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 ($\text{FiO}_2$) has shown clinical benefits in reducing SSI in colorectal resection surgeries, the true clinical benefits of $\text{FiO}_2$ 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 ($\text{FiO}_2 > 60\%$) versus low ($\text{FiO}_2 < 40\%$) 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 ($\text{FiO}_2 = 80\%$) versus a standard low ($\text{FiO}_2 = 30\%$) oxygen in reducing SSI with *P. aeruginosa* – one of the most prevalent Gram-negative pathogens, responsible for nosocomial SSIs. Our data demonstrate that 5 h perioperative treatment with 80% $\text{FiO}_2$ is significantly more effective in reducing SSI with *P. aeruginosa* compared to 30% $\text{FiO}_2$ treatment. We further show that whilst 80% $\text{FiO}_2$ treatment does not affect neutrophil infiltration into *P. aeruginosa*-infected muscles, neutrophils in the 80% $\text{FiO}_2$-treated and infected animal group are significantly more activated than neutrophils in the 30% $\text{FiO}_2$-treated and infected animal group, suggesting that high oxygen perioperative treatment reduces SSI with *P. aeruginosa* by enhancing neutrophil activation in infected wounds.

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.,...
High fraction of inspired oxygen (FiO\textsubscript{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\textsubscript{2} (Greif et al., 2000). However, the true clinical benefits of FiO\textsubscript{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; Brax 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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{2}=80\%) on SSI, to match the landmark clinical study by Greif et al. (2000), which demonstrated that 80\% FiO\textsubscript{2} is significantly more effective in reducing SSI than the standard low 30\% FiO\textsubscript{2}. Finally, these animal studies also did not provide any insights into possible mechanism(s) responsible for enhanced antimicrobial defences imparted by FiO\textsubscript{2} treatments, although the authors again postulated that elevated oxygen levels may enhance neutrophils’ bacterialid 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\textsubscript{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\textsubscript{2}’ (Belda et al., 2014).

In this report, we examined the effects of a short-term (5 h) 80\% versus 30\% FiO\textsubscript{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\textsubscript{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 x 10\textsuperscript{7} CFU ml\textsuperscript{−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 anesthetized 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 x 10\textsuperscript{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 FiO₂ (80% oxygen) or standard FiO₂ (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 FiO₂=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 71 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% FiO₂ 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 10⁻¹–10⁻⁴ dilutions in 1 ml volume, plus an undiluted sample 10⁻¹. 100 μl aliquots of each muscle solution (10⁻¹–10⁻⁴) 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. Hematoxylin 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 densitometry 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% FiO₂ and 30% FiO₂ 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% FiO₂ is significantly more effective in reducing SSI with P. aeruginosa than 30% FiO₂
In order to evaluate the impact of high (80%) and low/standard (30%) FiO₂ therapies on SSI, we injected either saline (mock) or 5×10⁵ 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% FiO₂ or 30% low (standard) FiO₂ 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% FiO₂ was more effective in reducing SSI in patients than the 30% FiO₂. P. aeruginosa was chosen because of its clinical

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Fig. 1. Treatment with 80% FiO₂ is significantly more effective in reducing SSI with P. aeruginosa than 30% FiO₂. After surgery, 5×10⁵ bacteria were injected into biceps femoris muscles. Infected rats then received either 80% or 30% FiO₂ for 5 h. After FiO₂ treatments, rats were placed in normoxia conditions (21% O₂). 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).
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% FiO₂ was more effective than 30% FiO₂ in reducing SSI in muscle by ~1.4 log order (Fig. 1) (mean CFU count for 80% FiO₂ = 5.1 × 10⁴ ± 3.4 × 10³; mean CFU count for 30% FiO₂ = 7.1 × 10⁵ ± 2.1 × 10⁵; P = 0.017; n = 8 animals/group). These results confirmed the clinical findings which demonstrated the more beneficial effect of 80% FiO₂ in reducing SSI in comparison to 30% FiO₂ (Greif et al., 2000).

**Fig. 2.** Treatment with 80% FiO₂ does not affect neutrophil migration into infected muscle. Rats treated with 80% or 30% FiO₂ were either injected with saline or infected with 5 × 10⁶ *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 polymorphonuclear (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).

**Treatment with 80% FiO₂ 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% FiO₂ 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% FiO₂-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<sub>2</sub>-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<sub>2</sub>-treated animal group, compared to 30% FiO<sub>2</sub>-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<sub>2</sub> by Western immunoblotting. A representative Western blot gel is shown in Fig. 3(a), and the MPO levels, as determined by densitometer and normalized to GAPDH, are shown in Fig. 3(b). As shown in Fig. 3, there was no difference in MPO levels between 80% FiO<sub>2</sub>+saline (2.23±0.46) and 30% FiO<sub>2</sub>+saline (2.11±0.60), indicating that additional oxygen exposure in the 80% FiO<sub>2</sub> 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<sub>2</sub> group (8.36±0.50) as compared to the 30% FiO<sub>2</sub> group (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<sub>2</sub>-treated animals were substantially higher than their uninfected saline-treated counterparts (P<0.001).

**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 5h perioperative treatment with 80% (high) FiO<sub>2</sub> was significantly more effective in reducing SSI than the standard low 30% FiO<sub>2</sub> (Geirf 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<sub>2</sub> had a significant reduction in the risk of wound infection when compared to the 30% FiO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> therapy is also more effective in reducing SSI than 30% standard FiO<sub>2</sub> therapy in the absence of prophylactic antibiotic treatment. Our data support the clinical findings by Geirf et al. (2000) and demonstrate that 5h perioperative treatment with 80% FiO<sub>2</sub> is also significantly more effective than 30% FiO<sub>2</sub> 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<sub>2</sub> on SSI.

FiO<sub>2</sub> treatment at normobaric conditions is reported to have only modest effect on PO<sub>2</sub> in the blood (Sjoberg & Singer, 2013). In contrast, FiO<sub>2</sub> treatment has been shown to result in significant increases in tissue PO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> perioperative treatment results in substantial increases in MPO levels in infected surgical site (Fig. 2), 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<sub>2</sub> treatment in patients? Although future clinical studies are needed to evaluate all potential adverse impacts of high FiO<sub>2</sub> 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 %) FiO₂ treatments (Hovaguimian et al., 2013). In fact, high FiO₂ 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 % FiO₂ therapy may be safe. We found that enhanced activation of neutrophils at surgical site in response to 80 % FiO₂ only occurred in infected muscles and not when the muscle wound was sterile (Fig. 3), indicating that 80 % FiO₂ 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 % FiO₂ should be adopted in clinical settings, as it may be more beneficial in reducing SSI than low 30 % FiO₂.

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