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AAO Foundation Final Report Form
(a/o 5/30/2021)

In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:

- *Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)*
- *Does this Final Report provide the level of detail, etc. that you would expect, if you were the reviewer?*

Please prepare a report that addresses the following:

Type of Award, e.g., Orthodontic Faculty Development Fellowship Award, Postdoctoral Fellowship Award, **Biomedical Research Award**, Center Award, Educational Innovation Award, Program Award, Research Aid Award

Name(s) of Principal Investigator(s)
Pae, Eung-Kwon

Institution
University of Maryland, Baltimore

Title of Project
Orofacial Bone Growth Defects Triggered by Neonatal Breathing Disturbance

Period of AAOF Support (e.g. 07-01-2021 to 06-30-2022):
07-01-2019 to 01-31-2022
(The support period was extended significantly due to COVID-19)

Amount of Funding
$30,000

Summary/Abstract

Morphometric studies reported a high incidence of skeletal malocclusions occurring among who were born preterm (Paulsson et al., 2008; Rythén et al., 2013). Prevalence of preterm birth (before 37 weeks of pregnancy) rate includes 10% of total newborns in the US (Martin et al., 2017). Disturbed breathing, expressed as apnea, is common in infancy, and appears near-universally in younger preterm infants that constitute 20% of 15 million preterm babies born globally per year (Lawn et al., 2013, 2014). The consequences of these "stopped breathing" periods include neural damages resulting in cerebral palsy, epilepsy and behavioral abnormalities. We propose that the preterm infants who experienced disturbed breathing are predisposed to compromised bone development, resulting in defective craniofacial growth, and skeletal malocclusions in later age. If the link between disturbed breathing in preterm and craniofacial defects in later age is confirmed, orthodontists are to diagnose and treat such skeletal malocclusions from a new perspective etio-pathophysiology. To date, no *mechanistic study* has carried out on the cause-effect relationship between breathing disturbances in preterm infants and long-term skeletal defects. Our rat model provides a means to study skeletal defects triggered by disturbed breathing in preterm infants. The central *hypothesis* is that long-term defective craniofacial growth and poor bone quality are induced by elevated sympathetic tone due to disturbed breathing. We will: 1) Study characteristics of orofacial bones in rat pups exposed to intermittent hypoxia (IH, alternating oxygen desaturation and saturation repeatedly) at postnatal day 0 (P0) to simulate preterm disturbed breathing, 2) Understand the potential pathophysiology of the skeletal defects induced by elevated sympathetic tone resulting from disturbed breathing. Neural development in rat pups at P0 is equivalent to that of human infants at gestational week 25 (Semple et al., 2013). Because IH results from apnea of prematurity and periodic breathing is common in preterm infants, we will expose P0 rat pups to one-time 1h IH alternating ambient O_2 levels between 21% and 10% to mimic disturbed breathing. We will address the following aims: **Aim 1:** Define morphometric characteristics of the orofacial growth of the IH pups using micro-computed tomography (μCT) with the *hypothesis* that one-time neonatal brief IH exposure at P0 will disrupt normal growth of the mandible, maxilla and cranial base. The rationale is that increased levels of sympathetic discharge after IH-challenge (Prabhakar et al., 2015) favor bone resorption, and decrease bone apposition and endochondral bone formation due to increased local β2-adrenergic activity as reported previously (Elefteriou et al., 2014, 2018). **Aim 2:** IH-treated P0 pups will show elevated sympathetic discharge that triggers orofacial bony defects. The damaged skeletal growth can be reversed by β-adrenergic blockers. The *rationale* is that IH exposure increases sympathetic discharge, activating adrenal glands, and elevates circulating noradrenaline (NA). Elevated levels of NA in blood will be sustained high in the IH rats. Yet, these animals treated with the β-blocker will exhibit improved bone quality and jaw size. ***Plans for Future:*** Based on the outcomes, we will examine the cause-effect relationship between breathing and bone growth in human children since the expected outcomes can help understand why orthodontic patients who were prematurely born tend to have malocclusions with an increased prevalence.

Detailed results and inferences:

1. If the work has been published please attach a pdf of manuscript OR
2. Describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis, and interpretation of results should be included.

The manuscript has been prepared and revised several times; however, it has not been published yet. The results are summarized as follow:

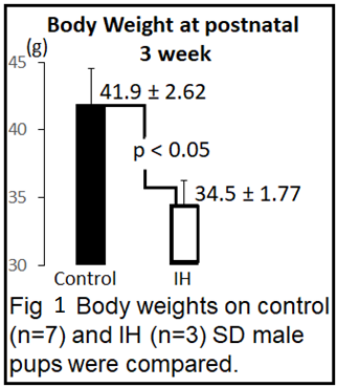

Fig 1 Body weights on control (n=7) and IH (n=3) SD male pups were compared.

One hour IH exposure alternating between 21% and 10% every 4 min on Postnatal Day 0 (in 2 h after parturition) to rat pups resulted in following summary: Data were collected at 3, 4 and 5 weeks postnatal. At 3 weeks postnatal, body weight showed a significant difference (See Fig 1). IH treated rats loosed approximately 30% of their body weight. As the pups grew older, the weight differences between control vs. IH-treated pups have reduced. This finding is supported by our as well as others' previous research results.

The main reason for the significant weight loss would be a lack of bone mass in the IH-groups as shown in Fig 2. IH-treated animals showed an increased bone marrow spaces with elevated osteoclastic activity (Fig 2). Fig 2 exhibited an increased TRAP activity in subcondylar areas in the tissues obtained from male pups.

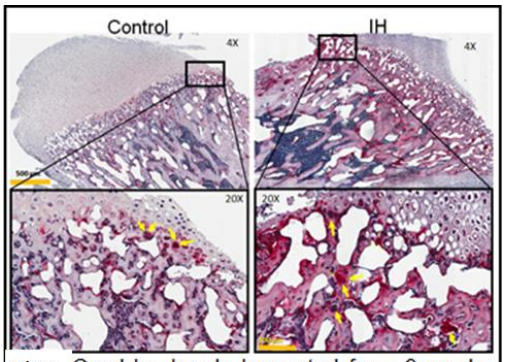

Fig 2 Condylar heads harvested from 3 weeks old male control and IH-treated (at P0) pups are compared. IH condyle shows more bone marrow (BM) spaces and an increased TRAP activity with more osteoclasts (yellow arrows) in the ROI. Scale bar in 20X panel = 100 µm (unpublished data)

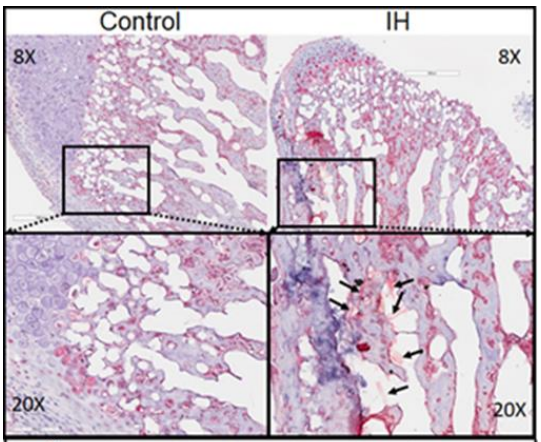

Fig. 3 Immunohistochemical stain using tyrosine hydroxylase (TH) antibody. Sprouting TH-positive sympathetic nerve fibers indicated by arrows are evident in osteochondral interface of IH-treated mandibular condyle as exhibited in the lower right panel. (Unpublished data)

We suspected an increased sympathetic function; thus, we stained the same condyle areas with tyrosine hydroxylase (TH) antibody to stain sympathetic fibers (See Fig 3). As shown, an increased density of TH-positive sympathetic fibers was evident in pink color. Our immunohistochemistry studies conclude that subchondral areas of the condyle region clearly demonstrate a lack of bone density with the increased osteoclastic activity due to increased sympathetic nerve activity.

Next, we compared IH pups vs. control pups in the results from assays on central blood serum taken from the heart. We performed ELISA assays on nor-epinephrine (NE) levels in serum obtained from 68 pups (33 males and 35 females) (See Fig 4). The male IH pups shows higher NE levels until 5 weeks old marks. However, female pups showed an elevated NE levels in IH-pups at 3 week old only. This sexual disparity deems further investigations.

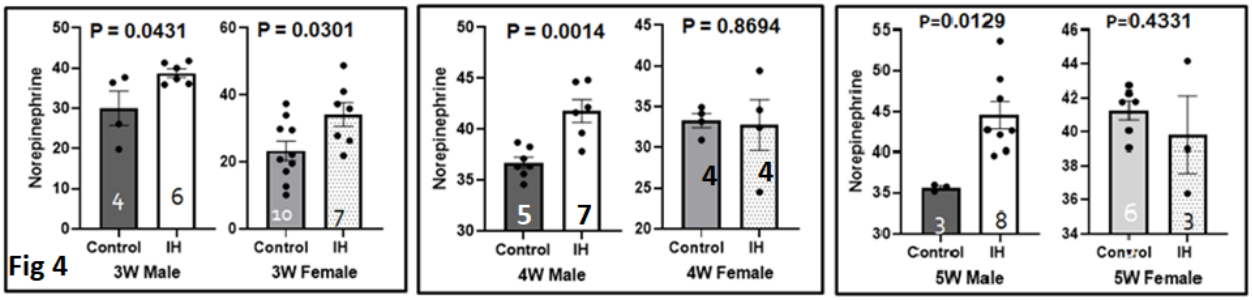

Fig 4

Then, we evaluated morphometric differences in the facial bones at 3 week-old time point. First, we report a significant transverse defect in the maxilla *i.e.*, a lack of intermolar widths (control vs. IH; 6.47 (n=5) vs. 5.98 (n=5), p = 0.0194 in males; 6.43 (n=7) vs. 6.16 (n=5), p = 0.0243 in females). Interestingly, any other anatomical structures including condyle width showed a significant defect (15.39 vs. 14.40, p = 0.0125 in males; 16.29 vs. 14.06, p = 0.0003 in females). As pups grow, a significance in size differences decreased. This finding supports a previous clinical report by Objois and Gebeile-Chauty (2019) showing a significant lack of palatal width among orthodontic patients born preterm.

In conclusion, one-time IH-exposure for 1 hour immediate after birth disturbed the bone remodeling process for at least 5 weeks postnatal. This disturbance is probably due to an increased sympathetic activity from IH exposure. We observed increased NE levels in the blood in IH-exposed groups when male and female are 3 weeks old. IH-exposed male pups showed elevated NE levels in 4 weeks and 5 weeks postnatal also. We conclude that one-time short IH exposure can result in defects in bone quality and size of the facial bones particularly in male rats.

Respond to the following questions:

1. Were the original, specific aims of the proposal realized?

The original aims of the proposal are realized; yet the project could not carry out morphometric studies using µCT due to the significant time delay on accessing the facility and their unexpected high charge for use. Thus, the research material was shipped out to an external facility and defleshed by beatles. So a lot of tissues and materials were wasted inevitably. Some analyses were not performed.

2. Were the results published? **Not yet**.
    a. If so, cite reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers
    b. Was AAOF support acknowledged? **Yes.**
    c. If not, are there plans to publish? **Revision of the manuscript is underway**. If not, why not?
3. Have the results of this proposal been presented? **Not yet**.
    a. If so, list titles, author or co-authors of these presentation/s, year and locations
    b. Was AAOF support acknowledged?
    c. If not, are there plans to do so? **Yes**. If not, why not?
4. To what extent have you used, or how do you intend to use, AAOF funding to further your career?

AAOF funds have been providing a chance to investigate etio-pathophysiology of various skeletal malocclusions by means of animal models. Otherwise, clinicians have to rely on conjectures without any direct cause-effect connections between etiological conditions and malocclusions. Because most of my studies in orthodontics have not been 'sufficiently fundamental' to draw attention from NIH reviewers, I would continue to rely on AAOF.

Accounting for Project; (i.e.), any leftover funds, etc.

As of the project ended by 01-31-2022, the leftover amount of the fund was returned to AAOF.