THE EFFECTS OF PROPYLPARABEN ON PREIMPLANTATION EMBRYO DEVELOPMENT

BY

NASTASIA ZHEN EE LAI

THESIS

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Master's Committee:

Professor Romana A. Nowak, Chair and Director of Research
Professor Jodi A. Flaws
Associate Assistant Professor Ayelet Ziv-Gal

ABSTRACT

Parabens are chemicals that have been widely used in personal care products and food as antimicrobial preservatives. They are synthesized as a series of parahydroxybenzoates or esters of parahydroxybenzoic acid. Parabens are known to have potential estrogenic activity as they can bind to both estrogen receptor 1 (ESR1) and -2 (ESR2) and are therefore classified as endocrine disrupting chemicals. With substantial numbers of women consumers exposed to parabens daily, there is a need to investigate the effects of parabens on the female reproductive system. A previous study in a mouse model found that development of the ovaries was impacted by exposure to parabens, with an increase in cystic follicles, decrease in corpora lutea, and thinning of the follicular epithelium. The effects of parabens on embryo development have not been studied extensively. The goal of the present study was to determine the impact of propylparaben exposure on preimplantation embryo development, our specific endpoints were the rate of development to the hatched blastocyst stage; number of inner cell mass and trophectoderm cells; and distribution of cytoskeletal F-actin in cultured mouse embryos. To analyze propylparaben effects on embryo development, concentrations of 0, 0.5μg/mL, 5.0 μg/mL, 10 μg/mL and 15 μg/mL propylparaben were tested. Five- to six-week-old female CD-1 mice were superovulated and placed with B6D2F1 male mice for mating. After 21 hours, the embryos were collected and transferred to either control medium, vehicle control (DMSO 0.075%) or various doses of propylparaben in treatment drops for embryo culture and monitored until the hatched blastocyst stage. Hatched blastocysts were collected and immunofluorescence staining was performed. Primary antibodies OCT-4 and CDX-2 were used to identify inner cell mass (ICM) and trophectoderm (TE) cells respectively. Phalloidin staining was used to identify the F-actin network in the hatched blastocyst. The

percentage hatched blastocysts were significantly decreased at 10 μg/mL (23.40% hatched) and 15μg/mL propylparaben (13.04% hatched) compared to vehicle control (40.76% hatched). Propylparaben treatment had no significant effects on TE number. However, treatment with 0.5 μg/mL and 15μg/mL propylparaben significantly decreased the numbers of ICM cells when compared to vehicle control. The intensity of the phalloidin fluorescence staining was significantly decreased at the 10μg/mL and 15μg/mL propylparaben treatments when compared to vehicle control. In summary, our findings show that propylparaben exposure, at certain doses, disrupts ICM formation, impacts cytoskeletal F-actin network formation and alters hatched blastocyst development rate. These results suggest that exposure of embryos to elevated levels of parabens either *in vivo* or *in vitro* needs to be minimized.

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INTRODUCTION

Hormones are produced by specialized glands and tissues and function as autocrine, paracrine, or endocrine signals to the same cells, nearby cells or circulate in the bloodstream and may act on distant target tissues. This interaction is crucial as it controls several categorical functions: growth and development, reproduction and sexual differentiation, metabolism and maintenance of homeostasis (Nussey and Whitehead, 2001). In particular, the reproductive gonadal axes of both males and females are highly dependent on the various signaling components of the hypothalamic-pituitary gonadal (HPG) axis (Chrousos, 2017). In the environment, there are naturally and synthetically occurring chemicals that have the ability to interfere with hormone signaling in the body of humans and animal species as well. These groups of compounds are known as endocrine disrupting chemicals (EDCs) and their effects were first studied more than 50 years ago. (Kahn *et al*, 2020). Their ability to interfere with endocrine signaling involves various mechanisms including nuclear receptors, nonnuclear steroid hormone receptors, nonsteroid hormone receptors, and metabolic enzymatic pathways that may eventually alter endocrine and reproductive systems (Diamanti-Kandrakis *et al.,* 2009; Lind *et al.*, 2014; Sifakis *et al.*, 2017; Yilmaz *et al.*, 2019). Previous research has shown that EDCs have contributed to the increased prevalence of cancer, diabetes, obesity, metabolic syndrome, and infertility (Swan *et al*, 2000; Fernandez *et al.*, 2011; Bouglogne *et al.*, 2012; Shafei *et al.*, 2018; Tang *et al.,* 2021). In the last decades, there has been a decline in fertility and a higher incidence of reproductive disorders such as breast cancer, endometriosis, reduced birthweight, preterm birth, and leiomyomas (Weng, Y.I *et al.*, 2010; Sobinoff *et al.*¸2010; De Coster and van Larebeke, 2012; Delclos *et al.*, 2014, Gao *et al.*, 2019).

The female reproductive system requires careful coordination of hormone release, follicle development and ovulation, fertilization, implantation, and maintenance of pregnancy for successful procreation of the species (Christensen *et al.*, 2012). Successful implantation depends on a variety of synchronized processes such as structural and functional remodeling of the uterus, secretion of growth factors into the uterine lumen, sex steroid hormone secretion for proper uterine decidualization, and signaling that involves autocrine, paracrine, and endocrine pathways (Guzeloglu-Kayisli *et al.*¸ 2009; Kim, S.K and Kim J.S, 2017). A failure of these processes can lead to early loss of pregnancy and almost 75% of pregnancy losses are due to failure of implantation (Commins-Boo *et al.*, 2016).

Early embryo development is time- and environment- sensitive as it involves epigenetic reprogramming, occurring over a continuous series of cell divisions that include embryonic genome activation between the 4- and 8- cell stage (Niakan *et al.*, 2012). During this time, the embryo continuously divides and develops into a blastocyst, containing approximately 100 cells. This process of preimplantation embryo development takes around 4 to 5 days as the embryo navigates through the oviduct (Ikawa, *et al.*, 2010; Su and Fazleabas, 2015). The early cell divisions of embryo development lead to the establishment of two distinct cell lineages in the blastocyst: the inner cell mass (ICM) and trophectoderm (TE).

As the blastocyst continue to progress, it undergoes morphologic changes that lead to the development of a fluid-filled blastocoele cavity and an ICM that is surrounded by the TE (Niakan *et al.*, 2012). The TE plays an important role in interaction with the maternal endometrium during implantation as well as forming a large part of the placenta (Tocci, 2020). The ICM give rise to the fetus and contains embryonic stem cells that have the potential to differentiate into a variety of cell lineages: ectoderm (epidermis skin cells, neurons), mesoderm (connective tissues) and

endoderm (respiratory tract, gastrointestinal tract) as well as fetal and adult cells (Yamanaka *et al.*, 2008; Yusuke M. and Alarcón V.B, 2009).

The cytoskeleton of cells is a network of proteins that provides cells, including those in embryos, motility, facilitation of cell division, and organization of the nuclear structures (Okuno *et al.*, 2020). One of the most important cytoskeletal proteins is filamentous actin (F-actin), the most abundant protein in most eukaryotic cells (Dominguez and Holmes, 2011). Proper cell division is vital during early embryogenesis and these microfilament proteins regulate this process to ensure organization of nuclear structures and expansion (Baarlink *et al.*, 2017). Timely dissociation of F-actin is vital for proper developmental progression in the zygote. Disruption of F-actin may interfere with progressive embryo development for genomic structure organization and impact nuclear functions, specifically chromatin structures (Okuno *et al.*, 2020). If the embryo fails to complete development under optimal conditions over a specific timeline, it has a lower chance to successfully implant in the uterus and this may lead to a failed pregnancy.

Parabens are esters of parahydroxybenzoic acid (PHBA) and propylparaben is one of the most commonly used antimicrobial preservatives in food, cosmetic products and drugs (Darbre and Harvey, 2008; USDA, 2020). Parabens have been detected and identified in various types of biological samples that include serum, urine and amniotic fluid in women, indicating that women and embryos may be constantly exposed to propylparaben (Hines *et al*, 2015; Martinez *et al.*, 2019; Philippat *et al.*, 2013). Parabens have been found to have weak estrogenic properties as they have the ability to bind to estrogen receptor 1 (ESR1) and estrogen receptor 2 (ESR2) and are therefore classified as EDCs (Okubo *et al*, 2001; Darbre *et al.*, 2004; Charles and Darbre, 2013; Engeli *et al.*, 2017). Previous studies have shown that parabens may have the potential to disrupt the female reproductive system as female rats exposed to parabens showed an increase in cystic follicles,

decrease in corpora lutea, and thinning of the follicular epithelium (Vo *et al.*, 2010). It is well documented that propylparaben is widely used in personal care products and women are one of the largest consumers of these products. With the substantial demographic of women consumers exposed to propylparaben daily and lack of studies, there is a need to investigate the effects of propylparaben on other aspects of reproductive function including embryo development.

Preimplantation development of the embryo is a critical and sensitive period for successful implantation and pregnancy and environmental toxicants circulating in the bloodstream and body can affect the viability and development of the embryo. The overall hypothesis of this work is that propylparaben disrupts early preimplantation embryo development. The specific endpoints investigated in this study were the rate of development to the hatched blastocyst stage, difference in number of ICM and TE cells, presence of micronucleation, and the distribution of cytoskeletal F-actin in mouse embryos in the different treatment groups.

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CHAPTER 1: LITERATURE REVIEW

Endocrine disrupting chemicals

Since the highlighting of the effects of dichlorodiphenyltrichloroethane (DDT) on sexual development and reproduction by Rachel Carson's 'Silent Spring' in 1962, there has been a growing interest in human exposure to endocrine-disrupting chemicals (EDCs) and their effects on human health. With the exponential growth of mass manufacturing and production, there has been an increase in production of synthetic chemicals which are compounds capable of interfering with endogenous hormonal action, as they have the ability to mimic hormones. Studies have suggested that EDCs contribute to human health problems such as obesity, infertility, cancer and metabolic syndrome (De Coster and van Larabeke, 2012; Bourguignon *et al.*, 2016; Gao *et al.*, 2019; Kawa *et al.*, 2021). Humans and other animals are exposed to EDCs via inhalation, dermal uptake, and ingestion. EDCs comprise a wide range of chemicals, which exist either naturally or are synthetically produced. The presence of EDCs is ubiquitous in the environment, and humans and animals are constantly exposed to EDCs on a daily basis.

Several examples of EDCs are industrial solvents/lubricants and their by-products such as (polychlorinated biphenyls (PCBs)), plastics (bisphenol A (BPA), alkylphenols), plasticizers (phthalates), pesticides (methoxychlor (MXC), dichlorodiphenyltrichloroethane (DDT)), preservatives (parabens), polyfluoroalkyl (PFA) substances, and fungicides (vinclozolin) (Chou and Wright, 2006; Jurewicz and Hanke, 2011; Mao *et al.*, 2012; Nohynek *et al.*, 2013, Palioura and Diamanti-Kandarakis, 2016; Yilmaz *et al.*, 2020). EDCs have been shown to have adverse effects on antral follicle growth, cardiovascular diseases, endometriosis, and spermatogenesis dysfunction (Oishi, S., 2002; Giampietri *et al.*¸2005; Gore *et al.*, 2015; Zhou and Flaws, 2017;

Yilmaz *et al.,* 2020). The mechanisms by which EDCs interact and interfere with hormone action may be complex as hormones have different effects at various time points during the life cycle and the timing and duration of EDC exposure may be variable. These are several important factors to consider when studying EDC effects on endocrine systems (Zoeller *et al.*¸2012, Gore *et al*., 2015).

Prenatal exposures to EDCs have indicated that these chemicals increase the risk of health concerns later in life. For example, in both human and animal studies, prenatal exposure to nicotine from cigarette smoking from pregnant females was found to restrict fetal intrauterine growth that led to obesity in later life of the offspring (Das and Sysyn, 2004; Gluckman *et al.*, 2005; Holbrook, 2016). Most EDCs have low water and high lipid solubility which leads to accumulation of these chemicals in fatty adipose tissue that may contribute to the long-term effects that are seen in adulthood (Ünüvar and Büyükgebiz, 2012). Phthalate and phenol prenatal exposure also increased the risk of asthma and allergic diseases in children, suggesting that the immune and respiratory system development were disrupted (Casas and Gascon, 2020). A study was performed on 499 prepubertal boys that were exposed to organochlorine chemicals, lead, and non-dioxin-like-PCBs, where EDC blood concentrations were assessed at ages 8-9 years and annual physical examinations were performed until the age of 18-19 years. At entry, each boy's parent or guardian answered health and lifestyle questions that included birth and medical history, demographic, and socioeconomic status. It was found that EDCs were persistent in collected blood samples from childhood to young adulthood, where PCBs accelerated puberty timing, and organochlorine chemicals lead to delayed puberty development (Sergeyev *et al.,* 2017). These data provide insight that the peripubertal window is sensitive to organochlorine chemicals and lead that disrupt male puberty and growth. Based on scientific literature and given that EDCs are ubiquitous in the

environment, it is imperative to continuously investigate the potential effects on the endocrine system.

Effects of endocrine-disrupting chemicals in the female reproductive system

In the female reproductive system, sex steroid hormones play a vital role in timing of puberty, reproductive cycles, pregnancy, mammary gland development, and lactation (Macias and Hinck, 2012; Cooke et al., 2021). Normal puberty is essential for achieving proper reproductive function. Several studies have shown that EDCs can affect the onset of pubertal timing and could be an indicator of future fertility issues (Sergeyev *et al.*, 2017; López-Rodriguez *et al*, 2021; Spaziani *et al.*, 2021). Researchers have reported that earlier breast development in girls, disruption of neuroendocrine sexual maturation and hypothalamic control of sex hormone secretion are associated with higher EDC exposure (Aksglaede *et al.*, 2009; Parent *et al.*¸2015; Franssen *et al*., 2016; López-Rodriguez *et al*., 2019).

There have been several studies that have investigated the effects of EDCs on the female reproductive system. Urinary samples with higher BPA concentrations were reported to be associated with decreased blastocyst formation and number of normally fertilized oocytes (Ehrlich *et al.*, 2012). Bovine oocytes that were cultured in phthalates had a decrease in developmental competence of oocytes that cleave to 2- to 4-cell stage embryos and had altered gene expression associated with embryonic pluripotency and DNA methylation which suggests that phthalates may impact oocyte maturation and early embryonic development leading to low-quality embryos (Kalo and Roth, 2017). BPA, phthalates, pesticides and diethylstilbesterol (DES) are some examples of EDCs that have been linked to premature ovarian failure and early menopause (Hoover *et al*, 2011; Souter *et al*, 2013; Grindler *et al.*, 2015). Hormone-sensitive cancers of endometrium, ovary, thyroid and breast in humans have showed an increased incidence over the past few decades. This

suggests that EDCs in the environment may affect reproductive systems by potentially altering hormone receptor binding, leading to improper feedback signaling or competition for endogenous hormone (Jobling *et al.,* 2012). BPA has been shown to affect estrous cycles in adult females through reduced aromatase expression, increased follicle loss and delayed onset of female puberty (Zhou *et al.*, 2016; Rochester, 2013; Viguié *et al*., 2018). Reproductive function is tightly regulated by the coordination of hormone release which requires integration of the HPG axis and an extensive network of neuronal and glial cell signaling components (Christensen *et al.*¸2012; Coss, 2017). BPA altered pituitary function in rats, with lower gonadotropin-releasing hormone (GnRH) pulsatility and signaling, where adult rats treated neonatally with BPA were found to have continued disruption of GnRH pulsatility, suggesting that BPA may permanently affect estrous cycles in adulthood (Fernández *et al*., 2009).

Endocrine disrupting chemicals can also affect the uterus. Abnormalities have been seen in uterine structure, fundus length and function in 33 young girls exposed to dioxins and polychlorinated biphenyls (PCBs) (Su *et al*., 2011). Girls who were exposed to higher than median PCB levels had a significantly shorter fundi and uteri lengths when compared to controls. This suggests that exposure to PCBs may delay reproductive development in these girls (Su *et al*., 2011). Mono (carboxy-isooctyl) phthalate exposure in maternal women and subsequently their adolescent female offspring resulted in significantly increased uterine volume (Hart *et al.*, 2014). Results suggests that maternal exposure to phthalates during pregnancy may have long-term estrogenic or anti-androgenic effects on the reproductive development of their daughters (Hart *et al*., 2014). These studies and others suggest that EDCs that exist in the environment can adversely affect the female reproductive system and it is complex to fully assess their potentially harmful effects on humans and animals.

Parabens

Parabens are chemicals that are widely utilized as preservatives in many personal care products, food, and medications (Nowak *et al*., 2018). Parabens are synthesized as a series of parahydroxybenzoates or esters of parahydroxybenzoic acid (PHBA), a known phenolic derivative of benzoic acid. All widely used commercial parabens are synthetically produced, but PHBA is known to exist naturally in low concentrations in some plants such as chokeberries, coconut and is one of the main catechin metabolites after green tea consumption in humans (Szopa *et al*, 2013; Dey *et al*, 2005; Pietta *et al*, 2008). The antimicrobial potency increases with increasing alkyl chain length from methyl-, ethyl-, propyl-, butyl-, isopropyl-, to benzylparaben (Golden, Gandy, and Vollmer, 2005). The most commonly used parabens are methylparaben, propylparaben, butylparaben and ethylparaben due to their low cost and low known toxicity values (Bajkacz and Słocka, 2020, Nowak *et al*, 2018). They are often combined in a single formulation in products (Cherian *et al*, 2020).

Since it is considered to be low in toxicity, paraben usage has been permitted as an additive in a variety of products in many countries (Golden, Gandy & Vollmer, 2008; Fransway *et al.*, 2019). The European Scientific Committee on Consumer Products (SCCP) allows paraben usage at a maximum of 0.19% concentration of propylparaben and butylparaben in finished products but the use of these two preservatives is prohibited in leave-on personal care products (Denis *et al.*, 2015). In 2011, Denmark banned the use of propyl- and butyl-paraben as well as their isoforms in the formulation of personal care products for children up to three years of age (Scientific Committee on Consumer Safety, 2011). Based on the Cosmetic Ingredient Review Expert Panel (CIR) in the United States, a mixture of parabens (up to 0.8%) or a single paraben (up to 0.4%) is allowed in cosmetic and personal care product preparations (CIR, 2008). Currently, there are no

strict regulations in the United States and manufacturers are not obligated to follow guidelines from the CIR or Health Canada. Both organizations recommend that manufacturers follow SCCP guidelines in the European Union (Kirchhof *et al*., 2013; Health Canada, 2020). It is estimated that humans (average weight of 60kg) are exposed to parabens at ranges between 1.2-2.4mg/kg/day from cosmetic products, food, and medication (Andersen, 2008; Nowak *et al.*, 2018).

Humans are exposed to parabens via inhalation, ingestion, and dermal contact. Exposure to parabens has been broadly detected in a variety of human biological samples. For example, urinary concentrations of methyl-, ethyl-, *n*-propyl-, butyl- and benzyl- parabens were detected in nearly all samples from a demographically diverse group of 100 U.S male and female adults between the years 2003 to 2005. It was found that methyl- and *n*-propyl-parabens were found at the highest median concentrations (43.9 ng/mL and 9.05 ng/mL respectively) in more than 96% of the samples (Ye *et al,* 2006). The presence of parabens and their conjugates excreted in urine suggests that these levels could be used as exposure biomarkers and provide insight into risk assessment of these potentially harmful chemicals. In a study performed in Brazil, parabens (methyl-, ethyl-, and propyl-paraben) were detected in 100% of breast milk samples from 16 lactating women (Souza *et al*, 2016). Parabens and their main metabolite (PHBA) have been shown to be able to cross the placental barrier and this suggests that there is the possibility of fetal exposure to parabens during pregnancy (Andersen *et al*, 2021). Recent studies found that parabens with increasing chain length accumulate in placental tissue at increased level (Andersen *et al*, 2021).

Parabens are known to have potential estrogenic activity as they bind both estrogen receptor 1 (ESR1) and -2 (ESR2) and are therefore classified as endocrine disrupting chemicals where the estrogen receptor binding affinity of parabens is 10,000- to 100,000-fold lower than for

17β-estradiol (Okubo *et al*., 2001; Gomez *et al*., 2005; Wróbel and Gregoraszczuk, 2014). In human breast cancer-cell line MCF-7, methyl-, butyl- and propyl-paraben were found to increase 17β-estradiol secretion in these cells and proliferation of MCF-10A human breast epithelial cells (Wróbel and Gregoraszczuk, 2013). The potential estrogenic impact of parabens was studied by assessing histopathological abnormalities in pre-pubertal female mice that were orally treated with parabens (methyl-, ethyl-, propyl-, isopropyl-, butyl-, and isobutyl-paraben). Results showed that there was a decrease of corpora lutea, increase in the number of cystic follicles, and thinning of the follicular epithelium (Vo *et al*., 2010). The oral exposure also resulted in changes in circulating estradiol and testosterone in treated mice. The long-term exposure to parabens in these pre-pubertal mice induced morphological and physiological alterations in uteri and ovary development as parabens seem to have suppressive effects on female reproductive organs at critical developmental stages, where sex steroids that include estrogen play an important role in progressing this development (Nakamura *et al*., 2008; Vo *et al*., 2010). A separate study investigated the potential effects of ethyl- and butyl-paraben on steroidogenesis and found that there were significant effects on gene expression in female fetuses. Results showed that ESR-2, IGF and Complex C3 expression was significantly decreased, which suggests that it could be a response to paraben estrogenic effects (Sundstrom *et al*., 1989; Suzuki *et al*., 2007; Taxvig *et al*., 2008). From this study, it could be hypothesized that the estrogenic effect of parabens could be disrupting the cholesterol transport to the mitochondrion that affects downstream targets such as steroidogenesis.

Parabens have also been shown to have adverse effects in the male reproductive system. Previous reports have indicated that several parabens demonstrated spermatocidal activity, negatively impact testicular structure, reduced sperm counts and quality in mice (Song *et al*, 1989, 1991; Kang *et al*, 2002; Oishi, 2001, 2002a; Riad *et al*., 2018). In addition, maternal exposure to

butyl-paraben had significant effects on sperm production in F1 male rats, where sperm motility was decreased at 10 mg/kg dose and sperm abnormalities were higher in all 10, 100 and 200 mg/kg butylparaben doses (Guerra et al., 2016). Butylparaben concentrations of 200 mg/kg were found to significantly decrease androgen receptor (AR) immunostaining in Sertoli cells, which correlated with data that showed an increased percentage of abnormal sperm head count (Guerra *et al*., 2016). AR from Sertoli cells play an important role in proper functional responses for normal spermatogenesis (Smith and Walker, 2014). These data suggest that parabens may impact AR expression leading to abnormal sperm head counts. Parabens also possess antiandrogenic properties as it has been shown that propylparaben decreased accessory male sex organ weights in prepubertal male rats, modified luteinizing hormone (LH) and follicle-stimulating hormone (FSH) serum levels, and increased abnormalities in tissue histology (Özdemir *et al*, 2018). Several parabens (methyl-, butyl-, and propyl-) have also been shown to inhibit testosterone-induced transcriptional activity in human embryonic kidney cell-based assays by as much as 40% at 10μM concentrations, which also supports the anti-androgenic property in parabens (Chen *et al*, 2007). Parabens have also been shown to affect respiratory parameters and testis mitochondrial membrane potential. Propylparaben and butylparaben have also been shown to significantly decrease testis mitochondrial enzyme activity of succinate cytochrome C reductase (Complexes II-III) at all tested concentrations (50 μM, 100 μM, and 250 μM) used. This demonstrates that parabens may inhibit testis mitochondrial respiratory chain complex function and cause dysfunction in the male reproductive system.

Urine and semen samples collected from 315 men who attended an infertility clinic in Poland showed a positive correlation between urinary paraben concentrations and an increase in abnormal sperm morphology (Jurewicz *et al*., 2017). It was observed that butylparaben exhibited

a greater reproductive toxicity than the other parabens examined (methyl-, ethyl-, propyl-, butyl- and iso-butyl-paraben), with an increase in sperm DNA damage, decrease in sperm motility, abnormal sperm head morphology, and decreased testosterone levels. However, in recent years contrasting results were reported in a separate experimental study involving parabens and the male reproductive system. A study performed in Murcia, Spain found no associations between urinary parabens (methyl-, ethyl-, propyl-, and butyl-paraben) and semen quality parameters such as motility, semen volume, sperm concentration, total sperm count, and morphology (Adoamnei *et al*., 2018). Blood samples were also collected from the same 215 participants and there was no association with serum reproductive hormone concentrations (estradiol, luteinizing hormone (LH), sex hormone-binding globulin (SHBG), follicle-stimulating hormone (FSH) and testosterone) and urinary concentrations of parabens. Due to variable paraben exposure in different populations and differing sample size, studies are limited and future research in different populations would greatly expand these findings.

Propylparaben

Propylparaben is san ester of parahydroxybenzoic acid (PHBA) and occurs naturally in many plant and insect species. Propylparaben is one of the commonly used antimicrobial preservatives in cosmetic and personal care products (Fransway et al, 2019). Propylparaben is a known EDC as it has been reported to have negative impacts on male and female reproductive systems (Oishi, 2002; Gal *et al*, 2019). Propylparaben exposure has been shown to negatively impact the male reproductive system. Daily sperm reserves and concentrations in the cauda epididymis decreased in prepubertal male rats with increasing propyl-paraben exposure (12, 125, 1290 mg/kg/day) for 4 weeks, reaching significance at doses of 125 and 1290 mg/kg/day (Oishi, 2002b). The sperm counts were also lower at doses of 125 and 1290 mg/kg/day and there was a

significant decrease in serum testosterone concentrations in serum at 125 mg/kg/day dose (Oishi, 2002b). The results suggest that propylparaben may impact spermatogenesis, adversely affect hormonal secretion and delay sperm development in males. However, there are some discrepancies in this study as the testosterone levels were significantly increased at the highest propyl-paraben concentration but there were no collected data on FSH and LH serum levels to determine if this was a central or peripheral effect of propylparaben. A subsequent propylparaben dosing study included a low-dose exposure (3 mg/kg/day), larger sample sizes (10-25 per treatment), longer dosing duration intervals (8-weeks and second sub-group of 26-week), and a more comprehensive hormone analysis (Gazin et al., 2013). The investigators did not find any significant effects of propylparaben exposure on male reproductive organs (testes and epididymis), epididymal spermatid count and motility, or hormone levels (LH, FSH, and testosterone). This study contrasts with the data presented in Oishi's 2002 study but the levels of propylparaben exposure were also much lower over a longer duration of exposure. In light of these somewhat contradictory data, it is important to investigate further the potential for propylparaben toxicity in the male reproductive system.

Studies investigating the effects of propylparaben in the female reproductive system have also been initiated in recent years. In female mice, propylparaben treatments were shown to suppress mouse antral follicle growth and steroidogenic function *in vitro*. Expression of cell cycle regulators (*Cdk4* and *Cdkn1a*), steroidogenic regulator (*Star*) and apoptotic factor (*Bax*) increased in cultured mouse antral follicles in response to 100 µg/mL propylparaben treatment, suggesting that propylparaben interferes with mammalian ovarian follicle function and disrupt steroidogenic function (Gal *et al.*, 2019). Ovarian function has also shown to be negatively affected by propylparaben exposure. Mice were injected subcutaneously with propylparaben at doses of 100

and 1000 mg/kg/day during superovulation and it was found that there was a significant decrease in oocyte retrieval and corpus luteum appearance compared to control, which suggests that excessive propylparaben injection resulted in ovulatory dysfunction (Jiao *et al*, 2021).

Propylparaben also negatively affects embryo implantation and endometrial decidualization. Critical markers of decidualization such as bone morphogenetic protein 2 (BMP-2), homeobox A10 (HOXA10), progesterone receptor (PR), and matrix metalloproteinase 9 (MMP9) and expression of decidual/trophoblast prolactin-related protein (DTPRP) in the endometrium were found to be significantly decreased in mice treated with 2500 mg/kg propylparaben (Wang et al, 2021). These results suggest that propylparaben exposure can impair the decidualization process and subsequent embryo implantation. Propylparaben concentrations relevant to human exposure doses (7.5, 90, and 450 mg/kg/day) were found to accelerate ovarian aging in 46-week-old adult mice which are equivalent to the age of 40 years old in women. Additional findings indicated disrupted estrous cycles, reduced primordial follicles, increase in atretic follicles and decreased serum estrogen and progesterone in these female mice (Li *et al*, 2021).

Additionally, propylparaben exposure resulted in a decreased volume of mammary gland ductal epithelium and periductal collagen (Mogus *et al*., 2021). Oral doses of propylparaben (20, 100 and 10,000 µg/kg/day) administered to pregnant female mice from pregnancy day 0 until lactation day 21 led to alterations in mammary gland morphology, downstream targets of estrogen ER-mediated genes and several immune cell populations in the mammary gland (Mogus *et al*., 2021). Downstream ER-genes (insulin-like growth factor 1 (IGF1) and transforming growth factor beta 2 (TGFβ2) showed an alteration that suggested propylparaben-treatment effects on gene expression of these proteins in the mammary gland. Moreover, T cells and M2 macrophages

showed increases in cell numbers from the control groups, suggesting that propylparaben may influence immune cell populations. However, this warrants further studies as the mammary stroma samples for evaluation of immune cell populations were collected weeks after the last propylparaben dosage, suggesting long term effects (Mogus *et al*, 2021).

With the evidence of previous and current studies demonstrating the potential endocrine disrupting effects of parabens, there is a concern that parabens may affect developing fetuses during pregnancy. Since women are one of the largest consumers of personal care products that contain propylparaben, it is important to determine the effects of propylparaben on developing preimplantation embryos.

Early Embryo Development

Early embryo development is a complex process as it involves a series of events that involves cellular divisions, genomic activation, functional protein transcription, and formation of cell lineages (Niakan *et al.*, 2012; Firmin and Maître, 2021). The zygote undergoes morphological, molecular, and genomic activation changes during this process of rapid cellular division (Niakan *et al.*, 2012). The first few days after fertilization is crucial as the embryo needs to attain implantation competencies for successful pregnancy, where it involves compaction, cavitation and determination of cellular lineages (Watson and Barcroft, 2001; Maître *et al*., 2015). During the first five to six days of preimplantation development, the embryo undergoes rapid cellular division inside the zona pellucida (ZP) (Zhao *et al*, 2018). The ZP is comprised of glycoproteins that encompasses the entire egg and is involved in oocyte growth support, binds to spermatozoa to initiate the acrosome reaction for successful fertilization, and protects the developing preimplantation embryo before implantation to a receptive uterus (Wassarman and Litscher, 2018). A developing, healthy, and expanded blastocyst must hatch out of the ZP in a timely manner before

implantation as ectopic pregnancies (early hatching) or missed implantation (late hatching) can occur (Jun *et al*., 2004; Diedrich *et al*., 2007; Leonavicius *et al*., 2018). Furthermore, failure of zona hatching results in early embryonic loss and infertility (Enders and Schafke, 1967; Petersen *et al*., 2005; Seshagiri *et al.*, 2009; Shafei *et al*., 2017)

An important factor that contributes to proper early embryo development is cytoskeletal proteins that play a role in establishing nuclear structures during this progressive stage (Simon and Wilson, 2011). The development from morula to blastocyst is a defining transition of preimplantation embryos, where the embryo forms a permeable paracellular barrier to allow blastocyst cavity expansion (Zenker *et al.*, 2018). Embryonic cells establish adherens and tight junctions that function to create a permeable barrier that seals the embryo from the exterior which progresses the blastocoel expansion and morula to blastocyst transformation process (Eckert and Fleming, 2008). Filamentous actin (F-actin) serves as a structural support for the developing embryo, cellular mobility during cell division, and modulating transcription activity on embryo *hox* genes (Sun and Schatten, 2006; Miyamoto *et al.*, 2011; Miyamoto *et al.* 2013; Okuno *et al*., 2020). A recent study investigated whether the disruption of F-actin would affect *in vitro* produced (IVP) mouse blastocysts and it was indicated that F-actin disorganization negatively impacted IVP preimplantation embryo development (Tan *et al*., 2015). Culture medium supplemented with cytochalasin B (CB) which specifically inhibits actin organization was found to have significantly reduced blastocyst development rate which suggests that proper actin organization and function is required for embryonic development (Tan *et al*., 2015).

During the blastocyst stage, cell fate decision of the blastomeres in the zygote will be determine if they will form the inner cell mass (ICM) or the trophectoderm (TE) of the blastocyst. ICM is a cluster of pluripotent stem cells in the primordial embryo that develops into a variety of

cell lineages that result in definitive structures of the developing fetus (Yamanaka *et al.*, 2008; Coticchio *et al.*, 2019). The TE forms a layer of epithelial cells that encompasses a hollow fluid-filled blastocoel and the ICM which eventually forms a large portion of the embryonic placenta (Marikawa, 2012; Saiz and Plusa; 2013; Posfai *et al.*, 2019). The TE plays an important role in the implantation interaction with the maternal uterus and proceeds to differentiate into extraembryonic tissues to progress development of the fetus in pregnancy (Marikawa, 2012). This process of cell fate lineage decision requires the differential expression of transcription factors. The pluripotency characteristic in ICM is ensured through expression of octamer-binding protein 4 (OCT4) during early embryo development (Saiz and Plusa; 2013). OCT4 expression is encoded by the POU5F1 gene. Its expression is vital for primordial germ cell survival and it is specifically expressed in the ICM and embryonic stem cells (Scholer *et al*., 1989; Pesce and Scholer, 2001; Kehler *et al.*, 2004). Developing embryos that are absent of the transcription factor OCT4 will not successfully achieve implantation in the uterus due to a lack of pluripotent ICM cells and a decrease in OCT4 expression has resulted in lower quality embryos (Nichols *et al.*, 1998; Van Thuan *et al.*, 2006). Knockdown of OCT4 expression has shown to induce embryonic stem cells to differentiate into TE cells while overexpression of OCT4 induces embryonic stem cells differentiation to primitive endoderm and mesoderm (Niwa *et al*., 2000). Therefore, there is a very precise OCT4 expression level that is required to maintain pluripotency and determinacy of embryonic stem cell fate (Zarafarana *et al*., 2009; Li *et al.*, 2010).

Another transcription factor that is crucial in early embryo development is the caudal-type homeobox-2 gene (CDX2), which regulates the formation and maintenance of TE cell lineage (Niwa *et al.*, 2005; Yamanaka *et al.*, 2006). CDX2 is one of the major regulators of embryonic stem cell fate and instructive in formation of TE cells in the developing embryo (Niwa *et al.*, 2005;

Huang *et al.*, 2017). Embryonic expression of CDX2 is confined to the TE cells in the blastocyst stage and is absent in ICM cells (Sritanaudomchai *et al.*, 2009; Baines and Renaud, 2017). A reduction in CDX2 expression impacts cell polarity and results in developmental issues that lead to a reduced blastocyst development rate (Chawengsaksophak *et al*., 1997; Jedrusik *et al.*, 2010; Jedrusik *et al*., 2015). A depletion of CDX2 also arrested porcine embryo development at the blastocyst stage, high rate of apoptosis and reduced cell proliferation (Bou *et al*., 2017). These data suggest that proper expression of CDX2 is necessary for blastocyst development and TE formation where dysregulation of this process can be one of the factors that lead to failed pregnancy.

The interaction between CDX2 and OCT4 expression is complex as they have a reciprocal relationship in repressing the other gene transcription in embryonic stem cells. This co-expression of transcription factors leads to the first cell fate differentiation in mammalian embryonic development during the blastocyst stage (Dietrich and Hiiragi, 2007; Bou *et al.*, 2016). Previous studies have shown that increased OCT4 expression in dysregulated TE cells of CDX2 knockout mice embryos and increased CDX2 expression in OCT4 knockout mice embryos resulted in reduced blastocyst development ratet, which provides further insight into the complex molecular regulatory interaction between CDX2 and OCT4 which is crucial during ICM and TE cell fate determination (Jedrusik *et al*., 2010; Strumpf *et al*., 2005; Wu *et al*., 2010; Wu *et al*; 2013). It has been shown that these two transcription factors bind to the other's regulatory regions and reciprocally suppress transcription of each other, allowing them to form a protein complex for mutual antagonism during cell fate determination into TE or ICM cells (Niwa *et al*., 2000; Velkey and O'Shea, 2003; Bou *et al*., 2016). Overall, OCT4 and CDX2 play an important role in regulating downstream transcription factors and target gene expression during early embryo development (Uesaka *et al*., 2004; Baines and Renaud, 2017).

Currently, there is a knowledge gap as to whether propylparaben, a known EDC has effects on developing preimplantation embryos, the number of ICM and TE cells and cytoskeleton F-actin network. Considering the well-established data that TE and ICM morphology and F-actin filaments are crucial for successful embryo implantation and progressive development, the concern of the direct effects of propylparaben on these important markers have not been reported in current studies. This study aims to investigate the direct effects of propylparaben on the development of early preimplantation embryos, the number of ICM and TE cell population, and the formation of F-actin in embryonic cells.

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CHAPTER 2: EFFECTS OF PROPYLPARABEN ON PREIMPLANTATION EMBRYO DEVELOPMENT

Introduction

Synthetically produced chemicals have seen increased usage in mass manufacturing and production practices over the past 50 years. With the surge in mass production and introduction of new chemicals, many of these chemicals have not been investigated extensively for their potential toxicological effects on human and animal health. Synthetic chemicals are found in many daily-use products such as plastic containers, metal food cans, food, medicine, cosmetics, and children's toys (Benjamin *et al.,* 2017). Many of these chemicals have been reported to negatively impact human and animal health (DeCoster and van Larabeke, 2012; Sifakis *et al.*, 2017; Marinello and Patisaul, 2021; Streifer and Gore, 2021). One of the most common class of chemicals that humans are exposed to are added preservatives that are prevalent in many daily use products.

Parabens have been widely used as preservatives since the 1920s due to their effective antimicrobial properties and low known toxicity values (Nowak *et al,* 2018). They are synthetically synthesized but can also be found naturally in bacteria and plants, where it is thought they prevent growth of fungi and Gram-positive bacteria (Ali *et al.*, 1998; Bais *et al*, 2003; Li *et al*, 2003; Peng *et al.*, 2006; Nowak *et al.*, 2018). However, they are present in very low levels in nature while the vast majority of parabens in the environment are derived from industrial and mass manufacture-scale chemical synthesis (Nowak *et al*., 2018). Synthesized parabens are wide-ranging as they are produced with differing akyl chain lengths of methyl-, ethyl-, propyl-, butyl-, isopropyl- and benzyl-paraben, with increases in chain length leading to increased antimicrobial efficacy (Routledge *et al*., 1998, Darbre *et al*., 2002). Parabens are often used together in combination in a single formulation due to their broad spectrum of antimicrobial action (Chen, 2016). Based on the

high level of efficacy as preservatives and the low production cost, parabens are used extensively as a preservative in food, pharmaceutical products, and personal care products. Propylparaben is one of the more commonly used preservatives in products and is known to be an endocrine disrupting chemical (Nowak *et al*., 2018). Propylparaben has been reported to be found in human urine, breast milk, serum, placental tissue, and breast tumor tissue (Schlumpf *et al*., 2010; Meeker *et al*., 2011; Jiménez-Díaz *et al.*, 2011; Smith *et al.*, 2012). Urinary levels of propylparaben have been detected in more than 95% of adult and children populations studied in the United States, Spain, Denmark, Korea, and Japan (Calafat *et al*, 2010; Frederiksen *et al*, 2011; Casas *et al*, 2011; Shirai *et al*, 2013; Kang *et al*, 2013).

Following successful fertilization between an oocyte and sperm, the zygote undergoes a series of cleavage divisions that is regulated by the maternal environment (Yurttas *et al*., 2010). During this time, the embryo undergoes genomic activation, compaction, cavitation, zona pellucida (ZP) hatching, and finally implantation into the maternal uterus (Watson and Barcroft, 2001; Rossant and Tam, 2018). This process involves several key proteins including filamentous actin (F-actin) that are essential for the remodeling dynamics in the developing embryos including expansion of the blastocoelt cavity, cytokinesis, and reorganization of the nuclear compartment within the blastocyst (Rawe *et al*., 2006; Baarlink *et al.*, 2017; Zenker *et al.*, 2018). F-actin is a cytoskeletal protein that is integral for chromatin organization and nuclear expansion as this process involves multiple rounds of cellular division processes during the progressive development from fertilization to the blastocyst stage (Baarlink *et al*., 2017; Okuno *et al*., 2020). Disorganization of this cytoskeletal network in early preimplantation embryos may be detrimental for successful implantation where inhibited actin organization was shown to result in a significantly reduced rate of blastocyst development (Tan *et al*., 2015).

Successful establishment of embryo implantation and pregnancy requires a time-sensitive interaction between a healthy, competent blastocyst and a receptive maternal uterus (Zhang *et al.*, 2012; Coticchio *et al.*, 2019). One of the crucial events during this pre-implantation timeline involves the hatching of the blastocyst from the zona pellucida (ZP), which happens around day 5 of *in vitro* development (Zhao *et al.*, 2018). The ZP surrounds mammalian oocytes and acts to envelope oocytes and developing embryos (Moros-Nicolás *et al*., 2021). The ZP is a polymeric cross-linked glycoproteinaceous matrix that is involved in polyspermy prevention and protecting the developing embryo within the oviduct prior to implantation (Wassarman and Litscher, 2008; Gupta *et al.*, 2009; Moros-Nicolás, 2021). Mammalian embryos must hatch out of the ZP before implantation for establishment of successful pregnancy and failure of blastocyst hatching results in early embryonic loss and infertility (Enders and Schlafke, 1967; Petersen *et al.*, 2005; Seshagiri *et al*., 2009, Shafei *et al*., 2017).

Endocrine disrupting chemicals have been documented to negatively impact embryo development, embryo quality, and embryonic genome activation (Choi *et al*., 2016; Caserta *et al*., 2021; Xu and Yang, 2021). Urinary and serum BPA concentrations have been correlated with lower rates of normally fertilized oocytes, lower blastocyst formation, and overall oocyte quality in human IVF patients (Fujimoto *et al*., 2011; Ehrlich *et al.*, 2012). Volatile organic compounds (VOCs), that are ubiquitous in the environment since they are present in various everyday materials such as polyvinyl chloride (PVC) flooring and cosmetics, have also been found to exert detrimental effects on extended embryo culture in an *in vitro* fertilization (IVF) laboratory (Agarwal *et al*., 2017; Mahalingaiah, 2018; Xu and Yang, 2021). One study reported that by reducing VOCs in the IVF laboratory, there was an increase of approximately 18% in good quality blastocyst formation including timely appearance of expanded blastocoels, increased cell number of the ICM, and

increased compactness of TE cells (Agarwal *et al*., 2017). Previous studies have indicated that TE morphology, ICM grading, and embryo stage at specific time points are reliable predictors of live birth in clinical IVF pregnancies (Gardner *et al*., 2000; Thompson *et al*., 2013; Chen *et al*., 2014; Ai *et al*., 2021). In human treatments, blastocyst grading and quality parameters include several criteria such as blastocyst expansion, hatching status at specific time points, morphology and number of cells in the inner cell mass (ICM) and trophectoderm (TE) layers (Gardner *et al*., 2000; Zhao *et al*., 2018; Ai *et al*., 2021). How differences in these morphological parameters alter the physiological mechanisms of blastocyst hatching and maternal implantation remains poorly understood, and this is an important relationship to investigate in the very early stages of pregnancy (Araki *et al*., 2016).

Studies have shown that propylparaben inhibits steroidogenic function and antral follicle growth, disrupts endometrial decidualization, and interferes with embryo implantation (Gal *et al*, 2019; Wang *et al*, 2021). Recent studies have reported the negative effects of propylparaben on the female reproductive tract, but very few have focused specifically on early embryo development and implantation (Nowak *et al.*, 2018; Gal *et al.*, 2019; Wang *et al.*, 2021; Li *et al.*, 2021). The goal of this study was to examine the direct effects of propylparaben concentrations on several embryonic developmental endpoints such as hatching ability, number of ICM and TE cells, and formation of F-actin cytoskeleton.

Materials and Methods

Chemicals

Propylparaben powder was purchased from Sigma (CAS#94-13-3; purity >99%). Four stock solutions of propylparaben were prepared, utilizing dimethylsulfoxide (DMSO) from Sigma-Milipore (#D2650) as the vehicle diluent. Concentrations of 0.5µg/mL, 5 µg/mL, 10 µg/mL, and 15 µg/mL were used for this study. The doses used were selected based on study designs by Gal *et al.,* (2019) and in a dose range that is more environmentally relevant according to daily exposure levels of propylparaben from dermal contact, inhalation, and ingestion. Propylparaben is usually used in combination with other parabens for increased preservative efficacy in products. Humans are estimated to be exposed daily to parabens at ranges between 1.2-2.4 mg/kg/day from medicine, personal care products, and food (Andersen, 2008; Nowak *et al*., 2018). For this study, the dose ranges were selected to mimic exposure to propylparaben observed in reproductive fluids such as amniotic fluid (0.3-1.4 µg/mL), umbilical cord (< 0.27 µg/L), and breast milk (0.1-0.2 µg/L) (Philippat *et al*., 2013; Hines *et al*., 2015; Pycke *et al*., 2015).

Pregnant mare serum gonadotropin (PMSG) was purchased from Prospec (#HOR-272), human chorionic gonadotropin (HCG) from MilliporeSigma (#230734), and hyaluronidase from bovine testes from MilliporeSigma (#H4272). Fetal bovine serum (S11150) was purchased from Atlanta Biologicals. Global culture medium for embryo culture was purchased from Life Global (#LGGG-050). Mineral oil was purchased from Target (Up&Up) and later on replaced with sterile mineral oil for tissue culture from CooperSurgical (ART-4008-5P) for subsequent experiments. Normal donkey serum (017-000-121) was purchased from Jackson Immuno Research. Primary antibodies rabbit anti-OCT4 (#D6C8T) were purchased from Cell Signaling and mouse anti-CDX2 (#MU392A) were purchased from Biogenex. Primary antibodies CY3 donkey anti-rabbit 555nm

(#711-165-152) and CY5 donkey anti-mouse 690nm (#715-175-151) were purchased from Jackson Immuno Research. Fluorescent-labeled secondary antibodies cyanine 3 (CY3) donkey anti-rabbit 555nm (#711-165-152) and cyanine 5 (CY5) donkey anti-mouse 690nm (#715-175-151) were purchased from Jackson Immuno Research. CytoPainter Phalloidin-iFluor 488 Reagent (#ab176753) was purchased from Abcam. Global culture medium for embryo culture was purchased from Life Global (#LGGG-050).

Animals

Animal experiments followed guidelines of the National Institutes of Health standards for the use and care of animals and were approved by the University of Illinois Institutional Animal Care and Use Committee (#21002). Female CD-1 and male B6D2F1 mice were purchased from Charles River Laboratories (Wilmington, MA). The mice were housed at the University of Illinois Urbana-Champaign at the Carl R. Woese Institute for Genomic Biology Animal Facility. These animals were provided food and water *ad libitum* and housed in a controlled animal room environment, maintained at a temperature of 22 ± 1 ℃ and on 12-hour light-dark cycles.

Culture Dishes and Handling Medium Preparation

Propylparaben stock solutions for 0.5 µg/mL, 5 µg/mL, 10 µg/mL, and 15 µg/mL were freshly prepared using a propylparaben stock solution of 100 ug/mL and diluted accordingly with dimethylsulfoxide (DMSO). One mL of LifeGlobal culture medium were added into autoclaved capped tubes for treatment preparations. 0.75 µL of the respective concentrations (DMSO (0.075%), 0.5 µg/mL, 5 µg/mL, 10 µg/mL, and 15 µg/mL were added to the 1mL capped tubes and thoroughly mixed.

Treatment wash dishes were prepared to ensure there was no further dilution and cross-contamination in the final culture drops. For this, 500µL of the respective treatments were placed in individual Falcon 60mm center well organ culture dishes and covered with 1 mL of mineral oil to prevent dehydration. Culture dishes were made for the embryo development in treatment drops. In each culture dish, 5-6 droplets of 20 µL for each treatment group were added in a Falcon 35 mm dish and then covered with 9 mL of mineral oil. Handling medium (3-(*N*-morpholino) propanesulfonic acid) (OMOPS) was freshly prepared before each collection. 9.5 mL of OMOPS and 500µL of 5% fetal bovine serum (FBS) were combined in a Falcon 14mL capped tube which was then placed into an incubator set at 37℃. Wash and culture dishes were placed in a ThermoFisher 8000 WJ CO_2 incubator at 37.5℃, 6% CO_2 and 75% RH settings and left to acclimate overnight.

Embryo Culture and Development

Female CD-1 mice (35 days old) were allowed to acclimate for at least 3 days before undergoing the superovulation procedure. These female mice were injected intraperitoneally (IP) with 6 IU (120 µL) of pregnant mare serum gonadotropin (PMSG) at 3:30-4:00 pm and 6 IU (120 µL) of human chorionic gonadotropin (HCG) were injected at the same location 45-46 hours later at 1:00pm. After the HCG injection, females were placed with B6D2F1 males for natural mating overnight (1:1) until the next morning. The next day, 19 hours later at 10:00 am, the females were euthanized with carbon dioxide and cervical dislocation. The ovaries, oviducts and uterine horn section were collected and placed into a Falcon 14mL tube containing OMOPS handling medium supplemented with 5% FBS to transport back to the laboratory.

Using an Olympus SZX12 stereo microscope warmed at 37℃, the contents were placed into a Falcon 35-mm plate. All subsequent steps were done using a plate warmer at 37℃. The

ampulla of the oviducts was perforated with a 25G sterile needle to release the cumulus-oocyte complexes, containing presumptive zygotes. They were then transferred into a separate Falcon 60 mm center well organ dish containing 500 µL of the same OMOPS handling medium + 5% FBS. After pooling the embryos, 500 µL of hyaluronidase was added to aid in dissociating the cumulus cells from the embryos for 30 seconds. The embryos were aspirated repeatedly with a 10 µL pipette to thoroughly remove all cumulus cells before transferring the embryos into a new Falcon 60mm center well organ dish containing OMOPS. Embryos were washed twice and counted, once in OMOPS and followed by once in Global culture medium. These embryos were then graded based on quality (intact ZP, no fragmentation, and visible polar bodies) and selected for final culture. Selected embryos were transferred into respective treatment wash dishes of 0.5 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL, DMSO (0.075%) or control (Global medium only) covered with mineral oil. Following this final washing step, the embryos were moved to culture droplets for each respective treatment.

Embryos were assigned randomLy into treatment culture drops in the culture dishes prepared at least 24 hours before the collection. A total of six to fifteen embryos were placed in each 20µl culture drop of their respective treatment groups (0.5µg/mL, 5µg/mL, 10µg/mL, 15µg/mL, DMSO 0.075% (vehicle control) and control (embryo culture media)) and were allowed to develop in a ThermoFisher 8000 WJ CO_2 incubator at 37.5°C, 6% CO_2 and 75% RH settings, for the next six days in culture. On days four, five, and six of culture, embryos were observed for blastocyst development and ZP hatching at 11 am. Images of the embryos were taken using an Olympus IX70 contrast phase microscope on day six of culture and fixed in freshly prepared 4% paraformaldehyde.

Quantification

The rate of overall embryo development to the blastocyst stage was calculated for each treatment. On day two (one day after embryo retrieval and collection) at 11 am, oocytes that did not develop to 2-cell embryos were excluded to avoid miscounting unfertilized oocytes in the final count for the analysis of embryo development. On days four, five, and six, embryo development stages were observed and recorded (blastocysts, hatching blastocysts, hatched blastocysts and fragmented). The percentages of blastocysts formed, hatching blastocysts, hatched blastocysts and fragmented embryos on day six were calculated by totaling up the results from 8 separate experimental trials.

Immunofluorescence Staining

To investigate the effects of propylparaben on early cell lineage commitment, the numbers of ICM and TE cells were counted in hatched blastocysts. This was determined by immunofluorescence staining with antibodies to detect OCT4 (specific for ICM) and CDX2 (specific for TE) transcription factors and visualized utilizing a confocal microscope.

Hatched embryos were fixed in 4% paraformaldehyde for 20 minutes and stored in holding medium that consisted of phosphate-buffer saline (PBS) supplemented with 0.5% bovine serum albumin (BSA) and covered with mineral oil to prevent dehydration at 4℃ until ready for immunofluorescence staining. Embryos were washed in 500 μL of washing buffer (PBS, 0.1% Triton X-100, and 0.1% polyvinylpyrrolidone (PVP) three times for 10 minutes each time, following permeabilization with 1% Triton X-100 in PBS for 30 minutes. Following another series of washing steps, embryos were blocked in 500 μL in blocking buffer (PBS, 0.1% Triton X-100, 1% BSA, 0.1M Glycine) supplemented with 50μL normal donkey serum for two hours and

followed by another washing step for 25 minutes. The primary antibodies used were rabbit anti-OCT4 (1:800 dilution) and mouse anti-CDX2 (1:300 dilution). Volumes of 125µL of each antibody (total 250 µL/well) were used and the embryos were incubated overnight at 4℃.

The next day, embryos were washed three separate times for 10 minutes each and incubated in 250 µL/wells with fluorescent-labeled secondary antibodies CY3 donkey anti-rabbit 555 nm (dilution 1:500) and CY5 donkey anti-mouse 690 nm (dilution 1:400) in the dark for 2 hours. All antibodies were diluted in antibody buffer (PBS, 0.1% Triton X-100, 1% BSA) except the anti-CDX2 antibody that was diluted in a diluent provided by its manufacturer. Following this step, light exposure on embryo samples was minimized to prevent any photobleaching. Embryos were washed with two rounds of antibody buffer for 20 minutes each and finally a washing buffer step for 1 hour. For confocal microscope imaging, 35 mm glass-bottomed dishes with a 14 mm micro-well (#D35-14-1.5-N, Cellvis) were used and 20 µL of mounting medium containing DAPI (#H-1200, Vector Laboratories) was placed in the middle of the well. Embryos were placed in the mounting medium, covered with 200 µL of mineral oil, and a glass coverslip was placed on top. Stained samples were kept in the dark at 4℃ for up to 7 days until imaging.

The CytoPainter Phalloidin-iFlour 488 reagent (dilution 1:1000) was used for F-actin detection in blastocysts using a protocol that was modified from the immunofluorescence protocol. The embryos were washed three times for 10 minutes in 500µL of washing buffer and then followed by 500 µL permeabilization buffer incubation for 15 minutes. After that, embryos were washed again for three times for 10 minutes each time. After the washing steps, embryos were incubated with 250 µL CytoPainter Phalloidin-iFluor 488 reagent for one hour in the dark at room temperature. The embryos were washed once more, and finally incubated in mounting medium containing DAPI, covered with 200 µL of mineral oil and coverslipped in glass-bottomed dishes.

All immunofluorescence-stained samples were imaged with a Zeiss LSM880 confocal microscope located at the Core Facility at the Carl R. Woese Institute for Genomic Biology at the University of Illinois Urbana-Champaign. A Z-stack was performed in ZEN software to obtain images of the complete spherical structure of the embryos. Samples stained with Phalloidin were imaged on the same day to avoid variation.

Analysis of micronucleation in hatched blastocysts

The nuclei in the hatched blastocysts were stained with DAPI and visually appeared gray. Using Imaris x64 9.6.0 software and the orthogonal slicer tool, a three-dimensional image was created that allowed rotation for clear visualization of micronucleation in the embryo.

Trophectoderm and inner cell mass cell counting

Trophectoderm cells express the CDX2 transcription factor and visually appeared red while inner cell mass cells express OCT4 transcription factor and visually appeared green, and DAPI stained nuclei appeared blue when imaged using Zeiss LSM 880 confocal microscope. With the use of ZEN software, a Z-stack procedure was performed to obtain the entire spherical structure of the embryo and subsequent three-dimensional reconstructions using the Z-stack images were obtained using Imaris x64 9.6.0 software. By using the channel selector, orthogonal slicer, and measurement tool, the number of each cell respective cell type was counted.

Analysis and quantification of F-actin expression

Hatched blastocysts were stained for F-actin and images were obtained using the Plan-Apochromat 20x/0.8 objective lens, 488 nm laser excitation, with a set laser power of 1.0 and gain of 500. Using ZEN 2.3 Lite software, the fluorescence intensity of F-actin was measured by generating a cross-section line that encompassed the whole area of the embryo. A fluorescence

intensity profile graph was produced and average values of the peaks for the Z-stack plane were recorded. Distances ranging between 8 µm and 21 µm planes of the blastocyst from a total of 6 planes were analyzed for each hatched blastocyst.

Statistical Analysis

All statistical analyses were done using GraphPad Prism 9.1.0. Data presented in this study were collected from 15 independent cultures. The overall developmental parameters (blastocysts, hatching, hatched, and fragmented embryos) were analyzed by chi-square tests and multiple comparisons were done using Fisher's exact test (two-sided) and considering Bonferroni correction for multiple tests. Continuous variables, presented as mean ± standard error of the mean (SEM), were tested for normality of residuals using the Shapiro-Wilk test and compared between groups using one-way ANOVA and Tukey's post-hoc test when data were normally distributed; or Kruskal-Wallis test and Dunn's post-hoc test when data were not normally distributed. The percentage of micronucleation was analyzed by chi-square and multiple comparisons using Fisher's exact test. The number of micronuclei was analyzed using ANOVA with Bonferroni's multiple comparisons test. The number of ICM and TE cells and F-actin immunofluorescence intensity data were analyzed using ANOVA and Tukey's multiple comparisons test. Statistical significance was assigned at $p < 0.05$ unless otherwise stated respectively.

Results

Propylparaben reduces blastocyst hatching and increases rate of embryo fragmentation

After six days of *in vitro* culture in different propylparaben concentrations, the overall percentage of hatched blastocysts were analyzed and calculated (Table 1). Visual representation experimental design in a mouse model is shown in Figure 1. Representative images of the embryos on day 6 of culture are shown in Figure 2. To compare the developmental differences in observational day 5 and 6, the percentages of embryos from several stages were determined. The overall percentages of embryos in three distinct developmental stages were graded based on blastocysts completely hatched from the zona pellucida, mid-hatching from the zona pellucida, blastocysts, and fragmented embryos were calculated (Table 1). These developmental parameters were then grouped into three distinct developmental stages: completely hatched (teal bars), hatching (pink bars), blastocyst (black bars), or fragmented embryos (purple bars) are presented in Figure 3.

On day 5, there were several embryos that successfully hatched in all treatment groups. Furthermore, propylparaben did not seem to affect the rate of blastocyst development and there were no significant differences between treatments for hatching percentage on day 5. However, on day 6, results indicated that propylparaben reduced blastocyst hatching. Specifically, the percentages of hatched blastocysts for embryos cultured in 10 µg/mL (23.40%) and 15 µg/mL (13.04%) propylparaben treatments were significantly lower than DMSO 0.075% control (40.76%). Furthermore, the percentage of fragmented embryos were greater than 50% in the 10 ug/mL and 15 ug/mL propylparaben treatments respectively, in comparison with DMSO 0.075% control (33.15%).

Propylparaben exposure increases incidence of micronucleation in blastocysts

The incidence of micronucleation was calculated by analyzing micronucleation that was present or absent in embryos (Figure 5A). Embryos cultured with propylparaben treatments had a significantly increased incidence of micronucleation compared to DMSO 0.075% control. Micronucleation was present in all treatment groups with at least 60% incidence of micronucleation. Further, there was a statistically significant difference between DMSO 0.075% control and 5 µg/mL ($p < 0.0001$) and 10 µg/mL propylparaben treatments ($p < 0.001$) treatments respectively. The number of micronucleation present per embryo in treatments were also calculated (Figure 5B). The average number of micronucleation per embryo was also influenced by propylparaben treatment. Specifically, the number of micronucleation between blastocysts cultured in DMSO 0.075% control (2.4 ± 0.763) and 15 µg/mL (7.3 ± 1.578) propylparaben treatment were statistically different ($p < 0.01$).

Propylparaben alters inner cell mass but not trophectoderm cell numbers

To quantify the number of trophectoderm (TE) cells and inner cell mass (ICM) cells, immunofluorescence staining was performed. Immunofluorescent staining using OCT4 and CDX2 protein markers identified ICM and TE cell populations in hatched blastocysts as shown in Figure 7. The total number of analyzed embryos per treatment, number of ICM and TE cells, and ratio between the two cell types is shown in Table 2. Interestingly, the ratio between TE:ICM in 0.5 µg/mL propylparaben treatment (6.221 ± 0.5) is the highest among the propylparaben treatments in comparison with DMSO 0.075% control (3.659 ± 0.41) (Table 2). There was no significant difference between treatment groups for total number of TE cells (Figure 8A). However, there was a statistically significant difference in ICM cell numbers between DMSO 0.075% control and 0.5 µg/mL propylparaben treatment ($p < 0.01$) (Figure 8B). There was also a trend toward significance

between the DMSO 0.075% control and the 15 µg/mL (*p*-value = 0.0713) propylparaben treatments for ICM cell numbers (Figure 8B).

Propylparaben affects F-actin expression in hatched blastocysts

Fluorescent staining of F-actin in hatched blastocysts were performed to determine the effects of propylparaben (Figure 9). We observed a noticeably lower fluorescence intensity in 10 µg/mL and 15 µg/mL propylparaben treatments compared to DMSO 0.075% controls using the same laser power of 1.0 and gain of 500 for all analyzed blastocysts. Results indicated that F-actin fluorescence intensity was significantly lower in blastocysts exposed to 10 µg/mL ($p < 0.001$) and 15 µg/mL ($p < 0.001$) propylparaben treatments compared with DMSO 0.075% control (Figure 11).

Figures & Tables

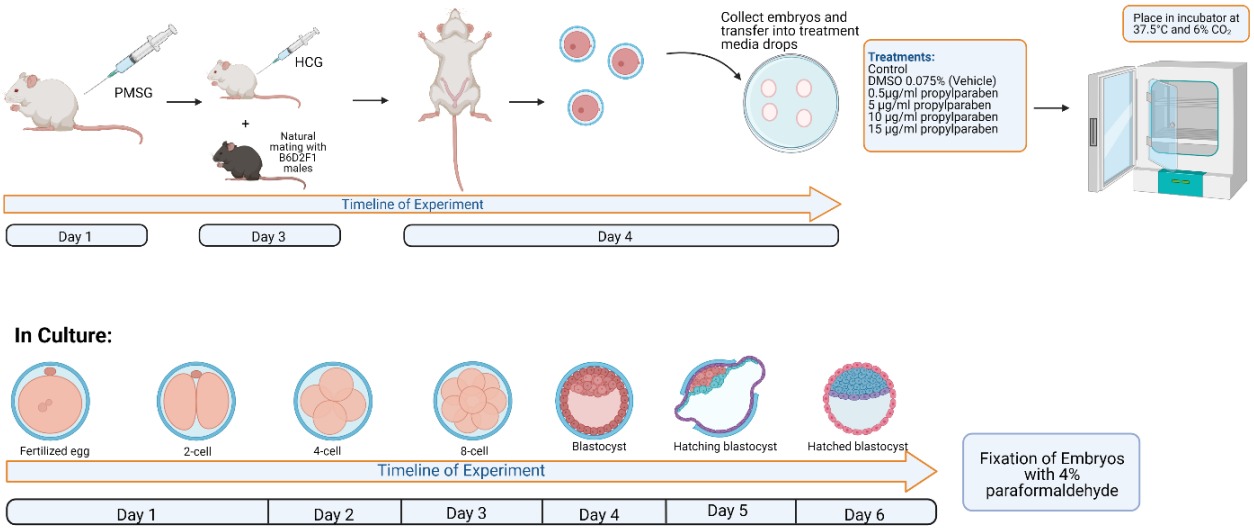

Figure 1: Visual representation of *in vitro* culture of mice embryos and chemical treatment during embryo development. After embryo collection and transfer into treatment culture drops, dishes are placed in a ThermoFisher 8000 WJ CO_2 incubator at 37.5°C, 6% CO_2 and 75% RH settings for the next six days in culture. Development progression is recorded at 1100 hours on days 5 and 6 at the same timepoint.

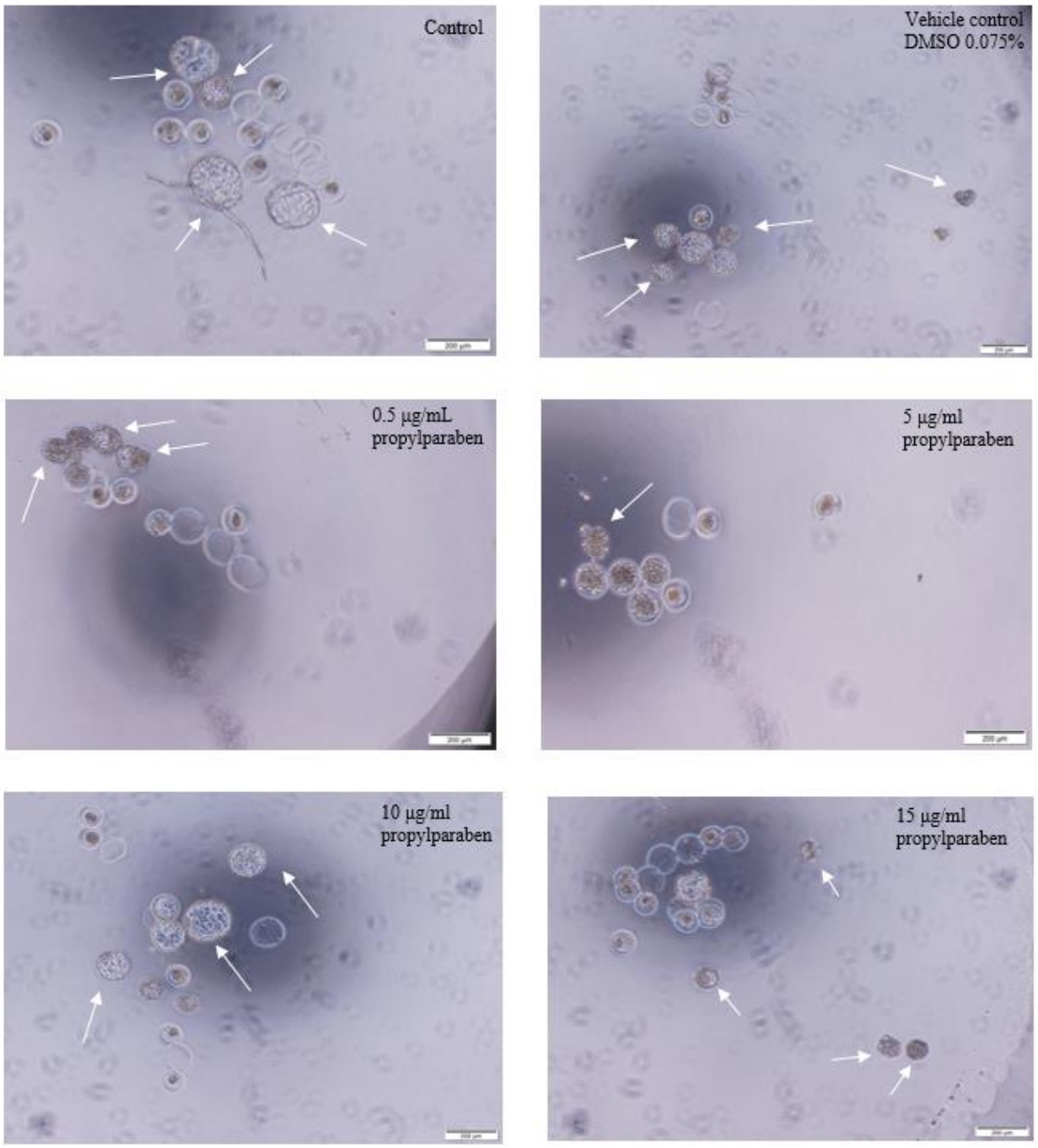

Figure 2: Development of embryos after 6 days in culture from various treatment groups. White arrows indicate hatched and expanded blastocysts. Images were obtained from an Olympus IX70 inverted phase-contrast microscope. Scale bars: 200 µm.

Table 1: Embryonic developmental parameters analyzed in embryos cultured in different concentrations of propylparaben.

	Total number of embryos (n)	Blastocysts (%)	Hatching (%)	Hatched (%)	Fragmented	*p*-value
Control	210	15 (7.14%)	32 (15.24%)	84 (40.00%)	79 (37.62%)	0.6827
DMSO	184	31 (16.85%)	17 (9.24%)	75 (40.76%)	61 (33.15%)	
0.5 µg/mL propylparaben	137	17 (12.41%)	16 (11.68%)	51 (37.23%)	53 (38.58%)	0.5641
5 µg/mL propylparaben	151	20 (13.25%)	14 (9.27%)	65 (43.05%)	75 (34.43%)	0.7385
10 µg/mL propylparaben	141	12 (8.51%)	21 (14.89%)	33 (23.40%) **	75 (53.20%)	< 0.0013
15 µg/mL propylparaben	138	24 (17.39%)	22 (15.94%)	18 (13.04%) ****	74 (53.63%)	< 0.001

Percentages were analyzed using chi-square and multiple comparisons by Fisher's exact test considering Bonferroni adjustment. Values are compared with vehicle control DMSO 0.075%. Values are compared with vehicle control DMSO. Analyses were performed in GraphPad Prism 9.1.0, *p*-value < 0.01 was considered significant and marked with an ** and *p*-value < 0.0001 was marked with an ****.

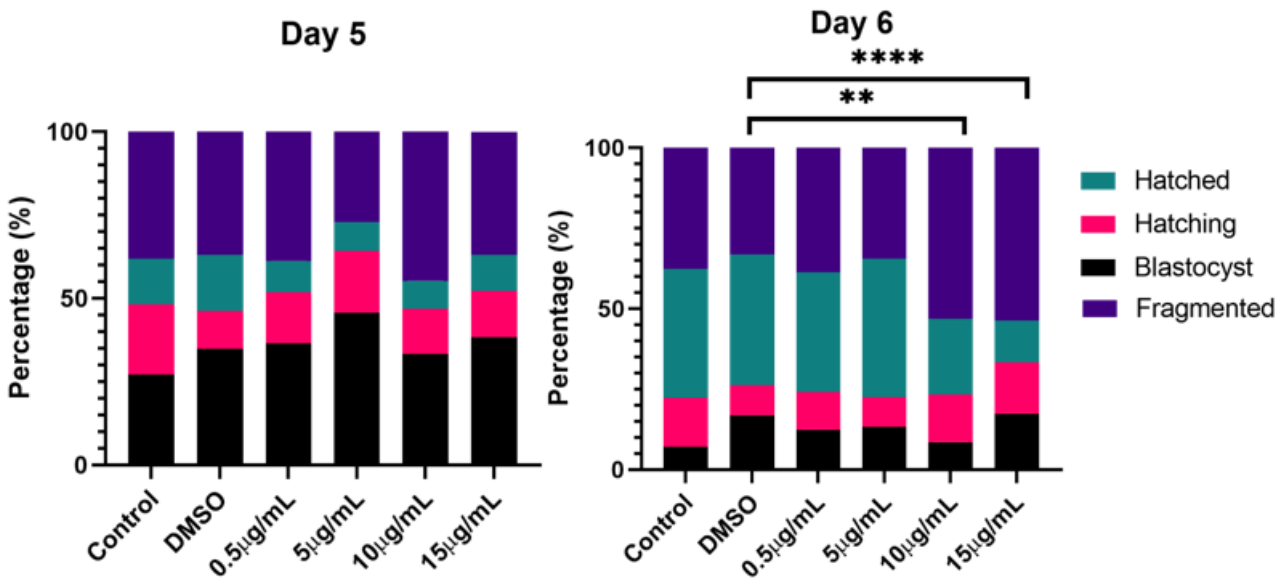

Figure 3: Effect of propylparaben on blastocyst hatching at days 5 and 6 of culture. The percentages of fragmented, zona pellucida intact blastocysts, hatching blastocysts, and completely hatched blastocysts are shown for each treatment. Percentages were analyzed by chi-square and multiple comparison by Fisher's exact test considering Bonferroni adjustment. Control n=210, DMSO n=184, 0.5 µg/ml n=137, 5 µg/ml n=151, 10 µg/ml n=141, 15 µg/ml n=138. Values are compared with vehicle control DMSO. Analyses were performed in GraphPad Prism 9.1.0, p-value < 0.01 was considered significant and marked with an ** and p-value < 0.0001 was marked with an ****.

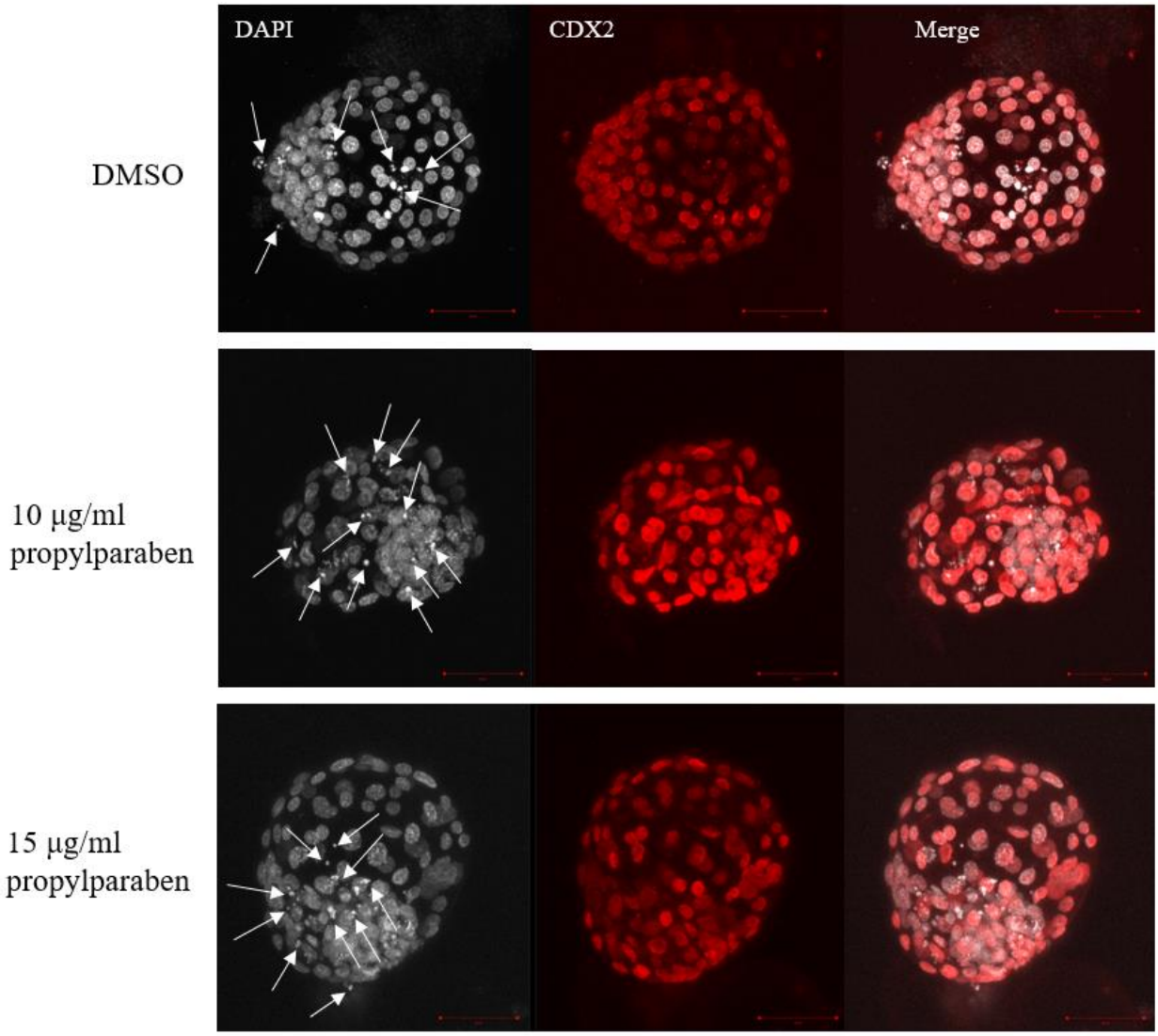

Figure 4: Immunofluorescence for DAPI and CDX2 in hatched blastocysts in various treatments. Nuclei are stained (gray) by DAPI and trophectoderm (TE) cells are stained (red) by CDX2. Images obtained from Zeiss LSM 880 confocal microscope. Scale bars: 50 µm.

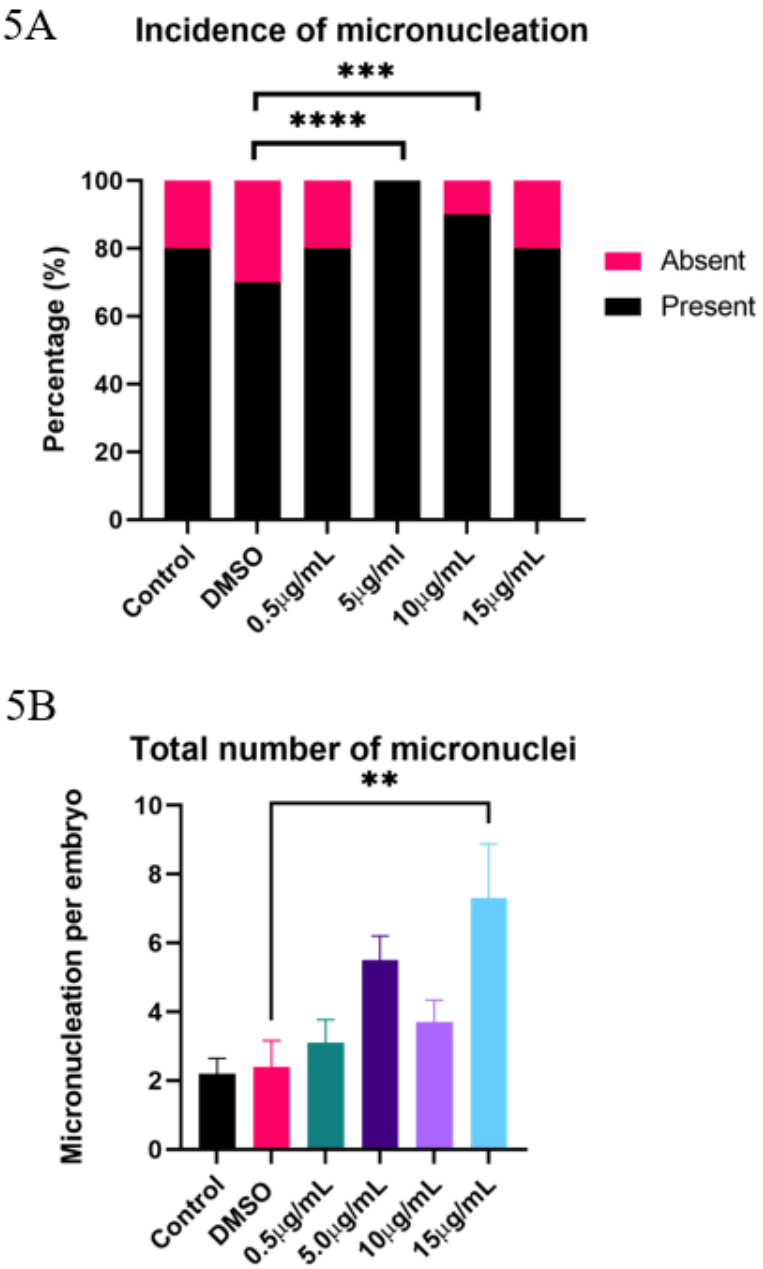

Figure 5: The effects of propylparaben on incidence of micronucleation (5A) and total number of micronuclei (5B). There were significant differences in the occurrence of micronucleation between propylparaben treatments and DMSO 0.075% controls. Incidence of micronucleation was analyzed by chi-square and multiple comparisons using Fisher's exact test. Numbers were compared using ANOVA and Bonferroni's multiple comparisons test. Values are compared with vehicle control DMSO. Analyses were performed in GraphPad 9.1.0, p-value < 0.01 was considered significant and marked with an ** and *p*-value < 0.0001 was marked with an **** and n = 10 for all treatments.

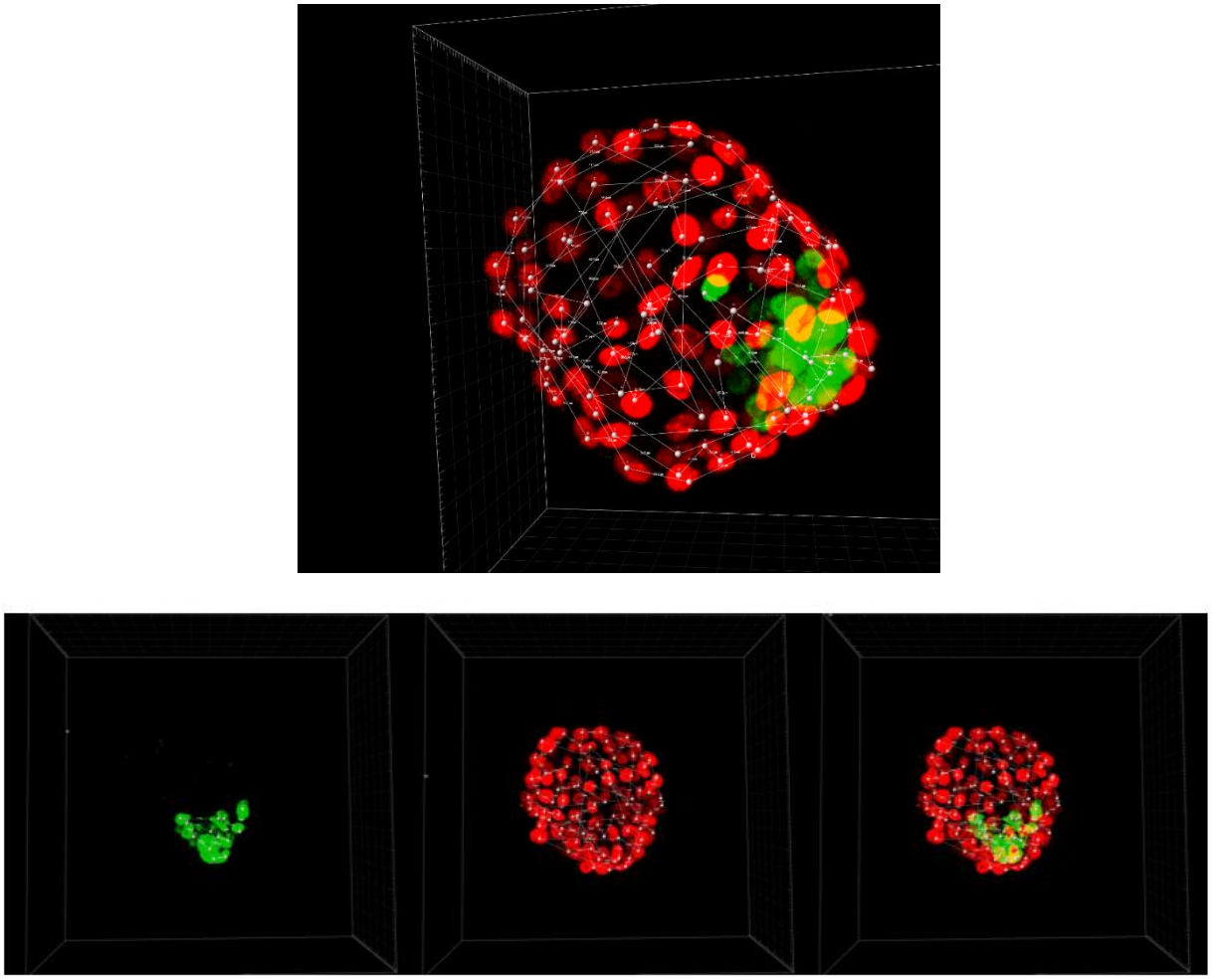

Figure 6: Representative images of analyses performed with Imaris software. Cell numbers were counted in the inner cell mass (ICM) and trophectoderm (TE) cells respectively. To accurately count the number of cells, the specific channel displaying the immunofluorescence (green or red) weas selected and each cell was counted using measurement points (white spheres). The green channel is specific for OCT4 and indicates ICM cells and the red channel is specific for CDX2 indicating TE cells.

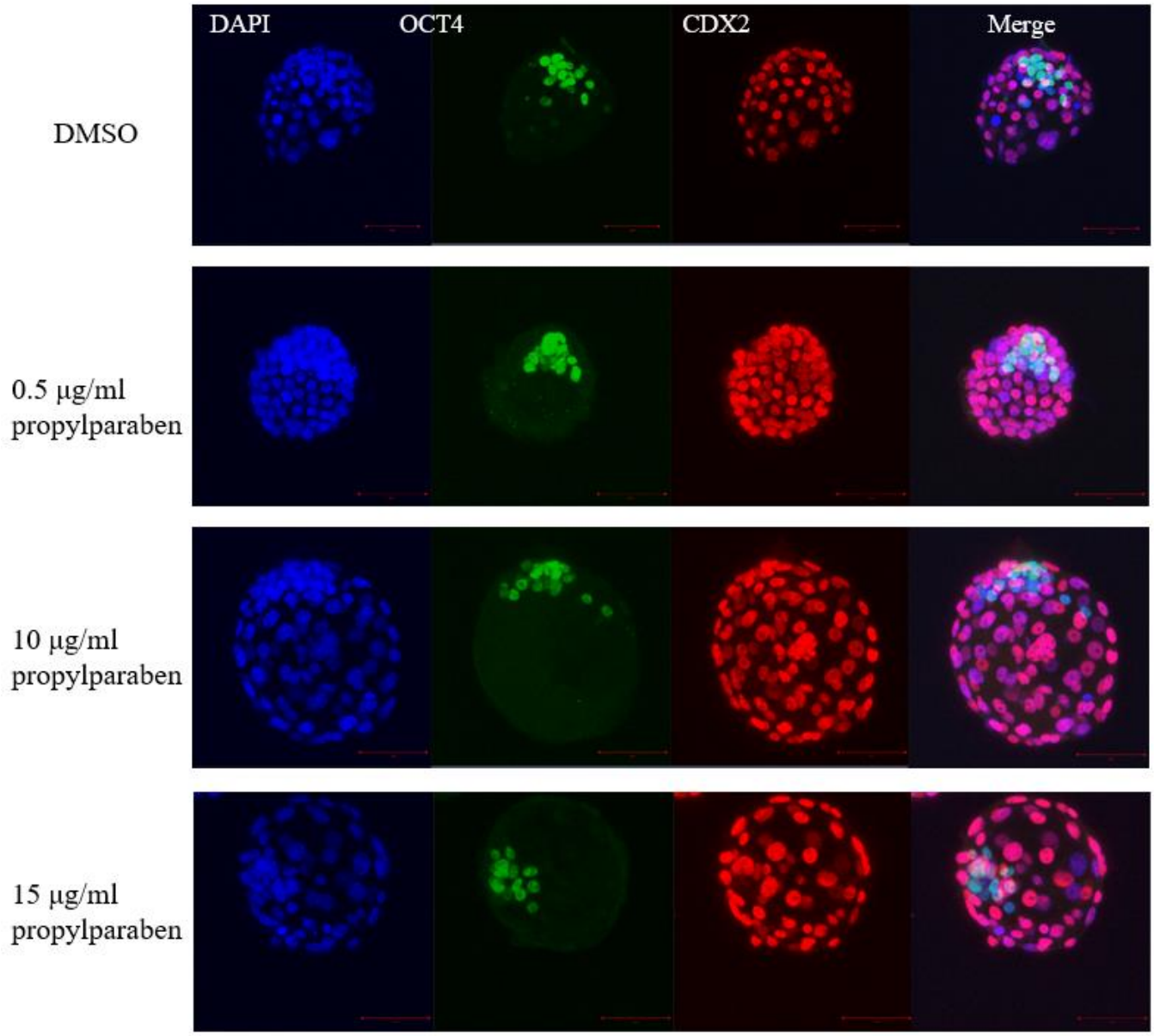

Figure 7: Immunofluorescence staining of trophectoderm (TE) and inner cell mass (ICM) cells in the various treatment groups. Nuclei are stained by DAPI in blue, ICM is stained by OCT4 in green, and TE is stained by CDX2 in red. Images were obtained from Zeiss LSM 880 confocal microscope. Scale bars: 50µm.

Table 2: Number of cells from inner cell mass (ICM) and trophectoderm (TE) cell populations, and ratio between TE and ICM in analyzed hatched blastocysts.

	N	Inner cell mass (ICM)	Trophectoderm (TE)	Ratio TE:ICM
Control	15	22.65 ± 3.036	63.35 ± 5.838	3.335 ± 0.3723
DMSO	23	22.39 ± 1.985	69.57 ± 4.334	3.659 ± 0.4112
0.5 µg/mL PP	15	13.42 ± 1.109 *	75.32 ± 3.504	6.221 ± 0.4967
5 µg/mL PP	26	16.77 ± 2.009	63.77 ± 5.470	4.789 ±0.7639
10 µg/mL PP	15	19.53 ± 2.176	73.78 ± 5.530	4.211 ± 0.3603
15 µg/mL PP	18	14.87 ± 1.412 ^	70.57 ± 4.157	5.413 ± 0.4150

Values presented are mean ± SEM. TE and ICM cell population numbers were analyzed by ANOVA and Tukey's multiple comparisons test. Values are compared with vehicle control DMSO. Analyses were performed in GraphPad Prism 9.1.0, significance was considered at p-value < 0.05 and marked with * and ^ for trending significance respectively.

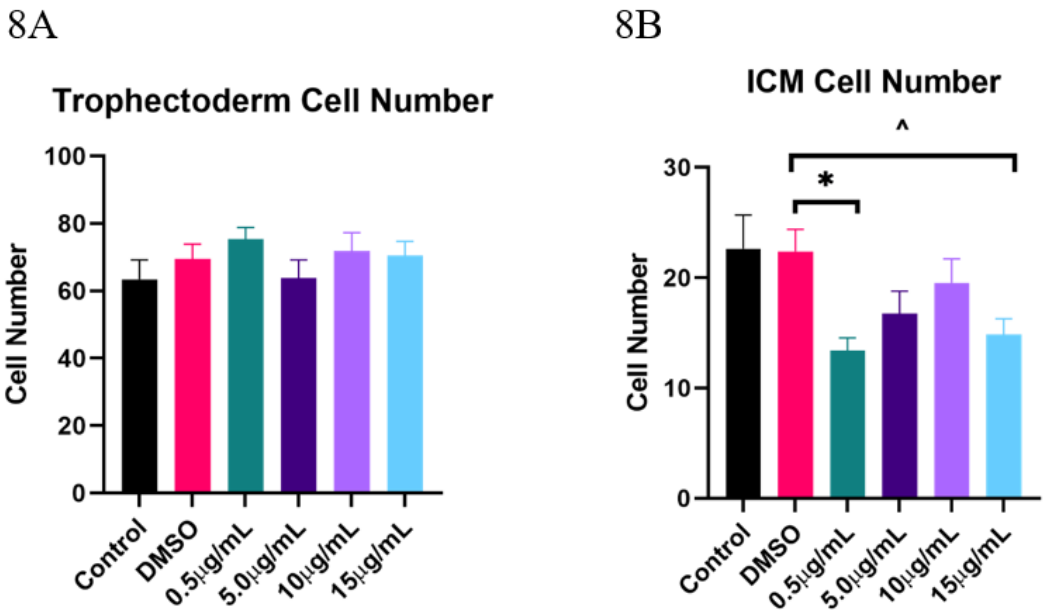

Figure 8: Effect of propylparaben on TE (8A) and ICM (8B) cell populations in hatched blastocysts. There was no significant difference in TE cell numbers between any treatments. There was statistical significance between DMSO 0.075% control and 0.5 µg/ml propylparaben (*p*-value = 0.0261). There was a trend toward significance between DMSO 0.075% control and 15 µg/ml propylparaben (*p*-value = 0.0713). Control n=20; DMSO 0.075% n= 23; 0.5 µg/mL n=19; 5 µg/mL n=22; 10 µg/mL n=18; 15 µg/mL n=23 for both analyses. Cell numbers were analyzed using ANOVA and Tukey's multiple comparisons test. Values are compared with vehicle control DMSO. Analyses were performed in GraphPad 9.1, *p*-value < 0.05 was considered as significant and marked with * and trend toward significance marked with ^.

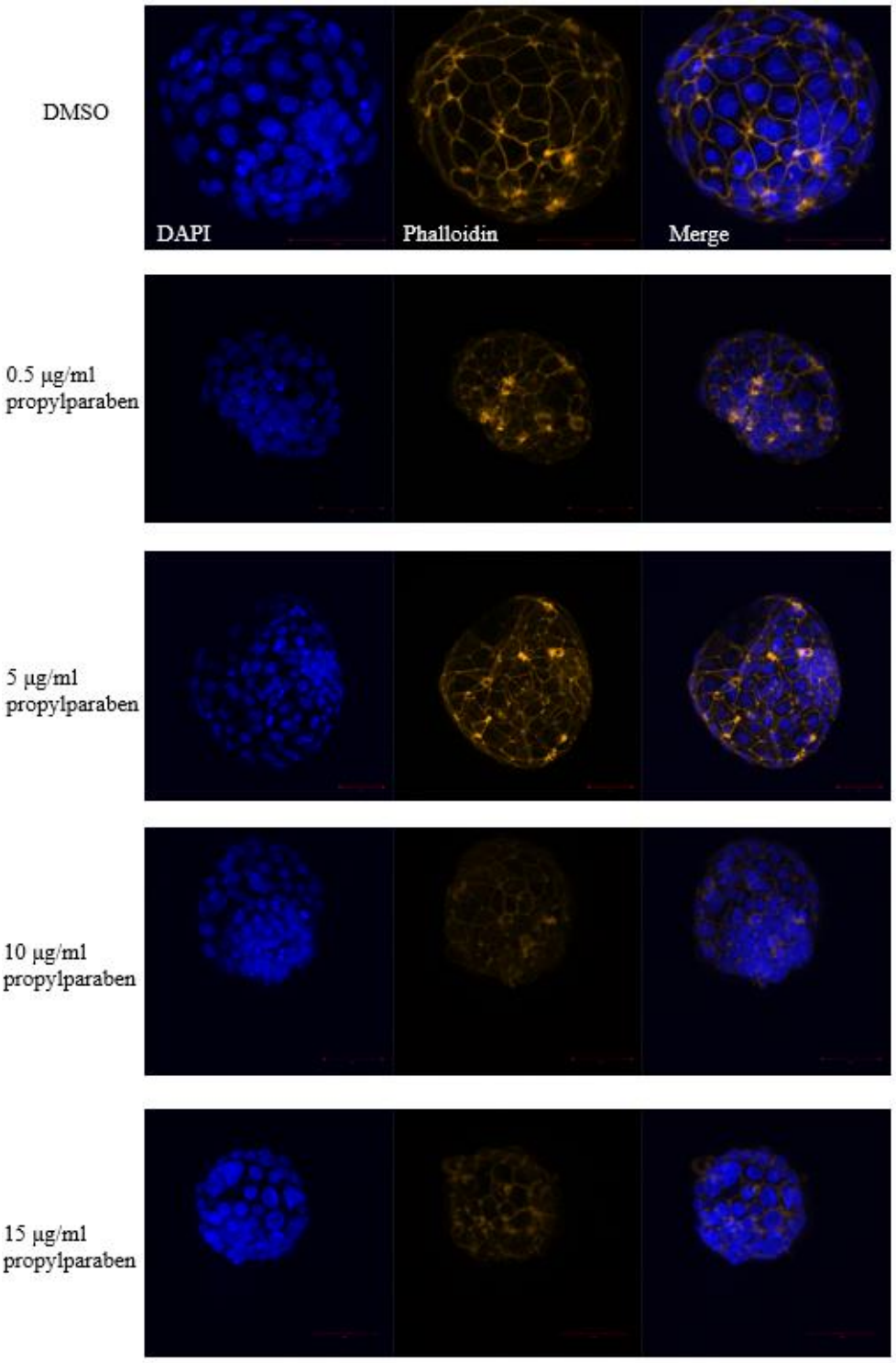

Figure 9: Fluorescence staining of F-actin in hatched blastocysts from various doses of propylparaben and vehicle control DMSO. Representative images show phalloidin staining for F-actin in orange and nuclei stained with DAPI in blue. Images were obtained using a ZEISS LSM 880 confocal microscope. Scale bars: 50 µm.

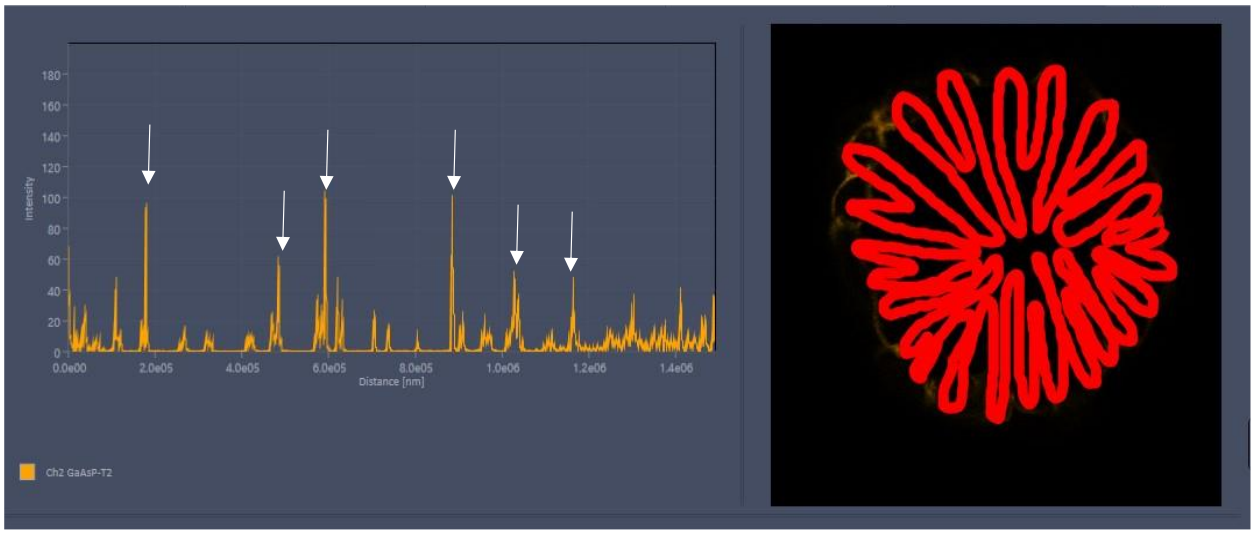

Figure 10: Analysis and quantification performed for F-actin expression in blastocysts using ZEN Lite software. A drawn line was marked across the area of the blastocyst and a fluorescence intensity graph was generated. Peaks of fluorescence intensity (marked with white arrows) were obtained at distances between 8 µm and 21 µm planes of the blastocyst to obtain an average of the intensity for a total of six planes per blastocyst.

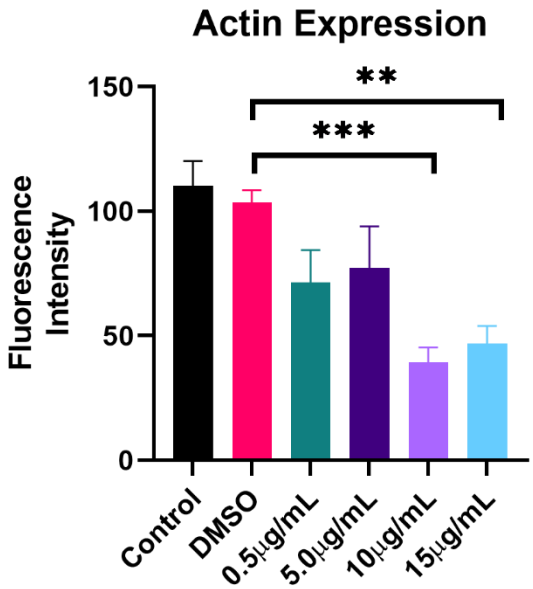

Figure 11: Analysis of cytoskeleton protein F-actin expression in hatched blastocysts. Fluorescence intensity of F-actin in hatched blastocysts was significantly affected by propylparaben exposure at 10 µg/mL (*p*-value = 0.0004) and 15 µg/mL PP (*p*-value = 0.0035) in comparison with vehicle control DMSO. Control n=18; DMSO n= 22, 0.5 µg/mL n=15; 5 µg/mL n=20; 10 µg/mL n=16; 15 µg/mL n=15. Values are compared with vehicle control DMSO. Data were analyzed by ANOVA and Tukey's multiple comparisons test, performed in GraphPad 9.1, *p*-value < 0.01 was considered significant and marked with an ** and *p*-value < 0.001 was marked with an ***.

Discussion

Propylparaben is a known endocrine disrupting chemical, with detrimental effects on embryo development. The goal of this study was to investigate the direct effects of propylparaben on early preimplantation embryo development utilizing a mouse model to expand on the limited scientific literature regarding the effects of propylparaben on the female reproductive system. Here we have demonstrated that propylparaben reduces blastocyst hatching ability, increases the incidence of micronucleation, negatively impacts F-actin expression in the cytoskeleton, and decreases inner cell mass cell numbers in embryos.

To successfully achieve pregnancy, the blastocyst needs to hatch from the zona pellucida (ZP) in a timely manner for implantation within the maternal uterus. Blastocyst hatching and uterine implantation are coordinated events that are critically linked for establishment of pregnancy at a specific time. The timing of hatching from the ZP is critical as early hatching leads to ectopic pregnancies and late hatching results in a missed implantation window (Jun *et al*., 2004; Diedrich *et al*., 2007; Leonavicius *et al*., 2018). Blastocyst hatching is thought to be regulated by growth factors, cytokines, and proteases with the dynamics of actin-based trophectodermal projections (TER). Yet, the understanding of the specific key molecular networks and molecular regulators that mediate ZP hatching are not fully understood (Seshagiri *et al*., 2015). Results from this study demonstrate that propylparaben exposure (10 µg/mL and 15 µg/mL) reduced the percentage of successful hatching in preimplantation embryos compared to the DMSO 0.075% control. There was also an increase in the numbers of fragmented embryos in the two highest propylparaben treatments compared to DMSO 0.075% control. Embryos that were exposed to any of the concentrations of propylparaben (0.5µg/mL, 5 µg/mL, 10 µg/mL, and 15 µg/mL) were able to develop into blastocysts but not many were able to hatch from the ZP in a timely manner on day

6 compared to embryos cultured in the DMSO 0.075% control. These findings suggest that the ability of developing embryos to successfully hatch from the ZP is reduced by exposure to propylparaben.

Based on previous studies, it is well known that embryos undergo fragmentation during development due to chromosome loss, apoptosis, and improper cell division during early stages of embryo development (Gardner *et al*., 2004; Daughtry *et al*, 2019; Tan *et al*., 2019; McCollin *et al*., 2020). Improper cleavage divisions linked to DNA damage is thought to cause fragmentation in human preimplantation embryos (Kort *et al.*, 2016). Endocrine disrupting chemicals such as pesticides (atrazine, DDT, and vinclozoline) have been reported to cause aneuploidy, disrupted spindle formation, and DNA abnormalities in preimplantation embryos (Campagna *et al*., 2001; Marques-Pinto *et al*, 2013; Yuan *et al*., 2017). Gal *et al*., 2019 reported that levels of *Bax* (pro-apoptotic factor) were significantly increased in mouse antral follicles exposed to propylparaben (100 µg/mL) after 72 hours. Our study's result observed a substantial increase in embryo fragmentation in mouse embryos cultured in the two highest propylparaben treatments (10 µg/mL and 15 µg/mL). Therefore, it is possible that propylparaben exposure disrupts chromosomal functionality, causing aberrant cleavage patterns, and leading to cellular arrest during early embryo development. Yet, more studies are needed to confirm and explore embryonic development mechanisms that may be affected by propylparaben exposure

Micronucleation is a commonly observed nuclear abnormality in preimplantation embryos resulting from DNA errors and mitotic chromosomal abnormalities during cellular division (Kort *et al*., 2016). Micronuclei are small extranuclear chromatin fragments or whole chromosomes that lag and become excluded during the telophase stage of cell division (Kort *et al*., 2016; Hintzsche *et al*., 2017). This occurrence arises from lagging chromosomal assembly, unrepaired fragmented

DNA double strand breaks from DNA replication, and improper nuclei assembly during mitotic exit (Krupina *et al*., 2021). Previous studies have indicated that the presence of micronucleation in embryos had a significantly negative impact on live birth rates in *in vitro* fertilized embryos, reduced implantation rate, and aneuploidy (Royen *et al*., 2003; Desch *et al.*, 2017). Our results demonstrated that embryos exposed to propylparaben *in vitro* at 5 µg/mL and 10 µg/mL had increased incidence and number of micronuclei at the hatched blastocyst stage. This suggests that exposure to propylparaben may cause chromosomal instability, genotoxic stress, and delayed chromosomal assembly during early embryo development.

Cell lineage commitment and differentiation during early embryo development is a crucial event, where cell fate is committed initially to the trophectoderm (TE) or inner cell mass (ICM) lineage. The TE is involved directly with implantation of the embryo within the maternal uterus and progressively develops into tissues of the placenta (Marikawa *et al.*, 2009; Posfai *et al*., 2019). The ICM consists of the cells from which the three germ layers of the developing embryo originate after successful implantation, and these layers will give rise to all fetal tissues (Marikawa *et al*., 2009; Weberling and Zernicka-Goetz, 2021). The expression of these two cell types is determined by octamer-binding protein (OCT4) for ICM and caudal-type homeobox-2 gene (CDX2) for TE cells. We utilized OCT4 and CDX2 as protein markers to accurately count the number of ICM and TE cells in hatched blastocysts respectively, but their overall mRNA expression levels were not quantified. Immunofluorescence staining procedures were performed to visualize the different cells and accurately quantify the cell numbers in each respective cell lineage. There were no significant differences in the numbers of TE cells in propylparaben treated embryos compared to controls. However, there was a significant decrease in ICM cell number in 0.05 µg/mL propylparaben treatment compared to DMSO 0.075% control. There was also a trend toward

significant difference between the DMSO 0.075% control and 15 µg/mL propylparaben (*p*-value = 0.0713) treatment in for ICM cell numbers. Previous studies have shown that the absence of OCT4 will result in failed implantation and that leads to lower quality embryos (Nichols *et al*., 1998; Van Thuan *et al*, 2006). The loss of OCT4 expression results in primordial germ cell apoptosis and loss of mammalian germline viability (Kehler *et al*., 2004). These results suggest that propylparaben exposure of embryos may negatively impact OCT4 expression and may lead to subsequent problems in cell fate specification.

The direct effects of propylparaben on F-actin expression in the cytoskeletal network of mouse blastocysts were also examined. F-actin is a cytoskeletal protein that plays an important role in contributing to progression of meiosis and subsequent embryo development (Simon and Wilson, 2011; González *et al*., 2012). Previous research has shown that actin disorganization may lead to impaired preimplantation embryonic development (Tan *et al*., 2015). Dysregulation of actin encoding genes suggests that it may also lead to increased embryo mortality. The *Bcl2* gene is involved in inhibiting cell adhesion by enhancing actin polymerization and deficiency in *Bcl2* leads to oocyte maturation arrest and apoptosis during early embryonic development (Boumela *et al*., 2011). *Palld* is an actin-associated protein that is required for proper actin cytoskeleton organization and impairment results in embryonic lethality from severe defects in cranial neural tube closure (Luo *et al*., 2005). Our results demonstrate that there was a significant decrease in F-actin expression in embryos treated with propylparaben (10 µg/mL and 15 µg/mL) compared to DMSO 0.075% control. Immunofluorescence images provided visual comparisons that revealed that F-actin was diffusely distributed in embryos treated with the two highest concentrations of propylparaben when compared with DMSO 0.075% control. As mentioned above, blastocyst hatching involves several processes that include the dynamics of actin-based trophectodermal

projections (TER) (Seshagiri *et al*, 2015). Results show that the two highest propylparaben treatments (10 µg/mL and 15 µg/mL) had a lower hatching rate and a reduced F-actin expression. This suggests that the reduced F-actin expression and networking may be directly involved in the lower success rate of hatching in propylparaben exposed embryos. Overall, our findings suggest that propylparaben may alter important actin-related proteins during pre-implantation embryo development.

Conclusion

Our findings demonstrate that propylparaben exposure during early embryonic preimplantation has negative impacts on developmental parameters and expression of several proteins that play critical roles for successful implantation and pregnancy. Additional studies are needed to further investigate the propylparaben effects on preimplantation embryo health and subsequent effects on pregnancy outcomes.

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CONCLUSION AND FUTURE DIRECTIONS

This study examined the direct effects of propylparaben on cultured preimplantation embryo development utilizing a mouse model. Propylparaben was first utilized in pharmaceutical products in 1923 and since it is commonly used as an additive preservative in food, drugs, and cosmetics. Generally, the use of propylparaben is viewed as safe to consumers. Yet, there is limited number of scientific studies on the effects of propylparaben in male and female reproductive systems. Preimplantation embryo development is a critical timepoint to investigate as disruption during this phase can lead to reduced embryo viability, aneuploidy, apoptosis, and failed implantation.

Results from this study provide insights that embryonic exposure to propylparaben concentrations of 10 µg/mL and 15 µg/mL increases fragmentation rate and reduces blastocyst hatching competence from the zona pellucida. There was also an increase of micronucleation in blastocysts cultured with all propylparaben treatments in comparison with vehicle control, indicating that propylparaben may cause chromosomal instability and delayed cellular division during embryogenesis. Blastocyst hatching involves mechanical processes, internal osmolarity, and secretion of lytic enzymes for chemical dissolution of the zona. Thus, based on our findings, investigating transcription factors and the mechanisms stated above are some optional endpoints for future studies to pursue. Regulatory molecules such as embryotrophic factors which include transcription factors, proteases, growth factors, cytokines, and secondary messengers also warrant further analysis.

This study demonstrates that propylparaben disrupted various protein markers related to implantation and cell fate decision. Results show that propylparaben exposure reduced OCT4

expression in the hatched blastocysts. In 0.5 µg/mL propylparaben treatment, there was a significant decrease in F-actin fluorescence intensity and in 15 µg/mL propylparaben treatment, there was a trend to significance. It was also expected that there would be a decrease in CDX2 in propylparaben treated embryos but there were no significant differences in comparison with vehicle control. This provided insight that propylparaben had an effect on the ICM but not TE cell numbers in hatched blastocysts. OCT4 and CDX2 have a reciprocal interaction in repressing each other gene transcription in embryonic stem cells, and this complex interaction should be investigated further. Since results demonstrate a decrease in OCT4 expression, it would be worth analyzing genes that are associated with cell proliferation and cell cycle control such as *Mif, Mybl2, Myc,* and *Mcyn* that are directly activated by POU51 gene. In addition, analyzing the levels of OCT4 and CDX2 expression in hatched blastocysts will also provide more data to further confirm the results of this study. Another key gene marker worth studying is estrogen receptor 1 (ESR1) and androgen receptor (AR). Considering that propylparaben has known weak estrogenic and antiandrogenic properties, this hormone mimicking action may have an effect on sex steroid receptors that resulted in a decrease in genetic response of OCT4 or other downstream molecular pathways.

Furthermore, F-actin expression was also significantly decreased in propylparaben treated embryos. Based on visual fluorescent imaging, it was apparent that it was diffusely distributed in propylparaben treated embryos when compared with the vehicle control where the F-actin networking was clear and well-organized. The two highest propylparaben treatments had a significantly reduced F-actin expression as well. F-actin are polymerized cytoskeletal filaments that are highly involved in cellular support, mobility, and morphogenesis regulation during cellular division. The regulation of F-actin cytoskeleton involves dynamic regulation with cadherin

junctions and various protein complexes. Previous research has shown that dynamin has direct interactions with microtubules and cytoskeletal filaments. Dynamin interacts with actin-related assembly factors, cellular scaffolding, and signal transduction enzymes that ultimately regulates actin filaments. Cadherin junctions are involved in junctional actin cytoskeleton and a decrease in cadherin reduce cell-cell junctions of F-actin. It would expand the F-actin findings if these protein complexes that directly influence F-actin organization and development are analyzed.

Overall, the results of this study indicate that environmentally relevant doses of propylparaben disrupt preimplantation mouse embryo development and several protein markers crucial for cell fate decision and cytoskeletal structural support. This study presents the novel findings of propylparaben and its effects on developing preimplantation embryos, and this warrants further assessments to determine the possible toxic effects of this synthetic chemical. Considering the established fact that propylparaben is commonly used in cosmetic products and women make up one of the largest consumers of these products, it is imperative that the effects of propylparaben in female reproductive function are investigated and explored further.

FIGURES

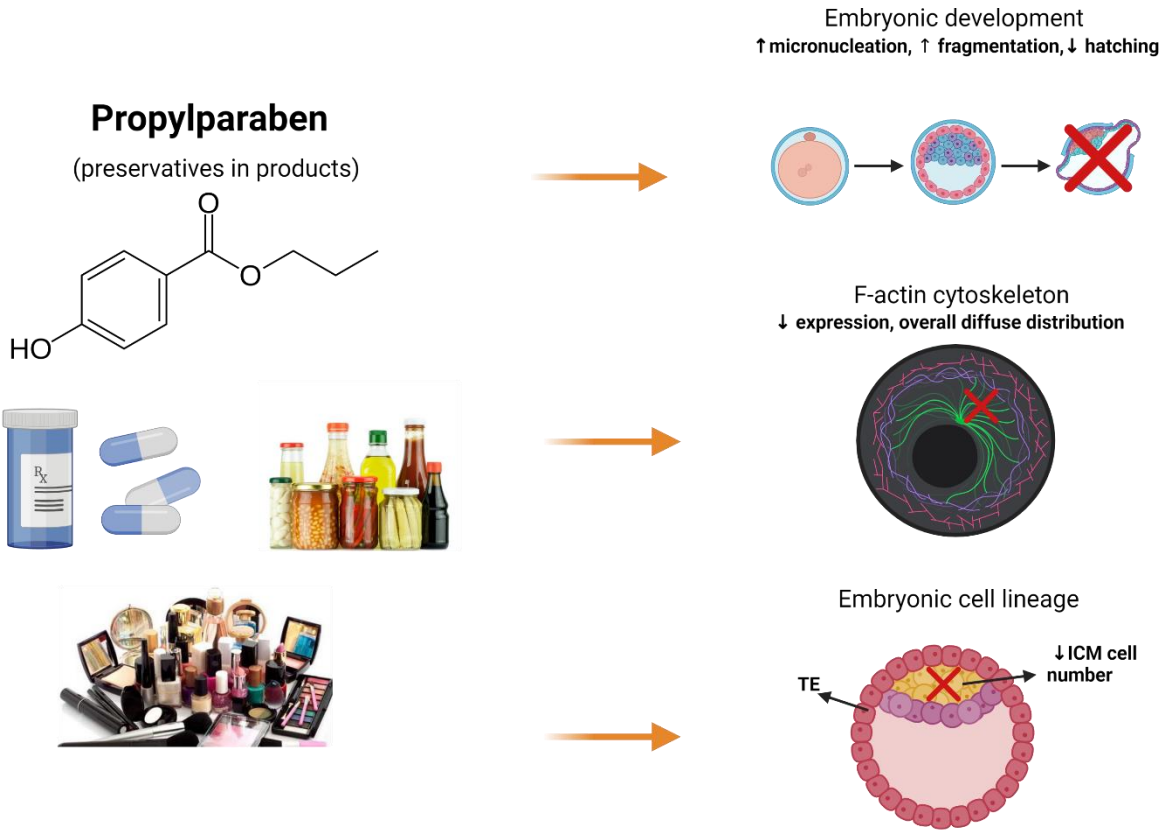

Figure 12: Graphical abstract of research study. The aims of the study were to investigate the effects of propylparaben during preimplantation embryo development. From the study, it was determined that propylparaben reduced blastocyst hatching ability, increased fragmentation rate, increased the incidence of micronucleation, decreased F-actin and inner cell mass cell numbers in hatched blastocysts.

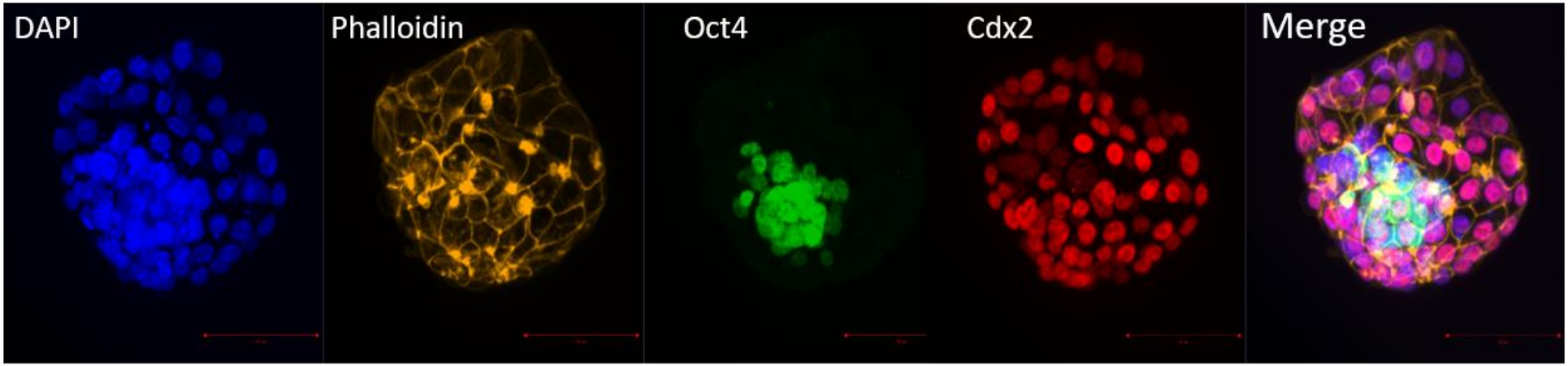

Figure 13: Representative image of immunofluorescence staining of hatched blastocyst for inner cell mass (ICM), trophectoderm (TE), and actin cytoskeleton of a blastocyst in Control. DAPI (blue) stains for cell nuclei, phalloidin (orange) detects cytoskeleton actin, OCT4 (green) stains the ICM, and CDX2 (red) stains the TE cell population. Pictures were obtained using a Zeiss LSM 880 confocal microscope. Scale bars: 50µm.

APPENDIX: PROTOCOLS

Optimized Embryo Culture Medium Recipes

(From Dr. Krisher Laboratory – CCRM/Revised October 18 2016 – JH)

The following solutions were prepared with sterile Milli-Q water and can be stored at 4℃ for 1 month

OEC Base was filtered with a 0.22µm syringe into a clean sterile glass bottle.

Optimized Embryo Culture Medium (OEC) Base

		MW	Final mM	g/100 mL	g/500 mL	g/1 L
NaCl	S6191	58.44	100	5.8440	29.2200	58.4400
KCl	60128	74.55	5.0	0.3728	1.8640	3.7280
KH_2PO_4	60218	136.1	0.50	0.0681	0.3406	0.6810
$CaCl_2 2H_2O$	21097	147.0	1.70	0.2499	1.2496	2.4990

*Omit KH_2PO_4 for Modified/PO_4-free OEC Base.

$MgSO_4$ Stock

		MW	Stock mM	
$MgSO_4$-$7H_2O$	Sigma M2773	246.5	50.0	0.1233 g/10 mL 1.2330 g/100 mL 3.0826 g/250 mL

Glucose Stock

		MW	Stock mM	
Glucose	Sigma G6152	180.2	100.0	0.1802 g/10 mL 1.8020 g/100 mL 4.505 g/250 mL 9.0100 g/500 mL 18.0200 g/1 L

L-Lactate Stock

		MW	Stock mM	
L-Lactate	MPbio 190228	90.08	100.0	0.0901 g/10 mL 0.9008 g/100 mL 2.252 g/250 mL 4.5040 g/500 mL 9.0080 g/1 L

Taurine Stock

		MW	Stock mM	
Taurine	Sigma T8691	125.1	50.0	0.0626 g/10 mL 0.3130 g/50 mL 0.6260 g/100 mL

EDTA Stock

		MW	Stock mM	
$Na_2EDTA\text{-}2H_2O$	JT Baker 8994-01	372.24	2.0	0.0075 g/10 mL * 0.0375 g/50 mL #

*dissolve in 220 µL 1.0 M NaOH and QS to 10mL (9.78 mL MilliQ H_2O)

#dissolve in 1.1 mL 1.0 M NaOH and QS to 50 mL (48.9 mL MilliQ H_2O)

Weekly Stocks

- Store at 5°C for **1 week**

Pyruvate Stock

		MW	Stock mM	g/10 mL
Na-Pyruvate	Sigma P4562	110.0	20.0	0.0220

Citrate Stock

		MW	Stock mM	g/10 mL
Citrate	Sigma C2404	192.12	50.0	0.0961

alpha Lipoic Acid (10 µM)

Sigma T1395, MW = 206.33

Prepare both stocks fresh before each use- DO NOT STORE

1) Stock A = dissolve 0.0083 g (8.3 mg) in 1 mL 100% ethanol
   Mix well, do not filter.
2) Stock B = add 600 µL Stock A to 5.4 mL MilliQ water.
   Mix well, do not filter.
3) 2.5 µL of Stock B per mL of medium = 10 µM

1.0 M NaOH

Sigma 71687

1) Weigh 2.0000 to 2.4000 g of NaOH into a 50 mL flask and record the exact weight.
2) Divide the weight in grams by 40 (formula weight of NaOH).
3) The result is the amount of MilliQ H_2O to be added in liters.
4) For example, if you have exactly 2.0000 g NaOH, divide by 40, you get 0.050 L, so you need to add 50 mL MilliQ H_2O to the flask.
5) This solution does not need to be filtered.
6) Store at room temperature.

Other Chemicals Used:

Glutagro (200 mM Ala-Gln) – cellgro/Mediatech 25-015-CI

Non-Essential Amino Acids (100x, MEM) – cellgro/Mediatech 25-025-Cl

Hyaluronidase

Sigma H-4272 (Embryo Tested), 30 mg/vial (~750-1500 U/mg)

1) Dilute to 10 mg/mL with 3 mL of OMOPS.
2) Aliquot 30 µL in sterile 0.5 mL tubes and store at -80 ℃.
3) Use 5 µL of this stock per 100 µL of OMOPS = 500 µg/mL (~375-750 U/mL).

MOPS-buffered OEC Handling Medium – OMOPS

1) Place approximately 25 or 250 mL MilliQ H_2O into a large beaker with a stir bar. Add the following stocks:

Stock (Final Concentration)	***mL of stock per***	
	100 mL	***1 L***
OEC Base	10	100
$MgSO_4$ (1.2 mM)	2.4	24
Glucose (0.5 mM)	0.5	5
L-Lactate (6.0 mM)	6	60
GlutaGRO (Ala-Gln, 1.0 mM)	0.5	5
Taurine (0.1 mM)	0.2	2
NEAA (1x)	1	10
EDTA (0.01 mM)	0.5	5
Alpha Lipoic Acid (10 µM)	0.25 mL stock B	2.5 mL stock B
Undiluted Gentamicin (10 µg/mL)	20 µL	200 µL
Hyaluronan (0.125 mg/mL)	2.5	25
1.0 M NaOH	2.1	21

2) Add the following ingredients while stirring:

Stock (Final Concentration)	***100 mL***	***1 L***
$NaHCO_3$ (5.0 mM)	0.0420 g	0.4200 g
MOPS (20.0 mM) *Sigma PHG0007, MW = 209.26*	0.4185 g	4.1852 g
Pyruvate (0.2 mM)	1 mL stock	0.0220 g
Citrate (0.5 mM)	1 mL stock	0.0961 g
FAF BSA (4.0 mg/mL)	0.4000 g	4.0000 g

3) Stir until all ingredients, including BSA, has dissolved.
4) QS to final volume in a volumetric flask. Pour the solution down the side of the flask SLOWLY to avoid bubbles.
5) Filter (0.22 µm), label, and store at 5°C. ***Expires in 1 month*****.**

Observation: We did not include hyaluronan in the MOPS.

Embryo glassware dishwashing procedure

- Always wear gloves when washing dishes (to protect yourself from chemicals that may be on the glassware), autoclaving, and when handling clean dishes (to keep them clean).
- DO NOT wash Embryo culture glassware with Alconox or other detergents (embryos are sensitive to soap). Keep away from glassware/chemicals. We have a plastic bin to put them inside (close to the sink facing the wall).
    - Rinse with MilliQ water (from Dr. Dean's lab). Remove laboratory tape; however, be careful to leave "Embryo Only" label in place (and other labels that are attached with transparent tape).
    - Soak in MilliQ water overnight inside the bin. Rinse 3 times with MilliQ water.
    - Leave to dry inside the bin.
    - Autoclave as you would other glassware.
    - Once autoclave is done, put glassware back in the Embryo Room.

Reviewed: Rachel Arcanjo and Katy East 9/6/2019

Reviewed: Katy East and Nastasia Lai 12/10/2019

Immunofluorescent Staining of Trophectoderm and Inner Cell Mass

[Original from Krisher's lab with modifications suggested by Shiv (IGB core), troubleshoot by Rachel Braz Arcanjo. Modified by Katy East (1/16/2020) and Nastasia Lai (2/12/2020).]

Solutions:

Holding media:
50 mL PBS + 0.25 g BSA (0.5%)

*4% Paraformaldehyde (4% PFA – **Make day before or day of fixation**):*
47.5 mL DI water + 2.5 mL 20X PBS + 2 g paraformaldehyde. **Must be done in the fume hood!**

1) Add 2g solid paraformaldehyde to DI water. Dissolve on heated stir plate inside the fume hood at level 5 spin and lowest level heat.
2) Add 5-10 drops 1M NaOH with small dropper until powder dissolves.
3) Adjust pH to 7.2-7.4. Filter with filter paper and let cool. Store at 4ºC for up to a week.

Washing buffer (WB):
500 mL PBS + 500 µl Triton X-100 (0.1%) + 0.5 g PVP (0.1%)
(PVP is poly-vinyl-pyrrolidone from Sigma PVP40 CAS#9003-39-8)

Permeabilization Buffer (PB):
50 mL PBS + 500 µl Triton X-100 (1%)

Blocking (BB):
50 mL PBS + 50 µl Triton X-100 (0.1%) + 0.5 g BSA (1.0%) + 0.3754 Glycine (0.1 M)
For each replicate, put 450 µl BB in a well and add 50 µl Horse Serum.

Antibody Buffer (AbB):
50 mL PBS + 50 µl Triton X-100 (0.1%) + 0.5 g BSA (1%)

Antibodies:

Primary: - Anti-OCT4 rabbit mAB (Cell Signaling - D6C8T)
100 µl | Suggested dilution 1:800 | Do not aliquot
- Anti-CDX2 mouse monoclonal antibody (Biogenex MU392A-100 (sample)
100 µl | Suggested dilution 1:300 | Shipped with a diluent (HK941-04K)

Secondary - CY3 donkey anti-rabbit (555 nm)
Jackson ImmunoResearch 711-165-152 | Suggested dilution 1:500
- CY5 donkey anti-mouse (690 nm)
Jackson ImmunoResearch 715-175-151 | Suggested dilution 1:400

Notes:
Primary/secondary antibodies are found in Rachel's box in -20ºC fridge. Use Agtech 6-well dishes (plastic) and glass round bottom plates for this protocol.

FIXATION (done: _____/_____/_____)

1. Prepare fresh 4% paraformaldehyde using above protocol.

2. Fix embryos by incubating in 4% paraformaldehyde for 20 minutes at room temperature.

3. Transfer embryos to Holding Media (cover drops/wells with oil) and maintain at 4ºC until staining.

Date: _____/_____/_____ Start time: _____:_____ End time: _____:_____

Day 1	Procedure	Time	Notes
	Wash in Washing Buffer	3x 10 minutes	**500 µl/well for each buffer step**
	Incubate in Permeabilization Buffer	30 minutes	
	Wash in Washing Buffer	10 minutes	
	Incubate in Blocking Buffer + donkey serum	2 hours	**450 µl of blocking buffer + 50 µl of donkey serum/well**
	Wash in Washing Buffer	25 minutes	
	Incubate with primary: anti-Oct-4 antibody (dilution 1:800) Diluted in AbB anti-CDX-2 antibody (dilution 1:300) made with respective diluent	Overnight at 4ºC	**250 µl/well. Transfer embryos to wells. Wrapped 6-well dish in parafilm and put inside the fridge**
		4 hours	

Incubation time was _____:_____

Date: _____/_____/_____ Start time: _____:_____ End time: _____:_____

Day 2	Procedure	Time	Notes
	Wash in Washing Buffer	3x 10 minutes	
	Incubate with secondary antibody: CY3 donkey anti-rabbit (1:500) Diluted in AbB CY5 donkey anti-mouse (1:400) Diluted in AbB	2 hours in dark at RT	Make one solution with both secondaries. Put 200 µl/well of that solution and transfer embryos to well. Cover with foil. ***Protect from light now and all subsequent steps!***
	Wash in Washing Buffer	3x 10 minutes	
	Incubate with actin stain: Phalloidin (1:1000) Diluted in AbB	90 minutes in dark at RT	200 µl/well. Cover with foil.
	Wash in Antibody Buffer	2x 20 minutes	
	Wash in Washing Buffer	1 hour	
	Mount embryos in a 35 mm Glass bottom dish with 14 mm micro-well. Make 1 dish/treatment (all embryos from same treat can be together) Incubate with DAPI.	30 minutes in dark at RT	Add 10-20 µl of mounting media with DAPI. Put embryos in mounting media then put 80 µl of mineral oil around the mounting media. Ensure no air is left in the well.
		7 hours	
	Coverslip with the help of a curved forceps.		
	Keep in at 4ºC in the dark for 24-48 hours before imaging (in a box inside the fridge).		

OCT-4 - should show green → specific for inner cell mass

CDX-2 - should show red → specific for trophectoderm

Immunofluorescent Staining of F-Actin

[Original from Krisher's lab with modifications suggested by Shiv (IGB core), troubleshoot by Rachel Braz Arcanjo. Modified by Katy East (1/16/2020) and Nastasia Lai (2/12/2020).]

Solutions:

Holding media:
50 mL PBS + 0.25 g BSA (0.5%)

*4% Paraformaldehyde (4% PFA – **Make day before or day of fixation**):*
47.5 mL DI water + 2.5 mL 20X PBS + 2 g paraformaldehyde. **Must be done in the fume hood!**

1) Add 2g solid paraformaldehyde to DI water. Dissolve on heated stir plate inside the fume hood at level 5 spin and lowest level heat.
2) Add 5-10 drops 1M NaOH with small dropper until powder dissolves.
3) Adjust pH to 7.2-7.4. Filter with filter paper and let cool. Store at 4ºC for up to a week.

Washing buffer (WB):
500 mL PBS + 500 µl Triton X-100 (0.1%) + 0.5 g PVP (0.1%)
(PVP is poly-vinyl-pyrrolidone from Sigma PVP40 CAS#9003-39-8)

Permeabilization Buffer (PB):
50 mL PBS + 500 µl Triton X-100 (1%)

Blocking (BB):
50 mL PBS + 50 µl Triton X-100 (0.1%) + 0.5 g BSA (1.0%) + 0.3754 Glycine (0.1 M)
For each replicate, put 450 µl BB in a well and add 50 µl Horse Serum.

Antibody Buffer (AbB):
50 mL PBS + 50 µl Triton X-100 (0.1%) + 0.5 g BSA (1%)

Staining: Phalloidin-iFluor 488 Reagent (488 nm)
Abcam [ab176753] | 300 tests | Suggested dilution 1:1000

Notes:
Phalloidin are found in KE box in -20ºC fridge. Use Agtech 6-well dishes (plastic) and glass round bottom plates for this protocol.

FIXATION (done: _____/_____/_____)

1. Prepare fresh 4% paraformaldehyde using above protocol.

2. Fix embryos by incubating in 4% paraformaldehyde for 20 minutes at room temperature.

3. Transfer embryos to Holding Media (cover drops/wells with oil) and maintain at 4ºC until staining.

Nastasia's notes: Phalloidin is not an antibody-based stain. I decided to separate my phalloidin stains as I noticed it faded a few months after staining. To prevent imaging the embryos at different power under LSM 880 in IGB, I decided to stain the treatment embryos together in one procedure to ensure the same imaging conditions.

Day 1	Procedure	Time	Notes
	Wash in Washing Buffer	3x 10 minutes	
	Incubate in Permeabilization Buffer	15 minutes	
	Wash in Washing Buffer	3x 10 minutes	
	Incubate with actin stain: Phalloidin (1:1000) Diluted in AbB	90 minutes in dark at RT	200 µl/well. Cover with foil.
	Wash in Washing Buffer	1 hour	
	Mount embryos in a 35 mm Glass bottom dish with 14 mm micro-well. Make 1 dish/treatment (all embryos from same treat can be together) Incubate with DAPI.	30 minutes in dark at RT	Add 10-20 µl of mounting media with DAPI. Put embryos in mounting media then put 80 µl of mineral oil around the mounting media. Ensure no air is left in the well.
		7 hours	
	Coverslip with the help of a curved forceps.		
	Keep in at 4ºC in the dark for 24-48 hours before imaging (in a box inside the fridge).		

Phalloidin – stains green, but I choose orange in imaging → specific for F-actin