depending on distribution of the data. Statistical significance was set at P<0.05.

**Results**

**Patient characteristics**

Patients in 33°C and 36°C groups did not differ in previous medical history or disease severity (Table 1). After reaching target temperature at T=1, body temperature was 33.2 ± 0.7°C in patients with a target temperature of 33°C and 35.6 ± 0.9°C in patients with a target temperature of 36°C (p=0.002). At T=1, plasma samples could not be collected from 4 patients. One patient with a target temperature of 33°C died before the last time
Cardiac arrest patients have an impaired immune response, which is not influenced by induced hypothermia point of blood sampling (T=3). A confirmed infection within 24 hours after ICU admittance occurred in 2 patients, 1 patient in each group (Table 1).

Table 1:
Baseline characteristics of 11 patients with a target temperature of 33°C compared 9 patients with a target temperature of 36°C, after reaching target temperature. Data expressed as mean ± SD.

<table>
<thead>
<tr>
<th></th>
<th>33°C</th>
<th>36°C</th>
<th>P-value</th>
</tr>
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<tr>
<td>Gender (Male/Female)</td>
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<td>8/1</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>64 ± 15</td>
<td>61 ± 15</td>
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<tr>
<td>Length (cm)</td>
<td>173 ± 12</td>
<td>178 ± 7</td>
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<tr>
<td>Weight (kg)</td>
<td>81 ± 24</td>
<td>81 ± 13</td>
<td>0.95</td>
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<td>Maximal leukocyte count</td>
<td>15.8 ± 4.9</td>
<td>16.1 ± 4.0</td>
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<tr>
<td>SAPS II score</td>
<td>54 ± 18</td>
<td>59 ± 18</td>
<td>0.58</td>
</tr>
<tr>
<td>APACHE III score</td>
<td>80 ± 44</td>
<td>88 ± 34</td>
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Previous medical history

<table>
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<th>36°C</th>
<th>P-value</th>
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<td>Arrhythmia</td>
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<td>1</td>
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<td>COPD</td>
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<td>Chronic respiratory failure</td>
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<tr>
<td>Cirrhosis</td>
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<td>0</td>
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<td>AIDS</td>
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</tr>
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<td>Immune insufficiency</td>
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<tr>
<td>Malignancy</td>
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</table>

The effect of hypothermia on the SIRS reaction

Baseline inflammatory response was measured in plasma cytokines levels (figure 1). Levels of IL–1RA, IL–8, IL–10 and MCP–1 showed an increase in the patient group with a target temperature of 36°C compared to healthy controls, which was not apparent in the 33°C group. Levels of IL–1RA, but not of other cytokines, decreased after 24 hours of temperature management protocol (T=1 vs. T=2; figure 1) in the group with a target temperature of 36°C. Levels of IL–1RA, IL–8, IL–10 and MCP–1 were higher in the 36°C group compared to the 33°C group shortly after reaching target temperature, but these
Cardiac arrest patients have an impaired immune response, which is not influenced by induced hypothermia differences disappeared during temperature management over time (T=1; figure 1). Plasma levels of IL-1β, MIP-1, soluble CD40 ligand and TNF-α levels were not increased in patients compared to healthy controls, nor were there differences between the 33°C group and the 36°C group.

Figure 1: Cytokine levels in plasma of healthy controls, cardiac arrest patients with a target temperature of 33°C and cardiac arrest patients with a target temperature of 36°C, after reaching target temperature (T=1), at the end of the target temperature protocol (T=2) and after reaching 37°C (T=3). Data expressed as median with range. *: P < 0.05; **: P < 0.01.
Cardiac arrest patients have an impaired immune response, which is not influenced by induced hypothermia.

Figure 2: Cytokine production after whole blood stimulation with LPS or LTA, in plasma of healthy controls, cardiac arrest patients with a target temperature of 33°C and cardiac arrest patients with a target temperature of 36°C, after reaching target temperature (T=1), at the end of the target temperature protocol (T=2) and after reaching 37°C (T=3). Healthy volunteers are marked by white bars; cardiac arrest patients with a target temperature of 33°C are marked by grey bars, cardiac arrest patients with a target temperature of 36°C are marked by black bars. Every measurement is preceded by a blank control stimulation. Data expressed as median with range. *: P < 0.05; **: P < 0.01.

The effect of hypothermia on the immune response to TLR ligands

Results of whole blood stimulated for 2 hours were not different from 24 hours stimulation. Therefore, only the results of 24 hours stimulation are shown (figure 2).

Response to LPS: The immune response to stimulation with Gram-negative antigen LPS resulted in lower levels of IL-6 and TNF-α in patients of the 36°C group compared to healthy controls, which increased towards the end of the temperature management protocol in the group with a target temperature of 36°C (figure 2, upper panel; T=1 vs. T=3). This increase was not observed in the 33°C group. TNF-α levels were decreased in the 36°C group compared to the 33°C group at baseline. However, no significant differences between the groups appeared during the 24 hours of temperature management and after rewarming.

Response to LTA: Production of IL-6 did not differ between patient groups and healthy controls, nor were there differences over time between the 33°C and 36°C groups (figure 2, lower panel). The TNF production in response to stimulation with the Gram-positive antigen LTA was decreased in patients with a target temperature of 36°C compared to
Cardiac arrest patients have an impaired immune response, which is not influenced by induced hypothermia.

Healthy controls at the start of the temperature management and increased again after regaining normothermia (T=1 vs. T=3; figure 2). TNF-α level was also lower in the 36°C group compared to the 33°C group at T=1, but this difference between groups disappeared during temperature management over time.

The effect of hypothermia on HLA-DR expression

HLA-DR expression on CD14-bright monocytes was reduced in patients compared to healthy controls at all the time points for both the 33°C and 36°C group (figure 3). HLA-DR expression on both bright and dull monocytes further decreased over time (T=1 vs. T=3; figure 3). HLA-DR expression showed no differences between the 33°C and 36°C groups.

Discussion

Patients after cardiac arrest demonstrate a systemic inflammatory response syndrome compared to healthy controls, which was most noted for those who were kept at 36°C and blunted for those who were cooled to 33°C. This SIRS reaction was associated with lower HLA-DR expression and an attenuated immune response to Gram-negative and Gram-positive antigens compared to healthy controls. Between patients with a targeted...
Cardiac arrest patients have an impaired immune response, which is not influenced by induced hypothermia.

An increased systemic inflammatory response following cardiac arrest compared to healthy controls has been shown before [22]. The inflammatory response in cardiac arrest patients treated with induced hypothermia, was previously studied by Bisschops et al, showing a temporary increase of IL–6 levels during hypothermia and increased levels of IL–8 and MCP–1 compared to baseline [26], with levels largely comparable to our patients. Anti-inflammatory cytokines IL–1RA and IL–10 were unaltered compared to baseline [26]. However, in this study, effects of body temperature could not be dissected from effects of ischemia–reperfusion injury following cardiac arrest. Our study expands on these findings by including a normothermic control group over time. We found that initial cytokine levels were reduced in patients with a target temperature of 33°C compared to 36°C, although differences were small. This decrease in inflammatory response is in line with multiple experimental studies demonstrating an inhibitory effect of hypothermia on cytokine levels [10-13, 19]. However, at the end of the 24 hours of the temperature management protocol, there were no differences between both groups in plasma cytokine levels. Thereby, any effect of hypothermia on systemic inflammatory response seems temporary.

The systemic inflammatory response in patients was accompanied by an initial decreased ability of immune cells to respond to TLR ligands LPS and LTA, although not all cytokines were affected. The ability to generate an immune response can also be measured by HLA-DR expression. Of interest, we found a clear reduction in the expression of HLA-DR following cardiac arrest, compared to healthy controls. A blunted host immune response to TLR ligands and a decreased HLA-DR expression have been shown before in septic shock patients [29, 34], which is thought to contribute to an increased risk of nosocomial infection. To the best of our knowledge, this attenuated immune response to bacterial antigens after cardiac arrest has not been reported before. Decreased HLA-DR expression together with a blunted response to TLR ligands suggests that also cardiac arrest patients have an impaired immune response to pathogens. In line with this, nosocomial infection occurs in up to 50-65% of cardiac arrest patients [35-37]. Thereby, cardiac arrest patients should be closely monitored for the development of nosocomial infection. Whether prophylactic antibiotics are warranted in this patient group remains to be determined.
To investigate effects of induced hypothermia on immune response in cardiac arrest patients, our study provided an appropriate experimental setting in a more or less homogenous patient population. We found that the decreased ability of immune cells to produce TNF-α in response to TLR ligands was more prominent in patients with a target temperature of 36°C compared to 33°C. Thereby, hypothermia does not seem to have an inhibitory effect on immune response to TLR ligands compared to normothermia. Also, we found no differences in HLA-DR expression between the 33°C and 36°C groups at any time points. These results are in line with the TTM trial which found no significant differences in infection rate between the 33°C and 36°C groups in a large cohort of cardiac arrest patients [23]. In contrast, a previous study in cardiac arrest patients found that induced hypothermia was an independent risk factor for infection [35]. However, that study lacked a normothermic control group. Also, a meta-analysis suggests that hypothermia increases risk of infection, but in a very heterogeneous patient population [25]. Taken together, our results suggest that having had a cardiac arrest may render the patient susceptible to infection by a decreased immune response, but this risk of infection is not further increased by induced hypothermia.

Limitations of our study are small sample size. However, larger sample sizes with these kinds of investigations including whole blood stimulations are intricate, since they are labour-intensive and expensive. However, our sample size is comparable to previous studies [24, 26]. Lastly, the inclusion period was extensive, but our standard of care protocol did not undergo relevant changes during this time period. The relevance of our findings is that lowering body temperature may be done safely without compromising the immune response in patient groups who are thought to benefit from regulating body temperature. This may be especially interesting for those patients who suffer from an increased inflammatory response. Currently, a large trial on hypothermia in sepsis patients is ongoing (Trial number: NCT01455116).

**Conclusion**

Patients after cardiac arrest have a modest systemic inflammatory response compared to healthy controls. Cardiac arrest was associated with lower HLA-DR expression and attenuated immune response to Gram-negative and Gram-positive antigens, indicative of an impaired immune response. A body temperature of 33°C did not influence immune reaction compared to 36°C, suggesting that induced hypothermia in itself does not affect immune response.
Cardiac arrest patients have an impaired immune response, which is not influenced by induced hypothermia. The hypothermia after cardiac arrest study group, (2002) Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. N Engl J Med 346: 549-556


Cardiac arrest patients have an impaired immune response, which is not influenced by induced hypothermia after cardiac arrest as a “sepsis-like” syndrome. Circulation 106: 562-568


