

Inflammation and Oxidative stress Impair Preimplantation Embryonic Morphogenesis in Allergic Asthma Model

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ABSTRACT

The effect of oxidative stress (OS) on embryonic development in allergic asthma model is under reported. Here, we investigated the impact of allergic asthma on the development of preimplantation embryos. Female BALB/c were subjected to ovalbumin (OVA) challenge. At the end of the treatment, 2-cell embryos were retrieved then cultured *in vitro* until the blastocysts hatched. Results showed reduced number of preimplantation embryos at all developing stages in all treated groups ($p \leq 0.0001$). Uneven blastomere size, partial compaction- and cavitation-activity, low formation of trophectoderm, as well as presence of fragmentation were noted in all treated groups. Maternal serum interleukin (IL)-4, immunoglobulin (Ig)-E and 8-hydroxydeoxyguanosine (8-OHdG) were notably high ($p \leq 0.0001$, $p \leq 0.01$) in contrast with low total antioxidant capacity (TAOC) ($p \leq 0.0001$). Our findings indicated that OVA-induced allergic asthma had compromised both quality and quantity of preimplantation embryos leading to embryonic cell death via OS mechanism.

KEYWORDS: allergic asthma, oxidative stress, ovalbumin, preimplantation embryos

INTRODUCTION

Allergic asthma is an inflammatory airway disease associated with T-helper type 2 (Th2) immune response. Current studies have reported various effects of ovalbumin (OVA), an allergic asthma inducer commonly used in laboratory model of asthma, on Th2 response, particularly on lung pathophysiological parameters. Sensitization of 20- and 100- μ g OVA had raised bronchoalveolar lavage fluid (BALF) Th2 cytokines and increased inflammatory cells infiltration into BALF or lung tissue in mice (Kim, Song and Lee, 2019; Ou *et al.*, 2021; Zhang *et al.*, 2021). On the other hand, reactive oxygen species (ROS) was reported to elevate following airway inflammation in allergic asthma models (Hajimohammadi *et al.*, 2020; Huang *et al.*, 2021). The findings suggest an association between inflammatory reaction and OS in allergen-induced asthmatic conditions, possibly leading to oxidation and cell death (Lushchak and Storey, 2021).

The development of preimplantation embryo involves distinct metabolic phases, protein synthesis, energy expenditure and amino acid uptake as it develops from fertilized zygote to blastocyst. Following fertilization, a zygote undergoes a series of cleavage division, where blastomere number determines the developmental stages of 2-, 4-, 8- or 16-cell. Across these stages, embryo quality can be graded based on blastomere number and cell size. Cleavage division promotes the embryos to become compacted, termed morula; and the changes are defined as compaction stage. For an embryo to undergo cell differentiation, the morula polarizes resulting in influx of fluid into blastocoel cavity concurrent with cavitation process where blastocoel cavity is formed (Kaneko, 2016). Cell differentiation then takes place to produce 2 different cells which are trophoblast (TE) and inner cell mass (ICM).

Preimplantation embryonic development is a vulnerable period of growth as it is susceptible to various endogenous and exogenous insults, such as oxidative stress (OS). OS arises when cellular antioxidant system is incapable to cancel the excess ROS leading to overabundance of superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) among others (Lushchak and Storey, 2021). Excessive ROS causes detrimental effects to cellular membranes, lipids, proteins including deoxyribonucleic acid (DNA) via electron scavenging (Pizzino *et al.*, 2017) with cell death or developmental cessation often reported as the ultimate negative outcome.

During preimplantation embryonic development, programmed cell senescence does not only contribute to embryo development in addition to cell proliferation, but also may indicate developmental cessation and failure. Cellular senescence in the maternal airway may contribute to poor embryo viability in the context of sequelae of allergic asthma. Thus, data particularly on the crosslink between maternal inflammatory-OS response and preimplantation embryonic development in studying allergic asthma, warrants to be elucidated.

This study hypothesized that OVA-induced allergic asthma may increase uneven blastomere cleavage division, partial compaction- and cavitation-activity, impairment of TE production, and fragmentation in preimplantation embryos. To test the hypothesis, 50-, 100- and 150- μ g/ 200 μ L OVA were challenged on female mice. Serum interleukin (IL)-4, immunoglobulin (Ig)-E, 8-hydroxydeoxyguanosine (8-OHdG) and total antioxidant capacity (TAOC) were measured, and *in vitro* embryonic development was evaluated. Results illustrated the effects of OVA-induced allergic asthma on embryo stage-specific characteristics, compaction- and cavitation-activity, degeneration of TE, as well as presence of fragmentation with OS as the prime mechanism. The stage-specific features are useful in determining the survivability of preimplantation embryos under this condition.

MATERIALS AND METHODS

Animal preparation

Animal care and experimental protocols involved were approved and conducted in accordance to the guidelines by Committee on Animal Research and Ethics (CARE), 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). Twenty-four female BALB/c mice aged 5 weeks-old weighed 20 – 25 grams were purchased from Laboratory Animal Facility and Management (LAFAM), Universiti Teknologi MARA, Puncak Alam Campus. Upon acclimatization, mice were randomly divided into 4 groups (n=6): a) PBS: phosphate buffer saline (control), b) OVA1: 50 μ g/200 μ L ovalbumin c) OVA2:100 μ g/200 μ L ovalbumin and d) OVA3: 150 μ g/200 μ L ovalbumin.

Allergic asthma model

Allergic asthma induction protocol was carried out based on methods by Paik *et al.*, (2014), with modification. OVA (Sigma-aldrich, USA) was used as an allergen that mimics allergic asthma while aluminium hydroxide [Al (OH)₃] (Sigma-aldrich, USA) was used as an adjuvant. Treated animals were sensitized with 50- or 100- or 150- µg OVA and 2 mg [Al (OH)₃] in PBS through intraperitoneal injection (i.p) before intra nasal instillation (i.n) of 75 µg OVA mixed in PBS. All animals were anesthetized by administering (i.p) a combination of 100 mg/mL ketamine: 20 mg/mL xylazine prior to the challenge protocol. Control animals underwent the same procedure by receiving PBS alone. Mch test was then conducted to confirm asthma by assessing the hypersensitivity of airways.

Superovulation and mating

Superovulation was carried out by administering (i.p) 0.1 mL of 5 IU pregnant mare serum gonadotropin (PMSG) (Merck Animal Health, Canada) on Day-21 followed by 0.1 mL 5 IU human chorionic gonadotropin (hCG) (Merck Animal Health, Canada) on Day-23 (Figure 1.0). 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. The day of successful mating was indicated as 0-day post coitum (d.p.c).

Serum collection

On Day-25 or 1.5-d.p.c, animals were euthanized through terminal cardiac puncture. 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 for Enzyme-linked Immunosorbent Assay (ELISA). Laparotomy was conducted to excise oviducts for 2-cell embryos retrieval. Timeline of the treatment is provided in Figure 1.0.

Two-cell embryo retrieval and *in vitro* culture

Two-cell embryos were flushed out of the oviducts by expiring approximately 0.1 mL M2 medium (Sigma-aldrich, USA) 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 into droplets of M2 medium before being cultured in a 35-mm culture dish containing pre-prepared 100 µL droplets of M16 medium (Sigma-aldrich, USA), overlaid with paraffin oil (Irvine

Scientific, USA). The embryos were then incubated in a humidified incubator (Binder, CB 170) at 37 °C (5 % CO₂) until Day-29 (5-d.p.c) where all cultured embryos were expected to hatch.

Assessment of preimplantation embryonic development

Embryo quality was determined by their morphology at each stage. The morphology was assessed under an inverted microscope (Leica DM IRB, BZ:05) connected to the PC software (PixeLINK) according to the ASEBIR's morphological scoring system (Saiz *et al.*, 2018). The embryos were categorized as normal or abnormal. The criteria for the embryo assessment were summarized in Table 1.0.

Table 1.0
Stage assessment and assessment time adopted from Saiz *et al.*, (2018).

Embryo stage	Assessment time (d.p.c)	Criteria
2-cell	1.5	• Size of blastomeres • Degree of fragmentation
4-cell	2.0	
8-cell	2.5	
Morula	3.0	• Blastomere compaction
Blastocyst	4.0	• Degree of expansion of the blastocoel • Characteristics of trophectoderm (TE)

Enzyme-linked immunosorbent assay (ELISA)

Serum interleukin (IL)-4, immunoglobulin (Ig)-E and 8-hydroxydeoxyguanosine (8-OHdG) were measured using ELISA (Elabscience, China, cat # E-EL-M0043, E-EL-M0691 and E-EL-0028), respectively. Briefly, a volume of 100- or 50-μL standard or sample were added into each respected 96-well microplate, respectively. Then, Biotinylated Detection and Avidin-HRP conjugate were then added following the respected protocols. Free components, excess conjugate and unbound sample or standard were washed, and the substrate solution was added into each respected microplate. Stop solution was finally added to terminate enzyme-substrate reaction. The optical density (OD) was read at 450 nm with microplate reader (Victor X5 Perkin Elmer). Serum total antioxidant capacity (TAOC) was measured using ELISA (Elabscience, China, cat # E-BC-K225-M). Briefly, a volume of 5 μL of each standard concentration was added into

different wells, respectively. 5 μ L of samples were then added into different wells. Next, 180 μ L of FRAP working solution was added into each well and finally the OD values of each well was read at 595 nm.

Statistical analysis

Data were analysed using a one-way analysis of variance (ANOVA) test or chi-square. 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.

RESULTS

Allergic asthma aggravated airway inflammation and oxidative stress

Increased level of IL-4 and Ig-E in all OVA groups indicated inflammatory response in mice (Figures 1.0 A & B). Results also indicated that the elevation in inflammation markers was dose dependant. The highest OVA dose (150 μ g/200 μ L) recorded the highest level of both IL-4 (139.0 ± 6.86 pg/mL, $p \leq 0.0001$) and Ig-E (2083.0 ± 338.9 ng/mL, $p \leq 0.0001$), respectively. The elevation of inflammation was followed by 100- and 50- μ g/200 μ L OVA dose, respectively ($p \leq 0.0001$).

In terms of the OS status, elevation of serum 8-OHdG concurrent with decreased serum TAOC indicated OS. The 8-OHdG level was increased in all OVA groups (Figure 1.0 C) However, there was no significance difference observed in the lowest dose (9.344 ± 0.95 , $p > 0.05$) in comparison to the control group. In 100 μ g/200 μ L group, the 8-OHdG level was higher than the lowest dose and control group (47.38 ± 8.29 , $p \leq 0.01$). The highest dose (150 μ g/200 μ L) recorded the highest 8-OHdG level (86.46 ± 34.09 , $p \leq 0.0001$). Concurrently, the TAOC level in all OVA groups was decreased compared to control and the effect was also dose dependent (Figure 1.0 D). The lowest TAOC status (0.63 ± 0.082 , $p \leq 0.0001$) was observed in the highest dose (150 μ g/200 μ L) followed by 100- and 50- μ g/200 μ L (1.45 ± 0.051 and 2.32 ± 0.30 , $p \leq 0.0001$), respectively.

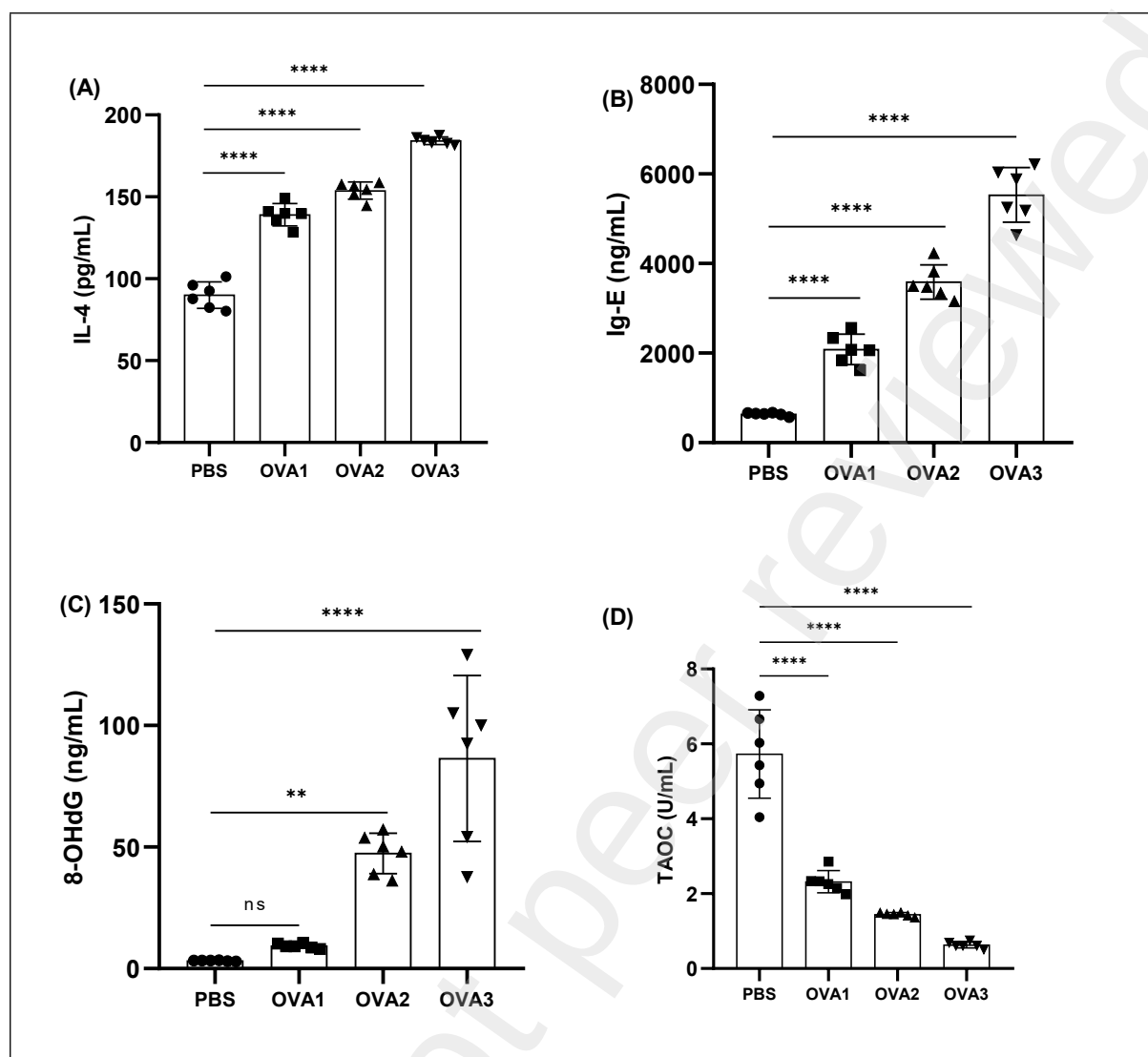


Figure 1.0 Inflammation and OS aggravated in OVA-induced asthma mouse. (A) Interleukin (IL)-4, (B) Immunoglobulin (Ig)-E, (C) 8-hydroxyguanosine (8-OHdG) (D) Total antioxidant capacity (TAOC). ● PBS, ■ OVA1: 50 $\mu\text{g}/200\mu\text{L}$, ▲ OVA2: 100 $\mu\text{g}/200\mu\text{L}$, ▼ OVA3: 150 $\mu\text{g}/200\mu\text{L}$. Data is presented as mean $\pm$ s.d and individual value was illustrated. **** $p \leq 0.0001$, ** $p \leq 0.01$, ns: non-significant

Low number of retrieved 2-cell embryo in allergic asthma groups

Control group recorded 25.0 ± 3.03 ($N = 150$) of the retrieved 2-cell embryos. The mean number of re

Declined number of developing preimplantation embryos in allergic asthma groups

Out of 25.0 ± 3.03 ($N = 150$) cultured 2-cell embryos, 22.8 ± 3.77 ($N = 137$) were developed into 4-cell embryos at 2.0 d.p.c in control group. For treated groups, the number of 4-cell embryos decreased in dose-dependent manner. In $50 \mu\text{g}/200\mu\text{L}$ dose, 10.8 ± 2.32 , $N = 65$ ($p \leq 0.0001$) embryos developed to become 4-cell embryos whereas 7.7 ± 2.07 , $N=46$ ($p \leq 0.0001$) and 1.2 ± 1.47 , $N = 7$ ($p \leq 0.0001$) embryos were developed in 100- and $150\text{-}\mu\text{g}/200\mu\text{L}$ dose, respectively. At 2.5 d.p.c, 21.0 ± 3.69 ($N = 126$) of embryos developed to become 8-cell embryo in control group. In the highest OVA dose ($150 \mu\text{g}/200\mu\text{L}$), huge decrease was observed where only 0.3 ± 0.52 ($N = 2$) of embryos developed to become 8-cell embryos ($p \leq 0.0001$). The other OVA doses (100- and $50\text{-}\mu\text{g}/200\mu\text{L}$) also recorded low number of 8-cell embryos which were 6.0 ± 1.41 , $N = 36$ ($p \leq 0.0001$) and 8.5 ± 2.43 , $N = 51$ ($p \leq 0.0001$), respectively (Table 2.0).

At 3.0 d.p.c, the control group recorded 19.2 ± 3.97 ($N = 115$) morula. The number of morulae in 50- and $100\text{-}\mu\text{g}/200\mu\text{L}$ OVA was decreased where 7.8 ± 2.48 ($N = 47$) and 5.5 ± 1.87 ($N = 33$) morula was recorded ($p \leq 0.0001$), respectively. In $150 \mu\text{g}/200\mu\text{L}$ OVA, the number of morulae was 0.3 ± 0.52 ($N = 2$) ($p \leq 0.0001$). Following 4.0 d.p.c, 18.7 ± 3.33 ($N = 112$) embryos from control group had become blastocyst. In the lowest OVA dose ($50 \mu\text{g}/200\mu\text{L}$), 7.2 ± 2.04 ($N = 43$) embryos and 4.0 ± 0.89 ($N = 24$) embryos in $100 \mu\text{g}/200\mu\text{L}$ OVA had become blastocyst ($p \leq 0.0001$). However, only 0.2 ± 0.41 ($N = 1$) blastocyst was recorded in the highest OVA dose ($100 \mu\text{g}/200\mu\text{L}$) ($p \leq 0.0001$) (Table 2.0).

Table 2.0
Number of developing preimplantation embryos

Developmental stage	PBS (n=6)	OVA ($\mu\text{g}/200\mu\text{L}$) (n=6)		
		OVA1 (50 μg)	OVA2 (100 μg)	OVA3 (150 μg)
2-cell (1.5-d.p.c)	25.0 ± 3.03 ($N = 150$)	12.8 ± 2.14^a ($N = 77$)	8.2 ± 1.60^a ($N = 49$)	2.7 ± 2.88^a ($N = 16$)
4-cell (2.0-d.p.c)	22.8 ± 3.77 ($N = 137$)	10.8 ± 2.32^a ($N = 65$)	7.7 ± 2.07^a ($N = 46$)	1.2 ± 1.47^a ($N = 7$)

8-cell (2.5-d.p.c)	21.0 ± 3.69 (N = 126)	8.5 ± 2.43 ^a (N = 51)	6.0 ± 1.41 ^a (N = 36)	0.3 ± 0.52 ^a (N = 2)
Morula (3.0-d.p.c)	19.2 ± 3.97 (N = 115)	7.8 ± 2.48 ^a (N = 47)	5.5 ± 1.87 ^a (N = 33)	0.3 ± 0.52 ^a (N = 2)
Blastocyst (4.0-d.p.c)	18.7 ± 3.33 (N = 112)	7.2 ± 2.04 ^a (N = 43)	4.0 ± 0.89 ^a (N = 24)	0.2 ± 0.41 ^a (N = 1)

Poor cell cleavage division in allergic asthma groups

Following embryo retrieval, the morphology of the embryos and cultured 4- and 8-cell embryos was observed under inverted microscope through assessing blastomere and the presence of fragmentation (Table 3.0). The control group recorded 90.7 % possessed even sized blastomere and low fragmentation (≤ 10 %), indicating normal morphology. However, the morphology of majority of the embryos from all OVA groups were observed with uneven blastomere size and presence of > 10 % fragmentation specifically in the 150 $\mu\text{g}/200\mu\text{L}$ OVA group.

Incomplete morula compaction in allergic asthma groups

The majority of morula in all OVA groups was observed with abnormal morphology due to cells underwent incomplete compaction. The abnormal morula morphology in all OVA groups were observed with distinguished blastomere cells (Table 3.0).

Poor Blastocyst formation in allergic asthma groups

The majority of blastocysts (93.7 %) from control group showed homogenous, cohesive and many number of trophectoderm (TE) cells. However, in OVA groups, majority of the blastocysts observed was of less homogenous with a smaller number of TE cells. In addition, the blastocoel cavity of blastocyst in control group was observed bigger compared to OVA groups (Table 3.0).

Hatched blastocyst rate decreased in allergic asthma groups

To determine the developmental potential of the preimplantation embryos, the developmental rate was calculated (Figure 2.0). In the control group, the developmental rate was 71.33 % where out of 150 embryos, 107 embryos developed into hatched blastocyst stage. The developmental rate in all OVA groups was decreased compared to control group. In 50- and 100- $\mu\text{g}/200\mu\text{L}$ OVA, 46.75 % (36 out 77 embryos) and 48.98 %

(24 out of 49 embryos) were developed into hatched blastocyst stage, respectively. The lowest developmental rate was observed in the highest OVA dose (150 $\mu\text{g}/200\mu\text{L}$) which was 6.25 % (1 out of 16 embryos developed into hatched blastocyst).

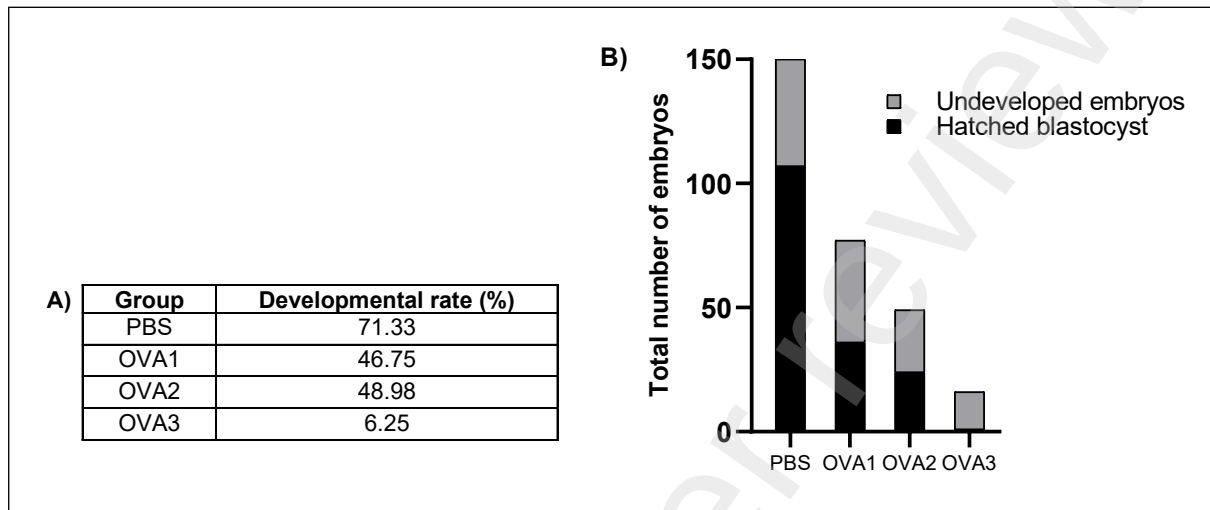
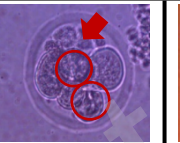
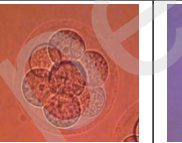
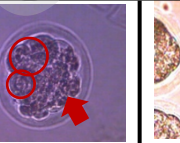
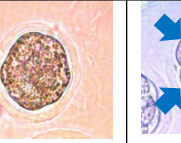
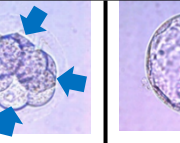
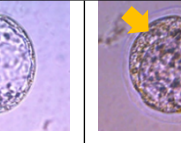


Figure 2.0 The developmental potential in preimplantation embryos. **A)** Developmental rate. **B)** Total number of hatched blastocysts. PBS: control group, OVA1: 50 $\mu\text{g}/200\mu\text{L}$, OVA2: 100 $\mu\text{g}/200\mu\text{L}$, OVA3: 150 $\mu\text{g}/200\mu\text{L}$.

Table 3.0
The morphology of preimplantation embryos at each developmental stage.

	2-cell Total number (%)		4-cell Total number (%)		8-cell Total number (%)		Morula Total number (%)		Blastocyst Total number (%)	
	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
PBS	136 (90.7)	14 (9.3)	118 (86.1)	19 (13.9)	115 (91.3)	11 (8.7)	107 (93.0)	8 (0.7)	105 (93.7)	7 (6.3)
OVA1	43 (55.9)	44.2 (7.8)	20 (30.8)	45 (69.2)	9 (17.6)	42 (82.4)	7 (14.9)	40 (85.1)	6 (14.0)	37 (86.0)
OVA2	11 (22.4)	38 (77.6)	2 (4.3)	44 (95.7)	2 (5.6)	34 (94.4)	2 (6.3)	30 (93.7)	2 (8.3)	22 (91.7)
OVA3	0 (0.0)	16 (100.0)	0 (0.0)	7 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)	2 (100.0)	0 (0.0)	1 (100.0)
										
	Embryo with 2 even sized blastomeres and presence of ≤10% fragmentation						Morula showing fully compaction. No blastomere can be distinguished		TE cells are homogenous, cohesive and many. Bigger blastocoel cavity	
	Embryo with 2 uneven-sized blastomeres and presence of >10% fragmentation						Morula showing partial compaction. Blastomeres can be distinguished		TE cells are less homogenous and fewer. Smaller blastocoel cavity	
	Embryo with 4 even sized blastomeres and presence of ≤10% fragmentation						* Circle indicates uneven-sized blastomere. Arrow indicates fragmentation		* Arrow indicates uncompacted blastomere	
	Embryo with 4 uneven-sized blastomeres and presence of >10% fragmentation								* A row indicates less homogenous TE	
	Embryo with 8 even sized blastomeres and presence of ≤10% fragmentation									
	Embryo with 8 uneven-sized blastomeres and presence of >10% fragmentation									

DISCUSSION

Our findings observed that the number of retrieved 2-cell embryos was lower in all allergic asthma groups and their morphology was abnormal (Tables 2.0 & 3.0). Subsequently, the number of 4- and 8-cell embryos was also reduced concurrent with abnormal morphology. In terms of blastomere, allergic asthma groups resulted in uneven blastomere size of cleavage embryos (Table 2.0 & 3.0). Formation of asynchronised and uneven blastomere size was determined by asymmetrical cleavage division.

During cytokinesis, organelles including mitochondria are equally distributed into each new cell (blastomere). Mitochondria involves in adenosine triphosphate (ATP) production necessary to further undergo cleavage division. Unequal mitochondria distribution might cause failure of the cleavage process resulting in uneven blastomere size and formation of 5-, 6- or 7-cell embryo. In addition, oxidative stress (OS) may also cause mitochondrial damage in ovalbumin (OVA)-induced asthma mice. Previous study using 20 µg OVA recorded reduction in ATP level (µmol/g prot) concurrent with mitochondria swelling and loss or disruption of mitochondria cristae in female BALB/c mice (Cui *et al.*, 2019). Thus, we postulate that OVA treatment had induced inflammatory-OS response causing unequal mitochondria distribution resulting in uneven blastomere size in cleavage embryos.

Maternal and paternal factors may contribute to asynchronised and uneven size blastomere. Previous study suggested that paternal factors such as sperm DNA abnormalities induced by OS may block embryonic cleavage division prior to embryonic genome activation (De Castro *et al.*, 2016). In another study, OS-induced actin and spindle defects in porcine oocytes had also resulted in low cleavage embryo rate (Park *et al.*, 2021). Blockage of cleavage division due to paternal and/or maternal factors could subsequently lead to cessation of preimplantation embryonic development due to OS. This is in line with our findings where we observed uneven blastomere size and low embryonic developmental rate in OVA-treated groups concurrent with elevation of OS.

At the morula stage, the blastomeres did not fully compact; instead, they underwent partial compaction (Table 3.0). Presently, there is no study that reported association between allergic asthma-inflammation-OS response with embryo compaction. However, several factors such as abnormal formation of tight junctions or inability to express proteins involved in cell adhesion have been proposed to cause partial compaction (Lagalla *et al.*, 2020). Prior to compaction, each blastomere express their adhesive junctions before increasing intercellular

adhesion through E-cadherin to attach with neighbour blastomeres (Sutherland, 2003; Nance, 2014). E-cadherin (multigene cadherin family) is known as cell–cell adhesion protein with roles in cellular adhesion and polarity maintenance (White *et al.*, 2016; Burandt *et al.*, 2021). Normal regulation of E-cadherin resulted in tightly bounded and indistinguishable morphologically blastomeres, in contrast to diminished cellular adhesion due to its downregulation (Burandt *et al.*, 2021). Diminished lung mRNA E-cadherin in OS model was recently reported (Yamamoto *et al.*, 2021). These findings might suggest that decreased mRNA E-cadherin in the respiratory system can also be reflected in the reproductive system. Thus, we postulate that activation of airway inflammatory-OS response might indirectly alter reproductive physiology via E-cadherin gene and protein downregulations in embryos leading to blastomeres undergoing partial compaction in this study. Confirming the regulation of E-cadherin gene or protein in mouse embryo will further explain the state of partial compaction as reported in this study.

At the blastocyst stage, the expansion of blastocoel cavity and formation of trophectoderm (TE) were deteriorated (Table 3.0). We postulated that it was due to partial compaction of morula. It is reported that partial compacted morula is prone to undergoing developmental arrest, embryonic degeneration, longer blastocyst developmental time and formation of poor blastocyst quality which eventually will impair preimplantation development (Lagalla *et al.*, 2020). Theoretically, apical domain (the surface of blastomeres that do not attach to neighbour blastomere) and basolateral domain (the surface of blastomeres that attach to neighbour blastomere) will be segregated during the late cleavage through asymmetrical division before forming two different daughter cells; TE and inner cell mass (ICM) (Sutherland, 2003; Nance, 2014). If blastomeres are unable to undergo compaction and polarization, it will also affect the formation of TE and ICM. Hence, compaction failure might subsequently alter pathways involved in cell fate-specification such as cell position-dependent Hippo signalling. A study reported that alteration of the signal lead to the defect of TE-fate specification resulted in low formation of TE cells in embryos of wild-type mice (Hirate *et al.*, 2013). Future targeted study particularly on the Hippo signalling during blastocyst formation might elucidate more detailed blastocyst formation in this study model.

Poor blastocyst quality particularly the quality of TE may increase implantation failure (Ohgi *et al.*, 2018). In addition, data on retrospective cohort analysis reported that poor grade of TE resulted in low clinical pregnancy rate (Zhao, Yu and Zhang, 2018), declined live birth rate (Hill *et al.*, 2013) and posed high risk for miscarriage (Shi *et al.*, 2020). To our knowledge,

there is no evidence indicating that allergic asthma may disrupt formation of TE. However, OS condition was proven to resulting in decreased number of TE observed under differential staining (Leite *et al.*, 2018). Thus, decreased TE cell numbers observed in our study may lead to blastocyst formation failure, and this reflects the decreased number of hatched blastocysts (Figure 2.0). More work including blastocyst genes screening will provide insight on the genetic materials involved in the formation of TE.

Throughout the development, we observed the presence of fragmentation at cleavage embryonic stage in all OVA-treated groups (Table 3.0). During early preimplantation embryonic development, organelles such as regulatory proteins, mRNA, cytoplasm, and mitochondria from each blastomere are distributed equally into each new daughter cell (blastomere). Organelles that fail to be distributed into each daughter cell will enter embryonic sub zonal space. Hence, undistributed organelles and embryonic contents remain in embryo cytoplasm forming fragmentation (Kim *et al.*, 2018). It is reported that high degree of fragmentation might not only compromise developmental potential but might also decrease implantation- and pregnancy-rate and increased abortion incidence (Kim *et al.*, 2018). A study by Daughtry *et al.*, (2019) reported that fragmented blastomere was highly likely to cause detrimental aneuploidy. In addition to this information, our findings show that allergic asthma via OS mechanism causes fragmentation at the earliest stage of embryonic development hence impaired the hatched blastocyst rate. Low rate of hatched blastocyst is associated with low rate of implantation subsequently might also alter pregnancy rate. Nonetheless, how OVA-induced allergic asthma is associated with fragmentation in preimplantation embryos is still not available. Nevertheless, a technique to remove fragmentation had been reported to assist embryonic development. A microsurgical fragment removal at day-2 of human embryo had improved implantation- and pregnancy-rate (Kim *et al.*, 2018) suggesting a potential technical procedure to reduce the degree of embryonic fragmentation to assist embryonic development.

Appropriate cytokines levels during pregnancy are crucial (Alvarez *et al.*, 2017). Elevation of maternal cytokines might lead to various female reproductive problems including impairment of preimplantation embryos. A study reports that lipopolysaccharide (LPS)-induced systemic inflammation at zygote stage reduced blastocyst ICM cell number, and ICM: TE cell ratio in mice (Williams *et al.*, 2011) confirming the effect of systemic inflammation on the developing embryos. Our findings of increased serum interleukin (IL)-4 and immunoglobulin (Ig)-E (Figures 1.0 A & B) also indicated systemic inflammation. In asthma, the Th cell production increases (Zarobkiewicz *et al.*, 2021). This may aggregate the nucleation

site of the reproductive tract causing imbalance homeostasis in the reproductive tract (Lee *et al.*, 2015). Our model also suggested some degree of DNA oxidative damage presented by elevated serum 8-hydroxydeoxyguanosine (8-OHdG) and reduced total antioxidant capacity (TAOC) (Figures 1.0 C & D) concurrent with impairment of embryonic development. In embryonic development, DNA oxidation at bases [guanine (G), adenine (A), cytosine (C), and thymine (T)] disrupt cell development and protein synthesis (Singh *et al.*, 2019). Thus, we suggest OS and DNA oxidative damage behind poor preimplantation embryo development recorded in this study.

In conclusion, OVA-induced allergic asthma activated inflammatory-OS response leading to uneven blastomere formation, partial compaction- and cavitation-activity, impairment of TE formation, and presence of fragmentation that consequently impacted the growth of preimplantation embryos. Further analysis such as preimplantation genetic testing (PGT) can be proposed to determine necessary pathways and/or genes associated with reduced embryonic viability in allergen-induced asthma model before intervention measures can be initiated.

DECLARATION OF INTEREST

Declaration of interest: none

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AUTHORS CONTRIBUTION STATEMENT

WCI conducted the experimental procedures, collected, analysed, and interpreted data, and involved in manuscript preparation. YSK planned study design, interpreted data and involved in manuscript revision. N-AMNK planned study design, interpreted data, and involved in manuscript revision. All authors have approved the final version of manuscript.

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