EFFECTS OF INTRAUTERINE INTERMITTENT HYPOXIA ON SKELETAL MUSCLE IN OFFSPRING RATS

จุฬาลงกรณ์มหาวิทยาลัย
CHULALONGKORN UNIVERSITY

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ผลของภาวะพร่องออกซิเจนเป็นพักๆในมดลูก ต่อกล้ามเนื้อลายของหนูรุ่นลูก

น.ส.วิรงรอง วงศ์กิติกำจร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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วิรงรอง วงศ์กิติกำจร : ผลของภาวะพร่องออกซิเจนเป็นพักๆในมดลูก ต่อกล้ามเนื้อลายของหนูรุ่นลูก. (EFFECTS OF INTRAUTERINE INTERMITTENT HYPOXIA ON SKELETAL MUSCLE IN OFFSPRING RATS) อ.ที่ปรึกษาหลัก : อ. ทพญ. ดร.สิริโฉม สาตราวาหะ

ภาวะพร่องออกซิเจนเป็นพักๆ ในครรภ์ เป็นอาการสำคัญของภาวะหยุดหายใจขณะนอนหลับ เปลี่ยนแปลงการควบคุมระบบหายใจทางระบบประสาท และการหดตัวของกล้ามเนื้อกะบังลมของรุ่นลูก ดังนั้นการศึกษานี้แสดงถึงผลของภาวะพร่องออกซิเจนเป็นพักๆ ในครรภ์ของหนูรุ่นลูกเพศชาย ต่อการเจริญเติบโตและเมทาบอลิซึมของกล้ามเนื้อเจนิโอไฮออยด์ ซึ่งถูกควบคุมโดยเส้นประสาทสมองคู่ที่ 12 ที่เกี่ยวข้องกับการหายใจ และมีบทบาทต่อการขยับลิ้น การกลืน เปรียบเทียบกับกล้ามเนื้อบดเคี้ยวขนาดใหญ่แมสซีเตอร์ หนูSprague-Dawley ถูกเลี้ยงในภาวะพร่องออกซิเจนเป็นพักๆ ระหว่างตั้งครรภ์ (ระดับออกซิเจน 4-21% ทุก 3 นาที) 8 ชั่วโมงต่อวัน ในช่วงวันที่ 7 ถึง 20 เปรียบเทียบกับภาวะออกซิเจนปกติ เมื่อหนูรุ่นลูกเพศชายอายุ 35 วัน กล้ามเนื้อเจนิโอไฮออยด์และแมสซีเตอร์ ถูกนำมาศึกษา (6 ตัว/กลุ่ม) และวิเคราะห์ข้อมูลทางสถิติด้วยการทดสอบทีของเวลช์ พบว่าขนาดไฟเบอร์ชนิด IIA ของเจนิโอไฮออยด์ลดลง แต่ไม่มีผลต่อกล้ามเนื้อแมสซีเตอร์ ผลของเวสเทิร์นบลอท พบว่าภาวะพร่องออกซิเจนเป็นพักๆ ในครรภ์ทำให้ปริมาณ PGC1α ลดลงอย่างมีนัยสำคัญในกล้ามเนื้อเจนิโอไฮออยด์เท่านั้น นอกจากนั้นปริมาณของโปรตีน optic atrophy 1 และ mitofusin-2 รวมถึง Mitochondrial ATP synthase subunit alpha และ transcriptional factor A (TFAM) ลดลงเช่นกัน ในขณะที่ mitochondrial fission 1 เพิ่มขึ้นในกล้ามเนื้อเจนิโอไฮออยด์ของหนูรุ่นลูกที่ได้รับภาวะพร่องออกซิเจนเป็นพักๆ ในครรภ์ งานวิจัยนี้แสดงถึงการบกพร่องของเมทาบอลิซึมของไมโทคอนเดรีย และการเปลี่ยนแปลงของไฟเบอร์กล้ามเนื้อเจนิโอไฮออยด์ชนิดออกซิเดทีฟในหนูรุ่นลูกช่วงก่อนวัยรุ่นที่ได้รับภาวะพร่องออกซิเจนเป็นพักๆ ในครรภ์ ซึ่งคาดว่าเป็นผลจากความไวต่อการเปลี่ยนแปลงของไมโทคอนเดรียในกล้ามเนื้อเจนิโอไฮออยด์ ต่อภาวะพร่องออกซิเจนเป็นพักๆ ในครรภ์ ซึ่งอาจเป็นผลจากการถูกควบคุมโดยเส้นประสาทสมองคู่ที่ 12

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KEYWORD: Obstructive sleep apnea, pregnancy, intermittent hypoxia, geniohyoid muscle, masseter muscles, offspring rat

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Gestational intermittent hypoxia (IH), a hallmark of OSA, alters the offspring's respiratory neural control and diaphragm contractile function. Thus, we aimed to investigate the effects of gestational IH on the muscle development and metabolism of geniohyoid (GH), which is innervated by the respiratory-related hypoglossal nerve and plays a role in tongue traction and suckling, in male offspring rats compared with masseter (MAS), the largest masticatory muscle. Pregnant Sprague-Dawley rats were exposed to IH (3-min periods of 4–21% O_2) compared to Normoxia for 8 hours/day during gestational days 7–20. GH and MAS from 35-day-old male offspring (n = 6 /group) were analyzed. Data were statistically analyzed with Welch's t-test. Gestational IH reduced type IIA fiber size in GH, but not in MAS. Western blot analysis showed that gestational IH induced significant downregulation of PGC1α protein in GH, but not in MAS. Moreover, optic atrophy 1 and mitofusin-2 proteins, Mitochondrial ATP synthase subunit alpha, and transcriptional factor A (TFAM) were decreased while mitochondrial fission 1 protein levels were increased in the GH of gestational IH-offspring. Our results suggest mitochondrial metabolism impairment and oxidative myofibers alteration of the GH from gestational IH-preadolescent offspring, owing to the susceptibility of GH mitochondria to gestational IH which might be influenced by hypoglossal nerve innervation.

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CHAPTER I INTRODUCTION

1.1 Background and Rationale

Obstructive Sleep Apnea (OSA) is "a disorder of breathing during sleep characterized by prolonged partial airway obstruction and/or intermittent complete obstruction (obstructive apnea) that interrupts normal ventilation during sleep and normal sleep patterns" as defined by American Thoracic Society [1, 2]. OSA causes intermittent hypoxia (IH), hypercarbia, sleep fragmentation, and negative intrathoracic pressure swing [3] which lead to cardiovascular disease, metabolic and neurocognitive morbidities and mortalities [3, 4]. The prevalence of OSA was found to be lower in women than men as 1.2% vs. 3.9% [5]. But from the study of Louis, pregnancies showed high prevalence of OSA which was up to 15.4% [6]. Pregnancies with OSA were older and had higher mean body mass index compared with non-OSA pregnancies. In addition, upper airway in pregnancies is smaller than non-pregnant women [7].

From the paradigm of Developmental Origins of Health and Disease (DOHaD), a multidisciplinary field that examines the influence of environmental factors during developmental period on organism capacity alteration to cope with later life's environment [8], intrauterine environment plays a critical role in offspring's development. Pregnancy IH causes decreasing of oxygen in both maternal and fetal circulation [9]. Intrauterine IH was previously reported about autonomic nervous system alteration in offspring rats with increasing blood pressure and pulse pressure [10], anatomical and functional changes in cardiovascular system as aortic thickening in fetal offspring and vascular dilatation impairment in adult offspring [11]. Prenatal IH also caused skeletal growth retardation during developmental period and increased osteoporosis in ovariectomy female rats [12].

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From previous study, postnatal IH caused ultrastructure adaptation as muscle deterioration which presented loss of muscle mass and reduction of oxygen uptake [13]. Moreover, in muscle metabolic adaptation, there was alteration of oxidative enzyme activity (in Kreb's cycle), glycolytic enzymes, glucose uptake, and lactic acid [13]. IH also has influences on diaphragm by autophagy induction and

muscle fiber type alteration [14]. Anyway, no studies showed the effect of intrauterine IH on skeletal muscle development in offspring.

1.2 Research Questions

Does intrauterine IH alter muscle morphology in offspring rats' skeletal muscle?

Does intrauterine IH alter muscle fiber type in offspring rats' skeletal muscle?

Does intrauterine IH induce autophagy in offspring rats' skeletal muscle?

Does intrauterine IH affect growth and atrophy in offspring rats' skeletal muscle?

Does intrauterine IH affect mitochondria in offspring rats' skeletal muscle?

1.3 Objectives of this Study

To clarify effects of intrauterine IH on morphology, fiber type disproportion and fiber size, autophagy marker, hypertrophy and atrophy marker, mitochondrial metabolism of skeletal muscle in offspring rats.

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1.4 Research Hypothesis

Null hypothesis: Intrauterine IH does not affect muscle fiber morphology, fiber type disproportion and fiber size, autophagy process, and skeletal muscle growth and atrophy, muscular mitochondrial metabolism in offspring rats.

Alternative hypothesis: Intrauterine IH affects muscle fiber morphology, fiber type disproportion and fiber size, autophagy process, and skeletal muscle growth and atrophy, muscular mitochondrial metabolism in offspring rats.

1.5 Benefits of this Study

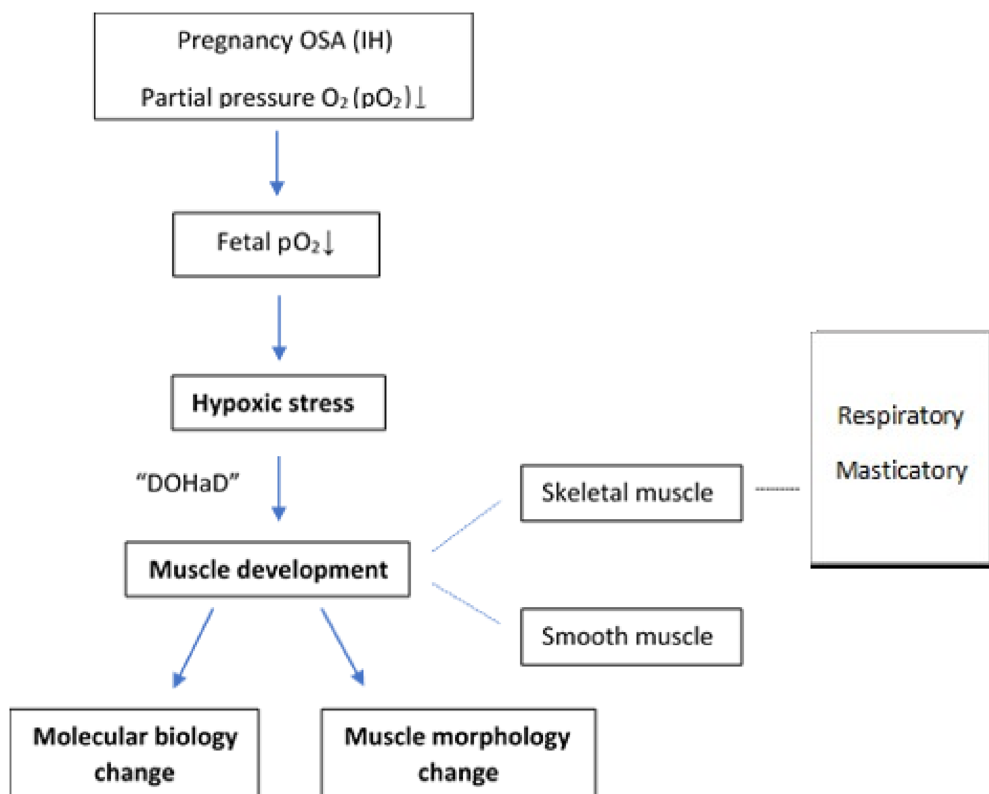
This study will clarify the effects of intrauterine IH on craniofacial skeletal muscles including of respiratory and masticatory skeletal muscles. Alteration of skeletal muscles from intrauterine IH might indicate as one of epigenetic cause of

congenital muscular dystrophy which presents hypotonia since childhood. Identifying pathology process in skeletal muscle could lead to further specific treatment in congenital muscle pathology.

1.6 Limitation of this study

Due to various protocols of IH has been used in different studies, this model might not explain as the result of different IH protocol. Moreover, human does not contain MHC IIb which is shown in rat skeletal muscle, human skeletal muscle's alteration might not be as same as in rat model. Additionally, this study is cross-sectional study, some expression might not reveal at the sacrificed time point or the pathology might be concealed with later skeletal muscle adaptation.

1.7 Conceptual Framework



CHAPTER II REVIEW LITERATURE

2.1 Obstructive Sleep Apnea – pregnancy

During pregnancy, pregnant women are at risk of obstructive sleep apnea (OSA) more than non-pregnant women [15]. Sleep-disordered breathing as an OSA is developed from anatomical and physiological changes to support the fetus. Not only the pregnancy hormones are elevated, but the maternal weight is also gain as well as the uterine volume is increased and elevated the diaphragm. The pregnancies encounter the changes in upper airway remodeling, reduced chest wall compliance due to elevated diaphragm which impair normal respiration. Impaired respiration is at its peak in the third trimester of pregnancy due to highest pregnancy hormones and largest womb [16]. Figure 1. Alteration of anatomy and physiology in pregnancies lead to sympathetic activation, inflammation, hypertensive disorders, increased cardiovascular risk, and gestational diabetes in maternal [17].

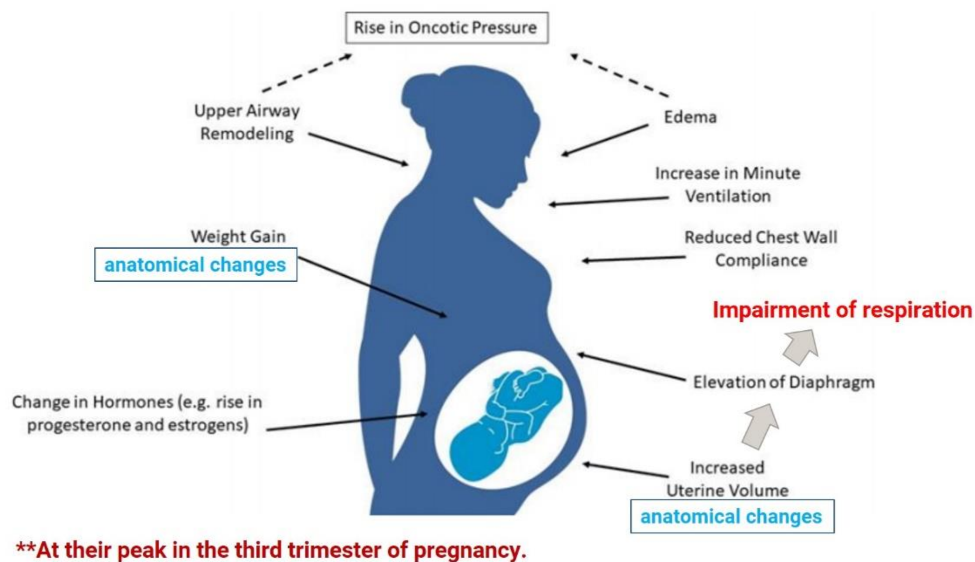


Figure ***1 Anatomical and physiological changes in pregnancy women. (modified from Martin et al. [16])***

Pregnancy hormones, rise in oncotic pressure, weight gain, increased uterine volume, and reduced chest wall compliance causes impairment of respiration in women which is worsen during third trimester of pregnancy.

Not only maternal but also fetal is affected from these consequences. Maternal OSA is associated with placental weight which is shown positive correlation with severity of maternal OSA and neonatal adiposity [18]. Increased placental weight also results in decreased birth weight/placental weight ratio. Bourjeily et al [19] have found correlation between OSA and increasing risk of congenital anomalies especially musculoskeletal anomalies with odd ratio 1.89. Neonates from OSA mothers had higher prevalence of preterm birth and were more frequently require intubation, intensive care unit admission, and longer hospital stay than non-OSA mothers. Hypercapnia or hypoxia in maternal results in placental dysfunction and hypoxia which induce fetal complications [19, 20].

2.2 Gestational IH

Fetal development is susceptible to acute changes in maternal oxygen levels, in rodents and humans [18, 20]. Fetal breathing activities has been reported since 24 weeks of gestational age [21]. Lung was developed since 3 weeks of embryo until 18 years postnatal growth which circum-environment in both prenatal and postnatal period are important for long development [22]. Respiratory rhythm and ventilatory responses were determined since embryonic development from different timing of intermittent hypoxia (IH) [23, 24]. Gozal et al [24] showed gestational IH from embryonic day 5 until delivery caused higher normoxic ventilation in postnatal age when observed until 4 months of age. While the ventilatory responses including peak hypoxic ventilatory response and hypoxic ventilatory depression were significantly reduced at 5 days postnatal age. However, ventilatory equivalents of IH gestationally exposed rats were significantly attenuated in both peak hypoxic ventilatory response and hypoxic ventilatory depression at all postnatal ages [24].

IH, a hallmark of gestational OSA, represents with brief (equal to or more than 10 seconds), alternatively exposure of hypoxia and normoxia (equal to or more than 5 episodes/hour) during sleep [25]. Pattern of IH is shown as airflow limitation through flatten nasal pressure while respiratory effort (esophageal pressure) is increased as shown in Figure 2 [25]. Maternal IH affects long-term postnatal development and increases the risk of cardiovascular and respiratory dysfunctions in

the offspring [26]. Oxidative stress was increased with thicker aortic wall in offspring from hypoxic pregnancies in animal model of Giussani et al.[26]. Also, there were vascular dilatation impairment and myocardial workload elevation.

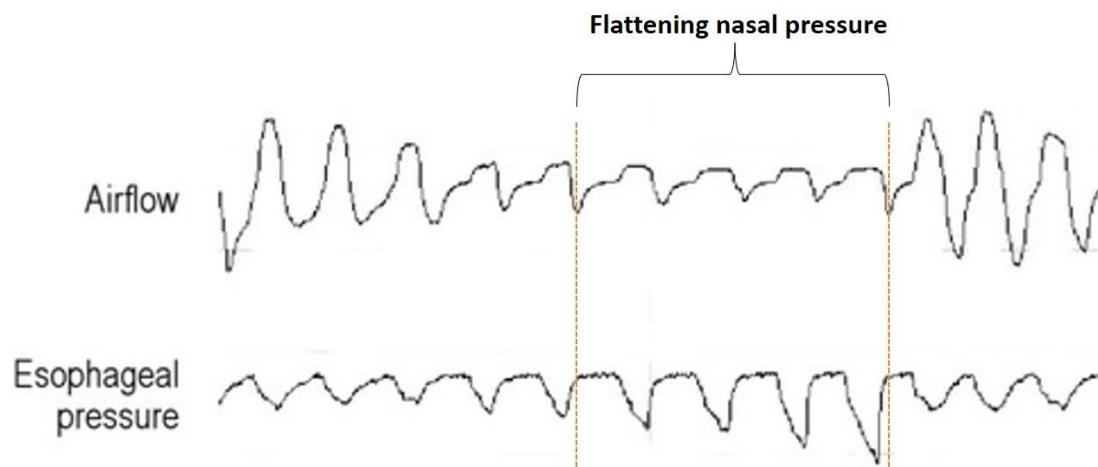


Figure 2 ***Obstructive hypoxia characteristics. (Modified from Berry et al [25])***
Obstructive of nasal airway attenuates airflow which is shown as flattening nasal pressure pattern. As the same time, esophageal pressure is rising from respiratory effort.

Johnson et al. have shown that gestational IH increases susceptibility to neuroinflammation and alters respiratory motor control in the offspring [23]. Neurogenesis, astrogenesis, and synaptogenesis begins in the gestational period. The onset of respiratory system development including of respiratory-related neurons, diaphragm, respiratory rhythmic is shown from gestational till postnatal period [27]. Figure 3. Gestational IH also decreases the contractile function of the diaphragm muscle in offspring rats, which suggests that IH reduces hypoxic tolerance of the diaphragm muscle during postnatal development [28].

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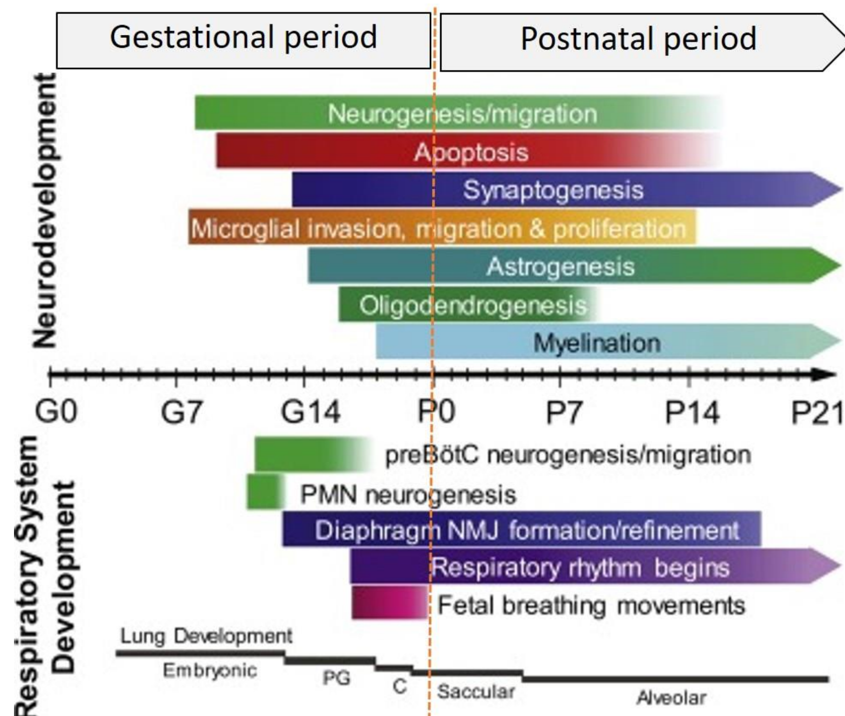

Figure 3 ***Timeline of neurodevelopment and respiratory system development. (Modified from Johnson et al. [27])***

Upper section illustrates timeline of neurodevelopment. Lower section illustrates timeline of respiratory system development. The preBötzinger Complex (preBötC) and phrenic motoneurons (PMN) are respiratory-related neurons which begins developing in gestational period. Diaphragm development and respirator rhythm are also shown since gestational age.

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2.3 Skeletal muscle

Skeletal muscle is derived from myogenic cells which differentiates into myoblasts before proliferation and fusion into primary myotubes. After that, they synthesize more contractile proteins and become muscle fibers [29]. During 3rd trimester of pregnancy, the skeletal muscle formation is in fiber hypertrophy stage. Placental insufficiency during this period causes reduction of myonuclei, DNA, RNA, and protein accumulation and differentiation [30].

Skeletal muscle is classified into 4 types of muscle fibers from low to high level of oxidative capacity; type I (slow-twitch oxidative), type IIA (fast-twitch

oxidative), type IIX/D (fast-twitch oxidative glycolytic) and type IIB (fast-twitch glycolytic), respectively. Mitochondria rich fibers (type I and IIA) are known as resistance to fatigue but less powerful function compared with glycolytic fibers (type IIX/D and IIB). Masticatory muscle is a large skeletal muscle in craniofacial compartment. Masseter (MAS) is the strongest masticatory muscle which composed of mainly type IIA, IIX/D, and IIB with no type I in rats masseter [31]. Muscle fiber type relates with powerful, daily usage [32], and oxidative capacity. Slow muscle (type I) shows higher daily use than fast muscles.

2.4 Geniohyoid muscle

Respiratory neurons in the reticular formation project to the hypoglossal motor nucleus in the brainstem, which in turn transmit the respiratory drive signal to the genioglossus and geniohyoid (GH) muscles of the tongue [33] via the medial branch of the hypoglossal nerve and GH muscle is also innervated by the fibers joined from the first cervical nerve (C1). Figure 4. Electromyographic studies of rodent models and humans revealed that hypoxia or hypercapnia increases respiratory drive to hypoglossal motoneurons and tongue muscles [34].

The GH muscle is a short, paired muscle which originates from inferior mental spines (genial tubercle) on the posterior surface of the mandibular symphysis and inserts to anterior surface of the body of the hyoid bone. Figure 5. GH muscle becomes one of suprahyoid muscles and locates inferiorly to genioglossus muscle which is the root of the tongue. GH muscle pulls hyoid bone in upward-forward direction dilating the pharynx which supports the respiration. Inversely, GH muscle opens the mouth, depresses and retracts the mandible when the hyoid bone is fixed. This motor behavior of GH muscle coordinately functions with other muscles in chewing motion. Additionally, GH muscle pulls larynx superiorly-anteriorly during swallowing.

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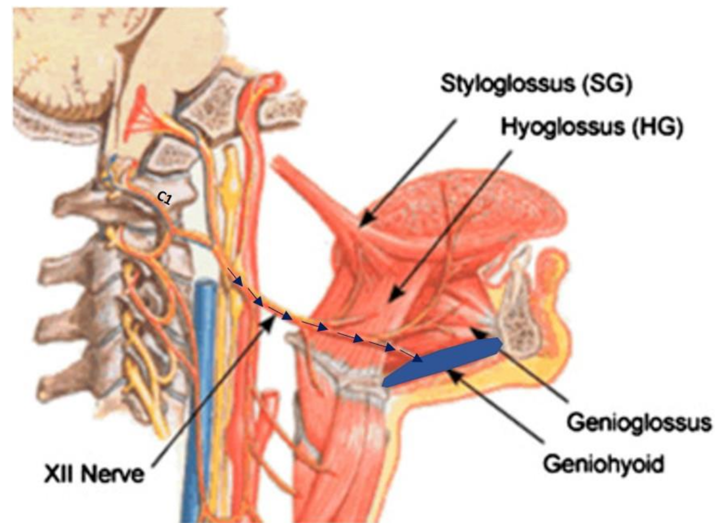

Figure 4 ***Innervation to Geniohyoid muscle. (Modified from Durand et al. [35])***

Medial branch of hypoglossal nerve (CNXII) after joining with C1 innervates the geniohyoid muscle.

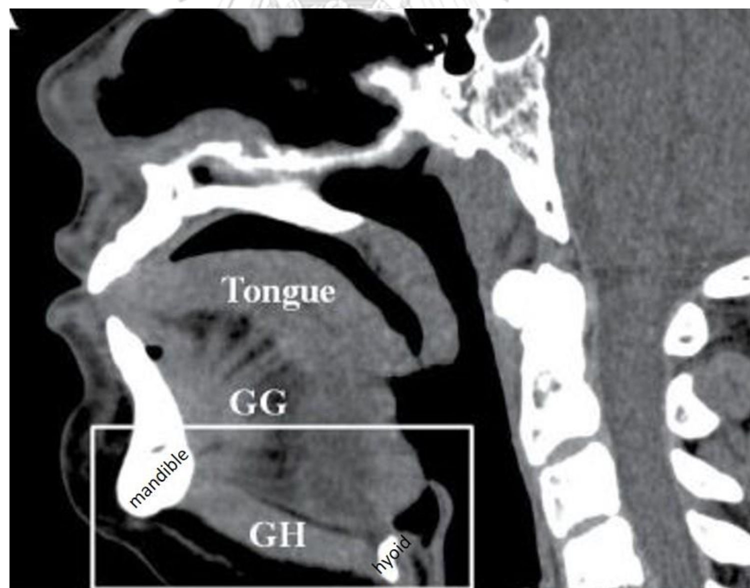

Figure 5 ***Location of GH. (Modified from Feng et al.[36])***

2D-computed tomography scan illustrates location of Geniohyoid (GH) muscle. GH muscle locates inferiorly to Geniohyoid muscle (GG) and connects between mandible and hyoid bone.

Gestational IH may increase impaired respiratory drive to hypoglossal motoneurons, which affects growth and development of GH muscles in young offspring. Thus, respiratory motor control of the GH muscle casts a doubt on whether gestational IH affects postnatal development of the GH muscle, thus contributing to suckling and feeding in young offspring.

2.5 Factors influence adaptation of skeletal muscle

Although skeletal muscle is a highly adaptive organ which could tolerate environmental changes [37], environmental and genetic factors could stimulate muscle adaptation in changing of muscle fiber size and composition [38], muscle mass [39], muscle strength [40] sarcomere number, and extracellular matrix composition [41]. About 30–50% of muscular variance in mass and strength are influenced by genetic while environmental factor has more influence on muscle [42]. Increasing age in human study showed lesser number of fiber and smaller fiber size especially in type II fibers of vastus lateralis muscle [38]. Muscle change from aging associates with activation of proteolytic cleavage, caspase-3, by mitochondria dysfunction [43]. In muscle development process, growth hormone and testosterone are important in muscle development by upregulation of insulin growth factor-1 (IGF-1) which stimulates cell proliferation in many tissues including skeletal muscle results in muscle protein synthesis [39, 40]. In addition, environmental factors such as nutrition and exercise are essential for muscle growth in postnatal life. Both protein intake from nutrition and protein metabolism after exercise could stimulate muscle growth [44]. ROS is an oxidative stressor which has both positive and deleterious roles on skeletal muscle depends on the levels and persistence of ROS on the tissue site [45]. The levels and persistence are due to level and duration of ROS, source or site of ROS generation, antioxidant status and their DNA repair capacity of the target cells. Low level or short duration of ROS activates specific signaling for controlling muscle adaptation such as Peroxisome proliferator-activated receptor-γ (PPAR) coactivator 1α (Ppargc-1α or PGC-1α) and 5' adenosine monophosphate-activated protein kinase (AMPK) which involve oxidative metabolism and mitochondrial biogenesis, and antioxidant enzyme. All of these signaling pathways are to prevent oxidative damage

from oxidative stress. In contrast, high level of ROS induces oxidative damage by increasing intracellular calcium and promoting autophagy or apoptosis through NF-kB or Forkhead box (FoxO) pathway. Thus, Oxidative damaging results in mitochondrial dysfunction, muscle atrophy, myopathies, and inhibit muscle regeneration.

2.6 Hypoxia and Skeletal muscle development

Gestational hypoxia was reported in association with the plasticity of muscle development [46-48]. Rozance et al reported muscle growth reduction in late-gestation hypoxemia fetal sheep. According to the study gestational hypoxemia didn't change the total body weights but decreased the hindlimb muscle length and muscle weight of tibialis anterior, and flexor digitorum superficialis. Also, the study found reduction of mRNA expression of muscle regulatory factors including Pax7, *MYOD*, *MYF6* in biceps femoris which correlates to reduced myoblast proliferation [48].

The fetal myogenesis begins from the myogenic precursors before developing into myoblasts and myotube differentiation. Lastly the myofibers grow by hypertrophy since late gestation and continuing throughout the postnatal life. Figure 6. The placental insufficiency has been concerned for a while of induction the pathology as an epigenetic factor. Not only nutrients but also oxygen related to fetal organ development. The placental insufficiency was reported of impairment myoblast function and skeletal muscle metabolism [49, 50]. Different timing of nutrient restriction during gestational period also caused different effect to muscle development according to the stages [50].

Chronic IH for 14 days was reported of higher oxidative stress which impaired the sternohyoid muscle in male rats [51]. Sternohyoid muscle is the upper respiratory muscle which was reported in previous study of increasing NADPH oxidase after IH exposure in rat model. According to the study, the IH exposure increased the oxidative stress in sternohyoid muscle and decreased the muscle power and force. However. Hypoxia-inducible factor expression wasn't affected according to the study model.

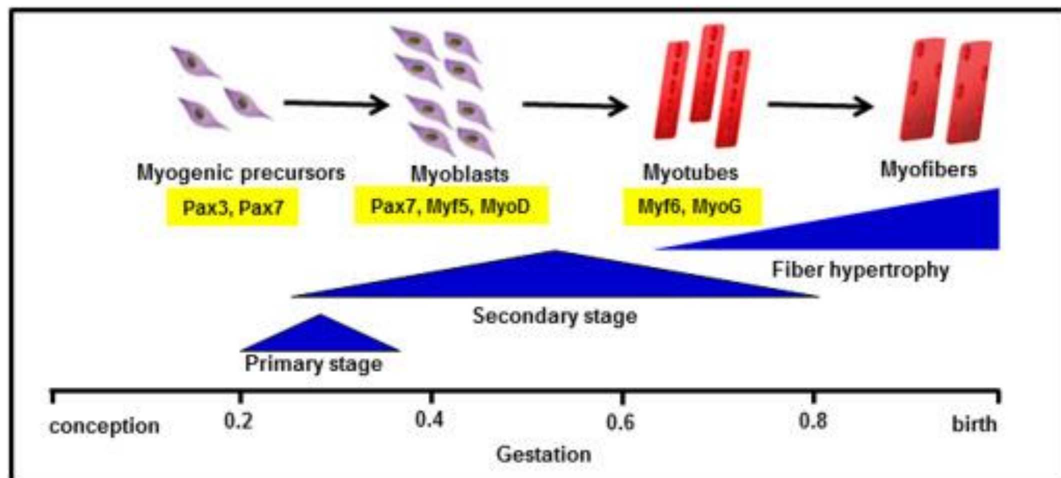

Figure 6 ***myogenesis diagram (from Brown and Hay [49])***

The muscle development begins since embryonic period. The muscle regulatory factors control the migration of the muscle progenitor cells which differentiate and mature to the specified muscles. Different muscles develop its myogenesis and synaptogenesis in different timing corresponding to the functional demands. As to feeding, tongue muscle develops faster than masseter muscle to support the suckling and swallowing function after birth while completion of the masseter development is later after birth to support the jaw movement for biting [52].

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2.7 Mitochondria

Mitochondria is noticeable in skeletal muscle which is the largest organ in human body. Mitochondria helps skeletal muscle maintenance by controlling the oxidative metabolism and energy transduction for skeletal muscle function [53, 54]. Mitochondria is a principal organelle for cellular energy production. The dysfunction of mitochondria has been found in relationship with metabolic diseases such as diabetes mellitus [55], neurological disorders [56] such as Alzheimer disease or Ischemic stroke, aging [57], skeletal muscle pathology such as sarcopenia [58]. Hiona et al reported dysfunction of mitochondria which involved the electron transport chain (ETC) complexes impaired the cellular energy production of mitochondria. Although no oxidative damage was found in this study model, there were skeletal muscle apoptosis and sarcopenia [58].

Exposure to epigenetic factors could induce mitochondria dysfunction. Decker et al reported long-term cigarette smoking altered mitochondria function [59]. They found upregulation of oxidative stress marker with downregulation of mitochondrial complexes. In recent years, Chronic IH has also been reported of its effect on the mitochondrial dysfunction involving central nervous system injury [60], vascular endothelial injury [61], liver injury [62], skeletal muscle disorder [63]. Bannow et al reported alterations in neuromuscular junctions and mitochondrial integrity after chronic IH in rat model. Soleus muscle showed reduction of neuromuscular junction size and type IIA fiber cross-sectional area. In addition, fraction of damaged mitochondria was significantly upregulated in chronic IH exposure group [63].

Mitochondria could be altered from intrinsic or extrinsic stressors such as genetic, metabolic, biochemical, environmental or infectious. Therefore, the mitochondrial quality control and oxidative capacity homeostasis are investigated through the cellular process such as mitochondrial dynamics which involves mitochondrial fission and mitochondrial fusion [64]. Figure 7. Mitochondrial dynamics illustrates the mitochondrial response to the stimuli. Mitochondrial DNA mutation have been found to be associated with diseases in various organs including brain and nervous system, muscles, cardiovascular, liver, kidney, endocrine and exocrine disorders, and systemic problems [65].

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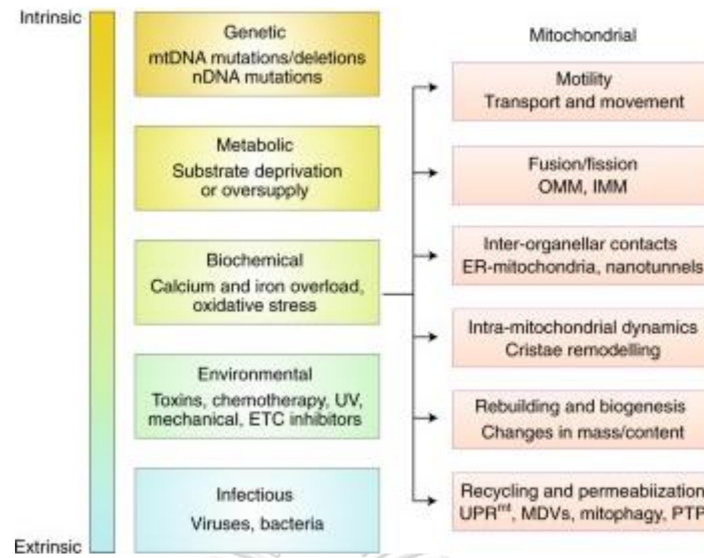

Figure ***7 Stressors induce alteration in mitochondria. (Modified from Eisner et al. [66])***

Intrinsic and extrinsic stressors on the left columns are shown the relatively induction of changes in mitochondrial involves the motility, mitochondrial fusion/fission, inter-organelle contacts, intra-mitochondrial dynamics, rebuilding and biogenesis, recycling.

Mitochondria is a small organelle with outer and inner mitochondrial membrane. The outer membrane is freely transportation of ions and small molecules. Thus, there is no membrane potential. The inner membrane is more tightly barrier which allows transportation through the membrane by specific membrane transport protein and selective ion channel. The oxidative phosphorylation or ATP synthesis occurs at the inner membrane from electrochemical gradient transportation through the protein complex. In the mitochondrial matrix, DNA replication, transcription, protein biosynthesis, and enzymatic process takes place [67]. Transcription factor A, mitochondrial (TFAM) is a DNA-binding protein which is necessary for transcriptional activation and mitochondrial DNA organization. Ngo et al. reported TFAM dimerization promotes DNA bending and enhances mitochondrial DNA compaction into nucloids [68]. Figure 8. TFAM protein levels controls the mitochondrial DNA expression. Bonekamp et al. reported the moderate level of TFAM overexpression increases the mitochondrial DNA copy number without affect the gene expression and animals were

well-tolerate to this condition [69]. However, too high level of TFAM also found as a repressor for mitochondrial DNA replication and leads to detrimental effect to the animals.

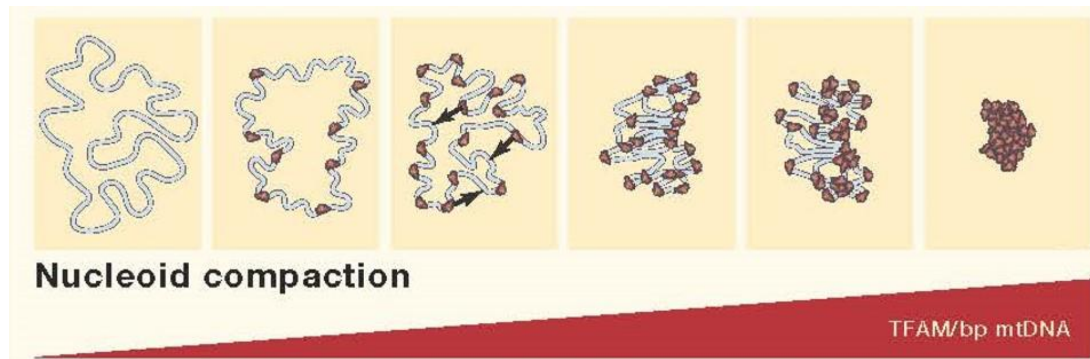

Figure 8 ***Nucleoid compaction (Modified from Bonekamp et al. [70])***
Transcription factor A, mitochondria (TFAM) binds to DNA leading to dimerization and compaction of the mitochondrial DNA. Lastly, the nucleoid is formed as a compaction from 1 mitochondrial DNA.

The inner membrane of mitochondria forms mitochondrial cristae which is the main site for energy conversion by complexes of the electron transport chain and the ATP synthase as shown in Figure 9. In the organs with high energy demand as skeletal muscle, the cristae are densely formed within the matrix of mitochondria [67]. The mitochondrial respiratory chain complexes comprise of 5 complexes including (i) Complex I, NADH dehydrogenase; (ii) Complex II, succinate dehydrogenase; (iii) Complex III, ubiquinol cytochrome c oxidoreductase; (iv) Complex IV, cytochrome C oxidase; and (v) Complex V, ATP synthase which encoded with ATP5A1. Previous studies reported the mitochondrial involvement diseases are caused from mutation in structural protein complex I which could be identified from autosomal genes encoding the complex I subunits or chromosomal genes encoding complex I subunits or autosomal genes encoding complex I assembly such as NDUFAF [71]. Swalwell et al reported mutation in mitochondrial DNA genes causes affects the complex I, III, IV and causes complex I deficiency [72]. Greggio et al. reported the necessary of respiratory chain complex assemblies in skeletal muscle exercise [73]. Respiratory supercomplex

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assemblies were upregulated in order to increase the muscle respiration for augmented energy demand in exercise.

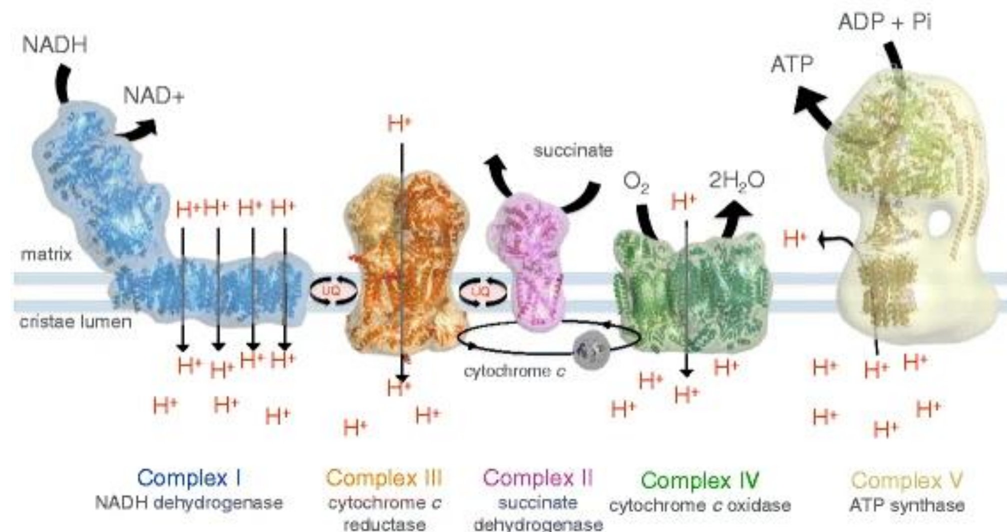

Figure ***9 Mitochondrial respiratory chain complexes. (from Kühlbrandt [67])***

Mitochondrial fission is necessary for cell division and proliferation while mitochondrial fusion was shown to be essential in preventing loss of mitochondrial DNA nucleoids which protects mitochondrial function [74]. In stress-induced environment, fusion is activated to complement the damaged mitochondria while mitochondrial fission might be inhibited to prevent from autophagic process [75].

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Postnatal IH induces mitochondrial dysfunction, with a significant decrease in peroxisome proliferator-activated receptor-gamma coactivator 1 (PGC1α) in rat genioglossal muscle [76]. Mitochondria are extremely sensitive to environmental stress, such as hypoxia and ischemia-reperfusion, and mitochondrial dysfunction causes a progressive loss of muscle strength and fatigue resistance. Moreover, environmental stress frequently causes mitochondrial fusion and fission, to maintain their functionality. PGC1α increases the expression of pro-fusion proteins optic atrophy 1 (OPA1), mitofusin (MFN)1, and MFN2; increase in the levels of MFN1 and MFN2 enhances mitochondrial fusion. PGC1α also decreases the levels of the pro-fission

protein guanosine triphosphatase dynamin-related protein 1 and mitochondrial fission 1 (FIS1), thus inhibiting mitochondrial fission in vitro [77, 78]. However, the effects of gestational IH on postnatal development and metabolism of the GH muscle are unclear.

2.8 Sex differences and Hypoxia

The sex differences showed different susceptibility to the effect of hypoxia [79]. Botek et al. reported the oxygen desaturation and sympathetic activity in males were more affected from resting hypoxia condition compared to females [79]. In the same way, the study of sex-differences responsiveness from 7 days-IH in rats showed higher heart rate and blood pressure in male rats [80]. Badran et al. reported the effects of gestational IH (GIH) on metabolic and vascular function in adult male mice and female offspring mice. According to Badran et al., male offspring exposed to GIH showed (i) low body weights in the first week followed by later increases in body weights and food consumption; (ii) dyslipidemia, hyperleptinemia and insulin resistance; (iii) increased systemic oxidative stress and inflammation; (iv) impaired endothelial function in the abdominal aorta; (v) loss of anti-contractile activity of perivascular adipose tissue (PVAT); (vi) low circulating and PVAT adiponectin levels, as well as increased inflammatory gene expression in PVAT; and (vii) hypermethylation of the adiponectin gene promoter. Female offspring did not exhibit any differences in weight after 3 weeks, food consumption, lipid profile, fasting insulin and glucose levels, or endothelial function at week 16 [81].

2.9 Ventilatory Stimuli

Ventilatory stimuli activate the jaw-closing muscles, as well as the genioglossus and GH muscles, to stabilize the mandible and upper airway patency in humans [82]. The hypoglossal nucleus contains motoneurons that innervate tongue muscles, while the motor trigeminal nucleus contains motoneurons that control jaw closing muscles, including the masseter (MAS) muscles. Interneurons of the motor trigeminal nucleus are part of the lateral tegmental field projections to the hypoglossal nucleus [83]. Thus, the MAS muscle not only functions as the jaw-closing muscle, but

its activity is also modulated by the respiratory inputs. However, the GH muscle shows respiratory-related cyclic activity even at rest [84], in contrast to the MAS muscle [85], and there may be some differences in physiological responses between the GH and MAS muscles as accessory muscles of respiration.

2.10 Hypoxia induction factor- α

Under hypoxic condition such as CIH, hypoxia induction factor-α (HIF-α), the oxygen homeostasis factor, is activated. In mammals, there are three isoforms of HIF-α (HIF-1α, HIF-2α, HIF-3α). HIF-1α and HIF-2α shares the same sequences as much as 48% but HIF-2α, also known as endothelial PAS-1 (EPAS1), is specifically found in endothelial cells [31]. HIFs are degraded by ubiquitin-proteasome system during normoxia and is formed as heterodimer with HIF-β in nucleus and activates transcription of hypoxia-responsive genes through hypoxia-responsive elements (HRE) during hypoxia [32, 33]. HIF-1α could be induced by CIH as a result of Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent ROS generation, increased intracellular calcium, and mammalian target of rapamycin (mTOR) activation [34, 35]. The target genes of HIF-1 encoded proteins increase oxygen delivery to the target by regulating genes involved in angiogenesis such as vasodilation, increased vascular permeability, extracellular matrix remodeling and proliferation [36, 37]. Target genes of HIF-1 also reduce oxygen consumption by tricarboxylic acid cycle (TCA) cycle metabolism inhibition, anaerobic metabolism promotion, and proliferation and apoptosis regulation. As a consequence, mitochondria reduce oxygen consumption and increase hypoxic ROS.

2.11 Autophagy pathway

Autophagy is one of proteolytic process that is found in skeletal muscle. Its role is to maintain muscle mass [2]. As usual, autophagy is at basal level for myofiber homeostasis [86] but upregulation as responsiveness to stress and hypoxia [87]. Exposure of IH on skeletal muscles in mice stimulated light chain 3B *(LC3B)* gene expression, LC3 is a soluble protein in mammalian tissues which becomes part of autophagosome and has been used as major autophagy biomarker, but did not

stimulate muscle-specific E3 ubiquitin ligase, one of muscle proteolysis pathway [14]. Thus, IH stimulates proteolysis pathway through autophagy process. Although mTOR is essential in muscle growth process [88], it does not play a significant role in autophagy process [89]. Autophagy pathway could be inhibited though FoxO by protein kinase B (Akt). In contrast, Akt inhibition showed significantly increased of autophagy gene expression such as LC3 [89]. As a consequence, hypoxia, glucose deprivation, and oxidative stress causes energy depletion which regulates AMPK and inhibits Akt to decrease energy demand and stimulate autophagy [90].

2.12 Mitochondrial related gene expression

Ppargc-1α or PGC-1α expressed in skeletal muscle especially in red oxidative fiber (type I) [91]. PGC-1α resides in mitochondrial matrix at subsarcolemmal and intermyofibrillar mitochondria [92]. PGC-1α is a master regulator of mitochondrial biogenesis and respiratory capacity in skeletal muscle [93]. PGC-1α is upregulated by muscle contraction. Expression of PGC-1α in skeletal muscle induces mitochondrial biogenesis for higher basal oxygen consumption such as during exercise activity [92]. During exercise, cellular energy stress increases AMPK, the energy sensor, to regulate mitochondrial biogenesis through PGC-1α [92]. As oxygen deprivation in hypoxia, PGC-1α is stimulated to regulate angiogenesis in skeletal muscle [94]. Upregulation of PGC-1α as a result of hypoxia causes lower intracellular oxygen due to increase of mitochondrial oxygen consumption [95]. Moreover, PGC-1α overexpression results in HIF-1α upregulation and stabilization [95].

2.13 Genes involve muscle atrophy and muscle growth

Akt involves both protein synthesis and degradation mechanism (Fig 1). Stimulation of IGF1-Akt pathway regulates protein synthesis through mTOR. While Akt inhibits protein degradation through FoxO pathway [96]. In contrast, suppression of IGF1-Akt pathway contributes to muscle atrophy and upregulates its downstream which causes protein degradation. F-Box Protein 32 *(Fbxo32)* or Atrogin-1 and Tripartite Motif Containing 63 *(Trim63)* or muscle RING-finger protein-1 (MuRF1) are E3 ubiquitin ligases that are increased in muscle atrophy [97]. Bodine et al. showed atrophy

conditions such as immobilization, denervation, unloading on skeletal muscle regulated *Fbxo32* and *Trim63.*

Myostatin (*Mstn* gene), a growth factor that belongs to transforming growth factor-beta (TGF-β) superfamily, is essential in muscle growth. Previous study in myostatin-null myoblasts showed higher satellite cell number which is myogenic progenitor for postnatal muscle growth, and proliferating myoblasts with delayed differentiation of myoblasts [98].

Neuronal nitric oxide synthase (nNOS or NOS1) is critical in skeletal muscle growth. nNOS generates nitric oxide (NO) which affects mitochondrial respiration and muscle development. In normal contractile condition, nNOS stimulates muscle growth through mTOR pathway [99, 100]. In disuse condition, nNOS induces oxidative stress and enhances muscle atrophy through FoxO pathway [100, 101]. nNOS null mice showed increasing autophagy in mitochondria, activating FoxO catabolic factors in skeletal muscle, and lowering muscle performance [99].

The aim of this study was to investigate the effects of gestational exposure to chronic IH on the muscle development and metabolism of GH and MAS muscles in male offspring rats. In this study, we characterized different responses of the GH and MAS muscles to gestational IH in male offspring rats.

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CHAPTER III MATERIALS AND METHODS

3.1 Experimental model

Six pregnant Sprague Dawley rats (225–250 g) were randomly exposed to normoxia (N, n=3) and IH (n=3), at a rate of 20 cycles/hour (nadir), the IH chamber was controlled with the valve with a timer as to alternatively allow mixing the flow of N_2 generator and air compressor in order to set 4 to 21 % of oxygen with 0 % carbon dioxide per cycle (1 cycle = from the minimum 4% of oxygen to maximum 21% of oxygen until it reduced to the minimum baseline again) for 8 hours/day during the 12-h “lights on” period, from gestation days 7 to 20, as previously described [102]. Figure 10. Blood oxygen saturation (SpO_2) levels were measured using a pulse oximeter (MouseOx; STARR Life Sciences Corp., Oakmont, PA, USA) placed on the neck of pregnant rats during IH cycles at gestation day 20. Mother rats were given ad libitum access to food and water throughout the experiment. All pups from both the groups were born naturally under normoxia (gestational normoxia with postnatal normoxia; N/N, prenatal intermittent hypoxia with postnatal normoxia; IH/N) and kept with their mothers until weaning. At the age of five weeks, we randomly chosen male pups from each mother rat, and six pups per each group were anesthetized with isoflurane and euthanized. Figure 11.

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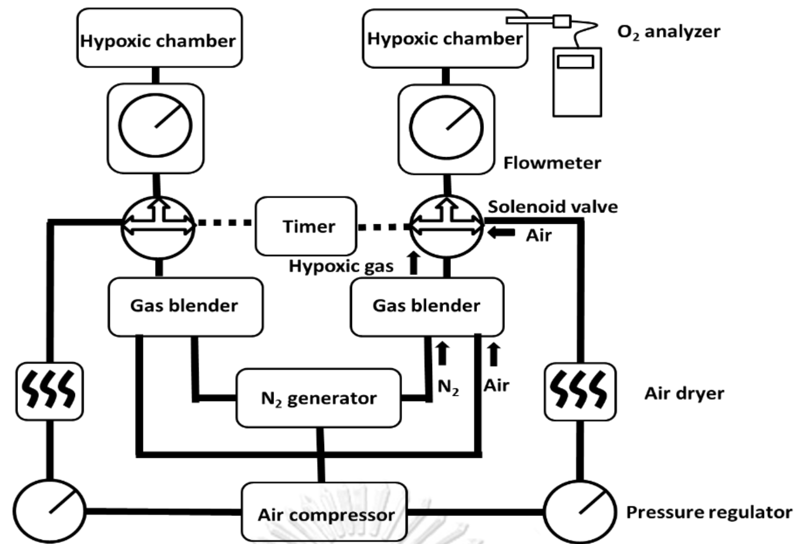

Figure 10 ***Illustrator of IH generator in this study. (as in Nagai et al. [103])***

Solenoid valve was opened/closed as setting time in timer. The gases were blended into the chamber through flowmeter. The oxygen concentration was analyzed with O_2 analyzer.

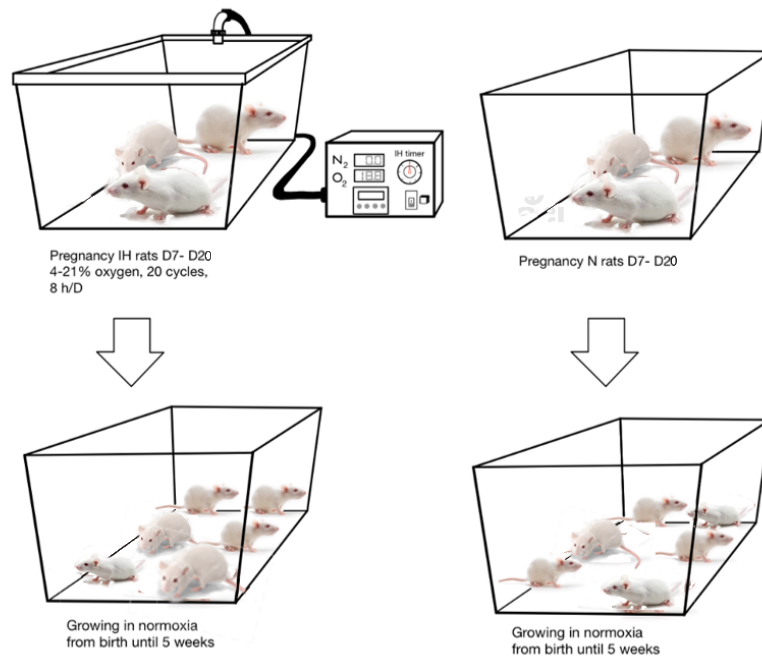

Figure 11 ***Illustrator of rat model in this study in IH and Normoxic condition***

The experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of Tokyo Medical University (ethics approval number: H31-0011).

3.2 Sample preparation and histological analyses

GH and MAS muscles were collected immediately from the euthanized male pups, frozen with isopentane in liquid nitrogen, and stored at -80 °C for histological analyses. Transverse 10 μm thick cryosections were stained with hematoxylin and eosin, modified Gomori trichrome, and nicotinamide adenine dinucleotide reductase (NADH) stains. Identification of GH and MAS of the rats was shown as in Figure 12.

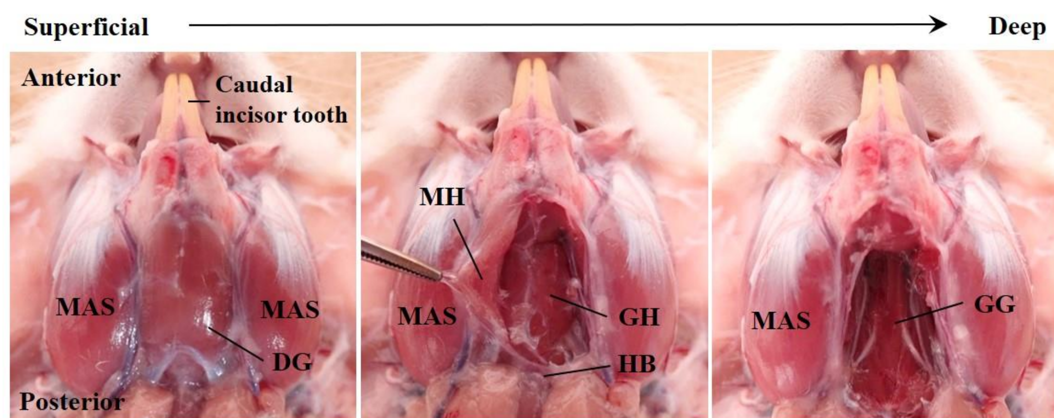

Figure 12 ***Anatomical images of rat - GH and MAS muscles.***
MAS, masseter muscle; DG, digastric muscle; HB, hyoid bone; MH, mylohyoid muscle; GH, geniohyoid muscle; GG, genioglossus muscle.

3.3 Immunohistochemistry

Transverse 8 μm-thick serial cryosections of the muscles were collected and blocked with 2 % bovine serum albumin in phosphate buffered saline. Each section was stained with primary antibodies against Myosin Heavy Chain (MHC) type I, BA-F8, IIA, SC-71, IIX/D, 6H1, or IIB; BF-F3 (DSHB, Iowa City, IA, USA) (Table 1), with a muscle cell membrane with laminin at 37 °C for 80 min.

For fiber size analysis, Alexa Fluor 488 anti-mouse and 568 anti-rabbit secondary antibodies (1:1000 dilution; Thermo Fisher Scientific, Waltham, MA, USA)

were used for detection. The sections would be illustrated with two color-staining to identify the membrane and the specific fiber type.

For fiber type distribution staining, anti-mouse Immunoglobulin G2b Alexa Fluor 350, anti-mouse Immunoglobulin G1 Alexa Fluor 488, and anti-mouse IgM Alexa Fluor 555 (1:1000 dilution; Thermo Fisher Scientific) were used for detection.

All staining images were acquired using a fluorescence microscope (Zeiss, Oberkochen, Germany). The sections for fiber size analysis were captured using the IN Cell Analyzer 2200 imaging system for calculating the muscle fiber size (diameters in minor axis) with IN Cell Developer Toolbox software (GE Healthcare, Chicago, IL, USA). The basal membrane was detected by laminin staining to calculated fiber size, and each myosin heavy chain-positive fiber was automatically selected by intensity. Muscle fiber size was assessed by quantifying the short diameters on the cross-sectional images. Data were analyzed as frequency distributions by comparing the N/N and IH/N groups. The sections for fiber type distribution were analyzed using Image J software and evaluated as the fiber distribution ratio.

Table 1 ***Primary antibodies list***

Antibody name	Target	Catalogue number	Manufacturer	Application	Concentration	Host species
BA-F8	MHC type I	BA-F8	DSHB	IHC	1:50	Mouse
SC-71	MHC type IIA	SC-71	DSHB	IHC	1:600	Mouse
6H1	MHC type IIX/D	6H1	DSHB	IHC	1:50	Mouse
BF-F3	MHC type IIB	BF-F3	DSHB	IHC	1:100	Mouse
L9393	Laminin	L9393	Sigma-Aldrich	IHC	1:200	Rabbit
15H4C4	ATP5A	ab14748	Abcam	WB	1:500	Mouse
6C5	GAPDH	ab8245	Abcam	WB	1:10000	Mouse
13798-1-AP	MFN1	13798-1-AP	Proteintech	WB	1:500	Rabbit

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12186-1-AP	MFN2	12186-1-AP	Proteintech	WB	1:500	Rabbit
EPR2796	NDUFAF1	ab79826	Abcam	WB	1:500	Rabbit
NB110-55290	OPA1	NB110-55290	Novus Biologicals	WB	1:500	Rabbit
NBP1-04676	PGC1α	NBP1-04676	Novus Biologicals	WB	1:500	Rabbit
ab131607	TFAM	ab131607	Abcam	WB	1:500	Rabbit
NB100-56646	TTC11/FIS1	NB100-56646	Novus Biologicals	WB	1:500	Rabbit

IHC, immunohistochemistry; WB, western blot.

3.4 Quantitative polymerase chain reaction (qPCR) analysis

Total RNA was extracted from frozen sections of the muscles using the RNeasy Plus Universal Mini kit (QIAGEN, Hilden, Germany), and converted to complementary DNA with the help of reverse transcription random primers using the SuperScript IV VILO Master Mix (Thermo Fisher Scientific), following the manufacturer's instructions. Real-time PCR was performed using 10 ng of cDNA template for each gene analysis and quantified using an Applied Biosystems QuantStudio3 real-time PCR system (Thermo Fisher Scientific). SYBR Green probes and primers were obtained from Takara Bio (Takara Bio, Otsu, Shiga, Japan) (Table 2). Lamin B receptor (Lbr) was used as an internal control, and gene expression levels were calculated using the $2-\Delta\Delta$CT relative quantitation method.

Table 2 ***Real-time reverse transcriptase-PCR primer sequences***

Gene	Forward primer	Reverse primer
Myh1	5′-TGTGGACAAACTGCAATCAAAGG-3′	5′-CTGGATCTTGCGGAACTTGG-3′
Myh2	5′-TCAGGCTTCAAGATTTGGTGGAT-3′	5′-GCAGCTTGCGGAACTTGGA-3′
Myh4	5′-GCGACCTCAATGAAATGGAAATC-3′	5′-CTTTCAAGTCATCCTGGCCTCTG-3′
Hif1a	5′-TCTAGTGAACAGGATGGAATGGA-3′	5′-TCGTAACTGGTCAGCTGTGGTAA-3′
Epas1	5′-CGCCTCATGTCTCCATGTTCA-3′	5′-CCAGCTGGCGCTTTAGCTTC-3′
Ppargc1a	5′-CACCGTAAATCTGCGGGATG-3′	5′-TATCCATTCTCAAGAGCAGCGAA-3′

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Atp2a1	5′-TCATTGCTCGGAACTATCTGGA-3′	5′-GCTGAAGACGCCTTGCCATTA-3′
Atp2a2	5′-GGTCAGTCTTAACGGCAGTGTG-3′	5′-CCCAAGCTCAGTCATGCAG-3′
Igf1	5’-GCACTCTGCTTGCTCACCTTTA-3	5’-TCCGAATGCTGGAGCCATA -3
Mtor	5’-GCTTATCAAGCAAGCGACATCTC-3’	5’-TCCACTGGAAGCACAGACCAAG-3’
Nos1	5’-TCAAAGCCATCCAGCGCATA-3’	5’-ACGTTCTGAGGGTGACTCCAAAG-3’
Mstn	5’-ATTATCACGCTACCACGGAAACA-3’	5’-AGCTGGGCCTTTACCACTTTG-3’
Trim63	5’-GACTCCTGCCGAGTGACCAA-3’	5’-TTCTCGTCCAGGATGGCGTA-3’
Fbxo32	5’-CAACATGTGGGTGTATCGAATGG-3’	5’-TGATGTTCAGTTGTAAGCACACA-3’
Lbr	5′-GCTTCAACCACATCCTGCCTTA-3′	5′-TGGTGTTCATCACGGGCTTC-3′

3.5 Western blot analysis

Samples from cryosections of GH and MAS muscles were homogenized in the sample buffer solution, with radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors, and centrifuged at 15,000 rpm at 4 °C for 5 min. Then, 30 μg of proteins for each sample was loaded on Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE) gels and blotted onto a PolyVinylidene DiFluoride (PVDF) membrane. The blots were incubated with primary antibodies against ATP5A1, TTC11 (FIS1), MFN1, MFN2, PGC1α, OPA1, NADH:Ubiquinone Oxidoreductase Complex Assembly Factor 1, mitochondrial transcription factor A, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1). Autophagy primary antibodies were blotted to MAS muscle samples including LC3I, LC3II, AKT, P-AKT, AMPK, P-AMPK. Horseradish peroxidase-conjugated secondary antibodies (Thermo Fisher Scientific) were used for chemiluminescence detection. All bands were detected using Clarity Western ECL Substrate (Bio-Rad) and visualized with the Image Lab 5.0 software. All data were normalized to GAPDH and analyzed as relative band intensities using Image Lab 5.0 software.

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3.6 Statistical analysis

Data are shown as mean ± standard deviation and analyzed with the Shapiro–Wilk normality test and a Welch's t-test after. Statistical significance was considered when the p-value was lower than 0.05. All statistical analyses were performed using IBM SPSS statistics 22.0 (Chicago, IL, USA).

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CHAPTER IV RESULTS

4.1 Chronic IH-induced changes in maternal blood oxygen saturation during pregnancy

Chronic IH-induced cyclical changes in maternal SpO_2 levels, responding to the IH cycle (3-min periods of 4–21 % O_2 in each chamber) are shown in Table 3. At baseline, IH mother rats showed stable SpO_2 levels, similar to normoxic mother rats. All pups from both the groups were born naturally and had increased body weight after birth and weaning. There was no statistical difference in the body weights of rats from the N/N and IH/N groups (the N/N group, 114.5 ± 5.9 g; the IH/N group, 106.4 ± 14.9 g) at day 35 after birth.

Table 3 ***Blood oxygen saturation (SpO_2) of intermittent hypoxia (IH) and normoxic pregnant rats at gestation day 20***

	Baseline	IH cycle	
		Hypoxia	Reoxygenation
IH pregnant rats (n = 3)	96.3 ± 1.5 (%)	67.3 ± 6.6 (%)	97.4 ± 1.3 (%)
Normoxic pregnant rats (n =3)	95.9 ± 0.6 (%)	N/A	N/A

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4.2 Characteristics of GH and MAS muscles in offspring rats exposed to gestational IH

The muscle fiber histological images (Figure 13) showed that both GH and MAS muscles were comparable between IH/N and N/N rats, at the age of five weeks. Skeletal muscle fiber type is characterized by slow fiber type to fast fiber type (type I ⟶ IIA ⟶ IIX/D⟶ IIB). Among type II fibers, type IIA fibers have a higher oxidative capacity and fatigue more slowly than type IIX/D and IIB [104].

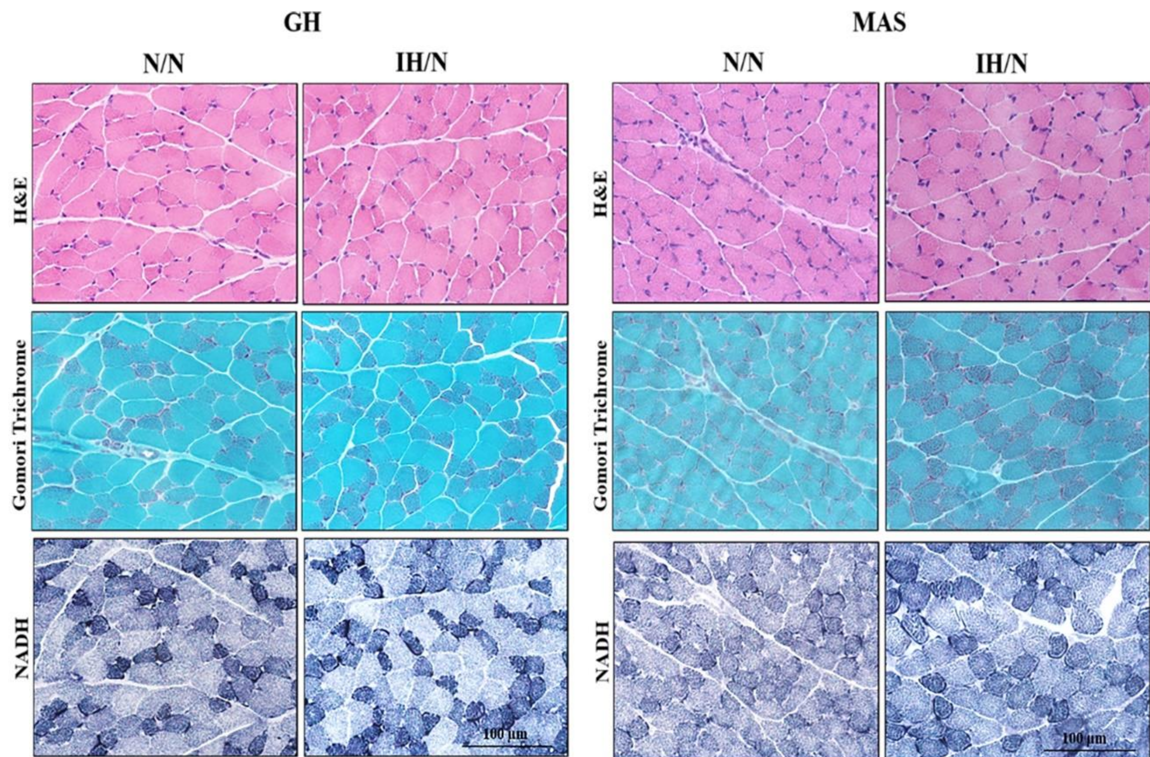

Figure ***13 Histological images of the geniohyoid (GH) and masseter (MAS) muscles of gestational intermittent hypoxia (IH) offspring.***

The upper panel, Hematoxylin & Eosin stain; the middle panel, modified Gomori Trichrome stain; the lower panel, NADH stain. GH, geniohyoid muscle; MAS, masseter muscle. Data represent male offsprings (n = 6) in each group. Scale bar: 100 µm.

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Immunohistochemically, both GH and MAS muscles consist predominantly of fast-type fibers, and only a few slow type fibers (Figure 14a and 15a). The baseline fiber type proportion was heterogeneous between both muscles in N/N rats. There was no significant difference in the MAS muscle between the N/N and IH/N groups, while the GH muscle tended to decrease type IIB (fast-glycolytic) fibers in the IH/N group compared to the N/N group, but the difference was not statistically significant (Figure 14b and 15b). Moreover, a smaller diameter of type IIA fiber was observed in the GH muscle of the IH/N group compared to that of the N/N group, while the other fiber types were comparable between both the groups (Figure 14b). In

the MAS muscle, there were no significant differences in the fiber size of each fiber type (Figure 15b). Thus, histological data suggest that gestational IH decreases the size of type IIA fibers (oxidative fibers) in the GH muscle of the offspring, but the MAS muscle remains unaffected.

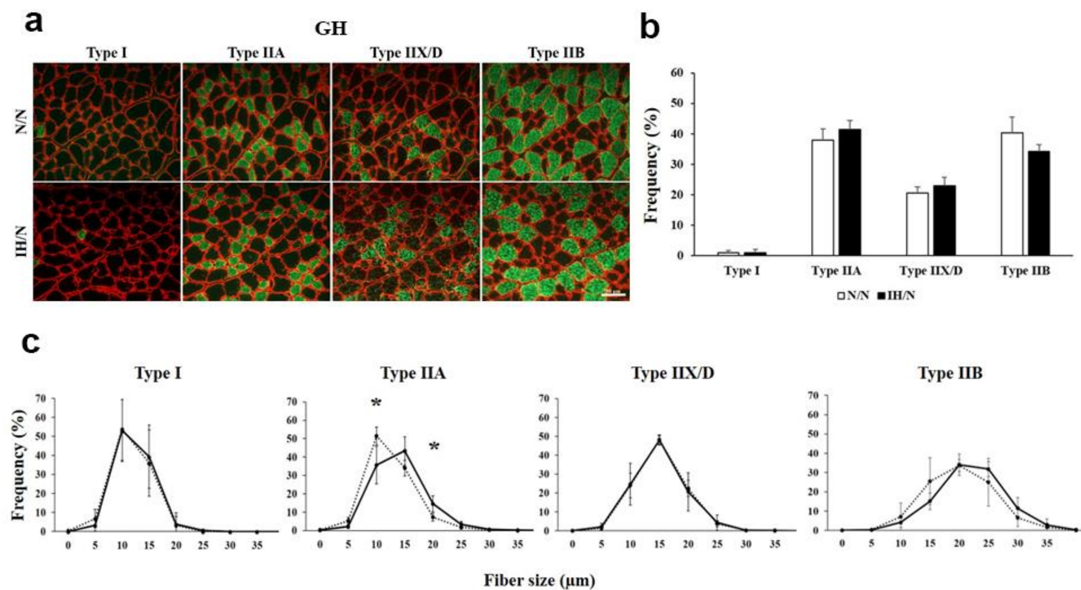

Figure 14 ***Distribution pattern of muscle fiber type in the GH muscle of gestational IH offspring rats.***

(a) Fiber type-specific immunohistochemical staining for type I, type IIA, type IIB, and type IID fibers with skeletal muscle membrane protein, laminin (red). Each panel shows a cross-sectional image of the GH muscle. Green areas indicate immuno-positive muscle fibers. . (b) The graph indicates the percentage of muscle fiber type distribution in GH muscle from each group. (c) Histogram of fiber size distribution of each muscle fiber type. Solid line and dotted line show N/N and IH/N groups, respectively. Data represent male offsprings (n = 6) in each group. Scale bar: 50 μm. *p < 0.05 vs. the N/N group.

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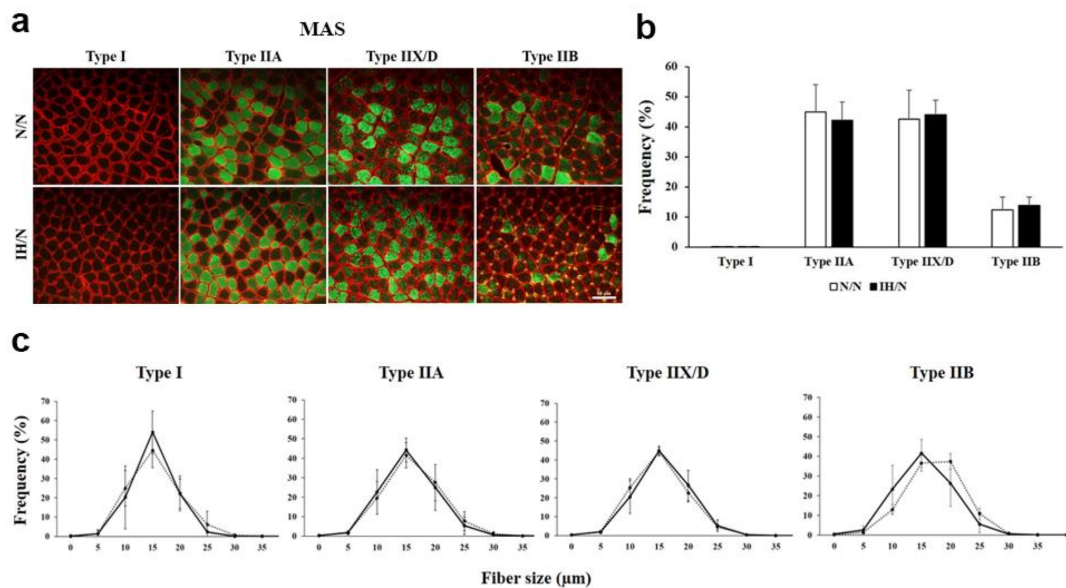

Figure 15 ***Distribution pattern of muscle fiber type in the MAS muscle of gestational IH offspring rats.***

(a) Fiber type-specific immunohistochemical staining for type I, type IIA, type IIB, and type IID fibers with skeletal muscle membrane protein, laminin (red). Each panel shows a cross-sectional image of the MAS muscle. Green areas indicate immuno-positive muscle fibers. (b) The graph indicates the percentage of muscle fiber type distribution in MAS muscle from each group. (c) Histogram of fiber size distribution of each muscle fiber type. Solid line and dotted line show N/N and IH/N groups, respectively. Data represent male offsprings (n = 6) in each group. Scale bar: 50 μm.

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4.3 Gestational IH-induced changes in genes related to fiber type characteristics in offspring rats

qPCR analysis confirmed that the expression of Myh2 was significantly decreased only in the GH muscle of the IH/N group (Figure 16a). In addition, Atp2a2 mRNA, encoding slow-type sarcoendoplasmic reticulum calcium ATPase (SERCA2), was significantly downregulated in the GH muscle of the IH/N group, in contrast to Atp2a1 mRNA (encoding first type-specific SERCA1). mRNA levels of both Atp2a1 and Atp2a2 were comparable in the MAS muscle between the IH/N and N/N groups. Hypoxia-inducible factors, HIF1 (encoded by Hif1a mRNA) and HIF2 (encoded by Epas1 mRNA)

are often assessed in perinatal animal models of hypoxic neuronal injury and impaired responsiveness to hypoxia during postnatal life. In our experimental model, mRNA levels of Hif1a and Epas1 were comparable between the IH/N and N/N groups in the GH muscle (Hif1a, 1 ± 0.097 in the N/N group vs. 1.034 ± 0.151 in the IH/N group, p = 0.658; Epas1, 1 ± 0.179 in the N/N group vs. 0.871 ± 0.081 in the IH/N group, p = 0.140) (Figure 16a), and the MAS muscle also showed no significant differences mRNA levels of the two hypoxic markers between the IH/N and N/N groups (Figure 16b). The GH qPCR data suggest that gestational IH affects mitochondrial biogenesis in the GH muscle.

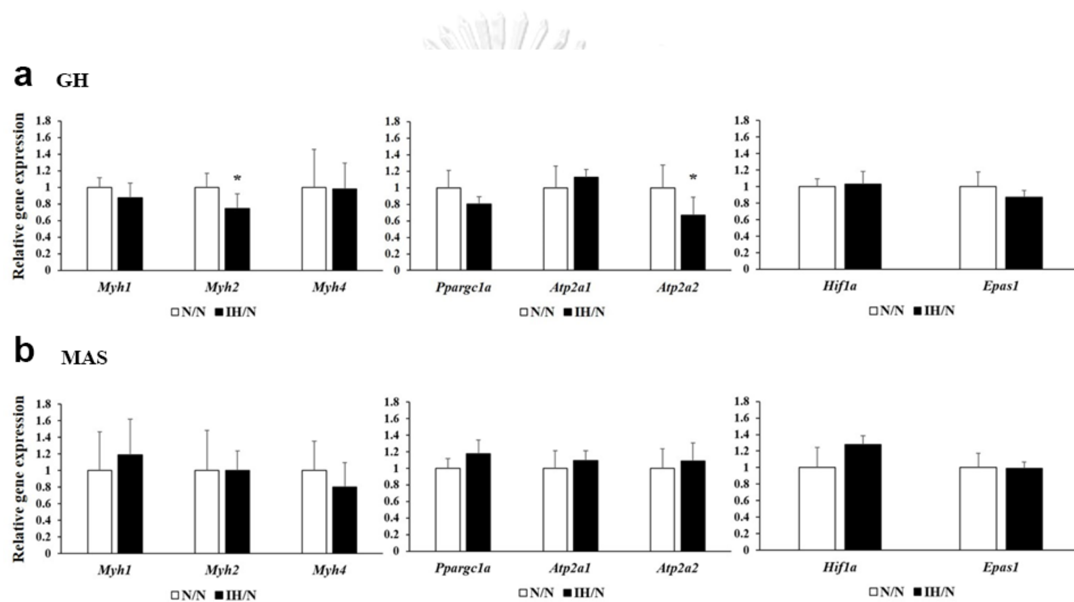

Figure 16 ***Quantitative polymerase chain reaction analysis in the GH and MAS muscles of offspring rats.***

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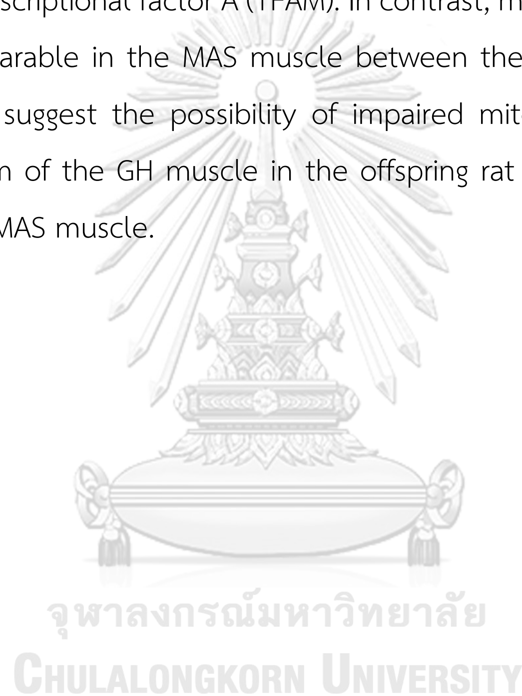
Gene expression of muscle fiber type-related factors (*Myh1*, *Myh2*, *Myh7*, *Atp2a1*, and *Atp2a2*) and hypoxic-inducible factors (*Hif1a* and *Epas1*) in GH **(a)** and MAS **(b)** muscle from N/N and IH/N groups. Relative expression level of each gene was normalized to the level of *Lbr* expression, and the relative expression levels were set to 1 for the N/N group. Data represent male offspring (n = 6) in each group. *$p < 0.05$ vs. the N/N group.

4.4 Downregulation of mitochondrial biogenesis and fusion proteins in the GH muscle of gestational IH offspring

Western blot analysis revealed that gestational IH induced significant downregulation of PGC1α protein in the GH muscle, but not in the MAS muscle (Figure 17a and 17b). The levels of mitochondrial fusion proteins, such as OPA1 and MFN2, were significantly decreased and the level of mitochondrial fission protein, FIS1, was significantly increased in the GH muscle of the IH/N group (Figure 17a). Data were supported by decreased levels of ATP synthase subunit alpha (ATP5A1) and mitochondrial transcriptional factor A (TFAM). In contrast, mitochondria-related protein levels were comparable in the MAS muscle between the N/N and IH/N rats (Figure 17b). These data suggest the possibility of impaired mitochondrial biogenesis and energy metabolism of the GH muscle in the offspring rat exposed to gestational IH, compared to the MAS muscle.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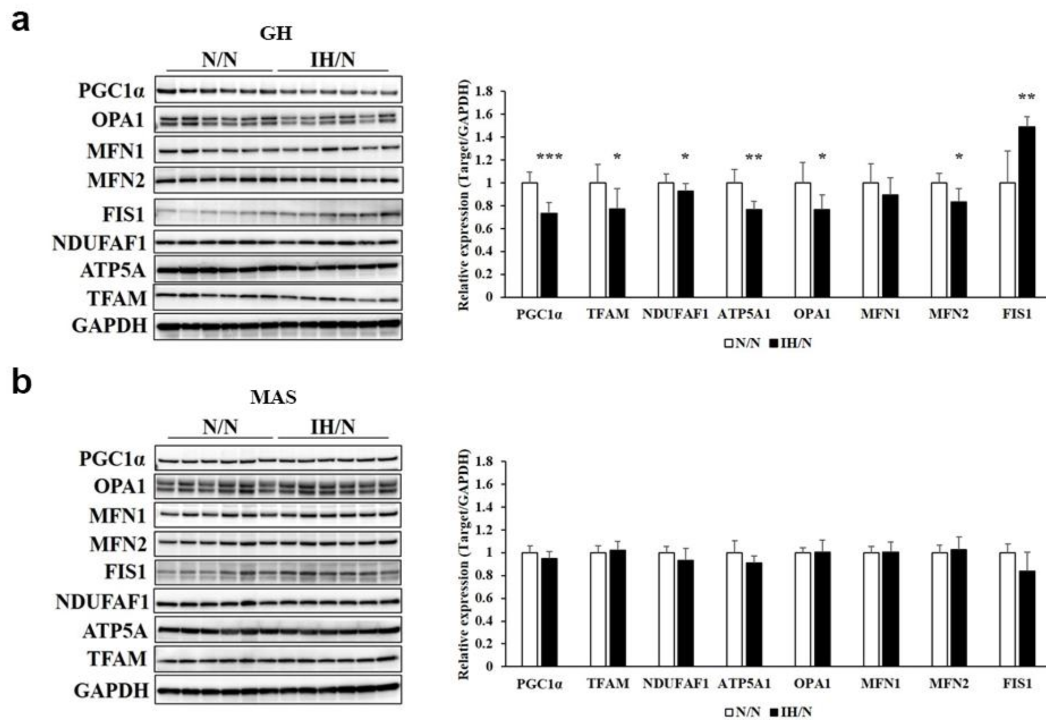

Figure ***17 Protein levels of mitochondrial metabolic markers in the GH and MAS muscles of offspring rats.***

Western blot images for peroxisome proliferator-activated receptor-gamma coactivator 1 (PGC1α), mitochondrial transcription factor A (TFAM), NADH:Ubiquinone Oxidoreductase Complex Assembly Factor 1 (NDUFAF1), ATP5A1, optic atrophy 1 (OPA1), mitofusin (MFN)1, MFN2, mitochondrial fission 1 (FIS1) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in GH **(a)** and MAS **(b)** muscles. The expression levels of the long and short isoforms of OPA1 were quantified together. The expression levels of the long and short isoforms of OPA1 were quantified together. Relative expression level of each protein was normalized to the level of GAPDH expression, and the relative expression levels were set to 1 for the N/N group. Data represent male offspring (n = 6) in each group. **p* < 0.05, ***p* < 0.01 and ****p* < 0.001 vs. the N/N group.

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4.5 Upregulation of skeletal autophagic activity in the MAS muscle of gestational IH offspring

Western blot analysis revealed significant upregulated of autophagic activity, LC3II/LC3I, in MAS after gestational IH (IH/N; 1.59 ± 3.16, N/N; 0.89 ± 0.17, $p < 0.01$) (Figure 18). Autophagosome is one of the autophagy processes in skeletal muscle. In autophagy process, LC3-I, the cytosolic form, transforms to LC3-II on the autophagosomes which further fuse with lysosomes for autophagic activation. AKT, P-AKT, P-AKT/AKT, AMPK, P-AMPK, P-AMPK/AMPK were comparable between the IH/N and N/N groups in the MAS muscle but no significant difference was detected.

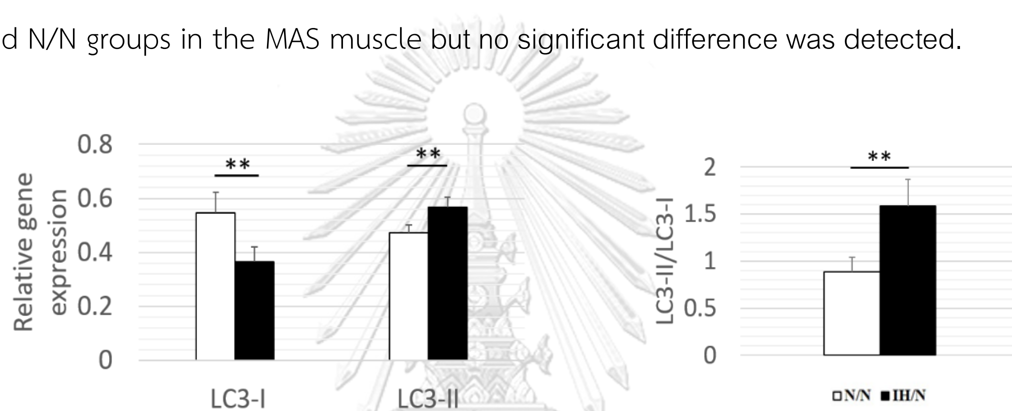

Figure 18 ***Protein levels of autophagic markers in the MAS muscles of offspring rats.***

Relative expression level of light chain 3-I (LC3-I) and LC3-II proteins were normalized to the level of GAPDH expression. LC3-II/LC3-I ratio represent autophagic activity within the MAS muscle. Results of the N/N and IH/N groups were comparable. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. the N/N group.

CHAPTER V DISCUSSION

In utero exposure to environmental stress impairs the regulation of mitochondrial dynamics in the rat placenta and skeletal muscles of the offspring, with a reduction in transcriptional regulators (PGC1α and PGC1β) [78]. However, it is unknown about the effects of gestational IH on postnatal development of the GH muscle which has a key role in suckling, a motor behavior that then gives way to chewing in young offspring.

The GH muscle has only a few type I muscle fibers (only 1.0% in total muscle fibers from our analysis) and has a low aerobic capacity owing to a high percentage (98 –100 %) of type IIA, IIX/D, and IIB muscle fibers [105]. Our data was consistent with the findings of a previous study on the muscle fiber composition of rat GH muscle [105] (Fig. 2a). Slower muscle fibers have a higher concentration of mitochondria than fast fibers, and type IIA fibers have a higher concentration of mitochondria than fast fibers and type IIX/D and IIB fibers [106]. In our model, a decreased fiber diameter of type IIA fibers was observed in the GH muscle of the gestational IH group. Myh2 is a specific maker gene for type IIA fiber, and qPCR analysis suggests that gestational IH leads to small type IIA fiber via the decreased Myh2 expression. Reduction of type I and/or type IIA fiber size correlates with decreased mitochondrial oxidative enzyme activity in muscle fibers [107]. Alteration in the diameter of type IIA fibers may reflect mitochondrial metabolism in the GH muscle of offspring of rats exposed to gestational IH.

PGC1 regulates energy metabolism through mitochondrial biogenesis. Western blot analysis showed that the protein level of PGC1α decreased in the GH muscle of preadolescent rats exposed to gestational IH. In our model, a subunit of the mitochondrial ATP synthase protein, ATP5A1, was also decreased in the GH muscle of the offspring of rats exposed to gestational IH. Silencing of the PGC1α gene induces mitochondrial fragmentation by decreasing the levels of OPA1, MFN1, and MFN2 and increasing the levels of mitochondrial fission proteins [77]. We observed decreased levels of OPA1 and increased levels of FIS1 in the GH muscle of the offspring with

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gestational IH. Mitochondrial fission separates the dysfunctional or damaged components from the healthy mitochondrial network [108]. However, excessive mitochondrial fission generates isolated mitochondria that are less efficient in ATP production and are dysfunctional because, under defects in mitochondrial fusion, they consume ATP to maintain their membrane potential [109]. Increased fission and/or decreased fusion leads to dysfunctional fragmented organelles, which results in decreased muscle fiber size and metabolic shifting [110]. Moreover, we observed the decreased protein levels of TFAM in the GH muscles of IH offspring, which reflects the decrease in mitochondrial biogenesis. Our findings suggest that gestational IH prompts muscular mitochondrial fission in the GH muscle, which leads to a decline in skeletal muscle mitochondrial function.

Postnatal IH induces mitochondrial fission via decreased Mfn2 expression and increased mitochondrial fission protein expression in cardiomyocytes, which leads to left ventricular hypertrophy and impaired contractile function in male rats [111]. Thompson et al. showed that gestational IH decreases cardiac contractile function in the heart of a male offspring due to the reduction of mitochondrial maximal respiration, respiratory reserve capacity, and complex IV activity rates in cardiomyocytes [112]. Gestational hypoxia during the advanced stages of fetal development decreases the mRNA levels of Mfn2 and Pgc1α and increases Fis1 and Drp1 mRNA levels in the heart of the rat offspring, which show mitochondrial structural abnormalities, dysfunction, decreased biogenesis, and mitochondrial fission/fusion imbalance [113]. Our findings suggest that gestational IH induces mitochondrial fission/fusion imbalance and impairment in the GH muscle of offspring rats.

The MAS muscle was insusceptible to gestational IH, unlike the GH muscle. We observed specific type distribution, fiber size and frequency of type I, IIA, IIX/D and IIB in rat GH and MAS muscles at baseline (Fig. 2 and 3). Gestational IH offspring showed a change in fiber size of type IIA fibers of the GH muscles, in contrast to the MAS muscle (Fig. 2b and 3b). Fogarty and Sieck showed different force and fatigue properties between intrinsic (superior and inferior longitudinal and transversalis) and extrinsic (genioglossus) muscles in the rat tongue in relation to muscular fiber type percentage, and they indicated that rat genioglossus muscle has more highly fatigue

resistant due to a higher proportion of type I and IIA fibers, than the intrinsic tongue muscles [114]. Their demonstration based on muscular fiber type percentage may provide one possible explanation for different metabolic responses of the GH and MAS muscles to gestational IH. Moreover, functional development of the MAS and GH muscles occurs after birth, which may elicit different responses of each muscle in the offspring. The development of rodent MAS muscles is closely associated with facial development and feeding after birth [115].

The MAS and GH muscles have different embryonic origins that arise at spatially distinct locations during the early embryonic period. A previous study on orofacial muscle formation in mouse embryos have shown the initiation of trigeminal innervation to the MAS muscle at embryonic day (ED) 10.5, in the first pharyngeal arch and a significant change in MHC composition of embryonic MAS muscle from EDs 14 to 18 [115]. On the other hand, the myogenic cells of the murine tongue start to migrate a long distance from the occipital somites toward the pharyngeal arches around ED 10.5 and form tongue muscles [116]. Moreover, hypoxia and increased mitochondrial respiration, with the concomitant production of reactive oxygen species, are known to affect morphogenic processes and cell function during embryonic development [117]. Early fetal hypoxia causes tissue-and cell type-specific growth restriction and cell proliferation in murine embryonic cells of the myocardium, but not in the cells of the spinal cord and the brain [118].In this study, the IH period from EDs 7 to 20 covered the period of early embryonic development for the MAS and GH muscles in rodents. The susceptibility of MAS and GH muscles to gestational IH may depend on tissue-specific effects of hypoxia on myogenic cells, which have different origins and formation patterns during early embryo development.

The MAS was investigated in muscle growth and atrophy markers, also autophagy marker to identify muscle homeostasis within the MAS. Although gestational IH did not induce mitochondrial impairment in MAS but the autophagic activity was higher. Autophagy plays vital roles to provide energy during deprivation [119] and degrade damaged proteins within the cells. In order to maintain muscle mass, muscle homeostasis controls the balance between catabolic and anabolic process. Its imbalance leads to muscle growth and atrophy condition. Postnatal IH-exposure in

skeletal muscle and cardiac muscle enhance autophagy in previous studies [14, 120]. Impairment of autophagy induces myofiber degeneration [121], mitochondria deformities, and protein aggregation in the muscle cells [122]. The upregulation of autophagic activity in MAS might be the adaptation to the gestational IH for maintenance of muscular homeostasis.

CHAPTER VI CONCLUSION

In conclusion, gestational IH reduces the size and mRNA expressions of selective muscle fiber IIA and also induces mitochondrial impairment in the GH muscle of male offspring, but not in the MAS muscle. Although we did not investigate autophagy or muscle growth and atrophy markers in GH, gestational IH induces autophagy in MAS without significant change in growth and atrophy. These results suggest the different susceptibility of prenatal IH to mitochondrial metabolism of the GH muscle in male offspring rat, compared to the MAS muscle.

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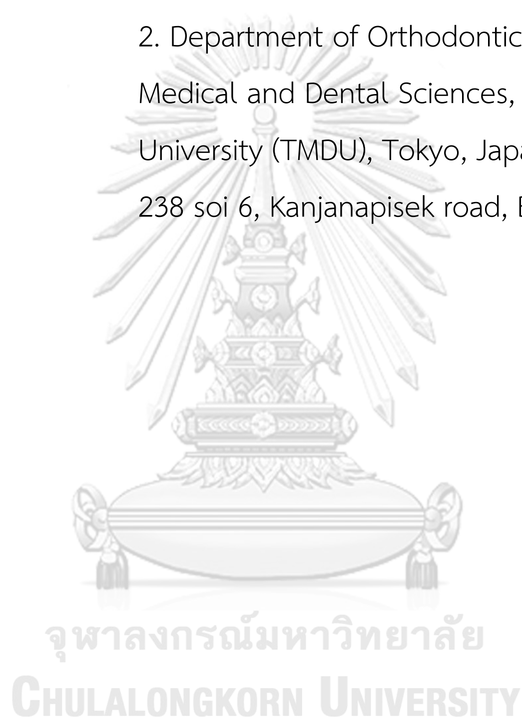
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