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**This work is dedicated to the memory of Dr. Domenico Spina. He passed away on the 5th December 2016. Dr Spina was a Reader in Pharmacology, and Head of Pharmacology and Therapeutics Research group in the Institute of Pharmaceutical Science at King’s College London. His death was premature and he will be greatly missed by his many colleagues, students and friends around the world.
ABSTRACT

Rational: Acute lung injury (ALI) is a common complication after intestinal ischemia and reperfusion (I/R) injury that can lead to acute respiratory distress syndrome (ARDS). We have previously demonstrated that females are protected against lung damage induced by intestinal I/R through an estrogen mediated mechanism.

Objectives: to investigate the effect of obesity on ALI induced by intestinal I/R in female mice.

Methods: C57Bl/6 female mice were fed with a standard low-fat diet (SD) or a high-fat diet (HFD) for 9 weeks. Intestinal I/R injury was induced by a 45 min occlusion of the mesenteric artery followed by 2 and 24 h of reperfusion.

Results: Significant increase in lung myeloperoxidase expression (MPO) and neutrophil numbers of SD and HFD mice occurred at 2 h and 24 h of reperfusion. Furthermore, HFD mice presented a significant increase in lung eosinophil peroxidase (EPO) expression and eosinophil numbers compared to SD mice. Lung wet/dry weight ratio was significantly greater in HFD mice at 2 and 24 h of reperfusion, accompanied by a significant increase in the expression of inducible NO in the lung tissue and a significant decrease in arterial oxygen saturation at 24 h of reperfusion relative to SD mice.

Conclusion: Obesity predisposes female mice to increased pulmonary oedema and deterioration in gas exchange, which is accompanied by an increase in iNOS expression in the lung.

Key words: Obesity, estrogen, lung injury, intestinal ischemia and reperfusion, inflammation, female mice.
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**Introduction**

The absence of oxygen supply and nutrients caused by ischemia creates a condition in which the restoration of blood circulation (reperfusion) results in oxidative damage of the tissue, release of inflammatory mediators and influx of inflammatory cells to local and remote organs [1-4]. In this scenario, acute lung injury (ALI) is a common complication after intestinal ischemia (I/R) that, when severe, can lead to acute respiratory distress syndrome (ARDS) and death [5]. We have previously demonstrated a significant increase in myeloperoxidase activity (MPO) in the lung and intestines, an increase in lung vascular permeability and the release of significant amounts of serum IL-6, IL-1β and IL-10 following experimental induction of I/R in normal weight rodents [3, 6, 7].

It is well established that obesity is an important co-morbidity in inflammatory diseases such as asthma, COPD, and diabetes [8, 9]. However, the effect of obesity in ALI induced by ischemic trauma is controversial. Early epidemiological data show that obesity is associated with worse outcomes in acute lung injury [10]. It has also been suggested that chronic low grade inflammation observed in obesity primes the lung for injury, increasing the risk of developing ARDS following an ischemic episode [11, 12]. In contrast, other authors have reported that increasing body mass index (BMI) is associated with both decreasing plasma inflammatory biomarkers and increasing white blood cells count, but not with increasing mortality, in patients who are critically ill with ALI [13]. More recent studies have confirmed these observations, suggesting that obesity is associated with higher morbidity, but not with an increased risk of mortality in critically ill patients [14].
Interestingly, whilst clinical findings suggest that obesity is an important factor in the severity of lung injury following ischemic trauma there is also evidence suggesting that this interaction may be gender influenced. Sex differences in ALI/ARDS have been documented with reports of higher incidence and higher mortality in male patients when compared to females [15-17]. Similarly, another study has demonstrated that male patients are more likely to receive mechanical ventilation than females when in intensive care units [18]. In addition, our group and others have demonstrated that estrogen has a protective effect in experimental models of acute lung injury induced by ischemic trauma [19-21].

In this study, we have investigated whether obesity alters the protective state conferred to female mice in ischemic events. To this end, we have measured inflammatory responses and arterial oxygen levels following intestinal I/R in mice fed either a standard or a high-fat diet.
**Material and Methods**

**Animals:** Female C57/Bl6 between 20-25 g, bred in the animal facility of the Institute of Biomedical Sciences-University of São Paulo. This study was approved by the Ethic Committee of Animals Experimentation of the Institute of Biomedical Sciences-University of São Paulo, following the guidelines of the National Council of Animal Experimentation that regulates animal research according to Brazilian Federal Law (Report no. 111/10/03, 2013).

**Diet:** Mice were fed for 9 weeks with a 30% fat diet (Pragsoluções Biociências, Brazil) or with a standard low-fat diet (Nuvilab CR-1, Brazil). All animals were weighed daily and their food consumption in grams recorded.

**Cholesterol and Triglycerides levels:** Levels of circulating serum cholesterol and plasma triglycerides were determined by a specific enzymatic assay, following the instructions of the manufacturer (Colesterol Liquiform, Triglicérides Liquiform both from Labtest, São Paulo, Brazil).

**Induction of intestinal ischemia and reperfusion (I/R):** Mice were anesthetized with ketamine/xylazine (100/20 mg/kg, i.p, respectively.) The superior mesenteric artery was obstructed for 45 min using a surgical clamp. After clamp removal, the abdomen was sutured and mice were humanely killed 2 or 24 h later with an over dose of anesthetic.

**Wet/dry weight ratio:** Wet weight of the lung tissue was recorded immediately after collection and dry weight after 24 h incubation in a dry oven at 60°C. Edema was assessed calculating the ratio between wet and dry weights of the samples.

**Immunohistochemistry:** Expression of neutrophil myeloperoxidase (MPO), eosinophil peroxidase (EPO) and inducible nitric oxide synthase (iNOS) was measured using specific primary antibody against mouse MPO (1:200 MPO bs-4943R, Bioss
Antibodies, US), mouse EPO (1:250 EPX bs-3881R Bioss Antibodies, US) and mouse iNOS (1:500 iNOS Novus Biologicals, US). Appropriate anti-rabbit biotinilated secondary antibody was used (1:200 or 1:500, Sigma, UK) and expression determined using 3,3'-Diaminobenzidine (DAB, Sigma, UK). Quantification was made using Image Pro Plus software, v5.5.0.29. Data represent average of percentage of positive DAB staining areas in the lung tissue, measured as 5 fields per sample, 4-5 animals per group.

**Cytokine levels:** Serum cytokine levels were measured using a 96-well magnetic beads multiplex plate, following manufacturer’s instructions (R&D, Abingdon, UK)

**Neutrophil and eosinophil number in lung tissue:** Formalin-fixed lung tissue was processed and embedded in paraffin blocks (Chandon tissue processor, Thermo-Ficher, UK). The tissue blocks were cut into 5 µm slices and stained using conventional hematoxylin and eosin staining. Tissue neutrophils were enumerated manually using a 50 µm$^2$ grid and the Image J cell counter plug-in software (v. 1.45S). Data represent the average number obtained from 3 random fields per image, 5 images per sample, and 5 animals per group. Eosinophils were identified using the eosinophil-specific stain Luna, which confers a bright red color to the cells [22]. Quantification of eosinophils was made automatically using the Image J plug-in particle quantification software (v. 1.45S). Data represent average number of 5 images per sample, 5 animals per group.

**Oximetry:** Before each time point of reperfusion, mice were anesthetized with ketamine/xylazine (100/20 mg/kg, i.p, respectively.) and linked to an oximeter by an infra-red collar sensor (MouseOx, Starr Life Science, USA). Arterial oxygen saturation was measured every 10 seconds for the period of 1 minute, totaling 6 recordings per mouse.
**Statistic analysis:** Data are represented as means ± SEM. Comparisons between groups were made by two-way ANOVA followed by Bonferroni post-test or Student’s t test. (GraphPad InStat software ver.5.0). Values of $P<0.05$ were considered statistically significant.
Results

Characterization of the obesity model

Mice maintained on high-fat diet for 9 weeks were characterized by a significant increase in body weight and abdominal fat deposition in comparison to mice fed with a standard low-fat diet (SD) for the same period of time (Fig. 1A and B). The high-fat diet (HFD) also induced a significant increase in circulating triglycerides (Fig. 1C) and cholesterol levels (Fig. 1D) when compared to mice fed a standard diet. There was no significant difference in basal plasma levels of estrogen between diets (0h) (Fig. 1E).

Lung Inflammation

Intestinal I/R caused a significant increase in lung tissue neutrophil number after 2 h of reperfusion that remained significant at 24 h in both groups when compared to the 0 h control (Fig. 2A). Immunohistochemistry measurements of MPO expression shows that HFD animals presented higher expression of MPO in the lung tissue in comparison to normal weight mice at 2h of reperfusion. At 24 h of reperfusion, levels of MPO expression were similar between diets (Fig. 2C). HFD mice presented significantly higher number of eosinophils relative to SD mice at 2 and 24 h of reperfusion (Fig. 2B). This difference was confirmed by measurements of EPO expression in lung tissue (Fig.2D).

Wet/dry lung weight ratio, inducible nitric oxide in the lung and arterial oxygen saturation

Mice fed a standard diet did not demonstrate a significant change in wet/dry lung weight ratio after 2 h or 24 h of reperfusion when compared to their controls at 0 h (Fig. 3A). In contrast, HFD mice developed a significantly greater wet/dry lung weight ratio after 2 h and 24 h of reperfusion when compared to 0 h levels and to SD mice (Fig.3A).
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The percentage of expression of inducible nitric oxide (iNOS) in the lung tissue was quantified by immunohistochemistry. Results show that HFD mice presented significantly higher levels of expression of iNOS in lung tissue at 0 h in comparison SD mice (Fig. 3B). After 24 h of intestinal reperfusion we observed a significantly higher percentage of expression of iNOS in the lung of HFD mice in comparison to SD mice.

There was no significant difference in arterial oxygen saturation between SD mice and HFD mice at 0 h (Fig. 3C). We also did not observe significant differences in arterial oxygen saturation in SD mice at 2 h or 24 h of reperfusion when compared to basal levels at 0 h (Fig. 3C). Similarly, there was no significant deterioration in arterial oxygen saturation in HFD mice at 0 and 2h of reperfusion. In contrast, there was a significant decrease in arterial O$_2$ levels in HFD mice at 24 h of reperfusion compared to SD mice and to 0 h (Fig.3C).

**Cytokine levels in the serum**

Intestinal I/R induced a significantly higher concentration of TNF-α in mice fed a standard diet at 2 h of reperfusion when compared to 0 h (Fig 4A). In addition, there was also a significantly higher level of the keratinocyte chemoattractant (KC) at 24 h of reperfusion when compared to 0 h (Fig. 5B). In contrast, HFD mice had a significant increase in levels of IL-6 and IL-10 at 24 h of reperfusion, when compared to 0 h and mice fed a SD (Fig. 4C and D). Mice fed high-fat diet presented significantly higher levels of circulating leptin in comparison to SD mice at 0h of reperfusion (Fig. 4E). We did not detect significant differences among groups regarding the levels of MIP-2 (Fig. 4F). Nor did we detect any differences in IL-17, VGEF, IL-1β or IL-5 in any of the samples analyzed (data not shown).
Discussion

In this study we have measured important biomarkers of lung injury induced by intestinal I/R, including leukocyte migration to the lung, oedema formation and arterial oxygen saturation measured at 2 h and 24 h after reperfusion [23]. Even though female mice fed a standard diet showed a significant increase in neutrophil numbers and MPO expression in the lung at 2 h and 24 h of reperfusion, SD female mice did not develop a significant increase in oedema, measured as wet/dry lung weight ratio, and did not show important alterations in arterial oxygen saturation, suggesting that the inflammatory state of their lungs was not severe enough to affect the gas exchange between lungs and circulation. These findings are consistent with previous observations suggesting that female rodents have an attenuated response to ischemic events, frequently attributed to a protective effect of estrogen [19, 24].

In mice fed a high-fat diet, neutrophil numbers and MPO expression were significantly increased post intestinal I/R, similar to those of the SD mice. However, wet/dry lung weight ratio and iNOS expression in the lung tissue was significantly higher in HFD mice compared to SD. This was accompanied by a significant deterioration of oxygen saturation in the blood at 24 h of reperfusion suggesting that obesity primes the lung of mice to the inflammatory effects of intestinal I/R.

Furthermore, a significant increase in lung eosinophil numbers, accompanied by significantly higher levels of eosinophil peroxidase expression was observed in the HFD mice relative to SD mice, after intestinal I/R treatment. This was an unexpected observation since the lung inflammatory response induced by intestinal I/R has been described as essentially mediated by neutrophils [23, 25]. Eosinophils, like neutrophils, are usually recruited to the site of inflammation from the circulation and the bone.
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marrow compartments. Some data indicate that they also reside in visceral adipose tissue under non-inflammatory conditions and are involved in fat tissue metabolism [7] [26]. Clinical and experimental evidence show that obesity correlates to exacerbated eosinophilic responses in the lung and to a general low grade inflammation status [27] [28, 29]. However, to the best of our knowledge evidence of an eosinophilic component of lung inflammation in mice after intestinal I/R has not been previously described.

We did not find significant differences in the serum levels of IL-5 between HFD mice and their normal weight counterparts (supplemental data). However, the basal level of leptin in serum was found to be significantly elevated in HFD mice and also in their adipose tissue after 2 h of intestinal I/R (supplemental data). Elevated levels of leptin are considered a biomarker of obesity, but it is also important in the maintenance of the energy balance in healthy individuals [6]. Leptin exerts pro-inflammatory effects and modulates functional activity of immune cells [30]. In fact, leptin levels in serum have been found to be elevated during eosinophilic inflammatory responses in the airways and IgE-associated atopic dermatitis in non-obese subjects [31] [32]. Taking this into account, it is possible to speculate that the elevated basal levels of leptin found in HFD animals could be influencing the influx of eosinophils to the lung tissue observed in our model. Interestingly, other authors have reported that obese asthmatic patients, mainly women, present a mixed neutrophil/eosinophil influx to the lung in comparison to lean asthmatic patients who present a predominantly eosinophilic inflammation [33, 34]. Our results show that a neutrophilic inflammation in HFD mice occurs with a concomitant influx of eosinophils, suggesting obesity may induce an abnormal mixed leukocyte response in the lung that does not depend on the nature of the stimuli.
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Data from the literature correlates nitric oxide with the development of remote organ injury, notably in lung after intestinal I/R [35]. Previous studies from our group have shown that the iNOS inhibitor, aminoguanidine, inhibits the increase of vascular permeability induced by intestinal I/R but does not affect neutrophil migration to the lungs of rats [36, 37]. In this study, we have found that intestinal I/R induced a significant increase in wet/dry lung weight ratio at 2 and 24 h of reperfusion in HFD but not in SD mice. In addition, we have also measured the levels of arterial oxygen saturation. This parameter reflects gas exchange function of the lungs and is one of the major markers used to diagnose, stratify, and monitor patients with ALI or ARDS [38]. Our results show that after 24 h of intestinal I/R, HFD mice have significantly reduced arterial oxygen saturation, in comparison to SD mice, that coincides with a significant increase in wet/dry weight ratio of the lung and iNOS expression. Overall, our results suggest a correlation among iNOS expression, lung edema and the deterioration of blood oxygenation observed after 24 h of intestinal I/R in obese mice.

Remote organ dysfunction, as observed in the lung after intestinal I/R, can be accompanied by a systemic inflammation. In this context, we have quantified plasma cytokines levels before and after the induction of intestinal I/R. Our results show that at 24 h of intestinal reperfusion, HFD mice presented a significant and robust increase of IL-6, IL-10 and a significantly increased level of leptin before induction of intestinal I/R. Clinical data obtained from obese patients with multiple risk factors for the development of acute lung injury (sepsis, trauma, pneumonia, etc) and experimental data from a murine model of LPS-induced lung injury have suggested that obese subjects release lower levels of pro-inflammatory cytokines in response to acute lung injury in comparison to lean controls [13, 39]. Our results do not support these observations and demonstrate that obesity is not attenuating the release of cytokines in
our model at the time points measured. This discrepancy is likely to be due to differences in model of lung injury and also time points when the measurements were performed.

In female rodents and premenopausal women, estrogen controls the deposition of adipose tissue by increasing subcutaneous fat accumulation and decreasing visceral fat deposition, primarily through an estradiol receptor (ER)-α-mediated mechanism [40]. Interestingly, in our model the level of estrogen in the circulation of HFD mice is not significantly different from SD mice, suggesting that in our model, obesity is not directly affecting the release of this sex hormone to the system. However, constitutive levels of circulating leptin are significantly higher in HFD mice in comparison to SD mice. It has been shown that leptin gene expression is highly influenced by estrogens, which are also produced in higher amounts in subcutaneous adipose tissue [41]. The leptin receptor on the hypothalamus co-localizes with the estrogen receptor-α and estrogen treatment decreases the expression of the leptin receptor on the arcuate nucleus, where leptin exerts its catabolic actions, increasing energy expenditure [42]. As we pointed out earlier, apart from its metabolic roles, leptin is also an important mediator of immune responses and inflammation [30]. In this scenario, it is possible that in our model the high levels of leptin found in HFD mice interact with estrogens. Such an interaction may indirectly override the protective effect observed in ALI induced by I/R injury in female rodents by mechanisms that have yet to be clarified.

In summary, our study demonstrates that obesity worsens the response to intestinal I/R in the lung of female mice. Obese female mice develop a significant increase in lung edema, iNOS expression and, more importantly, a significant decrease in arterial oxygenation at 24 h post intestinal I/R, suggesting a rapid deterioration of gas
exchange in the lungs of these animals that is not observed in normal weight female mice.

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References

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Legend of the figures

Figure 1: C57Bl/6 mice were fed with a standard low-fat diet or 30% high-fat diet, starting at weaning, for 9 weeks. (A), comparison of body weight gain; N=9 and 10, respectively, *p< 0.05 (B), abdominal fat weight, N=9 and 11, respectively. **p<0.001 (C) circulating cholesterol levels, N=6 and 8, respectively. *p<0.05 (D) Circulating triglyceride levels, N=12 and 7, respectively; *p < 0.05, (E) Basal levels of estrogen measured in plasma. N=9 and 11, respectively. All data analyzed by non-parametric Student’s t test. All data expressed as mean±S.E.M.

Figure 2: (A) neutrophil numbers in lung tissue. N= from left to right 5, 5, 5, 4, 5 and 4. (B) Eosinophil numbers in lung tissue. N= from left to right 5,5,5,4,5 and 4 (C) MPO expression in lung tissue. N= from left to right 5, 5, 4, 5 and 4 (D) EPO expression in lung tissue. N=5,5,5,4,5 and 4. *p<0.5 in comparison to basal levels, ***p<0.001 in comparison to basal levels, ****p<0.0001 in comparison to basal levels, α p<0.0001 in comparison to SD mice. All data was analyzed by 2-way ANOVA followed by Bonferroni post hoc test. All Data are expressed as mean±S.E.M.

Figure 3: (A) Wet/dry weight ratio of lung tissue. N=9 per group. *p< 0.05 in comparison to basal levels, α p<0.001 in comparison to SD mice. (B) Expression of iNOS in lung tissue. N=4/group **p<0.01 in comparison to SD mice. ****<p 0.0001 in comparison to basal levels. α p<0.0001 in comparison to SD mice. (C)Arterial oxygen saturation expressed as percentage. From left to right N= 11, 8, 6, 7 and 6, respectively. *p<0.05 in comparison to SD mice. All data was analyzed by 2-way ANOVA followed by Bonferroni post hoc test. All data are expressed as mean±S.E.M.

Figure 4: Levels of circulating cytokines measured in the serum by multiplex ELISA. From left to right N=15, 12, 11, 9, 9 and 7, respectively. *p<0.05 in comparison to mice fed a SD (A, B and C). *p<0.05 in comparison to mice fed a HFD (D and E). 2-
way ANOVA followed by Bonferroni post hoc test. All data are expressed as mean±S.E.M.
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