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Transdermally administered nitric oxide by application of acidified nitrite increases blood flow in rat epigastric island skin flaps

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Abstract

Surgical flaps are commonly used in the reconstruction of tissue defects after tumour surgery and trauma. Flap failure continues to be a clinical problem and the underlying causes are not fully understood.

In the present study a system that generates nitric oxide (NO) in a non-enzymatic fashion was created through the acidification with vitamin C of a cream containing increasing concentrations (0.125%, 0.25%, 0.5%, 1.25% and 2.5%) of nitrite (NO_2^-). The cream was applied for 30 min to a modified epigastric island skin flap in the rat. Blood flow in the supplying artery was measured by transit-time ultrasound flowmetry throughout the experiment and superficial skin blood flow was measured by laser Doppler perfusion imaging before and after treatment. Mean arterial blood pressure was also monitored. NO and the gas nitrogen dioxide (NO_2), which is formed when NO reacts with atmospheric oxygen, were measured above the cream using chemiluminescence.

In flaps treated with the NO generating cream, a concentration-dependent increase in blood flow in the supplying artery and flap skin of up to 130% was observed. Cream base alone or cream base acidified with vitamin C had no effect on blood flow. Also, concentration-dependent formation of both NO and NO_2 was seen.

NO increases both supplying artery blood flow and superficial cutaneous blood flow in an epigastric island skin flap model in the rat indicating that NO is of importance in flap physiology and possibly also for flap survival.

Keywords: Surgical flaps; Nitric oxide; Laser Doppler; Transit-time ultrasound flowmetry

1. Introduction

The gaseous molecule nitric oxide (NO) is known to play a part in several physiological processes including immune responses, neurotransmission and regulation of blood pressure and regional blood flow (Moncada, 1999; Thippeswamy et al., 2006). NO has also been shown to influence numerous pathological conditions (Naseem, 2005; Thippeswamy et al., 2006) and pharmacological applications are constantly being added (Low, 2005). Besides the synthesis of NO by the different isoforms of NO synthase (NOS) (Knowles and Moncada, 1994), non-enzymatic NO production from nitrite (NO_2^-) has also been identified (Lundberg and Weitzberg, 2005; Suschek et al., 2006). Here, NO_2^- is converted to NO through a multiple step reaction involving nitrous acid (HNO_2) as an intermediate. The reaction requires acidic conditions as seen naturally in for example the gut, on the skin and in ischemic tissues. Reducing agents enhance the reaction and ascorbic acid (vitamin C, pKa 4.2) reacts with HNO_2 to form dehydroascorbate and NO (Lundberg and Weitzberg, 2005; Suschek et al., 2006).

NO produced in the blood vessel either enzymatically by endothelial NOS (eNOS) or non-enzymatically from NO_2^- diffuses to the smooth muscle cells surrounding the vessels causing relaxation, vessel dilatation and increased blood flow. On a molecular level NO stimulates the soluble guanylate

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cyclase inside the smooth muscle cells, which leads to increased levels of cGMP, protein kinase activation and decreased Ca^{2+}-levels, thus causing smooth muscle relaxation (Carvajal et al., 2000; Cary et al., 2006).

When NO is mixed with atmospheric oxygen, spontaneous formation of the gas nitrogen dioxide (NO_2) occurs (Schedin et al., 1999; Weinberger et al., 2001). NO_2 can be harmful both through acute exposure causing hypoxemia, acidosis, pulmonary oedema and pneumonitis and after continuous exposure to minute concentrations causing inflammation, increased sensitivity to irritants and increased immune sensitisation in the airways. In addition, NO_2 might be carcinogenic (Schedin et al., 1999; Weinberger et al., 2001).

During ischemia–reperfusion injury, NO is thought to have both positive and negative effects (Guix et al., 2005; Moro et al., 2004; Schulz et al., 2004; Thippeswamy et al., 2006). In reconstructive surgery, surgical flaps are used to reconstruct defects after tumour surgery and trauma. Flap necrosis continues to be a clinical problem and its causes are, in spite of extensive research, still not fully understood (Khalil et al., 2006). The role of NO in flap pathophysiology has been postulated to be two-fold. The continuous production of NO by endogenous NOS is thought to be beneficial by promoting blood flow and inhibiting thrombocyte aggregation and leukocyte adhesion (Gribbe et al., 1997). However, large amounts of NO produced by inducible NOS (iNOS) during ischemia are thought to lead to the production of tissue damaging free radicals (Gribbe et al., 1999; Topp et al., 2005).

The aim of the present study was to evaluate a system for local, transdermal NO administration and to investigate its effects on local circulation in experimental skin flaps. An island flap in the rat based on the epigastric vessels, and containing skin and subcutaneous tissue, was positioned on top of a mount of hard plastic. The flap was hereby stabilised making the study of supplying and superficial blood flow convenient. A NO generating system for topical application on the flaps was created by acidifying a NO_2^- containing cream with vitamin C. In addition, the formation of NO and NO_2 was measured in the gas phase.

The epigastric island flap model as well as other flap models in the rat have also been used to study flap survival (Angel, 1993; Antonini et al., 2007; Chafin et al., 1999). In the present study flap survival was not studied. However the study was in part intended as preparatory for future flap survival studies by investigating different concentrations of the NO generating cream.

2. Materials and methods

2.1. Animals

Forty-five male albino rats (Sprague–Dawley) weighing between 350 and 470 g were used in the study. The rats were housed in environmentally controlled rooms with 12 h of light and offered standard rat chow and water ad libitum. Permissions were obtained from the local ethical committee for all animal experiments.

2.2. Anaesthesia and body temperature

A single intra-peritoneal injection of pentobarbital sodium (40 mg/kg) was used as anaesthesia during hair removal and later as induction of anaesthesia before surgery. During surgery, anaesthesia was maintained through a continuous intra-venous infusion of pentobarbital sodium (20 mg/kg/h) in saline, using a cannula in the left superficial jugular vein. The chosen dose created adequate anaesthesia and analgesia while maintaining cardiovascular functions (Yang et al., 1996).

Body temperature was monitored with a rectal probe and maintained at 37.5 °C using a heating pad.

2.3. Hair removal

The rats were anaesthetized as described above one day before surgery and the hair of the left part of the abdomen was removed by first using an electrical shaver and then a hair removing cream (Veet, Reckitt & Coleman, France) for 10 min. The rats were then allowed to recover for 24 h.

2.4. Surgical procedure

The rats were anaesthetized as described above and a 4×6 cm island flap based on the inferior superficial epigastric nerve and vessel pedicle was raised (Finseth and Cutting, 1978). The flap was outlined using a template and the borders cut out using a scalpel. Using a sharp pair of scissors the flap, consisting of skin and subcutaneous fat, was elevated from the underlying tissue. Care was taken to raise each flap in the same way in each animal and in all flaps the lateral branch of the artery in the flap was included (Petry and Wortham, 1984). The epigastric artery and vein were carefully freed under an operating microscope using microsurgical instruments. The nerves innervating the flap were cut in order to mimic the physiology of a free flap. The flap was then positioned on top of a hard plastic mount above the rat's body with the blood vessels reaching the flap through a hole in the hard plastic. The flap hereby continued to maintain its circulation via the freed vessel pedicle.

2.5. Measurement of supplying and superficial blood flow and blood pressure

The supplying blood flow (blood flow in the inferior superficial epigastric artery) was measured using transit-time ultrasound flowmetry (Transonic Systems, Ithaca, NY, USA; 0.5 mm V probe connected to a T206 Animal Research Flowmeter, data being collected at 200 Hz sampling rate).

Superficial flap skin blood flow (capillary blood flow) was measured using laser Doppler perfusion imaging technique (PIM II laser Doppler perfusion imager; Perimed AB, Stockholm, Sweden). The laser Doppler technique generates perfusion units that in previous studies have been shown to be closely correlated with blood flow (Johnsson, 1990).

Blood pressure was measured through a heparinized cannula in the left common carotid artery connected to a pressure

transducer (Statham, Puerto Rico) and the signal was amplified (Grass Instruments, Quincy, MA, USA).

The signals from the ultrasound flowmeter and blood pressure recordings were digitalised in an AD-converter (Perimed 472, Perimed) and subsequently recorded and analysed in a personal computer using the Perisoft software (Perimed).

2.6. Drug administration and treatment groups

A NO generating system was created by mixing 2 ml of cream base (K-Y Jelly), containing increasing concentrations of NO_2^- (0.25%, 0.5%, 1%, 2.5% or 5%; weight/volume), with 2 ml of K-Y Jelly, containing the same increasing concentrations of vitamin C. The NO_2^- and vitamin C creams were applied and mixed on the flap surface to give the final NO_2^- concentrations of 0.125%, 0.25%, 0.5%, 1.25% and 2.5%. Thus, five different treatment groups were designed ($n=7$ for each group). Two control groups were created: one where 2 ml+2 ml of K-Y Jelly were mixed on the flap surface ($n=7$) and one where 2 ml of K-Y Jelly and 2 ml of K-Y Jelly containing 5% vitamin C ($n=3$) were mixed on the flap surface to yield a final concentration of 2.5% vitamin C. In total, seven different study groups were thus created.

2.7. Measurement of NO and NO_2 release

Separate tests were undertaken to measure the release of NO and NO_2 from the cream. This was conducted in vitro and as in the animal experiments, 2 ml of K-Y Jelly containing increasing concentrations of NO_2^- (0.25%, 0.5%, 1% or 2.5%; weight/volume) was mixed with 2 ml of K-Y Jelly containing the same increasing concentrations of vitamin C. Hereby the final NO_2^- concentrations of 0.125%, 0.25%, 0.5% and 1.25% were reached. The creams were mixed on a piece of plastic of the same size as the skin flap template and then immediately placed in an air-tight chamber (volume 6.5 l) with a sample outlet and an air inlet supplied with air free from nitrogen oxides. Gas samples were drawn to a NO/NO_2 analyser (Monitor Labs 9840 Nitrogen Oxides Analyzer; Monitor Labs, Englewood, CO, USA) at a rate of 640 ml/min. The gas concentrations from each specimen of cream were measured for 30 min and for each concentration, 7 fresh specimens of cream were created.

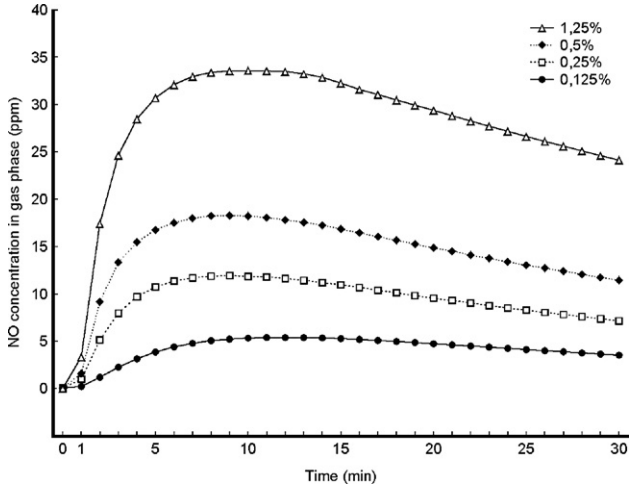

Fig. 1. Dose-dependent increasing NO release from a cream containing increasing concentrations (0.125%, 0.25%, 0.5% and 1.25% weight/volume) of nitrite and vitamin C ($n=7$ for each concentration). NO concentration in part per million (ppm) and release-time in min (significant increase <0.0001 using ANOVA with repeated measures design).

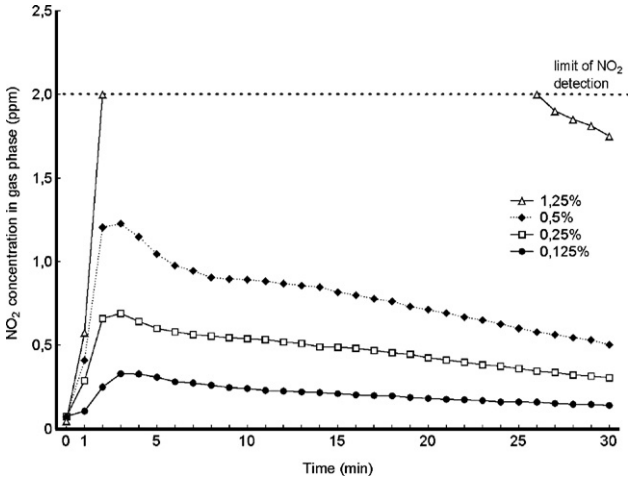

Fig. 2. Dose-dependent increase in NO_2 release from a cream containing increasing concentrations (0.125%, 0.25%, 0.5% and 1.25% weight/volume) of nitrite and vitamin C ($n=7$ for each concentration). NO_2 concentration in part per million (ppm) and release-time in min. The 2.5% concentration caused a NO_2 release exceeding the capacity of the analysis apparatus (significant increase $P<0.0001$ using ANOVA with repeated measures design).

2.8. Drugs and chemicals

The following compounds were used: K-Y Jelly (Johnson & Johnson, Arlington, TX, USA), heparin sulphate (Heparin Leo, Kabi-Vitrum, Stockholm, Sweden), sodium nitrite (Sigma Chemical CO, St. Louis, MO, USA), L-ascorbic acid (Sigma), and pentobarbital sodium (Pentobarbitalnatrium, Apoteksbolaget, Stockholm, Sweden).

2.9. Statistical analysis

The ANOVA with repeated measures design was used to test for differences between independent treatment groups (four to seven groups depending on experimental set-up). The ANOVA was used for testing the outcome of NO, NO_2, blood pressure and supplying and superficial blood flow. The ANOVA analysis also included interaction effects between time and the different independent groups. Post hoc comparison between effects of two different concentrations on superficial blood flow was performed using the Tukey's honest significance difference (HSD) test. All tests were two-sided and $P<0.05$ was regarded as statistically significant.

3. Results

3.1. Gas analysis for NO and NO_2

With increasing concentrations of NO_2^- and vitamin C in the cream, a dose-dependent release of NO and NO_2 to the air above

the cream was seen (Figs. 1 and 2). Both gases displayed rapid formation resulting in a maximum concentration of NO after approximately 8 min and of NO_2 after 3 min. Subsequently, a slow decrease in the formation of both gases was noted. The 0.125% concentration of NO_2^- and vitamin C cream released maximally 5.4 ppm of NO and 0.3 ppm of NO_2, the 0.25% concentration of the cream released maximally 11.9 ppm of NO and 0.6 ppm of NO_2, the 0.5% concentration of the cream released maximally 18.3 ppm of NO and 1.2 ppm of NO_2 whereas the 1.25% concentration of the cream maximally released 33.6 ppm of NO and more than 2 ppm of NO_2. It was not possible to determine the peak concentration of NO_2 from the 1.25% cream since the NO_2 concentration exceeded the maximum analysis capacity of the measuring apparatus, which is maximally 40 ppm NO*x* (the sum of NO and NO_2). However, the NO_2 concentration in the gas released from the 1.25% cream exceeded 2 ppm for more than 20 min (Fig. 2).

Significant increments in NO and NO_2 ($P<0.0001$) were found in all groups.

3.2. Recordings of flap supplying arterial blood flow by transit-time ultrasound flowmetry

In the epigastric artery supplying the flap, a significant increase in blood flow after treatment with the NO generating cream ($P<0.0001$) was measured by transit-time ultrasound flowmetry (Fig. 3). The effect became more marked with increasing concentrations of NO_2^- and vitamin C in the cream. In the control groups with either the cream base K-Y Jelly only or K-Y Jelly with vitamin C, no effect was seen. The 0.125% concentration caused a 70% maximum increase in blood flow and the 0.25% concentration caused a 90% maximum increase in blood flow (both significantly different $P<0.0001$ as compared to controls). The three concentrations 0.5%, 1.25% and 2.5% caused an approximately 130% maximum increase in blood flow (all significantly different $P<0.0001$ as compared to the 0.125% and 0.25% concentrations). There was no statistical evidence for differences between the two control groups, between the treatment concentrations 0.125% and 0.25% or between the three treatment concentrations 0.5%, 1.25% and 2.5%.

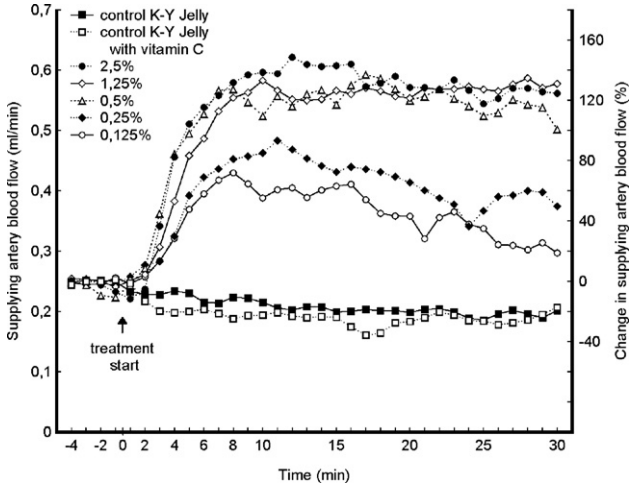

Fig. 3. Blood flow (ml/min and change in %, transit-time ultrasound flowmetry) in the supplying epigastric artery in an skin flap model in the rat during treatment with a NO generating cream containing increasing concentrations (0.125%, 0.25%, 0.5%, 1.25% and 2.5% weight/volume) of nitrite and vitamin C ($n=7$ each concentration). Cream base (K-Y Jelly, $n=7$) or cream base acidified with vitamin C ($n=3$) were used as control groups. Arrow indicates start of 30 min treatment. The treatment caused a significant increase in blood flow ($P<0.0001$ using ANOVA with repeated measures design).

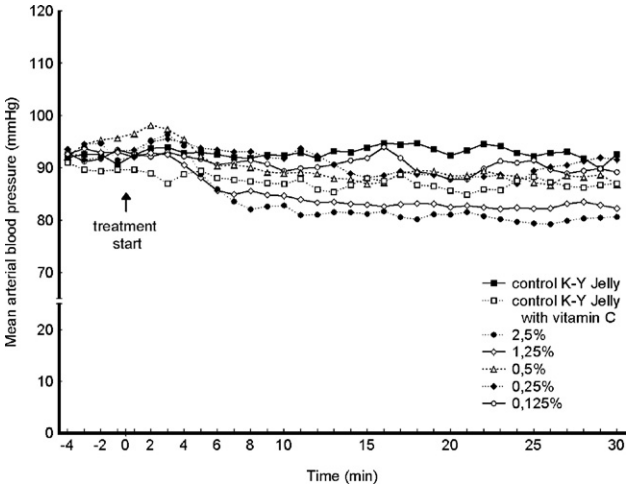

Fig. 4. Systemic mean arterial blood pressure (mm Hg) in rats operated with an epigastric skin flap treated with a NO generating cream containing increasing concentrations (0.125%, 0.25%, 0.5%, 1.25% and 2.5% weight/volume) of nitrite and vitamin C ($n=7$ each concentration). Cream base (K-Y Jelly, $n=7$) or cream base acidified with vitamin C ($n=3$) were used as controls. Arrow indicates start of 30 min treatment. The higher concentrations of the cream (1.25% and 2.5%) caused a significant decrease in blood pressure as compared to controls ($P<0.0001$, ANOVA with repeated measures design).

3.3. Blood pressure recordings

As seen in Fig. 4, the systemic blood pressure was unaffected in controls and the groups treated with the 0.125%, 0.25% and 0.5% concentrations of the NO generating cream. In the groups treated with 1.25% and 2.5% concentrations, a statistically significant decrease in blood pressure was noted ($P<0.0001$) as compared to controls.

3.4. Superficial blood flow by laser Doppler perfusion imaging

Examples of laser Doppler perfusion imaging of the superficial blood flow in the epigastric skin flap are shown in Fig. 5. Fig. 5A shows an image before and Fig. 5B shows an image after topical treatment with the NO releasing cream containing 2.5% NO_2^- and vitamin C. Fig. 6 depicts the changes in superficial blood flow for the five different treatment concentrations and for the two control groups. For each concentration, the relative change in laser Doppler perfusion caused by the treatment was calculated. As explained above, changes in perfusion are closely correlated to changes in blood flow (Johnsson, 1990). During treatment with increasing concentration of the NO generating cream, increasing superficial blood flow was observed ($P=0.0002$). No significant change in

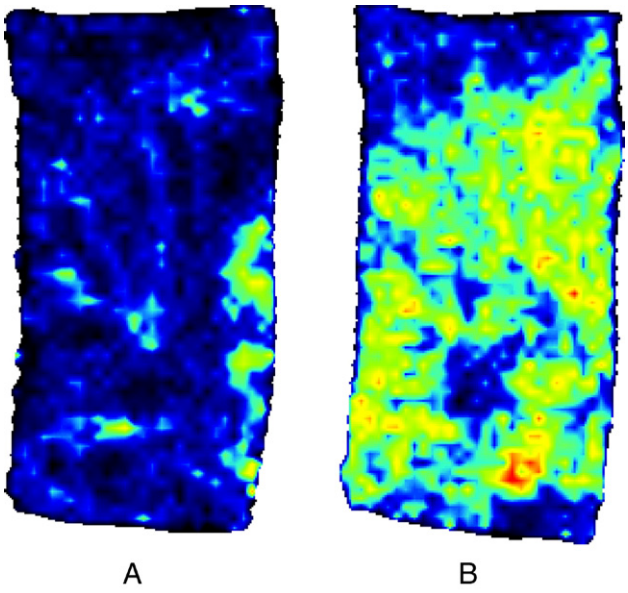

Fig. 5. Laser Doppler perfusion image of an epigastric skin flap in the rat (A) before and (B) after 30 min of treatment with a NO releasing cream containing 2.5% (weight/volume) nitrite and vitamin C. The flap is visualized from the epidermal side. The supplying vessel entered the flap from the subcutaneous side (away from the viewer) in the lower right quadrant where a strong Doppler signal is visible in panel b.

superficial blood flow was seen in controls. When comparing with the control groups no significant change was seen after treatment with the 0.125% or 0.25% concentration of the NO_2^- and vitamin C cream. However, after treatment with the three higher concentrations statistically significant increases in blood flow were demonstrated. The 0.5% concentration caused a 48% increase ($P=0.047$), the 1.25% concentration caused a 77% increase ($P=0.026$) and the 2.5% concentration caused a 119% increase ($P=0.001$). The highest treatment concentration (2.5%) thus led to the highest increase in blood flow.

4. Discussion

This study presents a model in the rat suitable for flap-circulation studies. Furthermore, a non-enzymatic NO delivering system was created and was shown to increase blood flow in flap-tissue.

In order to study the circulation in flap-tissue we have modified the frequently used epigastric island skin flap model in the rat (Petry and Wortham, 1984). In our modification, we placed the flap flat on top of a hard plastic mount just above the rat's body while letting the flap maintain its normal circulation. The flap hereby continues to receive its blood supply from the superficial epigastric artery and continues to be drained by the corresponding vein through a hole in the plastic mount. This set-up provides an immobilised island flap where monitoring of blood flow in the supplying artery and flap surface is possible without the interference of breathing pattern artefacts. Transit-time ultrasound flowmetry was used to study the blood flow in the supplying artery and laser Doppler perfusion imaging was used to study the circulation in the flap surface. Besides the study of flap circulation the model could be used for metabolic studies using microdialysis and possibly also intra-vital microscopy.

As previously described, NO can be formed in a non-enzymatic fashion through the reduction of NO_2^- under acidic conditions (Lundberg and Weitzberg, 2005; Suschek et al., 2006). In the present study a NO generating system was created by mixing NO_2^- and vitamin C (pK_a 4.2) in a cream (Tucker et al., 1999). Formation of NO was confirmed by chemiluminescence and the cream was applied to the skin flap. As NO is very soluble in tissues, it most likely passed through the flap skin, leading to vasodilatation and to the observed increase in blood flow (Tucker et al., 1999). The increase in blood flow was seen both in the supplying epigastric artery, as measured by transit-time ultrasound flowmetry, and in the superficial capillaries, as measured by laser Doppler perfusion imaging. This indicates that blood flow was not only shunted to the surface of the flap but that an increase in total flap blood flow occurred. At the lower concentrations, the NO generating cream caused a dose-dependent increase in supplying blood flow. During treatment with the three highest concentrations (0.5%, 1.25% and 2.5%) however, a plateau was seen. This plateau could be explained by the reduction in blood pressure observed at these higher treatment concentrations, indicating a systemic effect. A decrease in blood pressure most probably counteracts the increase in flap blood flow through a reduced perfusion pressure.

The NO generating cream used in the present study not only produced NO but also NO_2, as measured by chemiluminescence. NO_2 is a noxious gas with acute effects such as pulmonary oedema and pneumonitis, and exerts long term effects of inflammation and increased sensitivity to irritants in the airways (Schedin et al., 1999). In several countries, the workplace

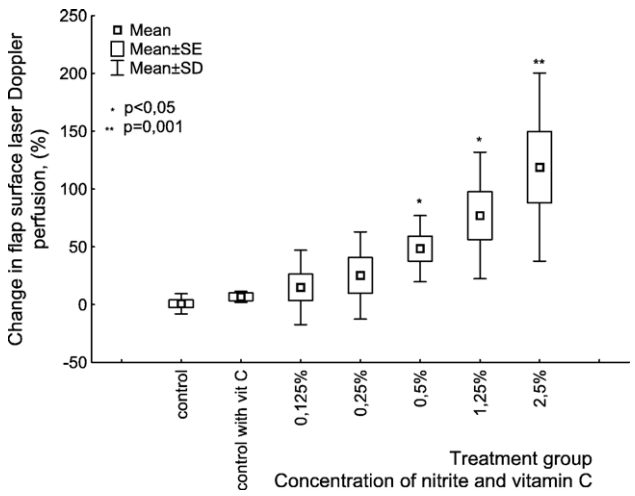

Fig. 6. Change in superficial flap blood flow as measured by laser Doppler perfusion imaging, expressed as the relative change in perfusion units (values post-minus pre-treatment). Treatment for 30 min with a NO generating cream containing increasing concentrations (0.125%, 0.25%, 0.5%, 1.25% and 2.5% weight/volume) of nitrite and vitamin C ($n=7$ each concentration). Cream base (K-Y Jelly, $n=7$) or cream base acidified with vitamin C ($n=3$) were used as controls. Treatment caused a significant increase in blood flow ($P<0.0001$, ANOVA with repeated measures design). Post hoc comparison using Tukey's HSD test showed significant difference between controls and the treatment concentrations 0.5% ($P=0.047$), 1.25% ($P=0.026$) and 2.5% ($P=0.001$).

environmental 8-hour time-weighted limit is 2 ppm (International Occupational Safety and Health Information Centre) and is under reconsideration at least for the EC.

The NO generating cream used here could prove useful in a clinical setting for the salvage of surgical flaps. However, before this becomes possible, further research on and modification of the cream is needed to reduce NO_2 in order to limit airway exposure to patients and hospital staff. After the cream has been modified accordingly its effect on flap survival could be studied.

In summary, we have shown that NO and NO_2 are generated dose-dependently from a cream containing increasing concentrations of NO_2^- and vitamin C and that this cream increases the supplying and superficial blood flow in a new model of the epigastric skin flap in the rat. The results indicate that NO is of importance in flap physiology and possibly also for flap survival.

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