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Perioperative high inspired oxygen fraction therapy reduces surgical site infection with *Pseudomonas aeruginosa* in rats

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Surgical site infection (SSI) remains one of the most important causes of healthcare-associated infections, accounting for ~17 % of all hospital-acquired infections. Although short-term perioperative treatment with high fraction of inspired oxygen (FiO_2) has shown clinical benefits in reducing SSI in colorectal resection surgeries, the true clinical benefits of FiO_2 therapy in reducing SSI remain unclear because randomized controlled trials on this topic have yielded disparate results and inconsistent conclusions. To date, no animal study has been conducted to determine the efficacy of short-term perioperative treatments with high (FiO_2>60 %) versus low (FiO_2<40 %) oxygen in reducing SSI. In this report, we designed a rat model for muscle surgery to compare the effectiveness of short-term perioperative treatments with high (FiO_2=80 %) versus a standard low (FiO_2=30 %) oxygen in reducing SSI with *Pseudomonas aeruginosa* – one of the most prevalent Gram-negative pathogens, responsible for nosocomial SSIs. Our data demonstrate that 5 h perioperative treatment with 80 % FiO_2 is significantly more effective in reducing SSI with *P. aeruginosa* compared to 30 % FiO_2 treatment. We further show that whilst 80 % FiO_2 treatment does not affect neutrophil infiltration into *P. aeruginosa*-infected muscles, neutrophils in the 80 % FiO_2-treated and infected animal group are significantly more activated than neutrophils in the 30 % FiO_2-treated and infected animal group, suggesting that high oxygen perioperative treatment reduces SSI with *P. aeruginosa* by enhancing neutrophil activation in infected wounds.

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INTRODUCTION

Surgical site infection (SSI) remains one of the most common and important healthcare-associated infections, accounting for ~17 % of all hospital-acquired infections (Klevens *et al.*, 2007; Magill *et al.*, 2012). Despite many advances in infection control practices – including improved operating room ventilation, barriers, sterilization methods, improved surgical techniques and administration of appropriate antimicrobial prophylaxis – SSI remains a significant cause of morbidity, prolonged hospitalization and death with a mortality rate of ~3 % (Awad, 2012). According to the Centers for Disease Control and Prevention (CDC), SSIs are estimated to cost between $3.5 billion and $10 billion annually in healthcare expenditures in the USA alone (Scott, 2009). It is not surprising that the US Department of Health and Human Services has identified combatting SSI as a top national priority.

Neutrophils are the first inflammatory leukocytes infiltrating into the wound where they play a crucial role defending wound tissue from invading pathogens (Martin, 1997; Nauseef & Borregaard, 2014). One of the most important mechanisms by which neutrophils destroy invading pathogens is through generation of antimicrobial oxidant species, such as HOCl, which is dependent on the availability of oxygen (Brinkmann *et al.*, 2004; Dovi *et al.*, 2004; Arsalan *et al.*,

Abbreviations: CDC, Centers for Disease Control and Prevention; CFU, colony-forming unit; FiO_2, fraction of inspired oxygen; H&E, haematoxylin and eosin; MPO, myeloperoxidase; SSI, surgical site infection.

2014). *In vitro* studies have demonstrated that neutrophil oxygen consumption and antimicrobial oxidant production are substantially impaired at low oxygen tension, which is often the condition found in wounds, suggesting that reduced oxygen availability may play a pivotal role in attenuating neutrophils' bacterial killing at surgical sites, leading to SSI (Allen *et al.*, 1997; Greif *et al.*, 2000; Anderson, 2011).

High fraction of inspired oxygen (FiO_2=80 %) therapy given perioperatively for 5 h has been shown to be effective in reducing the incidence of SSI after colorectal surgery compared to the standard low 30 % FiO_2 (Greif *et al.*, 2000). However, the true clinical benefits of FiO_2 therapy in reducing SSI remain uncertain because (i) randomized controlled trials on this topic have yielded disparate results with inconsistent conclusions – presumably owing to differences in protocols, surgical sites and/or insufficient power (Chura *et al.*, 2007; Al-Niaimi & Safdar, 2009; Qadan *et al.*, 2009; Brar *et al.*, 2011; Togioka *et al.*, 2012; Hovaguimian *et al.*, 2013) – and (ii) since all surgical patients also receive prophylactic antibiotics, the real efficacy of FiO_2 therapy in reducing SSI remains unknown. Moreover, no study has directly examined the impact of high inspired oxygen therapy on neutrophil influx and/or its activation at infected surgical sites, although it has been postulated that FiO_2 therapy-induced reduction in SSI may be due to increased tissue oxygen tension within surgical wounds – leading to increased oxidative capacity of neutrophils and enhanced neutrophil killing capacity in surgical sites (Knighton *et al.*, 1984, 1986; Allen *et al.*, 1997). Since infection studies cannot be performed in patients, animal modelling could help address these gaps in our understanding of the impact of FiO_2 therapy in reducing SSI.

The beneficial effects of oxygen therapy on SSI control have been demonstrated in animal models in three relatively old studies, which demonstrated that long-term (2–3 days) exposure to moderate oxygen levels (FiO_2=45 %) reduced SSI compared to low oxygen levels (21 % or 12 %) (Hunt *et al.*, 1975; Knighton *et al.*, 1984, 1986). Although these studies have provided strong evidence to support the notion that oxygen therapy may be effective in reducing SSI in animals, it remains unclear whether they truly recapitulate the clinical SSI situations – given that 2–3 day long exposure to FiO_2, used in these animal studies, is neither practical to apply to human patients in clinical settings nor advisable. Moreover, these studies did not examine the impact of high inspired oxygen (FiO_2=80 %) on SSI, to match the landmark clinical study by Greif *et al.* (2000), which demonstrated that 80 % FiO_2 is significantly more effective in reducing SSI than the standard low 30 % FiO_2. Finally, these animal studies also did not provide any insights into possible mechanism(s) responsible for enhanced antimicrobial defences imparted by FiO_2 treatments, although the authors again postulated that elevated oxygen levels may enhance neutrophils' bactericidal activity through the increased production of oxygen free-radical intermediates (Hunt *et al.*, 1975; Knighton *et al.*, 1984, 1986). A recent letter on the benefits and risks of high inspired oxygen stated that 'we can only tentatively conclude that applying high FiO_2 is very likely to reduce SSI', but 'further research is still needed if we are to clarify the specific effects of perioperative high FiO_2' (Belda *et al.*, 2014).

In this report, we examined the effects of a short-term (5 h) 80 % versus 30 % FiO_2 exposure on reducing SSI with *Pseudomonas aeruginosa*, using a rat thigh muscle SSI model that we described previously (Kroin *et al.*, 2015). We further evaluated the impact of 80 % versus 30 % FiO_2 treatments on neutrophil influx and its activation status at surgical sites in response to *P. aeruginosa* infection.

METHODS

Surgical infection model

All experimental procedures in this study were approved by the Animal Care and Use Committee of Rush University Medical Center and conformed to the Guide for the Care and Use of Laboratory Animals (National Research Council). The bacterial species chosen to induce a muscle surgical infection was *P. aeruginosa*, which is the most prevalent Gram-negative pathogen in all wounds, and it represents 25 % of surgical wound infections (Giacometti *et al.*, 2000; Greif *et al.*, 2000). In line with the importance of *P. aeruginosa* infection in SSI, we and others have demonstrated that *P. aeruginosa* uses a variety of virulence mechanisms to inhibit wound healing both *in vivo* and *in vitro* in order to propagate its favourite niche 'the wound' (Garrity-Ryan *et al.*, 2004; Shafikhani & Engel, 2006; Zhao *et al.*, 2010; Goldufsky *et al.*, 2015b; Wood *et al.*, 2015a, b). The strain of *P. aeruginosa* used in this study was PA103, which we and others have described previously (Shafikhani & Engel, 2006; Wood *et al.*, 2013; Goldufsky *et al.*, 2015a, b). Cultures were propagated in tryptic soy broth. The day before surgery and bacterial injection, frozen stock from the initial propagation of bacteria was grown overnight (37 °C incubator). Bacterial titres were determined as colony-forming units (CFU) by serial dilution and plating, as previously described (Shafikhani & Engel, 2006; Shafikhani *et al.*, 2008), to provide a 2.5×10^7 CFU ml^{-1} concentration (based initially on OD and confirmed by serial dilution and plating) on the morning of surgery.

To induce infection, male Sprague–Dawley rats (300 g, Sasco; Charles River Laboratories) were anaesthetized with 1.5 % isoflurane in oxygen provided via a nose cone. Surgery was performed with sterile instruments, sterile surgical gloves and aseptic techniques as follows (Kroin *et al.*, 2015). The left thigh was shaved and disinfected with alcohol swabs (three times), followed by a topical antiseptic solution (chlorhexidine gluconate 4 %) applied to the skin. A 2 cm long skin incision was made with a #15 scalpel blade to expose the biceps femoris muscle. The skin margins were retracted, and a 7–0 polypropylene suture was placed on the surface of the muscle for later identification of the injection site. Using a 25G needle and 1 ml syringe, 5×10^6 CFU of PA103 *P. aeruginosa* was slowly injected into the biceps femoris muscle in 0.2 ml volume (Lin *et al.*, 2005) adjacent to the 7–0 suture. A plastic tubing sheath over most of the needle limited the injection depth to 2 mm. At the end of surgery, all skin margins were closed with 4–0 nylon sutures. The topical antiseptic solution was again applied to the skin, and the animal recovered from anaesthesia. In the experiments to determine muscle neutrophil activity, control mock rats were injected with 0.2 ml sterile saline (no bacteria).

Oxygen exposure

Immediately after surgery, animals were placed in a closed clear plastic chamber (four rats per chamber) with a ventilated lid and a high-flow oxygen–air mixture (Knighton *et al.*, 1984, 1986), with either high FiO_2 (80 % oxygen) or standard FiO_2 (30 % oxygen) for 5 h. The 5 h perioperative exposure time was based on the study by Greif *et al.* (2000), in which patients received perioperative exposure to a total of 5 h of FiO_2=80 % or 30 % oxygen. Oxygen levels were measured with a Datex Capnomac Ultima gas monitor. The volume of the chamber was 20 l, and the flow of the oxygen–air mixture was 7 l min^{-1} so that the gases turned over every 3 min, assuring that there were normal carbon dioxide and water vapor levels (Knighton *et al.*, 1984, 1986). Preliminary experiments verified that the body temperature of four control rats maintained in the chamber at 80 % or 30 % FiO_2 was normal over the 5 h. At the end of the 5 h inspired oxygen exposure, rats were returned to the vivarium and normal room air (21 % oxygen).

Postoperative outcome measures

Bacterial muscle burden determination. At 24 h after surgery and *P. aeruginosa* muscle injection, rats were euthanized with carbon dioxide in a closed chamber. Under aseptic conditions, the left biceps femoris muscle was exposed, and a section was selected around the previously implanted 7–0 suture. A 7×7 mm area of infected muscle, 4 mm thick, was then removed from the body and used for determination of bacterial loads as follows (Kroin *et al.*, 2015). The muscle was weighed (typical value=0.20 g), minced with a razor blade and homogenized (PowerGen 125; Fisher Scientific; 7 mm probe, at full speed for 10 s three times) in 1 ml of sterile PBS. The bacterial loads were determined by serial dilution and plating, as previously described (Shafikhani & Engel, 2006; Shafikhani *et al.*, 2008). Briefly, the homogenized muscle mixture underwent 10-fold serial dilution in PBS to produce 10^{-1}–10^{-5} dilutions in 1 ml volume, plus an undiluted sample 10^0; 100 µl aliquots of each muscle solution (10^{-5}–10^0) were plated on tryptic soy agar plates and incubated for 24 h at 37 °C. The next day, muscle tissue bacterial counts (CFU) were determined from plates with 30–300 colonies. The final bacterial burden is expressed as CFU per tissue wet weight.

Neutrophil count determination and activity assessment. Haematoxylin and eosin (H&E) histological analysis was performed as described (Wood *et al.*, 2014; Goldufsky *et al.*, 2015b). Briefly, at 24 h after surgery and *P. aeruginosa* or saline muscle injection, rats were euthanized, and the biceps femoris muscle was removed as described (Kroin *et al.*, 2015). The muscle was placed on a glass plate over ice and bisected at the injection site (previously implanted suture). One piece of muscle per rat was placed in a tissue cassette and dropped into 10 % buffered formalin for H&E histology. The other piece of muscle per rat (0.10 g) was minced with a razor blade and homogenized (PowerGen 125; Fisher Sci; 7 mm probe, at full speed for 5 s five times) in 1 ml ice-cold lysis buffer [PBS with 0.2 % Triton X-100, plus protease inhibitor cocktail (COMPLETE Mini; Roche)]. The homogenate was centrifuged (5000 ***g***, 10 min, 4 °C), and the supernatant was frozen at −80 °C for later myeloperoxidase (MPO) Western blot analysis. After Western blotting, MPO levels were determined by densitometer using Image J and normalized to GAPDH loading control, as we described (Wood *et al.*, 2015a, b).

For H&E histological studies, muscle tissues were harvested by resecting the injected area of muscle before cross-sectioning the muscle at the injection site. Once collected, muscle samples were fixed in 10 % formalin for 48 h and embedded in paraffin. Muscle sections were transversely cut into 6 µm thick sections from the edge of the injection site and stained with H&E, and slides were visualized on a Nikon Eclipse Ti microscope using NIS-Elements AR software.

Statistical analysis

Difference in bacterial muscle burden between the 80 % FiO_2 and 30 % FiO_2 rats after surgery and bacteria muscle injection was compared with the two-tailed Student *t*-test. Differences in MPO activity from Western blot data were compared amongst four groups with ANOVA and the LSD post hoc test.

RESULTS

Treatment with 80 % FiO_2 is significantly more effective in reducing SSI with *P. aeruginosa* than 30 % FiO_2

In order to evaluate the impact of high (80 %) and low/standard (30 %) FiO_2 therapies on SSI, we injected either saline (mock) or 5×10^6 CFU of PA103 bacteria – [a wild-type *P. aeruginosa* strain described previously (Ohman *et al.*, 1980; Wood *et al.*, 2013; Goldufsky *et al.*, 2015a, b)] – into biceps femoris muscle, using a rat muscle surgical model for infection that we described previously (Kroin *et al.*, 2015) (for more detailed protocol, see Methods). Immediately after surgery and infection, animals were exposed to either high 80 % FiO_2 or 30 % low (standard) FiO_2 for 5 h, as described in Methods. The 5 h oxygen exposure time was chosen to match the landmark study by Greif *et al.* (2000), which demonstrated that 5 h perioperative exposure to 80 % FiO_2 was more effective in reducing SSI in patients than the 30 % FiO_2. *P. aeruginosa* was chosen because of its clinical

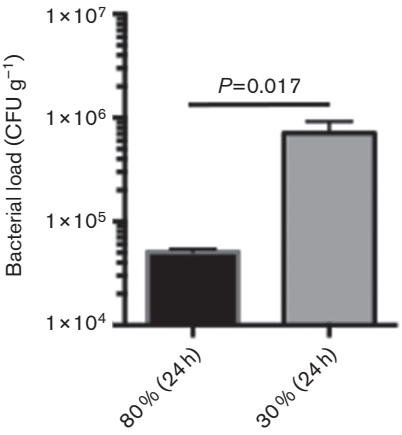

Fig. 1. Treatment with 80 % FiO_2 is significantly more effective in reducing SSI with *P. aeruginosa* than 30 % FiO_2. After surgery, 5×10^6 bacteria were injected into biceps femoris muscles. Infected rats then received either 80 % or 30 % FiO_2 for 5 h. After FiO_2 treatments, rats were placed in normoxia conditions (21 % O_2). Twenty-four hours later, infected muscles were removed, and their bacterial load was determined by serial dilution and CFU counts. Data are presented as mean±SEM (P=0.017; n=8 rats/group, Student *t*-test).

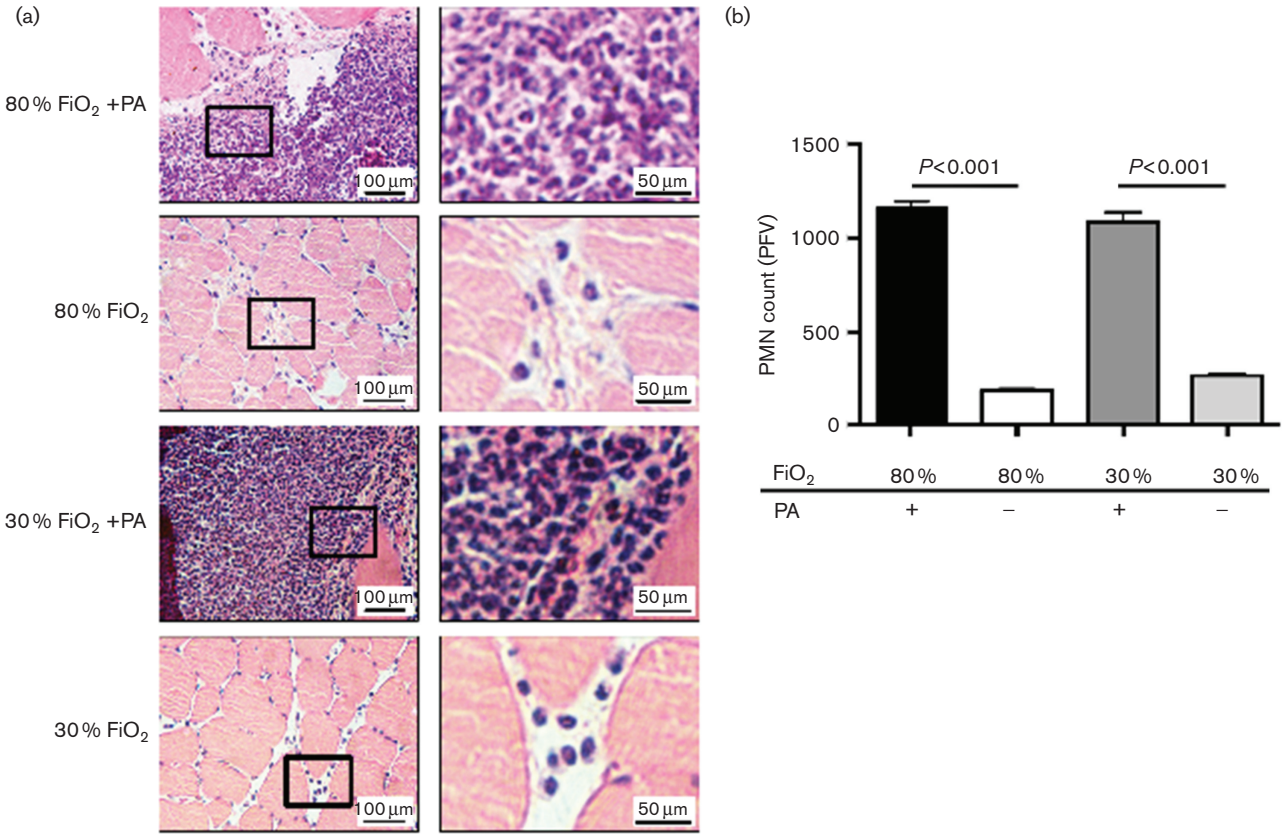

Fig. 2. Treatment with 80 % FiO_2 does not affect neutrophil migration into infected muscle. Rats treated with 80 % or 30 % FiO_2 were either injected with saline or infected with 5×10^6 *P. aeruginosa* (PA) into biceps femoris muscle after surgery. (a) Twenty-four hours later, muscle sections were fixed and stained with H&E. Enlarged areas of the section are indicated by a box inset. (b) The corresponding tabulated number of polymorphoneuculear (PMN) cells per field of view is shown as mean ±SEM. *P*-values are indicated (n=4 rats/group, 6 random fields/rat, ANOVA with the LSD post hoc test).

importance to all wound infections including SSI. *P. aeruginosa* accounts for ~25 % of SSIs, and its presence in wound correlates with a poor prognosis for healing (Halbert *et al.*, 1992; Madsen *et al.*, 1996; Giacometti *et al.*, 2000; Winstanley *et al.*, 2005; Gjødsbøl *et al.*, 2006; Ramakant *et al.*, 2011; Malik *et al.*, 2013).

Twenty-four hours after surgery and infection, infected muscles were then harvested, and the level of infections in the muscles was determined by determining the *P. aeruginosa* bacterial CFU counts per gram of infected muscles, as we described (Goldufsky *et al.*, 2015b; Kroin *et al.*, 2015). Our data indicated that treatment with 80 % FiO_2 was more effective than 30 % FIO_2 in reducing SSI in muscle by ~1.4 log order (Fig. 1) (mean CFU count for 80 % FiO_2=$5.1\times10^4\pm3.4\times10^3$; mean CFU count for 30 % FiO_2=$7.1\times10^5\pm2.1\times10^5$; P=0.017; n=8 animals/group). These results confirmed the clinical findings which demonstrated the more beneficial effect of 80 % FiO_2 in reducing SSI in comparison to 30 % FiO_2 (Greif *et al.*, 2000).

Treatment with 80 % FiO_2 does not affect neutrophil influx into infected muscle but increases neutrophil activation in *P. aeruginosa*-infected muscle

Given that neutrophils (a.k.a. PMNs) are the primary leukocytes in innate immune defences against *P. aeruginosa* and, without their function, tissues are completely vulnerable to *P. aeruginosa* infection (Tsai *et al.*, 2000), we sought to evaluate the impact of 80 % and 30 % FiO_2 treatments on the influx of neutrophils at surgical sites in the presence and absence of *P. aeruginosa* SSI. To this end, we harvested muscle tissues 24 h after saline treatment or *P. aeruginosa* infection and analysed their neutrophil contents by H&E staining, as we described (Wood *et al.*, 2014) (Methods). As expected, *P. aeruginosa* infection significantly increased neutrophil infiltration in the infected muscles in both 80 % and 30 % FiO_2-treated animals, compared to their uninfected counterpart animal groups (Fig. 2; P<0.001; n=4 animals/group, 10 random fields/animal). However, there

were no differences in the neutrophil levels between the 80 % and 30 % FiO_2-treated animal groups regardless of whether they were infected with *P. aeruginosa* or treated with PBS.

We wondered if increased antimicrobial defences against *P. aeruginosa* in the 80 % FiO_2-treated animal group, compared to 30 % FiO_2-treated animal group (Fig. 1), may be due to enhanced neutrophil activation in these infected muscles. The heme enzyme MPO is a marker for activated neutrophils, and this enzyme is required for the production of antimicrobial oxidants in activated neutrophils (Klebanoff *et al.*, 2013; Björnsdottir *et al.*, 2015; Winterbourn *et al.*, 2016). Twenty-four hours after surgery and *P. aeruginosa* infection, we evaluated the MPO protein levels of infected muscles or saline-treated uninfected muscles in animals treated with either 80 % or 30 % FiO_2 by Western immunobloting. A representative Western blot gel is shown in Fig. 3(a), and the MPO levels, as determined by densitometer and normalized to GAPDH loading control, are shown in Fig. 3(b). As shown in Fig. 3, there was no difference in MPO levels between 80 % FiO_2+saline (2.23±0.46) and 30 % FiO_2+saline (2.11±0.60), indicating that additional oxygen exposure in the 80 % FiO_2 group does not result in increased neutrophil activation in these animals in the absence of infection (P=0.112; n=6 rats/group). In contrast, MPO levels were significantly higher in the 80 % FiO_2+*P. aeruginosa* (8.36±0.50) as compared to the 30 FiO_2+*P. aeruginosa* (5.90±0.15) (P=0.001; n=6 rats/group), indicating that additional oxygen exposure results in enhanced neutrophil activation in infected wounds. Of note, the MPO levels in *Pseudomonas*-infected wounds in both the 80 % and the 30 % FiO_2-treated animals were substantially higher than their uninfected saline-treated counterparts (P<0.001).

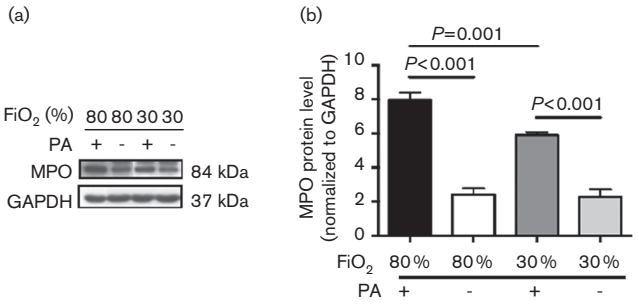

Fig. 3. 80 % FiO_2 increases neutrophil activation in *P. aeruginosa*-infected muscle. Rats treated with 80 % or 30 % FiO_2 were either injected with saline or infected with 5×10^6 *P. aeruginosa* (PA) into biceps femoris muscle after surgery. (a) Muscle tissue lysates were collected from the different treatment groups 24 h following surgery and probed for myeloperoxidase (MPO) by Western blotting. The experiment was performed in duplicate and repeated over four trials. (b) For each sample measured, the MPO levels were normalized to GAPDH. Data are shown as mean±SEM, ANOVA with the LSD post hoc test.

DISCUSSION

Despite many advances in infection control practices, SSIs are common complications with potentially devastating morbidity and mortality rates (Scott, 2009; Awad, 2012). A landmark clinical study, involving 500 patients undergoing colorectal resection, demonstrated that 5 h perioperative treatment with 80 % (high) FiO_2 was significantly more effective in reducing SSI than the standard low 30 % FiO_2 (Greif *et al.*, 2000). These findings were subsequently confirmed by another clinical study involving 300 patients undergoing colorectal resection, which again showed that patients receiving perioperative 80 % FiO_2 had a significant reduction in the risk of wound infection when compared to the 30 % FiO_2 group (Belda *et al.*, 2005). However, since all patients also had received prophylactic antibiotic therapy, there remained the possibility that the beneficial impact of high FiO_2 on SSI reduction may be indirect through enhancement of the prophylactic antibiotic activity.

We designed a rat muscle surgical site infection model to address whether 80 % high FiO_2 therapy is also more effective in reducing SSI than 30 % standard FiO_2 therapy in the absence of prophylactic antibiotic treatment. Our data support the clinical findings by Greif *et al.* (2000) and demonstrate that 5 h perioperative treatment with 80 % FiO_2 is also significantly more effective than 30 % FiO_2 in reducing SSI with *P. aeruginosa*, even in the absence of prophylactic antibiotic treatment (Fig. 1). It would be interesting to use this rat model to study the effect of antibiotic treatment, with and without 80 % perioperative FiO_2 on SSI.

FiO_2 treatment at normobaric conditions is reported to have only modest effect on PO_2 in the blood (Sjoberg & Singer, 2013). In contrast, FiO_2 treatment has been shown to result in significant increases in tissue PO_2 and shown to be predictive of the risk of wound infection in surgical patients (Hopf *et al.*, 1997). These findings have led to the general acceptance in the field that the beneficial impact of FiO_2 therapy on SSI reduction is due to its enhancement of neutrophil oxidative killing (Hunt *et al.*, 1975; Knighton *et al.*, 1984, 1986). To date, however, there are no published data to support this hypothesis. To our knowledge, for the first time, we provide strong evidence in support of this hypothesis. Our data demonstrate that high FiO_2 perioperative treatment results in substantial increases in MPO levels *in vivo* (Fig. 3), without affecting the neutrophil influx into infected surgical site (Fig. 2). MPO is a critical enzyme that is required for the production of reactive antimicrobial oxidants in neutrophils (Klebanoff *et al.*, 2013; Björnsdottir *et al.*, 2015; Winterbourn *et al.*, 2016).

How safe is it to use high FiO_2 treatement in patients? Although future clinical studies are needed to evaluate all potential adverse impacts of high FiO_2 therapy in patients, there are encouraging signs to suggest that this therapeutic approach may be safe in patients. A recent meta-analysis of randomized controlled trials found no evidence of atelectasis (lung collapse) or any other detrimental effect on postoperative gas exchange with high

(80–100 %) FiO_2 treatments (Hovaguimian *et al.*, 2013). In fact, high FiO_2 therapy has been shown to reduce the incidence of postoperative nausea and vomiting (Greif *et al.*, 1999; Hovaguimian *et al.*, 2013). Our data also support the notion that 80 % FiO_2 therapy may be safe. We found that enhanced activation of neutrophils at surgical site in response to 80 % FiO_2 only occured in infected muscles and not when the muscle wound was sterile (Fig. 3), indicating that 80 % FiO_2 therapy only primes neutrophils to exhibit heightened response to infection and in itself is insufficient to activate neutrophils, which could have undesirable consequences.

In summary, our data suggest that short-term perioperative treatment with 80 % FiO_2 should be adopted in clinical settings, as it may be more beneficial in reducing SSI than low 30 % FiO_2.

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