Transient neonatal exposure to hyperoxia, an experimental model of preterm birth, leads to skeletal muscle atrophy and fiber type switching.

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Abstract

Individuals born preterm show reduced exercise capacity and increased risk for pulmonary and cardiovascular diseases, but the impact of preterm birth on skeletal muscle, an inherently critical part of cardiorespiratory fitness, remains unknown. We evaluated the impacts of preterm birth-related conditions on the development, growth, and function of skeletal muscle using a recognized preclinical rodent model in which newborn rats are exposed to 80% oxygen from day 3 to 10 of life. We analyzed different hindlimb muscles of male and female rats at 10 days (neonatal), 4 weeks (juvenile) and 16 weeks (young adults). Neonatal high oxygen exposure increased the generation of reactive oxygen species and the signs of inflammation in skeletal muscles, which was associated with muscle fiber atrophy, fiber type shifting (reduced proportion of type I slow fibers and increased proportion of type IIb fast-fatigable fibers), and impairment in muscle function. These effects were maintained until adulthood. Fast-twitch muscles were more vulnerable to the effects of hyperoxia than slow-twitch muscles. Male rats, which expressed lower antioxidant defenses, were more susceptible than females to oxygen-induced myopathy. Overall, preterm birth-related conditions have long-lasting effects on the composition, morphology, and function of skeletal muscles; and these effects are sex-specific. Oxygen-induced changes in skeletal muscles could contribute to the reduced exercise capacity and to increased risk of diseases of preterm born individuals.

Key words: Skeletal muscle; Preterm birth; Muscle atrophy; Oxidative stress
Clinical perspectives:

- Young adults who were born preterm show a reduction in exercise capacity and muscle endurance that has been associated with reduced cardiopulmonary function; however, the impact of preterm birth on the development, growth, and function of skeletal muscle, an inherently critical determinant of exercise capacity and cardiopulmonary health, remains unknown.

- Using a rodent model of neonatal exposure to hyperoxia, we show that preterm birth-related condition induces an imbalance in the redox status in skeletal muscle associated with chronic inflammation, muscle atrophy, fiber type switching, and impaired muscle function. These alterations are sex-specific (more severe in males) and persist until adulthood.

- This study demonstrates that preterm birth-related conditions alter skeletal muscles, which opens a novel field of investigation in understanding the pathophysiology of chronic cardiopulmonary disease risks of individuals born preterm. Understanding the impact of preterm birth-related conditions on skeletal muscles is needed to optimize prevention and develop therapeutic approaches to the specific physiology of individuals born preterm.
Introduction

Improved neonatal care over the last 3 decades has allowed the survival of the vast majority of infants born preterm (< 37 weeks). Consequently, about 10% of current children and young adults were born preterm, including 1.5% very preterm (< 32 weeks) [1]. Preterm birth and related conditions can alter the development of the pulmonary and cardiovascular system [2,3], and adults born preterm show an increased risk of chronic lung and cardiovascular diseases. These dysfunctions have traditionally been evoked to explain the reduced exercise capacity in children and adults born preterm [4,5]. However, the impact of preterm birth on skeletal muscles, an inherently critical part of exercise capacity and of cardiorespiratory health, has been overlooked.

Skeletal muscles are mostly constituted of long cylindrical multinucleated cells, called myofibers, which are responsible for muscle contraction. The formation of myofibers during prenatal development is divided into primary myogenesis (until week 10 of gestation), during which a small number of large primary myofibers emerge and serve as templates for the formation of smaller myofibers during secondary myogenesis (between weeks 11-20 of gestation) [6,7]. Thereafter, during the end of the second and the beginning of the third trimester, at the time of preterm birth, the skeletal muscles undergo a critical phase of maturation characterized myofiber growth, fiber typing determination (slow vs fast fibers), and maturation of the excitation-contraction coupling system. Physiological fetal hypoxia plays a critical role in the proper regulation of these processes [8]. In vitro experiments have shown that the levels of oxygen have a decisive impact on the establishment of fiber typing [9]. Upon preterm birth, infants are exposed to higher blood oxygen levels (versus relative hypoxia in utero), which could interfere with muscle growth, fiber typing, and contractile function.

Few studies have provided indirect evidence of the contribution of skeletal muscles to
exercise capacity in preterm born individuals. These studies suggest a reduced skeletal muscle mass, with lower maximal power output, and altered strength and endurance in individuals born preterm [4,5,10,11], that is proportional to the decrease in gestational age [12]. Supporting the clinical observations, experimental rodent model of bronchopulmonary dysplasia (BPD) showed that transient neonatal exposure to high oxygen levels is associated with higher body weight without increase in muscle mass, lower muscle mitochondrial biogenesis, lower oxidative activity and compensatory higher glycolytic enzyme expression [13]. However, the impact of preterm birth-related conditions on skeletal muscle growth, composition, fiber typing, and contractile function remains unknown. Considering that skeletal muscle atrophy was shown to be associated with chronic obstructive pulmonary diseases and contributes negatively to the patient’s prognosis [14], and that very preterm birth is associated with higher risk of BPD and reduced exercise capacity, it is essential to investigate further the direct contribution of preterm birth-related conditions on skeletal muscles.

In the context of preterm birth, newborns are exposed to high levels of oxygen, especially compared to in utero oxygen tension. This relative “hyperoxic” environment is often exacerbated by neonatal oxygen supplementation. The sudden rise in oxygen levels induces an increase in reactive oxygen species (ROS) production while the antioxidant system is still immature [15]. Elevated ROS production can activate canonical Nuclear Factor-kappa B (NF-κB) signaling and the expression of downstream inflammatory genes, such as Tumor Necrosis Factor-alpha (TNF-α), Interleukin (IL)-1β, and IL-6 [16]. These changes have been shown to promote muscle wasting and fibro-fatty replacement in different pathological conditions [17-20] including sarcopenia (aging) and chronic obstructive pulmonary diseases [21]. Therefore, we hypothesize that neonatal hyperoxia induces changes in the redox balance and the inflammatory status that are associated with long-lasting alterations in muscle fiber structure,
typing, and function. We used a rodent model of transient neonatal exposure to high oxygen, which is well-established to mimic prematurity-associated conditions such as BPD, retinopathy of prematurity, cardiovascular, and renal complications [3,13,22]. This model is highly relevant to study the impact of preterm birth on the development of skeletal muscle, as the skeletal muscles of newborn rats are in a stage of development equivalent to that of humans born very preterm [23,24]. Our findings demonstrate that transient neonatal exposure to high oxygen induces an oxidative stress and a chronic inflammatory state in skeletal muscles, which is associated with reduced muscle mass and fiber size, shift in the proportion of fiber types toward fast fatigable fibers, and impaired contractile properties of the motor units.
**Materials and Methods**

**Animals**

Sprague-Dawley (Charles River, St-Constant, Québec, Canada) pups were maintained with a dam in 80% O₂ (OI, oxygen-induced injury group) in an oxycycler (A82OCV, Biospherix) or in room air (CTRL, control group) from day 3 to 10 of life. Dams in hyperoxia were interchanged every 12 h with a dam from a litter maintained in room air to avoid maternal O₂ toxicity. Both males and females were studied. In total 8 litters of 12 rats were used (4 litters per group: OI and CTRL) and no more than 4 animals per litter (2 males and 2 females, chosen randomly) were used for each experimental procedure. No mortality was observed during the transient neonatal hyperoxia exposure and no pups were excluded during the experimental procedure. All animals were housed, and all experimental procedures have taken place in the animal care facility of the Centre Hospitalier Universitaire Sainte-Justine. All experiments followed a protocol respecting the principles of the Canadian Council on Animal Care Guide for the Care and Use of Experimental Animals. All protocols were approved by the animal care committee of the Centre Hospitalier Universitaire Sainte-Justine.

**Experimental procedures**

Animals were measured and weighted prior to sacrifice (decapitation under anaesthesia by inhalation of isoflurane, 2-3%/L O₂) at 10 days (neonatal), 4 weeks (juvenile) and 16 weeks (adult). The Lee index (surrogate marker for body mass index [25]) was calculated with the equation: weight x 0.33/naso-anal length. The tibialis anterior (TA, fast-twitch muscle), and the soleus (slow-twitch muscle) were collected, flash frozen in liquid nitrogen, or embedded in Tissue-Tek® optimal cutting temperature compound (OCT compound, Sakura Finetek USA Inc., Torrance, CA) and frozen in 2-Methylbutane (Sigma-Aldrich, Missouri, USA) cooled in liquid nitrogen. For ex vivo muscle function at 4- and 16 weeks, animals were...
anesthetized with i.p. injection of pentobarbital sodium (50 mg/kg) and maintained
anesthetized during the dissection to keep the perfusion in the extensor digitorium longus
(EDL, fast-twitch muscle).
For all following experiments and analyses, the experimenters were blinded to the identity of
the samples.

Histology and immunofluorescence
Histological and immunostaining were performed on transversal cryosections (10 μm) from
proximal, medial, and distal parts of the TA and soleus muscles. Coloration by hematoxylin
and eosin (Sigma-Aldrich) was used for morphological analysis, Sirius Red (kit Sirius Red
24901-500, PolySciences, Toronto, Canada) to assess the collagen content, and lipidTox
green neutral lipid stain reagent (Invitrogen Thermo Fisher, Toronto, Canada, H34475) to
assess lipid droplets. For the minimal fiber diameter (minimal distance between the two
parallel tangents of the muscle fiber; as described in protocol DMD_M.1.2.001) [26], 8-10
images were randomly selected from different parts of the muscle section (10X magnification;
Axio-scan. Z1). A minimum of 400 fibers per muscle per rat were quantified using the image
J software (NIH, Bethesda, MD). For the Sirius Red staining, 8 to 10 images at 10X
magnification were acquired under polarized light with the Leica DMi 8 microscope (Leica
Biosystems). The percentage of Sirius Red-positive pixels over the total area was quantified
with the ImageJ software. For lipidTox staining 8-10 images at 63X magnification were
acquired with the Leica SP8 confocal microscope (Leica Biosystems) and the percentage of
green-positive pixels over the total tissue area was quantified with the ImageJ software.
For immunostaining, sections were incubated with primary antibodies overnight at 4°C,
washed, and incubated with secondary fluorescent antibodies for 1 hour at room temperature.
Co-immunofluorescence was used to assess fiber typing with antibodies (DSHB, Iowa, United States) for myosin heavy chain (MyHC) type I (slow oxidative fibers; BA-F8, dilution: 1/6), IIA (fast oxidative fibers; SC-71, dilution: 1/3), and IIB (fast glycolytic fibers; BF-F3, dilution: 1/3) [27]. Six images per muscle per rat were randomly selected and acquired at 10X magnification; a minimum of 900 fibers per muscle and per rat were analysed. The proportion of myofiber stained for MyHC type I, IIA, and IIB was expressed as a percentage of total fiber count (myofibers not stained by MyHC I, IIA, or IIB, were considered as IIX fibers; as described here [27]). Co-immunofluorescence with mouse anti-rat CD68 (Abcam, Toronto, Canada, ab125212, dilution: 1/500) and rabbit anti-rat CD163 (Bio-rad, Mississauga, Canada, MC342, dilution: 1/300) antibodies was performed to assess total (CD68+), resident (CD68+/CD163+), and infiltrating (CD68+/CD163-) macrophages in skeletal muscle. Immunofluorescence with mouse anti-rat CD43 (Abcam, Toronto, Canada, ab22351, dilution: 1/500) was performed to assess neutrophil count. Pictures were acquired with a Leica DMi 8 microscope and analysed with ImageJ. Each section was examined at 40X magnification and 12 images were randomly acquired. A minimum of 50 macrophages per muscle and per rat was analysed. The number of labelled cells was counted, and the surface area of each image was quantified to express the data as the number of cells per mm².

Western blot

Western blot procedure was adapted from a protocol described previously [28]. Briefly, flash frozen muscles homogenized in RIPA buffer were used for western blotting. Forty micrograms of proteins were electrophoretically separated on SDS gels and transferred to PVDF membrane. Membranes were blocked (5% non-fat dry milk or 5% BSA for antibodies targeting phosphorylated antigen) and incubated with the following primary antibodies overnight at 4°C: rabbit anti-Atrogin-1 (anti-Fbx32, Abcam, ab168372, dilution: 1/1,000),...
rabbit anti-MuRF-1 (anti-TRIM63, ProteinTech, USA, 55456-1-AP, dilution: 1/1,000), rabbit anti-superoxide dismutase (SOD)1 (Enzo Life Sciences, Ontario, Canada, ADI-SOD-101-E, dilution: 1/1,000), rabbit anti-catalase (Abcam, ab52477, dilution: 1/1,000), sheep anti-glutathione peroxidase (Novus biological, CO, USA, S-072-100, dilution: 1/1,000), goat anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz, CA, USA, sc20357, dilution: 1/1,000) mouse anti-rat p65 (Cell signaling, Danvers, USA, 6956S, dilution: 1/1,000) and rabbit anti-rat p-p65 (Cell signaling, 3031S, dilution: 1/1,000) diluted in 3% of bovine serum albumin solution. Corresponding secondary horseradish peroxidase-conjugated antibodies (goat anti-rabbit, rabbit anti-goat, or donkey anti-sheep, Abcam) were added (dilution: 1/2,000) to the membrane for 1 hour at room temperature. Immunoreactivity was detected using the ECL western blot detection (Pierce™ ECL Plus Western Blotting Substrate, Thermo Fisher, 32132) in G: BOX Syngen. Protein expression was normalized to GAPDH.

**Ex vivo muscle functional testing**

The proximal and distal tendons of the EDL muscles were attached with a 3-0 silk suture. Muscles were carefully dissected and placed in the 300A Test System organ bath (Aurora Scientific Inc., Ontario, Canada) filled with buffered physiological salt solution (Krebs-Ringer supplemented with glucose) continuously perfused with carbogen bubbling (5% CO₂, 95% O₂), and thermostatically maintained at 25°C. After calibration of the optimal muscle length, muscles were stimulated (25 V, 500 ms) at increasing frequencies: 1, 25, 50, 80, 100, 150 Hz. The muscle was allowed to rest for 3 min between two stimulations [29]. The specific muscle force was calculated as follow: [maximal force × optimal fiber length (0.44 x muscle length) × muscle density (1.06 g/cm³)]/muscle mass. At the end of the protocol, the length and weight of the muscle were measured. The twitch-to-tetanus (Pt/P₀) ratio was calculated as the ratio of
single twitch force (1 Hz) to the maximal tetanic force [30]. Data were analyzed with Dynamic Muscle Data Analysis software version 6.1 (Aurora Scientific, Inc.).

Oxidative stress

Oxidative fluorescent dye dihydroethidium (DHE, Life technologies, D1168) was used for detection of superoxide (O$_2^-$) on frozen sections. Ten to twelve images were immediately acquired with a Leica DMi 8, and analysed with Image J software to quantify the proportion of red pixels/total muscle surface. The general indicator of ROS, 2’’7’’-dicholorofluorescein diacetate (H$_2$DCF-DA, Invitrogen, D399), was incubated with whole TA or soleus muscle homogenates for 30 min at 37°C and 5% CO$_2$. Fluorescence was detected and measured by spectrophotometry [31,32].

Statistics

Comparisons between groups were performed by two-way ANOVA with Tukey’s honest statistical difference for multiple comparison or 2-tailed Student’s t test to compare quantitative data populations with normal distribution and equal variance with GraphPad Prism version 6.0 (GraphPad Software, La Jolla, CA). The two-way ANOVA was used for comparing sex and treatment group, and Tukey’s honest post-tests were performed only when either of the main effects were significant by overall ANOVA. The level of significance was set at p< 0.05. Results are expressed as mean ± SEM.
Results

Relevance of transient neonatal hyperoxia as a model to study skeletal muscle in preterm birth conditions.

Neonatal rats were exposed to high concentrations of oxygen to mimic the sudden rise in pO\textsubscript{2} levels associated with preterm birth. Even though newborn rats are not preterm, the maturational stage at birth for many organs including the eyes, brain, kidneys, lungs, heart, and skeletal muscles correspond to those of a human infant born very preterm in the second trimester [22,33,34]. Our analysis of newborn rats at day 3 (immediately before the hyperoxia period) showed that their skeletal muscles contain high proportion of developmental MyHC (>80%) and absence of adult fast type fibers, which is identical to what is observed in 24 weeks human fetuses (Supplemental Figure 1A) [23,24,35,36]. Conversely, skeletal muscles of term babies express low levels of developmental MyHC (10%) and high levels of adult fast fibers (60-70%) [24]. Moreover, we observed the presence of large fibers remaining from primary myogenesis, as well as centro-nucleated fibers (newly-formed fibers), and myofibers scattered in the interstitial tissue in skeletal muscle of newborn rats (Supplemental Figure 1B, C). These features are characteristics of skeletal muscles at very preterm stage (20-26 weeks of gestation), and are not observed in term newborns [6,24].

Impact of transient neonatal exposure to high oxygen on oxidative stress and inflammation markers in skeletal muscles

To assess the impact of transient neonatal exposure to high oxygen on the redox status, we measured ROS production and the expression of key antioxidant enzymes. In OI vs CTRL males, DHE staining showed an increase in the levels of anion superoxide (O\textsubscript{2}\textsuperscript{-}) in the TA at 10 days and 4 weeks (Figure 1A-D). Similar results were observed in the EDL, another fast-twitch muscle, at 4 weeks (Supplemental Figure 2A, B). An increase in DCF-DA, a general
indicator of ROS, was also noted in the TA at 4- and 16 weeks (Figure 1E-G). This increase in ROS was associated with reduced antioxidant SOD1 enzyme expression at 10 days and 4 weeks (Figure 1H-K). In the slow-twitch soleus muscle, oxidative stress was much less robust, as the DHE staining was only increased at 10 days in OI vs CTRL males, and DCF-DA was similar between groups at all ages studied (Supplemental Figure 2C-H). In females, all the ROS markers in the TA and soleus were similar between OI and CTRL at all ages. In the TA from OI vs CTRL female rats, SOD1 expression was similar between groups at all ages studied. Analysis of sex differences in the TA of OI males vs OI females showed higher levels of DHE staining at 4 weeks in males, and of DCF-DA at all time points studied, indicating stronger oxidative response in males (Figure 1A-G). For antioxidant enzymes, lower levels of catalase and glutathione peroxidase protein expression was observed at 10 days in the TA of male vs female OI and CTRL rats, respectively (Supplemental Figure 2I, L).

Considering that ROS are known to stimulate inflammation, we next investigated the activation of NF-kB, a key inflammatory pathway, in skeletal muscle. Our results indicate an increase in the phosphorylation of p65 in OI males at 4 weeks, which is known to induce the nuclear translocation of p65 and increase the expression of NF-kB target genes (Figure 2A-C). To further characterize the impact of transient neonatal high oxygen exposure on inflammation, we assessed the accumulation of neutrophils and macrophages, which play a pivotal role in skeletal muscle physiopathology [37]. A 1.5- to 2-fold increase in the number of CD43+ cells (neutrophil marker) was observed in the TA of both males and females OI vs CTRL at 4 weeks, which remained elevated at 16 weeks only in males (Figure 2D-F). Similarly, we observed in both males and females OI vs CTRL, a 1.5- to 2-fold increase in the number of CD68+ cells (pan-macrophage marker) in the TA at 4 weeks. This increase in macrophages content remained significant only in males at 16 weeks (Figure 2G-I). In the soleus, the number of CD68+ macrophages were similar between CTRL and OI groups for
males and females at 4- and 16 weeks (Supplemental Figure 3A, B). The density of CD163+
cells, a marker for a subset of resident/anti-inflammatory macrophages, was increased in the
TA of OI males and females at 4 weeks, and only for males at 16 weeks (Figure 2G, J, K).
The proportion of anti-inflammatory macrophages relative to total macrophages
(CD163+/CD68+ cells) was significantly reduced in OI male rats at 16 weeks, but not in
females (Supplemental Figure 3E-H). Analysis of sex differences showed a higher level of
CD68+ and CD163+ macrophages in the TA of OI males vs OI females, suggesting a stronger
inflammatory response in males (Figure 2G-K).

**Impact of transient neonatal exposure to high oxygen on muscle atrophy**

To assess muscle atrophy, we first measured the body weight and the Lee index (surrogate
marker for body mass index) and observed that they were similar between the groups for both
males and females throughout the experimental period (Supplemental Figure 4A-C). At 10
days (end of the hyperoxia exposure period), the ratio of both TA and soleus muscle weight to
body weight were not different between CTRL and OI groups for males and females (Figure
3A and Supplemental Figure 4D). This ratio was significantly reduced in the TA of OI vs
CTRL males 4- and 16 weeks and in the EDL at 4 weeks, but not in the soleus muscle (Figure
3B,C and Supplemental Figure 4D-H). In OI vs CTRL females, the TA, EDL, and soleus
weight to body weight ratio were similar at 4 weeks, but a significant reduction was observed
at 16 weeks only for the TA (Figure 3B, C and Supplemental Figure 4D-H). Analysis of sex
differences showed a lower ratio of muscle weight to body weight for the TA of OI males
compared to OI females at 16 weeks (Figure 3C, Supplemental Figure 4F).

Next, we assessed muscle fiber size as a direct measure of muscle atrophy. In OI vs
CTRL males, TA fiber size was similar at 10 days but was strongly reduced at 4- and 16
weeks (Figure 3D-F). Soleus muscle fiber size was similar between groups at all ages studied.
In OI vs CTRL females, the fiber size of both the TA and soleus muscles was unaffected by high oxygen exposure at any time point. (Figure 3G-I and Supplemental Figure 4L-N).

To further comprehend the mechanism of muscle atrophy induced by transient neonatal exposure to high oxygen, we evaluated at 4- and 16 weeks the expression of Atrogin-1 and MuRF-1, two ubiquitin ligases involved in muscle protein degradation. In OI vs CTRL males, the expression of both Atrogin-1 and MuRF-1 was increased in the TA at 4 weeks but was similar at 16 weeks (Figure 4A-E). No difference in MuRF-1 or Atrogin-1 expression was observed in the soleus at both 4- and 16 weeks (Supplemental Figure 5A-E). Females OI vs CTRL rats showed an increase in Atrogin-1 expression in both TA and soleus muscles only at 16 weeks (Figure 4E and Supplemental Figure 5E).

**Transient neonatal exposure to high oxygen affects fiber type determination**

Considering the importance of oxygen levels in fiber typing, we next examined whether transient neonatal exposure to high oxygen leads to long-lasting changes in muscle fiber typing. We assessed the proportion of type I fibers (slow oxidative), type IIA fibers (fast oxidative), IIX and IIB fibers (fast glycolytic) by co-immunofluorescence (Figure 5A). In the TA of OI vs CTRL males, the proportion of type I and IIA fibers was reduced at 4- and 16 weeks along with a concomitant increase in type IIB fibers (Figure 5B, C). A small proportion (3-4%) of hybrid IIA/IIB fibers was observed in both groups at 4 weeks. Similar changes were observed in the TA of OI vs CTRL females at 4 weeks; however, this switch was less pronounced in 16 weeks OI females, which only showed a significant reduction in the proportion of type IIA fibers (Figure 5D, E). This switch from slow to fast fibers was also observed in the extensor digitorum longus (fast-twitch muscle), in which a reduction in the proportion of type I fibers was associated with an increase in type IIB fibers at 4- and 16
weeks in OI vs CTRL male and female rats (Supplemental Figure 6A-D). In the soleus, high oxygen exposure did not affect fiber typing in both males and females at any time point. (Supplemental Figure 6E-H).

**Changes in fibro-adipose tissue deposition after transient neonatal exposure to high oxygen**

To determine whether muscle atrophy is associated with accumulation of fibro-adipose tissue, skeletal muscles were stained with lipidTox, a marker of neutral lipid. We did not observe the presence of interstitial adipose tissue deposition in the TA and soleus muscles in OI and CTRL rats at any time point. However, we quantified the intracellular lipid droplets within the myofibers and observed a reduction in OI male rats at 4 weeks, which is consistent with the changes in fiber typing toward fast twitch fibers (Figure 6A-C). Sirius Red staining, a marker of fibrosis, was markedly increased in the TA of OI vs CTRL rats, in both males and females, at 4- and 16 weeks (Figure 6D-F). Fibrosis was also increased in the soleus muscles of male and female OI rats at 4 weeks, but it remained elevated only in males at 16 weeks (Supplemental Figure 7D-F). Analysis of sex differences showed that collagen content was significantly higher in OI males vs OI females in TA and soleus muscles at 16 weeks (Figure 6E,F and Supplemental Figure 7E,F).

**Impact of transient neonatal exposure to high oxygen on skeletal muscle function**

To investigate whether the changes in muscle morphology and composition induced by transient neonatal exposure to high oxygen were associated with an alteration in muscle function, we assessed the contractile properties of the EDL muscle *ex vivo*. Maximal tetanic force did not significantly differ between groups for both males and females at 4- and 16 weeks (Figure 7 A, B, D, E). As expected, maximal contractile force was increased in CTRL.
and OI male rats compare to CTRL and OI females at 16 weeks (Figure 7D,E). Next, we assessed the twitch-to-tetanus ratio (Pt/P₀ ratio), which compares the muscle force generated by a single electrical stimulus to its maximal contractile force. This indicator of the physiological properties of single motor units is directly correlated to fiber typing and fatiguability (i.e. Pt/P₀ ratio is higher in fast fatigable muscles). In males, Pt/P₀ ratio was significantly increased in OI vs CTRL rats at 4 weeks, but not at 16 weeks (Figure 7C, F). In females, Pt/P₀ was similar between groups at both 4- and 16 weeks (Figure 7C, F). Analysis of sex differences showed that the Pt/P₀ ratio is higher in OI males compared to OI females at 4 weeks.
Discussion

In this study, we showed, using a well-recognized animal model of BPD and preterm birth-related conditions, that transient neonatal exposure to high oxygen leads to long-term alterations in the skeletal muscles. These changes are characterized by an increased production of ROS and a chronic inflammatory state, alongside with signs of muscle atrophy, fibrosis, fiber type shifting, and impairment in contractile properties of the motor unit. These detrimental changes are observed in juvenile rats and are maintained until adulthood. Fast-twitch muscles were more vulnerable to the effects of hyperoxia than slow-twitch muscles. Moreover, male rats are more susceptible than females to oxygen-induced myopathy. Overall, these findings demonstrate that preterm birth-related conditions have long-lasting effects on the composition, morphology, and function of skeletal muscle, which could contribute to the development and prognosis of long-term cardiopulmonary consequences of prematurity (Figure 8).

Transition from intrauterine to extrauterine life requires a well-orchestrated cascade of events. Particularly, mechanisms must be in place to protect the newborn from the rapid and robust rise in pO₂ level (from 20-30 mmHg in utero to 90-100 mmHg ex utero) [38]. In preterm birth, this increase in oxygen levels happens in a context of immature antioxidant defenses [39-41], which is associated with deleterious effects on different tissues such as lungs, retina, brain, and cardiovascular system [2,42-44]. Our findings indicate that skeletal muscles are also strongly affected by this pathological condition. In the TA muscle of male rats, we observed a strong rise in ROS production immediately after the high oxygen exposure (at 10 days) that was still significantly higher than control at 4- and 16-weeks. A similar increase was also observed in the fast-twitch EDL muscle at 4 weeks. However, this increase in ROS production was milder in the soleus muscle of OI males, suggesting that the muscle type may affect its capacity to maintain its redox balance. The soleus muscle is mainly
constituted of slow-twitch fibers, which have a higher mitochondrial content and possess a potent ROS scavenging system that could help to compensate for the oxidative stress induced by hyperoxia [45]. Contrarily to males, ROS production was virtually unaffected in skeletal muscles of OI female rats. It could be postulated that this discrepancy between males and females is explained by differences in the maturation of their antioxidant system. Indeed, a decrease in the expression of the key antioxidant enzyme SOD1, which converts anion superoxide to hydrogen peroxide, was observed in the TA of males but not in females OI rats along with an increase in DHE staining, a marker of anion superoxide, at 10 days and 4 weeks. Several studies in mammalian species support the notion that upon birth, and especially preterm birth, females have superior antioxidant defense mechanisms that translate into a better neonatal adaptation and reduced incidence of prematurity-associated complications [46,47]. Similarly, a previous study showed that mitochondrial dysfunction in the plantaris muscle (composed mainly of type II fibers) is only observed in male rats, and not in female rats, one year after transient neonatal hyperoxia [48]. Overall, these findings indicate that the impact of transient neonatal exposure to high oxygen on the redox balance in skeletal muscles is sex-dependent and is related to fiber type.

Our data showed that transient neonatal exposure to high oxygen leads to a reduction in muscle mass and fiber size. These findings have important implications considering that skeletal muscle atrophy contributes to the pathogenesis and is an indicator of poor prognosis in various conditions such as aging, chronic obstructive pulmonary diseases, cancer, and chronic heart failure [49-51]. The atrophic changes were not observed immediately after the high oxygen period but developed overtime and persisted until adulthood. These observations indicate that elevated pO2 does not induce muscle atrophy per se, but rather induces chronic detrimental changes that impair muscle growth. Noteworthy, these signs of muscle atrophy were more severe in male OI rats. Accumulating evidence suggest that the lower antioxidant
defenses of males could explain, at least partially, the muscle wasting observed. Supporting this possibility, a reduction in the expression of the antioxidant enzymes SOD1 and catalase was associated with the accumulation of ROS in the skeletal muscles of aged mice, which accelerated age-related loss of muscle mass and function [52,53]. Elevated ROS production was shown to activate canonical NF-κB signaling, a key pathway involved in different cellular process such as inflammation, survival/apoptosis, and stress response [16]. NF-κB target genes such as TNF-α, IL-1β, and IL-6 are known to promote muscle atrophy in chronic diseases [54,55]. Moreover, NF-κB can directly bind to the promoter region of the muscle-specific ubiquitin ligases MuRF-1 and atrogin-1. It has been shown that NF-κB-induced MuRF-1 expression promotes muscle atrophy [56]. Conversely, mice lacking the Nfkb1 gene or the activating kinase inhibitor of NK-κB kinase 2 (IKK2) are protected from disuse atrophy and display reduced fibrosis [57,58]. In accordance, our results indicate that NF-κB overactivation is associated with an upregulation of the ubiquitin ligases MuRF-1 and Atrogin-1 in OI rats, particularly in males. These enzymes are master regulators of protein breakdown in skeletal muscles, and they play key roles in muscle atrophy [59]. Different evidence also suggests that the increase in ROS production could contribute to this upregulation in the ubiquitin proteasome system. In vitro experiment showed that ROS directly upregulate the expression of the MuRF-1 and Atrogin-1 and their ubiquitin conjugation activity [60]. In vivo, it was shown in different muscular disorders, such as aging or chronic obstructive pulmonary diseases, that ROS production is associated with increased expression of Atrogin-1 and MuRF-1 [61,62]. Altogether, these results suggest that the immature antioxidant defenses and increased susceptibility to oxidative stress in males significantly contribute to the activation of the ubiquitin proteasome system, through ROS production and/or NF-κB activation, which promotes muscle atrophy.
Analysis of distinct types of muscle revealed that oxygen-induced muscle atrophy is fiber-type specific. In the fast-twitch TA muscle that has lower antioxidant defenses there is a stronger rise in ROS production, increased Atrogin-1 and MuRF-1 expression, and reduced muscle mass and size, compared to the slow-type soleus muscle. Fast-fiber specific muscle atrophy has also been observed in other acquired myopathies such as cancer cachexia, diabetes, chronic heart failure, and sarcopenia [63]. In addition, transient neonatal hyperoxia induced a switch in fiber typing toward fast fibers in the TA and EDL muscles (reduced proportion of oxidative type I and/or IIA fibers and increased proportion of fast glycolytic type IIB fibers). Slow-to-fast fiber type switching is a hallmark of different muscular disorders such as disuse [6]. This shift in fiber typing is consistent with in vitro experiments showing that high oxygen levels promote fast-fatigable fiber type characteristics in developing myofibers [64]. Noteworthy, during fetal development, skeletal muscles are predominantly constituted of fast fibers and their maturation to slow fiber type only occurs during the third trimester [65]. Therefore, a rapid increase in pO$_2$ associated with preterm birth in this critical period of muscle maturation could impair the development of slow fiber type, which would increase muscle fatigability.

Assessment of maximal muscle force did not reveal significant differences in the OI vs CTRL rats; however, at 4 weeks of age the interindividual variability in the maximal force was high and precluded any conclusions. This heterogeneity in the phenotype and the prognosis is observed in preterm born individuals (i.e. some individuals being highly affected while others have no apparent complications) [66]. It could be hypothesized that transient neonatal exposure to hyperoxia induce a predisposition to reduced muscle force, which could be exacerbated by additional deleterious conditions and/or aging. Nevertheless, the absence of muscle force deficit was unexpected considering the strong evidence indicative of muscle atrophy in OI male rats. Importantly, force generation requires a complex system that is not
solely driven by muscle size. Changes in the excitation-contraction coupling could compensate for the loss of muscle mass. In this regard, further analysis of contractile properties showed that the twitch-to-tetanus (Pt/P0) ratio was increased in OI male rats. A similar increase in the Pt/P0 ratio was observed in a variety of conditions such as disuse and aging [67-69]. This ratio represents a physiological property of motor units that is correlated to fiber typing and fatigability (i.e. higher Pt/P0 ratio in fast-fatigable muscle) [30]. The rise in Pt/P0 ratio is coherent with the switch that we observed in OI rats in favour of fast fiber type.

The higher content of fast fibers in OI rats could help to partially mitigate the loss of muscle force induced by muscle atrophy considering that fast-twitch fibers are more powerful than slow-twitch fibers; however, they are also more fatigable. Taken together, these findings suggest that the reduced exercise capacity of individuals born preterm does not solely depend on reduced cardiopulmonary capacity but also on impaired contractile properties of their skeletal muscles [68,69].

In summary, this study shows that a transient exposure to high oxygen levels in newborn rats induces an imbalance in the redox status associated with chronic inflammation, muscle atrophy, fiber type switching, and impaired muscle function. These alterations in skeletal muscles closely mimic the ones observed in premature aging [70,71] and patients with chronic obstructive pulmonary disease [14,72-74]. Moreover, our findings indicate that these oxygen-induced myopathic changes are long-lasting and sex-specific (i.e. males are more affected than females), which is consistent with clinical observations showing that males are more at risk of developing preterm birth-associated morbidities [75]. While our study focused on different hindlimb muscles it will be important as well in the future to examine the effect of transient neonatal exposure to high oxygen on the respiratory muscles including the diaphragm, especially considering the predisposition of preterm subjects to chronic lung diseases.
This study has some limitations and alternative explanations to consider. The transient neonatal hyperoxia rat model has been extensively used to study the complications of prematurity [3,22,76]; however, one limitation of this model is that it does not fully recapitulate the complexity of preterm birth-related conditions. For instance, contrarily to the rat model, preterm born babies are often treated with corticosteroids, which could have effects on skeletal muscle growth. Another limitation is that neonatal hyperoxia induces detrimental changes to various tissues. Therefore, it is difficult to determine what part of the defects on skeletal muscles come from the hyperoxia insult per se or through systemic effects. Our previous studies demonstrated that detrimental changes to the cardiac and vascular systems (e.g. blood pressure, heart rate, systolic and diastolic function) are absent or very mild at 4 weeks in OI rats, but develop overtime at 12- and 16-weeks [3,77]. Most of the changes observed in skeletal muscle (except fibrosis) reach their peak in 4 weeks old rats, indicating that the changes in skeletal muscles precede the ones on the cardiovascular system. On the other hand, detrimental changes on the lungs are rapid and are concurrent to the changes observed in skeletal muscles in OI rats [22]. Therefore, the reduction in respiratory function could influence the skeletal muscle. Conversely, skeletal muscle atrophy/dysfunction could also contribute to impaired cardio-respiratory function [78]. Overall, skeletal muscle defects induced by neonatal hyperoxia is a complex process, in which muscle atrophy/dysfunction is interconnected with cardiorespiratory defects similar to what is observed in other pathologies like aging [70,71] and chronic obstructive pulmonary diseases [14,72-74].

In summary, these novel findings set the foundation of a new field of research in the consequences of preterm birth and related risk of adult diseases. Skeletal muscles play a critical role in exercise capacity and are a strong determinant of cardiopulmonary health and of risk of morbidity and mortality. Determining the role of skeletal muscles in the risk of
disease of adults born preterm could help to better identify at-risk individuals and develop targeted and effective preventive and therapeutic strategies.
Data Availability Statement

Data is available for sharing upon reasonable request to the corresponding authors.

Conflict of interest statement

The authors have declared that they have no conflict of interest.

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Figure legends

**Figure 1: Oxidative stress and antioxidant defenses.** (A-C) Quantification of DHE (dihydroethidium) staining of tibialis anterior (TA) sections and (E-G) of DCF-DA (2′,7′-dichlorofluorescein diacetate) fluorescence intensity in TA cells homogenate from 10 days, 4- and 16 weeks (wks) old room air control (CTRL) vs. neonatal oxygen-induced injury (OI) male and female rats. (D) Representative images of DHE staining of TA from CTRL and OI 4 wks old male rats. (H) Representative images of western blots showing the protein expression of superoxide dismutase (SOD) 1 and GAPDH (as loading control) in TA from 4- and 16 wks old CTRL vs. OI male and female rats. All samples from a specific time point and sex were processed on the same gel/blot. Black bar delineates samples that are not next to each other on the same blot. (I-K) Protein expression of SOD1 (relative to GAPDH) in TA from 10 days, 4- and 16 wks old CTRL vs. OI males and females. Error bars represent means ± SEM; n=5-6 per group. Scale bars = 25 µm. Statistical analyses were performed using two-way ANOVA with Tukey’s post-test, *p<0.05; **p<0.01; ***p<0.001 vs group indicated.

**Figure 2: NF-κB and inflammatory cell infiltration.** (A) Representative images of western blots showing the protein expression of phospo-p65 (p-p65), p65 and GAPDH (as loading control) in the tibialis anterior (TA) from 4- and 16 weeks (wks) room air control (CTRL) and old neonatal oxygen-induced injury (OI) male and female rats. All samples from a specific time point and sex were processed on the same gel/blot. Black bar delineates samples that were not next to each other on the same blot. (B, C) Ratio of relative protein expression of p-p65/p65 in TA from 4- and 16 wks old CTRL vs. OI males and females. (D) Representative micrographs of immunostaining for CD43 (neutrophils markers, red) and DAPI (nuclei marker, blue). (E, F) Quantification of CD43+ cells per mm² in TA sections from 4- and 16 wks old CTRL vs. OI male and female rats. (G) Representative micrographs of immunostaining for CD68 (pan macrophages marker, green) and CD163 (resident macrophage marker, red) and merge of CD68, CD163, and DAPI on TA sections from 4 wks old CTRL and OI male rats. Quantification of the number of CD68+ (H, I), and double positive CD68+ and CD163+ (J, K) cells per mm² in TA sections from 4- and 16 wks old CTRL vs. OI male and female rats. Error bars represent means ± SEM; n=5-6 per group. Scale bars= 10 µm for CD68+ and CD163+ staining, and 25 µm for CD43. Statistical analyses were performed using two-way ANOVA with Tukey’s post-test, *p<0.05; **p<0.01; ***p<0.001 vs group indicated.

**Figure 3: Muscle mass and fiber size.** (A-C) Muscle weight to body weight ratio for the tibialis anterior (TA) from 10 days, 4- and 16 weeks (wks) room air control (CTRL) vs. neonatal oxygen-induced injury (OI) male and female rats. Evaluation of fiber diameter with the minimal fiber feret (µm) in TA from 10 days, 4- and 16 wks old CTRL vs. OI males (D-F) and females (G-I). Error bars represent means ± SEM; n=6-8 per group. Statistical analyses were performed using two-way ANOVA with Tukey’s post-test (A-C) or Student t-tests (D-I) *p<0.05; **p<0.01; ***p<0.001 vs group indicated (A-C) or CTRL vs. OI (D-I).

**Figure 4: Markers of protein degradation.** (A) Representative images of western blots showing the protein expression of atrogin-1, MuRF-1 and GAPDH (as loading control) in tibialis anterior (TA). All samples from a specific time point and sex were processed on the same gel/blot. (B-E) Protein expression of atrogin-1 and MuRF-1 (relative to GAPDH) in TA from 4- and 16 weeks (wks) old room air control (CTRL) vs. neonatal oxygen-induced injury (OI) male and female rats. Error bars represent means ± SEM; n=5-6 per group. Statistical
analyses were performed using two-way ANOVA with Tukey’s post-test **p<0.01; ***p<0.001 vs. group indicated.

**Figure 5: Skeletal muscle fiber types.** (A) Representative images of type I (purple), IIA (red), IIB (green) and IIX (black) myosin heavy chain immunostaining of the tibialis anterior (TA) sections from 4 weeks (wks) old room air control (CTRL) and neonatal oxygen-induced injury (OI) male rats. Proportion of each fiber type in the TA from 4- and 16 wks old CTRL vs. OI males (B, C) and females (D, E). Error bars represent means ± SEM; n=6 per group. Scale bars = 25 µm. Statistical analyses were performed using two-way ANOVA with Tukey’s post-test. *p<0.05; **p<0.01 vs. group indicated.

**Figure 6: Lipid deposition and fibrosis.** (A) Representative images of lipidTox staining (neutral lipid marker, green) and DAPI (nuclei marker, blue) of tibialis anterior (TA) sections from 4- and 16 weeks (wks) room air control (CTRL) and neonatal oxygen-induced injury (OI) male. White arrows identify muscle fibers positive for lipidTox staining. (B, C) Quantification of lipid droplet area per fiber (percentage of green pixels/area) of TA sections from 4- and 16 wks old CTRL vs. OI males and females. (D) Representative images of Sirius Red staining (collagen type I) TA sections from 4- and 16 wks old CTRL and OI males. (E, F) Quantification of Sirius Red staining (percentage of red pixels/area) of TA sections from 4- and 16 wks old CTRL vs. OI males and females. Error bars represent means ± SEM; n=5-6 per group. Scale bars = 25 µm. Statistical analyses were performed using two-way ANOVA with Tukey’s post-test *p<0.05; ***p<0.001 vs. group indicated.

**Figure 7: Muscle contractile properties.** (A, D) Maximum specific force, (B, E) force-frequency curve, and (C, F) Twitch-to-tetanus ratio (Pt/P0) of the extensor digitorum longus (EDL) muscle from 4- (A-C) and 16 wks old (D-F) room air control (CTRL) vs. neonatal oxygen-induced injury (OI) male and female rats. Error bars represent means ± SEM; n=3-6 per group. Statistical analyses were performed using two-way ANOVA with Tukey’s post-test *p<0.05; **p<0.01; ***p<0.001 vs. group indicated. # CTRL males vs CTRL females; † OI males vs OI females.

**Figure 8: Schematic overview of the mechanism proposed.** Preterm newborns are exposed to high levels of oxygen compared to in utero that induces oxidative stress and systemic inflammation in a context of immature tissue development. Using a rodent model of neonatal exposure to hyperoxia, we showed that preterm birth-related condition induced an imbalance in the redox status in skeletal muscles associated with the activation of the key inflammatory pathway NF-kB and chronic inflammatory cell recruitment. These changes were associated with muscle atrophy, fibrosis, fiber type switching (favoring fast-fatigable fibers), and impaired contractile properties. These changes develop overtime and persist until adulthood and are more severe in males than females.
### Table A

|          | 4 weeks | 16 weeks |
|----------|---------|----------|
|          | CTRL    | OI       | CTRL    | OI       |
| p-p65    |         |          |         |          |
| p65      |         |          |         |          |
| GAPDH    |         |          |         |          |
| p-p56    |         |          |         |          |
| p56      |         |          |         |          |
| GAPDH    |         |          |         |          |

**Males**

- p-p65: 65kDa
- p56: 65kDa
- GAPDH: 37kDa

**Females**

- p-p65: 65kDa
- p56: 65kDa
- GAPDH: 37kDa

### Figure B

**Tibial anterior 4 wks**

- Ratio p-p65/p65
  - MALE
  - FEMALE

### Figure C

**Tibial anterior 16 wks**

- Ratio p-p65/p65
  - MALE
  - FEMALE

### Image D

**CTRL**

- CD43 + DAPI

**OI**

- CD43 + DAPI

### Image G

**CTRL**

- CD68

**OI**

- CD68

### Image H

**Tibialis anterior 4 wks**

- Number of CD43+ cells/mm²
  - MALE
  - FEMALE
  - CTRL
  - OI

### Image I

**Tibialis anterior 16 wks**

- Number of CD68+ cells/mm²
  - MALE
  - FEMALE
  - CTRL
  - OI

### Image J

**Tibialis anterior 4 wks**

- Number of CD68+CD163+ cells/mm²
  - MALE
  - FEMALE
  - CTRL
  - OI

### Image K

**Tibialis anterior 16 wks**

- Number of CD68+CD163+ cells/mm²
  - MALE
  - FEMALE
  - CTRL
  - OI
A

|                | 4 weeks | 16 weeks |
|----------------|---------|----------|
| CTRL Atrogin-1 |         |          |
| OI Atrogin-1   |         |          |
| CTRL MuRF-1    |         |          |
| OI MuRF-1      |         |          |
| CTRL GAPDH     |         |          |
| OI GAPDH       |         |          |

B

Tibialis anterior Male

C

Tibialis anterior Male

D

Tibialis anterior Female

E

Tibialis anterior Female
A

CTRL

OI

Type I - IIA

IIx - IIB

B

Tibial anterior Male 4 wks

C

Tibial anterior Male 16 wks

D

Tibial anterior Female 4 wks

E

Tibial anterior Female 16 wks
