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# Lower limb ischaemia and reperfusion injury in healthy volunteers measured by oxidative and inflammatory biomarkers

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## Abstract

**Objective:** Ischaemia-reperfusion (IR) injury is partly caused by the release of reactive oxygen species and cytokines and may result in remote organ injury. Surgical patients are exposed to surgical stress and anaesthesia, both of which can influence the IR response. An IR model without these interfering factors of surgery is, therefore, useful to test the potential of antioxidant and cytokine-modulatory treatments.

The aim of this study was to characterize a human ischaemia-reperfusion model with respect to oxidative and inflammatory biomarkers.

**Materials and methods:** Ten male volunteers were exposed to 20 minutes of lower limb ischaemia. Muscle biopsies and blood samples were taken at baseline and 5, 15, 30, 60 and 90 minutes after tourniquet release and analysed for malondialdehyde (MDA), ascorbic acid, dehydroascorbic acid, tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-1 receptor antagonist (IL-1Ra), IL-6, IL-10, TNF-receptor (TNF-R)I, TNF-RII and YKL-40.

**Results:** We found no significant increase in MDA in the muscle biopsies after reperfusion. Plasma levels of oxidative and pro- and anti-inflammatory parameters showed no significant differences between baseline and after reperfusion at any sampling time.

**Conclusion:** Twenty minutes of lower limb ischaemia does not result in an ischaemia-reperfusion injury in healthy volunteers, measurable by oxidative and pro- and anti-inflammatory biomarkers in muscle biopsies and in the systemic circulation.

## Keywords

ischaemia-reperfusion; oxidative stress; inflammatory markers; cytokines; pneumatic tourniquet

## Introduction

Restoration of blood flow to an ischaemic organ is essential to prevent irreversible tissue injury. Reperfusion, however, results in a local and systemic inflammatory response that may augment tissue injury in excess of that produced by ischaemia alone.<sup>1</sup> Cellular damage after reperfusion of previously viable ischaemic tissue is defined as ischaemia-reperfusion (IR) injury.<sup>1</sup> IR of skeletal muscle leads to a production of reactive oxygen species (ROS) and pro-inflammatory cytokines, which not only affects the muscle, but may also cause remote organ injury.<sup>2–5</sup> Antioxidant and/or cytokine-modulatory therapies might, therefore, reduce morbidity.<sup>6,7</sup>

Clinical IR interventional studies are widely performed in patients with elective ischaemia, e.g. orthopaedic tourniquet surgery<sup>6,8–10</sup> or transplantations<sup>7,11</sup> or in acute ischaemia, e.g. acute myocardial infarction.<sup>12</sup> The studies apply a variety of interventions, such as

antioxidative treatment (N-acetyl cysteine, vitamin C), ischaemic preconditioning, anaesthetic intervention

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(propofol, ketamine, dexmedetomidine) and inflammation-modulating therapy (recombinant P-selectin glycoprotein ligand IgG) and report of both non-significant<sup>10,11</sup> and beneficial effects<sup>6-9,12</sup> of these interventions in reducing markers of oxidative stress and inflammation. However, the results of the reperfusion protective effect are difficult to interpret because of the added complexity of the clinical situation, e.g. surgical stress and influence of anaesthesia and a simple experimental model without these interfering factors is, therefore, warranted.

The time frame for enduring ischaemia is somewhere between 20 and 30 minutes if the subjects are to complete the study without the need of anaesthetics and analgesics.<sup>13</sup>

In this study, we investigated if 20 minutes of ischaemia of the lower extremity and 90 minutes of subsequent reperfusion was sufficient for the expression of biochemical markers of oxidative and inflammatory stress, both locally and in the systemic circulation.

## Materials and Methods

The study protocol was approved by the local ethics committee (H-4-2011-110) and The Danish Data Protection Agency. All participants gave written informed consent before enrolment in the study. The trial was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (NCT01486212).

### Study design

The study was a methodological study, including healthy subjects between the ages of 18 and 40. The trial consisted of one study day. The subjects served as their own controls.

Exclusion criteria were: smoking, known vascular disease, known hypertension, known metabolic diseases, known cardiovascular risk factors (familiar ischaemic heart disease, elevated total cholesterol, elevated triglyceride, body mass index (BMI) >30, daily alcohol intake >5 units), intake of prescription medicine or 'over-the-counter' drugs <3 weeks prior to study participation, infection treated with antibiotics <2 weeks before study participation or if no informed written consent was obtained.

The subjects were placed in a supine position in peaceful surroundings and relaxed for 20 minutes before the measurement of baseline blood pressure (BP) and heart rate (HR). An intravenous catheter was inserted into the cubital vein for blood sampling.

A baseline muscle biopsy was taken from the vastus lateralis muscle, approximately 10-15 cm above the patella. The biopsy was taken with a 5 mm Bergström muscle biopsy needle (Pelomi, Albertslund, Denmark).<sup>14</sup>

A pneumatic tourniquet was placed on the opposite leg and inflated to a pressure of 300 mmHg and this pressure was sustained for 20 minutes until deflation.

Before inflation (baseline), every fifth minute during inflation and until 15 minutes after deflation, the subject used a visual analogue scale (VAS) (0 mm = no pain, 100 mm = maximal pain) to score pain (eight scores in total).

Blood samples were drawn at baseline and 5, 15, 30, 60 and 90 minutes after reperfusion.

After 20 minutes of ischaemia, the pneumatic tourniquet was deflated (reperfusion). Thirty minutes after reperfusion, a muscle biopsy was taken from the vastus lateralis muscle just below the placing of the tourniquet.

### Primary outcome

**Malondialdehyde (MDA) in muscle biopsies.** Immediately after the biopsies were taken, they were weighed and placed in cryo tubes. They were then frozen in liquid nitrogen and afterwards stored at -80° C until analysis. Tissues were homogenized in ice-cold phosphate buffered saline (1:10 weight/volume) and analyzed in triplicate for MDA with a SpectraMax Plus<sup>384</sup> UV-spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) using the Morton-Stubb three-point correction as described previously.<sup>15</sup>

### Secondary outcomes

**Oxidative stress markers in blood samples.** For the determination of the concentration of MDA, ascorbic acid (AA), dehydroascorbic acid (DHA) and total ascorbic acid (TAA) blood was drawn into tubes containing heparin free of lipopolysaccharide (LPS) and centrifuged at 3100 rpm for 3 minutes and then stored at -80° C until analysis. AA, DHA and TAA were stabilized with 10% meta-phosphoric acid containing 2 mM disodium ethylenediaminetetraacetic acid (EDTA) before storage. Both MDA and AA were analyzed using high performance liquid chromatograph (HPLC) methods as described previously.<sup>15,16</sup>

**Inflammatory markers in blood samples.** For the determination of pro- and anti-inflammatory cytokines and cytokine receptors and the pro-inflammatory marker YKL-40, blood was drawn into tubes containing EDTA and centrifuged at 3100 rpm for 3 minutes, then stored at -80° C until analysis. The cytokines tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , IL-1 receptor antagonist (IL-1Ra), IL-6, IL-10 and the soluble TNF receptors (sTNF-R) I and II were measured in a Luminex 100 IS analyser (Luminex Corporation, Austin, TX, USA), using appropriate multiplex antibody bead kits purchased from Invitrogen

(Invitrogen Corporation, Carlsbad, CA, USA). Data were analyzed using StarStation version 2.0 software (Applied Cytometry Systems, Sheffield, UK). The lowest levels of detection were (pg/ml): TNF- $\alpha$ : 0.5, IL-1 $\beta$ : 1.0, IL-1Ra: <100, IL-6: 1.0, IL-10: 1.0, TNF-RI: 15 and TNF-RII: 15.

Kit precisions were (coefficient of variation (CV%)): TNF- $\alpha$ : 7.7, IL-1 $\beta$ : 4.4, IL-1Ra: 5.0, IL-6: 7.6, IL-10: 9.4, TNF-RI: 4.3 and TNF-RII: 7.9.

The concentration of YKL-40 in plasma was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) (Quidel, Santa Clara, CA, USA) as described previously.<sup>17</sup>

### Sample size

There are, to our knowledge, no other studies which have been performed with the same set-up and no pilot study was performed. Thus, our study population was based on a study in patients undergoing reconstruction of the ligamentum cruciatum genus anterior.<sup>18</sup> In this study, they used 10 patients and found a significant increase in MDA 20 minutes after reperfusion, following a tourniquet time of about 60 minutes.

### Statistics

Data were analyzed using non-parametric methods, with Friedman analysis of variance and Wilcoxon's signed ranks test. Data are presented as median (interquartile range, IQR) unless specified otherwise. Results with  $p$ -values <0.05 were considered statistically significant. Data were analyzed using SPSS version 20 software (SPSS, Chicago, IL, USA).

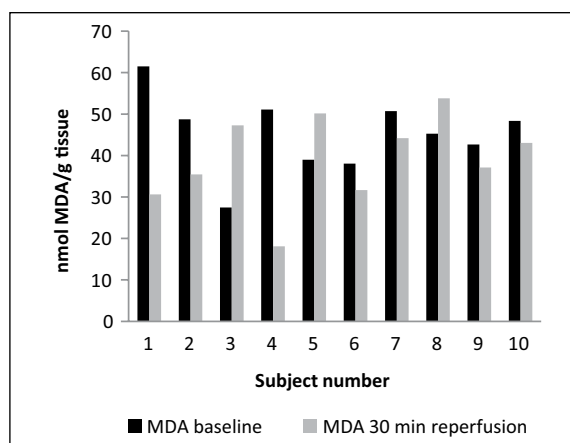
### Results

Ten healthy young men were included in the study, with a median age of 24 years (range 22-39) and BMI 21 kg/m<sup>2</sup> (range 19-27). All participants completed the study with no missing samples.

#### Primary outcome

**Malondialdehyde (MDA) in muscle biopsies.** The weight of the muscle biopsies was 54.5 (18.5-95.8) mg at baseline and 73.5 (36.5-117.5) mg after reperfusion ( $p = 0.037$ ).

The 20 minutes of ischaemia induced no significant difference between MDA values when comparing baseline 46.9 (38.8-50.8) nmol/g tissue with 30 minutes of reperfusion 40.1 (31.4-48.0) ( $p > 0.05$ ) (Figure 1).



**Figure 1.** Malondialdehyde (MDA) in muscle biopsies in each individual subject. No significant difference was found between baseline and 30 minutes after reperfusion ( $p > 0.05$ ). Data were analyzed using the Wilcoxon signed ranks test.

#### Secondary outcomes

**Oxidative markers in plasma.** None of the oxidative markers, MDA, AA or DHA, changed significantly from baseline to after reperfusion ( $p > 0.05$ ) (Figure 2).

**Inflammatory markers in plasma.** None of the pro- or anti-inflammatory cytokines or soluble cytokine receptors (TNF- $\alpha$ , IL-1 $\beta$ , IL-1Ra, IL-6, IL-10, sTNF-RI and sTNF-RII) showed significant changes from baseline to 90 minutes of reperfusion ( $p > 0.05$ ) (Figure 3).

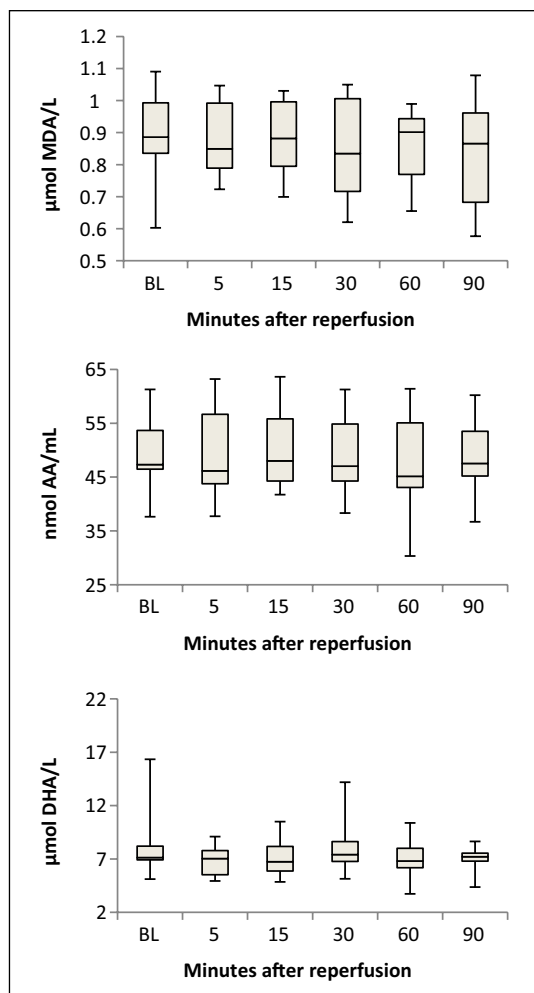
The pro-inflammatory marker YKL-40 showed no significant difference from baseline to after reperfusion at any sampling time ( $p > 0.05$ ) (Figure 3).

**Visual analogue scale (VAS) pain scores.** There were significant differences between pain scores, measured by VAS, at baseline value compared with 5, 10, 15 and 20 minutes after inflation ( $p = 0.005$ ) and also 5 minutes after reperfusion ( $p = 0.008$ ).

There were no significant differences in pain scores, measured by VAS, between 5 and 20 minutes of ischaemia to the lower extremity ( $p = 0.26$ ). The peak VAS score was at 20 minutes of ischaemia, with a median of 22 (10-56) mm (Figure 4). After 10 minutes of reperfusion, there were no significant differences in VAS scores compared to baseline values ( $p > 0.05$ ).

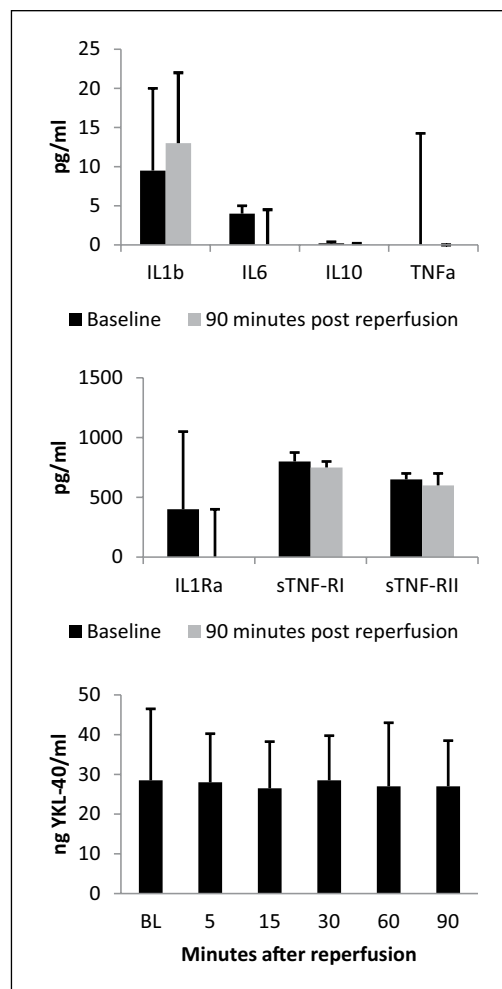
### Discussion

This study shows that 20 minutes of ischaemia of the lower limb did not produce a reperfusion injury that could be measured by a range of oxidative and inflammatory markers up to 90 minutes after reperfusion.



**Figure 2.** Oxidative stress markers in plasma. Box plot with median (black horizontal line), 25<sup>th</sup> and 75<sup>th</sup> percentiles (closed box) and ranges (bars) for oxidative stress markers in plasma. Malondialdehyde (MDA), ascorbic acid (AA), dehydroascorbic acid (DHA). BL = baseline. There were no significant differences in any of the oxidative markers between baseline and post reperfusion ( $p > 0.05$ ). Data were analyzed using the Friedman analysis of variance.

Several studies using the pneumatic tourniquet during surgery have documented the production of a significantly increased amount of MDA in plasma (measured at 5 and 30 minutes after reperfusion and compared to baseline) as an expression of lipid peroxidation by ROS.<sup>8,9,19,20</sup> Several antioxidant interventions (vitamin C, N-acetyl cysteine and propofol) have been tested and proved to lower the MDA level.<sup>6,20,21</sup> However, all these studies were performed in patients subjected to ischaemia for about 60 minutes, undergoing surgery and with the influence of different types of anaesthesia. It is known that several anaesthetic compounds, especially propofol, are powerful antioxidants, thus, making it difficult to distinguish the effect of the antioxidant intervention from the influence of anaesthesia in these models.<sup>9,19,22</sup> Surgery elicits local as well as systemic

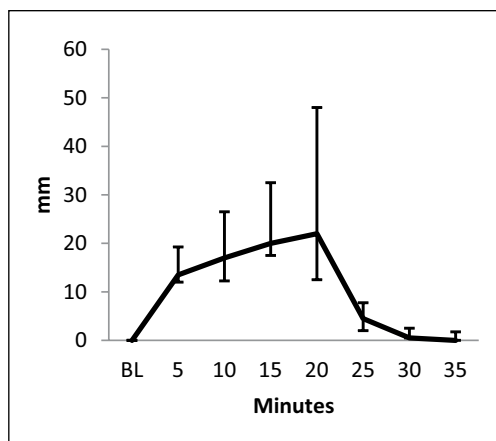


**Figure 3.** Pro- and anti-inflammatory markers in plasma. Column chart with median (top of column) and 75<sup>th</sup> percentiles (bars). There were no significant differences in any of the investigated markers between baseline and post reperfusion ( $p > 0.05$ ). Data were analyzed using the Wilcoxon signed ranks test. YKL-40 was analyzed using the Friedman analysis of variance. IL-1 $\beta$  (interleukin-1 $\beta$ ), IL-6 (interleukin-6), IL-10 (interleukin-10), TNF- $\alpha$  (tumor necrosis factor alpha), IL-1Ra (interleukin-1Ra), sTNF-RI and II (soluble tumor necrosis factor receptors I and II). pg = picogram ( $10^{-12}$ g). There were too few measurable amounts of TNF- $\alpha$  at baseline and after reperfusion and of IL-1Ra 90 minutes after reperfusion to express a median above 0.

inflammatory, endocrine/metabolic, humoral and immunological alterations which makes it difficult to interpret the results from interventional studies performed in patients undergoing surgery.<sup>23-25</sup> Hence, a simple model which excluded these influencing factors would be preferred for studies of IR-injuries.

In our study, we could not produce a reperfusion response upon which an intervention could be tested. This could be due to several reasons.

With regard to the oxidative markers, the ischaemic period (20 minutes) was probably too short for the production of detectable amounts of MDA as an expression



**Figure 4.** Median visual analogue scale (VAS) pain scores with error bars (25<sup>th</sup> and 75<sup>th</sup> percentiles) for the 10 healthy subjects. 0 mm = no pain, 100 mm = maximal pain. 5, 10, 15 minutes = ischaemia; 20, 25, 30, 35 minutes = reperfusion. Significant difference from baseline (BL) and until 15 minutes post reperfusion ( $p=0.005$ ). Data were analyzed using the Friedman analysis of variance.

of lipid peroxidation. For example, the duration of ischaemia-reperfusion is an important determinant of the degree of oxidative stress.<sup>26,27</sup> Since we found no significant difference in VAS pain scores between 5 and 20 minutes, we could probably have continued the ischaemic period up to at least 30-35 minutes without the need of analgesics.<sup>13</sup> Based on previous studies that have used a pneumatic tourniquet during surgery, this is probably a more realistic timeframe for the production of MDA.<sup>6,9,20,28</sup> In these models, however, the surgical stress might also have contributed to the MDA response.<sup>29</sup> Lipid peroxidation caused by IR of the extremities is one of the most important organic expressions of oxidative stress induced by the reactivity of ROS.<sup>30</sup> ROS and lipid peroxides are short-lived and difficult to measure directly. Thus, MDA, a more stable and longer living degradation product of lipid peroxides, is often used as reflecting the lipid peroxidation level.<sup>8,28,30</sup> The choice of MDA, as well as AA and DHA, as the oxidative stress markers in plasma and the sampling times for these markers in our setup is in accordance with other studies.<sup>8,9,19,20,31</sup>

With regard to the pro- and anti-inflammatory markers, cytokines and cytokine receptors were measured at 90 minutes after reperfusion. We chose the pro-inflammatory cytokines (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ), anti-inflammatory cytokines (IL-10, IL-1Ra) and soluble TNF-binding receptors (sTNF-RI and sTNF-RII) as the literature<sup>32,33</sup> supports that these (IL-1 and TNF- $\alpha$ ) are present at sites of local inflammation and their roles (IL-1, IL-6 and TNF- $\alpha$ ) are well documented in IR injuries.<sup>34</sup> However, 90 minutes after reperfusion is probably not sufficient time to detect a response of reperfusion

on cytokine production and release to the circulation. Indeed, other studies, measuring cytokines after tourniquet operations, have found a significantly increased level of the pro-inflammatory cytokine IL-6 between 4 and 24 hours after reperfusion.<sup>3,35,36</sup> TNF- $\alpha$  and IL-1 did not, however, increase significantly at this time. The tourniquet time in these studies was around 60-120 minutes. An experimental study has shown that the highest response of TNF- $\alpha$  following severely injured post-ischaemic extremities was observed one minute after reperfusion and reached control levels after 10 minutes of reperfusion.<sup>37</sup> Thus, it might have been more appropriate if we had also analyzed the 5-minute blood sample, as we might have missed the TNF- $\alpha$  peak at this time-point.

In a surgical setting, IL-6 increased 30-60 minutes after commencing surgery and the change in concentration became significant after 2-4 hours.<sup>25</sup> The degree of tissue trauma, i.e. 20 minutes of ischaemia, was probably also insufficient trauma to trigger an early cytokine response.<sup>38,39</sup> Notably, the surgical stress in the tourniquet studies may, by itself, have contributed to the systemic inflammatory response and these studies are not directly comparable to our setup, where we have avoided the influence of surgical stress.

In summary, the ischaemic period may have to be longer to provoke systemically detectable oxidative stress and cytokine responses and the post-reperfusion sampling times should be extended to measure a pro- and/or anti-inflammatory response in the circulation.

In our study, we measured the systemic response in blood taken from the antecubital vein contra-lateral to the reperfused leg. Some studies have shown that oxidative stress markers measured at the site of the reperfusion (local) are significantly increased compared to markers measured systemically.<sup>40,41</sup> In our setup, obtaining blood locally from the reperfused leg might have given a better response.

In conclusion, we were not able to show an effect of 20 minutes ischaemia in our model and we, therefore, recommend that the setup for a future methodological study should include an ischaemic period of 30-35 minutes and post-reperfusion sampling times ranging from 5 minutes up to a minimum of 4 hours after reperfusion. The proposed setup is still feasible without the use of anaesthetic supplement.

If the setup is successful in obtaining a detectable IR injury response, it could potentially be an interesting supplement in the aspect of testing antioxidant therapy upon IR injuries.

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### Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

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