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# Local Cooling Reduces Regional Bone Blood Flow

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Blood flow of various organs is affected by temperature decrease, leading to a decrease of organ perfusion.<sup>1-3</sup> The underlying mechanism is a vasoconstrictor response mediated by both reflex and local factors.<sup>4-8</sup> Intact sympathetic noradrenergic innervation enables blood vessels to reduce skin blood flow due to decreased local temperature, independent of change in core temperature. Cold-sensitive afferent nerves leading to release of norepinephrine from sympathetic active vasoconstrictor nerves sense local temperature reductions. Furthermore, unknown non-neural mechanisms seem to be part of a prolonged vasoconstriction during prolonged local cooling.<sup>5,8-10</sup>

Hypothermia is known to be protective in a number of conditions, especially in traumatic, ischemic, burn, and neurological injury.<sup>11</sup> It was shown in many experimental studies that hypothermia modulates the inflammatory response during endotoxemia. The release of pro-inflammatory cytokines is decreased due to hypothermia leading to protect organ damage.<sup>12-14</sup>

In contrast to other organs the effect of local cooling on bone tissue and its blood flow is poorly investigated. This may be attributed to the fact that measurement of bone blood flow poses various difficulties due to its compact bone mass and intraosseous bone vessels architecture.

Conflicts of interest: none.

Arne J. Venjakob and Stephan Vogt contributed equally to this study.

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The most reliable technique for measurement of regional bone blood flow (RBBF) is the fluorescent microsphere (FM) method.<sup>15-18</sup> This method is based upon the principle that left ventricular injected FM were homogeneously mixed within the blood and then distributed with the arterial blood. In the periphery MS were trapped in the capillaries due to their higher diameter (15  $\mu\text{m}$ ). The number of spheres lodged in an organ is proportional to its blood flow. The number of FM in a specific organ is determined indirectly by measuring fluorescence intensity. This method allows determining blood flow simultaneously in different regions within a bone. Furthermore cardiac output (CO) as well as blood flow of various organs can be determined by the same measurement.

In summary, the FM method is ideally suited to investigate the influence of temperature on bone blood flow. The null-hypothesis was tested: Local cooling has no effect on RBBF.

## MATERIALS AND METHODS

Six adult female New Zealand White rabbits with a mean body weight of  $3.6 \pm 0.4$  kg were used in this study which was authorized by the animal care committee at the Bavarian state authority (Munich, Germany). The animals were anaesthetized by means of intramuscular injection of ketamine (15 mg/kg, Ketavet®, BW, Pharmacia & Upjohn, Erlangen, Germany) and xylazine (2 mg/kg, Rompun®, BW, Bayer, Leverkusen, Germany) as well as intravenous injection to maintain anesthesia (ketamine: 10 mg/kg body weight/h; xylazine: 2.4 mg/kg body weight/h). The animals were positioned supine on a padded surface. After exposure of the trachea the animals were intubated and mechanically ventilated using a respirator (ventilation pressure, 12 mmHg; air to O<sub>2</sub> ratio, 0.21-0.40; respiratory rate,

25–30 breaths/min with an inspiratory to expiratory ratio of 1:2) (Sechrist IV-100 B Infant Ventilator, Anaheim, CA). The right common carotid artery was isolated and cannulated with a catheter passed into the left ventricle (Cavafix® MT, B. Braun Melsungen AG, Melsungen, Germany) with a maximum flow rate of 10 ml/min. The catheter was connected to an online pressure monitoring system (Sirecust 304 D, Siemens, Munich, Germany), correct position of the catheter's tip was confirmed by observing the typical waveform of the left ventricular pressure curve. The catheter served to control arterial blood pressure and was used for the injection of microspheres.

A second catheter (Cavafix® MT) for collection of the arterial blood sample was introduced into the left carotid artery and advanced into the descending aorta. Blood pressure and heart rate were monitored continuously throughout the experiment.

One hind limb of the animals was randomly placed in a water bath (Julabo SW, Seelbach/Germany) distal from the diaphysis of the femur so that the tibia was completely water-cooled. Water temperature was lowered in six steps (32, 26, 20, 14, 8, and 2°C) by adding ice water to the water bath. After 20 min of cooling at each temperature level intramuscular temperature of both cooled and uncooled hind limbs were measured by use of a thermometer (Thermistor SU, Diesen + Kern GmbH, Bad Bramstedt, Germany). Measurement was performed one centimeter distal from the tibial tuberosity between tibialis anterior muscle and ventral tibia before injection of FM was performed.

Arterial PaO<sub>2</sub>, PaCO<sub>2</sub>, and pH were determined before each injection.

#### Microsphere Methodology

Fluorescence-labeled microspheres with different colors such as yellow-green, blue-green, blue, orange, red and crimson (Molecular Probes, Eugene, OR; 15.5 ± 0.3 μm diameter, in 0.15 mol/l NaCl containing 0.05% Tween 20) were used for the experiments. 3 ml (1 × 10<sup>6</sup> Mio MS/per kg body weight) of the parent solution were aspirated into a syringe after

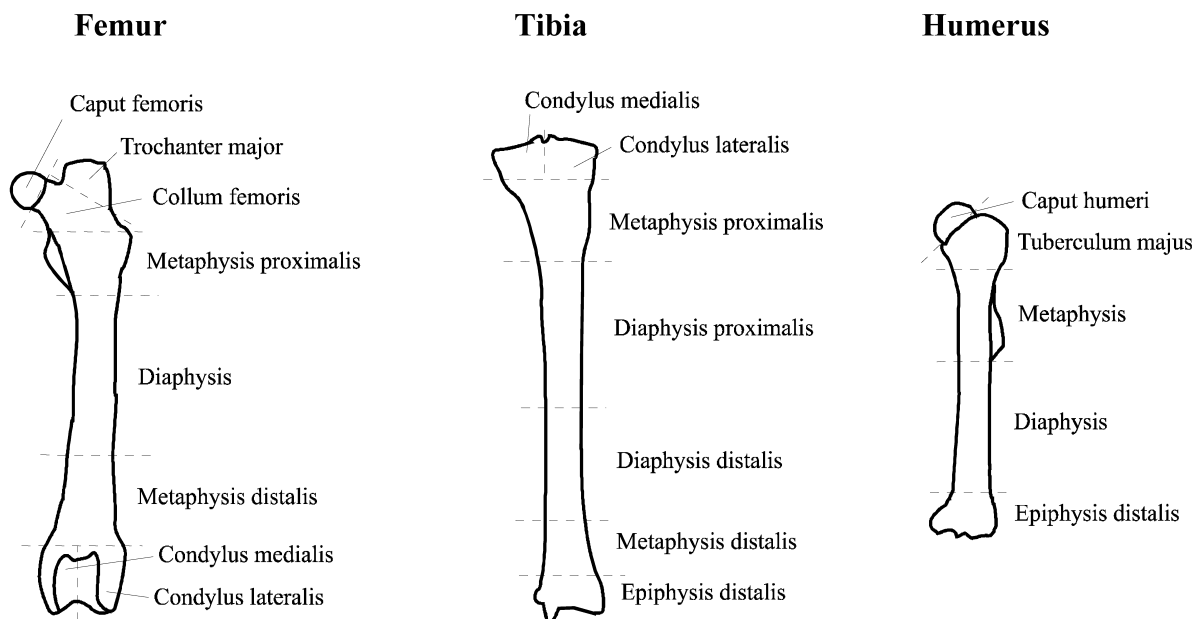
3 min of vortex mixing, 5 min of sonication, followed again by 3 min of vortex mixing, and diluted to a total volume of 10 ml with 0.9% NaCl. A 20 μl aliquot of the suspension was stored for later determination of the total number of spheres administered. Correct position of the injection catheter was verified by checking the left ventricular pressure curve. Blood pressure and heart rate were recorded before each injection. Arterial reference blood sampling was started 15 s before injection of the microspheres and was continued for 2 min. The withdrawal rate of the Harvard pump ('33'Syringe Pump, FMI, Egelsbach, Germany) was 3.54 ml/min. The same person throughout all experiments injected microspheres manually during a period of 1 min. The interval between two consecutive injections was approximately 30 min (microsphere preparation and constant water bath temperature for 20 min). The animals were sacrificed by an overdose of pentobarbital sodium (Narcoren®, Rhone Merieux GmbH, Laupheim, Germany) after the last injection of microspheres and the organs (long bones, kidneys) were retrieved. After removing connective and fatty tissues, each kidney was dissected into eight samples. Bones were cleaned carefully and dissected according to a standardized dissection scheme (Fig. 1). After dissection, the individual samples were weighed.

Fluorescence intensity of the bone samples was determined after dissolving the crystalline matrix for 3 weeks in hydrochloric acid (1 mol/L). Measurement of fluorescence intensity was carried out by an automated system<sup>19</sup> using the Sample-Processing-Unit; SPU (Gaiser, Kappel-Grafenhausen, Germany).<sup>20</sup>

Fluorescent data were used to calculate the blood flow values (ml/min/100 g) as follows:

$$F_{\text{sample}} = F_{\text{reference}} \times I_{\text{sample}} \times I_{\text{reference}}^{-1}$$

$F_{\text{sample}}$ , blood flow in the sample in ml/min;  $F_{\text{reference}}$ , withdrawal rate of the Harvard pump (3.54 ml/min);  $I_{\text{sample}}$ , fluorescence intensity of individual tissue sample;  $I_{\text{reference}}$ , fluorescence intensity of the reference blood sample.



**Figure 1.** Dissection scheme according to Ref.<sup>17</sup>

**Table 1.** Intramuscular Temperature of the Cooled and Uncooled Tibia in °C

Extremity	Temperature of the Water					
	32°C	26°C	20°C	14°C	8°C	2°C
Cooled	32.9 ± 0.2 <sup>#</sup>	28.5 ± 0.4 <sup>a,#</sup>	23.1 ± 0.3 <sup>a,b,#</sup>	17.5 ± 0.5 <sup>a,b,c,#</sup>	11.1 ± 0.4 <sup>a,b,c,d,#</sup>	4.7 ± 0.6 <sup>a,b,c,d,e,#</sup>
Uncooled	34.8 ± 0.2	34.4 ± 0.1	34.2 ± 0.3	33.9 ± 0.3	33.7 ± 0.3	33.6 ± 0.2

Values are means ± SEM ( $n = 6$ ). <sup>a</sup>Significantly different to 32°C ( $p < 0.05$ ). <sup>b</sup>Significantly different to 26°C ( $p < 0.05$ ). <sup>c</sup>Significantly different to 20°C ( $p < 0.05$ ). <sup>d</sup>Significantly different to 14°C ( $p < 0.05$ ). <sup>e</sup>Significantly different to 8°C. <sup>#</sup>Significant difference between both sides ( $p < 0.05$ ).

To allow the comparison of different samples, the calculated blood flow value of the individual tissue sample ( $F_{\text{sample}}$ ) was divided by the sample weight and normalized to 100 g. CO was determined as follows:

$$\text{CO} = F_{\text{reference}} \times I_{\text{injected}} \times I_{\text{reference}}^{-1} \times \text{bwt}^{-1}$$

CO, cardiac output in ml/min/kg;  $F_{\text{reference}}$ , withdrawal rate of the Harvard pump (3.54 ml/min);  $I_{\text{injected}}$ , fluorescence intensity of totally injected FM;  $I_{\text{reference}}$ , fluorescence intensity of the reference blood sample; bwt, body weight. Vascular resistance was calculated as follows:

$$R_{\text{organ}} = \frac{\text{MAP}}{\text{Blood flow}_{\text{organ}}} \text{ (ml/min/100 g)}$$

#### Statistical Analysis and Calculations

The statistical analysis was done using the SPSS software package for Windows (Version 19, SPSS Inc., Chicago, IL).

All data are reported as mean ± standard error of mean. By using the nonparametric Friedman test for related samples, variance on ranks was tested to determine significant differences between repeated measurements. Wilcoxon signed rank test was used to evaluate the differences between single measurements and comparison of blood flow values of right- and left-sided samples. Probability values <0.05 were considered significant.

#### Results of Local Cooling

For the cooled extremity intramuscular temperature was 32.9 ± 0.2°C at 32°C. At 26°C intramuscular temperature was 28.5 ± 0.4°C, at 20°C 23.1 ± 0.3°C, at 14°C 17.5 ± 0.5°C,

at 8° 11.1 ± 0.4°C and at 2°C intramuscular temperature was 4.7 ± 0.6°C. Temperature of the contra lateral extremity revealed constant values of 34.6 ± 0.4°C to 33.6 ± 0.2°C as shown in Table 1, muscle and bone temperatures at all temperature levels were stable after 11 ± 2 min.

#### Physiologic Parameters

Mean arterial blood pressure (MAP) and CO remained constant throughout the experiment, whereas heart rate showed a significant decrease from 166 ± 6 min<sup>-1</sup> at 32°C to 118 ± 4 min<sup>-1</sup> at 2°C water temperature. Arterial blood gases showed a slight variance, as well as hemoglobin and hematocrit (Table 2).

#### Blood Flow During Local Cooling

Blood flow of both kidneys (RBF) was identical at all time points ( $p > 0.05$ ; Wilcoxon Test). RBF varied between 162.0 ± 23.1 ml/min/100 g and 122.5 ± 11.8 ml/min/100 g. At 2°C a statistically significant decrease in RBF was found (Fig. 2).

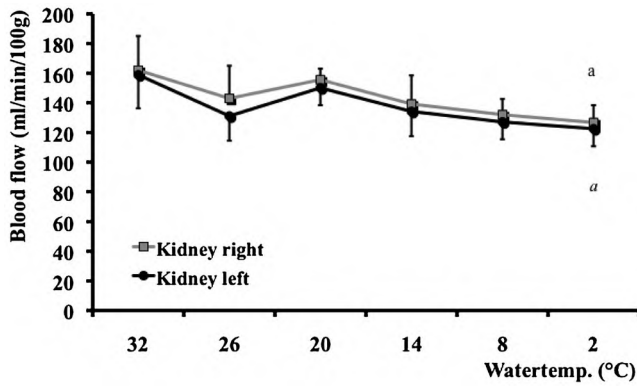
The upper extremity (both humeri) was not cooled and revealed no difference of blood flow between right and left specimens. At 32°C blood flow revealed values about 13 ml/min/100 g. Decreasing water temperature to 20°C led to increased blood flow values by 17.6 ± 2.1 ml/min/100 g (left: 16.4 ± 1.8 ml/min/100 g) ( $p < 0.05$ ; Friedman-Test; post hoc test: Wilcoxon Test). Between 20 and 2°C blood flow was stable and showed constant blood flow values.

Blood flow of the partially cooled femur at 32°C was 10.7 ± 0.9 ml/min/100 g and remained constant while decreasing water temperature to 14°C. At 8 and 2°C a statistically significant decrease of blood flow was found ( $p < 0.05$ ; Friedman-Test; post hoc test: Wilcoxon Test).

**Table 2.** Physiologic Parameters

Parameter	Temperature of the Water					
	32°C	26°C	20°C	14°C	8°C	2°C
MAP (mmHg)	71.5 ± 5.0	66.5 ± 3.6	62.7 ± 2.8	63.0 ± 2.7	60.7 ± 1.9	65.2 ± 3.5
HR (min <sup>-1</sup> )	165.8 ± 6.0	146.7 ± 6.3 <sup>a</sup>	136.8 ± 4.6 <sup>a,b</sup>	126.7 ± 7.6 <sup>a,b</sup>	115.8 ± 9.1 <sup>a,b,c</sup>	118.3 ± 4.1 <sup>a,b,c</sup>
CO (ml × min <sup>-1</sup> × kg <sup>-1</sup> )	110.9 ± 21.9	112.6 ± 17.9	123.8 ± 13.0	110.4 ± 12.0	118.4 ± 23.0	105.2 ± 16.9
Hb (g/dl)	9.2 ± 0.3	9.3 ± 0.5	8.2 ± 0.3 <sup>a,b</sup>	8.0 ± 0.5 <sup>a,b</sup>	7.5 ± 0.5 <sup>a,b</sup>	7.5 ± 0.4 <sup>a,b</sup>
Hct (%)	25.4 ± 3.0	25.1 ± 4.1	22.3 ± 3.6 <sup>a</sup>	25.9 ± 3.1 <sup>a</sup>	18.7 ± 4.1 <sup>a</sup>	20.6 ± 3.6 <sup>a</sup>
P <sub>a</sub> O <sub>2</sub> (mmHg)	141.8 ± 6.5	142.7 ± 8.6	149.9 ± 8.0	159.4 ± 7.3 <sup>a,b,c</sup>	167.6 ± 5.3 <sup>a,b,c,d</sup>	170.4 ± 7.9 <sup>a,b,c,d</sup>
P <sub>a</sub> CO <sub>2</sub> (mmHg)	32.2 ± 3.4	43.8 ± 1.8 <sup>a</sup>	41.9 ± 2.3	41.9 ± 1.8	43.0 ± 1.3 <sup>a</sup>	40.8 ± 1.9

Values are means ± SEM ( $n = 6$ ). MAP, mean arterial blood pressure; CO, cardiac output; Hb, hemoglobin; Hct, hematocrit; P<sub>a</sub>O<sub>2</sub> and P<sub>a</sub>CO<sub>2</sub>, arterial partial pressure of O<sub>2</sub> and CO<sub>2</sub>. <sup>a</sup>Significantly different to 32°C ( $p < 0.05$ ). <sup>b</sup>Significantly different to 26°C ( $p < 0.05$ ). <sup>c</sup>Significantly different to 20°C ( $p < 0.05$ ). <sup>d</sup>Significantly different to 14°C ( $p < 0.05$ ).



**Figure 2.** Blood flow of the kidney. Values of renal blood flow (ml/min/100 g) in both kidneys at local cooling of one hind limb. Values are means  $\pm$  SEM, ( $n = 6$ ). <sup>a</sup>Significantly different to 32°C, bolt: right kidney, italic: left kidney.

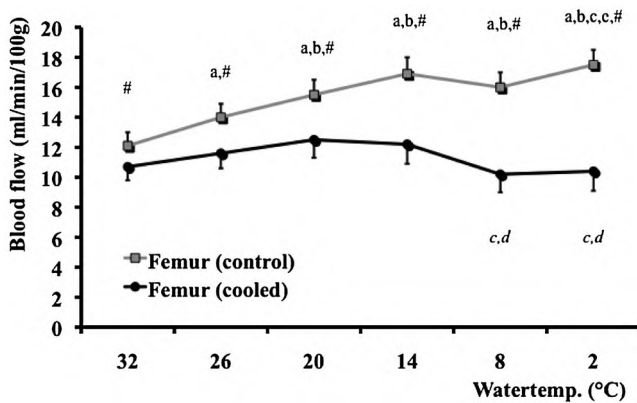
Blood flow of the control femur at 32°C was  $12.1 \pm 0.9$  ml/min/100 g and increased statistically significant to  $17.5 \pm 1$  ml/min/100 g ( $p < 0.05$ , Friedman-Test; post hoc test: Wilcoxon Test). RBBF of cooled and uncooled femur (control) showed a significant difference at all temperature levels ( $p < 0.05$ ; Wilcoxon Test) (Fig. 3).

RBBF of the tibia was  $6.5 \pm 0.6$  ml/min/100 g at 32°C. Cooling to 2°C revealed a statistically significant decrease of blood flow to  $1.1 \pm 0.1$  ml/min/100 g ( $p < 0.05$ ; Wilcoxon Test), being a decrease of 83% compared to the initial blood flow value.

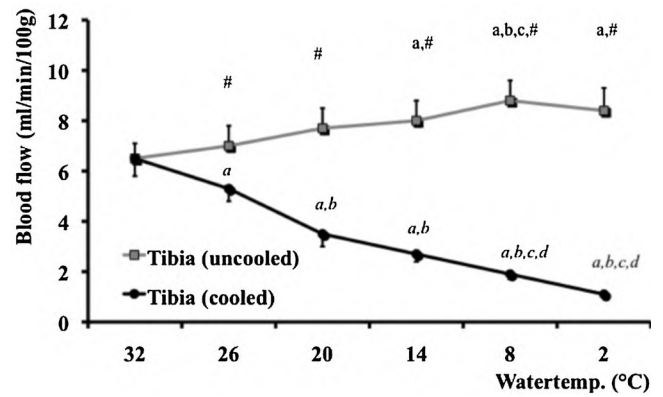
RBBF of the control extremity increased from  $6.5 \pm 0.6$  to  $8.4 \pm 0.9$  ml/min/100 g. At 14°C the increase of blood flow was found to be statistically significant ( $p < 0.05$ , Friedman-Test; post hoc test: Wilcoxon Test).

At 32°C blood flow of the cooled and uncooled tibia were identical. Decreasing temperature led to statistically significant decreased blood flow values of both sides ( $p < 0.05$ ; Wilcoxon Test) (Fig. 4).

Blood flow values of all organs are shown in Table 3.



**Figure 3.** Bone blood flow of the femur at partial local cooling ( $n = 6$ ); values of RBBF (ml/min/100 g), means  $\pm$  SEM, <sup>a</sup>Significantly different to 32°C ( $p < 0.05$ ), <sup>b</sup>Significantly different to 26°C ( $p < 0.05$ ), <sup>c</sup>Significantly different to 20°C ( $p < 0.05$ ), <sup>d</sup>Significantly different to 14°C ( $p < 0.05$ ), <sup>e</sup>Significantly different to 8°C ( $p < 0.05$ ), <sup>#</sup>Significant difference between control and treated specimens ( $p < 0.05$ ), bolt: control extremity, italic: cooled extremity.



**Figure 4.** Bone blood flow of the tibia at local cooling ( $n = 6$ ); values of RBBF (ml/min/100 g), means  $\pm$  SEM, <sup>a</sup>Significantly different to 32°C ( $p < 0.05$ ), <sup>b</sup>Significantly different to 26°C ( $p < 0.05$ ), <sup>c</sup>Significantly different to 20°C ( $p < 0.05$ ), <sup>d</sup>Significantly different to 14°C ( $p < 0.05$ ), <sup>e</sup>Significantly different to 8°C ( $p < 0.05$ ), <sup>#</sup>Significant difference between control and treated specimens ( $p < 0.05$ ), bolt: control extremity, italic: cooled extremity.

**Vascular Resistance**

Vascular resistance of both kidneys was constant by  $0.5 \pm 0.04$  mmHg/(ml/min/100 g).

In contrast vascular resistance of the long bones increased statistically significant ( $p < 0.05$ , Friedman-Test; post hoc test: Wilcoxon Test). Vascular resistance of the femur increased statistically significant from  $9.5 \pm 1.0$  to  $22.0 \pm 4.2$  mmHg/(ml/min/100 g), in the tibia from  $28.4 \pm 6.7$  (32°C) to  $87.9 \pm 11.3$  mmHg/(ml/min/100 g) (2°C).

Vascular resistance of the control extremity (femur and tibia) revealed a statistically significant decrease from  $7.7 \pm 0.7$  to  $4.3 \pm 0.2$  mmHg/(ml/min/100 g), respectively,  $21.8 \pm 5.3$  to  $13.7 \pm 1.9$  mmHg/(ml/min/100 g) ( $p < 0.05$ , Friedman-Test; post hoc test: Wilcoxon Test). Vascular resistance of both humeri (not cooled) also showed a statistically significant decrease from  $10.3 \pm 2.6$ , respectively,  $10.5 \pm 2.5$  mmHg/(ml/min/100 g) to  $5.5 \pm 0.7$  and  $4.3 \pm 0.2$  mmHg/(ml/min/100 g).

Vascular resistance of both kidneys and the long bones is shown in Table 4.

**RBBF Distribution in Long Bones**

RBBF of both humeri was highest in the greater tubercle (between  $22.3 \pm 4$  ml/min/100 g and  $30.1 \pm 3.5$  ml/min/100 g), metaphysis (between  $17.8 \pm 2.5$  ml/min/100 g and  $22.9 \pm 3.7$  ml/min/100 g) and humeral head (between  $13.4 \pm 2.1$  ml/min/100 g and  $21.4 \pm 2.1$  ml/min/100 g). In diaphysis and epiphysis statistically significant lower blood flow values were found. An increase of RBBF was found for all regions of the humerus. RBBF distribution of both uncooled humeri revealed no difference compared right and left specimens ( $p > 0.05$ ; Wilcoxon Test).

The distal femur was cooled, whereas the proximal regions have not been cooled. RBBF of the proximal regions (femoral head, greater trochanter, and femoral neck) showed an increase of blood flow values. In contrast distal regions (proximal metaphysis, diaphysis, distal metaphysis, medial, and lateral condyle) showed statistically decreased blood flow values ( $p < 0.05$ , Friedman-Test; post hoc test: Wilcoxon Test). Decrease of RBBF varied slightly between different

**Table 3.** Blood Flow Values

Organ	Temperature of the Water					
	32°C	26°C	20°C	14°C	8°C	2°C
Kidney right	162 ± 23.1	143 ± 22	155.6 ± 7.5	139.2 ± 19.3	132 ± 10.6	126.7 ± 11.7 <sup>a</sup>
Kidney left	158.9 ± 22.6	131.1 ± 16.7	150.1 ± 11.7	134.2 ± 16.7	127.1 ± 11.7	122.5 ± 11.8 <sup>a</sup>
Humerus right	13 ± 1.5	15.5 ± 1.9	17.6 ± 2.1 <sup>a</sup>	17.3 ± 2 <sup>a</sup>	15.6 ± 1.6 <sup>a</sup>	17 ± 1.6 <sup>a</sup>
Humerus left	13.2 ± 1.6	14.2 ± 1.6	16.4 ± 1.8 <sup>a</sup>	16.6 ± 1.8 <sup>a</sup>	16.1 ± 1.6 <sup>a</sup>	17.6 ± 1.9 <sup>a</sup>
Femur (control)	12.1 ± 0.9 <sup>#</sup>	14 ± 0.9 <sup>a,#</sup>	15.5 ± 1 <sup>a,b,#</sup>	16.9 ± 1.1 <sup>a,b,#</sup>	16 ± 1 <sup>a,b,#</sup>	17.5 ± 1 <sup>a,b,c,e,#</sup>
Femur (cooled)	10.7 ± 0.9	11.6 ± 1	12.5 ± 1.2	12.2 ± 1.3	10.2 ± 1.2 <sup>c,d</sup>	10.4 ± 1.3 <sup>c,d</sup>
Tibia (control)	6.5 ± 0.6	7 ± 0.8 <sup>#</sup>	7.7 ± 0.8 <sup>#</sup>	8 ± 0.8 <sup>a,#</sup>	8.8 ± 0.8 <sup>a,b,c,#</sup>	8.4 ± 0.9 <sup>a,#</sup>
Tibia (cooled)	6.5 ± 0.7	5.3 ± 0.5 <sup>a</sup>	3.5 ± 0.5 <sup>a,b</sup>	2.7 ± 0.3 <sup>a,b</sup>	1.9 ± 0.2 <sup>a,b,c,d</sup>	1.1 ± 0.1 <sup>a,b,c,d</sup>

Values of blood flow (ml/min/100 g) in different organs at local cooling. Values are means ± SEM ( $n = 6$ ). <sup>a</sup>Significantly different to 32°C ( $p < 0.05$ ). <sup>b</sup>Significantly different to 26°C ( $p < 0.05$ ). <sup>c</sup>Significantly different to 20°C ( $p < 0.05$ ). <sup>d</sup>Significantly different to 14°C ( $p < 0.05$ ). <sup>e</sup>Significantly different to 8°C ( $p < 0.05$ ). <sup>#</sup>Significant difference between control and treated specimens ( $p < 0.05$ ).

regions whereas extremely vulnerable bone regions could not be identified.

Blood flow of all cooled tibial regions showed a statistically significant decrease at most temperature levels ( $p < 0.05$ , Friedman-Test; post hoc test: Wilcoxon Test). Proximal metaphysis (−86%), proximal diaphysis (−84%) as well as both condyles (−84% and −82%) showed a distinctive decrease of blood flow at local cooling compared to the initial value at 32°C. The decrease of RBBF in the distal epiphysis (−66%) was less marked.

Blood flow of all control regions (not cooled) showed a slight increase of RBBF. Blood flow values of all anatomical regions of the long bones were listed in Tables 5–7.

## DISCUSSION

Although postoperative cooling is widely used in the daily clinical routine, the repercussions on bone blood flow and the underlying metabolism is poorly investigated so far. In our experimental setup we cooled the hind limb of a rabbit in a water bath and measured RBBF by means of the FM-method. Our data clearly demonstrate that local cooling leads to a proportional decrease of blood flow in long bones.

Determination of bone blood flow using the FM-method has yielded reliable data in bone tissue.<sup>17</sup> To minimise methodical errors such as inadequate preparation of FM or incorrect measurement of fluorescence intensity we utilized a standardized protocol proven effective in previous studies.<sup>15–17</sup> To exclude systematic errors, blood flow was determined in reference organs. The comparison of regional blood flow values of bilateral organs for example, kidney or upper arm revealed no differences indicating adequate mixing of FM in the blood. To exclude changes of bone perfusion caused by altered hemodynamic conditions, hemodynamic parameters should be constant during the experiment. In our study cardiac output and arterial blood pressure were stable over time. Heart rate showed a decrease of values during local cooling, which may be interpreted as a response to the local cooling procedure leading to mild hypothermia. However the decrease of heart rate did not affect cardiac output.

Reducing temperature was proven by measurement of intramuscular temperature of both cooled and

**Table 4.** Vascular Resistance

Organ	Temperature of the Water					
	32°C	26°C	20°C	14°C	8°C	2°C
Kidney right	0.4 ± 0.06	0.5 ± 0.05	0.4 ± 0.02	0.5 ± 0.05	0.5 ± 0.04	0.5 ± 0.06
Kidney left	0.4 ± 0.07	0.5 ± 0.05	0.4 ± 0.03	0.5 ± 0.05	0.5 ± 0.05	0.5 ± 0.06
Humerus right	10.3 ± 2.6	7.1 ± 1 <sup>a</sup>	7.2 ± 6.7	6.1 ± 1.1 <sup>a</sup>	6.7 ± 1.4 <sup>a</sup>	5.7 ± 0.9 <sup>a</sup>
Humerus left	10.5 ± 2.5	7.3 ± 0.9 <sup>a</sup>	6.7 ± 1.3	7.8 ± 2.4 <sup>a</sup>	7.1 ± 1.9 <sup>a</sup>	5.5 ± 0.7 <sup>a</sup>
Femur (control)	7.7 ± 0.7	5.8 ± 0.4 <sup>a,#</sup>	5 ± 0.3 <sup>a,#</sup>	4.5 ± 0.3 <sup>a,#</sup>	4.5 ± 0.3 <sup>a,#</sup>	4.3 ± 0.2 <sup>a,#</sup>
Femur (cooled)	9.5 ± 1	9.6 ± 1.3	9.7 ± 1.4	9.9 ± 1.3	13.3 ± 1.9 <sup>a,b,c,d</sup>	22 ± 4.2 <sup>a,b,c,d,e</sup>
Tibia (control)	21.8 ± 5.3	24.5 ± 5.9	27.9 ± 12.6 <sup>#</sup>	13.8 ± 1.8 <sup>a,b,c,#</sup>	23.4 ± 9.6 <sup>#</sup>	13.7 ± 1.9 <sup>a,b,c,e,#</sup>
Tibia (cooled)	28.4 ± 6.7	29.8 ± 7.8	67.5 ± 5.9 <sup>a,b</sup>	54.3 ± 12.8 <sup>a,b</sup>	57.4 ± 0.8 <sup>a,b</sup>	87.9 ± 11.3 <sup>a,b</sup>

Values of vascular resistance, mmHg ml<sup>-1</sup> min 100 g<sup>-1</sup> in different organs under local cooling. Values are means ± SEM ( $n = 6$ ). <sup>a</sup>Significantly different to 32°C ( $p < 0.05$ ). <sup>b</sup>Significantly different to 26°C ( $p < 0.05$ ). <sup>c</sup>Significantly different to 20°C ( $p < 0.05$ ). <sup>d</sup>Significantly different to 14°C ( $p < 0.05$ ). <sup>e</sup>Significantly different to 8°C ( $p < 0.05$ ). <sup>#</sup>Significant difference between control and treated specimens ( $p < 0.05$ ).

**Table 5.** Regional Blood Flow of the Humerus

Humerus	Temperature of the Water					
	32°C	26°C	20°C	14°C	8°C	2°C
Caput humeri	14.8 ± 2	17.6 ± 2.6	20.3 ± 2.7 <sup>a</sup>	20.7 ± 2.4 <sup>a</sup>	19.4 ± 1.1 <sup>a</sup>	21.4 ± 2.1 <sup>a</sup>
	13.4 ± 2.1	15.2 ± 2.6	19.9 ± 2.5 <sup>a</sup>	19.4 ± 2.5 <sup>a</sup>	19.2 ± 1.3 <sup>a</sup>	20.4 ± 2.2 <sup>a</sup>
Tuberculum majus	22.3 ± 4	25.9 ± 5.9	30.1 ± 6.4	28.5 ± 6.9	24.9 ± 3.7	25.6 ± 2.3
	22.9 ± 4	22.3 ± 3.7	27.2 ± 3.2	27.4 ± 3.4	27.1 ± 2.1	30.1 ± 3.5
Metaphysis	17.8 ± 2.5	21.3 ± 2.8	22.9 ± 3.7	22.6 ± 2.2	20 ± 1.8	22.3 ± 2.5
	17.8 ± 2.3	20 ± 2.6	20.9 ± 3.1	21.7 ± 2.3	19 ± 1.9	21.5 ± 3.2
Diaphysis	6.7 ± 1	8.9 ± 1.8	10.2 ± 2.2	9.9 ± 1.8	9.3 ± 1.7	10 ± 2.2
	6.4 ± 1.6	7.2 ± 1.3	7.6 ± 1.7	8 ± 1.2	8 ± 1.8	8.6 ± 1.5
Epiphysis distalis	5 ± 1.4	5.7 ± 1.1	6.8 ± 1.8	6.6 ± 1.5	6.2 ± 1.6	7 ± 1.1
	5.3 ± 1.2	6.1 ± 1.2	6.5 ± 0.9	6.7 ± 1.8	7.4 ± 1.5	7.6 ± 0.9

Values of regional blood flow (ml/min/100 g) in right and left humerus (not cooled). Values are means ± SEM ( $n = 6$ ). <sup>a</sup>Significantly different to 32°C ( $p < 0.05$ ).

control extremity. We could unequivocally identify the effect of local cooling on intramuscular temperature: The decrease of water temperature strongly correlates with the decrease of intramuscular temperature.

In femur and tibia a strong correlation between decrease of tissue temperature and decrease of bone blood flow was found. A more detailed view shows that the decrease of RBBF was more significant in tibia compared to femur, which is explained by the technical feasibility of cooling the hind limb only distal from the diaphysis of the femur. Proximal regions have not been cooled and showed—as well as upper extremity and the control—an increase of blood flow. For those regions, most likely, a reflective decrease of vascular resistance was found, leading to vasodilatation.

The regions of the femur being adjacent to cooling were diaphysis and distal metaphysis. Reduction of RBBF at 2°C compared with 32°C was -21% for the diaphysis and -51% for distal metaphysis, respectively. The decrease of blood flow was -81.5% for the medial femoral condyle and -88% for the lateral femoral condyle, respectively (cooled completely).

In contrast to the femur, the tibia was cooled completely and therefore showed a proportional decrease of RBBF at any step of cooling. The decrease of blood flow of all anatomic regions of the tibia was identical—especially vulnerable regions could not be found. Local cooling leads to an increase of vascular resistance followed by vasoconstriction of bone vessels resulting in decreased bone blood flow.

**Table 6.** Regional Blood Flow of the Femur

Femur	Temperature of the Water					
	32°C	26°C	20°C	14°C	8°C	2°C
Caput femoris	8.1 ± 1.9	10 ± 1.3	11.9 ± 1.9	13.2 ± 2.5 <sup>a</sup>	13.5 ± 2.2 <sup>a</sup>	14.6 ± 2.4 <sup>a</sup>
	8.2 ± 1.9	9.6 ± 1.9	11.8 ± 2.5	13.2 ± 2.5 <sup>a</sup>	12 ± 2.4 <sup>a</sup>	13.3 ± 0.9 <sup>a</sup>
Collum femoris	18.8 ± 3	20.2 ± 2.6	24 ± 3.5	25.4 ± 3.9 <sup>a</sup>	22.6 ± 3.1	23.3 ± 2.9
	17.5 ± 2.8	20.4 ± 2.7	23 ± 3.5 <sup>a</sup>	22.5 ± 4.1	19.1 ± 2.6	22.2 ± 3.1
Trochanter major	12.2 ± 2.6	14.9 ± 2.7	16.8 ± 4	21.8 ± 4.5	22.3 ± 3.6 <sup>a</sup>	24 ± 1.9 <sup>a</sup>
	11.6 ± 2.5	13 ± 3.2	17.2 ± 3.1	17.9 ± 4.6	18.2 ± 4.2 <sup>a</sup>	20.7 ± 2.2 <sup>a</sup>
Metaphysis proximalis	14.9 ± 2.3	18.3 ± 2	19.2 ± 2.7	20.1 ± 2.6	18.5 ± 2.4	20.5 ± 3.5
	11.7 ± 3.4	13.5 ± 3.6	16.3 ± 4.1	15.8 ± 4.3	13.4 ± 3.1	11.9 ± 2.7
Diaphysis	9.8 ± 1.7	11.8 ± 1.9	13.1 ± 2.1	13.2 ± 1.4	12.2 ± 1.6 <sup>#</sup>	12.8 ± 2.8 <sup>#</sup>
	9.2 ± 1.8	10.8 ± 2.1	10.6 ± 2.2	10.5 ± 1.7	8.1 ± 1.4 <sup>d</sup>	7.3 ± 1.6 <sup>d,e</sup>
Metaphysis distalis	13.1 ± 2	11.8 ± 2.3	14.6 ± 2	15.9 ± 2.3 <sup>#</sup>	14.3 ± 1.9 <sup>#</sup>	15.4 ± 2.2 <sup>#</sup>
	11.2 ± 2.2	12 ± 2.4	11.8 ± 2.2	10.2 ± 2.1	6.8 ± 1.3 <sup>a,b,c</sup>	5.5 ± 2 <sup>a,b,c,d</sup>
Condylus medialis	9.8 ± 1.8	15 ± 1.9 <sup>#</sup>	11.7 ± 1.4 <sup>#</sup>	12.4 ± 2.3 <sup>#</sup>	11.9 ± 1.8 <sup>#</sup>	14.2 ± 1.4 <sup>#</sup>
	8 ± 1.4	6.4 ± 1.3	4.6 ± 1.2	3.6 ± 0.5 <sup>a</sup>	2.2 ± 0.3 <sup>a,b,c,d</sup>	1.4 ± 0.2 <sup>a,b,c,d,e</sup>
Condylus lateralis	10.4 ± 1.7	10.6 ± 1.9 <sup>#</sup>	12.8 ± 1.6 <sup>#</sup>	13.6 ± 2.2 <sup>#</sup>	12.7 ± 1.5 <sup>#</sup>	15 ± 1.6 <sup>#</sup>
	8.3 ± 1.5	6.8 ± 1.1	4.4 ± 1	3.8 ± 0.8 <sup>a,b,c</sup>	2.2 ± 0.3 <sup>a,b,c,d</sup>	1 ± 0.2 <sup>a,b,c,d,e</sup>

Values of regional blood flow (ml/min/100g) in partially cooled and non-cooled femur. Values are means ± SEM ( $n = 6$ ). <sup>a</sup>Significantly different to 32°C ( $p < 0.05$ ). <sup>b</sup>Significantly different to 26°C ( $p < 0.05$ ). <sup>c</sup>Significantly different to 20°C ( $p < 0.05$ ). <sup>d</sup>Significantly different to 14°C ( $p < 0.05$ ). <sup>e</sup>Significantly different to 8°C ( $p < 0.05$ ). <sup>#</sup>Significant difference between control and treated specimens ( $p < 0.05$ ).

**Table 7.** Regional Blood Flow of the Tibia

Tibia	Temperature of the Water					
	32°C	26°C	20°C	14°C	8°C	2°C
Condylus medialis	9.6 ± 1.5	10.6 ± 3	11.2 ± 2.2 <sup>#</sup>	12.7 ± 2.1 <sup>#</sup>	13 ± 2.3 <sup>a,#</sup>	12.7 ± 2.1 <sup>#</sup>
Condylus lateralis	9.6 ± 1.1	8.2 ± 0.7	4.8 ± 1.3 <sup>a,b</sup>	3.5 ± 0.6 <sup>a,b</sup>	2.4 ± 0.4 <sup>a,b,c</sup>	1.5 ± 0.3 <sup>a,b,c,d</sup>
	6.8 ± 1.6	8.2 ± 2.9	9.2 ± 2.2 <sup>#</sup>	9.6 ± 2.6 <sup>#</sup>	10.6 ± 2.4 <sup>#</sup>	12.1 ± 2.4 <sup>a,b,c,#</sup>
Metaphysis proximalis	7.3 ± 1.3	5.5 ± 1.1	3.8 ± 1.8 <sup>a</sup>	3.1 ± 0.5 <sup>a,b</sup>	2.4 ± 0.7 <sup>a,b</sup>	1.3 ± 0.4 <sup>a,b,c</sup>
	11.4 ± 1.5	13.1 ± 2.2	13.2 ± 1.8 <sup>#</sup>	13.2 ± 2.2 <sup>#</sup>	13.9 ± 1.1 <sup>#</sup>	14.3 ± 2.5 <sup>#</sup>
Diaphysis proximalis	12.9 ± 1.4	10.2 ± 0.9 <sup>a</sup>	5.8 ± 1.2 <sup>a</sup>	4.1 ± 0.6 <sup>a,b</sup>	2.5 ± 0.5 <sup>a,b,c</sup>	1.8 ± 0.4 <sup>a,b,c,d</sup>
	7.1 ± 0.7	8.5 ± 1 <sup>#</sup>	9.2 ± 1.1 <sup>#</sup>	9.2 ± 1 <sup>#</sup>	9.6 ± 1.2 <sup>#</sup>	7.6 ± 1.4 <sup>#</sup>
Diaphysis distalis	7 ± 0.9	6.1 ± 0.8	3.7 ± 0.8 <sup>a</sup>	3 ± 0.8 <sup>a,b</sup>	1.4 ± 0.4 <sup>a,b,c,d</sup>	1.1 ± 0.3 <sup>a,b,c,d</sup>
	2.5 ± 0.4 <sup>#</sup>	2.6 ± 0.8 <sup>#</sup>	2.6 ± 0.5 <sup>#</sup>	3.2 ± 0.8 <sup>#</sup>	3.2 ± 0.8 <sup>#</sup>	2.9 ± 0.3 <sup>#</sup>
Metaphysis distalis	2 ± 0.5	1.8 ± 0.5	1.2 ± 0.5	0.9 ± 0.3 <sup>a</sup>	0.9 ± 0.4 <sup>a</sup>	0.5 ± 0.2 <sup>a</sup>
	4.2 ± 1.1	2.9 ± 0.9	4.3 ± 1.4	4 ± 1.1 <sup>#</sup>	4.9 ± 1.6 <sup>#</sup>	3.9 ± 0.6 <sup>#</sup>
Epiphysis distalis	3.5 ± 1.2	2.1 ± 0.7	2.4 ± 1.5	1 ± 0.4 <sup>a</sup>	2 ± 1.1	0.8 ± 0.4 <sup>a</sup>
	3.7 ± 0.9	3.2 ± 1.4	3.7 ± 0.9	4.4 ± 0.6	6.5 ± 1 <sup>a,b,c,#</sup>	5.1 ± 0.4 <sup>#</sup>
	3.2 ± 1.2	3.4 ± 1	2.6 ± 1.1	3.3 ± 1.5	1.5 ± 0.8 <sup>a,b,d</sup>	1.1 ± 0.5 <sup>a,b,d</sup>

Values of regional blood flow (ml/min/100 g) in cooled and non-cooled tibia. Values are means ± SEM ( $n = 6$ ). <sup>a</sup>Significantly different to 32°C ( $p < 0.05$ ). <sup>b</sup>Significantly different to 26°C ( $p < 0.05$ ). <sup>c</sup>Significantly different to 20°C ( $p < 0.05$ ). <sup>d</sup>Significantly different to 14°C ( $p < 0.05$ ). <sup>#</sup>Significant difference between control and treated specimens ( $p < 0.05$ ).

In contrast RBBF of the contra lateral bones (femur, tibia) and of the upper extremities showed increased blood flow values. This may be explained by a compensative vasodilatation under normal conditions due to severe vasoconstriction (excitation) of the cooled ipsilateral side. The underlying mechanism remains unclear. Further characterization of the underlying mechanism reaches beyond the scope of this study and will be explored in the future.

Our findings of decreased blood flow at local cooling are in accordance to those of other authors describing predominantly vasoconstriction of skin and blood vessels under local cooling<sup>8,9,21–23</sup> by release of norepinephrine.

Yamazaki et al.<sup>24</sup> pointed out that the rate of local cooling is an important determinant of the early non-adrenergic vasodilator response to local cooling and that functional nitric oxide synthase, adrenergic nerves, as well as other mechanisms play roles in vasoconstriction during prolonged local cooling of skin.

Furthermore it has been reported, that vasoconstriction can be locally initiated by axon reflex and subsequent release of norepinephrine.<sup>8,25,26</sup>

Ho et al.<sup>27</sup> measured bone blood flow by means of triple-phase technetium bone scans after 20 min of knee cooling. Cooling was performed by use of an ice wrap leading to decreased arterial blood flow by 38% in distal femur and proximal tibia.

The purpose of local cooling such as hypothermia is to decrease metabolism and energy consumption, apoptotic and necrotic cell death and oxidative stress.<sup>28</sup> Induction of hypothermia seems to decrease the release of pro-inflammatory cytokines believed to influence distant organ damage positively, in order that local cooling may have a comparable effect.

Our results are especially important for the after-care of surgical procedures which induce bone bleed-

ing, for example, after bone surgery such as open-wedge osteotomies or fracture fixation.

By local cooling hematoma formation can be reduced by decreasing RBBF. In clinical practice, bleeding control of cancellous bone can only be achieved by application of bone wax, Gelfoam paste, Amicar, and therapeutic angiography.<sup>29–31</sup> In this regard, local cooling would be a sufficient method to reduce bone bleeding reliably by decreasing bone blood flow after surgery—so far ice application is mainly performed to decrease pain, but not to affect blood flow.

Our results show that local cooling is a potential, easy, safe, and cost-effective method to substantially reduce bone blood flow. In our approach external temperature of 20°C decreased blood flow up to 50% illustrating the immense effect of this simple procedure.

## CONCLUSION

Local external cooling reduces bone blood flow up to 84% indicating the powerful reduction in bone blood flow levels in response to reduced temperature. Local cooling of extremities of surgical patients therefore offers the possibility to reduce pain and decrease bleeding complications. Local cooling offers a safe, straightforward and cost-effective way of modulating blood flow in bone tissue.

As a result the Null-Hypothesis needs to be discarded revealing that local cooling has an effect on RBBF.

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