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ALTERATION OF EXTRACELLULAR MATRIX COMPONENTS IN ANTERIOR PITUITARY
GLAND OF NEONATAL RATS INDUCED BY MATERNAL BISPHENOL A (BPA) DIET DURING
PREGNANCY



Miss Bumpenporn Sanannam

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Medical Sciences

Common Course

FACULTY OF MEDICINE

Chulalongkorn University

Academic Year 2021

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การเปลี่ยนแปลงของ extracellular matrix ในต่อมใต้สมองส่วนหน้าของลูกหนูแรทแรกเกิดจากแม่
ที่ถูกเหนี่ยวนำด้วยสาร bisphenol A (BPA) ในช่วงตั้งครรภ์



น.ส.บำเพ็ญภรณ์ สนั่นนาม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์ ไม่สังกัดภาควิชา/เทียบเท่า
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| | |
|-------------------|---|
| Thesis Title | ALTERATION OF EXTRACELLULAR MATRIX COMPONENTS IN ANTERIOR PITUITARY GLAND OF NEONATAL RATS INDUCED BY MATERNAL BISPHENOL A (BPA) DIET DURING PREGNANCY |
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 ลูกหนูแรทแรกเกิดจากแม่ที่ถูกเหนี่ยวนำด้วยสาร bisphenol A (BPA) ในช่วงตั้งครรภ์. (
 ALTERATION OF EXTRACELLULAR MATRIX COMPONENTS IN ANTERIOR PITUITARY
 GLAND OF NEONATAL RATS INDUCED BY MATERNAL BISPENOL A (BPA) DIET DURING
 PREGNANCY) อ.ที่ปรึกษาหลัก : ผศ. ดร.เดกษา จินดาทิพย์, อ.ที่ปรึกษาร่วม : ผศ. ดร.เทวฤทธิ์ สะ
 ระชนะ

สารบิสฟีนอล เอ หรือ บีพีเอ เป็นสารเคมีที่ใช้เป็นส่วนประกอบในการผลิตพลาสติกชนิดแข็ง
 สารเคลือบวาวบนภาชนะบรรจุภัณฑ์ต่างๆอย่างแพร่หลาย มีรายงานถึงการได้รับสารนี้ของทารกในครรภ์ ส่งผลต่อ
 การเปลี่ยนแปลงของเซลล์ที่ทำหน้าที่ผลิตฮอร์โมนในต่อมใต้สมองส่วนหน้า อย่างไรก็ตามยังไม่พบรายงานใน
 ปัจจุบันถึงผลต่อการเปลี่ยนแปลงของ extracellular matrix (ECM) ซึ่งมีบทบาทสำคัญในการควบคุมสมดุลการ
 ทำงานของเซลล์ต่างๆในต่อมใต้สมองส่วนหน้า ในงานวิจัยนี้ได้นำต่อมใต้สมองของลูกหนูแรทเกิดอายุ 1 วันที่เกิด
 จากแม่หนูแรทที่ถูกกระตุ้นด้วยการป้อนสารบีพีเอรายวันปริมาณ 5,000 ไมโครกรัมต่อน้ำหนักตัว 1 กิโลกรัม มา
 เพื่อศึกษาการเปลี่ยนแปลงที่เกี่ยวข้องกับ ECM ในต่อม รวมถึงการศึกษาการเปลี่ยนแปลงของเซลล์ที่ทำหน้าที่
 สร้าง ECM โดยศึกษาทั้งจำนวนเซลล์ และคุณลักษณะภายใต้กล้องจุลทรรศน์ การแสดงออกของยีนที่เกี่ยวข้อง
 ด้วยวิธีการ Real-time RT-qPCR และการศึกษาการเปลี่ยนแปลงภายในเซลล์ รวมถึงการจัดเรียงตัวของเซลล์
 ภายในต่อม โดยอาศัยการศึกษาภายใต้กล้องจุลทรรศน์อิเล็กตรอน ผลจากการศึกษาแสดงให้เห็นว่าสารบีพีเอ
 ส่งผลให้เกิดการลดลงของจำนวน pericytes รวมถึงปริมาณคอลลาเจนที่เซลล์ชนิดนี้สร้าง สอดคล้องกับผลของ
 folliculostellate cells มีจำนวนเพิ่มขึ้น ซึ่งเซลล์นี้ทำหน้าที่สร้างตัวควบคุม ECM มากมาย พบการแสดงออก
 ของระดับ mRNA ที่เพิ่มมากขึ้นของ *MMP2*, *MMP9* และ *TIMP 1* ถึง 4 นอกจากนี้ในกลุ่ม SLRPs พบว่ามีการ
 แสดงออกของ decorin และ osteoglycin เพิ่มขึ้น ส่วนการแสดงออกของ PRELP และ Tsukiji มีค่าลดลง
 นอกจากนี้การศึกษายภายใต้กล้องจุลทรรศน์อิเล็กตรอนแสดงให้เห็นถึงความบกพร่องของรูปแบบการจัดเรียงตัว
 ของเซลล์ภายในต่อม ข้อมูลจากงานวิจัยนี้ แสดงให้เห็นถึงผลจากการที่ได้รับสารบีพีเอในช่วงตั้งครรภ์ แม้อยู่
 ภายใต้ระดับความเข้มข้นของสารที่ไม่ก่อให้เกิดผลกระทบต่อสุขภาพที่ปรากฏอาการให้เห็น (no-observed-
 adverse-effect-level) แต่กลับส่งผลให้เกิดการเปลี่ยนแปลงของเซลล์ที่สร้าง ECM และปริมาณของ ECM ใน
 ที่นี้คือ คอลลาเจน รวมถึงยีนที่เกี่ยวข้องกับการควบคุมสมดุลของ ECM ในต่อมใต้สมองส่วนหน้าของทารก ด้วย
 เหตุนี้จึงควรตระหนักและเพิ่มความสนใจถึงผลกระทบที่ส่งผลเสียต่อสุขภาพของสารบีพีเอ โดยเฉพาะอย่างยิ่งใน
 กลุ่มของหญิงตั้งครรภ์

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5974758530 : MAJOR MEDICAL SCIENCES

KEYWORD: Anterior pituitary gland Bisphenol A Extracellular matrix Folliculostellate cell
Neonate Pericyte Matrix metalloproteinases Tissue inhibitor of matrix
metalloproteinases

Bumpenporn Sanannam : ALTERATION OF EXTRACELLULAR MATRIX COMPONENTS IN
ANTERIOR PITUITARY GLAND OF NEONATAL RATS INDUCED BY MATERNAL BISPHENOL
A (BPA) DIET DURING PREGNANCY. Advisor: Asst. Prof. DEPICHA JINDATIP, Ph.D. Co-
advisor: Asst. Prof. Tewarit Sarachana, Ph.D.

Bisphenol A (BPA) is a well-known endocrine-disrupting chemical that is widely used in the resin and plastic manufacturing sectors. Prenatal exposure to this chemical results in changes of pituitary hormone-producing cells. However, there have been no reports of the alterations of extracellular matrix (ECM) which play a role in controlling cell activities in the pituitary gland. In the present study, a daily oral dose of 5,000 µg/kg BW of BPA was administered to pregnant rats. ECM-producing cells, i.e., pericytes and folliculostellate cells, ECM products, and ECM regulators were investigated in the neonatal anterior pituitary at day 1 at the histology and molecular levels. The number of cells and immunosignal intensity were determined under light microscopy. Real-time RT-qPCR was used to investigate the mRNA expression of the ECM products and their controllers. The ultrastructural changes were observed by transmission electron microscopy. In BPA-treated rats, pericytes and their collagen syntheses were reduced, consistent with the increase in the number of FS cells that expressed several ECM regulators. Components of the ECM regulators were also adaptable. *MMP2*, *MMP9*, and *TIMPs* (*TIMP 1-4*) mRNAs were upregulated. In addition, mRNA levels of decorin and osteoglycin were increased, whereas those of PRELP and Tsukiji were dropped. Moreover, transmission electron microscopy showed the unorganized-cell cluster in the gland. This study revealed that although mother received BPA at a no-observed-adverse-effect level, the alterations of ECM-producing cells as well as collagen and related ECM balancing genes occur in the neonatal anterior pituitary gland. Therefore, awareness of the harm from this chemical should be raised, especially during pregnancy.

Field of Study: Medical Sciences

Academic Year: 2021

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ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude and thanks to my major advisor, Assist. Prof. Depicha Jindatip, who provided tremendous supports and guidance with patience through a lot of hard work and bad times. She offered excellent instruction, encouragement, and care, helping me improve myself in all aspects of my life, whether I was working or living. Without her tireless assistance, I would not have been able to complete this dissertation and Ph.D. life. I want to thank my co-advisor, Assist. Prof. Tewarit Sarachana, for guiding, supporting, and solving my molecular studies. He always backs me up and brings my wretched soul back to reality. Without his compassion and encouragement, I would not be able to get through the difficult times. Furthermore, I would like to sincerely thank all of his students in his unit for their help, guidance, and support, especially Ms. Surangrat Thongkorn and Mr. Songphol Kanlayaprasit.

When Prof. Dr. Vilai Chentanez was the welcoming chairman, that was my pleasure. I would like to express my gratitude for her kindness, guidance, and instruction. In addition, I'd like to express my gratitude to my thesis committee: Assoc. Prof. Dr. Vilai Anoma-siri, Prof. Dr. Sittiporn Agthong, and Assist. Prof. Dr. Suwadee Chaunchaiyakul for their invaluable guidance on my research, which contributed significantly to its success.

I'd want to express my gratitude to Ms. Sasikarn Looprasertkul, a wonderful colleague, coworker, and supporter. She always goes out of her way to assist me, even when I don't ask for it. Furthermore, she delivers her best abilities to all processes. Ms. Atitaya Roumwong assisted me in performing several tips and techniques in many procedures in various experiments. I'd like to express my gratitude to Mr. Preecha Reuangwechvorachai for his assistance and advice in an immunohistochemistry investigation and to Miss Wilawan Ji-au and Miss Sirinapa Srikam for their assistance in using an electron microscope. I'd want to express my gratitude to the Histo Center and Rushmore Company for providing the necessary equipment and program assistance.

Lastly, I wish to acknowledge the support and great love of my family and my friends. They kept me going on and this work would not have been possible without their care and encouragement. Lastly, I would like to thank Miss Apinya Buthlee for her assistance several times. This work was supported by The 90th Anniversary Chulalongkorn University Fund Graduate School, Chulalongkorn University and Ratchadapiseksompotch Fund, Faculty of Medicine, Chulalongkorn University.

Bumpenporn Sanannam

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LIST OF ABBREVIATIONS

| | |
|----------|---|
| ASD | Autism spectrum disorder |
| BPA | Bisphenol A |
| Col1a1 | Collagen type I |
| Col3a1 | Collagen type III |
| DES | Diethylstilbestrol |
| DIP cell | Novel desmin-immunopositive perivascular cell |
| ECM | Extracellular matrix |
| EDC | Endocrine-disrupting chemicals |
| EE | Ethinyl estradiol |
| ER | Estrogen receptor |
| ESFA | European Food Safety Authority |
| FDA | Food and Drug Administration |
| FS cell | Folliculostellate cells |
| GI | Gastrointestinal |
| IBD | Inflammatory bowel disease |
| MMP | Matrix metalloproteinase |
| NOAEL | No-Observed-Adverse-Effect level |
| PRELP | proline and arginine rich end leucine rich repeat protein |
| SLRP | Small leucine-rich proteoglycans |
| TEM | Transmission electron microscopy |
| TIMP | Tissue inhibitors of metalloproteinases |

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Bisphenol A (BPA) is one of the most acquainted and widespread endocrine-disrupting chemicals (EDCs) primarily used as a monomer of polycarbonate plastics and epoxy resin productions. BPA is approved as an endocrine disruptor owing to its estrogenic activity. A part of its structure is similar to estradiol which can bind and activate human estrogen receptors with a low-affinity effect compared to endogenous estradiol [1]. Invalid polymerization of residual BPA monomers in plastic containers and coatings can cause the potential of food contamination. Moreover, prolonged storage and heating processes are also other BPA environmental migration factors [2-4]. Several researchers reported the associations between BPA exposure and its adverse effects on prenatal and postnatal health status outcomes [5-9]. Since BPA has the potential to transfer through the umbilical blood circulation and the fetal metabolism of this chemical is limited, BPA is detected with a high concentration in both placenta and fetus [10]. Exposure to BPA in both embryonic and neonatal stages leads to a permanent decrease in fertility and an abnormal estrus cycle [6-8]. Moreover, BPA related to the decreasing in LH secretion in embryonic and postnatal BPA-exposed animals via the effect of gonadotropin-releasing hormone (GNRH) were also noted [8, 9]. This evidence indicated that the hypothalamo-hypophyseal system is also a target of BPA.

The anterior pituitary gland is the major organ that plays an important role in the endocrine system. This gland produces many essential hormones related to body homeostasis. Populations of the anterior pituitary gland are composed of hormone-producing cells, i.e., somatotrophs, lactotrophs, thyrotrophs, corticotrophs, gonadotrophs, and non-hormone cells including folliculostellate (FS) cells, endothelial cells, pericytes, and macrophages. The hormone-producing cells formed as clusters

intimately related to the FS cells and these clusters were separated by fenestrated capillaries. The areas around cell clusters (inter-parenchymal space) and capillaries (perivascular space) are occupied by the extracellular matrix or ECM [11]. Generally, ECM in almost all human organs was synthesized by fibroblasts. It is important to note that; however, ECM in the normal anterior pituitary gland is mainly produced by pericytes [12] and FS cells [13]. ECM displays potential roles in cellular interactions such as cell growth mediation, cell migration-proliferation, and cell differentiation [14]. Therefore, ECM, pericytes, and FS cells act as the controller of tissue remodeling [15]. Moreover, ECM also plays an essential role in controlling the function of cell populations and cell organization in the anterior pituitary gland, which is important to regulating hormone secretion [16]. The effects of BPA on the alteration of ECM components and characteristics were documented in some organs, for example, the liver fibrosis in rats [17] and the alters of ECM structure on cardiovascular function in mice [18]. In the anterior pituitary gland, BPA adverse effects on hormone-producing cells and their secretion changes were reported in several studies [19-21]. However, BPA-induced ECM changes, especially in the anterior pituitary of neonates, have not been clarified. Therefore, this study will be the first to evaluate the effects of BPA on the anterior pituitary gland of neonatal rats from BPA maternal exposure focusing on ECM alterations.

1.2 Research questions

- 1.2.1 Does BPA with NOAEL dose affect to ECMs in the neonatal anterior pituitary glands of maternal BPA-treated rats?
- 1.2.2 Do ECM-producing cells in the neonatal anterior pituitary glands alter their characteristics and numbers resulting from the BPA-NOAEL dose?
- 1.2.3 Does BPA alter ECM-related molecules in these neonatal anterior pituitary glands?

1.3 Research objectives

- 1.3.1 To demonstrate and clarify the effect of BPA with NOAEL dose on ECMs of neonatal anterior pituitary glands of maternal BPA-treated rats.
- 1.3.2 To observe the alteration of ECM-producing cells character and number resulting from BPA-NOAEL dose.
- 1.3.3 To evaluate ECM-related molecules alteration in these neonatal anterior pituitary glands after BPA treatment.

1.4 Research design

Animal experiment

1.5 Keywords

Anterior pituitary gland, Bisphenol A, Extracellular matrix, Folliculostellate cell, Neonate, Pericyte, Matrix metalloproteinases, Tissue inhibitor of matrix metalloproteinases

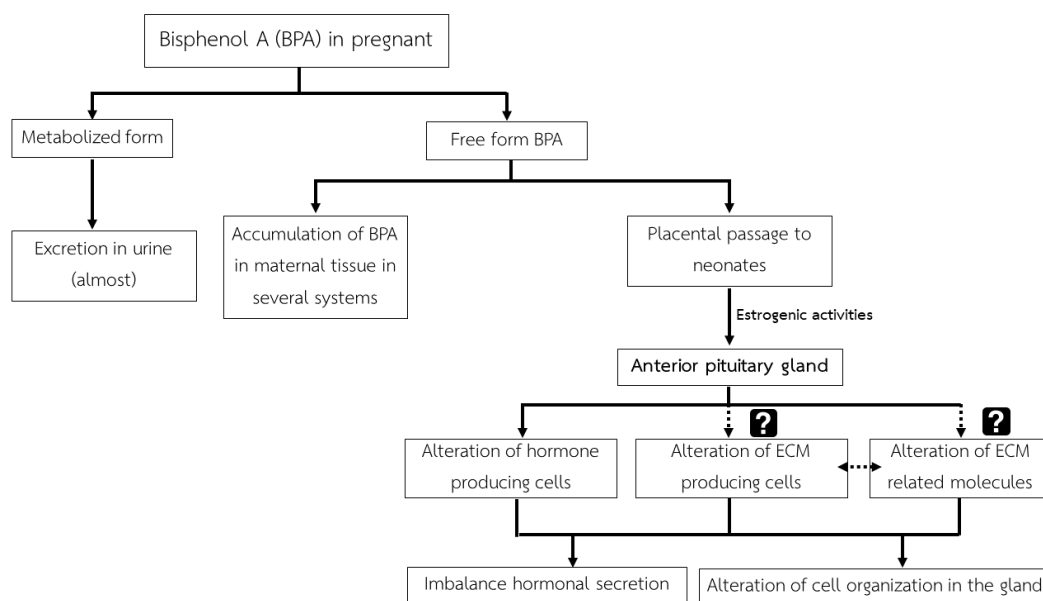
1.6 Hypothesis

Even at NOAEL level, maternal BPA exposure can affect the neonatal anterior pituitary gland ECM changes. This condition will cause ECM-producing cells to adapt, resulting in an imbalance of ECM product and ECM regulator components. ECM-producing cells can be changed on both structural and functional levels.

1.7 Benefit and application

This study revealed the effect of prenatal BPA exposure at NOAEL level on neonatal anterior pituitary gland focusing on the ECMs and their related controller alterations. This discovery data will be the basic knowledge that may further diagnose some pathology resulting from prenatal BPA exposure. Our findings are expected to raise awareness of the hidden dangers of BPA exposure in live people, particularly pregnant women.

1.8 Conceptual framework



CHAPTER II

LITERATURES REVIEWS

2.1 Endocrine-disrupting chemicals

Endocrine-disrupting chemicals (EDCs) are synthetic chemicals that can act as endogenous hormones. Several reports revealed that exposure to environmental EDCs can cause several additional effects such as alterations in metabolism, nervous system, cardiovascular system, and adaptation of behavioral status. Moreover, this compound is associated with abnormalities of reproduction and development in both wildlife and human [22]. Epidemiology about the relation of BPA's adverse effects on endocrine organs such as the pituitary gland and thyroid gland were also documented [23-25]. Exposures to this kind of compound can attack the endocrine system via interrupting simple biological behavior including the synthesis abilities, metabolic process, binding potential, and cellular responsiveness of natural hormones. From these epidemiological data, animal experiments, and human clinical investigations, EDCs are implied as public health concerns nowadays. One of the most widespread EDCs is bisphenol A or BPA.

2.2 Bisphenol A (BPA) structure

BPA is approved as an endocrine disruptor due to its estrogenic activity. BPA is an estrogen mimic structure formed by condensation of a couple of phenol groups and one acetone molecule (Figure 1). This compound can bind and activate the human estrogen receptor with a low-affinity effect compared to endogenous estradiol [1]. Dodds and Lawson mentioned that BPA is a weak estrogen [26].

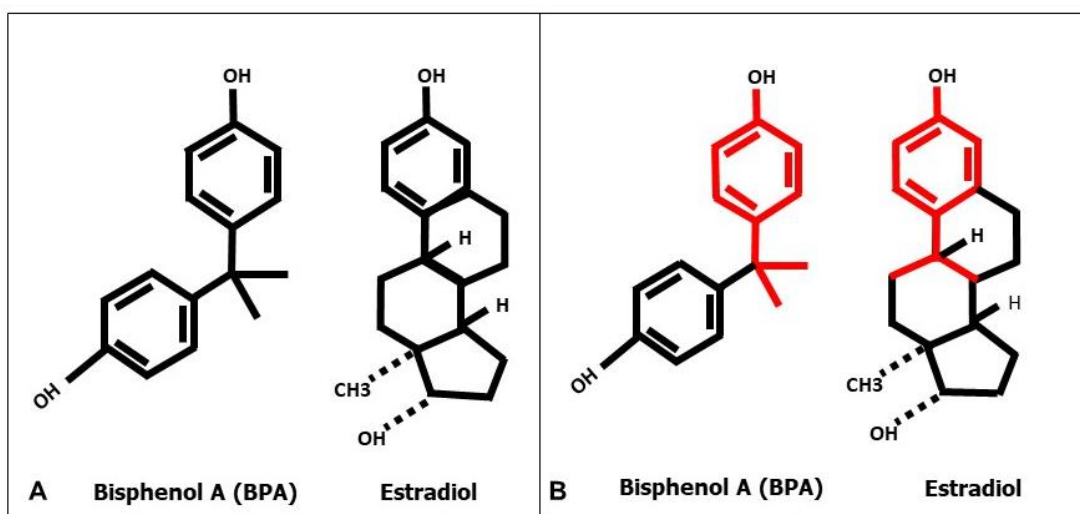


Figure 1 Chemical structure of BPA and estradiol (A, B), Note a part of similar form between BPA and estradiol (red) (B). Modified from Beverly, 2011 [27].

The structure of BPA (Figure 2) demonstrates largely binding sites that relate to hormonal activities. The result from EDC activities comparative study of BPA and various compounds revealed that the 4-hydroxyl group of phenyl ring (A, B binding site) is the crucial compartment for several hormonal bindings to influence their downstream activities. Moreover, other factors affect these activities including a replacement at 3,5-positions phenyl rings and alkyl bridging [1].

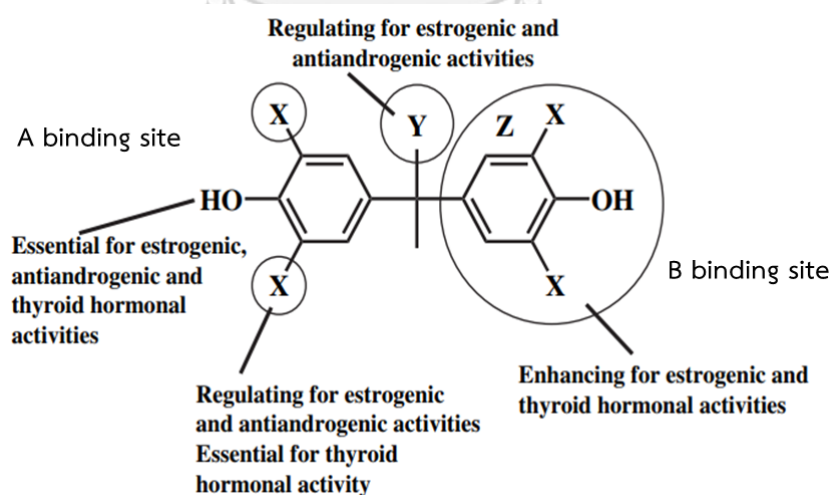


Figure 2 Requirement region of BPA structure related to endocrine-disrupting activities [1].

BPA is used in polycarbonate plastics and epoxy resin productions [18]. According to its characteristics, BPA is found in many consumer products such as reusable plastic bottles, baby feeding bottles, plates, goblets, cups, and microwave containers. Moreover, it is also used to coat internal surfaces lining the cans of food and beverage. These BPA applications may cause foodstuffs contact and contamination. Not only the foodstuffs industrial but several daily life products also contain BPA including sunglasses, building materials, compact disc, thermal receipts, medical equipment, and dental materials [3]. Calafat and colleagues reported in 2008 about an increase in BPA level with mean levels of 30.3 ng/ml were found in pediatric patients in the intensive care unit [28]. In addition, Olea and colleagues (1996) demonstrated BPA levels in the saliva of patients from 90 to 931 μg after receiving 50 mg of a dental sealant [29]. However, several incidents showed that BPA level increment was detected in persons who worked in a BPA-exposed environment [30-33].

2.3 BPA worldwide status

BPA can be leaked by several paths leading to a lot of BPA being detectable in humans. Industrial community wastewater, indoor and outdoor air can be the origin of environmental BPA contamination. Invalid polymerization of residual BPA monomers in plastic containers and coated surfaces can cause food contamination. Moreover, prolonged storage and heating processes are also other BPA environmental migration factors [2, 4, 34].

Since the 2000s, many researchers, under systematic reviews from more than 500 publications from many regions globally, have reported about contamination of BPA in the environment including water sources, ground (soil, sediment, mud), and air [35]. Corrales and colleagues (2015) evaluated BPA levels from the effluent sources.

The result revealed around 370 µg/l of BPA in these samples [35]. Moreover, the survey study found that BPA concentration was higher at the surface water more than the sub-surface area and the seabed [36]. It is interesting to note that surface water from Asia had the highest amount of BPA compared to reports from Europe and North America [35]. Studies of wastewater sedimentation from urban communities in Taiwan [37], China [38, 39] and Germany [40] demonstrated that the levels of BPA are 10,500 µg/kg, 3,400-3,600 µg/kg, and 1,630 µg/kg, respectively. Furthermore, the data about detected-BPA in tap water were reported by Colin et al. (2014) work. Their result showed the average value is 14 ng/l and the highest value is 1.3 µg/l [41]. In addition, BPA was also found in soil, sludge, and sediment. Staples et al. (2008) stated the amount of BPA in North American mud samples (780 µg/kg; dry weight) and Europe (160 µg/kg; dry weight). In the ground, the range of BPA level was around 1–150 µg/kg in Asia, Europe, and North America. The contamination might have occurred from several factors such as sewage sludge [42, 43], released effluent [44], and electronic landfill waste [45]. In the air, there were reports of BPA contamination in the atmosphere; at the house or working place, especially occupational exposure to BPA in plastics factories. A high level of BPA was observed in the air of resin-related industries ($> 50,000 \text{ ng/m}^3$) in China. Fu and Kawamura [46] reported comparing BPA levels between urban and countryside communities in India, China, Japan, and Germany. The result displayed urban community has BPA in the air more than 80 times compared to countryside communities.

2.4 Routes of human BPA exposure

Previously, ingestion was considered as the major route of human BPA exposure because BPA could leak out to the food or beverage containers. However, it probably has other exposure routes instead of ingestion [27]. Braun et al. (2010) report revealed exciting data about BPA levels in urine samples from pregnant women. They informed that the urinary BPA level was highest in pregnancy cashiers [47]. Thus, inhalation from

the air and absorption through the skin seems to be other routes of BPA exposure. Biedermann and colleagues (2010) evaluated the concentration of BPA in the thermal receipt from various shops. Their result revealed that 11 of 13 receipts contained BPA. They also examined the level of BPA that transferred to the finger by holding a thermal receipt. After 30 s, participants moved their fingers in the ethanol, and then BPA was extracted and measured. The mean of BPA transference on a single finger was 1.13 μg . Interestingly, they found that the BPA transfer rate was highly increased in humid and oily fingers [48]. In 2011, Zalko's research team evaluated BPA distribution and diffusion by using radioactivity after short-term culture of pig skin explant, human skin explant, and frozen pig's skin explant. This *ex vivo* model demonstrated a high percentage of BPA distribution in both skin explant and its culture media. The quantity results of BPA metabolites are also presented in the same phenomenon. Thus, they concluded that skin contact could contribute to the BPA exposure route in humans [49].

2.5 Effects of BPA on health status

In 2002, Scientific Committee on Food (SFA) defined No-Observed-Adverse-Effect level or NOAEL for BPA as 5 mg/kg BW/day (5,000 $\mu\text{g/kg BW/day}$) [50]. This dose resembles the European Food Safety Authority (EFSA) and Food and Drug Administration (FDA) who concluded a few years later [51]. It was considered NOAEL due to the results from an experimental three-generation study in rats [52] and a two-generation study in mice [53]. Therefore, this is the current NOAEL for BPA. However, many reports demonstrated the adverse effect of various BPA doses on human systems. The data are shown as follows:

2.5.1 Metabolism and obesity

Since BPA is a hydrophobicity compound, it was found with a ubiquitous level in adipose tissue [54]. BPA affects many structures related to the metabolism pathway and causes metabolic syndrome occurrence (Figure 3). The cross-sectional study in Chinese school children by Wang et al. (2012) demonstrated that 84.9% of samples

presented BPA level at 0.45 ng/ml. Their analyses showed increases in BMI associated with an increment of urine BPA concentration. In addition, there were several reports stated that higher BPA level was significantly associated with higher waist circumference [55, 56], abdominal obesity and insulin resistance [57], fat mass and serum leptin [58], and odds of overweight/obesity in boys [59]. Likewise, animal experiments also showed the association between BPA and obesity [8, 60].

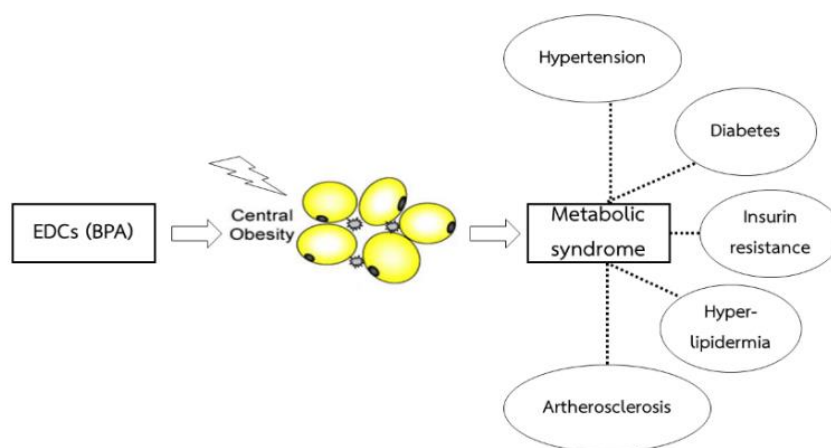


Figure 3 BPA affected obesity-related metabolic syndrome. [61].

2.5.2 Diabetes mellitus

As BPA showed the association with metabolic alteration and obesity, it also affected insulin level adaptation related to causes of diabetes mellitus (Figure 4). Alonso-Magdalena et al. (2005) conducted an in vivo experiment to evaluate the impact of BPA on the function of pancreatic β -cell (insulin-producing) cells in the pancreas compared to 17β -estradiol [62]. The results showed temporary hyperinsulinemia after acute treatments with either estradiol or BPA, whereas longer-term exposure stimulated insulin resistance with chronically increased insulin levels. These characteristics permit the development of diabetes mellitus. In addition, induction of glucose intolerance and insulin resistance in mice followed by BPA-treated were documented. Its effects are directly initiated via estrogen receptor (ER) [63]. In humans, Lang et al. (2008) studied the association between urinary BPA and

pathological conditions in adults. The result displayed the higher concentrations of this compound in the urine of diabetes samples [64].

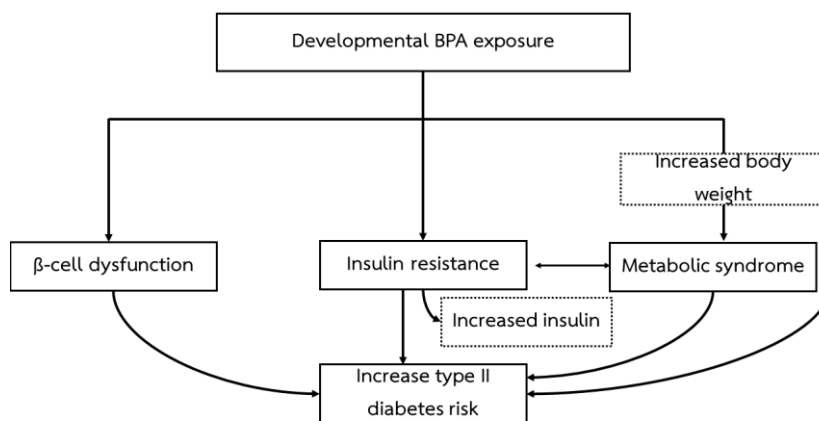


Figure 4 Proposed mechanisms of developmental BPA exposure leads to increased type 2 diabetes risk. Modified from Xu J, Huang G, and Guo TL., (2016) [65].

2.5.3 Digestive system

The digestive tract and liver are the primary targets of metabolized BPA. Lang et al. (2008) demonstrated that increasing BPA concentrations were associated with liver enzyme alteration [64]. Recently, Wang and colleagues (2018) showed that bio-accessible BPA decreased after digestion, but BPA degradation could not complete in the gastrointestinal tract [66]. Moreover, BPA exposure significantly altered microbial components and behavior in the colon. Javurek et al. (2016) revealed normal gut flora disturbance in the first-generation descendant of BPA and Ethinyl estradiol (EE) treated maternal mice. This phenomenon was associated with different gastrointestinal (GI) disorders such as inflammatory bowel disease (IBD), metabolic disorders, and colorectal cancer [67]. In addition, BPA can cause hepatotoxicity [68, 69], ROS production and lipid-peroxidation [70], abnormal histological liver tissue appearance, and abnormal levels of serum hepatic biomarkers [71].

2.5.4 Nervous system

There was evidence revealed that BPA was associated with the property of neuron synapse [72], developmental stage of the brain in the neonate [73], neurogenesis [74], learning [75], memory [76], anxiety [77], and autism spectrum disorder (ASD) [78, 79]. For example, Ishido and colleagues (2004) studied hyperactivity in 4- or 5-week Wistar rats after BPA administration. The spontaneous motor activity of rats was significantly increased. At the molecular level, they demonstrated that dopamine transporter in rat midbrain was suppressed by BPA more than three folds. Moreover, they found the reduction of tyrosin hydroxylase in substantia nigra. This indicated that dopaminergic neurons were degenerated [80]. Recently, Thongkorn et al. (2019) demonstrated that maternal exposure to 5,000 µg BPA during pregnancy related to neonatal hippocampal tissue alteration and a significant increase in ASD-related genes [81] in rats.

2.5.5 Reproductive system

There is evidence reported about BPA accumulation in abundant female reproductive tissues including ovarian follicular fluid, placenta, breast milk, and colostrum [82-84]. In recent years, several researchers also revealed that increased BPA is associated with female infertility [85-87]. BPA affects morphology and function of the uterus, for example, adaptation level of contraction-associated proteins and decreased contractility of the uterus [85], a decrease of endometrial cell proliferation [86], and decrease of uterine weight [87] were found in animal experiments. Since BPA has an androgenic binding property, it can interfere with male reproductive system organs. A recent publication demonstrated that dorsolateral prostatic cell proliferation and epithelial-mesenchymal alteration were observed in aged Sprague-Dawley rats with BPA oral administration. The researcher also showed that all groups of BPA treated had an increase in estrogen to androgen ratio with dose-dependent manner [88]. Moreover, there is evidence revealed increasing in weight and size of the prostate

gland in male mice exposed to BPA [89-91]. In addition, Chianese et al. (2018) reported the effect of BPA exposure on spermatogenesis. Their results showed that BPA altered testis function including blood-testis barrier impairment, DNA breaks in cells of the seminiferous tubules, decrease of SIRT1 expression, a decrease of oxidative stress defenses, and higher apoptotic rate in testis [92].

2.5.6 Endocrine system

As mentioned above, BPA treatment involved changes of an estrogenic signaling pathway. It may give effects on the alteration of the endocrine system. Katoh et al. (2004) demonstrated the effect of BPA on the anterior pituitary of male castrated sheep. Their result displayed BPA involved decreasing in GH release, GH mRNA expression, and cell number. These characters indicated that BPA suppresses the secretory function of anterior pituitary cells [20]. A recent publication also reported the effect of BPA on gonadotroph of zebrafish. After 14 days of BPA treatment, the histological data of light microscopy and transmission electron microscopy showed alteration of gonadotroph. Large vacuole formed by cisternae of reticulum fusion and several rERs were observed in BPA treated fish [21].

2.6 Relationship between BPA and pregnant women

According to environmental BPA contamination, general people and pregnant women are also exposed to this compound. Several reports demonstrated that BPA was observed in the circulation of pregnant women, amniotic fluid, neonatal blood, placental tissue, cord blood, and breast milk [83, 84]. The experimental data in animals also showed an association between maternal BPA exposure and pregnancy outcome. The BPA-treated rats showed long-lasting effects on the number of implantation sites and associated gene expression [93]. Rubin et al. (2001 and 2009) demonstrated that body weight increment of offspring related to perinatal BPA exposure [8, 60]. Alonso-Magdalena and colleagues (2010) showed that exposure to BPA in maternal mice affected to alteration of glucose metabolism in both maternal

and offspring (62). This indicated that BPA may provide metabolic disorders leading to an increase in diabetes risk. Vandenberg et al. (2008) examined structural changes of the mammary gland from offspring mice at 3, 9, and 12-15 months of age which maternal mice were exposed to BPA since GD8 through day 16 of lactation. The result demonstrated intraductal hyperplasia, the character of beaded ducts, and epithelial cells occupying the ductal lumen of female mice. These occurrences indicated that perinatal BPA exposure can alter the morphology of mammary gland rodents in adulthood [94]. Howdeshell and colleagues (1999) have reported that prenatal exposure to low dose BPA (2.4 µg/kg body weight) promoted weight gain and alter puberty period in female CF-1 mice [95]. In addition, prenatal exposure to BPA could induce abnormalities of follicles in the ovary and vaginal epithelium [96, 97]. Previous reports from rodent studies found that increase in T4 levels of offspring [98] and thyroid-specific gene expression [99] related to prenatal BPA exposure. Moreover, few epidemiological studies showed that exposure to BPA was associated with thyroid parameters alteration in maternal, neonatal, and adolescent [100-102]. The adverse effects of BPA on cells in the neonatal anterior pituitary gland were also documented. Brannick et al. (2012) showed an increase in gonadotroph cell proliferation, expression of gonadotropins, and their receptors in first-day neonatal mice of BPA treated mothers [19]. Systemic review data [30] demonstrated the correlations between maternal BPA exposure and abnormalities of descendants in various systems. Several studies reported higher maternal urinary BPA associated with abnormalities of behaviors in daughters [103], anxious and poorer emotional control [104], depressed and aggressive [105]. Moreover, Perera et al. (2012) revealed that maternal BPA also related to aggressive behaviors in boys [105]. Furthermore, incident of child wheeze associated with higher maternal urinary BPA was also documented [106]. The summarized phenomena of adverse effects of BPA are presented in table 1.

Table 1 Summary data about an incident of BPA adverse effects in several systems of animals who exposed to BPA.

| System | Incident | Reference | Model | Dose | Evidence |
|--------------|--|------------------------------------|------------|-------------------------|--|
| Metabolic | Obesity | Howdeshell et al., 1999 [95] | CF-1 mice | 2.4 µg/kg | - ↑ weight |
| | | Rubin et al., 2001 [8] | SD rats | 100 µg/kg 1200 µg/kg | - ↑ weight (*) - ↑ weight (*) |
| | Diabetes mellitus and glucose homeostasis | Alonso-Magdalena et al., 2010 [63] | OF-1 mice | 10 µg/kg | - ↑ insulin resistance |
| | | | | 100 µg/kg | - ↑ insulin resistance (*) |
| Digestive | Significantly alters microbial community in colons | Wang et al., 2018 [66] | HepG2 cell | 25 µg/l | - ↓ microbial community |
| | | | | 250 µg/l | - ↑ microbial community |
| | | | | 2500 µg/l | - ↑ microbial community |
| Nervous | Alteration of brain sexual dimorphisms | Kubo et al., 2003 [107] | Wistar rat | 0.1 mg/l 1 mg/l | - Alter brain dimorphisms and behavior in both 0.1 and 1 BPA |
| | | Rubin et al., 2006 [108] | CD-1 mice | 25 ng/kg 250 ng/kg | - ↓ TH positive neuron - Decrease TH-positive neuron (*) |
| | Changes in behavior, cognitive and autistic behavior | Ishido et al., 2004 [80] | Wistar rat | 0.02, 0.2, 2, 20 µg/kg | - Rat showed more active behavior especially in 2 and 20 decrease dopamine receptors (dose dependent). |
| | | Tian et al., 2011 [109] | Mice | 6, 18 mg/kg | - ↑ movement and activities - ↓ novel object recognition |
| | | Thongkorn et al., 2019 [81] | Wistar rat | 5,000 µg/kg | - Downregulation of ASD-related gene in hippocampus of the neonate |
| Reproductive | Time of puberty alteration | Howdeshell et al., 1999 [95] | CF-1 mice | 2.4 µg/kg | - ↓ number of days of vaginal opening and first estrus cycle |
| | | Honma et al., 2002 [110] | ICR mice | 2, 20 µg/kg | - 20 µg/kg involve earlier vaginal opening |
| | Changes of uterus, ovary and estrous cycles | Markey et al., 2005 [111] | CD-1 mice | 25 ng/kg 250 ng/kg | - ↓ weight of vagina (NS) - ↓ weight of vagina (*) and absolute ↓ lamina propria volume(*) |
| | | An et al., 2013 [85] | SD rat | 10, 100, 500 mg/kg | - ↑ uterine weight (dose dependent manner) - Alteration of targeted gene expression almost in 100(*) & 500(*) |
| | | Bosquiaz et al., 2010 [86] | Wistar rat | 0.5 mg/kg | ↓ BrdU express in endothelium (*) ↑ VWF positive vascular area ↓ VEGF mRNA expression (*) |

| System | Incident | Reference | Model | Dose | Evidence |
|------------------------|--|------------------------------|--------------------------------|---------------------------------------|--|
| Reproductive (Con.) | | | | 20 µg/kg | ↓BrdU express in endothelium (*) ↓VWF positive vascular area ↓VEGF mRNA expression (*) |
| | | | | 100 mg/kg | - ↓uterine weight (*) |
| | | | | 200 mg/kg | - ↓ right ovary weight |
| | | | | 500 mg/kg | - Expansion of uterine lumen |
| | | | | 1,000 mg/kg | - Expansion of uterine lumen |
| | Changes of prostate gland and testis | Nagel et al., 1997 [89] | CD-1 mice | 2, 20 µg/kg | - Both conc. of BPA increased prostate weight (*) |
| | | | | 2, 20 µg/kg | - Both conc. of BPA increased prostate weight (*) |
| | | Gupta et al., 2000 [91] | CD-1 mice | 100 ng/kg | - NT |
| | | | | 5 µg/kg | - ↑ prostatic weight/size |
| | | Huang et al., 2018 [88] | SD rat | 10, 30, 90 µg/kg | - All dose involves increasing in height of prostatic epithelium (*) |
| | | | | 10, 30, 90 µg/kg | - ↑ E2 expression (90 µg) (*) |
| | | Chianese et al., 2018 [92] | Wistar rat | 0.1 mg/l | - Both conc. Increase ROS, DNA damage, apoptosis in testis (*) |
| | | | | 0.2 mg/l | - Both conc. Increase ROS, DNA damage, apoptosis in testis (*) |
| | Mammary gland development and hyperplasia | Vandenberg et al., 2008 [94] | CD-1 mice | 0.25 µg/kg | - Presence of alveolar bud |
| | | | | 2.5 µg/kg | - ↑ alveolar bud (*) |
| | | | | 25 µg/kg | - Lack of bead duct at 12-15 mo. |
| Endocrine | Gonadotropic cell activated | Molina et al., 2018 [21] | Zebra fish | 1 µg/l | - Only activated gonadotroph cells ↓(*) |
| | | | | 10, 100 µg/l | - Change morphology, CYP19B ↑(*) |
| | | | | 1,000 µg/l | - Gonadotroph hypertrophy, CYP19B ↓(*) |
| | Stimulating of prolactin and TSH in anterior pituitary gland | Fujimoto et al., 2001 [22] | F344 rat | 0.1, 1, 10 mg/kg | ↑ pituitary weight, uterine weight, PRL (NS) in all dose |
| | | | | 0.1, 1, 10 mg/kg | ↑ pituitary weight, uterine weight, PRL (NS) in all dose |
| | Changes of growth hormone-releasing hormone (GHRH) | Katoh et al., 2004 [20] | Ovine anterior pituitary cells | 10 ⁻⁶ – 10 ⁻³ M | - ↓ GH release in dose dependent manner, most at 10 ⁻³ (*) |
| | | | | 10 ⁻⁶ – 10 ⁻³ M | - ↓ GH release in dose dependent manner, most at 10 ⁻³ (*) |

(*) the data showed significantly statistical analysis.

2.7 Anterior pituitary gland

The human anterior pituitary gland is a master gland that plays an important role in regulating other organs in the body. Populations of the anterior pituitary gland are divided into two major types: hormone-producing cells and non-hormone-producing cells which do not secrete any hormone.

2.7.1 Secretory cells in the anterior pituitary gland

The anterior pituitary gland consists of five endocrine or secretory cell types. Each type is defined by their hormone secretion as follows:

Somatotrophs; growth hormone (GH)

Lactotrophs; prolactin (PRL)

Gonadotrophs; luteinizing (LH) and follicle-stimulating hormone (FSH)

Corticotrophs; adrenocorticotrophic hormone (ACTH) producing cells,

Thyrotrophs; thyroid-stimulating hormone (TSH)

All hormone cells are formed in the characteristic of a cluster with FS cells. These cell clusters are surrounded by extracellular matrices (ECMs) and act as a functional unit in the anterior pituitary gland [11, 112].

2.7.2 Non-secretory cells in the anterior pituitary gland

Non-hormone-producing cells do not secrete any classical hormone, but they can interact with other cell types in the gland. These cells are FS cells, macrophages, endothelial cells, pericytes, and novel desmin-immunopositive perivascular (DIP) cells. In the present study, pericytes and FS cells were evaluated.

2.7.2.1 Pericytes

In 1871, Eberth documented the presence of pericyte as the perivascular cell [113]. Afterward, Charles-Marie Benjamin Rouget defines the discovery of pericytes as mural contractile cells encircle around the endothelial cells of micro-vessels (Figure 5). Then, these cells were called “Rouget cells” and also adapted to the present term “pericytes” [114, 115]. These cells play an important role in vascular tube forming via interaction with endothelium. Pericytes entrap and share their basement membrane

with endothelial cells. They were found at the branching points and their primary processes were often found along each branch [116]. Pericytes are the collagen-producing cells in the anterior pituitary gland, this character was found in both rats and humans [12, 112]. Pericytes can collaborate with other pituitary populations. They interact with endothelial cells in the process of angiogenesis via PDGF β -PDGFR β [117], Angiopoietin-1/Tie-2 [118, 119], TGF β [120, 121] interaction. Moreover, they also express ECM-related molecules including collagen I and III [14, 112], Tissue inhibitors of metalloproteinases (TIMPs) [122], and small leucine-rich proteoglycans (SLRPs) [13]. Since collagens, especially types I and III, are the principal component in ECM of the anterior pituitary [14], pericytes may play an important role in ECM adaptation.

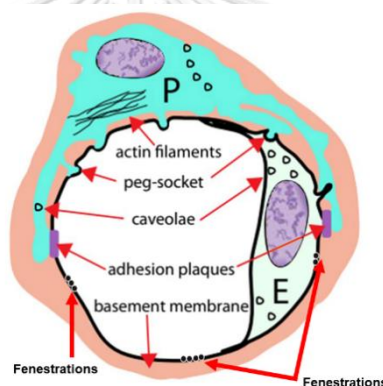


Figure 5 Ultra-structure and interaction between pericyte (P) and endothelial cell (E). Modified from Armulik et al., 2011 [116].

2.7.2.2 Folliculostellate cells (FS cells)

FS cells are agranular cells presenting in the anterior pituitary gland but do not secrete classical hormones. FS cells usually form a follicle within a cluster of mixed five types of endocrine cells (Figure 6) [123] and they have an elongated process [11] that interacts with other cells through gap junction communication [124, 125].

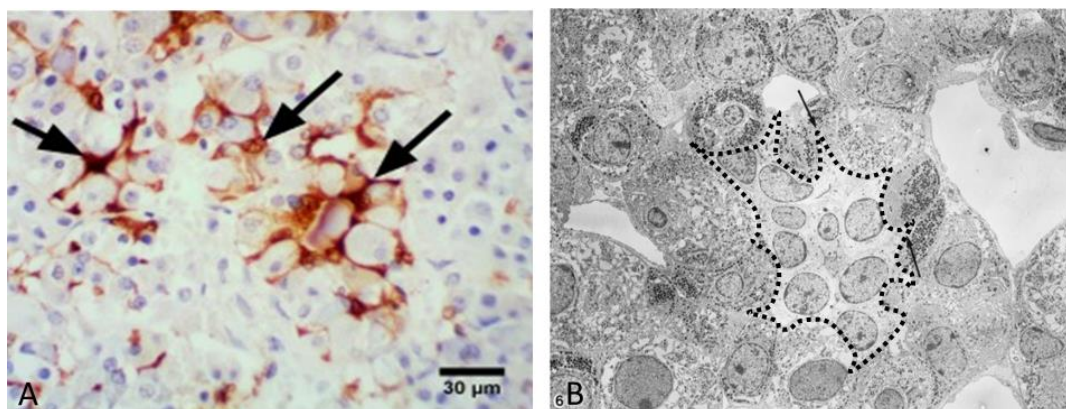


Figure 6 Schematic of folliculostellate cells (FS cells) in anterior pituitary cell clusters by immunohistochemistry of anti-s100 protein (A) [126] and transmission electron micrograph of FS cells (arrows) occupying within secretory cells cluster (B) [123].

FS cells of the anterior pituitary gland express several regulatory molecules. They can regulate hormone-producing cells, in terms of both production and secretion, via paracrine factors secretions [127, 128]. Moreover, they secrete several molecules such as fibroblast growth factor [129], vascular endothelial growth factor [130], interleukin-6 [131], annexin-1 [132] to interact with other cells. In addition, not only does cytokine modulate numerous cells in the anterior pituitary gland but FS cells also express matrix metalloproteinase 9, the family of ECM breaker, under the associated condition with laminin [133]. Tsukada and colleagues (2014) demonstrated that FS cells are related to collagen production via the interaction between FS cells and pericytes [134].

2.7.3 Extracellular matrix (ECM)

In recent years, it is known that ECMs not only contribute a framework for the tissues, but also play roles in cell-cell and cell-matrix signaling (145, 148). The cell-matrix interaction is an important factor to regulate cell proliferation and differentiation. Moreover, the diversity of ECM components, morphology, and behavior influence to whole structure and tissue network (Figure 7). For the anterior pituitary gland, Perez and colleagues (2005) reported the crucial role of ECM in the regulation of the function of cells in the gland and the cellular response along the hypothalamic

axis [135]. Evan and Chitcholtan (2011) also documented the adaptation of ECMs synthesis and their components. Their receptor molecules can regulate both the characteristic behavior of the hormone cells as well as hormone secretions which affect several systems [14]. It is noted that “matricrine signaling” is used as a technical term of the cell-cell and cell-matrix interactions in the anterior pituitary gland. The followings are the major and interested ECMs that involve in pituitary cell behavioral regulator via matricrine action.

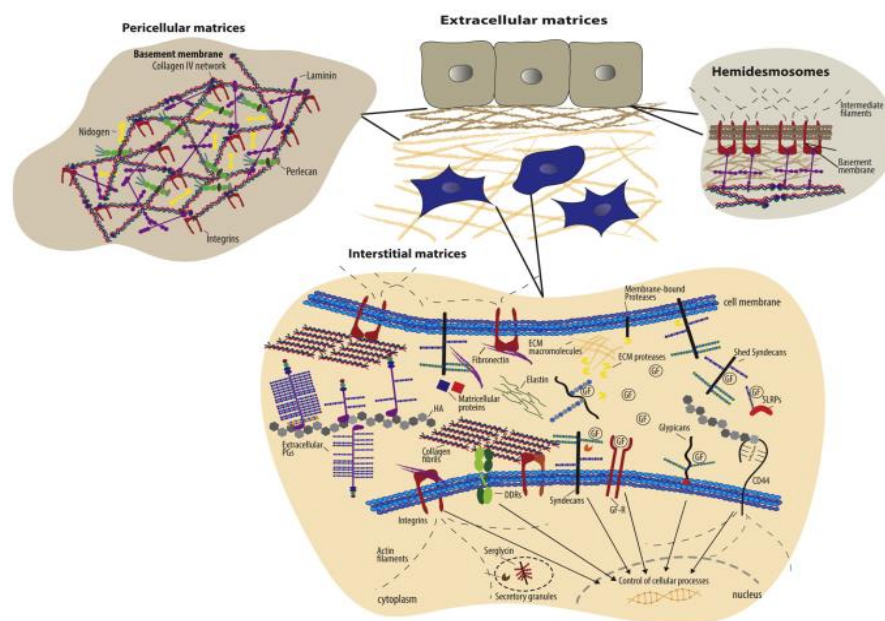


Figure 7 Schematic of two main ECM types; pericellular matrices and interstitial matrices [136].

2.7.3.1 Collagens

Collagens (Figure 8) are divided into fibrillar and non-fibrillar forms. They provide tensile strength to the ECMs limiting the distensibility of tissues [15]. Type I collagen is a fibrillar ECM protein presenting in almost all organs. The adaptation of collagen and its producing cells is associated with pathologic conditions [137] such as liver fibrosis [138, 139], kidney fibrosis [140], and inordinate dermal scar formation [141]. In the anterior pituitary gland, collagens show several profiles that can drive the behavior and function of local populations. They can induce FS cell proliferation [13],

regulate small leucine-rich proteoglycan (SLRP) production [142], alter the morphology of pituitary cells [143], and facilitate hormone production [144]. Alteration of collagen-producing cells and abnormal collagen composition are related to pituitary adenomas [12, 145]. In addition to collagen type I, type III is also the principal ECM component in the anterior pituitary gland [11, 14, 112].

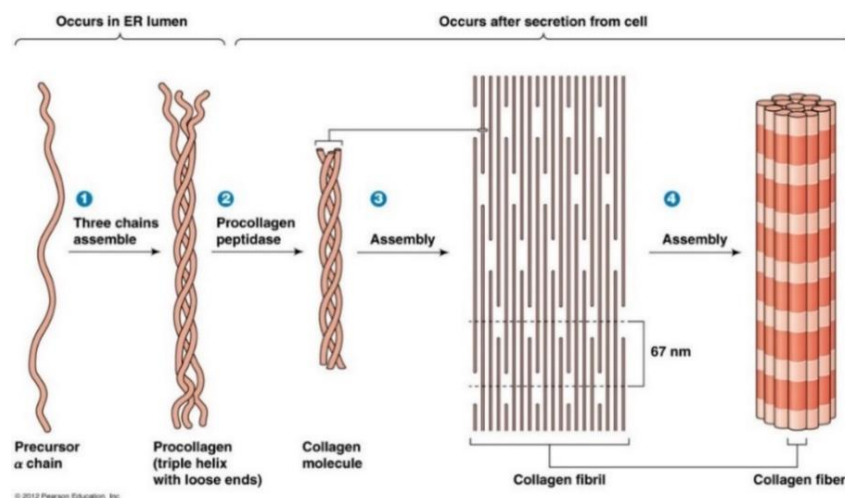


Figure 8 Collagen structure and collagen fibrils [146].

2.7.3.2 Small leucine-rich proteoglycans (SLRPs)

SLRPs (Figure 9) are one of the major families of the proteoglycans and are the key to regulating the cellular signaling pathways. Several tissues including the tendon, skin, liver, kidney, and heart have SLRPs as the biological regulator [147]. They have the potential to control collagen fibril formation [148, 149]. In the pituitary gland, the major members of SLRPs consist of decorin, biglycan, fibromodulin, lumican, proline, and arginine-rich end leucine-rich repeat protein (PRELP), and osteoglycin (Table 2). Notably, SLRP-expressing cells in the gland are either FS cells or pericytes [13].

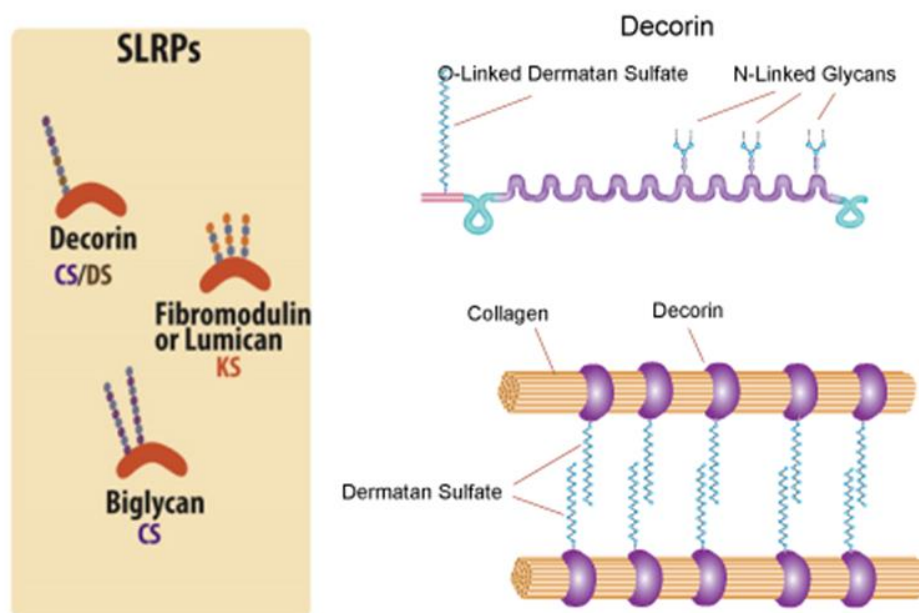


Figure 9 The structure of small leucine-rich proteins (SLRPs) and their relation to collagen fiber [136].

Decorin, biglycan, and asporin were documented as the candidate molecules associated with the collagen-binding phenomenon [147]. Especially, decorin showed high-affinity collagen-binding effect and could shape collagen fibril and diameter [150]. SLRPs are related to collagen fibrogenesis and adaptation of collagen and ECM affect to anterior pituitary gland's population behavior. Knowing their roles and interaction may clarify or discover hallmarks of ECM action on anterior pituitary gland alterations.

Table 2 Class and member of small leucine-rich proteoglycans (SLRPs) [151] and their expression in the anterior pituitary gland [13].

| Class | Member | Character |
|-------|---|--|
| I | <i>Decorin, biglycan, asporin, ECM2, ECMX</i> | Predominant: Decorin Moderate: biglycan, lumican, PRELP, and ostomodulin Barely: asporin, fibromodulin, osteoglycin, and tsukushi Not detectable: keratocan, epiphygan, opticin, chondroadherin, nyctalopin, and podocin-like protein 1 |
| II | <i>Fibromodulin, lumican, proline/arginine-rich end leucine-rich repeat protein (PRELP), keratocan and osteoadherin</i> | |
| III | <i>Epiphygan, opticin, and osteoglycin</i> | |
| IV | <i>Chondroadherin, nyctalopin, and tsukushi</i> | |
| V | <i>Podocan and podocin-like1</i> | |

*Italics are SLRPs that were evaluated in Horiguchi's work.

As mentioned in table 2, SLRPs are classified into five classes according to their structural properties by Shaefer and Schaefer in 2010 [151]. Horiguchi's research group (2013) evaluated the expression of SLRPs in the rat anterior pituitary gland. They found that class I-IV SLRP genes are expressed in the gland of adult rats. The predominant expression was decorin which was restrictively detected in FS cells and pericytes. Biglycan, fibromodulin, lumican, PRELP, and osteoglycin were also expressed in most pericytes and some FS cells. Thus, SLRPs probably regulate the anterior pituitary functions via paracrine or matricrine between FS cells, pericytes, and hormone-producing cells [13].

2.7.3.3 Matrix metalloproteinases (MMPs)

There are various factors involved in ECM balance including ECM-producing structure, enzymes of ECM degradation, and ECM degradation inhibitor [152]. ECM degradation is the main process along with ECM remodeling. Matrix metalloproteinases (MMPs) are enzymes that act as the key of ECM degradation in physiological conditions and accelerate their activity during pathological adaptation (tissue repairing or remodeling). The well-known proteolytic enzyme is MMP-9 [133]. Elswefy and colleagues (2016) reported the alteration of MMP9 and its inhibitor (TIMP2) in rat liver fibrosis [17]. MMP9 but MMP2 and MMP3 were also documented in liver fibrosis progression [153-155]. In the anterior pituitary gland, Ilmiawati et al. (2012) demonstrated that FS cells expressed MMP9, promoting their function on laminin [133]. Moreover, FS cells could interact with pericytes to provide collagen synthesis in the gland [121]. Therefore, alteration of both cell types might influence on behavior and biological profiles of ECMs.

2.7.3.4 Tissue inhibitors of metalloproteinases (TIMPs)

TIMPs regulate ECM via MMPs-TIMPs interaction. They inhibit ECM degradation which occurs by metalloproteases to maintain the balance of ECM remodeling [156]. In vertebrates, there are four members of TIMPs, i.e., TIMP1, TIMP2, TIMP3, and TIMP4 [157]. Upregulation of TIMP1 level related to pituitary adenoma fibrosis that led to immoderate collagen deposition [158]. Additionally, TIMP3 and TIMP4 may be the leading members who act as controllers of fibrosis. TIMP3 regulated fibrosis through several pathways, including mediating inflammation and inducing pericyte to transform into activated-myofibroblast-like cells [167], whereas TIMP4 was related to fibrosis enhancement following injury [159]. In addition to fibrosis, tumor and cancer were also occurred by ECM dysregulation [15]. In the anterior pituitary gland, the TIMP family is generally expressed in rats [165] and humans [122]. TIMP1 and TIMP2 were related to pericytes and FS cells in the human and rat pituitary gland, whereas TIMP3 exhibited

a close relationship with endothelium and pericytes. In adenomas, TIMP3 was upregulated while TIMP1 and TIMP2 seemed to be similar between control and adenomas. Table 3 is the summarized expression of ECM-related molecules in pericytes and FS cells.

Table 3 ECMs and ECMs-related molecules in the anterior pituitary gland and their producing cells expression in humans (control and adenomas) and rats.

| Pericyte | FS cell |
|--|---|
| Col1a1 ^{H, R} , Col3a1 ^{H, R} | MMP9 ^R |
| TIMP1 ^{H, HA} , TIMP2 ^{H, HA} , TIMP3 ^{H, HA, R} | TIMP1 ^{H, R} , TIMP2 ^{H, R} , TIMP3 ^R , TIMP4 ^H |
| SLRP ^R (Decorin, biglycan, fibromodulin, lumican, PRELP, osteoglycin) | SLRP ^R (Decorin, biglycan, fibromodulin, lumican, PRELP, osteoglycin) |

H: Human with normal, HA: Human with adenomas, R: Rat)

2.8 Effect of BPA on ECM

There were few studies reported about the effect of BPA on ECMs. Elswefy and colleagues (2016) reported the effect of BPA on the Wistar rat's liver. Their results demonstrated that BPA caused increased inflammation, oxidative stress, and cellular apoptosis. Focusing on ECM adaptation, they examined the expression of MMP9 and TIMP2. Their result revealed upregulation of MMP9 while TIMP2 was downregulated. The imbalance of MMP-TIMP in this phenomenon led to liver fibrosis [17]. In 2015, Belcher and colleagues evaluated CD-1 mice heart alteration followed by varied BPA-treated dosages. At dose 300 ppm of BPA (around 5000 µg/kg BW), the expression of ECM and collagen-related genes showed downregulations of collagens and MMPs whereas decorin and TIMP3 were upregulated [18].

All information in this literature review shows that BPA has effects on several organs, including the anterior pituitary. Although the studies of the effects of BPA on this gland focusing on the adaptation of endocrine cells and their secretion behavior

were already reported. But the changes of ECMs in the anterior pituitary gland after BPA exposure have not been elucidated. Besides, BPA can pass to the newborn via the placenta. Therefore, we hypothesize that BPA treatment in rats during pregnancy may influence the ECM alteration of the neonatal anterior pituitary gland. Moreover, we would like to challenge whether BPA with No-Observed-Adverse-Effect level (NOAEL) can generate alterations in the neonatal anterior pituitary gland.



CHAPTER III

Research methodology

3.1 Animal procedures

3.1.1 Maternal rats BPA treatment

In this investigation, eight-week-old maternal Wistar rats were used. Animals were fed and watered (*ad libitum*) freely and kept at a constant temperature with a 12:12-hour light/dark cycle. The vaginal plaque of the maternal rats was observed after normal inbreeding and then divided into BPA and control groups. In the BPA group, maternal rats were given 5,000 $\mu\text{g/kg}$ BW of BPA every day. The control rats were given corn oil by mouth for the duration of their pregnant periods (~21 days) (Figure 10). Each day's amount of treated BPA was calculated based on the maternal weight. Both groups of rats were housed independently in biohazard system environmental at Chulalongkorn University Laboratory Animal Center (Animal Use Protocol No. 1773011, and the Ethical Committee of the Faculty of Medicine 010/2562). Oral gavage equipment was kept separate and solely utilized by each group. Reusable stainless steel oral gavage needles were thoroughly cleaned with ethanol followed by Milli-Q deionized water wash several times before subsequent use.

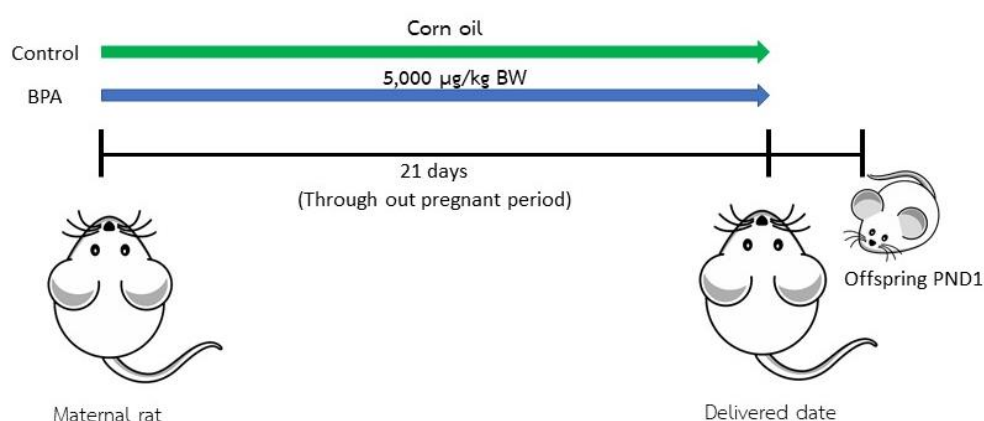


Figure 10 Animal BPA administration procedure.

3.1.2 Neonatal rats euthanasia

The BPA treatment stopped at the date that neonates were delivered. After parturition, post neonatal rats at day 1 (PND 1) were euthanized. These rats were injected with sodium pentobarbital via intraperitoneal injection. Then, rats were put into hypothermic conditions (one minute within the icebox) followed by decapitation.

3.2 Sample size calculation

The sample size is calculated using the data from the pilot study with the following formula. Standard deviation and mean of TIMP4 mRNA expression level was used to calculate the number of the neonatal anterior pituitary gland in each experiment.

$$n = \frac{(\sigma_1^2 + \sigma_2^2)(Z_{1-\alpha/2})}{(\mu_1 - \mu_2)^2}$$

$$n = \frac{(0.60^2 + 0.85^2)(1.96 + 0.84)^2}{(3.19 - 1.17)^2}$$

$$n \approx 3$$

σ = Variances (SD)

μ = Population mean

α = Type I error = 0.05

β = Type II error = 0.2

3.3 BPA preparation

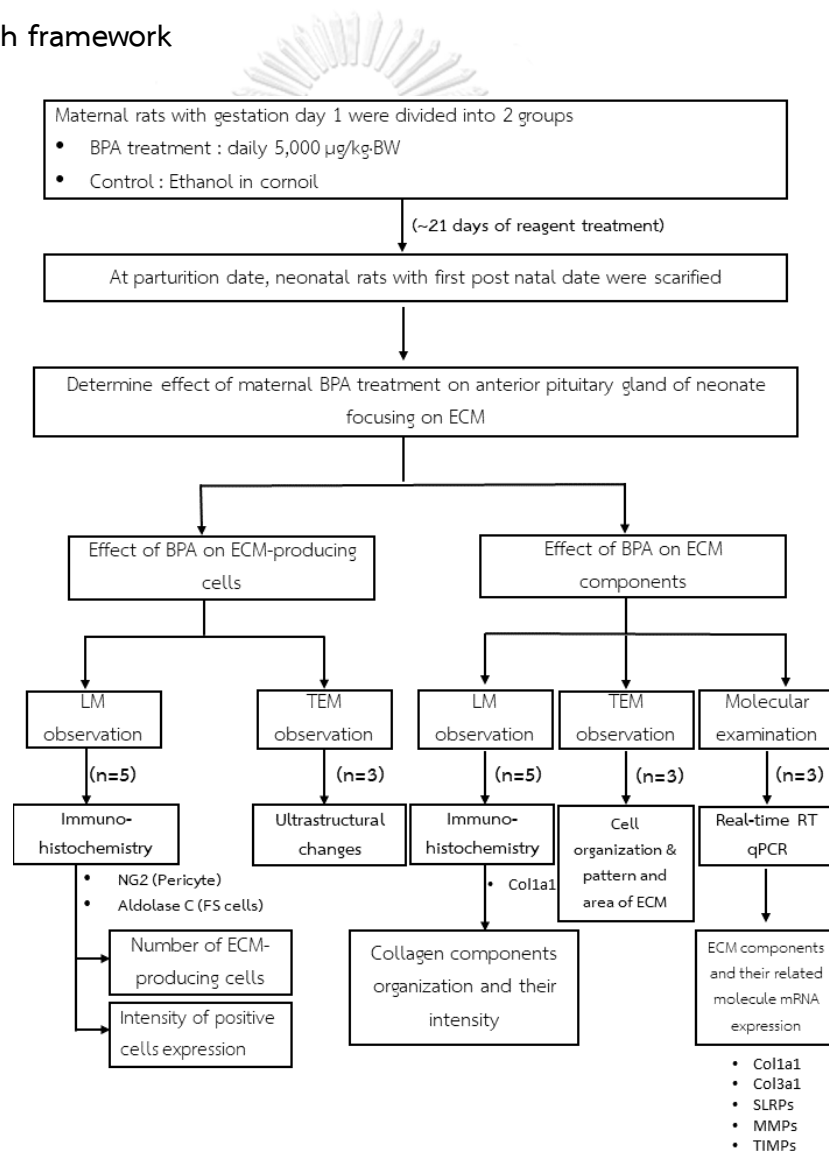
BPA (Sigma-Aldich, USA) was first dissolved by absolute ethanol (Merck millipore, USA) to make the BPA stock at 250 mg/ml. Then, it was diluted with corn oil to 5,000 $\mu\text{g/kg}$ BW of BPA for each rat treatment as the below formular. Therefore, $\frac{x}{50}$ μl of stock BPA was mixed with corn oil to 200 μl as a daily volume. The vehicle control was prepared by a combination between absolute ethanol and corn oil.

Maternal rats 1000 g BW will be received BPA 5000 μg
 If maternal rats X g Then they will be received BPA $\frac{5000x}{1000}$
 = 5x μg or $5x \times 10^{-3} \text{ mg}$

Stock BPA prepared as 250 mg/ml

BPA 250 mg dissolved in absolute ethanol alcohol 1 ml.
 Target BPA $5x \times 10^{-3} \text{ mg}$ Then final volume of BPA is $\frac{5x \times 10^{-3}}{250} \text{ ml} = \frac{x}{5 \times 10^4} \text{ ml}$
 $= \frac{x}{50} \mu\text{l}$

3.4 Research framework



3.5 Tissue collection

A stereo microscope was used to identify the pituitary gland (Figure 11) at the rat's skull base (SMZ800 Zoom, Nikon, Japan). All anterior pituitary glands were taken and processed with the appropriate procedure for each experiment.

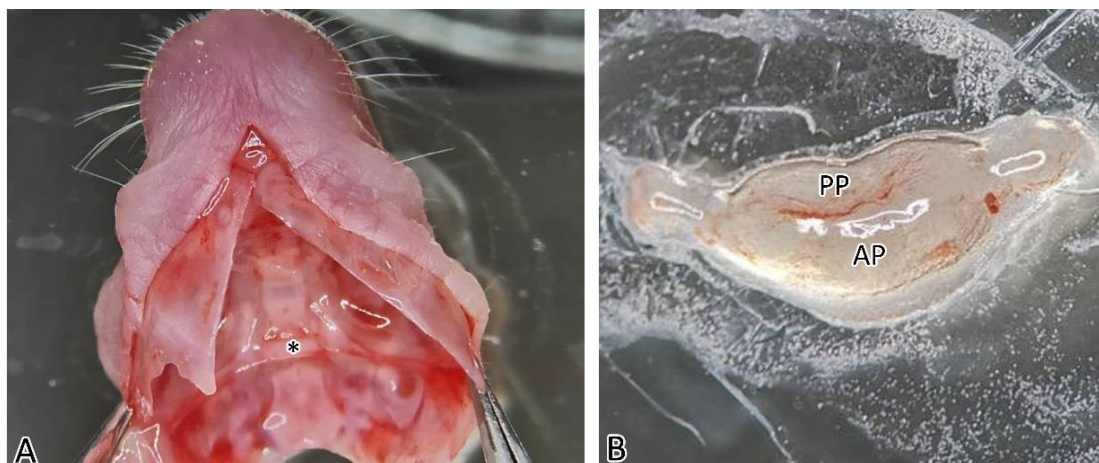


Figure 11 Demonstration of neonatal rat's skull base after brain removal (A) and pituitary gland (B) visualized via stereo microscope. Note sella turcica (asterisk), posterior lobe (PP) and anterior lobe (AP).

For light microscopic observation, pituitary glands were immediately immersed into 4% paraformaldehyde overnight, followed by 30% sucrose in 0.1M phosphate buffer (PB, pH 7.4) for 48 hours. After preservation in sucrose solution, the glands were embedded in Tissue-Tek compound (Sakura Finetechnical, Tokyo, Japan), immediately frozen, and continually stored at -80°C until they were used in the experiment. Tissues were sectioned by a cryostat (CM1950; Leica Biosystems, Wetzlar, Germany) and placed on a coated slide (PlatinumPRO; Matsunami, Osaka, Japan). Neonatal anterior pituitary glands were observed under the light microscope (ICC50 HD on DM1000, Leica, Germany). All sections were processed through the whole gland scan at 40X level using Axioscope7 (ZEISS, Oberkochen, Germany). In addition, tissues processed for the TEM study were immediately immersed into 2% glutaraldehyde in 0.1M PB after harvested. Then these tissues have proceeded along with the standard protocol of TEM (more detail in TEM topic). Only pituitary glands considered for the molecular study were cut

along the cleft for anterior pituitary removal and collected. This anterior lobe was then immediately immersed into *RNAlater* (AM7024, Thermo Fisher Scientific Inc., Vilnius, Lithuania for further RNA extraction. All replicates were obtained from independent litters.

3.6 Light microscopic observation using immunohistochemistry evaluations

Five glands from neonates (PND 1) in each group were harvested for morphological examination. Under a light microscope, all morphological evaluations were calculated from four slides of each rat. The order of slides was chosen from one of every five sections. These selected sections displayed the whole gland which consisted of anterior, intermediate, and posterior lobes.

3.6.1 Immunohistochemistry of ECM-producing cells

Regarding pericytes and FS cells are the rat anterior pituitary gland ECM-producing cells, the numbers, and their immunosignal intensity were determined. For pericytes and FS cells identification, sections were stained with NG2 (1:600 dilution) and aldolase C (1:200 dilution). Protocol of immunohistochemistry was described, as follows;

- Bring slide from -80°C storage and air dry.
- Wash by PBS (on shaker) for 5 minutes (3 times).
- Block the non-specific background by using 2% normal goat serum in filtered PBS (20 minutes, RT in moist chamber).
- Gentle dip wash in PBS several times.
- Incubate in primary antibody (overnight, RT in the moist chamber).
- Wash by PBS (on shaker) for 5 minutes (3 times).
- Incubate in secondary antibody for 30 minutes in the moist chamber with 30°C.
- Wash by PBS (on shaker) for 5 minutes (3 times).

- ABC method (30 minutes, in the moist chamber with 30°C). **This reagent must be prepared 30 minutes before use.*
- Wash by PBS (on shaker) for 5 minutes (3 times).
- Detect immunoreactive signal expression of DAB. Pull slides one by one from the last step of the PBS beaker. Then soak in the beaker containing DAB working solution.
- Observe the immunoreactivity under a light microscope.
- Stop the reaction with distilled water.

The number of immunoreactive cells was counted from the whole gland. The scope areas of calculation were divided into three parts related to the width of the posterior lobe, as shown in Figure 12. Pericytes or FS cells were counted when the researcher observed cell cytoplasm containing the nucleus.

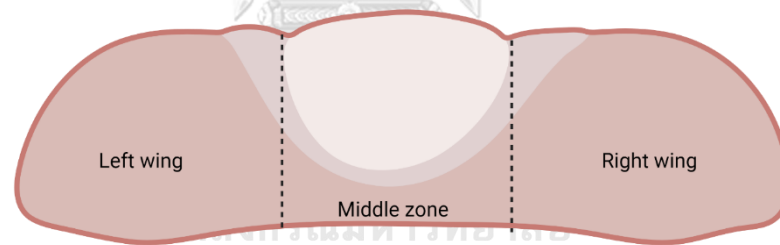


Figure 12 Demonstration of three parts of the anterior pituitary gland for cell counting.

ZEN 3.0 (Blue version) program was used as an immunosignal intensity evaluator. The mean intensity was evaluated (Figure 13). Furthermore, collagen percentage area distribution was calculated by Fiji ImageJ program (NIH, Bethesda, Maryland, US).

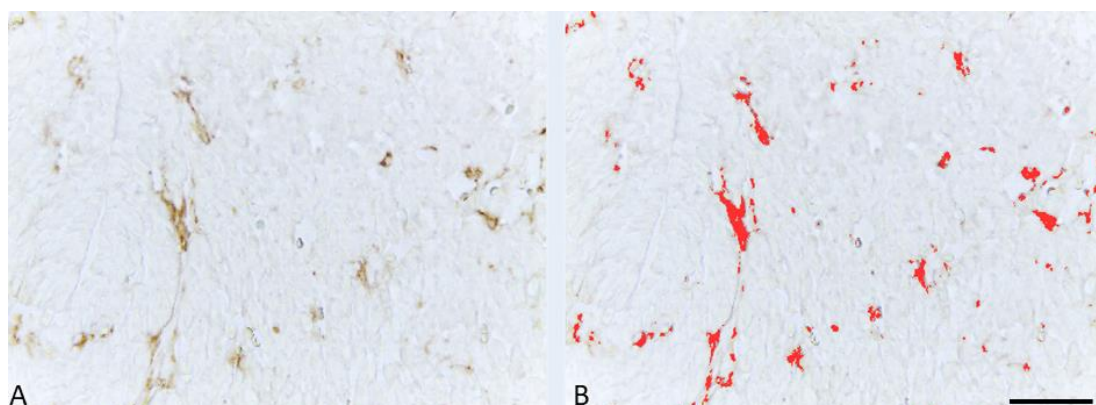


Figure 13 Demonstration of immunohistochemistry section(A) and intensity evaluation by the software (B). Scale bar: 50 μ m.

3.6.2 Immunohistochemistry of ECM components

The predominant ECM in the rat anterior pituitary gland is collagen. Firstly, this study planned to evaluate the ECM components using Masson's trichrome staining for collagen distribution demonstration. However, Masson's trichrome staining was not suitable for our tissue. Therefore, immunohistochemistry of collagen type I expression was used instead of Masson's trichrome technique. Sections were stained with col1a1 immunohistochemistry protocol similar to the protocol mentioned in the previous experiment. The collagen percentage area distribution and immunosignal intensity were examined.

3.7 Transmission electron microscopic (TEM) observation

TEM was performed to evaluate the changes of ultra-structure of ECM-producing cells, cell cluster organization, and ECM components.

3.7.1 Specimen preparation and processing

Following neonatal decapitation, three collected pituitary glands per group were fixed for 2 hours in 0.1M PB (pH 7.4) with 2% glutaraldehyde (EMS, Hatfield, PA, USA), followed by 30 minutes of PB washing on ice (4 times). Before immersion, the pituitary glands were chopped until we got 1-2 mm³ tissues. The protocol was described, as follows;

- Postfix with 1% osmium tetroxide (OsO_4) on ice for 90 minutes.
- Wash specimens in cold distilled water (several times.)
- Dehydrate tissues by using the series of EtOH:50%, 70%, 80%, 90%, 95% (5 minutes for each session).
- Gradually penetrate 100% EtOH (5 times; 45 minutes in total).
- Discard half volume of absolute EtOH, then add an equal amount of propylene oxide into the bottle for 10 minutes. Repeat this step twice.
- Then immerse specimens into pure propylene oxide for 10 minutes
- Prepare 2/3 Epon:propylene oxide (2/3 epon resin solution) reagent for replacement of propylene oxide to 2/3 epon resin solution.
- Discard half volume of pure propylene oxide, then add an equal amount of 2/3 epon resin solution and incubate for 15 minutes. Repeat this step twice.
- Then, discard all volumes of the last step solution and add pure 2/3 epon resin solution.
- Pull out a tissue from 2/3 epon resin solution. Put tissue on the Kim wipe, roll over it to remove the excess 2/3 epon resin solution.
- Transfer the tissue into the vial containing new epon resin for 20 minutes. Repeat this step twice.
- Transfer tissue from the last step and embed it into the sample block. Check that there was no bubble within the block.
- Incubate the sample in a 60°C incubator for at least 2-3 days.

3.7.2 Tissue sectioning and processing for TEM observation

Resin blocks were sectioned at semithin level (1500 μm) by an ultramicrotome (EM UC7, Leica, Vienna, Austria) to locate the interest area in the anterior lobe and primary cellular profile observation. These sections were stained with 1% toluidine blue and then visualized under light microscopy. The selected area was then trimmed to one mm^2 for further ultrathin sectioning. Ultrathin sections with 70 nm in thickness were cut by an ultramicrotome (EM UC7, Leica, Vienna, Austria) with a diamond knife. Sections were placed on the copper grid, dried, and then stored in the grid case until TEM observation.

3.7.3 Tissue evaluation

Ultrastructural changes of pericytes and FS cells were examined under the transmission electron microscope (JEM 2100 plus, JEOL, Massachusetts, USA). Cell organization, characteristics of capillaries, perivascular area, and ECM pattern were also observed.

The copper grids containing sections were stained with the metal before observation. Uranyl acetate – lead citrate staining was done, as follows;

- Drop uranyl acetate and lead citrate solution on parafilm placed in the close petri dish. Note that the petri dish containing lead citrate was added NaOH tablets.
- Stain sections on the grid with uranyl acetate for 5 minutes (put the grid on the top of the drop). Then wash the grid by gently dipping it in distilled water.
- Wipe distill water from the bottom of the grid. All steps were applied again with a lead citrate solution.

3.8 Determination of mRNA expression by RT-qPCR

This procedure was performed to examine the mRNA expression of ECM and ECM-related molecules in the anterior pituitary gland. For the RNA extraction process, a microtube containing frozen anterior pituitary lobe in *RNAlater* was removed from the freezer then melted at room temp. Total RNA extraction was done according to the steps below;

- Remove the gland from the *RNAlater* tube and place it on Kimwipe gentle roll tissue to remove the *RNAlater*. Then, put it into the microtube containing 100 μ l of Trizol. (15596026, Invitrogen, Carlsbad, CA, USA)
- Refrigerate the magnetic bead and put it into the tissue tube. Add another 100 μ l of Trizol, then shake the tube several times to homogenize the tissue until the mixture is well-prepared.
- Discard the mixture in the new tube. Add 100 μ l of Trizol to wash the excess mixture from the bead and transfer it to the RNA extraction tube. The final volume of the lysate mixture is 300 μ l. Incubate for 5 minutes.
- Add 60 μ l of chloroform, incubate for 3 minutes, then shake the tube.
- Centrifuge the sample for 15 minutes at 12,000Xg at 4°C. The mixture was isolated into three layers. Transfer the clear supernatant of the RNA mixture to the new tube.
- Add and incubate 150 μ l of isopropanol for 10 minutes, then centrifuge the sample for 10 minutes at 12,000Xg at 4°C.
- Discard the supernatant and resuspend the RNA pellet in 300 μ l of 75% EtOH and vortex, then centrifuge for 5 minutes at 7,500 Xg at 4°C.
- Discard the supernatant, then air dry the tube with a covered Kimwipe.

- Add 20-30 μL of Hypure water, then heat the mixture in the heat box at 60°C for 10 minutes.

The quality and concentration of RNA were evaluated using a NanoDrop 1000 spectrophotometer (Thermofisher, Friendship, ME, USA) by measuring the A260/280 ratio. The RNA with a ratio of more than 1.0 was further used for cDNA synthesis as below steps by using RevertAid First Strand cDNA Synthesis Kit (K1621, Thermo Fisher Scientific Inc., Rockford, IL, USA).

- Pipette an RNA mixture containing 500 ng of RNA.
- Prepare premix mixture at 12 μL final volume of 500 ng RNA, 1 μL Random Hexamer primer, and Hypure water.
- Incubate at 65°C for 5 minutes after mixing and spinning down. Then, chill for 2 minutes on ice.
- Prepare master mix for reverse transcription. (1 reaction containing 4 μL of 5X Reaction Buffer, 1 μL of RiboLock RNase Inhibitor (20U/ μL), 2 μL of 10 mM dNTP Mix and 1 μL of Revert Aid.
- Then, pipette this master mix into the premix mixture.
- Perform the reverse transcription using a thermocycler. Incubate the mixture for 5 minutes at 25°C followed by 60 minutes at 42°C, 5 minutes at 70°C, the last step was set at 4°C ∞ . The final concentration of cDNA was 25 ng/ μL .

Quantitative PCR analysis (Figure 14) was performed with triplicate. AccuPower® 2X GreenStar™ qPCR MasterMix (K-6253, Bioneer, Daejeon, Korea) was introduced for qPCR running process by using the manufacturer's instructions. The reaction was incubated and detected the expression of each gene by Bio-Rad CFX Connect Real-Time System (Bio-Rad, USA). cDNA was diluted to 5 ng/ μL for amplification template. The master mix of qPCR for one reaction contained 6.25 μL of master mix from the kit, 0.5 μL of 10 pmol forward and reverse primers, and 4.25 μL of

Hypure water. The final master mix volume per one reaction is 11.5 μL . Then this master mix was added of 1 microliter of 5ng/ μL cDNA.

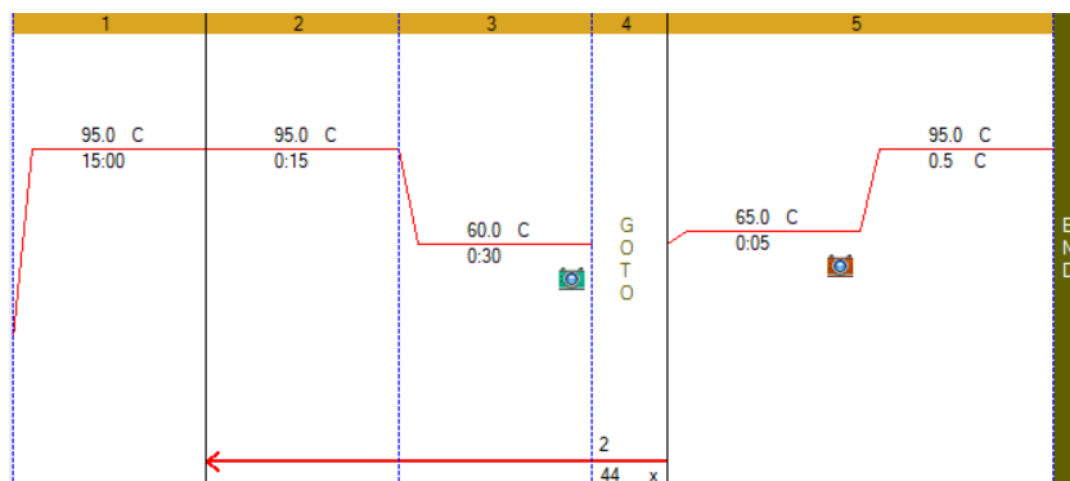


Figure 14 RT-qPCR protocol :(1) Heating process for denaturing, 45 cycles of (2) 15 sec at 95°C for denaturing, (3) 30 sec at 60°C for annealing/extension step, and (5) product confirmation by melting curve analysis (65 to 95°C). * (4) is the repetition of denaturing, annealing and extension until the 45 cycle is complete. Primers for RT-qPCR (Table 4) were explored by using USCS Genome Browser (<https://genome.ucsc.edu/>) and Ensembl (<https://asia.ensembl.org/index.html>). The sequences of primers were created by Primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0/>).

Table 4 Primers of targeted genes

| Genes | Forward primer (5'>-3') | Reverse primer (5'>-3') |
|---|-------------------------|-------------------------|
| Collagen type 1 (Col1a1) | CCTGACGCATGGCCAAGA | CTGGGCAGAAAGGACAGCA |
| Collagen type 3 (Col3a1) | TGCCACCCTGAACTCAAGAG | CACCAGCATATGTCCACCA |
| Small leucine-rich proteoglycans (SLRPs) | | |
| Decorin (Dcn) | GGATTGAAAATGGAGCCTTG | TCCACAACGGTGATGCTATT |
| PRELP | AGACACAGACACGCACCAAC | GGCCAAGATGAGGAGAAGTG |
| Osteoglycin (Ogn) | GCCCACCAAGAAAGAAAATG | TTTTGGCAGTGGTGGTACAG |
| Tsukushi (TSU) | CGGGACTTCAACCTCACC | GCCAAACGTCTCTTCCTCAC |
| Matrix metalloproteinase 2 (MMP2) | TACTGGACCCACGCCTACAC | GTCACGTGGTGTCACTGTCC |
| Matrix metalloproteinase 9 (MMP9) | AGAGCGTTACTCGCTTGGA | CTGCAGGAGGTCATAGGTCA |
| Tissue inhibitor of metalloproteinase 1 (TIMP1) | CTGGCATCCTCTTGTGCT | AGGTGGTCTCGATGATTCTG |
| Tissue inhibitor of metalloproteinase 2 (TIMP2) | GACGTTGGAGGAAAGAAGGA | GGCTCTTCTTCTGGGTGATG |
| Tissue inhibitor of metalloproteinase 3 (TIMP3) | TGGGAAAGAAGCTGGTGAA | CACATGGGGCATCTTACTGA |
| Tissue inhibitor of metalloproteinase 4 (TIMP4) | CACGCCATTTGACTCTTCTC | CTCCCAGGGCTCAATGTAGT |
| 18S Ribosomal RNA (RN18S) | CTGGATACCGCAGCTAGGAA | GAATTTACCTCTAGCGGCG |

Results were analyzed by using the Relative Quantitation ($\Delta\Delta C_t$ method, Livak and Schmittgen 2001). The relative quantitative changes in steady-state mRNA between multiple samples and their expression levels were determined compared to an internal reference (RN18S).

$$R = 2^{-[\Delta C_{t\text{sample}} - \Delta C_{t\text{con}}]} = 2^{-\Delta\Delta C_t}$$

Where

$\Delta C_{t\text{sample}}$ = Ct (interest gene of sample) – Ct (internal reference gene of sample)

$\Delta C_{t\text{control}}$ = Ct (interest gene of control) – Ct (internal reference gene of control)

3.9 Data analysis

The results were presented as the mean \pm standard error of the mean (SEM). Statistical analyses were performed with unpaired Student's t-test between control and BPA groups and calculated by IBM SPSS Statistics version 23 (SPSS, Inc., Armonk, NY, USA). The probability values of less than 0.05 were considered statistically significant.



CHAPTER IV

RESULTS

This study evaluated the effect of maternal BPA diet during pregnancy on the alteration of the neonatal anterior pituitary gland; focusing on ECM. BPA at a dose of 5,000 µg/kg BW was used as the treatment group while control was fed by ethanol dissolved in corn oil. The results conducted in this present study were divided into two main parts, i.e., effects of BPA directly on cells that play an essential role in ECM production and the alteration of ECM components in the neonatal anterior pituitary.

4.1 Immunohistochemical study

4.1.1 Effects of BPA at NOAEL level on the ECM-producing cells

ECM components in the anterior pituitary gland were regulated via the interaction of pericytes and FS cells. Therefore, the numbers of pericytes and FS cells were examined by using immunohistochemistry. NG2 was used as a pericyte marker. The expression of NG2 was seen in both anterior and posterior lobes (Figure 15A, B). NG2-immunopositive cells were in close relation with the capillaries (Figure 15C, D). Focusing on the anterior pituitary lobe, the areas of counting were divided into 3 parts; left wing, right wing, and center (which related to the width of the posterior lobe). The results revealed that the total number of pericytes significantly decreased in BPA treated group ($p < 0.05$) (Figure 16A).

In addition, the intensity of NG2 expression was also examined. The neonatal anterior pituitary gland of the BPA treated group showed a significant decrement of NG2 intensity compared to the control (Figure 16B).

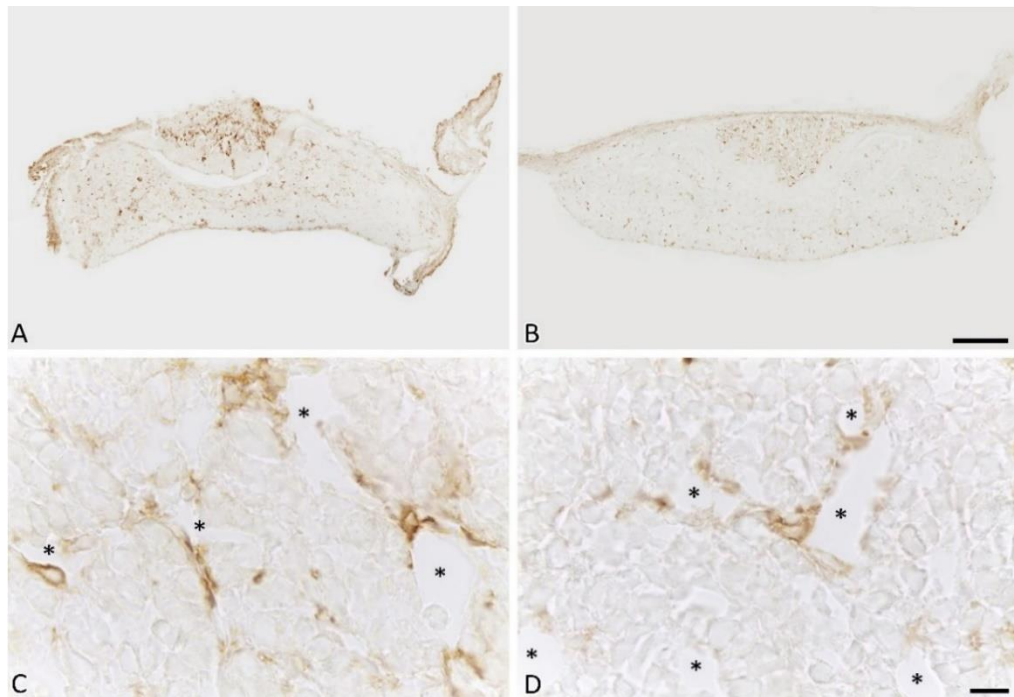


Figure 15 NG2-immunohistochemistry in the PND 1 neonatal anterior pituitary glands of control rats (A) (C higher magnification view of A) and BPA treated rats (B) (D higher magnification view of B). Note capillary lumens (asterisks). Scale bars: 100 μ m (A, B), 10 μ m (C, D).

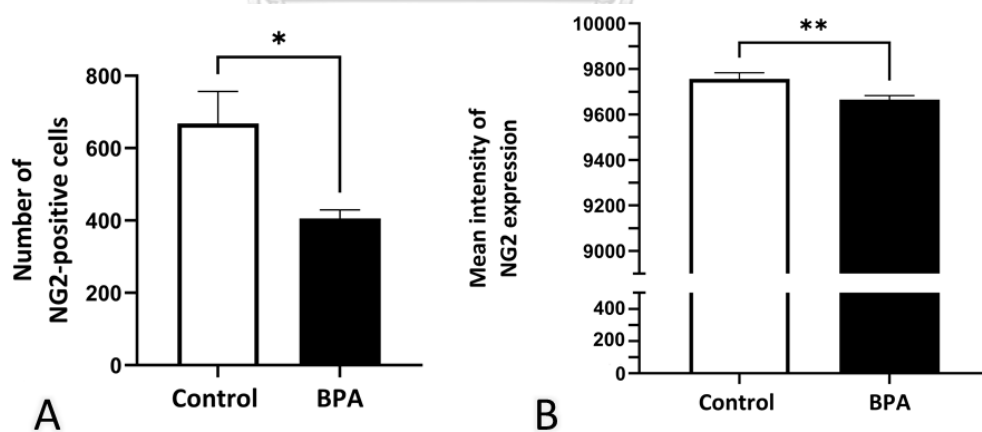


Figure 16 Number (A) and mean intensity (B) of NG2-positive cells in control and BPA treated groups. Note * $p < 0.05$, ** $p < 0.01$.

As pericytes interact with FS cells for ECM components balancing in the anterior pituitary gland, the effects of BPA on FS cells alteration were also examined. In general, S100 is commonly used as a marker of FS cells in several adult rodents. In the present study, however, the S100-immunopositive signal cannot be observed in the anterior lobe of PND 1 neonatal rats (Figure 17).

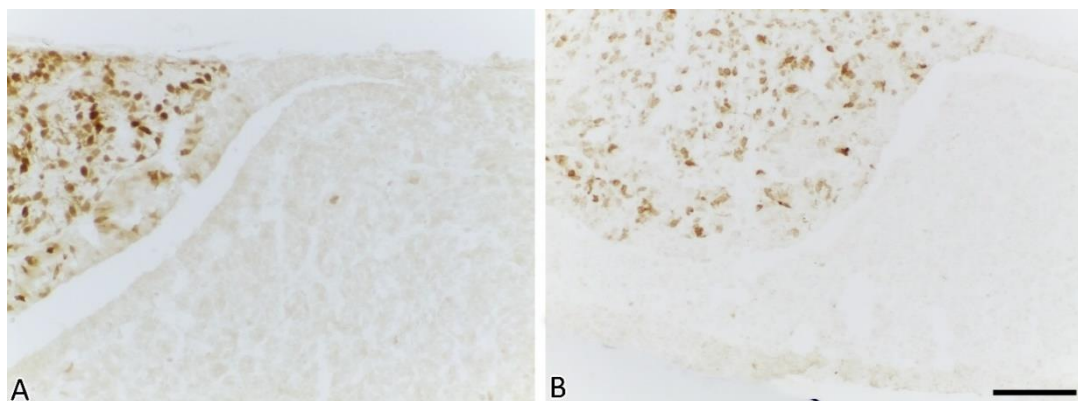


Figure 17 Absence of S100 expression in PND 1 neonatal anterior pituitary gland in both control (A) and BPA treated (B) groups. Scale bar: 100 μ m.

Recently, Fujiwara and colleagues (2020) [160] published the novel molecular marker for FS cells in mice pituitary; aldolase C. We did the pilot study of aldolase C in the adult and neonatal anterior pituitary gland according to this finding. The results demonstrated that FS cells were intensely stained by anti-aldolase C antibody (Figure 18). Therefore, aldolase C was selected for FS cell identification instead of S100.

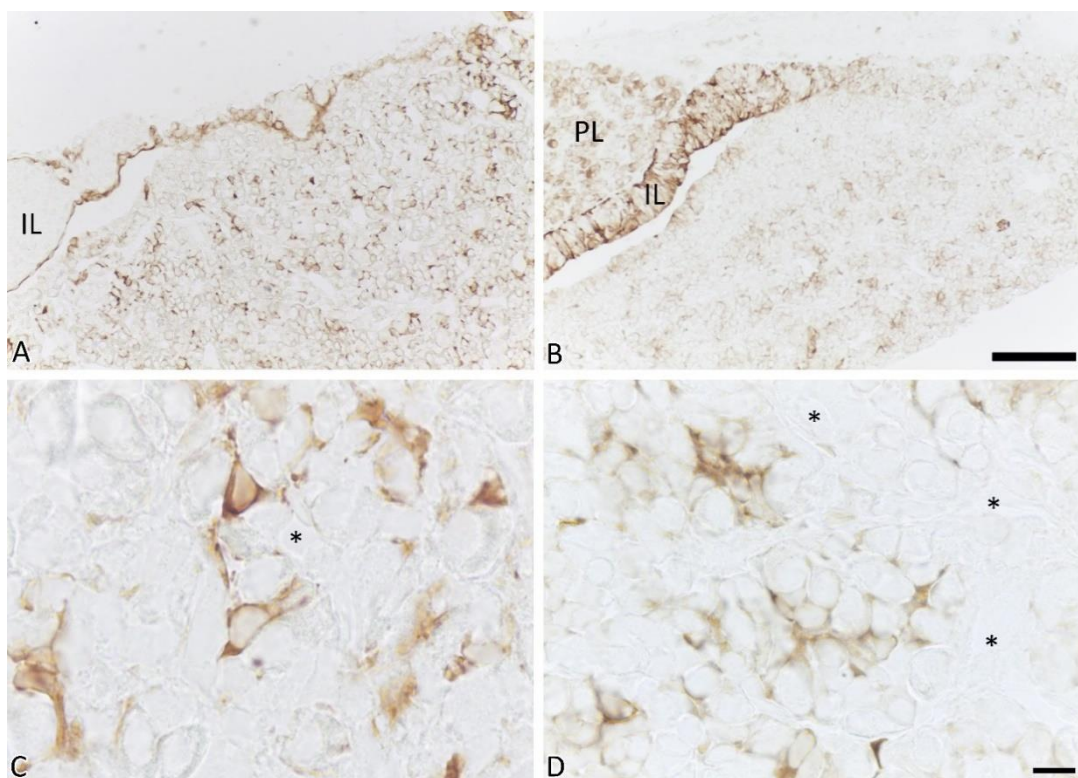


Figure 18 Expression of aldolase C in the anterior pituitary gland of adult rats (A) (C higher magnification view of A) and neonatal rats (B) (D higher magnification view of B). Note capillary lumens (asterisks), PL: Posterior lobe, IL: Intermediate lobe. Scale bars: 100 μ m (A, B), 10 μ m (C, D).

Using aldolase C immunohistochemical analysis in the present study (Figure 19), the results revealed that BPA involved higher activities of FS cells. An increase in the number of aldolase C-immunopositive cells (Figure 19D and 20A) and immunoreactive signal intensity (Figure 20B) were observed in all zones of the BPA treated group compared to the control.

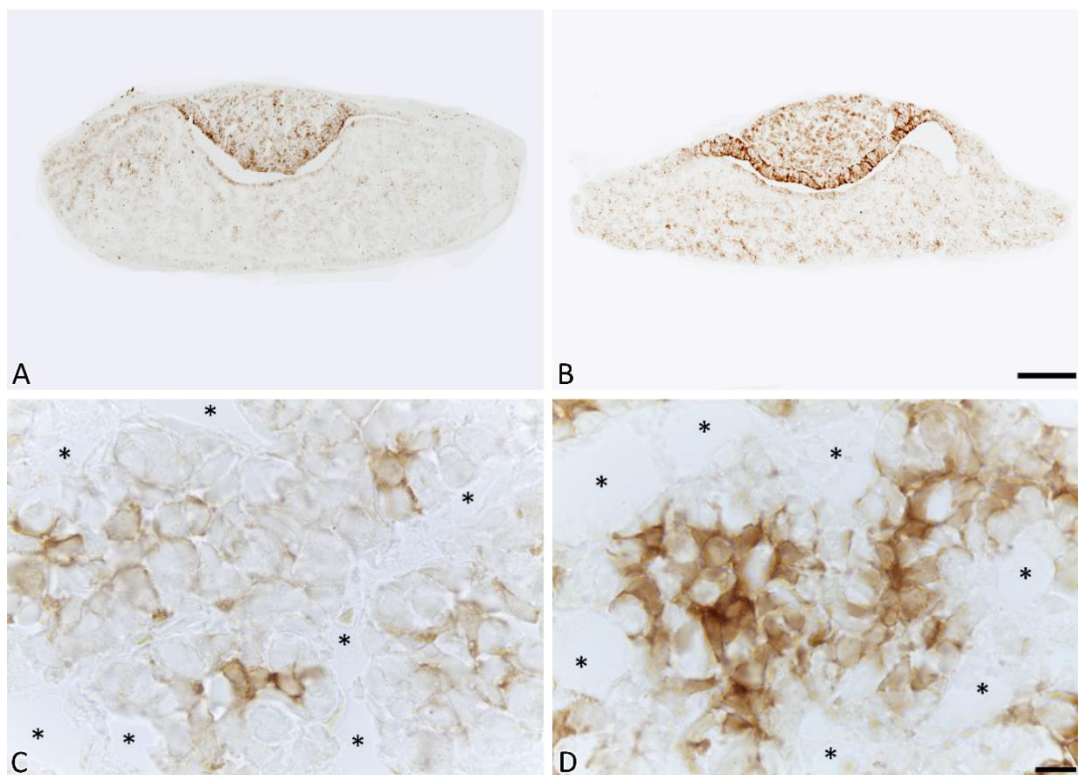


Figure 19 Aldolase C-immunohistochemistry in the PND 1 neonatal anterior pituitary glands of control rats (A) (C higher magnification view of A) and BPA treated rats (B) (D higher magnification view of B). Note capillary lumens (asterisks). Scale bars: 100 μ m (A, B), 10 μ m (C, D).

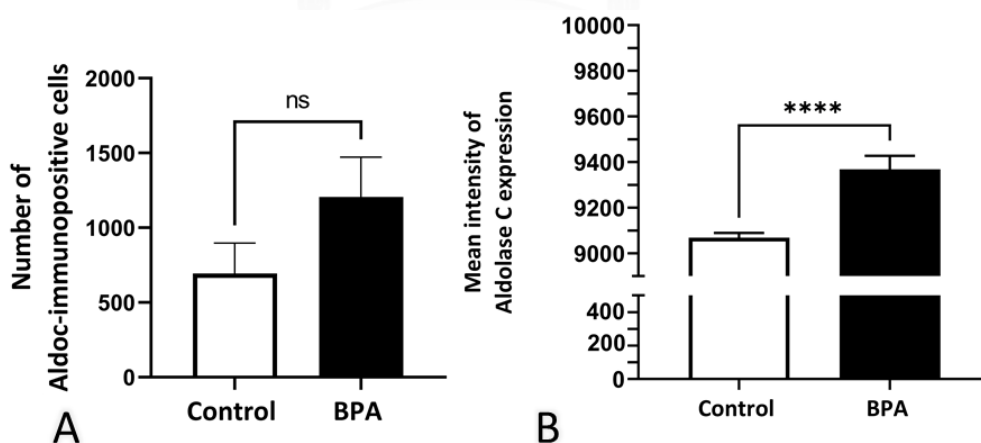


Figure 20 Number (A) and mean intensity (B) of aldolase C-immunopositive cells in control and BPA treated groups. Note no significant differences (ns), **** $p < 0.0001$.

4.1.2 Alteration of ECM-components focusing on the collagen

Collagens are the predominant ECM in the anterior pituitary gland, especially collagen type I. We examined the amount of col1a1 in the gland by using immunohistochemistry. The result showed that the pattern of collagen distribution in the BPA group seemed lesser than the control group (Figure 21). Both percentage area of collagen type I expression and their immunoreactive signal intensity were decreased in the BPA group (Figure 22).

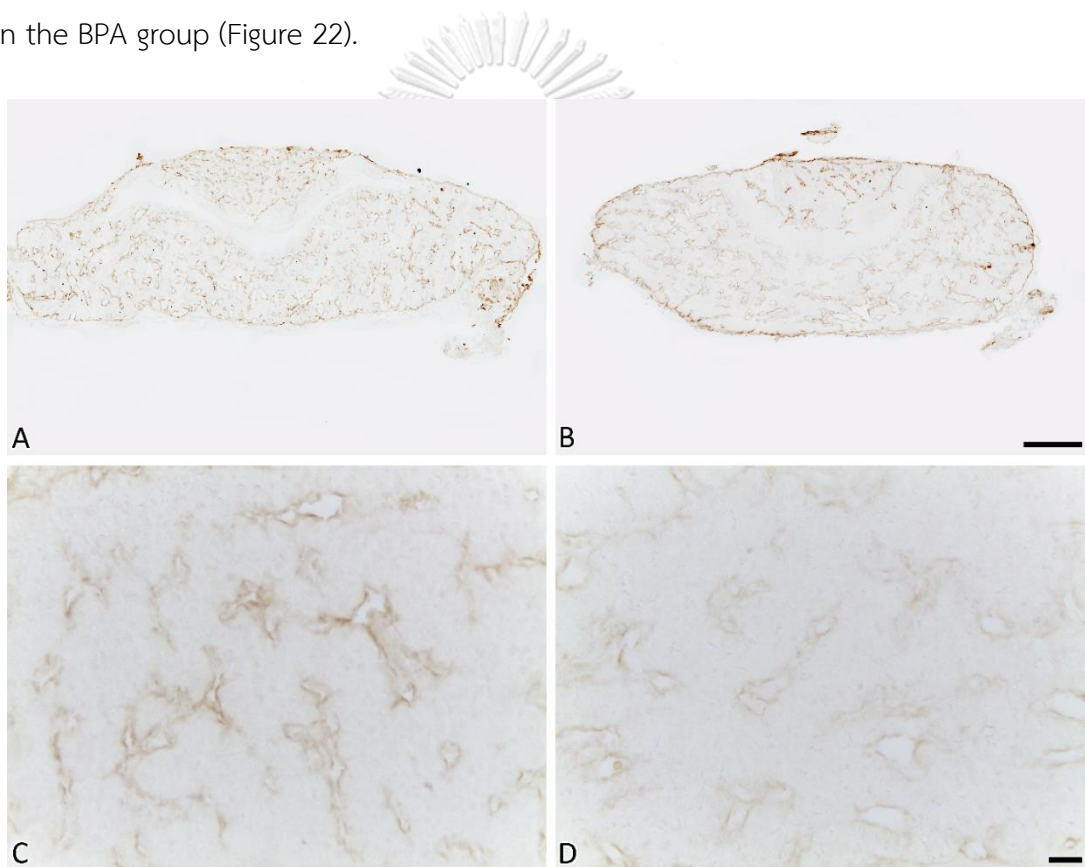


Figure 21 Collagen type I immunohistochemistry in the PND 1 neonatal anterior pituitary glands of control rats (A) (C higher magnification view of A) and BPA treated rats (B) (D higher magnification view of B). Scale bars: 100 μ m (A, B), 10 μ m (C, D).

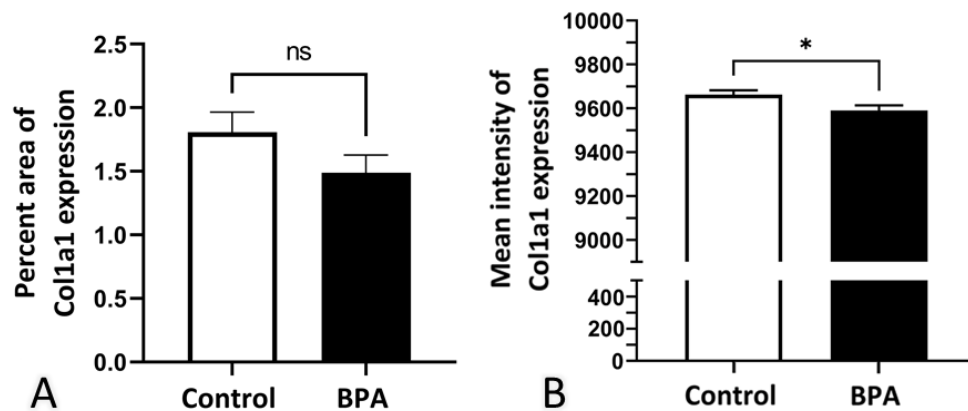


Figure 22 Percentage of collagen type I distribution area (A) and mean intensity (B) in control and BPA treated groups. Note no significant differences (ns), * $p < 0.05$.

4.2 Molecular study

The category of interested ECM mRNA examination was divided into 3 subclasses. First, the collagen types I and III which are the predominant ECM in the anterior pituitary gland. Second, the collagenases that are related to collagen synthesis in tissue, i.e., SLRPs. Third, the MMPs and TIMPs which are the ECM balancing regulators.

4.2.1 Alterations of collagen gene expression

Using RT-qPCR analysis, col1a1 and col3a1 were examined and 18S ribosomal RNA was used as housekeeping gene normalization. The mRNA expression of both col1a1 and col3a1 revealed downregulation in BPA treated group (Figure 23). Especially, collagen type I which is the most anterior pituitary gland ECM component demonstrated a significant decrease in BPA treated group compared to the control.

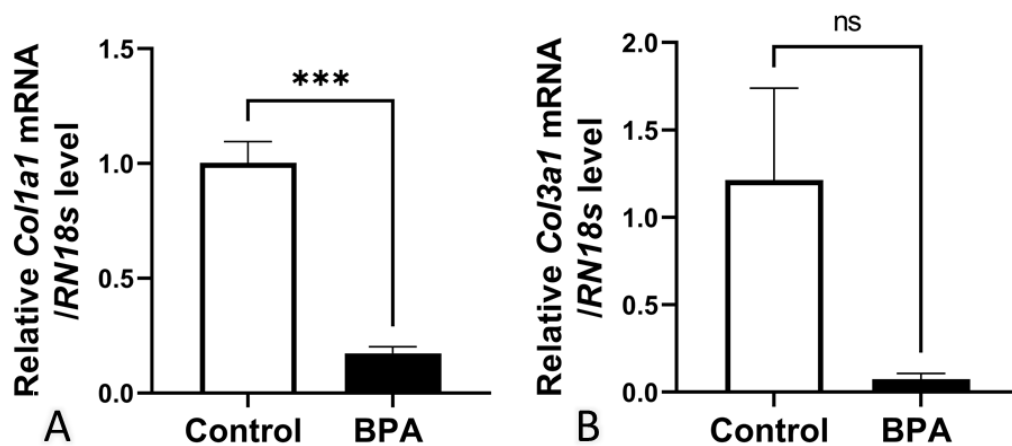


Figure 23 Collagen type I (A) and III (B) mRNA expression in control and BPA treated groups. Note no significant differences (ns), *** $p < 0.0001$.

4.2.2 Alterations of collagen related molecules

SLRPs are related to collagen fibrogenesis and adaptation of collagens and ECM that affect the anterior pituitary population behavior. Some reports demonstrated the relation between SLRPs and collagens in the anterior pituitary gland which presented 5 classes of SLRPs. We used one member notable in each class (1-4) to evaluate the effects of BPA treatment on the alteration of SLRPs. Therefore, the mRNA expression of decorin (Class I), PRELP (Class II), osteoglycin (Class III) and tsukushi (Class IV) were examined.

Decorin is the most predominant expression of SRLPs in the rat anterior pituitary gland. After maternal BPA treatment, the mRNA expression of decorin and osteoglycin in the neonatal anterior pituitary gland showed higher upregulation than the control (Figure 24A, C). However, only decorin showed a significant difference between control and BPA treated groups ($*p < 0.05$). While the upregulations of decorin and osteoglycin were detected, the mRNA expression levels of PRELP and tsukushi tended to be

downregulated in BPA treated group. There were no statistically significant differences between groups (Figure 24B, D).

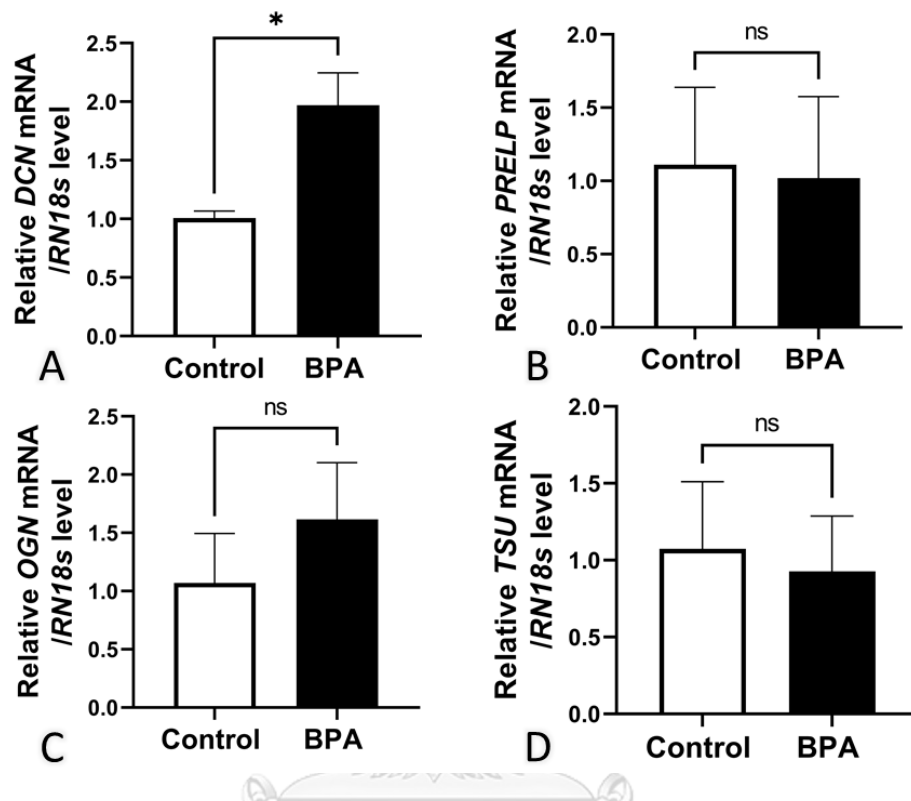


Figure 24 Decorin (A), PRELP (B), osteoglycin (C), and tsukushi (D) mRNA expression in control and BPA treated groups. Note no significant differences (ns), * $p < 0.05$.

4.2.3 Alterations of ECM balancing regulators

The ECM balancing in both general and pathological conditions is involved in MMPs and TIMPs interactions. Therefore, MMP2 and MMP9, the major MMPs in the anterior pituitary gland, were examined for their mRNA expression. Moreover, the expression of TIMP1, 2, 3, and 4 mRNA were also evaluated.

MMP2 and MMP9 demonstrated their high upregulation in BPA treated group (Figure 25). These expression levels showed statistically significant differences (* $p < 0.05$), especially that of MMP9.

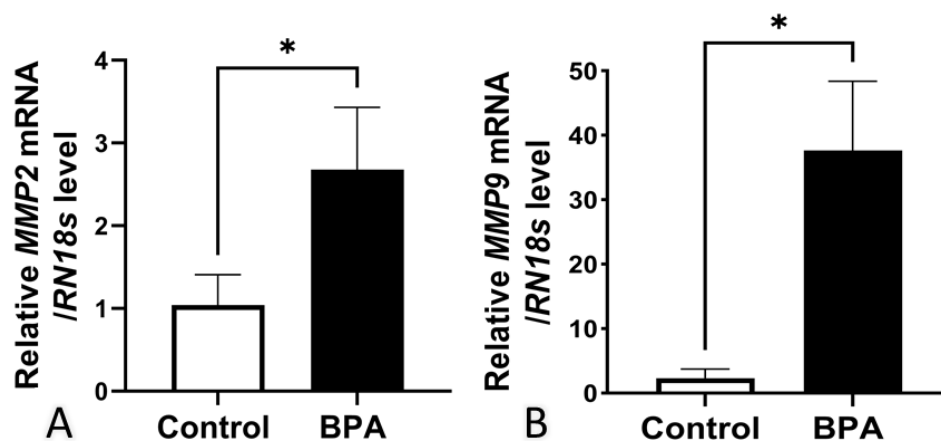


Figure 25 MMP2 (A) and MMP9 (B) mRNA expression in control and BPA treated groups.

* $p < 0.05$.

TIMP family mRNA expression (TIMP1, TIMP2, TIMP3, and TIMP4) was detected in the glands of both control and BPA-treated groups. The results revealed no discernible change in TIMP1 expression levels between the two groups, but TIMP2 and TIMP3 mRNA expression levels were increased in BPA-treated rats (Figure 26). TIMP4 mRNA levels were also increased, although the difference between groups was not statistically significant.

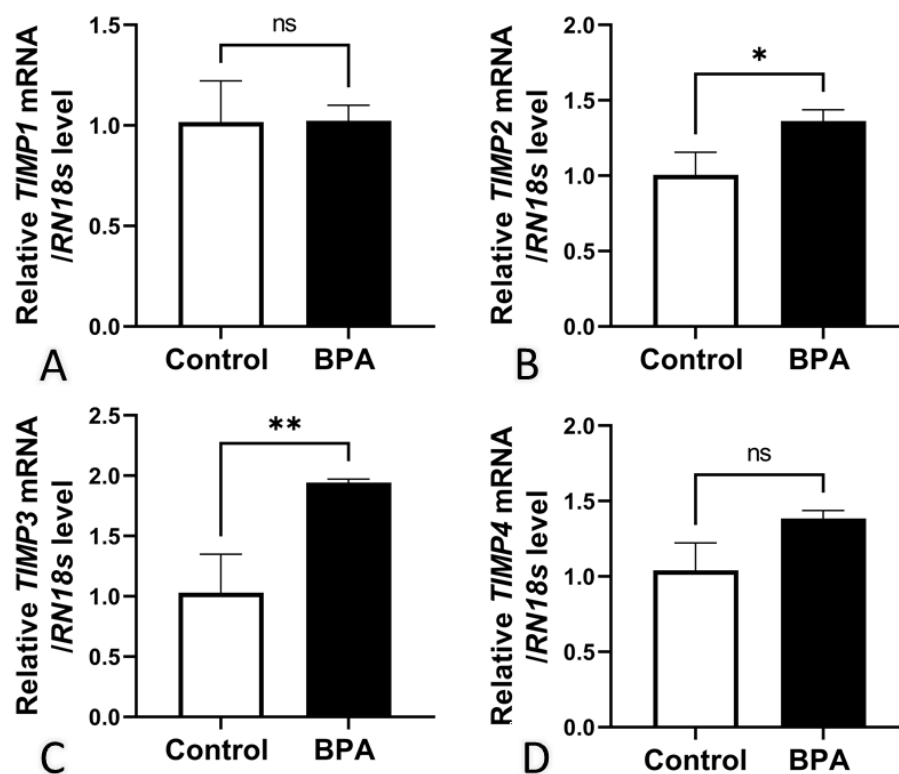


Figure 26 TIMP1 (A), TIMP2 (B), TIMP3 (C), and TIMP4 (D) gene expression in control and BPA treated groups. Note no significant differences (ns), * $p < 0.05$ and ** $p < 0.01$.

Transmission electron microscopic study

TEM was used to evaluate the changes of ultrastructure and pattern of tissue organization. According to semithin (1.5 μm thickness) observation to locate the targeted area for the ultrathin section, the sections in the control group exhibited more frequency of plenary cell cluster formation than the BPA-treated group (Figure 27).

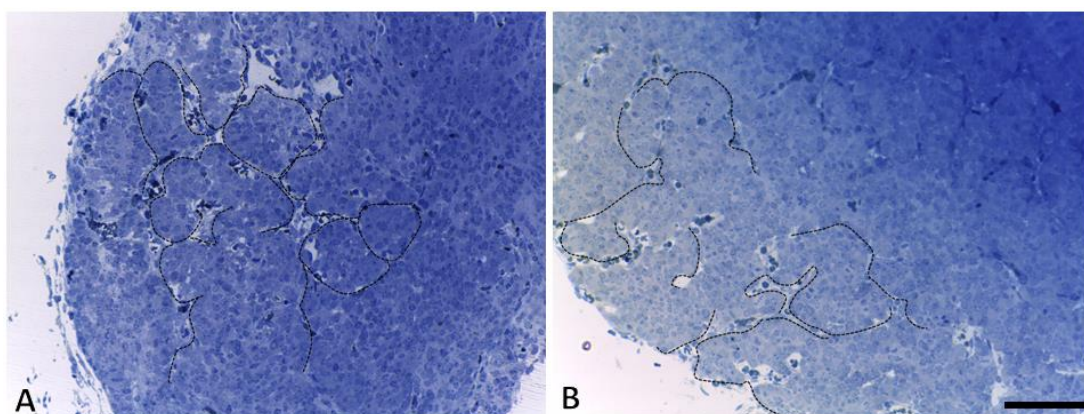


Figure 27 Semithin sections of control (A) and BPA-treated group (B) demonstrate poorer cell cluster formation in the BPA group. Note cell cluster formation (dash lines). Scale bar: 100 μm .

Under transmission electron micrographs of control rats (Figure 28), the anterior pituitary gland of the neonate demonstrated the character of cell cluster formation which was encircled by the vessels. The fenestrated capillary network forming and vasculature development were also observed. The hormone-producing cells displayed several varied sizes and shapes of granules in the cytoplasm. Another cell type that was situated in the parenchyma and had no secretory granule was called FS cells. In general, the parenchyma of the anterior pituitary gland is composed of hormone-producing cells and FS cells inserted within the cluster. Vessels and parenchyma were separated from each other by their basement membranes. and the potential space between them was the perivascular space. This space was the location of perivascular cells.

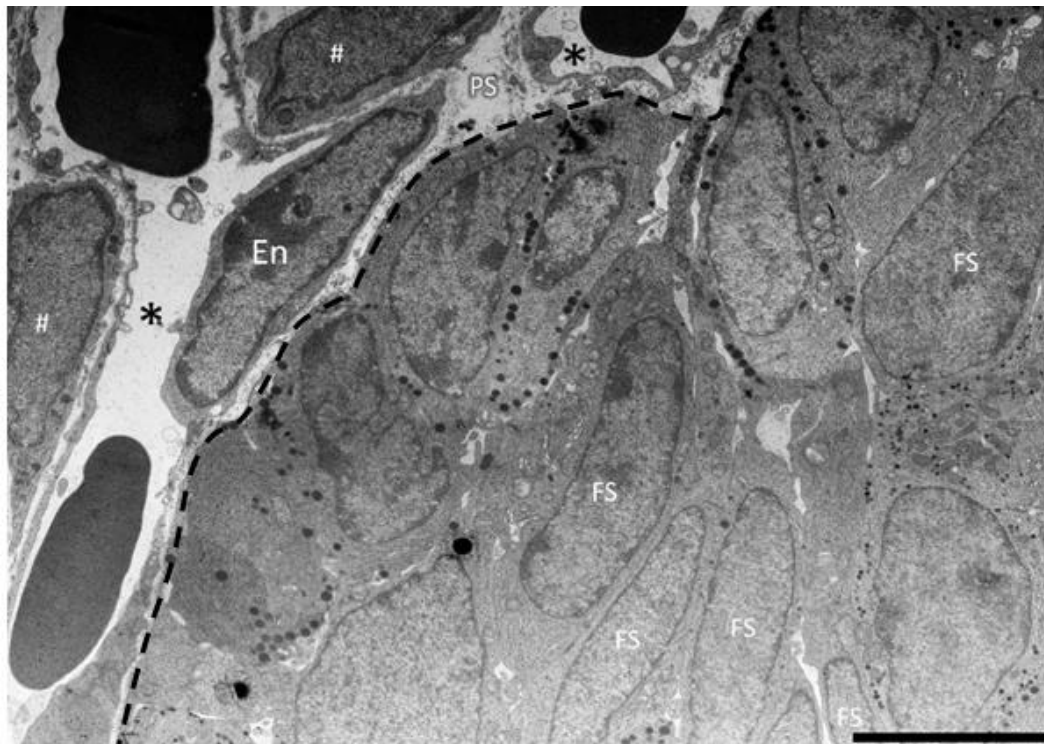


Figure 28 Transmission electron micrograph of parenchyma, vessels, and perivascular space of neonatal anterior pituitary gland of control rats. Note capillary lumen (asterisks), perivascular space (PS), perivascular cells (#), FS cells (FS), and cell cluster formation (dash lines). Scale bars: 5 μ m.

In BPA-treated neonatal rats, the general characteristics of parenchyma, perivascular space, and vascular component were also identified. In comparison to the control group (Figure 29A), the anterior pituitary in the BPA-treated group showed a poor cell cluster formation in numerous regions (Figure 29B). Furthermore, FS cells formed a follicle and possessed the pseudolumen including microvilli in the anterior pituitary of both control and BPA rats (Figure 29D, F). The development of follicles was related to junctional complexes between neighboring FS cells. FS cells had a packed cell clump with pale cytoplasm and no secretory granules in the control group (Figure 29C, E). They demonstrated a tight connection between neighboring cells held together by the junctional complex. These cells in the BPA-treated group formed many

clusters scattered across the whole parenchyma area. When compared to the control, FS cells showed numerous pseudolumens and cellular junctional complexes. In addition, many elongated microvilli were discovered (Figure 29F).

In the case of vascular and perivascular populations (Figure 30), the endothelium of neonates displayed well-developed organelles in their cytoplasm. The nuclei exhibited a huge oval shape. The populations of perivascular space are pericytes, novel DIP cells, and macrophages. Pericytes demonstrated their close relationship and shared basement membrane with endothelium (Figure 30A, B). While the histological and molecular studies demonstrated the changes in the number of pericytes and their function, transmission electron micrographs of pericytes did not show ultrastructural changes. The data demonstrated that the morphology of pericytes in BPA-treated rats resembled the neonatal control. For example, they were in a close relationship with endothelial cells, had several processes, and displayed the incomplete basement membrane (Figure 29B) as found in the characteristics of early postnatal development. Moreover, perivascular space demonstrated novel DIP cells that exhibited dilated cisternae rER in their pale cytoplasm (Figure 30C, D). Macrophages were also observed in PND 1 perivascular space and revealed several lysosome materials in the cytoplasm and well-developed organelles (Figure 30E, F). Both DIP cells and macrophages had no detectable alterations of cell morphology between groups. In addition, the collagen fibrils produced by pericytes seemed sparse in the perivascular space of the BPA treated group.

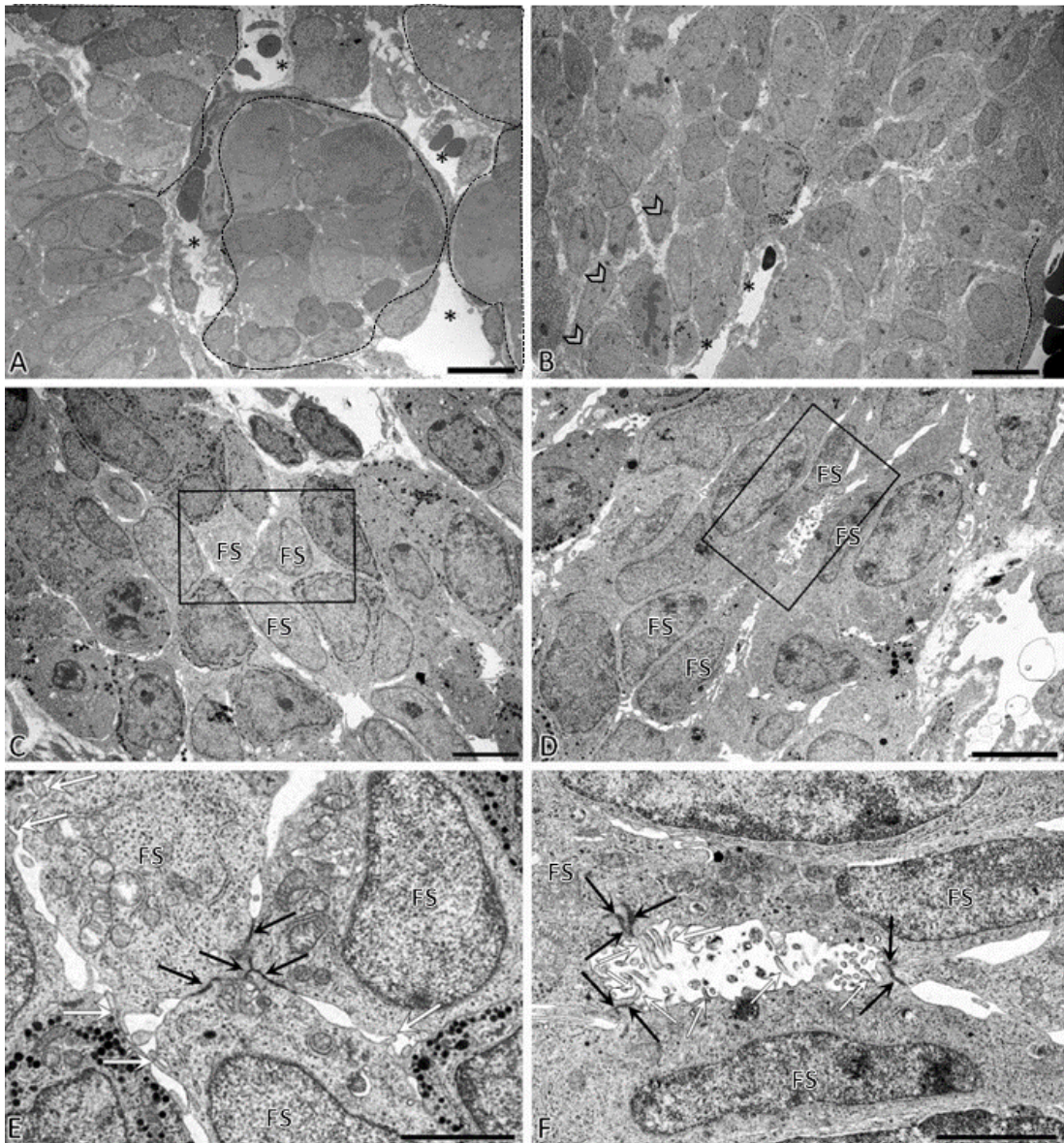


Figure 29 Transmission electron micrographs of the parenchyma of the neonatal anterior pituitary gland of control (A, C, E) and BPA treated group (B, D, F). The cell cluster organization is present in A and B. The characters of FS cells in control (C) (E higher magnification of C) and BPA treated group (D) (F higher magnification of D). Note capillary lumen (asterisks), junctional complex (black arrows), microvilli with in pseudolumen (white arrows), cell cluster formation (dash lines), and sparse parenchymal cell (gray arrow head). Scale bars: 10 μm (A, B), 5 μm (C, D), and 2 μm (E, F).

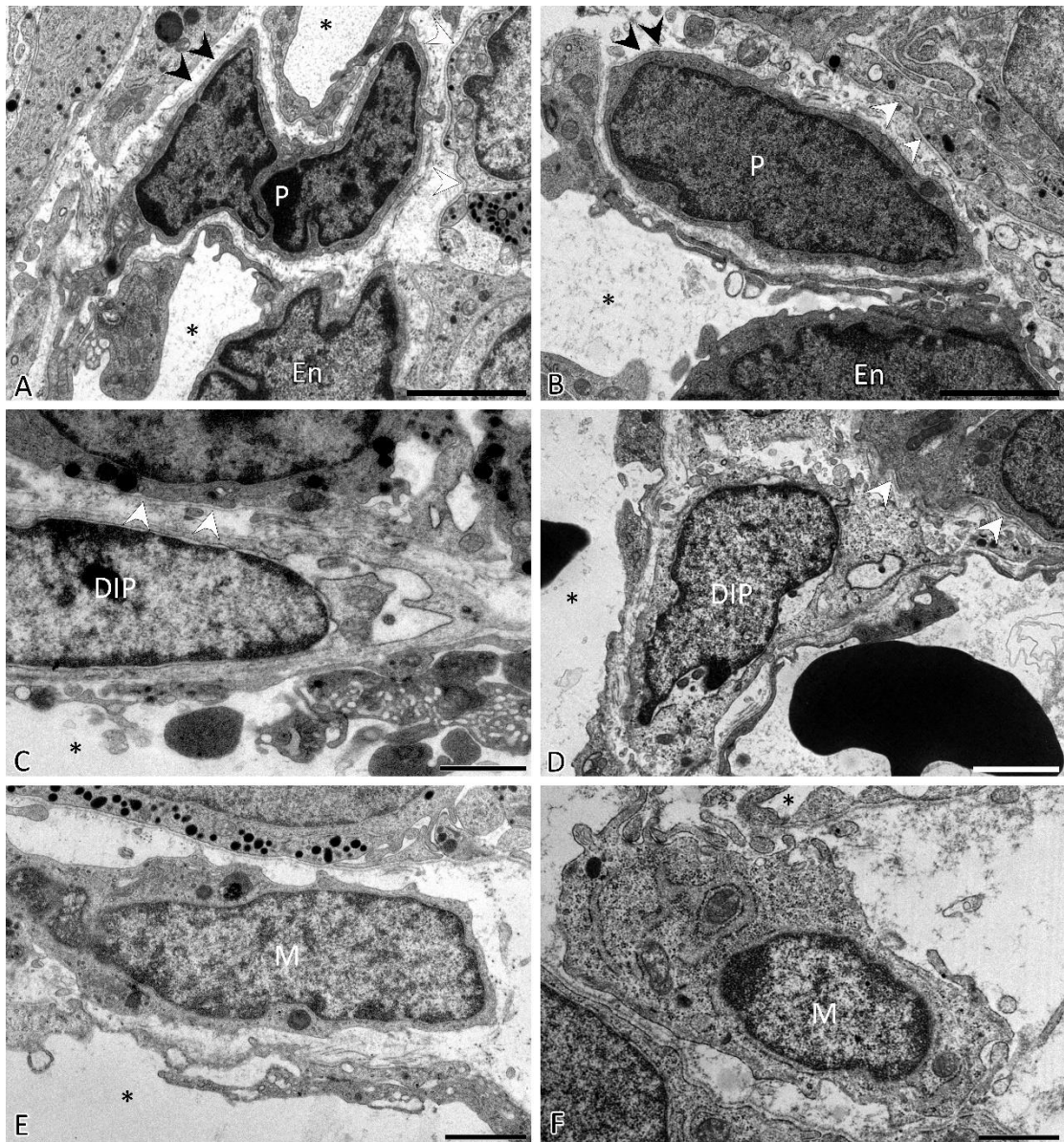


Figure 30 Transmission electron micrographs of perivascular cells of the neonatal anterior pituitary gland of control (A, C, E) and BPA treated group (B, D, F). There are three perivascular cell members, i.e., pericytes (A, B), novel DIP cells (C, D), and macrophages (E, F). Note capillary lumens (asterisks), vascular basement membrane (black arrowheads), parenchymal basement membrane (white arrow heads), endothelium (En), pericyte (P), novel DIP (DIP) and macrophage (M) Scale bars: 2 μ m.

CHAPTER V

DISCUSSION

According to our findings in the anterior pituitary gland of neonatal rats, NOAEL of BPA (5,000 $\mu\text{g/kg BW}$) affected ECM-producing cells and collagen components (Figure 30). The number of pericytes and FS cells changed in an inverse relationship, as revealed by immunohistochemistry. After BPA exposure, the number of pericytes, NG2-immunosignal intensity, and collagen immunohistochemical staining were decreased, consistent with the decrease in collagen mRNA expression. In addition, increases in FS cell numbers and their staining intensity correlated with alterations in ECM balancing regulators, i.e., collagen fibrillogenesis (SLRPs), MMPs, and TIMPs. We found that *DCN*, *OGN*, *MMP2*, *MMP9*, and *TIMP1-4* mRNAs were upregulated, whereas *PRELP* and *TSU* were downregulated. Moreover, transmission electron microscopy revealed predominantly unformed cluster characteristics in the anterior pituitary gland of BPA prenatal exposure rats.

From postnatal development through maturity, pericytes in the anterior pituitary gland are recognized as the host of collagen type I and III production. Jindatip et al. (2018) reported that diethylstilbestrol (DES) induced prolactinoma in rats with downregulation of collagen synthesis. The present study also showed that maternal BPA exposure reduced the number of pericytes and collagen expression in neonatal rats (Figure 16A, 23). DES and BPA have been categorized as estrogen-like endocrine-disrupting chemicals that can bind to estrogen receptors and cause hyperprolactinemia. We hypothesize that the effects of BPA on collagen suppression are conveyed through this mechanism since pericytes also have estrogen receptors [161]. Although BPA can promote prolactinoma by causing excessive cell proliferation in various cell types [162], the NOAEL of BPA used in this study did not trigger pathological tumor angiogenesis. Therefore, a high proliferation of vascular pericytes was not observed, unlike DES treatment.

S100 is a particular marker for FS cells in the anterior pituitary gland that is routinely utilized. In contrast, our preliminary evaluation found that S100

immunostaining was not seen in any part of the PND 1 anterior pituitary gland (Figure 17). This finding was related to Wada et al. (2014), who demonstrated that S100 immuno-positive signals were observed in the anterior pituitary gland around postnatal day 15 [162]. Recently, aldolase C has been identified as a new marker of FS cells in the pituitary glands of adult mice [160]. We also introduced aldolase C for FS cell identification and it can be used as an appropriate marker of FS cells in the early postnatal anterior pituitary gland of rat strain.

Endocrine-disrupting chemicals may trigger alterations in the characteristics of FS cells since they contain estrogen receptors [163]. In contrast to pericytes, BPA substantially enhanced the number and the immunostaining intensity of FS cells in this work. Brannick and colleagues reported that a low dosage of BPA in the prenatal period of mice increased gonadotrophs proliferation and SOX2-expressing progenitor cells in the neonatal anterior pituitary gland [19]. Moreover, there were several publications reported that SOX2 is one of the progenitor or stem cell markers expressed in FS cells [164-166]. Therefore, it is possible that FS cells in prenatal BPA-treated neonates in this present study are also one of the proliferation cells that increase in the number after BPA exposure. Additionally, since FS cells possess various pituitary hormone receptors, the changes of FS cells might be from other indirect local hormones adverse consequences [167]. Ahmed (2016), detected an increase in serum TSH in 20-day fetuses after the mothers were exposed to BPA from days 1 to 20 of pregnancy [168]. Moreover, Brokken et al. (2005) found that there were TSH receptors in the FS cell line and these cells produced several cell proliferation-related genes controlled by TSH [169]. However, BPA induced high TSH level is still debatable. According to Brannick et al. (2012), maternal BPA exposure since G10.5 to G18.5 did not alter the level of *TSH* mRNA in thyrotrophs of PND1 mice [19]. Consequently, more evidence is needed to confirm local hormone effects on FS cells resulting from BPA exposure. Pituitary pericytes interact with FS cells via $TGF\beta 2$ pathway for producing and controlling the collagens [170]. In addition, ECM proteolytic enzymes and their inhibitors, including MMP9 and the TIMP family, were synthesized from either pericytes

or FS cells. Ilmiawati et al. (2012) found that FS cells release MMP9 on laminin involvement, which later returned to boost their proliferation activity [133]. This data resembled our findings of many FS cells (Figure 19D) and a dramatic increment of MMP9 (Figure 25B). Thus, autocrine stimulation would be considered an additional route of FS cell proliferation triggered by BPA.

BPA may activate specific cells by binding to estrogen receptors, and because pericytes and FS cells include estrogen receptors, this approach may also involve them. However, the agonist and antagonist characteristics phenomena might explain why BPA causes them to respond in such a different way. Furthermore, estrogenic chemicals use genomic and non-genomic mechanisms to influence downstream signaling pathways. The stimulating protein-1 binds to the estrogen response element (ERE) transcription factor and is an essential mediator of non-genomic signaling [171]. The changes triggered by EDCs can be caused by stimulating genes containing ERE. There are pieces of information in the CHEA Database provided on genes containing ERE linked to the assessed genes in this study. Decorin, osteoglycin, tsukushi, and MMP2 contain EREs related to androgen receptor involvement. Collagen type I, decorin, osteoglycin, PERLP, and TIMP3 are, on the other hand, linked to estrogen alpha receptors. Relationships between TIMP4 and estrogen receptor beta have also been identified. However, we can not provide and clarify this issue at this time. More details should be examined in further study.

In general, collagen fibrillogenesis and adaptation involve the dynamic changes of ECM in various tissues. These properties affect connective tissue environmental network forming, cellular organization, and changes in cellular functions. SLRPs are the most well-known collagen fibrillogenesis proteoglycans and there are five family classes. In the rat anterior pituitary gland, only classes 1 to 4 were detectable. According to Horiguchi et al. (2013) report decorin, PERLP, osteoglycin, and tsukushi were chosen to represent class I, II, III, and IV, respectively [13]. In the present study, *DCN* mRNAs were significantly increased. The relationship between decorin and collagen type I have been reported in several studies. Under the condition of excessive

decorin, collagen type I had a smaller diameter, wider inter-fibrillar gap, shorter length of the fibril, and poor organization [172, 173]. For SLRP classes II, III, and IV, these classes are expressed at a very low level in the rat anterior pituitary gland. Therefore, the alterations of them involved by BPA are still unclear resulted from limited data of these classes in the gland.

In the rat and human anterior pituitary glands, MMP-TIMP interactions maintain ECM turnover. The TIMP family has four subtypes that serve as tissue inhibitors of MMPs. Azuma et al. (2015) reported that *TIMP1*, *TIMP2*, and *TIMP3* mRNAs were primarily expressed in FS cells, but *TIMP4* was not found in any cells of the adult rats anterior pituitary gland [157]. Unexpectedly, all TIMPs, including TIMP4, were observed in both groups of neonates in the present study. The increasing trend of all TIMPs was observed in the BPA-treated group while MMP persisted high level. From these results, we assume that even though the expression of TIMPs was elevated, it was not high enough to inhibit the increasing level of MMPs. Imbalance of actively dynamic MMP-TIMP properties might be the factor resulting in ECM imbalance, leading to poor cell organization as shown in Figure 28B. However, TEM observation of each cell type did not show critical fine-structural alteration, as is the case of immunohistochemistry and RT-qPCR results. It is possible that this NOAEL dose affects the functional and molecular levels rather than the cell morphological phenotypes.

Conclusion (Figure 31): The effects of BPA leading to ECM imbalance may cause by the following; BPA activates estrogen (and other hormones) receptors in ECM-producing cells, causing cellular and functional changes. The effect of BPA on collagen reduction may result from the depletion of pericytes. Furthermore, an increase in FS cells associated with high MMP9 production might be another factor related to a high rate of ECM turnover, resulting in less collagen. Changes in SLRP profiles, particularly significant decorin overexpression, might indicate an additional route of collagen reduction. The poor cell cluster formation shown in transmission electron micrographs might result from all the alterations caused by BPA on the ECM imbalance.

In summary, results indicated that modifications in ECM-producing cell characteristics, ECM products, associated ECM balance genes, and cell organization were detected in the anterior pituitary gland after fetuses were exposed to BPA at a level that had no adverse effects. Because this is one of the critical developmental stages, the findings of this study support the concept that the prenatal stage is especially vulnerable to endocrine-disrupting substances. It is also essential to increase awareness about the dangers of BPA, especially in pregnant women.

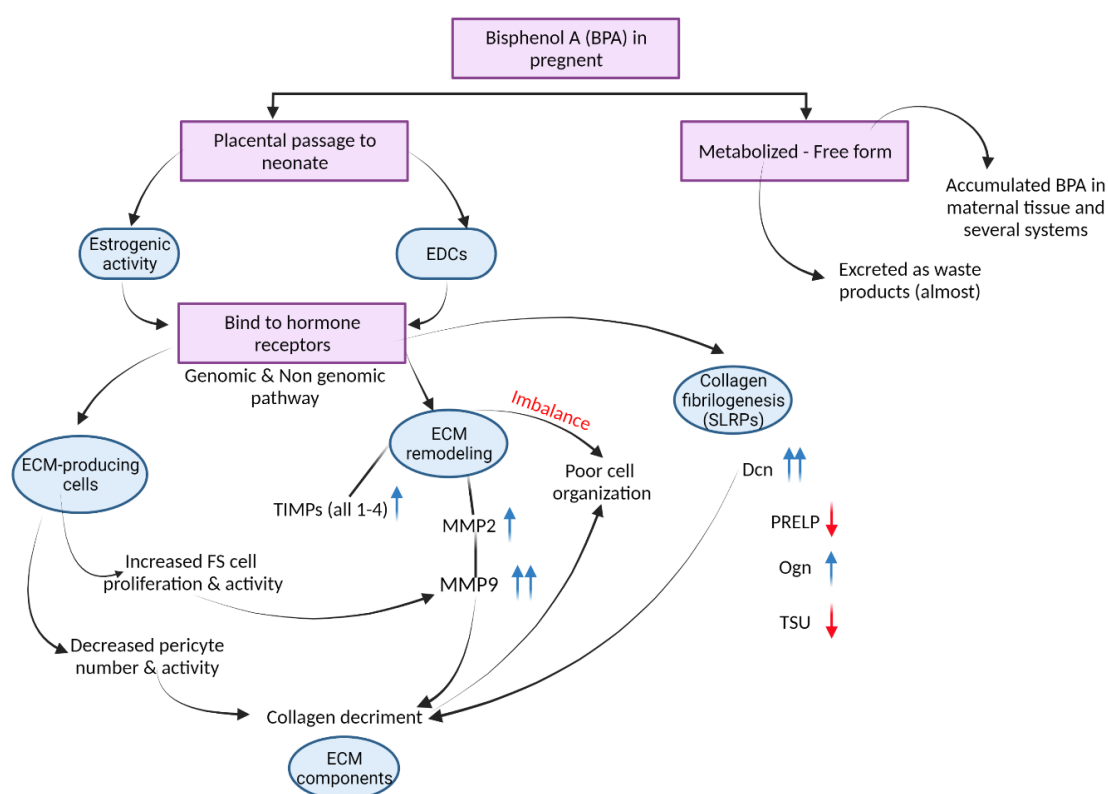


Figure 31 Summary of the present study

LIMITATIONS OF THIS STUDY

1. In early neonatal specimens, the basic FS cell identification marker (S100) does not function (PND1). Fortunately, Fujiwara's work on aldolase c expression in FS cells of mice anterior pituitary gland was published in 2020. The antibody is then validated in rat neonates. Finally, we discovered that aldolase C is a new marker for FS cell identification in the anterior pituitary gland of developing rats.
2. We intended to use Masson's trichome to assess the cellular organization and collagen deposition in the proposal draft. However, this did not work. Instead of Masson's trichome staining, we applied collagen type I immunohistochemistry.
3. In this work, fixative perfusion was not possible because each rat had to be performed with molecular evaluation, immunohistochemistry, and TEM observation. Nevertheless, this was resolved by immersing the specimen as quickly as possible to allow fixative penetration.
4. This study cannot prove how EDCs work on the cell since they can activate and influence downstream pathways in genomic and non-genomic ways. Further study should be performed to determine which route has the most impact on BPA. Including the cell culture experiments to evaluate the effects of BPA on each cell type.

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Reagent preparation

1. Real-time RT qPCR master mix per one reaction

| Reagent | Volume (μl) | Final concentration |
|---------------------------------|-------------|---------------------|
| 2X GreenStar Mater Mix | 6.25 | 1X |
| DNA template | 1 | 100 ng/μl |
| 10 pmol/μl Forward primer | 0.5 | 0.4 μM |
| 10 pmol/μl Reverse primer | 0.5 | 0.4 μM |
| Hypure water | 4.25 | |
| Total Master Mix volume (1 rxn) | 12.5 | |

2. Epon preparation

| Epon resin | For 50 ml | For 100 ml | For 150 ml | For 200 ml |
|------------|------------|------------|-------------|-------------|
| Quetol-812 | 23.8 | 47.6 | 71.4 | 95.2 |
| DDSA | 11.2 (=35) | 22.4 (=70) | 33.6 (=105) | 44.8 (=140) |
| NMA | 15 (=50) | 30 (=100) | 45 (=150) | 60 (=200) |
| DMP-30 | 0.9 | 1.8 | 2.7 | 3.6 |

3. Antibody dilutions

| Antibody | Clone | Dilution |
|-----------------|-------------------|----------|
| NG2 | Rabbit polyclonal | 1:600 |
| Aldolase C | Rabbit polyclonal | 1:200 |
| S100 | Rabbit polyclonal | 1:1000 |
| Collagen type I | Rabbit polyclonal | 1:400 |

4. DAB working solution

| Reagent | qty |
|-----------------------------------|-------|
| DAB | 12 mg |
| 0.05 M TRIS-HCL | 50 ml |
| 5 % H ₂ O ₂ | 50 μl |

Analytical data

1. Number of immune-positive cell

| Parameter | Group | n1 | n2 | n3 | n4 | n5 | Mean | T-test |
|------------|---------|------|------|-----|------|------|---------|--------|
| NG2 | Control | 556 | 961 | 572 | 780 | 471 | 668 | 0.022 |
| | BPA | 372 | 467 | 359 | 369 | 461 | 405.6 | |
| Aldolase C | Control | 642 | 490 | 318 | 1477 | 547 | 694.8 | 0.165 |
| | BPA | 1290 | 1137 | 651 | 785 | 2166 | 1205.96 | |

2. Percentage area of collagen

| Collagen type I | Control | BPA | T-test |
|-----------------|---------|-------|--------|
| n1 1/4 | 1.04 | 0.97 | 0.142 |
| n1 2/4 | 1.89 | 0.78 | |
| n1 3/4 | 1.57 | 0.80 | |
| n1 4/4 | 2.82 | 0.58 | |
| n2 1/4 | 0.95 | 1.19 | |
| n2 2/4 | 0.85 | 1.82 | |
| n2 3/4 | 0.76 | 1.62 | |
| n2 4/4 | 1.02 | 1.35 | |
| n3 1/4 | 3.16 | 2.57 | |
| n3 2/4 | 2.76 | 2.65 | |
| n3 3/4 | 2.05 | 1.50 | |
| n3 4/4 | 2.03 | 1.37 | |
| n4 1/4 | 2.55 | 1.87 | |
| n4 2/4 | 2.51 | 2.33 | |
| n4 3/4 | 2.23 | 0.65 | |
| n4 4/4 | 1.94 | 0.75 | |
| n5 1/4 | 1.61 | 1.93 | |
| n5 2/4 | 1.25 | 2.10 | |
| n5 3/4 | 1.38 | 1.66 | |
| n5 4/4 | 1.77 | 1.26 | |
| Mean | 1.807 | 1.488 | |

3. Intensity of immunoreactivity signal of NG2

| Collagen type I | Control | BPA | T-test |
|-----------------|----------|---------|--------|
| n1 1/4 | 9703.28 | 9667.53 | 0.007 |
| n1 2/4 | 9788.07 | 9526.83 | |
| n1 3/4 | 9691.59 | 9672.66 | |
| n1 4/4 | 9675.80 | 9635.80 | |
| n2 1/4 | 9715.18 | 9787.36 | |
| n2 2/4 | 9763.01 | 9693.30 | |
| n2 3/4 | 9787.21 | 9795.37 | |
| n2 4/4 | 9706.73 | 9671.32 | |
| n3 1/4 | 9675.20 | 9712.50 | |
| n3 2/4 | 9670.67 | 9717.73 | |
| n3 3/4 | 9730.62 | 9660.62 | |
| n3 4/4 | 9657.48 | 9702.6 | |
| n4 1/4 | 10005.80 | 9727.92 | |
| n4 2/4 | 9925.98 | 9683.32 | |
| n4 3/4 | 9980.12 | 9672.75 | |
| n4 4/4 | 9994.41 | 9727.08 | |
| n5 1/4 | 9647.27 | 9513.76 | |
| n5 2/4 | 9687.53 | 9613.10 | |
| n5 3/4 | 9673.19 | 9616.34 | |
| n5 4/4 | 9645.01 | 9504.01 | |
| Mean | 9756.16 | 9665.09 | |

4. Intensity of immunoreactivity signal of aldolase C

| Collagen type I | Control | BPA | T-test |
|-----------------|---------|----------|----------|
| n1 1/4 | 8988.00 | 9184.08 | < 0.0001 |
| n1 2/4 | 8974.00 | 9170.64 | |
| n1 3/4 | 8976.50 | 9200.16 | |
| n1 4/4 | 8973.70 | 9587.06 | |
| n2 1/4 | 9147.70 | 9263.33 | |
| n2 2/4 | 9149.80 | 9226.03 | |
| n2 3/4 | 9132.80 | 9272.05 | |
| n2 4/4 | 9338.10 | 9353.24 | |
| n3 1/4 | 8926.30 | 9106.83 | |
| n3 2/4 | 9008.30 | 9396.72 | |
| n3 3/4 | 9017.10 | 9262.99 | |
| n3 4/4 | 9018.20 | 9371.20 | |
| n4 1/4 | 9053.20 | 9154.68 | |
| n4 2/4 | 9137.00 | 9190.10 | |
| n4 3/4 | 9091.80 | 9211.82 | |
| n4 4/4 | 9077.70 | 9252.66 | |
| n5 1/4 | 9092.50 | 9746.79 | |
| n5 2/4 | 9147.20 | 9767.99 | |
| n5 3/4 | 9037.00 | 9506.36 | |
| n5 4/4 | 9081.70 | 10140.50 | |
| Mean | 9068.40 | 9368.41 | |

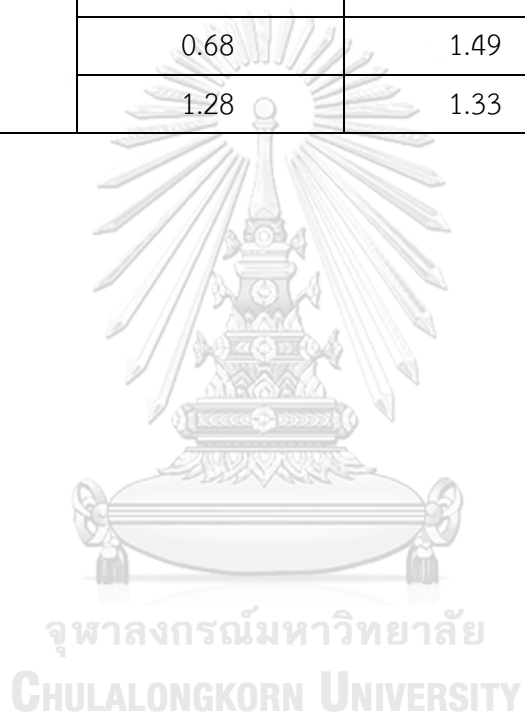
5. Intensity of immunoreactivity signal of collagen type I

| Collagen type I | Control | BPA | T-test |
|-----------------|---------|---------|--------|
| n1 1/4 | 9489.91 | 9465.74 | 0.022 |
| n1 2/4 | 9514.9 | 9464.14 | |
| n1 3/4 | 9585.72 | 9491.50 | |
| n1 4/4 | 9552.62 | 9487.09 | |
| n2 1/4 | 9655.85 | 9509.52 | |
| n2 2/4 | 9608.88 | 9531.49 | |
| n2 3/4 | 9646.97 | 9446.14 | |
| n2 4/4 | 9863.07 | 9495.72 | |
| n3 1/4 | 9737.50 | 9468.63 | |
| n3 2/4 | 9726.64 | 9637.29 | |
| n3 3/4 | 9724.91 | 9681.49 | |
| n3 4/4 | 9715.93 | 9628.68 | |
| n4 1/4 | 9612.13 | 9640.11 | |
| n4 2/4 | 9681.47 | 9698.27 | |
| n4 3/4 | 9700.21 | 9714.86 | |
| n4 4/4 | 9739.29 | 9739.84 | |
| n5 1/4 | 9669.37 | 9698.21 | |
| n5 2/4 | 9648.45 | 9717.63 | |
| n5 3/4 | 9700.02 | 9653.75 | |
| n5 4/4 | 9674.79 | 9636.87 | |
| Mean | 9662.43 | 9590.35 | |

6. Fold change of targeted gene expression from real-time qRT-PCR

| Gene | Control | BPA | T-test |
|-------------|---------|-------|---------|
| Col1a1 | 0.94 | 0.14 | 0.00012 |
| | 1.11 | 0.19 | |
| | 0.96 | 0.19 | |
| Col3a1 | 2.24 | 0.04 | 0.09664 |
| | 0.90 | 0.04 | |
| | 0.50 | 0.14 | |
| Decorin | 1.09 | 2.35 | 0.02742 |
| | 1.04 | 1.43 | |
| | 0.89 | 2.13 | |
| PRELP | 1.38 | 0.67 | 0.84888 |
| | 1.45 | 1.66 | |
| | 0.50 | 0.73 | |
| Osteoglycin | 1.33 | 1.47 | 0.21661 |
| | 1.30 | 2.16 | |
| | 0.58 | 1.22 | |
| Tsukuji | 1.29 | 0.68 | 0.67708 |
| | 1.36 | 1.34 | |
| | 0.57 | 0.76 | |
| MMP2 | 1.06 | 2.60 | 0.02762 |
| | 0.67 | 1.97 | |
| | 1.40 | 3.47 | |
| MMP9 | 0.78 | 55.97 | 0.02691 |
| | 1.35 | 19.95 | |
| | 0.95 | 34.39 | |
| TIMP1 | 1.07 | 0.96 | 0.96056 |
| | 1.19 | 1.00 | |
| | 0.79 | 1.11 | |

| Gene | Control | BPA | T-test |
|-------|---------|------|---------|
| TIMP2 | 0.88 | 1.39 | 0.02034 |
| | 1.17 | 1.28 | |
| | 0.97 | 1.42 | |
| TIMP3 | 0.98 | 1.91 | 0.00773 |
| | 0.74 | 1.96 | |
| | 1.37 | 1.96 | |
| TIMP4 | 1.16 | 1.33 | 0.1465 |
| | 0.68 | 1.49 | |
| | 1.28 | 1.33 | |



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