Journal of Plastic Surgery and Hand Surgery

ISSN: 2000-656X (Print) 2000-6764 (Online) Journal homepage: www.tandfonline.com/journals/iphs20

Subdermal nitrous oxide delivery increases skin microcirculation and random flap survival in rats

Merdan Serin, Dincer Altinel, Cem Leblebici, Burcu Biltekin, Onder Huseyinbas, Sevgi Kurt Yazar, Fatih Irmak, Ahmet Sonmez & Mehmet Bayramicli

To cite this article: Merdan Serin, Dincer Altinel, Cem Leblebici, Burcu Biltekin, Onder Huseyinbas, Sevgi Kurt Yazar, Fatih Irmak, Ahmet Sonmez & Mehmet Bayramicli (2019) Subdermal nitrous oxide delivery increases skin microcirculation and random flap survival in rats, Journal of Plastic Surgery and Hand Surgery, 53:1, 37-44, DOI: 10.1080/2000656X.2018.1531013

To link to this article: https://doi.org/10.1080/2000656X.2018.1531013

Published online: 29 Oct 2018.

JOURNAL OF PLASTIC SURGERY AND HAND SURGERY
2019, VOL. 53, NO. 1, 37–44
https://doi.org/10.1080/2000656X.2018.1531013

ARTICLE

Subdermal nitrous oxide delivery increases skin microcirculation and random flap survival in rats

Merdan Serin[a], Dincer Altinel[a], Cem Leblebici[b], Burcu Biltekin[c], Onder Huseyinbas[d], Sevgi Kurt Yazar[a], Fatih Irmak[e], Ahmet Sonmez[f] and Mehmet Bayramicli[g]

[a]Department of Plastic and Reconstructive Surgery, Istanbul Training and Research Hospital, University of Health Sciences, Istanbul, Turkey; [b]Department of Pathology, Istanbul Training and Research Hospital, University of Health Sciences, Istanbul, Turkey; [c]Department of Histology and Embryology, Cerrahpasa Medical School, Istanbul University, Istanbul, Turkey; [d]Animal Research Laboratory, Istanbul Bezmialem University Medical School, Istanbul, Turkey; [e]Department of Plastic and Reconstructive Surgery, Istanbul Sisli Etfal Training and Research Hospital, University of Health Sciences, Istanbul, Turkey; [f]Private practice in Plastic and Reconstructive Surgery, Istanbul, Turkey; [g]Department of Plastic and Reconstructive Surgery, Marmara University School of Medicine, Istanbul, Turkey

ABSTRACT

Random skin flaps are essential tools in reconstructive surgery. In this study, we investigated the effect of subdermal nitrous oxide (N_2O) application on random flap survival. In this experimental study, we used 21 female rats in three groups. In the N_2O and air groups, gases were administrated under the proposed dorsal flap areas daily for seven days. Following the treatment period, flaps were raised and inserted back into their place from the dorsal skin. In the control group, the flaps were elevated and inserted back to their place without any pretreatment. Calculation of necrotic flap areas, histological examination and microangiography was performed to evaluate the results 7 days after the flap surgery. The average of necrotic flap area in the N_2O, air and control group was 13.45%, 37.67% and 46.43%, respectively. (N_2O vs air $p = .044$; N_2O vs control $p = .003$). The average number of capillary formations identified in the histological analysis was 7.0 ± 1.58, 3.75 ± 2.36 and 4.4 ± 0.54 in the N_2O, air and control group, respectively. (N_2O vs air $p = .017$; N_2O vs control $p = .037$). The average number of capillary structures identified in the angiography images were 6.3 ± 1.52, 1.6 ± 1.15 and 1.3 ± 0.57 in the N_2O, air and control group, respectively. (N_2O vs air $p = .04$; N_2O vs control $p = .02$). We conclude that subdermal N_2O application increases random flap survival through an increase in the skin microcirculation and could be promising for future clinical applications.

ARTICLE HISTORY
Received 21 June 2018
Revised 14 September 2018
Accepted 26 September 2018

KEYWORDS
Nitrous oxide; random flap; skin flaps; rat; reconstructive surgery; microcirculation

Introduction

Random flaps are essential tools in reconstructive surgery in cases where axial pattern flaps are not available. Random flaps are usually limited in their design by a 2:1 to 3:1 length to width ratio. Various drugs and techniques have been shown to increase random flap survival [1,2].

Nitrous oxide is an oxide of nitrogen with a chemical formula N_2O. The gas was first discovered by Joseph Priestley back in 1772. It has been used as an anesthetic and analgesic since 1844 [3,4]. It has also been used as an alternative to carbon dioxide for laparoscopic surgery. N_2O has a low abuse potential and very few side effects at lower concentration even during systemic inhalation [5]. In room temperature, N_2O is a non-flammable gas although at high temperatures it becomes a powerful oxidizer.

The exact mechanism of action of N_2O is not entirely understood. Central nervous system effects such as anesthetic, hallucinogenic and euphoria effects are probably due to its effect on NMDA receptors and ion channels [6]. Its effects on GABA receptors could be responsible for anxiolytic effects [7]. The main purpose of the use of nitrous oxide in modern anesthesiology is to obtain analgesia and balanced anesthesia. Its analgesic effect is probably due to its interactions with the endogenous opioids and noradrenergic system [8]. The exact mechanism of how N_2O initiates the release of these endogenous opioid peptides is not known.

N_2O has also been described as mimicking the effects of Nitric oxide (NO) because of its similar structure in the central nervous system, which might be connected to its analgesic and anxiolytic effects. [9]. NO is a potent vasodilator, and its effect on random flaps has been well documented in several studies [10–13]. NO is classified as toxic/hazardous gas, and its subdermal application would not be practical compared with N_2O. It is only approved use is for the primary pulmonary hypertension treatment of neonates. In contrast, N_2O is a relatively safe gas, which is non-flammable and stable. Despite this, there are certain safety hazards of N_2O to be considered. The most obvious danger comes from the fact that it is stored as a compressed liquid gas and as it is a strong oxidizer, it can cause an explosion when in contact with fuels under high temperature or pressure.

Nitrous oxide has been shown to cause cerebral vasodilatation and an increase in the pulmonary capillary blood flow in previous studies [14]. In this study, we hypothesized that possible cutaneous vasodilator and angiogenetic effects of N_2O could increase

CONTACT M. Serin merdanserin@gmail.com Department of Plastic and Reconstructive Surgery, Istanbul Training and Research Hospital, University of Health Sciences, Org. Abdurrahman Nafiz Gurman Cad., Samatya, Fatih 34098 Istanbul, Turkey

skin microcirculation and flap survival similar to NO. The benefits of this effect could potentially be used in the treatment of diseases, which impair skin circulation with vascular, metabolic and traumatic origin. To our knowledge, this is the first study to examine the effect of subdermal nitrous oxide on random flaps.

Materials and methods

Twenty-one female Wistar rats with an average weight of 300 g were used for the study. Ethical comity approval was obtained prior to the experiment (ethical approval date and number: 18 August 2016/177)

Surgical anesthesia was induced with sevoflurane inhalation and was maintained with 90 mg/kg intraperitoneal Ketamine (Ketalar; Pfizer, New York, NY) and 10 mg/kg Xylazine (Rompun 2%; Bayer, Leverkusen, Germany) injection. An electrical razor was used to shave the back skin of the animals. Skin cleaning with povidone–iodine was performed prior to surgery.

The animals were arranged into three groups with seven animals in each:

Group 1: *Nitrous oxide group*. 100% N_2O was administrated under the skin of proposed flap areas, daily with a 22-gauge needle, which was connected to the regulator through a silicone tubing, for 7 days prior to the elevation of the flaps. Two points on the flap midline, one 3 cm from the caudal edge and the other 3 cm from the cranial edge, was marked for injection. Gas pressure valve was regulated to 0.1 bar provide a flow rate at about 5 ml/s to inflate approximately 10 ml volume of gas from each injection point (Figure 1). Gas flow rate was calculated using the flow rate formula taking into account the skin tensile stress and pressure drop from the tubing which was calculated from another formula (Appendix 1).

Group 2: *Air group*. Air was administrated under the proposed flap areas from two injection points in the flap midline, similar to the N_2O group, with a 20 ml syringe, daily for 7 days prior to flap elevation.

Group 3: *Control group*. These animals were kept under the same conditions with the others without any pretreatment.

Flap elevation

At the end of the treatment period caudally based dorsal skin flaps, 9 × 3 cm in dimension, were elevated and inserted back into their place in all of the animals [15,16]. The flaps were sutured with 4.0 polypropylene sutures (Figure 2). After the surgery, the flaps were photographed daily with a digital camera (Sony® Nex-3n, Tokyo, Japan). Flap areas were marked on graph papers and flap area calculation was performed with the VistaMetrix software

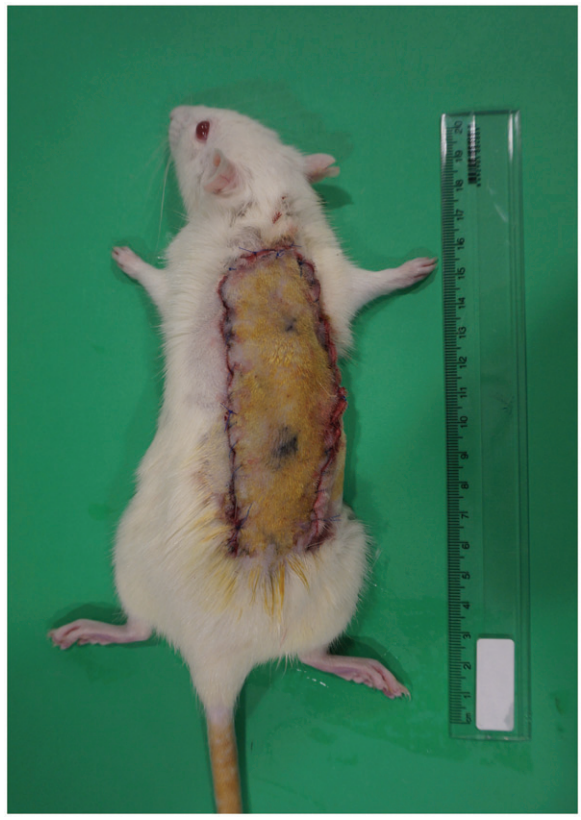

Figure 2. Caudally based dorsal skin flap after flap reinsertion.

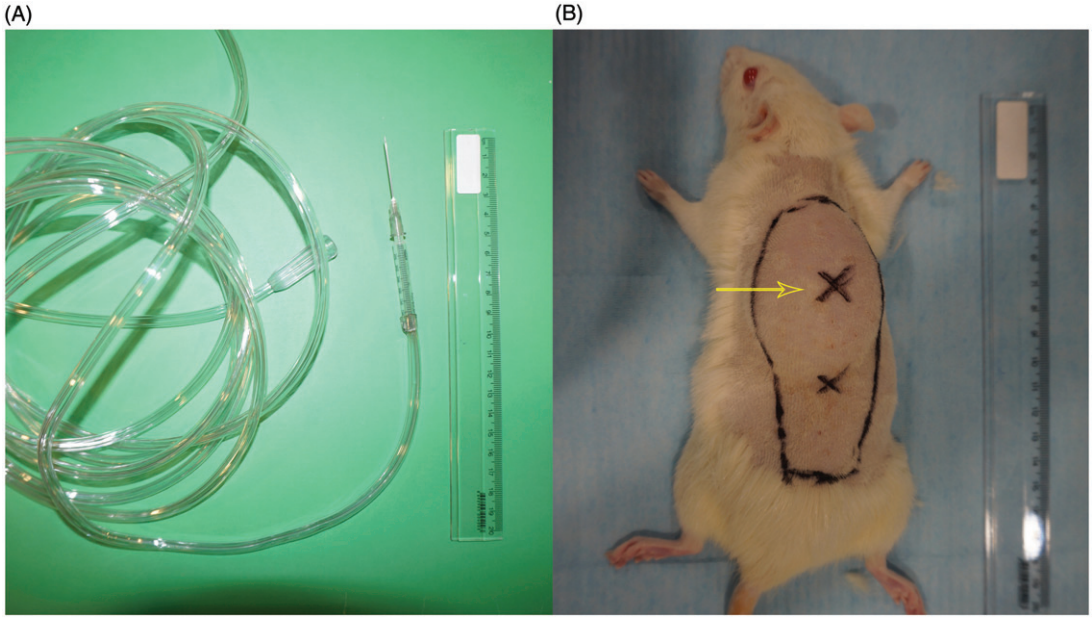

Figure 1. (A) Tubing and needle tip apparatus for the subdermal N_2O application. (B) Gas administration points are marked on the dorsal skin. Marked point with an arrow can be noted as inflated after the administration of the gas.

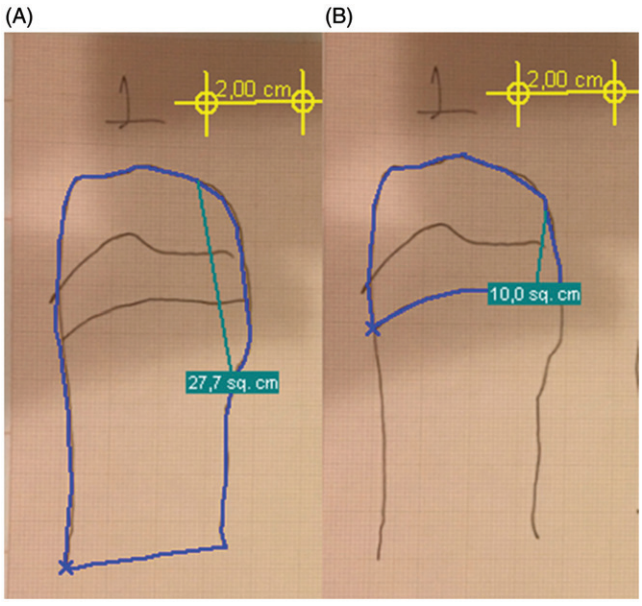

Figure 3. Flap area calculation example from an animal from the air group. (A) Necrotic flap area. (B) Total flap area.

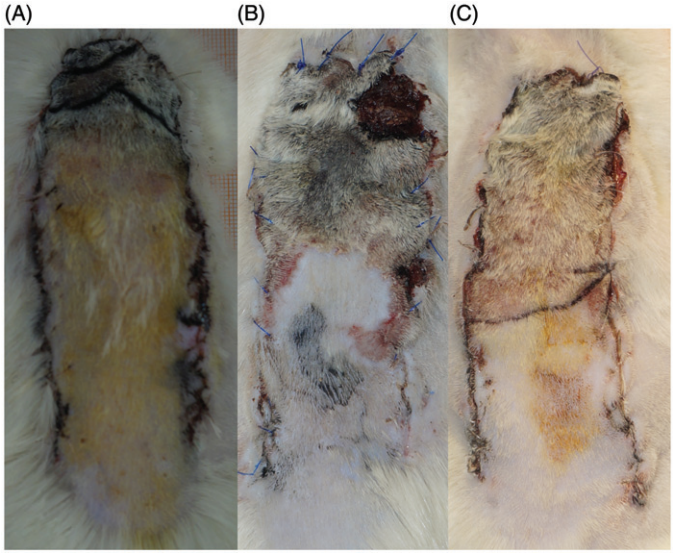

Figure 4. Photographs of flaps at the postoperative seventh day. (A) N_2O group. (B) Air group. (C) Control group.

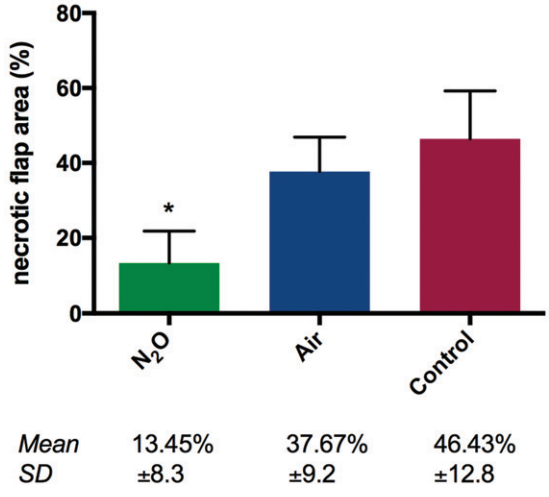

Figure 5. Average of necrotic flap area percentage for each group (*$p < .05$).

image processing software (Version 1.35.0; Skillcrest LLC®,Tucson, AZ, USA) (Figure 3) [17,18].

Microangiographic evaluation

At the end of the first week after the flap elevation, the abdominal cavity of the animals was opened and the aorta and inferior vena cava were exposed. Aorta was cannulated with the 22-gauge cannula and inferior vena cava was cannulated with a 20-gauge cannula. The remaining blood in the vascular system was cleaned with the injection of 300 ml of serum saline solution through both cannulas. 40 ml of %10 barium sulfate and 10 ml of gelatin was added into 100 ml serum saline to form a radiopaque solution. This solution was injected from the aorta cannula and the taps of cannulas were sealed. The body of the animal was wrapped in a plastic bag and was placed in a −18° C freezer for overnight. The next day the flap was separated from the rest of the body and stabled on cartoon board [19–21].

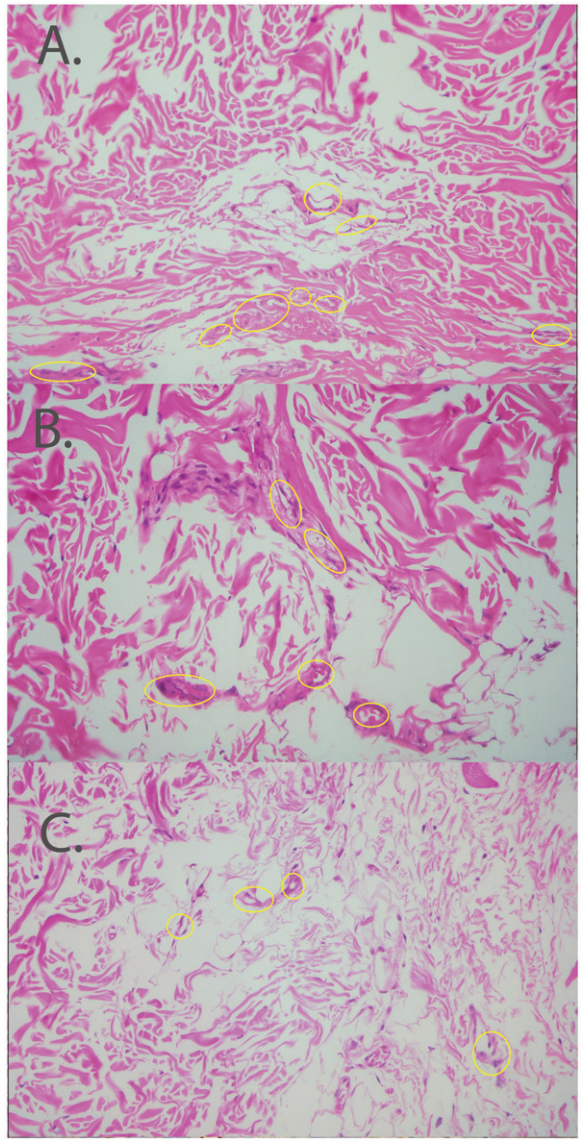

Figure 6. Evaluation of histological images for capillary formations. (A,B) N_2O group. (C) Control group. (Hematoxylin and eosin, A: ×100, B,C: ×200.) Capillary formations are marked with circles.

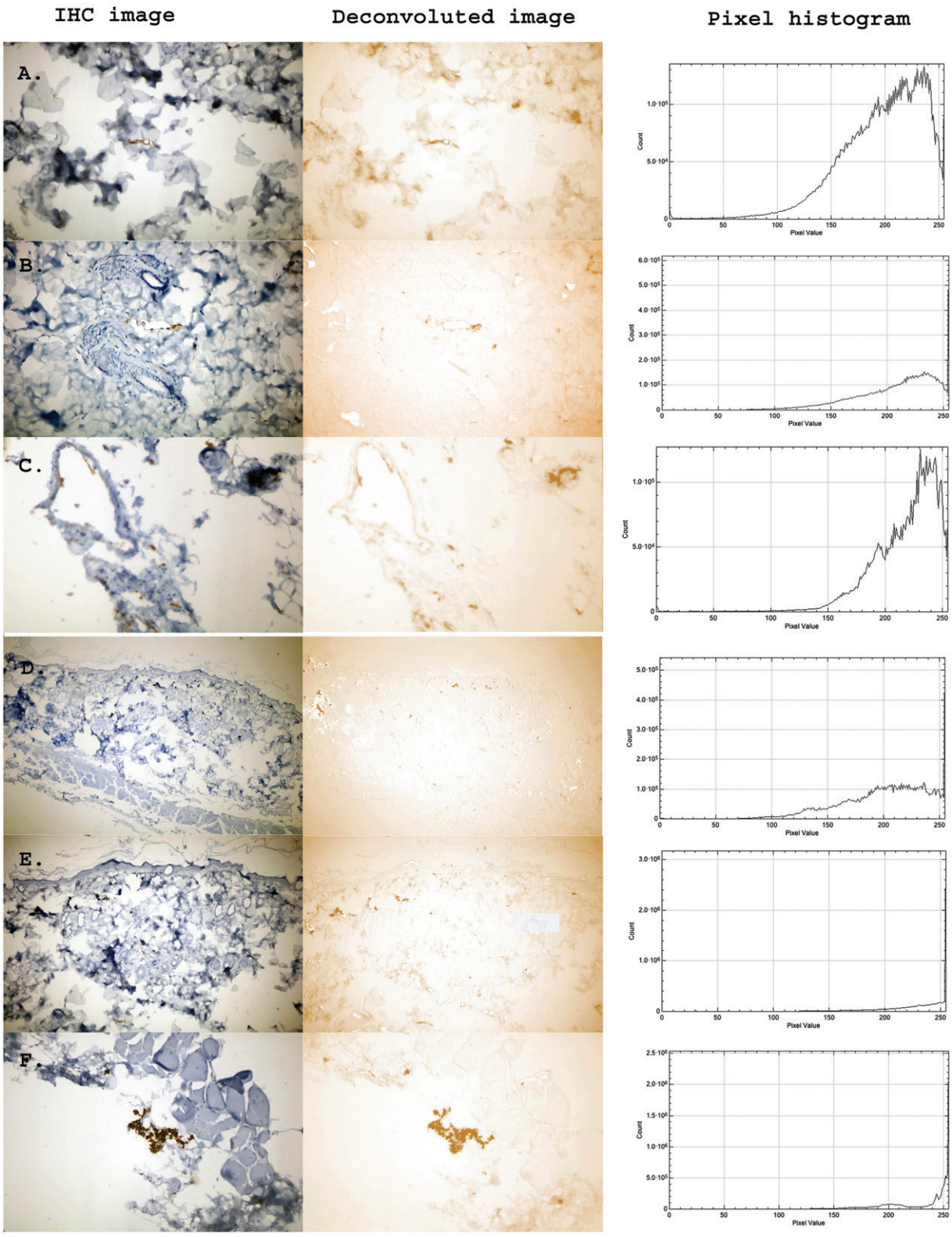

Figure 7. Histological images from IHC staining with CD31. [(A) N_2O group (×400), (B) Air group (×400), (C) Control group (×400)] and CD45 (LCA) [(D) N_2O group (×100) and € N_2O group (×200), (F) Control group (×400)]. Deconvoluted image and histogram are shown for each IHC image.

Histological analysis

Specimens taken from the viable flap areas were fixed in 10% formaldehyde and embedded in paraffin. Paraffin blocks were sectioned and stained with hematoxylin and eosin for routine histopathological observation under light microscopy. Immunohistochemical (IHC) staining with endothelial cells marker CD31 and leukocyte common antigen (LCA) CD45 was performed to better visualize capillary structures and inflammatory cells in selected samples. Image J software (U. S. National Institutes of Health, Bethesda, MD, USA) and IHC profiler plugin were used to

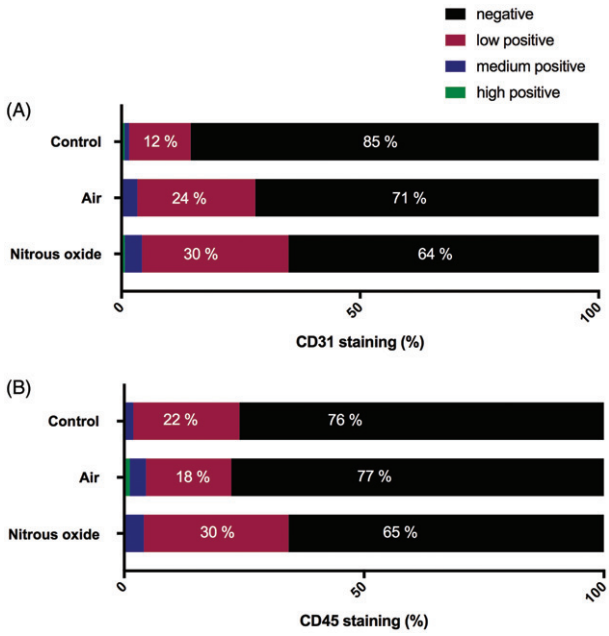

Figure 8. Statistical histograms for IHC staining. (A) CD31 staining. (B) CD45 staining. (Percentage of staining with negative, low, medium and high positive is illustrated in the bar graphs.).

create histograms and statistical analysis from IHC images [22]. The number of capillaries and inflammatory cells in the dermis was counted in three different high-power fields by the same pathologist. The mean of 3 separate counts are presented in the results and was included in the statistical analysis. The pathologist was blinded to the study groups to prevent any bias.

Statistical analysis

Flap necrosis area percentages and histological results in each group were compared using Kruskal–Wallis and Dunn's multiple comparisons test. Prism 7 software (version 7.00 for Windows; GraphPad Software, La Jolla, CA) was used for statistical analysis.

Results

Necrotic flap area calculations

The average of necrotic flap area compared with the whole flap area was 13.45%, 37.67% and 46.43% in the N_2O, air and control group, respectively. The variation between the N_2O and the control group was statistically significant. ($p = .003$). The variation between the N_2O and the air group was also statistically significant ($p = .044$). The variation between air and control groups was insignificant (Figures 4 and 5).

Evaluation of histological images

The average number of cells related to inflammation was very low in all of the groups and no significant variation between the groups was found. The average number of capillary formations were 7.0 ± 1.58, 3.75 ± 2.36 and 4.4 ± 0.54 in the N_2O, air and control group respectively. The variation between the N_2O and the control group was statistically significant. ($p = .037$). The variation between the N_2O and the air group was also statistically significant ($p = .017$). The variation between air and control groups was insignificant (Figures 6–9).

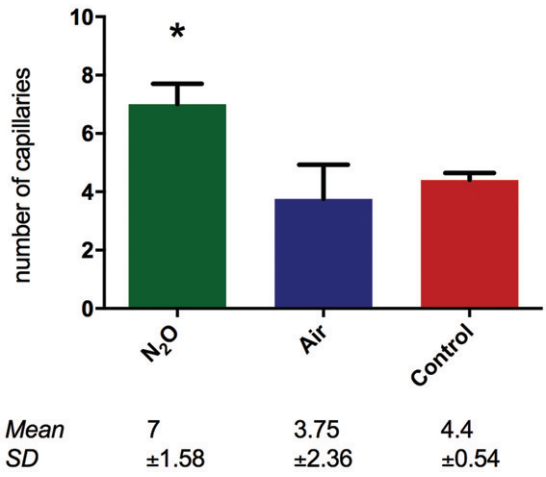

Figure 9. Average of a number of capillary formations counted in the histological analysis for each group. ($^*p < .05$). Left scale represents the exact number of capillaries.

Evaluation of angiography images

Images were evaluated with magnified high-resolution prints. In selected images from each group, vessels were identified. In the N_2O group, vessels were more prominent compared with the air and the control group. The average number of capillary structures identified in the images were 6.3 ± 1.52, 1.6 ± 1.15 and 1.3 ± 0.57 in the N_2O, air and control group, respectively. The variation between the N_2O and the control group was statistically significant ($p = .02$). The variation between the N_2O and the air group was also statistically significant ($p = .04$). The variation between air and control groups was insignificant (Figures 10 and 11).

Discussion

Transdermal and subdermal gas delivery has been investigated by various studies. Some of these studies involve slow and continuous gas delivery methods, whereas others have described faster and periodic gas delivery techniques. Said et al. has shown that continuous transdermal oxygen therapy increased epithelial wound healing in a rabbit ear wound model [23]. The efficacy of transdermal oxygen in pressure ulcers [24] and diabetic wounds [25] has been reported in other studies. Despite this successful result, another study has shown that 6 ml/h continuous transdermal oxygen was not effective for the treatment of sternal wounds compared with additionally inspired oxygen [26]. Carbon dioxide therapy has been previously shown to be useful in the treatment of cellulite. Sonmez et al. have shown that carbon dioxide therapy increased capillary formation in a rat random flap model. Despite this, there was no significant increase in the viable flap area [27].

In a previous study, Dohar et al. have compared the effects of different inhalation anesthetics on flap survival, which revealed a higher rate of flap survival with the combination of isoflurane and N_2O compared with a control group [28]. In another study, Dohar et al. have shown an increased flap survival with isoflurane compared with N_2O [29]. These results could be related to systemic effects caused of these gases. During anesthetic use, N_2O is excreted essentially unchanged from the lung and less than 0.004% is metabolized in humans. Hence, its systemic inhalation effects cannot be directly compared with a local subdermal application. These systemic effects would be far less prominent in the subdermal application.

NO donors [30] and NO synthases [31] have been shown to increase flap survival. In contrast, N_2O antinociception has been

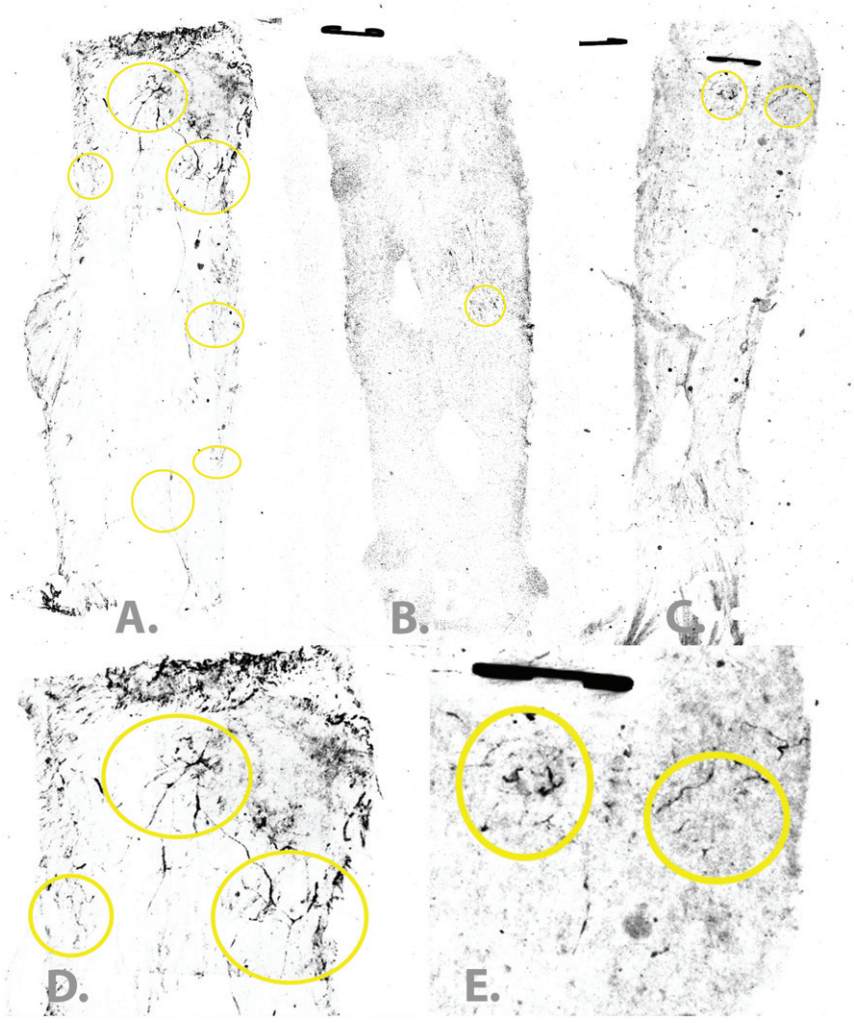

Figure 10. Angiography results from selected samples. (A) N_2O. (B) Air. (C) Control group. (D) Magnification of the N_2O image. (E) Magnification of the control image. Vessel formations are marked with circles.

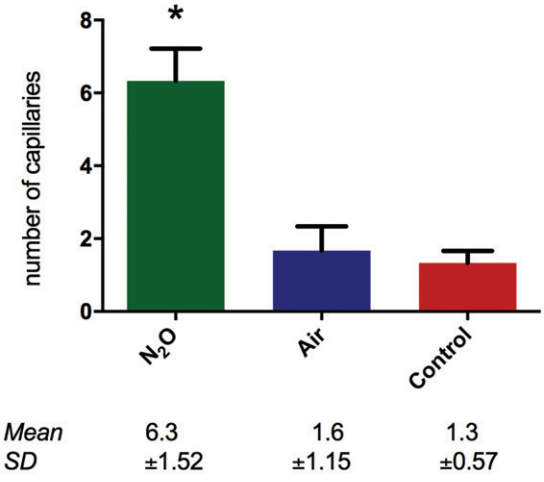

Figure 11. Average of a number of capillary formations counted in the angiography images in each group ($^*p < .05$). Left scale represents the exact number of capillaries.

demonstrated to be reduced with the use of NO synthase (NOS) inhibitors [32,33]. These results reveal the effect of NO in the action of N_2O, which might be due to the effect of NO which induces the release of endogen opiates [34]. However, the exact mechanism of how N_2O initiates this process is unknown. It is suspected that N_2O might be indirectly activating the NOS mechanism in the synaptic nerve terminals. Cerebral vasodilatation effect of N_2O could be related to these mechanisms. In this study, N_2O was shown to increase angiogenesis. It has been shown in previous studies that the NOS mechanism can induce angiogenesis [35,36]. NOS can trigger cell growth and differentiation through the activation of endothelial-NO synthase activation. Our findings support the idea that N_2O activates NOS mechanism.

N_2O a relatively safe gas, though there are several potential safety hazards to be aware of. The most important of these comes from the fact that it is stored as a liquefied compressed gas, which can rarely cause explosive accidents from defective tubes. Side effects have been reported from prolonged exposure to N_2O and should also be noted that there are occupational hazards involving N_2O use in un-ventilated rooms and risk of asphyxiation. There are also environmental hazards of N_2O, which comes from the fact that it accumulates in the stratospheric ozone layer of the atmosphere [37].

This study shows a potential benefit from subdermal nitrous oxygen application for random flap survival. One of the main problems with the injection of gases under the skin, that is, the fact that it has the potential to create a delayed phenomenon. To overcome this problem an "air" control group was used in this study. Although there was a slight increase in the flap survival rate in the air group that supports this idea,

the difference between the air and the control groups was not significant.

The results from the histological studies revealed an increase in the number of capillary formations in the deep dermis tissue in the N_2O group compared with the other groups. There was a lack of inflammatory response in all of the groups. Microangiography results also support the findings related to the increase in the number of capillary formations. More prominent vessel formations were found in the N_2O group images. Despite this, it should be noted that angiography in this study was highly demanding, obtaining clean pictures was difficult.

Conclusions

We conclude that nitrous oxide application can increase the flap microcirculation and thus causing an increase in the flap survival rate. These findings can be translated to other areas concerning microcirculation and tissue perfusions such as wound healing, free flap survival and replantation surgery.

Disclosure statement

No potential conflict of interest was reported by the authors.

References

[1] Hodgson RS, Brummett RE, Cook TA. Effects of pentoxifylline on experimental skin flap survival. Arch Otolaryngol Head Neck Surg. 1987;113:950–952.

[2] Vourtsis SA, Spyriounis PK, Agrogiannis GD, et al. VEGF application on rat skin flap survival. J Invest Surg. 2012;25:14–19.

[3] Maleck WH, Piper SN, Koetter K. Joseph Priestley, nitrous oxide and oxygen. Anaesthesist. 2005;54:395–396; author reply 96.

[4] Smith WD. A history of nitrous oxide and oxygen anaesthesia. I. Joseph Priestley to Humphry Davy. Br J Anaesth. 1965;37:790–798.

[5] Gerhardt RT, King KM, Wiegert RS. Inhaled nitrous oxide versus placebo as an analgesic and anxiolytic adjunct to peripheral intravenous cannulation. Am J Emerg Med. 2001;19:492–494.

[6] Mennerick S, Jevtovic-Todorovic V, Todorovic SM, et al. Effect of nitrous oxide on excitatory and inhibitory synaptic transmission in hippocampal cultures. J Neurosci. 1998;18:9716–9726.

[7] Hapfelmeier G, Zieglgansberger W, Haseneder R, et al. Nitrous oxide and xenon increase the efficacy of GABA at recombinant mammalian GABA(A) receptors. Anesth Analg. 2000;91:1542–1549.

[8] Gillman MA, Lichtigfeld FJ. Pharmacology of psychotropic analgesic nitrous oxide as a multipotent opioid agonist. Int J Neurosci. 1994;76:5–12.

[9] Emmanouil DE, Quock RM. Advances in understanding the actions of nitrous oxide. Anesth Prog. 2007;54:9–18.

[10] Engel H, Sauerbier M, Germann G, et al. Dose-dependent effects of a nitric oxide donor in a rat flap model. Ann Plast Surg. 2007;58:456–460.

[11] Taleb S, Moghaddas P, Rahimi Balaei M, et al. Metformin improves skin flap survival through nitric oxide system. J Surg Res. 2014;192:686–691.

[12] Tao XY, Wang L, Gao WY, et al. The effect of inducible nitric oxide synthase on multiterritory perforator flap survival in rats. J Reconstr Microsurg 2016;32:643–649.

[13] Topp SG, Zhang F, Chatterjee T, et al. Role of nitric oxide in surgical flap survival. J Am Coll Surg. 2005;201:628–639.

[14] Hancock SM, Eastwood JR, Mahajan RP. Effects of inhaled nitrous oxide 50% on estimated cerebral perfusion pressure and zero flow pressure in healthy volunteers. Anaesthesia. 2005;60:129–132.

[15] Hammond DC, Brooksher RD, Mann RJ, et al. The dorsal skin-flap model in the rat: factors influencing survival. Plast Reconstr Surg. 1993;91:316–321.

[16] Hurn IL, Fisher JC, Arganese T, et al. Standardization of the dorsal rat flap model. Ann Plast Surg. 1983;11:210.

[17] Serin M, Bayramicli M. Evaluation of scar penetrating neovascularisation in a rat epigastric flap model. J Plast Surg Hand Surg. 2015;49:295–299.

[18] Serin M, Altinel D, Leblebici C, et al. Preoperative subcutaneous sildenafil injection increases random flap survival in rats. Acta Cir Bras. 2018;33:216–222.

[19] Serin M, Bayramicli M. Evaluation of scar penetrating neovascularisation in a rat epigastric flap model. J Plast Surg Hand Surg. 2015;49:295–299.

[20] Tuncel A, Serin M, Bayramicli M. Venous component in scar penetrating neovascularisation. J Plast Surg Hand Surg. 2018;52:47–52.

[21] Ylmaz KB, Gurunluoglu R, Bayramicli M. Flap survival after previous vascular pedicle division and preexisting scar formation at the pedicle site: an experimental study. Ann Plast Surg. 2014;73:434–440.

[22] Varghese F, Bukhari AB, Malhotra R, et al. IHC Profiler: an open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. PLoS One. 2014;9:e96801.

[23] Said HK, Hijjawi J, Roy N, et al. Transdermal sustained-delivery oxygen improves epithelial healing in a rabbit ear wound model. Arch Surg. 2005;140:998–1004.

[24] Azimian J, Dehghan Nayeri N, Pourkhaleghi E, et al. Transdermal wound oxygen therapy on pressure ulcer healing: a single-blind multi-center randomized controlled trial. Iran Red Crescent Med J. 2015;17:e20211.

[25] Hirsh F, Berlin SJ, Holtz A. Transdermal oxygen delivery to diabetic wounds: a report of 6 cases. Adv Skin Wound Care. 2009;22:20–24.

[26] Bakri MH, Nagem H, Sessler DI, et al. Transdermal oxygen does not improve sternal wound oxygenation in patients recovering from cardiac surgery. Anesth Analg. 2008;106:1619–1626.

[27] Sonmez A, Yaman M, Yalcin O, et al. Carbon dioxide therapy increases capillary formation on random pedicled skin flaps in the rat. J Plast Reconstr Aesthet Surg. 2009;62:e236–e237.

[28] Dohar JE, Goding GS Jr, Maisel RH. The effects of the inhalational anesthetic agent combination, isoflurane-nitrous oxide, on survival in a pig random skin flap model. Arch Otolaryngol Head Neck Surg. 1994;120:74–77.

[29] Dohar JE, Goding GS Jr, Maisel RH. The effects of inhalation anesthetic agents on survival in a pig random skin flap model. Arch Otolaryngol Head Neck Surg. 1992;118:37–40.

[30] Kuntscher MV, Juran S, Menke H, et al. The role of pre-ischaemic application of the nitric oxide donor

spermine/nitric oxide complex in enhancing flap survival in a rat model. Br J Plast Surg. 2002;55:430–433.
[31] Furuta S, Vadiveloo P, Romeo-Meeuw R, et al. Early inducible nitric oxide synthase 2 (NOS 2) activity enhances ischaemic skin flap survival. Angiogenesis. 2004;7:33–43.
[32] Cattano D, Valleggi S, Abramo A, et al. Nitrous oxide discretely up-regulates nNOS and p53 in neonatal rat brain. Minerva Anestesiol. 2010;76:420–424.
[33] Zhang Y, Quock LP, Chung E, et al. Involvement of a NO-cyclic GMP-PKG signaling pathway in nitrous oxide-induced antinociception in mice. Eur J Pharmacol. 2011;654:249–253.
[34] Emmanouil DE, Dickens AS, Heckert RW, et al. Nitrous oxide-antinociception is mediated by opioid receptors and nitric oxide in the periaqueductal gray region of the midbrain. Eur Neuropsychopharmacol. 2008;18:194–199.
[35] Ziche M, Morbidelli L. Nitric oxide and angiogenesis. J Neurooncol. 2000;50:139–148.
[36] Vimalraj S, Bhuvaneswari S, Lakshmikirupa S, et al. Nitric oxide signaling regulates tumor-induced intussusceptive-like angiogenesis. Microvasc Res. 2018;119:47–59.
[37] Ravishankara AR, Daniel JS, Portmann RW. Nitrous oxide (N2O): the dominant ozone-depleting substance emitted in the 21st century. Science. 2009;326:123–125.

Appendix 1. Technical details of gas flow rate calculations.

Following gas flow rate formula was used to calculate the approximate gas flow rate;

$$\mathrm{Q(gas\ flow)} = \frac{1}{60} \times 4.17 \times \mathrm{C} \times \left(\frac{d}{4.654}\right)^2 \times p1 \times \left(1 - \frac{\frac{\Delta p}{p1}}{3 \times \mathrm{F}\gamma \times \mathrm{xT}}\right) \times \sqrt{\frac{\frac{\Delta p}{p1}}{\mathrm{T}}}$$

Q: air flow rate (Nm^3/min)
T: air temperature (°K)
$p1$: primary pressure (= 103.325 kPa absolute)
$p2$: secondary pressure (= 101.325 kPa absolute)
Δp: pressure difference (p1–p2)
C: discharge coefficient (= 0.7)
$F\gamma$: Specific heat ratio factor (= 0.935714286 for N_2O)
xT: Pressure differential ratio factor (= 0.72)
d: opening diameter (22 Gauge needle inner diameter 0.41 mm)

Pressure drop from silicone tubing was calculated with the following formula. These calculations revealed a 0.67 milibar pressure drop from the tubing. Pressure drop from sudden contraction from 5 mm silicone tube to 0.41 mm needle which was found to be significant at 76.75 milibar. This difference has been subtracted from $p1$ pressure and gas flow calculation was performed accordingly.

$$\Delta P\ (\ pressure\ drop) = \frac{\tau \times 4 \times \mathrm{l}}{d}$$

τ: Shear stress of the tubing wall (kg/m^2): Has been calculated from the dynamic viscosity value of N_2O. (= 0.01349 cp)
l: Length of the tube (= 4.65 meters of silicone tube)
d_1: diameter of tube (= 0.005 meters)
d_2: diameter of needle (= 0.00041 meters)
Final flow rate result from the calculations was 0.000310656 Nm^3/min.
(= 5.17 milliliter/second.)