



Ovalbumin causes impairment of preimplantation embryonic growth in asthma-induced mice

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ABSTRACT

Insufficient experimental studies have reported the effect of ovalbumin (OVA) as an allergen towards embryonic growth in asthma mouse model. The impact of 10 µg/200 µL OVA on maternal inflammatory and oxidative stress (OS) responses, and preimplantation embryonic development was investigated in this study. We first established OVA-induced asthma mouse model, and following superovulation, mated the females and challenged them with Methacholine (Mch) test. Upon embryo retrieval, only those with the highest implantation potential were cultured *in vitro*. Significant reduction in the number of embryos at each preimplantation stage was noted in the treated group. Uneven sized blastomeres at 2-, 4- and 8-cell stages were also evident in this group. Embryo fragmentation was significant at only 2-, 4- and 8-cell stages. We also found that OVA tended to raise maternal inflammatory and OS biomarker levels as well as to cause inappropriate levels of pregnancy hormones progesterone (P₄) and estrogen (E₂) although insignificant. The combined results indicate that 10 µg/200 µL OVA had altered both quality and quantity of the embryos in asthma mouse model although its effect on pregnancy hormones, inflammatory and OS responses were non-pathological.

1. Introduction

Asthma is a chronic disease where inflammation occurs in the airways causing it to become hyper-responsive with symptoms of wheezing, dyspnea, chest tightness and coughing (Mims, 2015). In Malaysia, mortality rate due to asthma is 1.29 % (Ministry of Health Malaysia, 2018). This respiratory condition is caused by factors such as allergies, tobacco smoking, environmental factors, obesity, stress or genetic (Brazier, 2020) among many others. Among types of asthma (including allergic, non-allergic or occupational); allergic asthma may be triggered by allergens such as pollen, dust or pet dander.

In animal model of asthma, ovalbumin (OVA), a protein component in egg white is commonly used to mimic allergic asthma due to its association with T-helper type 2 (Th 2) immune response. Activation of Th 2 immune response promotes production of Th 2 cytokines namely interleukin (IL)-4, IL-5 and IL-13 which play critical role in asthma

exacerbation (Casaro et al., 2019). IL-4 stimulates the release of immunoglobulin (Ig)-E and promotes mucus secretion in the airways. IL-5 on the other hand causes inflammatory cells (mainly eosinophil) to infiltrate, which induces airway hyper-responsiveness (Kim D.I et al., 2019; Kim S.-B. et al., 2019).

Presently, little is known about crosslink between OVA and oxidative stress (OS). However, literature indicated that inflammatory process is a key mediator behind OVA-induced OS effects. Inflammatory process is known to associate with OS and reduced cellular antioxidant capacity. The theory being overproduced free radicals impair cell membrane fatty acids and proteins, and compensate their functions is evident in the literature. In terms of cellular organelle integrity, access of free radicals to the DNA leads to mutation, single- or double strand breaks. This may be a predisposing factor for various genetic disorders.

Evidence of inflammatory cells invasion into the respiratory airways and the resulting lipid peroxidation in female BALB/c mice induced by

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OVA is available (Cui et al., 2019) suggestive of DNA oxidative damage as a result of OVA treatment. Another report suggested Ig-E binding to receptor FcεRI had activated mast cells and regulated reactive oxygen species (ROS) through IL-4, IL-13 and Ig-E positive feedback loop (Qu et al., 2017). It is suggested that allergen-asthma affects systemic inflammation which does not only confine in the lung tissues but also involve the lining of blood vessels of many internal organs. This inflammatory process is often characterized by active inflammatory response and tissue repair/destruction representing airways remodeling. Other studies have documented inflammatory activation in chronological aging (Sanada et al., 2018), cardiovascular disease (Zhang et al., 2017), diabetes (Yuan et al., 2019) and autoimmune diseases (Escárcega et al., 2018). However, at present, there is still considerable ambiguity with regards to the role of OVA as an allergic-asthma inducer, pro-inflammatory and OS mediator on the growth of embryo. This calls into question to investigate the allergen in this study.

Inflammation and hormonal changes may pose some roles in fertility. It was reported that 10 % of couples sustained infertility problem in which 10–15 % of them could be attributed to allergic disorders (Adamczak et al., 2019). However, whether allergic airways disorders determine infertility is still a subject of discussion between researchers. In mouse, disrupted ovulatory processes following ovarian inflammatory condition had cause damage to the ovarian follicles (Choi et al., 2017). Follicular development into several distinct phases are crucial before ovum release. Thus, failure in folliculogenesis may potentially lead to unsuccessful ovulation. Furthermore, over expressive inflammation via accumulation of neutrophil extracellular traps (NETs) during early gestation was documented to cause placental disorder and pregnancy loss in BALB/c mice (Erpenbeck et al., 2016).

The role of pregnancy hormones is crucial in sustaining embryonic growth, and hormonal imbalance may end with infertility. Progesterone (P₄) and estrogen (E₂) play vital roles during early embryonic development and throughout gestational period. E₂ aids in developing new blood vessels in the endometrium during follicular phase. However, its over production was associated with endometriosis (Burns et al., 2018). On the contrast, P₄ inhibits uterine cell proliferation hence repairing endometriosis in mouse (Li et al., 2016). As Th 2 cytokines and inflammatory cells in the airways are theroretically over secreted during allergic airway inflammation, this may pose detrimental effects to preimplantation embryonic growth. Embryonic preimplantation period is highly responsive to any inflammatory signal. However, at present, there is insufficient information about the effects of allergen-inflammation-OS association on preimplantation embryonic development. Such study will provide direct chemical communication between mother-embryo during asthmatic conditions and determine the mechanisms responsible for the indirect effects of maternal allergic asthma on embryonic growth.

This study hypothesized that 10 µg/200 µL OVA causes impairment of preimplantation embryonic development via OS as the responsible mechanism. To test the hypothesis, 10 µg/200 µL OVA was treated on female mice to induce asthma. Inflammatory (IL-4 and Ig-E) and OS (8-hydroxydeoxyguanosine, OHdG) responses were analysed. Pregnancy hormone levels of progesterone (P₄) and estrogen (E₂) were also evaluated to associate hormonal effects with inflammation and OS. *In vitro* embryonic growth (via developmental rate, cell number, size and fragmentation) was also evaluated. The study provided evidence of direct effects of OVA-induced asthma on the maternal parameters, and indirect effects of OVA-induced asthma on the growth of preimplantation embryos in mice.

2. Materials and methods

2.1. Animals

The experimental protocol adhered to the regulations and animal ethical procedures outlined by the Committee on Animal Research

and Ethics, Universiti Teknologi MARA (UiTM CARE: 318/2020). Sample size was calculated using a crude method based on the law of diminishing return (Charan and Kantharia, 2013). Twelve 4–5 weeks old female BALB/c mice weighed 20–25 g were housed in polypropylene cages of 290 mm × 220 mm × 140 mm. They were kept in a room maintained at 27 °C, with a 12 hours light-dark cycle. Animals were provided with standard laboratory pellets and water *ad libitum*.

2.2. Establishment of asthma mouse model

Animals were randomly divided into 2 groups (phosphate buffered-saline, PBS as control and 10 µg/200 µL ovalbumin, OVA as treated) with 6 mice in each group (n = 6). OVA (Sigma-aldrich, USA) induction protocol was conducted based on methods by Paik et al. (2014), with modification. OVA was used as an allergen that mimics allergic asthma while aluminium hydroxide [Al(OH)₃] was used as an adjuvant. On Day-0 and Day-14, treated animals were sensitized with 10 µg OVA and 2 mg [Al(OH)₃] in 0.2 mL PBS through intraperitoneal injection (i.p). On Day-21, Day-22 and Day-23, 50 µL of 75 µg OVA mixed in 1.0 mL of PBS was instilled intranasally (i.n) to challenge the airways. Control animals underwent the same procedure by receiving PBS alone.

On Day-24, to assess airway hyper-responsiveness, all animals were subjected to 50 mg/mL methacholine (Mch) test by inhaling (n.i) acetyl-β-methylcholine chloride projected from a nebulizer for 3 continuous minutes. Respiratory-, pulse-rate and oxygen saturation (SpO₂) were measured daily at the left limb from Day-0 to Day-25 using a pulse oximeter (Yongrow, China). The readings were also recorded prior- and post-Mch test. Reduced respiratory-, pulse-rate and SpO₂ post-test were the indications of deterioration of lung function in respiratory diseases including asthma (Milton et al., 2012).

2.3. Superovulation and mating

Animals were superovulated by administering 0.1 mL of 5 IU pregnant mare serum gonadotropin (PMSG) on Day-21 followed by 0.1 mL 5 IU human chorionic gonadotropin (hCG) (i.p) on Day-23 (Fig. 1). Following hCG injection, females were mated with fertile males at a ratio of 1:1. Copulation plug was inspected 24 hours later to confirm mating.

2.4. Collection of blood and oviducts

On Day-25, animals were euthanized through terminal cardiac puncture (Fig. 1). Whole blood was withdrawn and stored in 6.0 mL BD Vacutainer Plus tubes. The blood was then centrifuged at 1000×g, 4 °C for 20 minutes to separate serum. Both oviducts were retrieved via laparotomy and excised to obtain 2-cell embryos (Section 2.5).

2.5. Embryo retrieval and in vitro culture

Two-cell embryos were flushed out of the oviducts by expiring approximately 0.1 mL M2 medium through a 32-gauge hypodermic needle attached to a 1.0 mL syringe under a stereo microscope (Leica Zoom 2000, Z45 V). The retrieved embryos were rinsed by aspirating and expiring the embryos in droplets of M2 medium before being cultured in a 35-mm culture dish containing pre-prepared 100 µL droplets of M16 medium, overlaid with paraffin oil (Irvine Scientific, USA). The embryos were incubated in a humidified incubator (Binder, CB 170) at 37 °C (5 % CO₂) until Day-29 where all cultured embryos were expected to reach hatched blastocyst stage.

2.6. Embryo morphology and developmental rate

The morphology and developmental rate were assessed under an

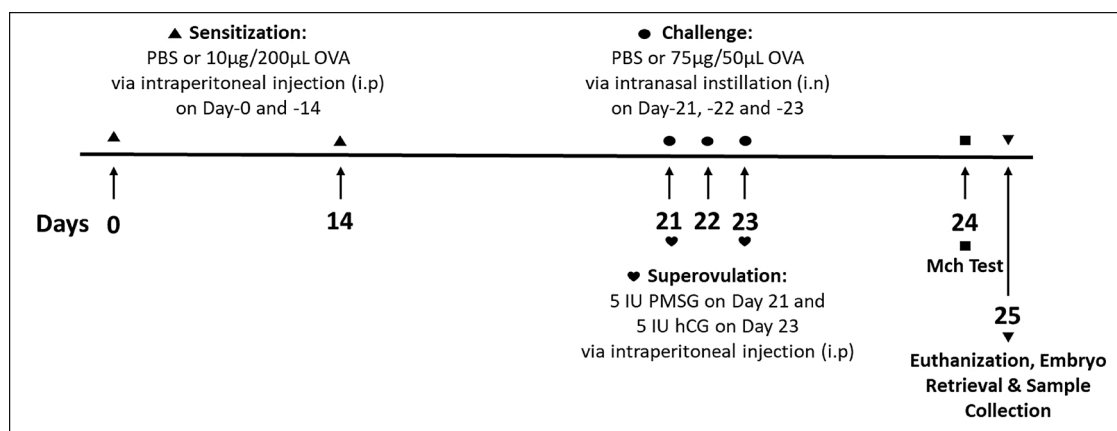


Fig. 1. Timeline of experimental design. PBS: Phosphate buffered saline. OVA: ovalbumin. PMSG: Pregnant mare serum gonadotrophin. hCG: Human chorionic gonadotrophin. Mch: methacholine.

inverted microscope (Leica DM IRB, BZ:05). Embryos were categorized according to Saiz et al. (2018). For 2- (1.5 days post coitum (d.p.c)), 4- (2 d.p.c) and 8-cell (2.5 d.p.c.) embryos, classification was based on the number and size of blastomere, and the degree of fragmentation. For morula (3 d.p.c), blastocyst (4 d.p.c) and hatched blastocyst stage (5 d.p.c), classification was based on the degree of fragmentation only. The degree of fragmentation was determined based on the presence of cellular debris (anucleated structures) inside the embryo. Embryos were assigned into one of these four fragmentation categories namely $\leq 10\%$, $>10\%$ to 25% , $>25\%$ to 35% and $>35\%$ (Saiz et al., 2018).

Developmental rate was calculated as a percentage (%) based on the total number of embryos developed from the previous developmental stage. The formula is as shown below:

$$\text{Developmental rate \%} = \frac{\text{Total number of embryo at current developmental stage}}{\text{Total number of embryo at previous developmental stage}} \times 100$$

2.7. Determination of serum IL-4, Ig-E, 8-OHdG, P₄ and E₂

Serum interleukin (IL)-4 and immunoglobulin (Ig)-E were measured using ELISA (Elabsience, China, cat # E-EL-M0043 and E-EL-M0691). A volume of 100 µL standard or sample were added into 96-well microplate pre-coated with antibody specific to mouse IL-4 or Ig-E, respectively. Biotinylated Detection Ab specific for mouse IL-4 or Ig-E, and Avidin-HRP conjugate were then added into each well. Free components were washed, and the substrate solution was added into each well. Stop solution was finally added to terminate enzyme-substrate reaction. The optical density (OD) was read at 450 nm.

Serum 8-hydroxydeoxyguanosine (8-OHdG) was measured using ELISA (Elabsience, China, cat # E-EL-0028). A volume of 50 µL standard or sample were added into 96-well microplate pre-coated with 8-OHdG. Biotinylated Detection Ab was added immediately. Excess conjugate and unbound sample or standard were washed, and the Avidin-HRP conjugate and substrate reagent were added, respectively. Stop solution was finally added to terminate enzyme-substrate reaction. The optical density (OD) was read at 450 nm.

Pregnancy hormones (progesterone, P₄ and estrogen, E₂) were measured using ELISA (Elabsience, China cat # E-EL-0090 and E-EL-0065). Standard or sample, HRP-labeled progesterone or estradiol, and anti-progesterone or anti-estradiol Ab were added into 96-well microplate pre-coated with Goat Anti-Rabbit IgG. The mixture was then washed, and the substrate solution was added into each well. Stop solution was finally added to terminate enzyme-substrate reaction. The optical density (OD) was read at 450 nm.

2.8. Statistical analysis

Data were analysed using an unpaired *t*-test and chi-square. A *P* value of less than 0.05 ($p < 0.05$) was accepted as significantly different. Statistical analyses were conducted using the SPSS version 26.0.

3. Results

3.1. Development of preimplantation embryos

Collectively, 74 embryos were retrieved from both PBS and OVA-treated animals. Treatment of 10 µg/200 µL OVA had caused significant decline in the number of retrieved 2-cell embryo ($p \leq 0.05$). Following *in vitro* culture, the total number of embryos were significantly decreased at 4-cell stage in OVA-treated group ($p \leq 0.05$). Similarly, the number of 8-cell embryos that developed *in vitro* was also reduced in OVA group ($p \leq 0.01$). This decreasing pattern was also true for morula, blastocyst and hatched blastocyst ($p \leq 0.01$). The developmental rate of embryos developed *in vitro* at each developmental stage was also reduced in OVA-treated animals (Table 1).

Table 1
Preimplantation embryonic development in 10 µg/200 µL OVA-induced asthmatic mice.

Embryo development	PBS (n = 6)		10 µg/200 µL OVA-treated animals (n = 6)	
	Mean ± SD	Developmental rate (%)	Mean ± SD	Developmental rate (%)
2-cell (1.5 d.p.c)	8.8 ± 1.64	100.00	6.0 ± 1.58 *	100.00
4-cell (2 d.p.c)	8.6 ± 1.52	97.73	5.6 ± 1.68 *	93.33
8-cell (2.5 d.p.c)	7.8 ± 1.64	90.70	4.6 ± 1.34 **	82.14
Morula (3 d.p.c)	7.4 ± 1.14	94.87	4.2 ± 1.48 **	91.30
Blastocyst (4 d.p.c)	6.6 ± 1.14	89.19	4.0 ± 1.23 **	95.24
Hatched blastocyst (5 d.p.c)	6.4 ± 1.52	96.97	3.4 ± 1.14 **	85.00

** : $p \leq 0.01$ compared to PBS group.

* : $p \leq 0.05$ compared to PBS group, d.p.c: days post coitum (1 d.p.c refers to *in vivo* pronuclear stage, which was not evaluated in this study).

3.2. Morphology of embryos

Table 2 shows the % of embryos with uneven blastomere size and fragmentation at each embryo developmental stage in 10 µg/ 200 µL OVA-induced asthmatic mice. The unevenness in blastomere size was evident at 2-cell stage in the treated group ($p \leq 0.0001$). Similar trend was also apparent at 4- ($p \leq 0.001$) and 8-cell stages ($p \leq 0.05$).

For the degree of fragmentation, ≤ 10 % of fragmentation was notable higher at each developmental stage in OVA-treated group. Significant increment of ≤ 10 % fragmentation was observed at 2- ($p \leq 0.001$), 4- ($p \leq 0.01$) and 8-cell stages ($p \leq 0.05$) in OVA-treated animals. Similar trend was also observed at morula and blastocyst stages, although insignificant. On the other hands, fragmentation was absent in the majority of embryos at each developmental stage in control group.

Figs. 2 and 3 show the morphological appearance in terms of blastomere size and presence of fragmentation. Uneven cell division and irregular size of blastomere with ≤ 10 % fragmentation were consistently observed in 2-, 4- and 8-cell embryos in 10 µg/ 200 µL OVA-treated group ($p \leq 0.05$). Less than or equal to 10 % fragmentation was also observed at morula, blastocyst and hatched blastocyst stages in the treated group although insignificant. On the other hand, equal cell division and regular size of blastomeres with absent of embryonic fragmentation were recorded at all stages in the control group.

3.3. Serum IL-4, Ig-E, 8-OHdG, P₄ and E₂ following 10 µg/200 µL OVA-induced asthma

Serum IL-4, Ig-E and 8-OHdG were notably higher in the treated compared to control group although insignificant (Fig. 4). Similar trend was also observed in P₄ and E₂ levels (Fig. 5)

4. Discussion

Data reporting associating ovalbumin (OVA)-induced maternal asthma and preimplantation embryonic development at present is still not well determined. In this study, OVA, an allergen that induces asthma was administered into healthy BALB/c mice. Asthma induction protocol was conducted based on Paik et al. (2014) with modification. We have employed the OVA sensitization dose of 10 µg/200 µL to our 4–5 weeks old female mice. In addition, a superovulation procedure was also introduced in this work. These had justified the selection of our dose in order to avoid unnecessary duress thus the probability for unsuccessful mating. The impact of OVA on inflammatory markers, oxidative stress (OS) and pregnancy hormones in the dams, as well as the growth of preimplantation embryonic development was subsequently recorded.

Following asthma induction using 10 µg/200 µL OVA, the total retrieved 2-cell embryos were significantly reduced (Table 1). The number of embryos at each developmental stage in this group was also

decreased (Table 1). Physiologically, a healthy female mouse may release between 8 and 10 oocytes in both ovaries during an estrous cycle with an average of 5.2 pups being delivered (Vaseghi et al., 2017). Our finding confirms embryonic deterioration effect of OVA in which not only the recovered 2-cells was reduced in number, but also the total hatched blastocysts. Reduced number of hatched blastocysts may subsequently cause low implantation rate leading to reduced parturition outcome.

Various other exogenous stressors such as nicotine (Kamsani et al., 2010), bisphenol A (Ferris et al., 2015) and pyrethroid (Babeřová et al., 2019) were also reported to produce similar impairment effect via OS mechanism. OS is defined as an imbalance level between reactive oxygen species (ROS) and endogenous antioxidants. ROS, free radicals with oxygen (O₂) molecules and uneven number of electrons, causes reactivity with lipid, protein and nucleic acid by scavenging their electrons causing oxidation (Pizzino et al., 2017). Uncontrolled lipid peroxidation diminishes cell membrane integrity (Gaschler and Stockwell, 2017) and cell mitochondria function due to poor mitochondrial distribution into the differentiated cells, resulting in low adenosine triphosphate (ATP) generation and utilization (Nor-Shahida et al., 2018).

We suggest a possibility that the reduced number of embryos observed in this study was due to OS as a result of OVA-induced asthma in our animals. Although studies concerning OVA on preimplantation embryonic development are less in literature, recent study has reported high ROS and malondialdehyde (MDA) levels in the lung cells of OVA-treated BALB/c mice, suggesting manifestation of OS in the maternal airways (Cui et al., 2019). Besides, the raised level of protein OS marker 3-nitrotyrosine (NT) in the lung tissues of mice treated with 20 µg OVA was also available (Wang et al., 2018). Further elevation in terms of lipid and DNA OS biomarkers of 8-isoprostene and 8-hydroxydeoxyguanosine (OHdG) in bronchoalveolar lavage fluid (BALF) of female BALB/c mice treated with 20 µg OVA had also corroborated OS in the respiratory airways (Wang et al., 2018). As our study focused on the growth of preimplantation embryos, we were interested to highlight nuclear- and mitochondrial-DNA damage using 8-OHdG as the specific biomarker, given the embryo's energy utilization and cell regulation are closely associated with mitochondria and nuclear functions.

Our findings show that the quality of 2-, 4-, 8-cell, morula, blastocyst and hatched blastocyst were affected in OVA-treated group (Table 2, Figs. 2 and 3). Embryos utilize energy generated from mitochondria to sustain their development. Thus equal division of mitochondria into each blastomere during cleavage division would ensure sufficient ATP important for embryonic growth (Nor-Shahida et al., 2018). Even though it is not yet certain that OVA may trigger OS leading to compromised embryo quality, a study reported previously confirms that 20 µg OVA had caused the disruption of cristae and swelling of mitochondria leading to cell dysfunction in female BALB/c mice (Cui et al., 2019). It is probable that OVA-induced asthma could also cause OS and disturb cell homeostasis, leading to compromised cell mitochondrial integrity and subsequent impaired growth of embryos as reported in this study.

Fragmentation was observed at 2-, 4- and 8-cell developmental stages in this study (Table 2, Figs. 2 and 3). This was probably due to the more distinguished anucleated structures and cellular debris in the embryos associated with less number of blastomeres; compared to morulae and blastocysts with multiplied number of blastomeres making those embryos more compacted. On the other hand, fragmentation may also occur if the organelles such as cytoplasm, mitochondria, mRNA and regulatory proteins not been well distributed during cleavage. Imbalance organelle distribution was documented to result in the formation of cellular debris, also known as anucleated structure (Kim S.-G. et al., 2018). Currently, how fragmentation impairs embryonic development is still less clear. However, cell fragmentation has been used as an indicator to unequal organelle distribution into new divided cells. Based on Saiz et al. (2018), the quality of embryo with high degree of fragmentation is poor and commonly being graded as B or C. A study by Bakri et al.

Table 2

Developed embryos with morphological defects in 10 µg/200 µL OVA-induced asthmatic mice.

Developmental stage	Embryo with uneven blastomere size (%)		Embryo with ≤ 10 % fragmentation (%)	
	PBS	10 µg/200 µL OVA-treated animals	PBS	10 µg/200 µL OVA-treated animals
2-cell	4.55	43.33 ****	4.55	40.00 ***
4-cell	4.65	39.29 ***	4.76	35.71 **
8-cell	5.13	26.09 *	5.13	26.09 *
Morula	–	–	2.70	19.05 ^{ns}
Blastocyst	–	–	3.03	20.00 ^{ns}

**** : $p \leq 0.0001$ compared to PBS group.

*** : $p \leq 0.001$ compared to PBS group.

** : $p \leq 0.01$ compared to PBS group.

* : $p \leq 0.05$ compared to PBS group.

^{ns} : non-significant.

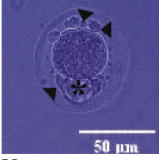
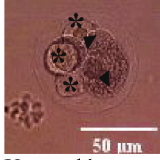
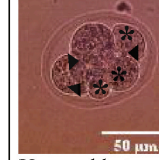
Morphology of embryos	2-cell	4-cell	8-cell
PBS	 Even blastomere size, with absent fragmentation	 Even blastomere size, with absent fragmentation	 Even blastomere size, with absent fragmentation
10µg / 200µL OVA-treated animals	 Uneven blastomere size, with ≤ 10 % fragmentation	 Uneven blastomere size, with ≤ 10 % fragmentation	 Uneven blastomere size, with ≤ 10 % fragmentation

Fig. 2. Morphology of 2-, 4- and 8-cell embryos in 10 µg/200 µL OVA-induced asthmatic mice. Images viewed under an inverted microscope at 40× magnification. □ Uneven size of blastomere. ▲ Fragmentation of embryos. PBS: Phosphate buffered saline. OVA: ovalbumin. Scale bar: 50 µm.

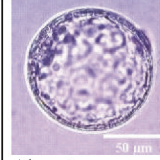
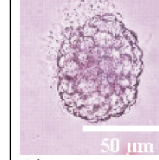
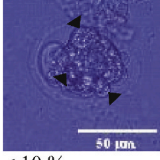
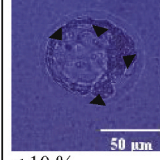
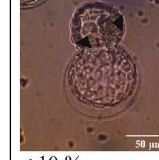
Morphology of embryos	Morula	Blastocyst	Hatched Blastocyst
PBS	 Absent fragmentation	 Absent fragmentation	 Absent fragmentation
10µg / 200µL OVA-treated animals	 ≤ 10 % fragmentation	 ≤ 10 % fragmentation	 ≤ 10 % fragmentation

Fig. 3. Morphology of morula, blastocyst and hatched blastocyst in 10 µg/200 µL OVA-induced asthmatic mice. Images viewed under an inverted microscope at 40× magnification.▲ Fragmentation of embryos. PBS: Phosphate buffered saline. OVA: ovalbumin. Scale bar: 50 µm.

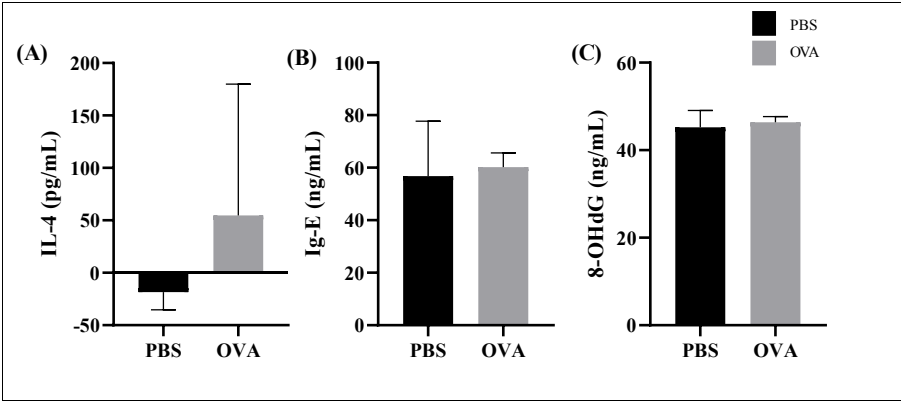


Fig. 4. Maternal serum IL-4 (A), Ig-E (B) and 8-OHdG (C) in 10 µg/200 µL OVA-induced asthmatic mice. Data is presented as mean ± SD. IL-4: Interleukin-4. Ig-E: Immunoglobulin-E. 8-OHdG: 8-hydroxydeoxyguanosine. PBS: Phosphate buffered saline. OVA: 10 µg/ 200 µL ovalbumin.

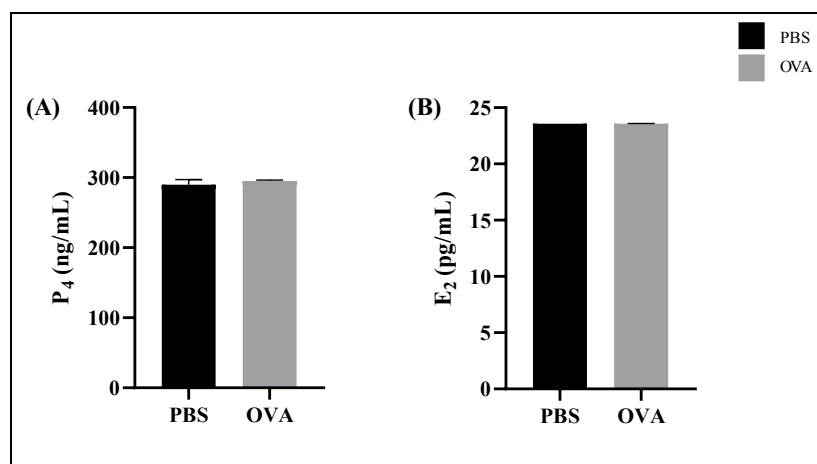


Fig. 5. Maternal serum P₄ (A) and E₂ (B) in 10 µg/200 µL OVA-induced asthmatic mice. Data is presented as mean ± SD. P₄: Progesterone. E₂: Estrogen. PBS: Phosphate buffered saline. OVA: 10 µg/ 200 µL ovalbumin.

(2016) had reported higher apoptosis probability in grade B and C as opposed to grade A cattle embryos. Therefore, our observation of embryonic fragmentation along with decreased embryo survivability in OVA-induced asthmatic mice could be due to cell apoptosis or other forms of programmed cell death, and this warrants further elucidation.

Inflammatory process is associated with the overproduction of free radicals, diminished antioxidant levels and OS. This condition is often characterized by active inflammatory response and tissue repair/destruction. The roles of inflammation in expressing aging processes (Sanada et al., 2018), cardiovascular diseases (Zhang et al., 2017), diabetes (Yuan et al., 2019) and autoimmune diseases (Escárcega et al., 2018) are available. In this study, maternal serum IL-4, Ig-E and 8-OHdG levels were increased, although the differences were not significant (Fig. 4). This is in contrast to Lee et al. (2019) whom had reported increased bronchoalveolar lavage fluid (BALF) and splenocyte IL-4, IL-5 and IL-13 in male BALB/c mice treated with 12.5 µg OVA. In allergic asthma, airway inflammation and the activation of Th 2 immune response have been the most common features when characterizing intracellular changes (Kim D.I et al., 2019; Kim S.-B. et al., 2019). Activation of Th 2 response leads to infiltration of inflammatory cells into the respiratory tree (Zainal et al., 2019). Thus, we postulate that 10 µg/200 µL OVA could probably have caused significant changes in BALF inflammatory cytokine levels rather than maternal serum IL-4, Ig-E and 8-OHdG. This may be the reason for the non-significant differences seen in the serum of our animals.

At 4–5 weeks old, 25–30 g of body mass is the range of weight for healthy, young, unmated female animals (Corre et al., 2017). Hence, the weight range was considered in our animal screening. Anatomically, organs of an adult mice within the same range of age are almost similar in terms of their structure, weight and function. However, other factors such as bioavailability, metabolism, clearance and absorption of OVA may also contribute to our findings. Physiologically, intraperitoneal injection (i.p) of OVA mixture will be absorbed by interstitial lymphatics and blood capillaries into the peritoneum, respectively. While one-half of the OVA mixture will be absorbed into the circulation. Thus, the effect of body mass on the bioavailability of OVA was insignificant in this study as one-half of the OVA mixture will be absorbed into circulation regardless of weight. It is clear that 10 µg/200 µL OVA is detrimental to embryonic development although not to the pathophysiological level. We suggest further OVA dose optimization study to comprehensively elaborate the results.

Our findings show slightly higher serum progesterone (P₄) and estrogen (E₂) levels in the treated group, although the difference was again not significant. Following fertilization, P₄ stimulates endometrium glands to nourish embryos, and establish circulation as well as nutrient

transport between mother-embryos. Alteration of both P₄ and E₂ causes imbalance in their levels leading to impaired embryonic growth. Although the effects of 10 µg/200 µL OVA on P₄ and E₂ were insignificant (Fig. 5), but strikingly the allergen's impact on embryonic growth was evident. This indicates that the role of OVA as an allergen that induces asthma, on maternal inflammatory and OS responses, P₄-E₂ ratio as well as embryonic growth may be dose-dependent. The possibility for OVA to mediate gestational outcome also requires proper elucidation. Likewise, whether OVA could also be associated with infertility in animal models with allergic airway inflammation, targeting OS as the responsible mechanism, is not yet known.

The Toxic Dose Low (TD_{Lo}) and Lethal Dose Low (LD_{Lo}), two principals that determine both toxic and lethal doses (Tsatsakis et al., 2018) are less described in OVA-associated allergic airway inflammation in mouse-embryo model. A systematic OVA dose-response-toxicity study is essential to be conducted to demonstrate its bioavailability, absorption and clearance. The route of entry, treatment duration and frequency of toxicity exposure will further recapitulate the role of OVA. This study shows that 10 µg/200 µL OVA produced detrimental effects on both quantity and quality of embryos (Tables 1 and 2; and Figs. 2 and 3). More apparent effects consistent with toxicity might be probable in a higher OVA concentration, and this needs further elaboration.

5. Conclusion

Ten µg/200 µL OVA impaired both quantity and quality of the developing preimplantation embryos in asthma mouse model. Inflammation, OS and appropriate levels of pregnancy hormones play important roles in the exacerbation of allergen-induced asthma. Further works on OVA dose optimization, OS and inflammatory biomarkers and associated functions of the organelles will provide greater insights into the current knowledge.

Declaration of Competing Interest

None.

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