Intrauterine growth restriction affects diaphragm function in adult female and male mice

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ABSTRACT

Background: *In utero* diaphragm development is critically important for postnatal respiratory function and disturbance to fetal development may lead to diaphragm dysfunction and respiratory complications in the postnatal period. Intrauterine growth restriction (IUGR) has been shown to affect respiratory function in a sex-dependent manner; however, the effect of IUGR on diaphragm function is unknown.

Aim: This study used a maternal hypoxia-induced mouse model of IUGR to investigate the impact of IUGR on diaphragm function and structure in male and female adult offspring.

Methods: Pregnant BALB/c mice were housed under hypoxic conditions (10.5 % O_2) from gestational days 11–17.5 and then returned to normoxic conditions. Control mice were housed under normoxic conditions throughout pregnancy. At 8 weeks of age, offspring were euthanized and diaphragms isolated for functional assessment in organ bath experiments and for histological analysis.

Results: IUGR offspring were lighter at birth and remained lighter at 8 weeks of age compared to Controls. While diaphragm force (maximal or twitch) was not affected by treatment or sex, the IUGR group exhibited a longer half relaxation time after twitch contractions compared to Control. Female offspring had a lower maximum rate of force development and higher fatigue resistance compared to males, independent of IUGR. There was no difference in the diaphragm myofibre cross-sectional area between groups or sexes.

Conclusion: Sex and IUGR independently affect diaphragm contraction in adult mice without changes in structure. This study demonstrates that IUGR affects diaphragm contractile function in later life and could impair respiratory function if exacerbated under conditions of increased respiratory load.

INTRODUCTION

In utero development of the diaphragm is critically important for the establishment of spontaneous, unsupported breathing at birth[1]. Contraction of the diaphragm, together with the intercostal muscles, creates a negative intrathoracic pressure which draws air into the lungs and enables respiration to occur[2]; conversely, relaxation of respiratory muscles facilitates expiration which is passive under resting conditions. The diaphragm fully develops in the womb[3], and fetal exposure to adverse *in utero* conditions such as inflammation[4] and undernutrition[5] impairs diaphragm contractile function.

Adverse conditions *in utero* can lead to intrauterine growth restriction (IUGR), a disorder that affects 6.5 % of the newborn population and is associated with future respiratory disease[6; 7]. The effects of IUGR have been examined in tissues other than the diaphragm. In a sheep model of IUGR produced by placental restriction that was initiated from the saccular phase (late gestation) until term, the number of alveoli was reduced and there was thickening of the pulmonary blood-air barrier[8]. In a rat model of hypoxia induced-IUGR administered during late gestation, cardiac remodelling and impaired post-ischemic recovery is observed in adult offspring[9]. We have also previously shown that IUGR affects airway structure and function in adult offspring and identified a sex-dependent response to IUGR that had not been previously considered in other studies[10]. Using a mid-gestation (GD 10.5 – 17) maternal hypoxia-induced IUGR mouse model, we showed that airways of IUGR male offspring were hypo-responsive during a bronchoconstrictor challenge, and in contrast, IUGR female offspring were hyper-responsive compared to Controls[10]. Whether IUGR affects diaphragm function in adult offspring is not known and is of high interest since changes in diaphragmatic function may directly affect ventilation in postnatal life and indirectly affect *in utero* development of other tissues and organs through altered mechanotransduction pathways produced by fetal breathing movements[12].

78 The aim of this study was therefore to determine the effects of IUGR induced within
79 the period of diaphragm development[3] on diaphragm function and structure in postnatal life.
80 Since IUGR has been shown to have sex-dependent effects in other respiratory tissues (i.e.,
81 airway wall)[10], the effects of IUGR on diaphragm structure and function were also compared
82 between males and females to determine whether IUGR impacts the diaphragm in a sex-
83 dependent manner.

METHODS

Maternal hypoxia-induced IUGR mouse model

All procedures involving animals were conducted at The University of Western Australia and approved by institutional Animal Ethics Committee (Approval Number RA/3/100/1570). Pregnant BALB/c mice were obtained from Animal Resources Centre (Murdoch, WA, Australia) at GD 7 and housed in a hypoxic chamber from GD 11 – 17.5 with oxygen levels maintained at 10.5 %[10]. Following hypoxic exposure, mice were returned to normoxic conditions (21 % O_2) until birth (GD 21). A Control group of dams were kept at normoxic conditions throughout the gestational period[10]. Offspring were kept with dams until weaning at 3 weeks of age and then housed with offspring of the same sex and group (IUGR or Control) with access to standard chow and water *ad libitum*.

Tissue sampling

One male and one female offspring were randomly chosen from each litter for analysis (IUGR male n = 8; IUGR female n = 10; Control male n = 8; Control female n = 8). Body weights of offspring were recorded at birth and again at 8 weeks of age, at which point mice were euthanized by an overdose of intraperitoneal injections of ketamine (240 mg/kg) and xylazine (12 mg/kg). The diaphragm was then excised and separate strips of fibres were taken for functional assessments in organ bath experiments or histological examination.

Organ bath experiments

A longitudinal section of muscle fibres from the costal diaphragm was mounted in an *in vitro* muscle test system (1205A, Aurora Scientific, Aurora, Canada) containing mammalian Ringer's solution, bubbled with carbogen at 25 ºC[1]. The muscle was stimulated electrically using two parallel electrodes situated on either side of the diaphragm[1]. Optimal muscle length

was determined and then muscle force was stabilised using 5 maximal 80 Hz isometric contractions for 500 ms at 3 min intervals. A twitch response was then elicited and contractile properties of peak twitch force (g), time to peak (ms), half relaxation time (time taken for the force to decrease to half of the peak twitch force, ms) and maximum rate of force development (g/s) were measured. A force frequency curve was then recorded at stimulation frequencies between 5 – 80 Hz[4].

The susceptibility to fatigue was evaluated by stimulating the muscle with a series of 900 tetanic contractions of 500 ms durations at 60 Hz repeated every 2 s. The fatigue index was determined by expressing the force produced during the 900th contraction as a proportion of the initial contraction where a higher number indicates a greater fatigue resistance. Force was normalised to cross-sectional area (CSA) calculated from optimal muscle fibre length, muscle mass and density (1.056 g.cm^{-3}) and presented as specific force (N.cm^{-2})[1].

Histology

A separate longitudinal strip of diaphragm fibres was dissected for histological analysis. The strip was fixed in tragacanth gum and frozen in isopentane cooled by liquid nitrogen and stored at -80 °C. Frozen sections (8 µm) were cut on a Leica (CM3050) cryostat (Leica Biosystems, Wetzlar, Germany) and stained with haematoxylin and eosin to measure fibre CSA. Myofibre CSA was determined from tiled images captured at 20 × using a Nikon Upright microscope (Nikon Corporation, Minato, Tokyo, Japan). Image analysis was performed using ImageJ (v1.51j8, National Institutes of Health, Bethesda, Maryland, USA) software and ImageJ cell counter plugin (https://imagej.nih.gov/ij/plugins/cell-counter.html). The outside of each fibre was manually traced and area averaged across 100 cells per sample to calculate fibre CSA (NB. if 100 cells were not present then every viable cell for the sample was measured)[13].

Statistical analysis

Student's t-test was used to compare birth weight of Control and IUGR groups. Two-way ANOVAs with Holm-Sidak *post-hoc* corrections were performed with treatment (Control vs IUGR) and sex (male vs female) as factors for eight-week weight, contractile measurements and myofibre CSA. Data were normally distributed and are presented as mean ± SEM. Statistical analyses were performed by SigmaPlot version 13.0 (version 13, San José, CA, USA) and figures prepared using GraphPad PRISM version 7 (version 7, La Jolla, CA, USA). n refers to the number of offspring and $P < 0.05$ was considered statistically significant.

RESULTS

Growth outcomes

As expected, IUGR offspring were born with significantly smaller body weights compared to Controls (Control, 1.61 ± 0.05 g, IUGR, 1.43 ± 0.04 g; $P = 0.013$; Figure 1A). At 8 weeks of age, there were significant differences between treatments with IUGR groups weighing less than their Control counterparts ($P < 0.001$; Figure 1B), and between sexes with males weighing more than females ($P < 0.001$; Figure 1B).

Diaphragm contractile properties

Maximum diaphragmatic specific force was not affected by treatment ($P = 0.478$) or sex ($P = 0.098$; Figure 2). There was no effect of treatment on peak twitch force or time to peak force (IUGR v Control); however, female offspring had lower maximum rate of force development compared to males (Table 1). The IUGR group had a significantly longer half relaxation time compared to the Control group ($P = 0.015$; Figure 3) independent of sex ($P = 0.368$; Figure 3). There was no difference in the force-frequency relationship between IUGR and Control groups in either males ($P = 0.995$; Figure 4A) or females ($P = 0.258$; Figure 4B). While females had a higher fatigue index (i.e., higher resistance to fatigue) than males ($P = 0.001$; Figure 5), there was no effect of treatment ($P = 0.993$; Figure 5). For all parameters outlined above, there were no interaction between treatment and sex.

Histology

Myofibre CSA of the diaphragm was not affected by treatment ($P = 0.859$; Figure 6) or sex ($P = 0.704$). There was also no interaction between treatment and sex ($P = 0.676$).

DISCUSSION

IUGR infants are characterised by low body weight at birth and represent 6.5 % of the Australian newborn population[6]. Individuals subject to IUGR may endure both long and short term consequences. Short term consequences include increased perinatal mortality rates and long term there is increased morbidity in 50 % of surviving infants[14; 15]. This is in line with the Development Origins of Health and Disease hypothesis stating that IUGR is the root cause of several health disorders in adult life[16]. Of relevance to the present study, strong associations have been found between IUGR and the development of respiratory disease, including COPD[10; 17]. Since had been show to affect other respiratory tissues, we hypothesised that IUGR would also impact diaphragm structure and function. Our main finding that the diaphragm of adult IUGR offspring had significantly slower relaxation rates indicates the critical impact of adverse exposures during fetal development on diaphragm contractile function in later life.

The mid-gestation phase of fetal development is an important determinant of proper diaphragm structure and function and therefore disruptions to diaphragm development during this phase may contribute to respiratory insufficiency in chronic respiratory diseases[3; 18]. Our previous rodent studies have shown that IUGR alters airway structure and function when IUGR was limited to the periods of peak airway development (GD 10.5 – 17)[10; 11]; a mid-gestation period which also corresponds to the development of other respiratory system components including the diaphragm. We have also demonstrated in an ovine model of chormioamnionitis that *in utero* inflammation impairs contractile function of the fetal diaphragm[4] and the severity of this contractile dysfunction is greater when the inflammatory stimulus is administered during early compared to late gestation[13]. Offspring from a maternal steroid exposure rat model during late gestation (GD 15 – 17) exhibited decreases in diaphragm force production and fatigue resistance and altered anabolic signalling that were not evident in the first week after birth, but manifested at a postnatal age of 3 weeks[19]. Data from a chronic maternal undernutrition rat

model showed a complex time course of diaphragm dysfunction; maximum force production in undernourished offspring was lower than controls at 7 d and 14 d after birth, not different to Controls at 21 d after birth, but significantly lower again in adulthood (12 weeks of age)[5]. These studies therefore demonstrate essential prenatal windows of susceptibility for abnormal diaphragm function which may manifest at various time points after birth.

As expected, offspring of hypoxia exposed dams weighed less at birth, and remained smaller in adulthood than their Control counterparts. This growth profile is in line with our previous maternal hypoxia-induced IUGR mouse cohort showing that IUGR offspring are not only born smaller than Controls but remain smaller into adulthood[10]. At 8 weeks of age (adulthood), a sex effect was also present with male offspring weighing more than females in both Control and IUGR groups.

There were no differences in maximum specific force or time to peak twitch force between IUGR and Control groups. These results were somewhat unexpected as low birth weight is associated with reduced muscle mass and strength in peripheral skeletal muscles in adulthood[20; 21]. In comparison to force development, there was a clear effect of IUGR on twitch half relaxation time which was evident for both male and female mice. Half relaxation time reflects the kinetics of relaxation after stimulation and is heavily influenced by muscle fibre type. The aforementioned maternal undernutrition study found that the proportion of type II fibres was lower in the undernutrition group during early postnatal development but was comparable with Control in adulthood[5]. However in our study, it is unlikely that a longer half-relaxation time in the IUGR group reflects changes in fibre type distribution as there were no changes in other time-dependent parameters (twitch time to peak and maximum rate of force development) or resistance to fatigue which would also be expected to change with alterations in fibre type proportions[22]. Half relaxation time is also dependent on the rate of detachment of myosin heads from actin and by the reduction in cytosolic Ca^{2+} by the action of the sarco

endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump[23]. The detachment of the myosin head from actin is relatively quick and therefore a less probable mechanism for increased relaxation time and we therefore favour changes in the actions of the SERCA pump[24].

It is possible that IUGR affects the expression or function of Ca^{2+} handling proteins including SERCA or plasma membrane Ca^{2+}-ATPase pumps as demonstrated in human placenta cells[25; 26]. Similar effects on the Ca^{2+} handling proteins in the diaphragm may reduce Ca^{2+} transport back into the sarcoplasmic reticulum and extending relaxation time[23; 27]. Two SERCA isoforms are expressed in skeletal muscle; SERCA1 in fast-twitch muscle fibres and SERCA2 in slow-twitch muscle fibres[28]. Both of these pumps have similar Ca^{2+} transport capacities and work in the same manner within their respective fibre type[28]. Disruption of SERCA1 expression severely impairs diaphragm function with SERCA1 null offspring dying of respiratory failure shortly after birth[29]. Furthermore, disruption of SERCA2 gene expression in mice prolongs muscle relaxation time without affecting contraction time or maximal force[27]. Therefore, a difference in SERCA pump expression could contribute to the prolonged muscle relaxation time without altering other contractile properties, as was observed in this study.

Interestingly, there were sex differences in the maximum rate of force development and susceptibility to fatigue that occurred independent of treatment. Firstly, the maximum rate of force development was lower in females than in males. This is in line with previous studies showing that the maximal rate of force development is significantly higher in males than females, determined by differences in myosin heavy chain isoforms that influence muscle function[30-32]. Sex differences were also evident in fatigability, with females having a higher fatigue index (greater resistance to fatigue) than males. The difference in fatigability may be due to sex steroids with both estrogens and androgens being potent regulators of postnatal skeletal muscle growth and development[33]. Estrogen has been shown to have positive effects on skeletal muscle contraction and protect against post-exercise muscle damage and

240 inflammation in rats[34]. Furthermore, estrogen deficiency has been shown to adversely affect
241 muscle oxidative metabolism and mitochondrial function illustrating the critical importance of
242 estrogen in maintaining efficient fuel metabolism and hence resistance to fatigue[35; 36].

243 Histological analysis revealed that there were no differences in myofibre CSA between
244 either treatment groups or sexes, suggesting that the observed functional differences occurred
245 independently of changes in myofibre size. This result is different from a study of chronic
246 maternal undernutrition-induced growth restriction which found a reduction in myofibre size
247 in the undernutrition offspring compared with Control, which may be attributed to type,
248 duration and timing of prenatal insults[5]. Our finding also contradicts previous studies in both
249 human and sheep that show IUGR fetuses have smaller skeletal muscle myofibres when
250 compared to Controls and another study showing that twin fetuses were born smaller with a
251 smaller myofibre CSA compared to singleton lambs[37; 38]. However, it is important to
252 acknowledge that these previous studies focused on peripheral skeletal muscles which are
253 known to have different functions and responses to adverse insults compared to the
254 diaphragm[39]. Furthermore, the assessments of myofibre CSA in these studies were performed
255 at birth, whereas we have examined diaphragm fibre CSA in adult mice. It is possible that
256 diaphragm fibre CSA was reduced at birth in our IUGR mice, but had recovered to be
257 indistinguishable from Control mice by adulthood despite a sustained reduction in body weight.
258 We also acknowledge that changes in diaphragm muscle mass could occur independently of
259 changes in individual myofibre CSA, for example due to hypoplasia[40], but this was not possible
260 to examine in the current study.

261 It is important to note that this study was conducted on young adult mice that were
262 otherwise healthy. Diaphragm dysfunction is evident in several respiratory diseases such as
263 COPD which primarily occurs later on in life[2; 41]. Therefore it may be worthwhile to study these
264 mice as older adults or to impose various challenges to the diaphragm in adulthood, such as an

inflammatory response to lipopolysaccharide exposure, to investigate if the observed IUGR effects persist or worsen when exposed to aging and stress[13; 42]. Furthermore, the effects of IUGR on the diaphragm could have been indirectly mediated by the impact of IUGR on airway function[10]; however, the effects of IUGR on airway function were primarily observed after bronchoconstriction induced to methacholine and were also sex-dependent. Since mice were not exposed to bronchoconstrictor triggers in the present study, nor was there was there any interaction between IUGR and sex, an indirect effect of IUGR on diaphragm is unlikely.

In summary, these data demonstrate that IUGR affects diaphragm function in otherwise healthy adult mice. Further research is required to determine if IUGR induced diaphragm dysfunction is exacerbated under conditions of increased respiratory demand, which might accompany normal ageing processes, or in response to other environmental insults.

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DISCLOSURE STATEMENT

None.

AUTHORS CONTRIBUTION

K.C.W.W., G.J.P., P.B.N. designed the study; M.R.F., K.C.W.W. conducted the study, M.R.F., G.J.P., K.C.W.W. were involved in data collection; M.R.F., G.J.P., K.C.W.W. were involved in data analysis; all authors were involved in data interpretation; M.R.F., K.C.W.W. drafted the manuscript and all authors contributed and approved the final manuscript writing.

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399

FIGURE LEGENDS

Figure 1. Body weight of offspring. Offspring body weights (g) at birth (A) and at 8 weeks of age (B). Data are mean ± SEM. * indicates a significant effect of treatment ($P < 0.05$), # indicates a significant effects of sex ($P < 0.05$). Control, open bars; IUGR, closed bars. IUGR; intrauterine growth restriction.

Figure 2. Diaphragm maximum specific force. Maximum specific force (N/cm^2) generated by diaphragm muscles of 8–week-old male and female mice. Data are mean ± SEM. Control, open bars; IUGR, closed bars. IUGR, intrauterine growth restriction.

Figure 3. Diaphragm twitch half relaxation time. Twitch half relaxation time (ms) in diaphragm muscles of 8-week-old male and female mice. Data are mean ± SEM. * indicates a significant effect of treatment ($P < 0.05$). Control, open bars; IUGR, closed bars. IUGR, intrauterine growth restriction.

Figure 4. Diaphragm force-frequency curves. Force generated at different stimulation frequencies by diaphragm muscles of 8-week-old male (A) and female (B) offspring presented as specific force (N/cm^2). Data are mean ± SEM. Control, open symbols; IUGR, closed symbols. IUGR, intrauterine growth restriction. NB. force-frequency data points in the male Control and IUGR groups (A) are almost identical, therefore the IUGR symbols are plotted behind the Control symbols.

Figure 5. Diaphragm fatigability. Fatigue index (force at 900^{th} contraction/initial contraction) of diaphragm muscles of 8-week-old male and female offspring. Data are mean ± SEM. # indicates significant difference between sexes ($P < 0.05$). Control, open bars; IUGR, closed bars. IUGR, intrauterine growth restriction.

Figure 6. Diaphragm myofibre cross-sectional area. Representative histological figure of diaphragm myofibre stained with haematoxylin and eosin in Control (A) and IUGR (B) offspring. Myofibre cross sectional area (μm^2) of diaphragm muscle in 8-week-old male and female mice (C). Data are mean ± SEM. Control, open bars; IUGR, closed bars. IUGR, intrauterine growth restriction.

TABLE

Table 1. Diaphragm twitch contraction.

	Control		**IUGR**	
	Male (n=8)	**Female (n=10)**	**Male (n=8)**	**Female (n=8)**
P_t (N/cm^2)	5.76 ± 0.73	5.45 ± 0.42	5.37 ± 0.38	4.87 ± 0.42
TTP (ms)	38.46 ± 1.15	42.50 ± 2.60	42.04 ± 1.04	40.58 ± 1.71
Maximum dF/dt (g/s)	144.10 ± 14.39	132.33 ± 18.41	158.79 ± 16.59	103.72 ± 13.17#

Data are mean ± SEM. # indicates a significant effect of sex. IUGR, intrauterine growth restriction; P_t, peak twitch force; TTP, time to peak; dF/dt, rate of force development.

FIGURES

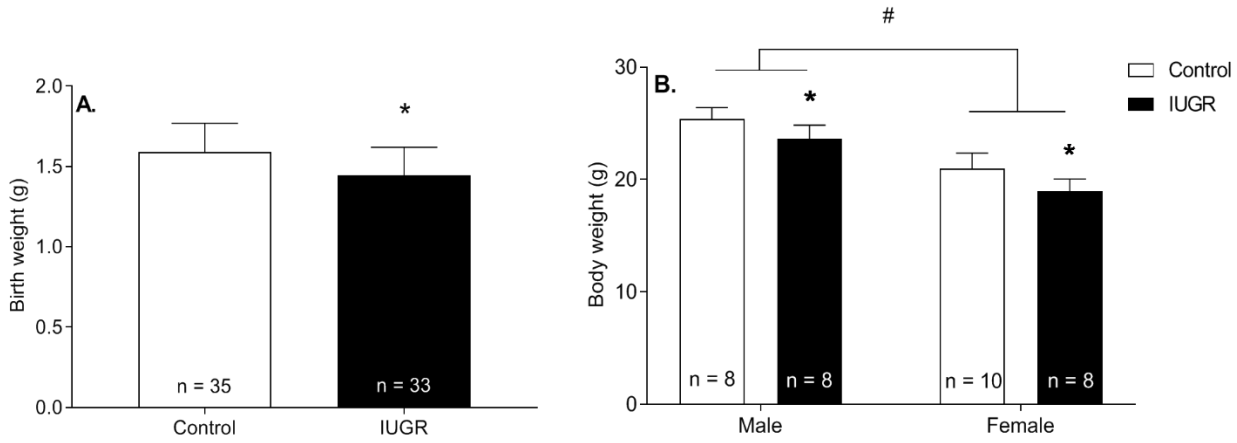
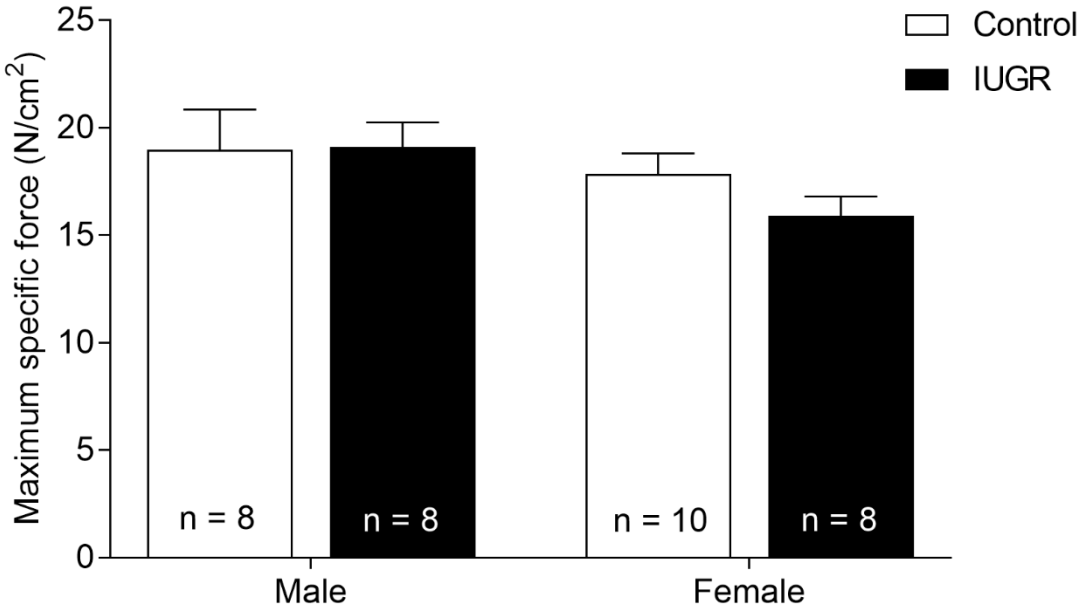


Figure 1.



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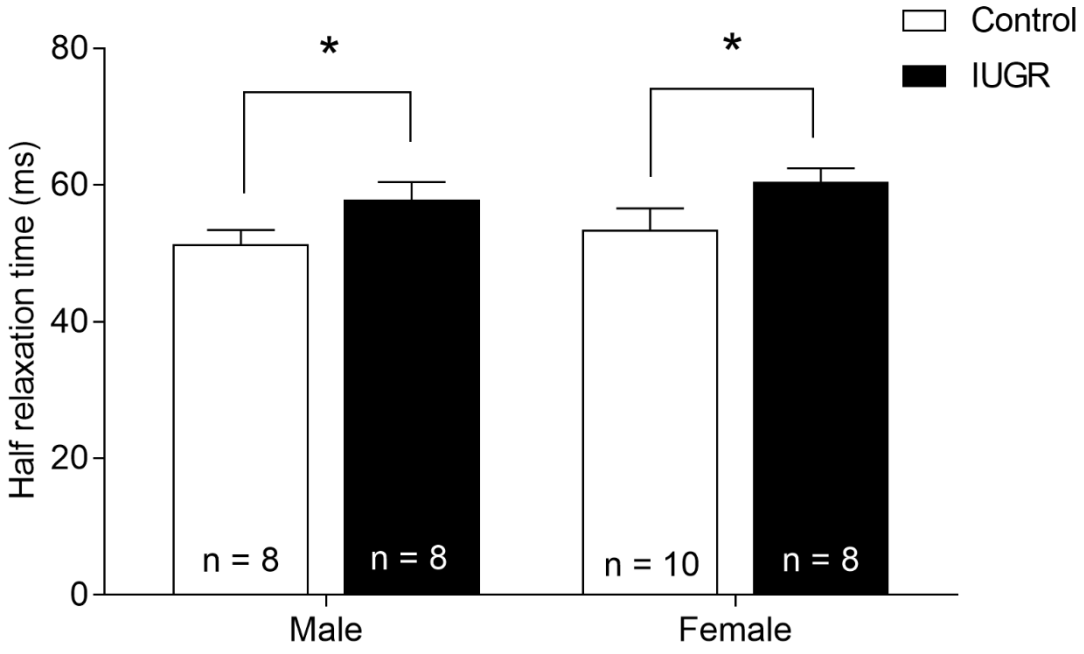
436 **Figure 2.**



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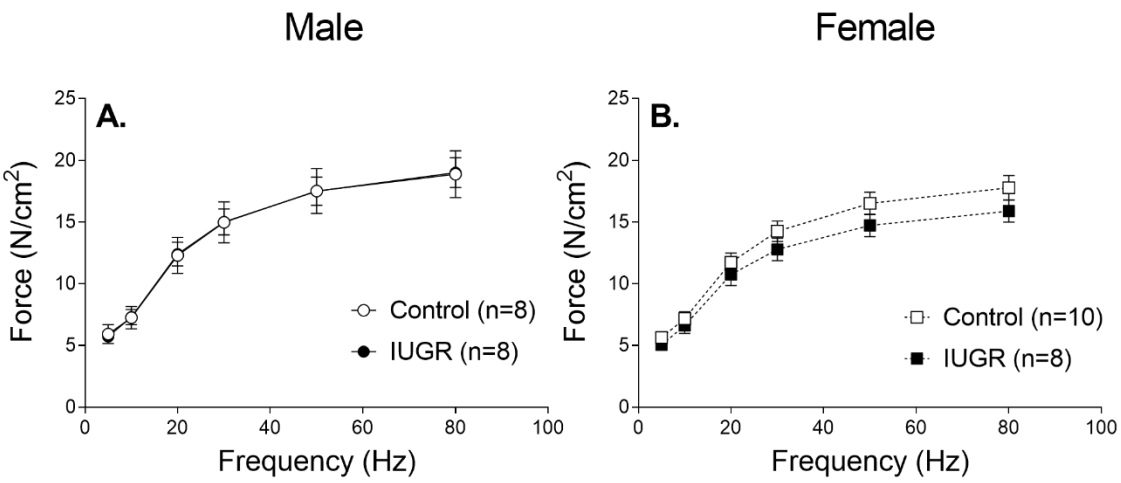
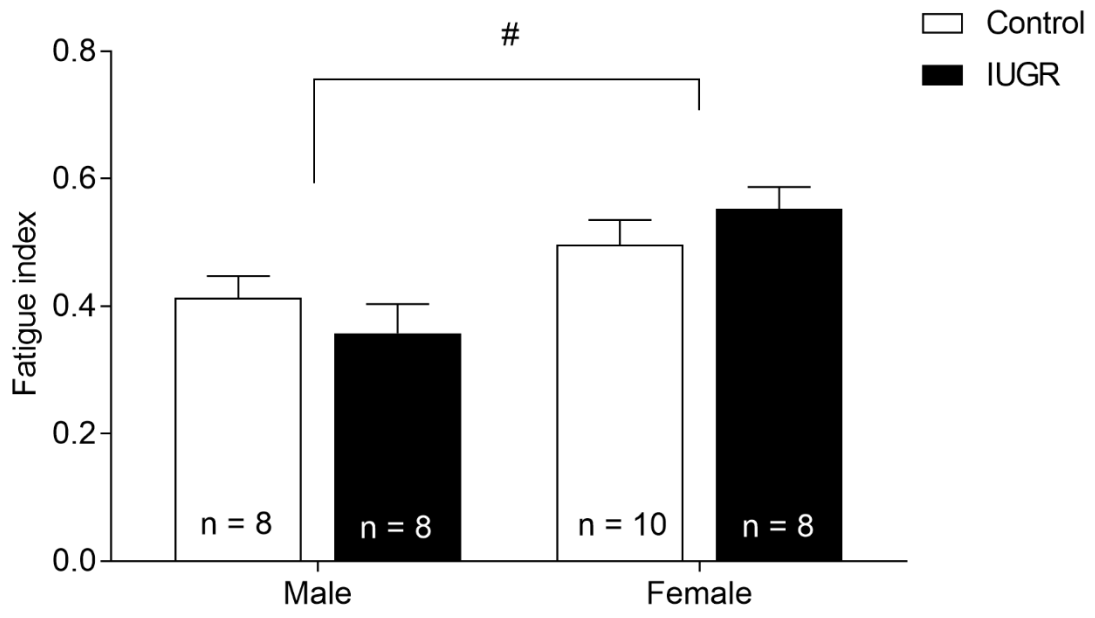
438 **Figure 3.**



439

440 **Figure 4.**

441



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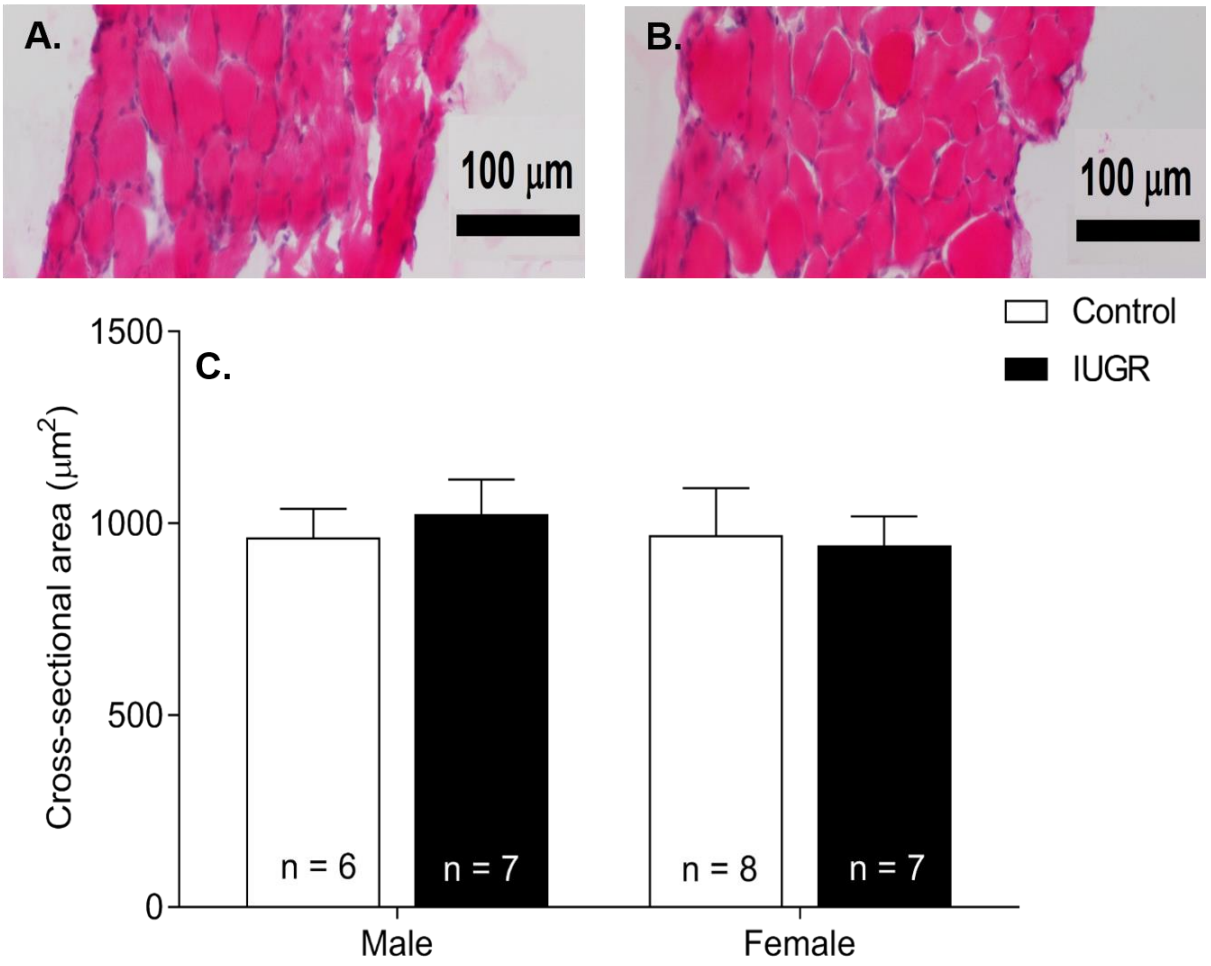
443 **Figure 5.**



444

445 **Figure 6.**