Review Article

Oxidative Stress and Antioxidant Activity in Hypothermia and Rewarming: Can RONS Modulate the Beneficial Effects of Therapeutic Hypothermia?

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Hypothermia is a condition in which core temperature drops below the level necessary to maintain bodily functions. The decrease in temperature may disrupt some physiological systems of the body, including alterations in microcirculation and reduction of oxygen supply to tissues. The lack of oxygen can induce the generation of reactive oxygen and nitrogen free radicals (RONS), followed by oxidative stress, and finally, apoptosis and/or necrosis. Furthermore, since the hypothermia is inevitably followed by a rewarming process, we should also consider its effects. Despite hypothermia and rewarming inducing injury, many benefits of hypothermia have been demonstrated when used to preserve brain, cardiac, hepatic, and intestinal function against ischemic injury. This review gives an overview of the effects of hypothermia and rewarming on the oxidant/antioxidant balance and provides hypothesis for the role of reactive oxygen species in therapeutic hypothermia.

1. Introduction

Hypothermia has been known as a possible therapeutic tool for millennia. However, it has only been used more systematically in the last two centuries, and it is recently that we have started to understand some of its mechanisms of action and side effects.

1.1. Characterization of Hypothermia. Normal body temperature in humans is maintained near a constant level of 36.5–37.5°C through homeostatic processes of thermoregulation. The hypothalamus controls body temperature through the preoptic and the posterior nuclei. The posterior nucleus is especially important, since it acts in regulating the physiological responses that allow the control of body temperature, such as vasoconstriction, shivering or increased intake of food to warm-up, sweating, and vasodilation. Heat is mainly generated in muscle tissue, including other thermogenic organs such as the heart and the liver, while it is lost through the skin (90%) and lungs (10%) and its rate is influenced by the physics involved in the mechanisms of convection, conduction, evaporation, and radiation [1]. In small mammals the brown adipose tissue (BAT) is known to act as a thermogenic organ allowing nonshivering thermogenesis. The presence and physiological relevance of BAT in adult humans were believed to be marginal. In recent years, however, it has been realized that a significant number of adult humans possess active BAT [2, 3] and that cold induces the activation of oxidative metabolism in BAT [4]. Moreover, an inverse relationship between BAT activity and shivering has been demonstrated in humans exposed to cold [5].

When the human body is exposed to cold and the homeostatic mechanisms are unable to compensate the heat that is being lost, there is a drop in body temperature. The symptoms and consequences of hypothermia may vary depending on the degree of hypothermia and have been associated according to the four degrees or stages of severity: mild 32–35°C; moderate, 28–32°C; severe, 20–28°C; and profound at less than 20°C [6]. Other authors such as Marion et al. [7] and Gentinello [8] included an additional category, the extreme hypothermia, when the temperature falls below 14°C.

Symptoms of mild hypothermia may be vague [1] and some physiological responses to preserve heat can be
observed with sympathetic nervous system excitation pro-
Voking shivering, hypertension, tachycardia, tachypnea, and
vasoconstriction. Additional symptoms that may be present
are cold diuresis, mental confusion, hepatic dysfunction, and
hyperglycemia due to the decrease in glucose uptake by cells,
a decrease in insulin secretion, and impaired tissue sensitivity
to insulin [6].

Moderate low body temperature results in a stronger
shivering. Due to a slower speed in nervous transmission
and lower brain blood flow, mild confusion, impaired mental
skills, and muscle miscoordination become apparent, and
movements are slow and labored [9]. Skin blood vessels con-
tract further as the body focuses its remaining resources on
keeping the vital organs warm. Microcirculation alterations
cause a reduction of blood flow, red cell sedimentation, and
an increase in blood viscosity (2% per degree heat loss), which
increases the reduced availability of oxygen in the tissues
leading to a hypoxic situation and acidosis [10].

Severe hypothermia occurs with decreasing temperature,
and other physiological systems begin to fail: heart rate,
breathing rate, and blood pressure decrease all. The hypotha-
lamus is not controlling anymore the thermoregulation. This
results in a heart rate of about 30 beats per minute with a
temperature of 28°C in humans [6]. Mental skills and
motor coordination are still more impaired with a difficulty
in speaking, sluggish thinking, incoherent behavior, and
amnesia starting to appear; lack of skill in using hands, poor
muscle coordination, difficulties in walking, and stumbling
are also usually present.

We must also consider the general effects of hypothermia
that occur in all categories, such as a decrease in metabolism
and oxygen consumption. The basal and activity metabolic
rates decrease between 3 and 7% by the fall of 1 degree
Celsius [7]. The Q10 or relative change in metabolic rate for
every 10 degrees of change in body temperature is about
2.3 [11]. Prakash [12] mentions that oxygen consumption is
reduced by 6% for each degree drop in body temperature.
Arrhythmias are also often accompanied by frequent atrial
fibrillation, bleeding, and coagulopathy due to mismatches
in platelet function [8]. Hypothermia has a strong immuno-
suppressive effect, increasing the risk of infections, specially
wound infections and pneumonia [13, 14]. On respiratory
function, hypothermia is also inhibitory, initially leading to
a rapid shallow breathing followed by a bradypnea,
bronchospasm, and hypoventilation. However, the oxygen
partial pressure is stable during hypothermia, indicating that
both cardiac output and oxygen consumption, despite being
reduced, are actually sufficient to meet metabolic needs.

A further problem derives from the subsequent pro-
cess of rewarming that necessarily follows in some time
hypothermia. Rewarming is a challenge for homoeothermic
organisms: a rise in temperature implies an increase in
metabolism and oxygen demand by tissues. A circulatory
collapse characterized by a decreased cardiac output and
blood pressure has been described during the rewarming
phase in victims of an accidental hypothermia [15]. The final
result will be influenced by the rate of rewarming. Polderman
and Callaghan [16, 17] advise rates of 0.2–0.5°C/h for cardiac
arrest and 0.1–0.2°C/h for other pathologies. After induction
of severe hypothermia (20°C) and rewarming in rats a high
mortality was found when rewarming at 0.35°C/min, while
all animals survived at a rate of 0.25°C/min [18].

1.2. The Therapeutic Use of Hypothermia. Despite the undesir-
able physiological effects of hypothermia, its therapeutic use
has been known since ancient times and more recently has
been revalued [13, 19, 20].

Mild to moderate hypothermia (35 to 32°C) appears to be
useful in preventing tissue damage, cell protection [21], and
survival [22]. Several international organizations such as the
American Health Association and the International Liaison
Committee on Resuscitation have recommended the use of
therapeutic hypothermia in patients with cardiac pathologies
among others [23]. In the European Resuscitation Council
Guidelines [24, 25], induced hypothermia is included in the
standard recommendations after cardiopulmonary resuscita-
tion.

Molecular and cellular pathways regulated by hypother-
ia have been recently reviewed [26]. Many studies on
the protective effects of hypothermia have been conducted
in cell cultures, for example, in endothelial cells [27], in
isolated organs such as heart [28], and in experimental
animals [29]. In this regard, the information obtained from
studying hibernating mammals is particularly relevant. The
hibernating mammals survive cyclical periods of torpor and
arousal with large fluctuations in body temperature. It has
been suggested that hibernation mitigates apoptosis [30] by
The elucidation of the molecular mechanisms occurring
during these periods of hypothermia can be helpful in future
human clinical studies of therapeutic hypothermia.

Some of the therapeutic and side effects of the application
of hypothermia are well documented [36]. However, it is
necessary to clarify the cellular mechanisms induced by cold
to enable its safe clinical use. In the present work we discuss
the role of RONS and antioxidants during hypothermia and
rewarming, and we hypothesize that if the beneficial effects
of therapeutic hypothermia could be due to RONS acting as
signaling molecules.

2. Hypothermia and Oxidative Stress

2.1. Tissue Oxygen Availability and Acid/Base Regulation dur-
ing Hypothermia. As we mentioned above, the decrease in
temperature affects all the physiological systems of the body.
The hypothermia process is associated with a reduction of
blood flow [37]. It is well known that cold exposure decreases
renal [38] and liver flow [39, 40]. We analyzed the correlation
between portal vein flows (PVF) as temperature drops from
37°C to 22°C in rats [33]. Figure 1 shows how the cooling
caused a decrease in PVF and we can see that the curve of PVF
versus temperature fits a second order polynomial regression.
This biphasic curve suggests that a homeostatic mechanism
is working in opposition to hypothermia and is capable
of maintaining PVF in mild and moderate hypothermia
close to normal ranges. However, the physiological regulator
mechanism can not control body temperature when this
Body temperature is not distributed uniformly. At 30°C, there is a peak in cooling (unshaded area) but it dropped drastically under 30 degrees. Blood flow is kept close to basal value during the beginning of the cooling phase. The curve was calculated by taking the mean of PVF values of all the animals (referred to as a percentage of the starting point) at 5 min intervals and plotting each of these as one point. Note that blood flow is kept close to basal value during the beginning of the cooling (unshaded area) but it dropped drastically under 30 degrees of body temperature (shaded area) [33].

In addition to changes in the availability of oxygen, hypothermia can affect other blood parameters like partial pressure of gases, electrolytes, and acid/base regulation. Alterations in pH are initially corrected by ventilation and then, in a slower process, by kidneys. When hypothermia develops the pH is altered. In fact, the combination of hypothermia and acidosis is seen as a critical point in the injured by trauma [42]. The failure of respiratory and/or renal functions, acid/base regulation, and ion regulatory mechanisms has been suggested as critical during severe hypothermia [43].

The hemoglobin oxygen affinity actually increases in hypothermia, and a restricted oxygen discharge in tissues could be expected. A reduction in the supply of oxygen to tissues can store oxygen in the blood for subsequent use in the tissues. Oxygen is stored in the blood as oxyhemoglobin. In physiological conditions there is a balance between the factors that promote the formation of free radicals and the levels of antioxidants. RONS are scavenged by enzymatic antioxidants like superoxide dismutase, glutathione peroxidase, and catalase [56] and by small molecular antioxidants such as reduced glutathione (GSH). GSH appears to be essential for the activation and maintenance of cellular defenses against oxidative stress, since it provides the substrate for glutathione peroxidase to detoxify peroxides. In rats acclimated to cold [57] lipid peroxidation increased and the activities and levels of antioxidants decreased in the erythrocytes.

We have studied the oxidant/antioxidant levels in vivo in rats at severe hypothermia and rewarming. After induction of anesthesia, animals were placed on a cooling/rewarming table and were mechanically ventilated with room air. Animals were cooled at a mean rate of ~0.25°C/min until they achieve 22°C. After one hour at hypothermia rats were rewarmed at a rate of 0.35°C/min to 37°C. Hypothermia and rewarming increased nitric oxide in plasma and liver and lipid peroxidation in plasma (Table 1). The erythrocyte antioxidant enzymatic activity decreased in hypothermia (superoxide dismutase and catalase) and rewarming (glutathione peroxidase). Results regarding the role of GSH in the hypothermia and rewarming process deserve a more detailed analysis. GSH appears to be essential for the activation and maintenance of cellular defenses against oxidative stress [58].

As we mentioned above, an important consequence of the hypothermia and rewarming process would be the reduction in oxygen delivery to some tissues. The effect of this decrease in cellular oxygen could resemble a hypoxic condition in which oxygen free radicals are produced [45] and released from the mitochondria [46].
Table 1: Oxidant/antioxidant status in rats after severe hypothermia and rewarming.

<table>
<thead>
<tr>
<th></th>
<th>Arterial Blood</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Hypothermia 1</td>
</tr>
<tr>
<td>NOx</td>
<td>10.76 ± 0.52</td>
<td>14.68 ± 0.59***</td>
</tr>
<tr>
<td>TBARS</td>
<td>3.60 ± 0.10</td>
<td>4.66 ± 0.36*</td>
</tr>
<tr>
<td>GSH</td>
<td>221.02 ± 5.53</td>
<td>230.61 ± 8.24</td>
</tr>
<tr>
<td>SOD</td>
<td>100 ± 3.58</td>
<td>82.86 ± 1.95**</td>
</tr>
<tr>
<td>GPx</td>
<td>100 ± 3.09</td>
<td>101.94 ± 3.38</td>
</tr>
<tr>
<td>CAT</td>
<td>100 ± 7.54</td>
<td>71.45 ± 4.39**</td>
</tr>
</tbody>
</table>

Animals were assigned to 3 groups of 6 individuals each. Sham animals were killed after anesthesia. In hypothermia group anesthetized animals were cooled for one hour at a mean rate of –0.25°C/min to achieve 22°C. Then they were killed. The rewarming group was cooled as described above and then it was rewarmed at a rate of 0.35°C/min to 37°C. Oxidative indicators were the concentration of nitric oxide derivatives (NOx) in plasma (nM) and liver (nmol/mg protein) and thiobarbituric acid-reactive substances (TBARS) in plasma (nM) and liver (nmol/mg protein). Antioxidant status was evaluated as thiols in plasma (GSH, μM) and in liver (GSH, μmol/g liver). The enzymatic antioxidant activities of Cu-Zn superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) were evaluated in erythrocytes and in liver and were expressed as a percentage of corresponding sham value. Data is mean ± SEM of six animals. Significantly different from corresponding sham values: * P < 0.05, ** P < 0.01, and *** P < 0.001. Significantly different from corresponding hypothermia values: †P < 0.05, ‡P < 0.01, and §§P < 0.001.

3. RONS and Apoptosis

With regard to radical generation there is increasing evidence that cell death is associated with an increase in intracellular RONS [69–72].

Apoptosis, or programmed cell death, is an accurately regulated mechanism whereby the cell actively uses a genetically controlled program to kill itself, with ATP being required to accomplish this process. Apoptosis is a key process that is involved in maintaining tissue homeostasis by removing senescent, genetically damaged cells or cells damaged by disease or noxious agents. Apoptosis is induced by two main ways: the activation of death receptors (the extrinsic pathway) or involving the mitochondria (the intrinsic pathway). In the extrinsic pathway signal arrives when “death ligands” such as TNFα, TRAIL, Apo3L, and Fas ligand (Fas L), bind to their specific membrane receptors causing their intramembrane domains to propagate the death signal intracellularly. RONS have been established as key participants in Fas-induced cell death [70,71]. In the mitochondrial-mediated pathway the signal is originated intrinsically [73] by the stress produced in organelles like the mitochondria (e.g., RONS) or endoplasmic reticulum (e.g., excess of misfolded proteins), inducing the release of pro-apoptotic factors into the cytosol or inhibiting antiapoptotic molecules that will ultimately trigger apoptosis.

Regardless of the signal origin, the formation of a permeability transition pore at contact sites between the mitochondrial outer and inner membranes and the release of cytochrome c into the cytosol are considered the “points of no return” in apoptotic process. Thus, mitochondria are the central organelle in the execution of apoptosis [74]. RONS generation in mitochondria increased prior to the onset of apoptosis [70], and the apoptotic process could be stopped by the addition of antioxidants [72].

4. The Role of RONS as Signaling Molecules

We must bear in mind that the formation of RONS is a physiological process. Indeed, RONS play a critical role in the cell, while at relatively high concentrations they become harmful; low levels can promote cell proliferation and survival [75, 76]. These dual effects of RONS, depending on their concentration, could explain why hypothermia through RONS generation sometimes gets involved in pathologies while when induced previous to or concomitant to an acute damage leads to a cellular protection. Some of the studies regarding the protective effects of hypothermia on oxidant/antioxidant parameters and against different types of injury are summarized in Table 2. While comparing different temperature levels in different species and affecting various tissues, some general conclusions can be drawn. Hypothermia increases oxidative stress, NO levels, and the GSH. When hypothermia is used in a model of injury, like ischemia or hypoxia (known to increase oxidative damage), paradoxically it causes a decreased oxidative stress and the maintenance or improvement of the antioxidant status.

Recently, we have described that the oxidative stress indicators were attenuated in rats with an acute damage (severe hypoxia) at hypothermia (at 22°C) compared with animals at normothermia (at 37°C) [33]. Similarly, in cardiomyocytes [77] it was described a hypothermic protection through...
Table 2: Selected data showing the protection induced by the experimental hypothermia. This table summarizes information about the impact of the experimental hypothermia on oxidant/antioxidant parameters. Despite the different levels of hypothermia, animal species, tissues, or injury models, the overall effects are as follows. First hypothermia by itself induces an increase in oxidative stress markers and in the reduced glutathione. Second, if hypothermia is applied during another injury there is a decrease in oxidative stress and the maintenance or improvement of antioxidants.

<table>
<thead>
<tr>
<th>HT level (°C)</th>
<th>Specie</th>
<th>Target</th>
<th>Injury model</th>
<th>Oxidative stress indicator</th>
<th>Injury effects</th>
<th>HT alone effects</th>
<th>HT-induced protection</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>Guinea pig</td>
<td>Heart</td>
<td>In vitro</td>
<td>ROS generation NADH⁺</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↓</td>
<td></td>
<td>[28]</td>
</tr>
<tr>
<td>20–22</td>
<td>Rat</td>
<td></td>
<td>In vivo</td>
<td>Plasma TBARS Liver TBARS</td>
<td>↑↑</td>
<td>=</td>
<td>↓</td>
<td>Intrahypoxic HT</td>
<td>[33]</td>
</tr>
<tr>
<td>23–24</td>
<td>Rat</td>
<td></td>
<td>In vivo</td>
<td>Catalase Vitamin E</td>
<td>ND</td>
<td>↓</td>
<td>ND</td>
<td>Ventilatory support not provided</td>
<td>[46]</td>
</tr>
<tr>
<td>25</td>
<td>Pig</td>
<td>Artery</td>
<td>In vitro</td>
<td>NO synthesis</td>
<td>ND</td>
<td>↑</td>
<td>ND</td>
<td></td>
<td>[60]</td>
</tr>
<tr>
<td>26</td>
<td>Rat</td>
<td></td>
<td>In vivo</td>
<td>TBARS SOD Catalase</td>
<td>= =</td>
<td>=</td>
<td>↓</td>
<td>Intraischemic HT followed by reperfusion at 37°C</td>
<td>[61]</td>
</tr>
<tr>
<td>30–32</td>
<td>Rat</td>
<td>Intestine</td>
<td>In vivo</td>
<td>TBARS GSH/GSSG</td>
<td>↑↓</td>
<td>=</td>
<td>↑</td>
<td>Ventilatory support using a mixture of O₂/NO*</td>
<td>[62]</td>
</tr>
<tr>
<td>32</td>
<td>Rat</td>
<td></td>
<td>In vivo</td>
<td>Heatstroke CAT (4°C)</td>
<td>↑</td>
<td>ND</td>
<td>↓</td>
<td></td>
<td>[63]</td>
</tr>
<tr>
<td>Mouse</td>
<td>CA1 hippocampus</td>
<td>MCAO</td>
<td></td>
<td>NOS expression CaM-KII</td>
<td>ND</td>
<td>ND</td>
<td>↑</td>
<td></td>
<td>[64]</td>
</tr>
<tr>
<td>33–35</td>
<td>Rat</td>
<td>Isolated Liver</td>
<td>In vitro</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Decreasing NAA = cell dysfunction and neuronal loss Intrainschemic HT is more effective than postischemic HT</td>
<td>[35, 66]</td>
</tr>
<tr>
<td>34</td>
<td>Rat</td>
<td>Ischemia</td>
<td>In vitro</td>
<td>Efflux rate TBARS</td>
<td>↑</td>
<td>=</td>
<td>↓</td>
<td></td>
<td>[67]</td>
</tr>
<tr>
<td>35</td>
<td>Mouse</td>
<td>Brain</td>
<td>In vivo</td>
<td>ALF GSH/GSSG</td>
<td>↓</td>
<td>ND</td>
<td>↑</td>
<td>GSH/GSSG Incremented over sham values</td>
<td>[68]</td>
</tr>
</tbody>
</table>

ALF: acute liver failure.
CaM-KII: Ca²⁺/calmodulin protein kinase II.
GSH/GSSG: reduced/oxidized glutathione.
HT: hypothermia.
Hx: hypoxia.
I/R: ischemia/reperfusion.
MCAO: middle cerebral artery occlusion.
MDA: malondialdehyde.
ND: not described.
NOS: nitric oxide synthase.
NOx: nitric oxide derived products.
O₂⁻⁺: superoxide radical.
SOD: superoxide dismutase.
TBARS: thiobarbituric acid-reactive substances.
↑, ↓: increase, decrease, respectively.
Figure 2: Modulation of apoptosis by hypothermia. After a serious insult the cell can trigger apoptosis, a highly regulated cell death mechanism. **Intrinsic Pathway**. Hypothermia increases ATP stores and slows ion channels then maintaining the integrity of the membranes. Hypothermia applied together or immediately after injury decreases the production of ROS. These events limit the rupture of the outer mitochondrial membrane and the release of proapoptotic molecules like cytochrome c into the cytosol. The hypothermia-induced increase in nitric oxide also avoids cytochrome c release and it is even reported that early NO production can exert a negative feedback regulation of iNOS [34]. Moreover, iNOS transcription activated by NF-κB was diminished after hypothermia [35]. Since catalase is absent in mitochondria, maintaining GSH redox cycle is critical to avoid H$_2$O$_2$ accumulation. There is abundant evidence that hypothermia keeps GSH pool. **Extrinsic Pathway**. It was found that hypothermia decreases the affinity of the death ligands-death receptors, with the consequent inhibition of the initiator caspases like caspase-8 or the NFκB-family molecules.

5. Role of RONS in the Beneficial Effects of Therapeutic Hypothermia

Therapeutic hypothermia has been used in the critically ill patients, and there is also abundant evidence from animal models of the protection induced by hypothermia when applied within minutes following ischemic damage (see Table 2). So far, the protective effects of hypothermia are believed to be a consequence of a reduction in the cellular hypoxia-inducible factor 1 (HIF-1) [84] which mediates many adaptive responses to hypoxia by regulating the expression of genes involved in glycolysis, mitochondrial function, cell survival, and resistance to oxidative stress [85]. Interestingly, activation of HIF-1α is correlated with better protection of fatty liver grafts after cold storage [86]. However, the addition of an inhibitor of NO in the preservation medium reversed that protection. This highlighted the role of NO in liver preservation.

As referred to hypothermia, our results [33] and others [77–80] lead us to propose the hypothesis that hypothermia through the generation of RONS and increasing GSH can induce protective mechanisms. The most remarkable of hypothermia is that protection is observed both when induced prior to injury, as a preconditioning model (experimental hypothermia), and when applied after damage (therapeutic hypothermia). In the latter case, the sooner hypothermia is applied, the better prognosis patient will have [17, 87].
metabolism and the retardation of destructive enzymatic reactions and the concomitant oxygen needs, thus conserving ATP levels [88].

More recently, the beneficial effects of hypothermia, when applied to prevent an ischemic episode, included a trigger level of RONS that can act as a mechanism for induction of signaling pathways and the modulation of the extrinsic and intrinsic pathways of apoptosis (see Figure 2). Hypothermia does not simply block cell signaling pathway of apoptosis and necrosis but selectively upregulates some protective genes after ischemia [89]. Many experimental assays showed that when hypothermia is applied during an ischemia or hypoxia episode, it is able to inhibit proapoptotic molecules and to induce an increase in antiapoptotic ones in ischemic tissues [90, 91].

Because of these different mechanisms of action, it can be suggested that hypothermia may be protective in many organs and against many kinds of injury.

6. Conclusion

The generation of RONS is a typical feature of hypothermia and more prominent in rewarming. There is increasing evidence showing that the beneficial effects of hypothermia included a trigger level of RONS that can act as a mechanism for induction of signaling pathways and the modulation of apoptosis.

Acknowledgments

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