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Oral ingestion of a novel oxygenating compound, Ox66™, is non-toxic and has the potential to increase oxygenation

Fan Zhang[a], Grace V. Aquino[a], Amjad Dabi[a], William H. Nugent[b], Bjorn K. Song[b], Erica D. Bruce[a,c,d,*]

[a] Baylor University, Department of Environmental Science, Waco, TX, 76798, USA
[b] Song Biotechnologies LLC, Baltimore, MD, 21030, USA
[c] Baylor University, Department of Biology, Waco, TX, 76798, USA
[d] Baylor University, Institute of Biomedical Studies, Waco, TX, 76798, USA

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ABSTRACT

Ox66™ is a novel solid state oxygenating compound. In order to support the use of Ox66™ as a potential oxygenating supplement to injured cells, this study evaluated the safety of Ox66™, its ability to withstand the conditions in the digestive tract, and its potential to increase oxygenation in the mesentery in rats. The toxicity of Ox66™ was evaluated by performing acute (10-day) and chronic (90-day) feeding studies on rats, the stability of the compound in the digestive tract was evaluated via *ex vivo* simulated digestion and subsequent CFDA viability assay on gut epithelial cells, and its capacity for oxygenation in the mesenteric microcirculation was determined by interstitial fluid pressure (P_{ISF}) O_2 measurements upon injection into the small intestine of rats. No toxicity was found associated with acute or chronic oral administration of the compound in rats, and the compound was able to withstand the environment of the digestive tract *in vitro*. Based on the acute animal feeding study, the NOAEL was considered to be 1000 mg/kg/day. This proof-of-concept study further demonstrates the potential of Ox66™ to function as an oxygenating supplement that might be useful for treating either pathological hypoxic-related conditions or to improve oxygenation levels during or after exercise under healthy conditions.

1. Introduction

The average human being metabolizes about 500 L of pure molecular oxygen (O_2) daily (Engelberg, 1996). One of the main roles of O_2 is that it functions as a bioenergetic that fuels the body by assisting cells in the conversion of nutrients into energy, mainly in the form of ATP. Inspired O_2 diffuses into the pulmonary capillaries where a majority of it is bound to hemoglobin within red blood cells and transported via the cardiovascular system to metabolically active organs and tissues. Upon reaching regions of low oxygen tension (PO_2), O_2 leaves hemoglobin and diffuses down its concentration gradient through the perivasculature to cellular mitochondria where it serves as the final electron acceptor in cellular respiration. The reduction of O_2 to water and CO_2 in the electron transport chain enables the conversion of biochemical energy from food into ATP, which functions as the cellular fuel that sustains daily human activities and provides approximately 2550 Calories (10.4 MJ) of energy (Wagner et al., 2011). The physiological role of O_2 extends well beyond mitochondrial respiration to numerous metabolic and biosynthetic pathways (Raymond and Segre, 2006). In fact, O_2 is estimated to be involved in over 10^3 metabolic reactions and the synthesis of macromolecules that carry out specialized cell functions in aerobes (Goldfine, 1965; Raymond and Segre, 2006).

Physical exercise produces a rapid increase in O_2 consumption from contracting skeletal muscle tissues due to the increase in metabolic activity (Nugent et al., 2016). This increased O_2 demand is met by a corresponding increase in O_2 delivery in the form of increased vascular perfusion (for review see (Golub and Pittman, 2013)). At some point, the O_2 delivery system cannot match demand and aerobic respiration becomes O_2 supply limited (Golub and Pittman, 2012). Anaerobic respiration involving glycolysis then takes over, but is far less efficient in converting organic macronutrients to ATP resulting in the increased accumulation of lactic acid, which interferes with muscle performance.

There are several gas-phase oxygenation products and treatments emerging in the supplementation market ranging from self-served oxygen-enabled supplements to pricier hyperbaric oxygen therapy. In this paper, we introduce a novel formulation of a solid-state oxygen-carrier, Ox66™, that may have the advantage of easier and safer handling than gaseous O_2. Ox66™ contains over 60% of oxygen in an

* Corresponding author. Baylor University, Department of Environmental Science, Waco, TX, 76798, USA.
E-mail address: erica_bruce@baylor.edu (E.D. Bruce).

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aluminum clathrate making it an ideal candidate to serve as a supplemental oxygen source to relieve hypoxic stress on compromised tissues, enrich oxygen content for improved organ functionality, or accommodate the high-oxygen demands of high endurance exercise.

Four studies that evaluate the toxicological and/or physiological effects of Ox66™ are presented in this paper. The aim of these studies was to determine if acute or chronic exposure to the compound result in adverse effects to human cells or rats. Previous research from our group has proven the safety of this compound in the *in vitro* setting (Gueldner et al., 2017). The first toxicity study presented here is an acute (10 day) oral gavage administration animal study that included single dose and multiple dose administrations. The second toxicity study is a chronic (90 day) oral gavage administration animal study. The third study evaluated the stability of Ox66™ in an *ex vivo* simulated digestive tract bio-fluids model and subsequently, the toxicity of the gut-digested compound on human gut epithelial cells *in vitro*. The fourth study was an *in vivo* proof-of-concept study that investigated the changes in oxygenation level across the small intestine to the mesentery of rats after injection of the compound into the small intestine.

2. Material and methods

The animal studies and experimental procedures performed here were approved by Charles River Laboratories IACUC (Study No. 20086532) and SoBran Biosciences, Inc IACUC (Protocol # SON-002-2016), and they are consistent with the National Institutes of Health guidelines for the humane treatment of laboratory animals, as well as the American Physiological Society's Guiding Principles in the Care and Use of Animals. Both the acute and chronic studies were performed at Charles River Laboratories, while the mesenteric study was performed by Song Biotechnologies, LLC, in strict adherance to the approved protocols and procedures. Animals were assigned to groups by a stratified randomization scheme designed to achieve similar group mean body weights. Males and females were randomized separately. Animals in poor health or outside the body weight range were not assigned to groups. In both studies, the dosing formulations were prepared in 0.9% physiological saline (Hospira) at approprtiate concentrations to meet dose level requirements. The dosing formulations were prepared weekly, stored in a refrigerator set to maintain 5 °C, and dispensed daily. The dosing formulations were removed from the refrigerator and stirred for at least 30 min before dosing and continuously during dosing. The dosing formulations were drawn into a syringe, and administered orally through the attachment of a gavage cannula.

2.1. Acute (10-day) oral gavage administration animal study

In this study, 17 male and 17 female Sprague Dawley rats (Charles River Laboratories, Raleigh, NC) were selected and divided into two study phases: 1) to determine dose tolerance, and 2) administer maximum dose tolerance for 10 consecutive days. Study animals were eight to nine weeks old, and the males weighed between 224 g and 317 g, while the females weighed between 180 g and 211 g.

Phase I: Rising-dose Study. For the rising-dose study, Ox66™ (a product of Hemotek, LLC) was administered to four groups, where each group consisted of three animals of each sex (total of six animals per group). Groups one to four each received 250, 500, 750 and 1000 mg/kg/day Ox66™, respectively, by oral gavage as a single dose. Before proceeding to the next dose level, any adverse effects were observed for a minimum 24-h after dosing the previous group. The day of dosing Group 1 was designated as Study Day 1, and the last dosing was completed on Study Day 4 with Group 4 (see Table 1). At the end of each observation period, the following parameters and end points were evaluated: clinically relevant signs (i.e. fur color, blood chemistry, hematology and coagulation, urinalysis, and food consumption), mortality, body weights, body weight changes, and gross pathology. Additionally, the heart, thyroid, and kidney were selected as secondary organs to evaluate potential adverse effects in offsite locations.

Table 1
Experimental design for 4-day rising-dose study.

Group No.	Test Material	Dose Level (mg/kg/day)	Dose Volume (mL/kg)	Dose (mg/mL)	Number of Animals	
					Rising-dose Study	
					Males	Females
1	Ox66™	250	5	50	3	3
2	Ox66™	500	5	100	3	3
3	Ox66™	750	5	150	3	3
4	Ox66™	1000	5	200	3	3

Table 2
Experimental design for 10-day multiple-dose study.

Group No.	Test Material	Dose Level (mg/kg/day)	Dose Volume (mL/kg)	Dose (mg/mL)	No. of Animals	
					Multiple-dose Study	
					Males	Females
1	Ox66™	1000	5	200	5	5

Phase II: Multiple-dose Study. For the multiple-dose study, Ox66™ was administered to 10 animals by gavage from Days 1–10 at the highest tolerable dose level determined from Phase I (1000 mg/kg/day). The first day of dosing was designated as Study Day 1 (Table 2). Same parameters and end points from Phase I were evaluated in the multiple-dose phase of this study.

Evaluations of clinical pathology and hematology from the acute study was made by comparing to historical control, which is the data pool of all historical negative control animals performed by Charles River Labrarotory in similar previous experiments. At the time of comparisons, the historical control consisted of data up to 713 animals. By incorporating information from historical control, it not only reduced the animal size in experiments, which is both ethical and practial, but it also provided a large database for results evaluation and statistical analysis (Kramer and Font, 2017).

2.2. Chronic (90-day) oral gavage administration animal study

In this study, 55 male and 55 female Sprague Dawley rats were received from Charles River Laboratories, Raleigh, NC. The animals were 8 weeks old and weighed between 235 and 282 g for males, and 185 and 226 g for females at initiation of dosing.

The appropriate animals were administered by once daily oral gavage from Days 1–91/92. The dose volume for each animal was based on the most recent body weight measurement. The doses were given using a syringe with attached gavage cannula. The first day of dosing was designated as Day 1 (Table 3).

Table 3
Experimental Design for 90-day feeding study.

Group No.	Test Material	Dose Level (mg/kg/day)	Dose Volume (mL/kg)	Dose (mg/mL)	Number of Animals	
					Main Study	
					Males	Females
1	Negative Control[a]	0	10	0	10	10
2	Ox66™	250	10	25	10	10
3	Ox66™	500	10	50	10	10
4	Ox66™	750	10	75	10	10
5	Ox66™	1000	10	100	10	10

[a] 0.9% Physiological Saline.

Table 4
Chemical constituents of the digestive tract fluids for *ex vivo* digestion.

	Saliva pH = 6.8 ± 0.1	Gastric juice pH = 1.3 ± 0.1	Duodenal juice pH = 8.1 ± 0.1	Bile juice pH = 8.2 ± 0.1
Inorganic solutions	896 mg KCl 200 mg KSCN 1021 mg $NaH_2PO_4.2H_2O$ 570 mg Na_2SO_4 298 mg NaCl 1694 mg $NaHCO_3$	2752 mg NaCl 1021 mg $NaH_2PO_4.2H_2O$ 824 mg KCl 302 mg $CaCl_2$ 306 mg NH_4C 6.5 mL HCl (37%)	7012 mg NaCl 3388 mg $NaHCO_3$ 80 mg KH_2PO_4 564 mg KCl 50 mg $MgCl_2.6H_2O$ 180 µL HCl (37%) 9151 mg $CaCl_2$	5259 mg NaCl 5785 mg $NaHCO_3$ 376 mg KCl 150 µL HCl (37%)
Organic solutions	200 mg urea 290 mg amylase 15 mg uric acid 25 mg mucin	650 mg glucose 20 mg glucuronic acid 85 mg urea 330 mg glucosamine-hydrochloride 1 g BSA 2.5 g pepsin 3 g mucin	100 mg urea 1 g BSA 9 g pancreatin 1.5 g lipase	250 mg urea 167.5 mg $CaCl_2$ 1.8 g BSA 30 g bile

The ame parameters and end points as in the acute study were evaluated in this study. Similarly, the pathology and hematology data were compared to historical controls.

2.3. *Ex vivo digestion and in vitro cytotoxicity of Ox66™ to human gut epithelial cells*

To evaluate the stability of Ox66™ in physiological digestive fluids, Ox66™ was subjected to an *ex vivo* physiological digestion simulating oral administration (i.e. ingestion). The particles first underwent simulated digestion through an *ex vivo* digestive tract model in which the particles were incubated in the four digestive fluids: saliva, gastric juice, duodenal juice, and bile juice. Next, 5-Carboxyfluorescein Diacetate (CFDA) cytotoxicity assays were performed on gut epithelial cells (Caco-2) exposed *in vitro* to digested Ox66™ particles.

Prior to incubation, the gastric fluids were heated to 37 °C. Each fluid was synthesized using the constituents listed in Table 4 (Ulleberg et al., 2011). In short, the complete digestion model consists of three steps: 1) incubating the particles in saliva (mixture A), 2) incubating mixture A in gastric juice (mixture B), and 3) incubating mixture B in duodenal juice and bile juice (mixture C). Partial digestions of the particles (mixtures A and B) were also used for comparison against the complete digestion (mixture C), in order to determine the point in the digestive tract at which the compound might experience physicochemical changes, if any.

The digestion procedure starts with the introduction of 6 mL of artificial saliva into 1 mL of 10 µg/ml of Ox66™ solution (in nanopure water). The mixture is rotated head-over-heels for 5 min. Subsequently, 12 mL of gastric juice are added and this mixture is rotated head-over-heels for 2 h. Finally, 12 mL of duodenal juice, 6 mL of bile juice, and 2 mL of $NaHCO_3$ solution are added for a final volume of 39 mL. After sequential incubation of Ox66™ in these fluids, the compound is extracted by centrifugation and resuspended in fresh culture medium. Gut epithelial cells seeded in dark 96 well-plates are then exposed to the digested particles in culture medium for 24 h, after which CFDA is performed to determine cellular viability.

2.4. *Effects of ingested Ox66™ on in vivo mesenteric microcirculation*

In this proof-of-concept study, anesethized, male Sprague Dawley rats had their small intestine intubated and flushed with either Ox66™ or phosphate buffered saline (PBS; vehicle and volume control). A bolus of either experimental solution or volume control was infused into the small intestine at a fixed rate through a pre-filled syringe that was connected to a syringe pump (Genie Touch™, Kent Scientific, Torrington, CT). Concurrently, the microcirculation of the mesentery, which is the network that transports nutrients from the small intestine to the body, was investigated for changes in interstitial fluid oxygenation ($P_{ISF}O_2$) using phosphorescence quenching microscopy. This animal protocol was approved by the SoBran Biosciences Inc. IACUC (Protocol # SON-002-2015) and was consistent with the National Institutes of Health guidelines for the humane treatment of laboratory animals, as well as the American Physiological Society's Guiding Principles in the Care and Use of Animals.

2.4.1. *Surgical preparation*

Male rats were inducted with 1–5% isoflurane in medical air for initial pre-operative preparation and cannulations. An intravascularly-delivered anesthetic, alfaxalone acetate (Alfaxan, Schering-Plough Animal Health, Welwyn Garden City, UK), was continuously infused at 0.1 mg/kg/min through a femoral vein cannula for maintenance of anesthesia during the remainder of the experiment. A femoral artery cannula was connected to a pressure transducer for monitoring of systemic circulatory variables with a multichannel physiological monitoring system (BIOPAC MP-150, BIOPAC Systems, Goleta, CA). A tracheal tube was inserted to maintain airway patency; however, animals continued to inspire room air and were not artificially ventilated. The small intestine was intubated with a tube that was connected to a syringe. After experimentation, animals were euthanized with a lethal dose of Euthasol (150 mg/kg, pentobarbital component, intravenously; Delmarva, Midlothian, Virginia).

2.4.2. *Mesentery preparation*

The rat mesentery muscle was isolated as previously described (Golub et al., 2007, 2011) on a thermostable animal platform adapted for intravital and phosphorescence quenching microscopy (Golub and Pittman, 2003; Nugent et al., 2016). Animals and exteriorized mesentery - isolated from atmospheric contamination by a transparent barrier film - were maintained at 37 °C during experimentation, which was monitored by rectal probe (BIOPAC; Part # SS7L, BIOPAC Systems, Goleta, CA).

2.4.3. *Microcirculatory parameters*

Observation and measurement of the mesentery were carried out with an intravital microscope (Axioimager2m, Carl Zeiss, Germany) configured for trans-illumination through a 20X/0.8 objective (Plan-APOCHROMATE, Zeiss, Germany) and custom modified for phosphorescence quenching microscopy. Phosphorescence quenching for the detection of oxygen in biological systems was originally described by Wilson et al. (Rumsey et al., 1988; Vanderkooi et al., 1987)). Adaptation of this technology for microscopic measurements of the $P_{ISF}O_2$ in the mesentery and other thin organs are described elsewhere (Golub et al., 2011; Pin et al., 1982).

Briefly, a palladium porphyrin 'oxygen probe' (Oxyphor R0; Frontier Scientific, Newark, DE) bound to bovine serum albumin was topically applied to the tissue and allowed to diffuse into the spinotrapezius muscle's interstitium. For measurements, the oxygen probe was excited by a xenon flash lamp (L11969, Hamamatsu, Japan), in an octagonal region 300 µm in diameter at a frequency of 1 Hz. The excitation light pulse was passed through a filter cube consisting of a narrow-band filter (525 CWL Narrowband, Edmund Optics, Barrington, NJ), a dichroic mirror (567 nm DMLP Longpass, Thorlabs, Newton, NJ), and a wide-band filter (Longpass Cut-on > 650 nm, Thorlabs, Newton, NJ) for selective collection of phosphorescence emission. Three interstitial sites were measured per animal. The phosphorescence signal was collected by a photomultiplier tube (R9110, Hamamatsu, Japan) and routed through a custom-built signal processor, collected by a data acquisition device (NI PCIe-6361, National Instruments, Austin, TX) and stored digitally on a computer. Each phosphorescence decay curve (which could be thought of as an exponential decay curve) was fitted to a rectangular distribution model presented in this paper (Golub et al., 1997) from which individual partial pressure of oxygen (PO_2) values for ISF PO_2 were calculated. Calibration of our PQM equipment and oxygen probe was performed as described by Golub and Pittman (2016).

2.4.4. Experimental protocol

Following surgical and mesentery preparations, animals were given 15–30 min to stabilize both temperature and plane of anesthesia. Once stable, a minute of baseline $P_{ISF}O_2$ was established. After baseline $P_{ISF}O_2$ measurements, $P_{ISF}O_2$ was recorded continuously as the syringe pump was utilized to perfuse the small intestine with a bolus of PBS, and then a bolus of Ox66™.

2.4.5. Sample size prediction

The peak amplitude data generated by this pilot study were subjected to a two-sided power analysis to predict sample sizes needed to obtain significance between PBS and Ox66™ mesenteric oxygenation. Parameters were: PBS mean – 0.20, Ox66™ mean – 0.42, SD – 0.2, and α – 0.05. To reach a desired power of 0.8, 14 experiments would be required, which is reasonable for an animal model.

2.4.6. Chemical characterization

All solid samples in chemical analyses were obtained by grinding raw Ox66™ in planetary mill (Restch PM200). Grinding parameters were 5 min under dry conditions using 4 mm stainless steel beads (filled to 2/3 volume of jar) in 50 mL stainless steel jar. A sample was prepared for Fourier transfer mass spectrometry (FTMS) (Thermo Orbitrap Discover, ThermoFisher, Electron spray ion source) by suspending 0.7 mg of prepared ground sample in 100 mL of nanopure water. 5–6 drops of ammonia solution (30.0 w/w %) were added to the suspension and left for a total of 20 min with intermittent inversions. 100 µl of this solution was then added to 1 mL of methanol, and the vial was thoroughly mixed. The methanol, ammonia, and ground sample solution was then run through the high-resolution FTMS instrument after background data for an ammonia-methanol blank was obtained. The resultant background spectrum was then subtracted from the sample spectrum. Infrared spectroscopy was performed using Thermo/Nicolet iS-10 FT-IR, with Smart™ iTX accessory using a germanium crystal.

2.5. Statistical analyses

2.5.1. Parametric/non-parametric

Data are expressed as mean ± standard error of the mean (SEM).

For the 90-day feeding study, a Levene's test was used to assess the homogeneity of group variances. One-way ANOVA was used to detect changes within groups. In cases where a significant difference ($p < 0.05$) was detected, an appropriate multiple comparison test (Dunn's test) was conducted.

2.5.2. Functional observational battery (FOB) data

For FOB count data, each data set was subjected to a statistical decision tree.

Data sets for each interval were initially analyzed for homogeneity of variance using Levene's test followed by the Shapiro-Wilk test for normality. A $p < 0.001$ level of significance was required for each test to reject the null hypothesis.

If both Levene's test and the Shapiro-Wilk test were not significant, a single-factor parametric ANOVA was applied, with animal grouping as the factor, using a $p < 0.05$ level of significance. If the parametric ANOVA was significant at $p < 0.05$, Dunnett's test was used to identify statistically significant differences between the control group and each test article-treated group using a minimum significance level of $p < 0.05$.

If either Levene's test and/or the Shapiro-Wilk test were significant, then the Kruskal-Wallis non-parametric ANOVA was applied, with animal grouping as the factor, using a $p < 0.05$ level of significance. If the non-parametric Kruskal-Wallis ANOVA was significant at $p < 0.05$, Dunn's test was used to identify statistically significant differences between the control group and each test article-treated group using a minimum significance level of $p < 0.05$.

Descriptive (categorical) and quanta FOB data was analyzed by Fisher's Exact test. If significance was detected, a group by group comparison was performed using the Chi-Square test.

3. Results

3.1. Acute (10-day) oral gavage administration animal study

Oral gavage administration of Ox66™ was well tolerated in rats at a single dose of up to 1000 mg/kg/day, or for 10 days at a level of 1000 mg/kg/day. No abnormal clinical observations or changes in body weight or body weight gains were noted during either phase of the study. The weight on Day 15 was measured to account for any possible delayed effects of Ox66 ™ that were not observed during the course of the experiment (4 days). Therefore, Day 8 which was the median point of 15 days was set as a another measurement. In the rising-dose study, the animals were dosed only once, each group with one concentration as specified in Table 1. In 10-day study, there was no control group. While the male and female animals in the rising-dose study both increased in weight over the course of the 10 days, no statistically significant differences were observed among the experimental groups. The average weights between males and females were different but normal (Fig. 1). Similarly, in the multiple-dose study, male and female rats increased in weight, with the males having a greater mean body weight compared to the females; which is typical for this species of rat (Fig. 2). There were also no changes in hematology, coagulation, or clinical chemistry parameters after 10 days of daily administration of 1000 mg/kg/day.

3.2. Chronic (90-day) oral gavage administration animal study

Oral gavage administration of Ox66™ once a day to rats for up to 91 days at doses of 250, 500, 750, or 1000 mg/kg/day resulted in no unscheduled deaths and no Ox66™-related changes in body weight, body weight gains, food consumption, FOB assessments, ophthalmology, clinical chemistry, or urinalysis parameters (data not shown). Non-adverse findings included mild hematological changes at ≥ 500 mg/kg/day in females and ≥750 mg/kg/day in males, and organ weight changes in heart (males only at 500 and 1000 mg/kg/day), kidney (females only at ≥ 750 mg/kg/day), and thyroid (females only at 500 and 1000 mg/kg/day), without any histological correlates. Tables 5 and 6 summarized organ weight changes for male and female animals respectively. All data were expressed as percentage change from control group means.

Clinical observations showed that there was a higher incidence of red fur staining in males at ≥ 750 mg/kg/day, and in females at all dose levels, compared to controls (which had no observations of red fur staining) (Data not shown). Clinical chemistry studies in males, showed

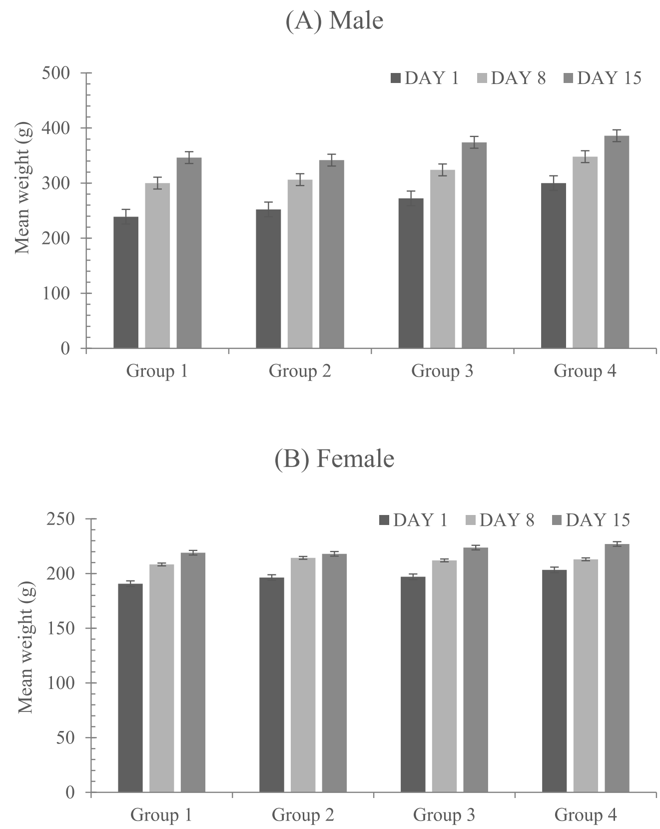

Fig. 1. Rat mean weights (g) for rising-dose study. Group 1 received 250 mg/kg-day Ox66™, Group 2 received 500 mg/kg-day Ox66™, Group 3 received 750 mg/kg-day Ox66™, and Group 4 received 1000 mg/kg-day Ox66™. Males (A) and females (B) were grouped separately and both show a steady increase in weight over the study period. However no statistically significant differences were observed among experimental groups, but the average weights between males and females were different, but normal.

that red blood cells (RBC) and red blood cell distribution width (RDW) were increased at 1000 mg/kg/day on Day 46 and at ≥ 750 mg/kg/day on Day 91. Reticulocytes were also increased at 1000 mg/kg/day on Day 91. Conversely, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were decreased at 1000 mg/kg/day on Day 46 and at ≥ 750 mg/kg/day on Day 91. Females showed similar trends with RBC significantly increased at 1000 mg/kg/day on Days 14 and 92, and RDW increased at 1000 mg/kg/day on Day 92. MCH and MCHC were decreased at 1000 mg/kg/day on Days 46 and 92, and MCV was decreased at ≥ 500 mg/kg/day in females on Day 92. Of these changes, MCV and MCH values fell below the range of historical controls in males on Days 46 and/or 91, and also MCHC on Day 91. MCH values fell below the range of historical controls in females on Day 46, and MCV, MCH, and MCHC values fell below the range of historical controls on Day 92.

Table 5
Summary of organ weight data for male rats – (Day 91/92).

	Males			
Group	**2**	**3**	**4**	**5**
Dose (mg/kg/day)	**250**	**500**	**750**	**1000**
No. Animals per Group	**10**	**10**	**10**	**10**
Heart (No. Weighed)[a]	10	10	10	10
Absolute value	14.275	18.042	9.0723	**19.2931**
% of body weight	8.45969	**14.1998**	8.41969	**22.07318**
% of brain weight	13.92631	17.72761	8.37586	**20.18199**
Kidney (No. Weighed)	10	10	10	10
Absolute value	6.8428	8.1856	9.4523	9.9286
% of body weight	1.49635	5.04022	9.16311	11.39194
% of brain weight	6.48097	7.94429	8.07226	10.51341
Thyroid Gland (No. Weighed)	10	10	10	10
Absolute value	5.00956	8.413	−5.08604	1.60612
% of body weight	−0.68497	6.91886	−4.92143	2.82236
% of brain weight	4.63327	8.09334	−6.62792	2.20695

Based upon statistical analysis of group means, values highlighted in bold are significantly different from control group – $p \leq 0.05$.
For other organs absolute weight values, please refer to Table 1S.
[a] All values expressed as percent difference from control group means.

Finally, urinalysis indicated that there were no Ox66 ™-related changes in urinalysis parameters. There were no significant differences in urine volume or specific gravity between the dose groups and control group values. Gross pathology showed no findings related to the test article. The gross findings at necropsy that were observed in the lungs (focus dark; area of darker tissue, focus pale; area of lighter tissue) are commonly associated with gavage-related acid reflux in the rats (Damsch et al., 2011); though the granulomas were considered to be procedure-related, and not test article-related, as a result of aspiration following gavage-related reflux (Damsch et al., 2011).

Fig. 3 shows the viability of Caco-2 cells after 24 h exposure to digested particles at a concentration of 10 ppm. No significant differences in fluorescence levels were observed between the control (no treatment with particles) and the cells treated with particles that underwent partial (i.e. digestion in saliva alone, or in saliva and gastric juice) or

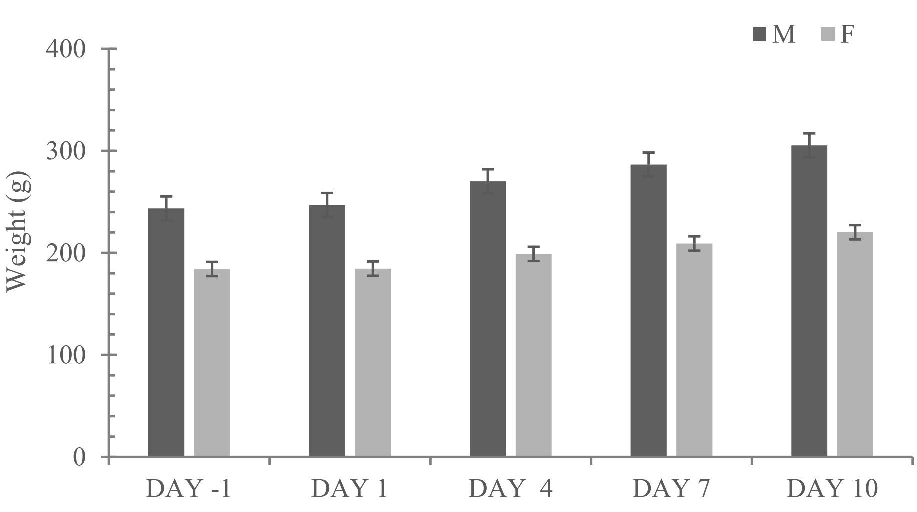

Fig. 2. Rat mean weights (g) for multiple-dose study. Male and female rat mean weights both show a slight, steady increase over the 10-day period of oral administration of 1000 mg/kg/day Ox66™ daily for 10 days. The male group has a greater mean body weight than the female group, which is typical for this species of rat.

Table 6
Summary of organ weight data for female rats – (Day 91/92).

	Females			
Group	**2**	**3**	**4**	**5**
Dose (mg/kg/day)	**250**	**500**	**750**	**1000**
No. Animals per Group	**10**	**10**	**10**	**10**
Heart (No. Weighed)[a]	10	10	10	10
Absolute value	−5.5535	−3.4721	1.5027	−1.596
% of body weight	−3.4721	0.18393	1.12435	5.82521
% of brain weight	−7.17822	−4.9338	0.99609	−1.10696
Kidney (No. Weighed)	10	10	10	10
Absolute value	1.2669	−0.7033	8.4497	1.1428
% of body weight	2.1199	3.35285	**8.87195**	**8.67246**
% of brain weight	−0.64693	−2.34136	7.82687	1.34172
Thyroid Gland (No. Weighed)	10	10	10	10
Absolute value	20.55687	**34.36019**	22.33412	**24.70379**
% of body weight	21.71759	**39.5369**	21.96292	**33.42728**
% of brain weight	18.42251	**31.94512**	21.24798	**24.36418**

Based upon statistical analysis of group means, values highlighted in bold are significantly different from control group – $p \leq 0.05$.
For other organs absolute weight values, please refer to Table 2S.
Ex vivo digestion and *in vitro* cytotoxicity of Ox66™ to human gut epithelial cells.
[a] All values expressed as percent difference from control group means.

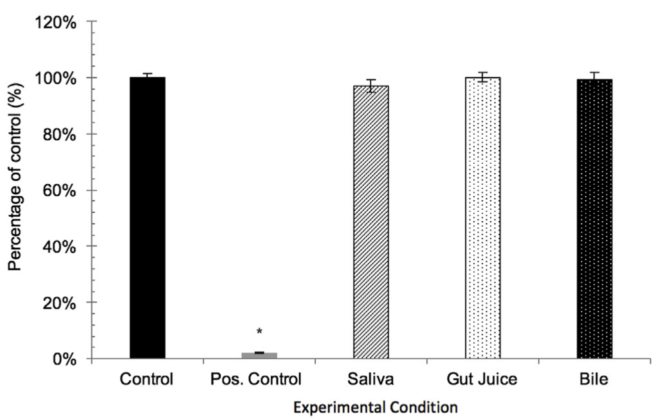

Fig. 3. Viability of CaCo-2 Cells after 24 h Exposure to Digested Ox66™ Particles. The viabilities of Caco-2 cells exposed to partial- and complete-*ex vivo* digested Ox66™ particles are unchanged compared to the control group (mean % fluorescence ± SD). Data has been normalized to control. 1% Triton X was used as positive control. Control was cell culture media.

complete digestion (saliva, gastric juice, duodenal juice and bile juice).

3.3. Effects of ingested Ox66™ on in vivo mesenteric microcirculation

The rat mesenteric microcirculation was placed under microscopic inspection and assessed for changes in $P_{ISF}O_2$ before and after intestinal perfusion of Ox66™ Low in PBS or PBS alone. Peak amplitude of the normalized $P_{ISF}O_2$ curve is reported in Fig. 4 and shows Ox66™ Low trending higher than PBS alone. Area under the curve, reported in Fig. 5, was also assessed and showed a similar relationship. It should be reiterated that this was a pilot or proof-of-concept study (N = 4)—due to the expense of *in vivo* experimentation—to drive a computational model (power analysis) that predicted sufficiently powered (0.8) significant differences would be detectable at fourteen animals per group.

4. Discussion

Ox66™ is a novel non-gaseous oxygenating compound, and shows

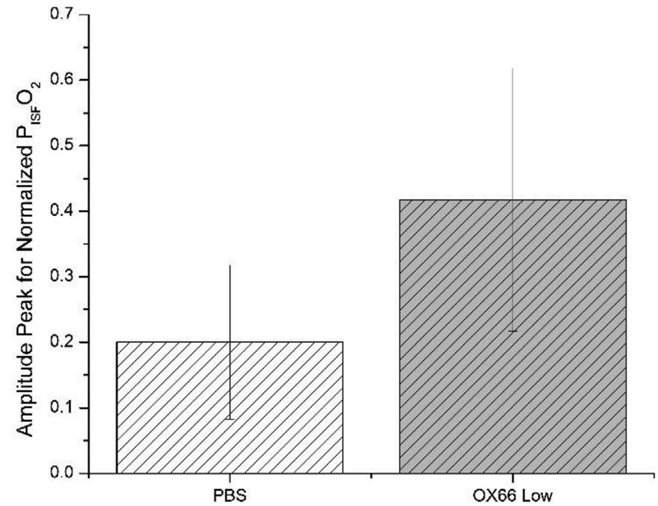

Fig. 4. Mesenteric Oxygenation Peak Amplitude. Discrete peak amplitudes in $P_{ISF}O_2$ during baseline and treatment phases were collected and normalized to an average of baseline values. Then, baseline was subtracted to produce a maximum rise in $P_{ISF}O_2$ over baseline during the treatment phases. Data are mean ± SEM with Ox66™ trending higher than PBS. N = 4.

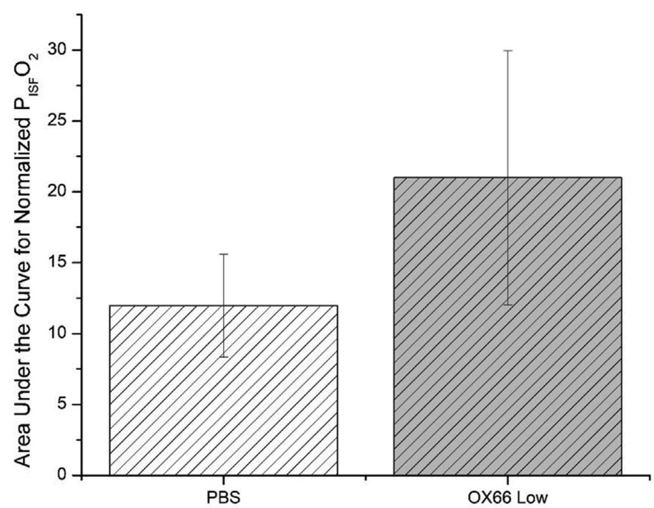

Fig. 5. Mesenteric Oxygenation Area Under the Curve (AUC). Each experiment was normalized to the 60s baseline (BL) measurement, which was recorded 1 min prior to infusion. BL AUC was calculated from 1 min of BL and multiplied by 4 to match the 4 min AUC measurement of treatments. BL AUCs were then subtracted from Treatment AUCs and plotted. Data are mean ± SEM with Ox66™ trending higher than PBS. N = 4.

potential as an oxygenating supplement to treat pathological hypoxic-related lesions or to improve oxygenation levels during or after exercise under healthy conditions. Therefore, the main purposes of this study were to evaluate the safety of Ox66™, its ability to withstand the conditions in the digestive tract, and its potential to increase oxygenation in the mesentery in rats. The toxicity of Ox66™ was evaluated by performing acute (10-day) and chronic (90-day) feeding studies on rats. The stability of the compound in the digestive tract was evaluated via *ex vivo* simulated digestion and subsequent CFDA viability assay on gut epithelial cells. Lastly, its capacity for oxygenation in the mesenteric microcirculation was determined by $P_{ISF}O_2$ measurements upon injection into the small intestine.

The toxicity studies presented here show overall no adverse effects to animals associated with acute (10-day) or chronic (90-day) ingestion of Ox66™, or to human epithelial gut cells exposed to partially or completely digested compound. The objective of the acute study was to determine the potential toxicity of Ox66™ when given orally for 10 days to rats. Reduced body weight is one of the primary signs of toxicological response (Chapman et al., 2013); however, the animals in this study sustained a normal body weight increase in both males and females (Fig. 1). The absence of abnormal clinical observations, hematology, coagulation, clinical chemistry parameters (data not shown) and body weight gains are good indicators of compound tolerability and that there appears to be no acute toxicity to the animals. Based on these results, the no-observed-effect level (NOEL) was considered to be 1000 mg/kg/day, and the compound was well tolerated by the rats.

A more protracted feeding study was conducted subsequently in which groups of rats were gavaged different doses, ranging from 250 to 1000 mg/kg/day for 90 days. The noted measures of mortality, body weight, body weight gains, clinical chemistry, food consumption, ophthalmology, hematology and coagulation, urinalysis, and functional observational battery (FOB) assessments all indicate that the compound does not produce adverse effects in rats (data not shown). Red fur in some rats might be an indicator of compound-related effects, due to the apparent relationship to dose and the lack of incidence in the concurrent controls (data not shown). However, these signs were not associated with any behavioral or morphological changes. The gross pathological changes in the lungs of the animals correlecated histologically to granulomas and atelectasis. Based on these results, the no-observed-adverse-effect level (NOAEL) was considered to be 1000 mg/kg/day.

Changes in organ weight may also be a general indicator of toxicity or pathology. Organ weight changes with an apparent relationship to the administration of the test article include an increase in heart weight (absolute, relative to body, and relative to brain) in 1000 mg/kg and 500 mg/kg (relative to body) males only. In females there was an increase in kidney weight (relative to body only) at > 750 mg/kg. An increase in thyroid gland weight was present at 500 mg/kg (absolute, relative to body, and relative to brain) and 1000 mg/kg (absolute, relative to body). There were no histologic correlates to any of the organ weight changes and no other test article-related organ weight changes were noted. The results of the acute and chronic feeding studies altogether suggest that the compound is safe for consumption by animals in the short-term and long-term scenarios, and that the compound can very likely be ingested by humans with little to no risk of adverse effects.

Following the toxicity (feeding) studies, an *ex vivo* digestion and *in vitro* cytotoxicity of Ox66™ to human gut epithelial cells were conducted to determine whether Ox66™ withstands the environment of a simulated *ex vivo* digestive tract and to determine any differences in viability of gut epithelial cells treated with the digested particles compared to that of untreated, healthy cells. CFDA is an intracellular uptake fluorescent dye used in cytotoxicity assays as a measure of cellular viability and enzyme activity; a greater fluorescence corresponds to a greater cellular viability. In this study, the results show that there is no statistically significant difference in cellular viability of cells not treated with Ox66™ and cells treated with partially or fully digested particles (Fig. 3). This means that the particles, regardless of any potential physicochemical changes they underwent when passing through the simulated digestive tract, did not increase in cytotoxicity to Caco-2 cells.

Once it was determined that there was no significant toxicity associated with feeding rats Ox66™, and the *ex vivo* digestive tract study demonstrated that Ox66™ withstands the environment of the gut, a separate proof-of-concept study was conducted. The purpose was to determine whether Ox66™ could oxygenate the mesenteric microcirculation, which is the network surrounding the small intestine and primarily responsible for transporting nutrients from the food to the rest of the body. Through a pilot study with few animals, we saw an elevation in both mean interstitial oxygenation peak value (Fig. 4) as well as the area under the curve (Fig. 5) in Ox66^{TM}-treated animals compared to those treated with phosphate buffered saline (PBS). These data were fed into a computational model which predicted a full study of fourteen animals in each group would show these trends significant. The elevated presence of interstitial oxygen is consistent with previous Ox66™ work (data not shown) where a model of extreme hemodilution was utilized. In those studies, animals underwent a 50% blood volume exchange, yet Ox66^{TM}-treated animals maintained baseline interstitial oxygen levels, whereas PBS-treated animals showed a significant decrease.

Several methods were used to identify the elements present in Ox66™ and its possible structure and mechanism of action. The first method used, Energy Dispersive Spectroscopy, revealed the presence of aluminum and oxygen as the two main elements of the therapeutic. Infrared Spectroscopy was then performed to further elucidate the configuration of the elements. Results from IR indicate an abundance of −OH ligands. The binding of −OH ligands to aluminum and oxygen is hypothesized to result in a structure that provides a source of non-gaseous oxygen. Additional support for this hypothesis was provided via mass spectrometry, the results of which indicate a dynamic behavior of the aluminum whereby a tetrahydroxoaluminate ion accepts methyl groups from methanol, forming a complex of different ions with varying numbers of bound methyl groups. Through inductively coupled plasma mass spectrometry (ICP-MS) and mercury analyzer analysis (QuickTrace™ M-8000 Mercury Analyzer), a detectable level of mercury was found in batches of Ox66™ (131–217 ppm). The mercury is believed to be a result of manufacturing adulteration, and not inherently part of the compound. However, had any toxicity been associated with this adulteration, it would have been detected in the studies performed. NOAELs of 0.93 and 0.23 mg mercury/kg/day are advised by ATSDR for acute- and intermediate-duration exposure to inorganic mercury for no observed renal effects in rats (ATSDR, 1999). Increased absolute and relative kidney weights are typical endpoints to evaluate mercury toxicity. The only statistical significant difference was observed in female rats after exposure to 750 and 1000 mg/kg/day in the kidney % of body weight (Table 6). However, no mercury toxicity was observed in the 90-day animal feeding studies upon examination of animals kidney weights to control.

Further characterization and testing will be carried out to better understand the exact mechanism of oxygen release and in what form the oxygen exchange with the heme group in blood takes place as suggested from the pilot mesentery data.

While O_2 is typically delivered in gaseous form through the capillary beds of the lungs into systemic circulation, based on the results of our proof-of-concept we speculate that Ox66™ -which is a non-gaseous form of O_2− can likewise provide oxygen to the tissues of the body through mesentery absorption via ingestion. The observed increase in $P_{ISF}O_2$ in the mesentery is evidence that Ox66™ can potentially supply added oxygen to tissues from the consumption of the compound. Based on these observations, Ox66™ holds promising applications as a nutritional supplement, energy enhancer, or as a treatment for hypoxia-related conditions or pathologies. For instance, Ox66™ can potentially serve as an oxygen source to reduce the relatively hypoxic conditions in compromised tissues such as in ischemia, inflammation, or cancer. In adipocyte hypoxia, for example, the lack of O_2 triggers hypoxia-inducible factor-1 alpha (HIF-1α), which leads to obesity-induced inflammation and insulin resistance. The inhibition of HIF-1α, however, either by genetic or pharmacologic method such as O_2 supplement, can prevent or reverse these pathophysiologic events, restoring insulin sensitivity and glucose tolerance (Lee et al., 2014). Additionally, as a natural detoxifier, O_2 therapy has been used to rid the blood and organs of toxic materials, and it is commonly performed to treat alcoholism and drug addiction or post-intoxication. A comparative clinical and psychopathological examination of patients who received hyperbaric oxygenation after intoxication with either drugs, narcotics or alcohol, showed significant improvement over psychoneurological and somatovegetative status during and after sessions, which decreased about two-fold treatment duration and the development of complications. Such favorable outcomes are believed to be the antihypoxic detoxifying and bioenergetics effects of oxygen supplement, potentially achievable with this novel oxygenating compound (Epifanova, 1995). Finally, as a potential source of oxygen, Ox66™ may be used to improve immune system performance since, according to Hatfiel et al., supplemental oxygenation stimulates the production of antitumor T cells and pro-inflammatory cytokines in mice, which ultimately improved lung tumor regression and longevity of the animals (Hatfield et al., 2015). These applications will be the subject of future studies using Ox66™ as an oxygen source.

Under healthy conditions, Ox66™ may be used to enrich oxygen content for improved organ functionality, or to accommodate the high-oxygen demand in post-exercise oxygen consumption (EPOC) or during intense periods of exercise. Following exercise O_2 consumption remains high as the O_2 “debt” accumulated from anaerobic processes is “repaid” (Borsheim and Bahr, 2003; Foureaux et al., 2006). The duration of increased oxygen uptake may last for several hours, with peak O_2 levels of up to 54% (Chad and Quigley, 1989), although it varies among different exercise modes, training status, and sex (Borsheim and Bahr, 2003). During exercise the metabolic demand of the body increases and when cellular respiration fails to meet the energy demand, the body resorts to anaerobic respiration which produces a build-up of lactic acid resulting in muscle fatigue and soreness. As a potential exercise or nutritional supplement, Ox66™ may supply increased oxygen level to the muscle, thereby assisting aerobic respiration, alleviating the effects of anaerobic respiration (fatigue and soreness), and shortening the body's post-exercise recovery time.

5. Conclusion

The purpose of this study was to examine any potential toxicities associated with the oral ingestion of Ox66™, its ability to withstand the environment of the digestive tract, as well as introduce this proof-of-concept study which found that the compound increased oxygenation to the mesentery in animals. No toxicity was found associated with the oral administration of the compound in rats, and the compound was able to withstand the environment of the digestive tract *in vitro*. To verify the effectiveness and evaluate other potential applications of this novel oxygenating compound, future studies involve measuring PO_2 in the mesentery of rats orally administered the compound to verify *in vivo* the *in vitro* results that indicate that Ox66™ remains viable after digestion, reaches the small intestine, and oxygenates the mesentery as well as other organs. In all, this proof-of-concept study demonstrates the potential of Ox66™ to function as an oxygenating supplement that might be useful for treating either pathological hypoxic-related conditions or to improve oxygenation levels during or after exercise under healthy conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2018.12.034.

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