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Tourniquet-induced Exsanguination in Patients Requiring Lower Limb Surgery

An Ischemia-Reperfusion Model of Oxidant and Antioxidant Metabolism

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Background: **Surgically induced ischemia and reperfusion is frequently accompanied by local and remote organ injury. It was hypothesized that this procedure may produce injurious oxidants such as hydrogen peroxide (H_2O_2), which, if unscavenged, will generate the highly toxic hydroxyl radical ($\cdot OH$). Accordingly, it was proposed that tourniquet-induced exsanguination for limb surgery may be a useful ischemia-reperfusion model to investigate the presence of oxidants, particularly H_2O_2.**

Methods: **In ten patients undergoing knee surgery, catheters were placed in the femoral vein of the limb operated on for collection of local blood and in a vein of the arm for sampling of systemic blood. Tourniquet-induced limb exsanguination was induced for about 2 h. After tourniquet release (reperfusion), blood samples were collected during a 2-h period for measurement of H_2O_2, xanthine oxidase activity, xanthine, uric acid (UA), glutathione, and glutathione disulfide.**

Results: **At 30 s of reperfusion, H_2O_2 concentrations increased ($\approx$90%) from 133 ± 5 to 248 ± 8 nmol · ml^{-1} ($P < 0.05$) in local blood samples, but no change was evident in systemic blood. However, in both local and systemic blood, xanthine oxidase activity increased $\approx$90% (1.91 ± 0.07 to 3.93 ± 0.41 and 2.19 ± 0.07 to 3.57 ± 0.12 nmol UA · ml^{-1} · min^{-1}, respectively) as did glutathione concentrations (1.27 ± 0.04 to 2.69 ± 0.14 and 1.27 ± 0.03 to 2.43 ± 0.13 μmol · ml^{-1}, respectively). At 5 min reperfusion, in local blood, H_2O_2 concentrations and xanthine oxidase activity peaked at 796 ± 38 nmol · ml^{-1} ($\approx$500%) and 11.69 ± 1.46 nmol UA · ml^{-1} · min^{-1} ($\approx$520%), respectively. In local blood, xanthine and UA increased from 1.49 ± 0.07 to 8.36 ± 0.33 nmol · ml^{-1} and 2.69 ± 0.16 to 3.90 ± 0.18 μmol · ml^{-1}, respectively, whereas glutathione and glutathione disulfide increased to 5.13 ± 0.36 μmol · ml^{-1} and 0.514 ± 0.092 nmol · ml^{-1}, respectively. In systemic blood, xanthine oxidase activity peaked at 4.75 ± 0.20 UA nmol · ml^{-1} · min^{-1}. At 10 min reperfusion, local blood glutathione and UA peaked at 7.08 ± 0.46 μmol · ml^{-1} and 4.67 ± 0.26 μmol · ml^{-1}, respectively, while the other metabolites decreased significantly toward pretourniquet levels. From 20 to 120 min, most metabolites returned to pretourniquet levels; however, local and systemic blood xanthine oxidase activity remained increased 3.76 ± 0.29 and 3.57 ± 0.37 nmol UA · ml^{-1} · min^{-1}, respectively. Systemic blood H_2O_2 was never increased during the study. During the burst period ($\approx$5–10 min), local blood H_2O_2 concentrations and xanthine oxidase activities were highly correlated (r = 0.999).**

Conclusions: **These studies suggest that tourniquet-induced exsanguination for limb surgery is a significant source for toxic oxygen production in the form of H_2O_2 and that xanthine oxidase is probably the H_2O_2-generating enzyme that is formed during the ischemia-reperfusion event. In contrast to the reperfused leg, the absence of H_2O_2 in arm blood demonstrated a balanced oxidant scavenging in the systemic circulation, despite the persistent increase in systemic xanthine oxidase activity. (Key words: Blood: glutathione; hydrogen peroxide; xanthine oxidase. Ischemia–reperfusion.)**

INTENTIONAL ischemia of the extremities occurs during peripheral vascular surgery, abdominal aneurysm resection, reimplantation of the extremities and during tourniquet application to facilitate a bloodless surgical

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field. Reperfusion, *i.e.*, restoration of blood flow, in the extremities has been associated with local and remote organ injury.[1–4] The exact cause of tissue injury secondary to reperfusion is uncertain, however, considerable evidence suggests the following toxic oxidant pathway: superoxide anion (O_2^-)→hydrogen peroxide (H_2O_2)→hydroxyl radical ($\cdot OH$).[5–9] The *in vivo* source of O_2^-, and hence H_2O_2, is phagocytes and endothelial cells, which generate these toxic metabolites from nicotinamide adenine dinucleotide phosphate oxidase and xanthine oxidase, respectively.[10] Other minor sources include cyclooxygenase, mixed function oxidases, and mitochondrial enzymes such as monoamine oxidase (fig. 1).[10]

Although no *in vivo* human study of ischemia and reperfusion has directly measured the purported toxic oxygen metabolites, there is evidence suggesting that xanthine oxidase is a significant source of O_2^-, a conclusion based on the observation that inhibitors of xanthine oxidase, *i.e.*, allopurinol and pterin aldehyde, effectively ameliorate tissue injury.[11,12] Friedl *et al.*[13] have shown increased blood xanthine oxidase activity after intentional ischemia (tourniquet)-reperfusion for bloodless upper limb surgery; however, these investigators did not measure a toxic metabolite. Conversely, that study did show that plasma contained evidence of products consistent with the formation of toxic oxidants, namely, the appearance of hemoglobin and fluorescent compounds predominantly in the reperfused limb.[13]

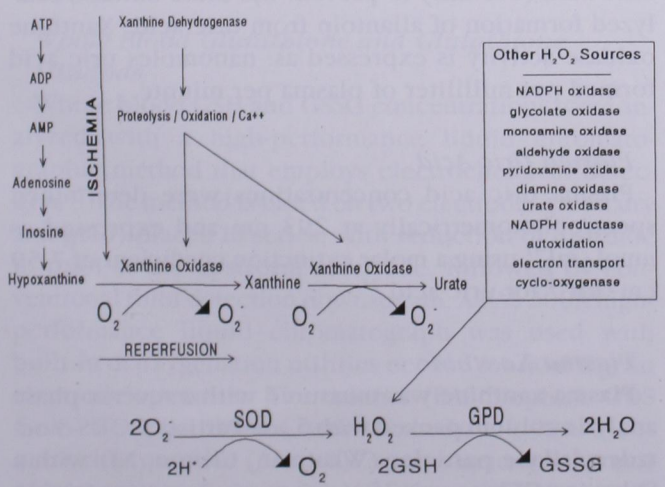

Fig. 1. Metabolic pathways of hydrogen peroxide (H_2O_2), xanthine oxidase, xanthine, uric acid, glutathione (GSH), and glutathione disulfide (GSSG).

We hypothesized that if xanthine oxidase is a major producer of toxic oxidants after intentional ischemia and reperfusion then blood H_2O_2 concentrations and xanthine oxidase activity should be increased. Furthermore, changes in blood H_2O_2 would be reflected by changes in its primary scavenger system, reduced glutathione (GSH) and glutathione disulfide (GSSG).[14] To study the ischemia-reperfusion event, patients undergoing elective knee surgery were chosen because they require a pneumatic tourniquet to facilitate a blood-free surgical field. This model has a well-controlled ischemic period ($\approx$ 2 h) and allows routine blood sampling during the reperfusion period. Although measuring H_2O_2 has been cumbersome in the past, a simple and highly sensitive radio-isotopic method recently became available to directly determine H_2O_2 in biologic fluids.[15] In addition to H_2O_2, GSH, GSSG, xanthine oxidase activity, and its end products, xanthine and uric acid, were measured.

Materials and Methods

The study was approved by the Institutional Review Board of the Hospital and informed consent was obtained from each patient participating in the study. Ten outpatients undergoing elective knee surgery with concurrent use of the pneumatic tourniquet were included in the study. Mean patient age 36.6 ± 3.6 yr (mean ± SE). No patient was receiving a vitamin supplement. All patients underwent general anesthesia including propofol (2 $mg \cdot kg^{-1}$) as an induction agent and maintained on nitrous oxide, oxygen, and isoflurane. Circumferential applications of elastic bands were applied to the extremity to be surgically treated to exsanguinate the extremity of blood followed by tourniquet application. An 18-G catheter filled with heparin was inserted into the femoral vein of the ipsilateral extremity (operated limb) and in an antecubital vein of the arm. The tourniquet was applied at a pressure approximately twice the systolic blood pressure (sufficient to prevent surgical bleeding at the surgical field). Tourniquet time was 126 ± 7 min. Blood specimens (10 ml) were obtained, from arm and leg, 5 min before tourniquet application and then after tourniquet release at these times: 30 s, and 5, 10, 20, 60 and 120 min.

Blood Sample Preparation

Blood samples (10 ml) were collected in tubes containing ethylenediaminetetraacetic acid (10 mM). For

H_2O_2 assay of whole blood,[15] a 100-μl aliquot was transferred directly to a microfuge tube containing 350 μl ice-cold 5% trichloroacetic acid solution. A protein-free supernatant was obtained by centrifugation in a refrigerated microfuge for 1 min. The deproteinized extracts were neutralized with 0.1 μl of ice-cold 1.25 M NaOH. For plasma xanthine oxidase activity and uric acid, an aliquot of blood was diluted immediately 1:1 (vol/vol) with an ice-cold solution containing 2.4 mM potassium phosphate, 150 mM sodium chloride, 10 mM dithiothreitol, and 1 mM phenylmethyl sulfonyl fluoride at a *p*H of 7.35.[13] This solution prevents conversion of xanthine dehydrogenase to xanthine oxidase.[13] Plasma was separated by centrifugation at 4°C within 5 min after sample collection. All samples were immediately frozen at −70°C and processed within 6 h. For plasma xanthine, an aliquot of blood was centrifuged without delay at 1,500–2,500*g* for 15 min at 4°C.[16] Plasma was stored at −70°C until assay. For GSH and GSSG assay of whole blood,[17] a 20-μl aliquot of blood was transferred directly to a microcentrifuge tube containing 800 μl of 0.1% Na_2 ethylenediaminetetraacetic acid. Two hundred microliters of 0.2 M $HClO_4$ was then added and the tube was vortexed briefly. After standing 10 min to precipitate the proteins, the sample was centrifuged 10 min at 1,600*g*, and the supernatant was filtered through a 0.2-μm membrane before assay.

Whole Blood Hydrogen Peroxide

Hydrogen peroxide in whole blood was determined by the method of Varma and Devamanoharan[15] with modifications according to Mathru *et al.*[18] Briefly, this is a radioactive method based on the decarboxylation of 1-^{14}C-alpha-ketoglutaric acid by H_2O_2. The liberated $^{14}CO_2$ was counted in a liquid scintillation counter. In our study, the reaction was carried out in a custom-designed test tube (8.5 × 1.5 cm) with a side arm (2.0 × 0.5 cm) situated 2.5 cm from the bottom (Supelco Separation Technologies, Belleconte, PA). A mixture of radiolabeled and nonlabeled alpha ketoglutarate was placed in the test tube, which was then covered with a CO_2 trap. A blood extract, diluted 1:20 with Tyrode buffer, was injected through a rubber stopper in the side arm. After an incubation period, the sample was acidified with trichloracetic acid and then incubated at 37°C for 60 min. The CO_2 trap was then transferred to a scintillation vial and counted. The H_2O_2 content in the blood sample was calculated as follows:

$$\text{hydrogen peroxide } (\mu M/L) = \frac{DPM_{sample} - DPM_{background} \times \text{dilution factor}}{DPM_{reference}}$$

where DPM_{sample} is the number of disintegrations per minute in the blood sample containing the radiolabeled analog of alpha-ketoglutarate, $DPM_{background}$ is the number of disintegrations per minute in the sample without the radiolabeled analog, and $DPM_{reference}$ is the number of disintegrations per minute in a quantity ($\mu M \cdot l^{-1}$) of the pure radiolabeled analog. In our study, the technician performing the assay was blinded to the identity of the sample. The lower limit of detection is 0.1 nmol. Specificity was greater than 90% as determined with *in vitro* control experiments in which known amounts of H_2O_2 were added to control blood. Minor interferences can be expected if other H_2O_2-dependent decarboxylating compounds are present in the blood.[16]

Plasma Xanthine Oxidase

Plasma xanthine oxidase activity was assayed spectrophotometrically by measurement of uric acid formation at 293 nm in the absence of NAD^+.[19,20] Allopurinol (50 μM), an inhibitor of xanthine oxidase will be used to confirm that the rates are due to this enzyme. The reaction mixture contained 100 μl xanthine (50 μM), 600 μl potassium phosphate (2.4 mM) and sodium chloride (150 mM) at *p*H 7.35 and 100 μl of plasma to a final plasma content of 5% (vol/vol). The reaction mixture contained 100 μl of the uricase inhibitor oxonic acid (0.1 mM) to prevent the urate oxidase-catalyzed formation of allantoin from uric acid. Xanthine oxidase activity is expressed as: nanomoles uric acid formed per milliliter of plasma per minute.

Plasma Uric Acid

Plasma uric acid concentrations were determined spectrophotometrically at 293 nm and expressed as $\mu mol \cdot ml^{-1}$ using a molar extinction coefficient of 7.59 $cm \cdot \mu M^{-1}$ for uric acid.[21]

Plasma Xanthine

Plasma xanthine was measured with a reverse-phase analytic column packed with 5-μm Partisil 5-ODS-3 octadecylsilane particles (Whatman, Clifton, NJ) with a Solvecon (Whatman) 25 × 4.6 mm column containing silica gel, 37–53-μm particle size, placed between the pump and the injector.[16] Protein-free plasma ultrafil-

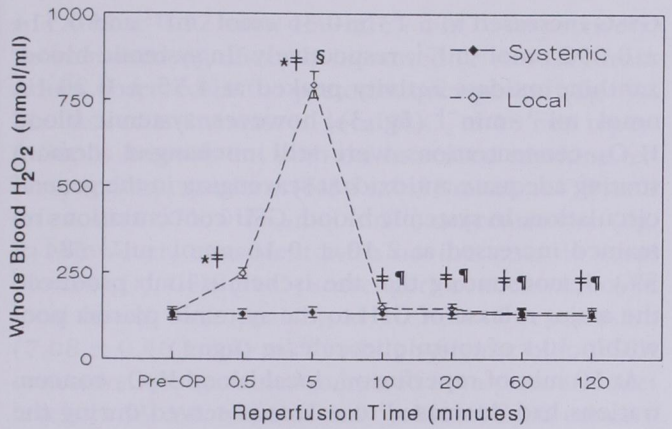

Fig. 2. Hydrogen peroxide (H_2O_2) concentrations in venous blood from the femoral vein of the leg (◇, local) before and after application of a pneumatic tourniquet for bloodless knee surgery and simultaneously from an antecubital vein of an arm (◆, systemic). Significance at $P < 0.05$; ****versus* preoperatively (Pre-OP); ‡ *versus* 0.5 min; ¶ *versus* 5 min; § *versus* systemic.

trate was obtained by passing the sample through an MPS-1 micropartition system *via* centrifugation. After equilibrating the columns for 1 h with mobile phase (5 mM heptane sulfonate, 10 mM monobasic phosphate (monohydrate), and 1% methanol; final *p*H 5.5), 10 μl plasma ultrafiltrate was injected onto the column using a BAS 200A HPLC/CMA Injector system (BAS, West Lafeyette, IN). Xanthine values ($nmol \cdot ml^{-1}$) were obtained from a standard curve of peak absorbances at 254 nm for known xanthine concentrations. The lower limit of detection was 0.1 $nmol \cdot ml^{-1}$.

Whole Blood Glutathione and Glutathione Disulfide

Whole blood GSH and GSSG concentrations were analyzed with a high-performance liquid chromatographic method that employs electrochemical detection.[17] The method is based on two electrodes (mercury and gold) placed in series, with reduction of disulfide to thiol at the upstream electrode, followed by conventional thiol detection downstream. A BAS 200A high-performance liquid chromatograph was used with built-in deoxygenation utilities needed for dual Hg/Au electrode operation. The column (BAS Biophase ODS 5 μm) was equilibrated with 1% methanol, 99% 0.1 M monochloroacetate (*p*H 3.0). Approximately 0.1 nmol of a nonretained thiol (cysteine) was added to each standard and sample to improve precision of the dual Hg/Au detector response. Minimum detectable quantities were 3.5 and 5.7 pmoles for GSH and GSSG, respectively.

Statistical Analysis

Significant differences between local and systemic blood parameter means were determined with a completely randomized block analysis of variance in conjunction with Student-Newman-Keuls test. A repeated measures analysis of variance was used for differences over time within the blood parameters. A Pearson correlation matrix was generated to determine a temporal relationship between H_2O_2 and xanthine oxidase activity over time. All values were expressed as means standard error of the mean. Normal distribution of data was verified with goodness-of-fit, W statistic, skewness, kurtosis, and mean-median symmetry. A P value of <0.05, Bonferroni-corrected for multiple comparisons, was considered significant.

Results

In the pretourniquet period, there were no significant differences in blood analyte concentrations between systemic (arm) and local (leg) samples (figs. 2–4). Thirty seconds after release of the tourniquet, local blood H_2O_2 concentrations increased 87 ± 4% (133 ± 5 to 248 ± 8 $nmol \cdot ml^{-1}$), however, systemic blood concentrations were not changed from baseline (fig. 2). In both local and systemic blood, xanthine oxidase activities increased (≈90%) from 1.91 ± 0.07 to 3.93 ± 0.41 and 2.19 ± 0.07 to 3.57 ± 0.12 nmol $UA \cdot ml^{-1} \cdot min^{-1}$, respectively, as did GSH concentrations increasing from 1.27 ± 0.04 to 2.69 ± 0.14 and 1.27 ± 0.03 to 2.43 ± 0.13 $\mu mol \cdot ml^{-1}$, respectively (figs. 3 and 4). This reflects a significant pooling of xanthine oxidase and GSH in the unperfused leg during ischemia with subsequent equilibration in the general circulation within 30 s of tourniquet release. Consistent with a lack of oxygen during ischemia, changes in local and systemic plasma xanthine, uric acid, and GSSG were not evident during the initial 30-s equilibration period (figs. 3 and 4).

At 5 min of reperfusion, local blood H_2O_2 concentrations and xanthine oxidase activity peaked at 796 ± 38 $nmol \cdot ml^{-1}$ (≈500%) and 11.69 ± 1.46 nmol $UA \cdot ml^{-1} min^{-1}$ (≈520%), respectively (figs. 2 and 3). In local blood, xanthine and UA increased from 1.49 ± 0.07 to 8.36 ± 0.33 $nmol \cdot ml^{-1}$ and 2.69 ± 0.16 to 3.90 ± 0.18 $\mu mol \cdot ml^{-1}$, respectively, while GSH and

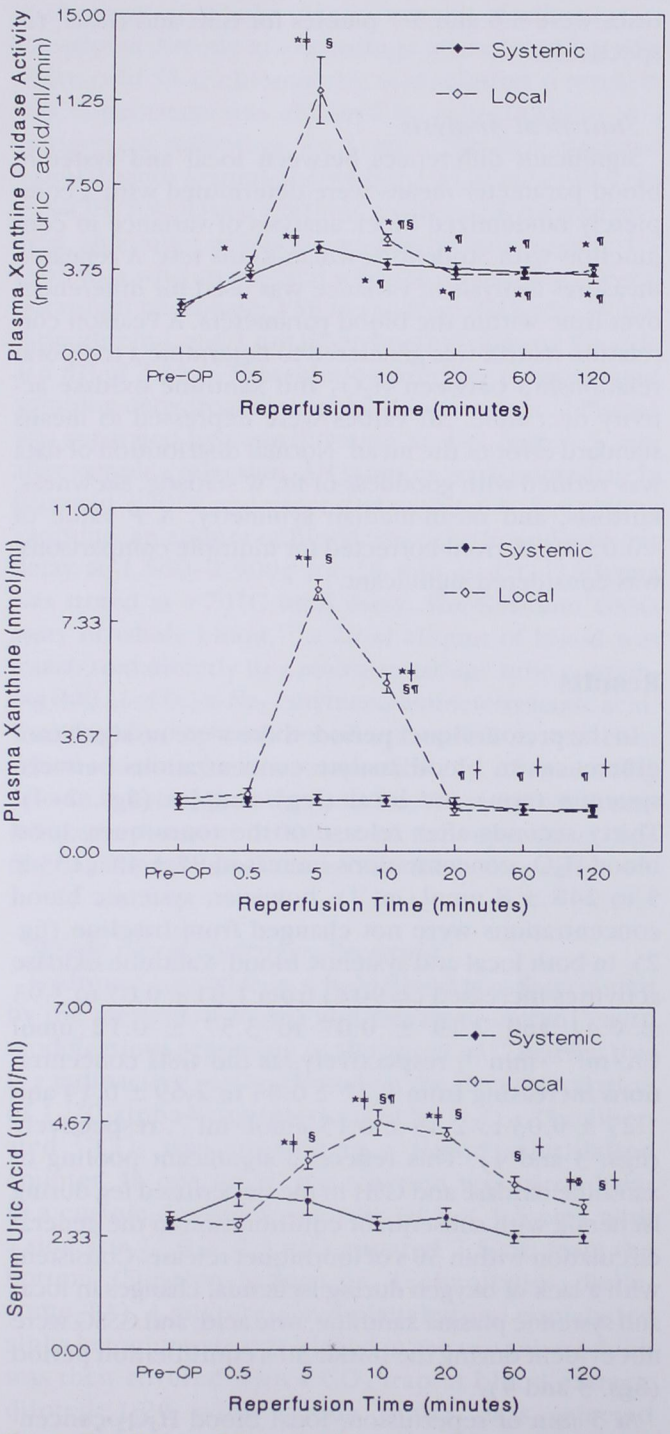

Fig. 3. Xanthine oxidase activity, xanthine, and uric acid concentrations in venous plasma from the femoral vein of the leg (◇, local) before and after application of a pneumatic tourniquet for bloodless knee surgery and simultaneously from an antecubital vein of an arm (◆, systemic). Significance at $P < 0.05$; **versus* preoperatively (Pre-OP); ‡ *versus* 0.5 min; ¶ *versus* 5 min; † *versus* 10 min; ϕ *versus* 20 min; § *versus* systemic.

GSSG increased to 5.13 ± 0.36 $\mu mol \cdot ml^{-1}$ and 0.514 ± 0.092 $nmol \cdot ml^{-1}$, respectively. In systemic blood, xanthine oxidase activity peaked at 4.75 ± 0.20 UA $nmol \cdot ml^{-1} \cdot min^{-1}$ (fig. 3), however, systemic blood H_2O_2 concentrations were still unchanged, demonstrating adequate antioxidant scavenging in the general circulation. In systemic blood, GSH concentrations remained increased at 2.10 ± 0.16 $\mu mol \cdot ml^{-1}$ (84 ± 5%) demonstrating that the ischemic limb produced the major release of GSH to the systemic plasma pool within 30 s of tourniquet release (fig. 4).

At 10 min of reperfusion, local blood H_2O_2 concentrations had decreased to values observed during the pretourniquet period (fig. 2). Local and systemic blood xanthine oxidase activities decreased to 5.10 ± 0.27

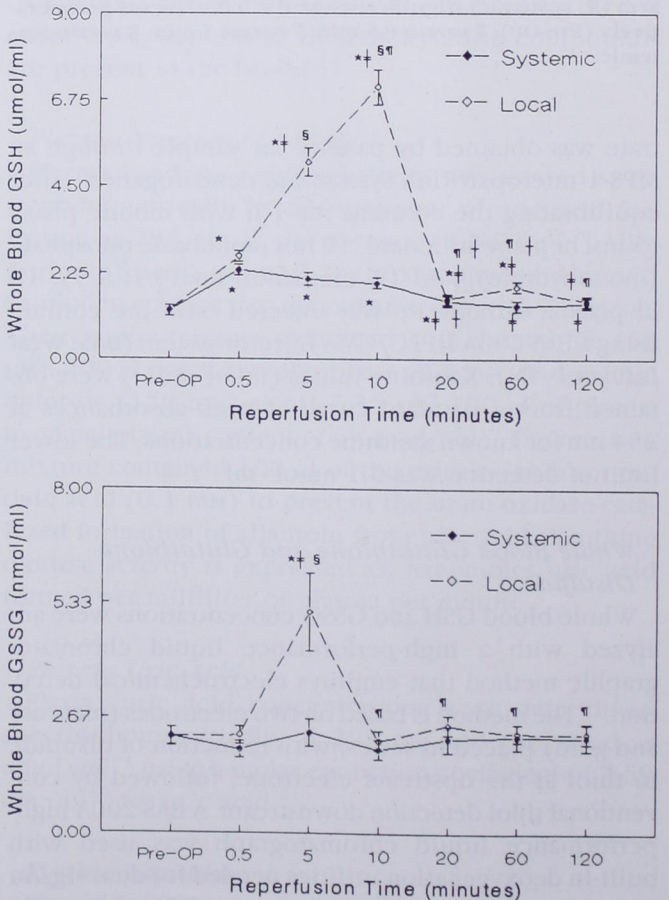

Fig. 4. Glutathione (GSH) and glutathione disulfide (GSSG) concentrations in venous blood from the femoral vein of the leg (◇, local) before and after application of a pneumatic tourniquet for bloodless knee surgery and simultaneously from an antecubital vein of an arm (◆, systemic). Significance at $P < 0.05$; **versus* preoperatively (Pre-OP); ‡ *versus* 0.5 min; ¶ *versus* 5 min; † *versus* 10 min; § *versus* systemic.

and 4.02 ± 0.12 nmol UA · ml^{-1} · min^{-1}, respectively, yet remained significantly increased ($P < 0.05$) from values observed in the pretourniquet period (fig. 3). The lack of an increase in local H_2O_2 at 10 min reperfusion is inconsistent with the increased xanthine oxidase activity, xanthine (5.36 ± 0.33 nmol · ml^{-1}), and uric acid (4.67 ± 0.26 μmol · ml^{-1}) concentrations (fig. 3). However, consistent with no increases in local blood H_2O_2, GSH (H_2O_2 scavenger) concentrations in local blood were peaking at 10 min of reperfusion (7.08 ± 0.46 μmol · ml^{-1}) while systemic blood GSH concentrations remained increased from pretourniquet levels (1.99 ± 0.13 μmol · ml^{-1}). Blood GSSG concentrations in local samples had returned to pretourniquet levels by 10 min reperfusion (fig. 3).

From 20 to 120 min of reperfusion, local and systemic blood H_2O_2 concentrations were not changed from values observed in the pretourniquet period, while both systemic and local xanthine oxidase activities remained significantly increased from values measured in the pretourniquet period (figs. 2 and 3). Blood GSH, GSSG, and plasma xanthine concentrations in local and systemic samples had decreased to values measured in the pretourniquet period (figs. 3 and 4). Throughout the entire 120 reperfusion period, systemic plasma xanthine and blood GSSG concentrations were not changed from values observed in the pretourniquet period. Plasma uric acid remained significantly increased in local blood from 5 to 120 min of reperfusion.

Table 1 presents matrices for correlation coefficients and the respective coefficient probabilities for the entire set of analyte data from baseline (pretourniquet) through completion of the reperfusion period. Local blood H_2O_2 was very highly correlated ($P \leq 0.016$) with xanthine oxidase activity (0.954) and GSSG (0.979) and highly correlated with local xanthine (0.846), reflecting a significant temporal association between H_2O_2 production and xanthine oxidase activity. Furthermore, local GSH and GSSG were highly correlated with local xanthine (0.829 and 0.806, respectively; $P \leq 0.028$), suggesting that H_2O_2 is scavenged mostly by the GSH-GSSG antioxidant system. Of all the analytes measured, xanthine oxidase activity had the greatest correlation between systemic and local blood (0.812; $P = 0.026$). Despite a lack of statistical difference in systemic H_2O_2 concentrations over time, there was good correlation (0.747–0.812; $P \leq 0.05$) between systemic H_2O_2 data and several local analytes (H_2O_2, xanthine oxidase, xanthine, and GSSG). Finally, systemic uric acid and GSH were highly correlated (0.810; $P = 0.027$).

Discussion

Tourniquet-induced ischemia and reperfusion was a potent generator of hydrogen peroxide in local blood as well as the cause of significant increases in xanthine oxidase activity of both local and systemic blood. Hydrogen peroxide is an integral component of the toxic oxygen pathway: superoxide anion (O_2^-) → hydrogen peroxide (H_2O_2) → hydroxyl radical (·OH). Although this pathway is believed to play a central role in ischemia-reperfusion injury to local as well remote organ tissue, no study has directly identified these compounds in human blood during the ischemia-reperfusion event. The current study demonstrated that H_2O_2 concentrations in blood of the reperfused leg increased almost 500% and peaked within 5 min, however, no changes were evident in systemic blood. Conversely, plasma xanthine oxidase activity (a superoxide, O_2^- generator) increased in both local and systemic blood samples. Xanthine oxidase in local blood increased and peaked in a manner similar to H_2O_2 (correlation coefficient of 0.954) however the activity of this enzyme was present long after (120 min) H_2O_2 levels returned to baseline. Furthermore, systemic blood xanthine oxidase activity was increased within seconds of reperfusion and remained elevated 85% for the duration of the study. These results suggest that in this human model of ischemia and reperfusion, xanthine oxidase initiates a toxic oxidant pathway leading to excessive H_2O_2 production and that antioxidant components in blood were critical for inactivation of H_2O_2 generated from the sustained increase in xanthine oxidase activities in both local and remote circulations during the reperfusion event.

Animal studies by Repine and coworkers[22–25] have demonstrated that xanthine oxidase contributes to injury of skeletal muscle, myocardium, renal, and lung tissue after ischemia and reperfusion. Moreover, in a study related to ours, Friedl *et al.*[13] reported a similar time course in xanthine oxidase activity, however, H_2O_2 was not measured. Evidence suggests that, during reperfusion, local tissue oxidative enzymes contribute to H_2O_2-mediated injury,[22,26,27] however, a neutrophil origin of H_2O_2 cannot be discounted.[28–30] In general, our correlation data are consistent with a xanthine-oxidase–mediated production of H_2O_2, however, two inconsistencies need to be addressed. First, blood xan-

Table 1. Pearson Correlation Matrix and Bonferroni Correlation Probabilities of Analytes Measured in Blood from the Ischemic-reperfused Leg (Local) and Normally Perfused Arm (Systemic) from Baseline (Pretourniquet) through the Reperfusion Period

	Local Blood						Systemic Blood					
	H_2O_2	X Oxidase	Xanthine	Uric Acid	GSH	GSSG	H_2O_2	X Oxidase	Xanthine	Uric Acid	GSH	GSSG
Correlations												
Local												
H_2O_2	1.000											
X oxidase	0.954	1.000										
Xanthine	0.846	0.917	1.000									
Uric acid	0.139	0.362	0.447	1.000								
GSH	0.427	0.607	0.829	0.612	1.000							
GSSG	0.979	0.944	0.806	0.214	0.355	1.000						
Systemic												
H_2O_2	0.747	0.812	0.778	0.697	0.619	0.748	1.000					
X oxidase	0.641	0.812	0.685	0.629	0.611	0.646	0.817	1.000				
Xanthine	0.548	0.546	0.721	0.429	0.678	0.466	0.752	0.366	1.000			
Uric acid	0.618	0.493	0.486	−0.052	0.289	0.490	0.588	0.294	0.778	1.000		
GSH	0.531	0.560	0.611	0.117	0.645	0.368	0.571	0.554	0.700	0.810	1.000	
GSSG	0.386	0.308	0.075	−0.059	−0.386	0.541	0.065	0.071	−0.334	−0.281	−0.557	1.000
Probabilities												
Local												
H_2O_2	0.000											
X oxidase	0.001*	0.000										
Xanthine	0.016*	0.004*	0.000									
Uric acid	1.000	1.000	1.000	0.000								
GSH	1.000	1.000	0.021*	1.000	0.000							
GSSG	0.000*	0.0001*	0.028*	1.000	1.000	0.000						
Systemic												
H_2O_2	0.052*	0.027*	0.040*	1.000	1.000	0.051*	0.000					
X oxidase	1.000	0.026*	1.000	1.000	1.000	1.000	0.025*	0.000				
Xanthine	1.000	1.000	1.000	1.000	1.000	1.000	0.049*	1.000	0.000			
Uric Acid	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.039*	0.000		
GSH	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.027	0.000	
GSSG	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.000

GSH = reduced glatathione; GSSG = oxidized glatathione.

* Significant correlation.

thine oxidase in the local and systemic samples increased essentially in parallel during the onset of reperfusion (30 s), however, H_2O_2 increased only in the local circulation. Furthermore, xanthine and uric acid (the substrate and product of xanthine oxidase activity, respectively) were not significantly different from preischemia concentrations in local or systemic samples until 5 min after reperfusion. This suggests that, initially, H_2O_2 may have been produced by a mechanism other than xanthine oxidase activity, possibly the activated neutrophil. In dog skeletal muscle previously made ischemic, Smith *et al.*[31] reported a 26-fold increase in neutrophil content and a 50% decrease in muscle GSH content within 1 h of reperfusion. Although we did not measure neutrophil content, there was an immediate and parallel increase in both local and systemic blood GSH concentrations suggesting significant injury to this tissue and pooling of GSH content. A local source of H_2O_2 production may be nicotinamide adenine dinucleotide phosphate oxidase activity in skeletal muscle mitochondria.

A second inconsistency arises after 10 min of reperfusion when xanthine oxidase activities in both local and systemic blood samples were above those at 30 s reperfusion, yet H_2O_2 concentrations had returned to values measured in the pretourniquet period. These results suggest that H_2O_2 production from xanthine oxidase may not detectable, *i.e.*, H_2O_2 is scavenged, until the enzyme activity reaches a certain threshold, and, that the initial burst of H_2O_2 generation in the limb at 30 s was due to its pooling in the absence of scavengers. The parallel increase in GSH concentrations in both local and systemic blood samples at 30 s indicate pooling during the ischemic period. The increase in blood GSH was not caused by increased red cell GSH because synthesis in red cells cannot occur over a short period of time ($\approx$2 h).[32,33] Thus, in general, the majority of H_2O_2 production during reperfusion was caused by enhanced xanthine oxidase activity, however, the source of the initial pooling of H_2O_2 during ischemia is uncertain.

Critical antioxidant mechanisms in the blood protect local and remote tissue from toxic oxygen metabolites such as H_2O_2 during reperfusion. Antioxidant scavenging of H_2O_2 was evident in the systemic blood throughout the study period, and, during the 10–120-min reperfusion in the local limb blood. The absence of an increase in systemic blood H_2O_2 was undoubtedly due to its inactivation by blood components, such as red cells. Studies have shown that intact red cells can scavenge plasma H_2O_2 and protect tissues from oxidant damage.[34,35] Conversely, the increased H_2O_2 in local blood at the onset of reperfusion (30 s) was the result of pooling and release in the absence of adequate antioxidants (red cells), immediately after ischemia as evidenced by the unchanged GSSG levels in that period. During scavenging by red cells, only GSSG, but not GSH, is released to the plasma under oxidative stress.[36] Thus, in the current study, when the GSH-GSSG cycling system was overloaded, *i.e.*, at 5 min reperfusion, changes in H_2O_2 and GSSG are observed. When the GSH-GSSG system is balanced, *i.e.*, 10 min reperfusion, changes in H_2O_2 and GSSG are not evident. Although we did not measure plasma H_2O_2, our data suggest that as reperfusion time progressed, reoxygenation increased xanthine oxidase activity and the accompanying H_2O_2, which is highly permeable, was picked up and scavenged by red cells as they traversed the reperfused limb. Apparently, scavenging of H_2O_2 in local blood was not effective when xanthine oxidase activity exceeded baseline by $\approx$90–125% (0.5–10 min reperfusion).

In conclusion, intentional ischemia (tourniquet) and reperfusion caused excess generation of H_2O_2 ($\approx$500% above baseline) in blood of the reperfused limb. Furthermore, from the correlation data we can infer that xanthine oxidase may be the primary source of H_2O_2, however, a secondary source was evident, possibly a neutrophil-mediated one. The absence of H_2O_2, GSSG, and xanthine in the systemic circulation suggests adequate scavenging by blood components despite a sustained increase in plasma xanthine oxidase activity ($\approx$85% above pretourniquet) during the 120-min study period. However, when xanthine oxidase activity exceeded baseline by $\approx$90–125% in the local circulation, H_2O_2 scavenging was not effective. These results suggest that inactivation of H_2O_2 in local circulations is limited and that the systemic circulation is readily able to scavenge this potentially toxic substance. This may not always be the case, however, because scavenging abilities may be decreased in certain situations. For example, in cases where the scavenging ability of blood components may be weakened or reduced such as during intentional hemodilution, circulating xanthine oxidase could produce unscavenged H_2O_2, which then may convert to the toxic hydroxyl radical to induce local or remote organ injury. This article demonstrates that the tourniquet-induced exsanguination procedure is a very accessible model to anesthesiologists and/or surgeons in which to study the balance between oxidant and antioxidant metabolism during the ischemia-reperfusion event.

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