Development of a single-cell droplet culture system for studying the effect of bisphenol A on the human granulosa cell line KGN

Master's thesis

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Üksikraku tilgakultuuri väljaarendamine bisfenool A testimiseks inimese granuloosa rakuliinis KGN

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Declaration

Hereby I declare that I have compiled the paper independently and all works, important standpoints and data by other authors have been properly referenced and the same paper has not been previously been presented for grading.

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Abstract

Continuous exposure to endocrine disrupting chemicals (EDCs) that have been shown to alter different hormonal processes has led to a concern about the safety of these compounds during chronic exposure. An EDC bisphenol A (BPA) has been shown to alter different signalling pathways necessary for regulating ovarian follicle growth and maturation. The effect of BPA has been mostly studied on granulosa cell lines which represent only one somatic cell type present in the follicle. Currently there are no methods for studying cellular response heterogeneity in the follicles in response to different EDCs by using single-cell culture.

The aim of this thesis was to study the feasibility of culturing KGN granulosa cells in a single-cell droplet culture by using droplet microfluidics and to evaluate its applicability in testing the effects of BPA.

As a result of the experimental part of this thesis, it was shown that KGN cells can be incubated in a single-cell droplet culture system for 24 hours with only minor effects on cell viability. In addition, it was also shown that physiological and supraphysiological doses of BPA can be used in droplet culture, since no droplet collapsing was observed after 24-hour incubation. BPA treatment in 2D cell culture also proved further its xenoestrogenic properties when treatment with supraphysiological concentrations of BPA and estradiol caused a significant decrease in the number of live cells.

Annotatsioon

Pidev kokkupuude endokriinsüsteemi häirivate ühenditega (*endocrine disrupting chemicals* – EDCs) on tõstatanud küsimuse nende ühendite ohutuse kohta kroonilise kokkupuute korral. Üheks selliseks kemikaaliks on bisfenool A (BPA), mille negatiivset mõju mitmetele munasarja folliikuli kasvamise ja küpsemise eest vastutavatele signaaliradadele on näidatud erinevates granuloosa rakuliinides, mis esindavad vaid üht somaatiliste rakkude tüüpi munasarja folliikulis. Teadaolevalt ei ole praegu kasutusel ühtegi meetodit, mis võimaldaks uurida erinevate munasarja folliikulis olevate rakutüüpide vastust EDC-dele üksikraku kultuuris.

Käesoleva töö eesmärgiks oli uurida tilga mikrofluidika meetodi sobivust granuloosa rakuliini KGN üksikute rakkude eraldamiseks ja inkubeerimiseks. Lisaks sellele oli eesmärk rakendada loodud meetodit , et uurida BPA mõju üksikutele rakkudele.

Töö tulemusena näidati, et KGN rakke on võimalik inkubeerida üksikraku kultuuris 24 tundi, säilitades rakkude kõrge elulemuse sarnaselt kirjanduses toodud tulemustele. Samuti näidati, et BPA lisamine tilkadesse ei mõjuta tilkade stabiilsust ning BPA mõju uurimine üksikutele rakkudele on tilgakultuuris võimalik. Lisaks sellele, näitasid katsed KGN rakkudega 2D kultuuris BPA ning östradiooli sarnast mõju rakkude proliferatsioonile, mis kinnitab veelgi enam BPA ksenoöstrogeenset mõju.

Abbreviations

BPA	bisphenol A
cAMP	cyclic adenosine monophosphate
CL	corpus luteum
DMSO	dimethyl sulfoxide
E2	estradiol
EDC	endocrine disrupting chemical
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
ERE	estrogen response element
EthD-1	ethidium homodimer-1
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
GATA4	GATA binding protein 4
GC	granulosa cell
GnRH	gonadotropin-releasing hormone
HPO	hypothalamic-pituitary-ovarian
IGF	insulin-like growth factor
LH	luteinizing hormone
PKA	protein kinase A
P	progesterone
PBS	phosphate-buffered saline
PCOS	polycystic ovarian syndrome
PF	primordial follicle
ROS	reactive oxygen species
SF-1	steroidogenic factor-1
UGT	uridine-5-diphospho-glucuronosyltransferase

Introduction

Endocrine disrupting chemicals (EDCs) are exogenous chemicals that can interfere with hormonal processes and cause negative effects on any part of the endocrine system. One of the most studied EDCs is bisphenol A (BPA) that has been shown to alter different pathways that regulate ovarian folliculogenesis. Although negative effects of BPA have been shown using various human cell lines as well as primary cells, the effects have not been studied on individual cells that are present in the ovarian follicle. Ovarian follicle consists of an oocyte surrounded by granulosa cells which during the follicular growth and maturation are further divided into subtypes of mural and cumulus granulosa cells, which are also accompanied by theca cells and leukocytes. In addition, the mural and cumulus granulosa cells have been shown to differentiate into further subpopulations. To understand the molecular initiating events that lead to reduced fertility after exposure to any EDC, the impact of all of these cell types present in the follicle have to be considered. This should also be done by using more relevant material – human primary cells, which could provide insights to the heterogeneous response leading to pathological outcome.

Droplet microfluidic methods have been used for isolating individual cells into nanolitre-sized droplets. These methods can be used to conduct single-cell analysis in a high-throughput manner where cellular heterogeneity would not be concealed as in bulk experiments. Even though droplet microfluidics has proven to be very useful for different applications, single-cell culturing of adherent eukaryotic cells in a suspension culture has not been used as widely.

Therefore, the main aim of this thesis was to establish a single-cell droplet culture method for granulosa cell line KGN that could be used for testing the effect of a common EDC BPA.

Results from the experimental part of this thesis suggest that KGN cells can be incubated in droplets for 24 hours without a significant effect on droplet quality and with retaining cell survival that is in accordance with the literature. In addition, droplet stability was retained after 24-hour incubation with physiological and supraphysiological concentrations of BPA which suggested its possible use for analysing its effects in single-cell droplet culture.

1. Literature overview

1.1. Ovarian follicle development

The process of ovarian folliculogenesis, that results in a mature oocyte available for fertilization, is very much dependent on the somatic cells present in the follicle. Two types of cells, granulosa and theca cells, surround the oocyte and support the growth and maturation of the follicle. Follicular development starts with an oocyte that is surrounded by squamous granulosa cells and is also called a primodial follicle (PF). Even though this is considered as the first stage of folliculogenesis, the effect of somatic cells on the oocytes is already apparent during embryogenesis, when the germ cell differentiation into oogonium is determined by the signals from surrounding somatic cells in the developing gonads (Telfer et al., 2023). These oogonia start to proliferate mitotically and result in a syncytium of germ cells due to incomplete cytokinesis. PFs are formed, when these germ cell clusters are broken and the surviving oocytes are surrounded with granulosa cells (GCs). These PFs, that have been formed by the end of fetal development, represent a finite pool of oocytes and an ovarian reserve throughout a woman's life (Edson et al., 2009). Most of PFs are kept dormant, having low transcriptional and translational activity, and majority of these undergo apoptotic demise of follicles, also called atresia. Activation of PFs occurs, when they are selected into the growing follicle pool and granulosa cells differentiate into cuboidal cells. The activation is controlled by intraovarian signals and is considered as a part of the gonadotropin independent stage of folliculogenesis (Figure 1). PF activation has been associated with the activation of different signalling pathways including phosphatidylinositol 3-kinases (PI3K)/Akt and Hippo pathway. PI3K/Akt pathway is important for cell growth, proliferation, survival, migration and metabolism, that can be activated by kit ligand (KL), insulin-like growth factor 1 (IGF-1) and epidermal growth factor (Telfer et al., 2023).

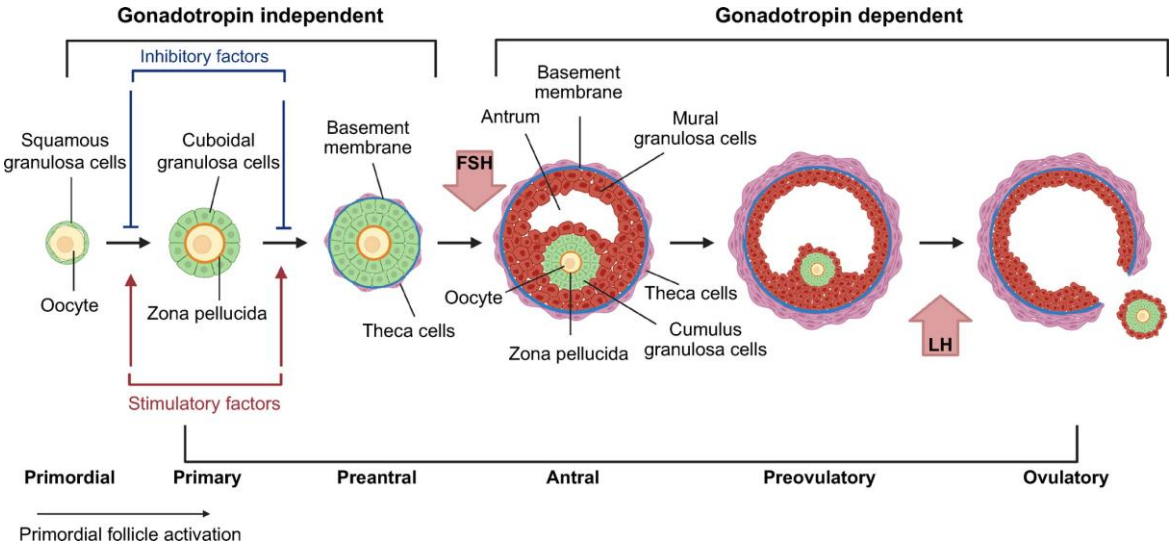


Figure 1. Stages of follicular development. Folliculogenesis can be divided into gonadotropin-independent phase, during which the follicle growth and maturation is dependent on local intraovarian signalling molecules, and gonadotropin dependent stage that is also dependent on the hypothalamus-pituitary-ovarian (HPO) signalling. HPO signalling is described by the hypothalamic release of gonadotropin-releasing hormone (GnRH) that triggers subsequent release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from anterior pituitary to bloodstream where the hormones are transported to ovaries. FSH binding to FSH receptor expressing granulosa cells has many effects on the follicle including stimulation of growth, differentiation and steroidogenesis, while the most apparent change in the follicle involves the formation of an antrum that contains the follicular fluid. LH surge at preovulatory stage is necessary for follicle rupture and cumulus-oocyte complex release (Telfer et al., 2023).

Primary follicle development into preantral follicles occurs when the proliferation of GCs has resulted in several layers of GCs that surround the oocyte. These GCs secrete IGF1 and KL that stimulate theca cell recruitment into the follicles. The oocyte secreted growth differentiation factor-9 (GDF-9) is important for theca cell differentiation. This theca cell layer that lines the outer part of basal lamina on the follicle, is necessary for providing blood supply to follicle and for sensitizing the follicle to gonadotropins (Orisaka et al., 2021). In preantral follicles, theca and granulosa cells express vascular endothelial growth factor (VEGF), mostly VEGF-A, that induces angiogenesis (Telfer et al., 2023).

Preantral to antral transition marks the beginning of the gonadotropin dependent phase of folliculogenesis, which is regulated by many signalling molecules that function in hypothalamic-pituitary-ovarian (HPO) axis. The HPO axis includes the release of gonadotropin-releasing hormone (GnRH) from hypothalamus that induces subsequent release of gonadotropins – follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland after entering the pituitary portal circulation. FSH and LH are secreted into circulation and bind to follicle-stimulating hormone receptor (FSHR) on granulosa cells and luteinizing hormone/choriogonadotropin receptor on theca and granulosa cells (Shoorei et al., 2023).

FSH is a glycoprotein hormone that stimulates antrum formation, maturation of antral follicles and granulosa cell proliferation (Padmanabhan & Cardoso, 2020). Acquisition of FSH dependence is induced by intraovarian regulators like androgen, IGF system and oocyte-derived factors GDF-9 and bone morphogenetic protein 15. Activin, which is also an important regulator of FSH-dependence and is produced by oocyte and GCs, promotes FSH production and secretion and GC expression of FSHR and aromatase. Connexin, that forms gap junctions which enable the exchange of ions, metabolites and other signalling molecules between oocyte and the adjacent GCs, are also believed to be crucial for antral follicle development (Orisaka et al., 2021).

The antrum is formed, when GCs produce hyaluronan and chondroitin sulphate proteoglycan which generate osmotic gradient that draws fluid from thecal vasculature (Rodgers & Irving-Rodgers, 2010). The resulting follicular fluid that makes up the antrum, also consists of steroids, metabolites, proteins, small peptides, antioxidant enzymes and other molecules that are used for intercellular signalling (Freitas et al., 2017). The antrum formation also results in two sub-populations of granulosa cells. Cumulus granulosa cells, which are adjacent to the oocyte, remain surrounding the oocyte until ovulation and fertilization. Mural granulosa cells that line the follicle wall, are responsible for corpus luteum (CL) formation together with theca cells, after ovulation occurs (Jozkowiak et al., 2023).

In the GCs that already expressed FSHR in the preantral stage, FSH induces the expression of aromatase that is necessary for estradiol (E2) synthesis (Orisaka et al., 2021). Steroidogenesis in these follicles occurs according to the ''Two-cell, two-gonadotropin'' theory, by which the two somatic cell types of the follicle are needed for producing hormones necessary for further follicular development. Follicular steroidogenesis starts in theca cells, where cholesterol is used as a precursor for androstenedione and testosterone synthesis through many intermediates and enzymes. Androstenedione and testosterone diffuse into GCs that convert these to E2 by aromatase (Shoorei et al., 2023). When the steroidogenesis has started and low amounts of hormones have been produced, the process is further accelerated by LH and FSH that induce intracellular cAMP rise

and protein kinase A (PKA) activation, that results in mobilization of free cholesterol in theca cells (Issop et al., 2013).

Antral follicle stage is followed by preovulatory stage. Only a small number of follicles, that are more sensitive to FSH than the surrounding antral stage follicles, continue to the preovulatory stage and the remaining undergo atresia. The selected follicles that survive, consist of two theca cell layers – *theca interna* on top of the basal lamina and *theca externa* surrounding the follicle outside (Georges et al., 2013).

Ovulation is triggered by LH surge, that drives the rupture of the follicle and the extrusion of the cumulus-oocyte complex towards fallopian tubes. This is accomplished by upregulating a cascade of proteolytic enzymes needed for extracellular matrix remodelling and follicle wall breakdown. LH also downregulates the expression and function of gap junction proteins connexins. An increase in protein and hormone levels responsible for further angiogenesis and leukocyte invasion is also seen after LH stimulation (Land et al., 2022).

During ovulation, the E2-producing follicle develops into a predominantly progesterone-producing CL. The remaining theca and granulosa cells in the follicle acquire luteal phenotype. In case of fertilization of the oocyte, CL remains and continues to produce progesterone (P). If the oocyte is not fertilized, P levels start to decline and CL degenerates (Land et al., 2022).

During folliculogenesis, usually only one dominant follicle is selected that results in ovulation. This is achieved by ovarian feedback on FSH secretion from the pituitary. When the follicles that have acquired the capacity to produce E2 in large amounts, the high levels of E2 and inhibins produced by the dominant follicle suppress FSH secretion that enhanced follicular growth and steroidogenesis. Now, only the follicles that most abundantly express FSHR can survive the low amounts of FSH, and the growth of other subordinate follicles is suppressed (Matsuda et al., 2012). Therefore, the majority of the follicles are destined to degenerate before reaching ovulation (Telfer et al., 2023).

Recently, the Reproductive Biology Research group at our department has revealed that by the ovulatory stage, several leukocytes have infiltrated the follicle. In addition, the mural granulosa cells have differentiated into sub-populations that possess somewhat different functional roles according to molecular signalling pathways present (Roos et al., 2022). The proportions of different somatic cell populations can be affected in women with reduced sensitivity to gonadotropins (Roos et al., 2022) or upon exposure to some endocrine disruptive chemicals (unpublished data from the research group).

1.2. Endocrine disrupting chemical bisphenol A

The Endocrine Society defines an endocrine disrupting chemical (EDC) as “an exogenous chemical, or mixture of chemicals, that interferes with any aspect of hormone action” (Zoeller et al., 2012). EDCs are mostly synthetic man-made chemicals but also include plant-derived compounds of natural origin. EDCs can affect any part of the endocrine system and therefore have been associated with the etiology of hormone-related diseases like diabetes, obesity or reproductive disorders such

as endometriosis, premature ovarian insufficiency (POI) and polycystic ovarian syndrome (PCOS) that can lead to reduced fertility (Marino et al., 2012, Jozkowiak et al., 2023). The risk of lifelong effects is enhanced when exposure to EDCs happens during formation and differentiation of organ systems in early development (La Merrill et al., 2020). This can be quite alarming considering that different EDCs have been detected from follicular fluid (Wang et al., 2017, Bellavia et al., 2023), serum (Jozkowiak et al., 2023), urine (Ehrlich et al., 2013), as well as from amniotic fluid (Shekhar et al., 2017), umbilical cord blood (Geer et al., 2017) and milk from breastfeeding mothers (Croes et al., 2012). Exposure to EDCs can occur by many routes since they are present for example in personal care products and plastics, agrochemicals and flame retardants that are applied to materials such as furniture, electronics and construction materials (Priya et al., 2021).

In terms of reduced fertility, bisphenol A (BPA) is one of the most studied EDCs due to its estrogenic properties and ubiquitous exposure (Palioura & Diamanti-Kandarakis, 2015, Ziv-Gal & Flaws, 2016). European chemicals agency (ECHA) has classified BPA as a substance that may damage fertility, causes serious eye damage and may cause an allergic skin reaction and respiratory irritation. In addition, it is very toxic to aquatic life with long lasting effects (ECHA, 2023).

BPA (4,4'-dihydroxy-2,2-diphenylpropane; CAS 80-05-7), first synthesized in 1891, was already characterized as a synthetic estrogen in 1936 (Dodds and Lawson, 1936, Rutkowska & Rachoń, 2014, Huo et al., 2015). Its rediscovery in 1950s and its use as a monomer in polycarbonate plastic, polyvinyl chloride and epoxy resin production has led to its wide use, with global consumption of approximately 10.6 million tonnes in 2022 (Cimmino et al., 2020, Shoorei et al., 2023). Epoxy resins are used for example as protective coatings in food and beverage cans and BPA has also been used in the production of reusable plastic food and beverage containers. It is also used in many consumer products like toys, electronics, medical equipment, lenses, dental monomers, water pipes and has been even detected from new and used clothes (Vandenberg et al., 2007, Wang et al., 2019).

Even though BPA has been shown to have low acute toxicity in humans, its exposure is considered to be continuous and ubiquitous and raises the question of its safety during chronic exposure (Hines et al., 2017). Oral exposure to BPA has been considered as a major source due to its leaching from food and beverage containers and in smaller amounts from dental sealants. BPA monomers are linked into polymers via ester bond, that can be broken by hydrolysis and leach out from cans and containers to food and beverages under acidic or basic conditions and at high temperatures (Vandenberg et al., 2007). The use of BPA in infant feeding bottles has been banned since 2010 in Canada and later also in USA and EU (Kang et al., 2014). Recently, European Food Safety Authority (EFSA) lowered the *Tolerable daily intake* (TDI) of BPA, setting a TDI 20 000 times lower than the one set in 2015 (EFSA, 2023).

Dermal exposure and inhalation of BPA have been rather associated with occupational settings. Dermal exposure has been described in case of contact with cash register receipts, paper money and other paper products, since BPA is used in production of thermal paper that is used in printing process. There are also several articles about how workers in different factories have higher urine concentrations of BPA and its metabolites than non-exposed humans (Liao & Kannan, 2011, Mendum et al., 2011, Hines et al., 2017).

BPA in the environment

BPA can be released into the environment by chemical manufacture, leaching from landfills, post-consumer release via effluent discharge from municipal wastewater treatment plants, combustion of domestic waste and breakdown of plastics in the environment (Flint et al., 2012). BPA has been detected from rivers, ground water and marine surface water samples, but higher concentrations of BPA have resulted from treatment plant influents and wastewater (Huang et al., 2017). BPA's reported log Kow values of 2.20-4.16 that represent its octanol/water partitioning, and water solubility of 200mg/L indicate moderate hydrophobicity and modest capacity for bioaccumulation, and the environmental compartments of BPA are more likely to be abiotic and associated with water and suspended solids, soil and sediments. Also, BPA has low volatility but in case of reaching into the atmosphere, it is believed to photo-oxidize and break down rapidly (Flint et al., 2012).

Associations with reduced fertility in women

Main causes of reduced fertility in women are endometriosis, problems with fallopian tubes, PCOS, poor ovarian response to hormonal stimulation and POI (Sturm & Virant-Klun, 2023). BPA has been shown to alter many pathways involved in folliculogenesis, influencing the regulation of follicular development on different levels including the hypothalamic and pituitary regulation (Huo et al., 2015).

BPA has been mostly associated with PCOS, since it has been detected from PCOS patients' follicular fluid (Wang et al., 2017), serum (Kandaraki et al., 2011, Konieczna et al., 2018) and urine (Lazúrová et al., 2021, Prabhu et al., 2023) in higher concentrations than from healthy women or in vitro fertilization (IVF) patients with other causes of infertility. PCOS is a highly heterogenous disease of unknown etiology, that is believed to be caused by genetic and environmental components. According to Rotterdam criteria, PCOS is diagnosed when two of the following conditions are present: 1) oligo- or anovulation, 2) clinical or biochemical hyperandrogenism, or 3) polycystic ovary morphology, defined as the existence of 12 or more follicles in ovary that are 2-9 mm visible on ultrasound. These conditions can be accompanied by hyperinsulinemia, obesity and chronic inflammation (Zuo et al., 2016, Jozkowiak et al., 2023).

In PCOS patients, higher urinary and serum BPA levels have positively correlated with increased androgen and testosterone levels (Takeuchi et al., 2004, Kandaraki et al., 2011). Oxidative stress, which is also one of the effects of BPA, has been shown to be a possible cause for PCOS and its associated hyperinsulinemia, obesity, hyperandrogenism and chronic inflammation (Zuo et al., 2016). Higher levels of anti-Müllerian hormone, necessary in the regulation of follicular growth and maturation inhibition, have also been detected from PCOS patients undergoing IVF procedure than from non-PCOS patients (Wang et al., 2017, Priya et al., 2021).

The cause-effect relationship between elevated BPA levels and PCOS needs to be further investigated. Although mostly linked to PCOS, it is important to note that BPA has been shown to affect different stages of follicular development and cause different pathological events observed in other diseases characterized by reduced fertility as well.

1.2.1. Bisphenol A metabolism and mode of action

BPA is rapidly and efficiently absorbed in the gastrointestinal tract after oral administration, and its half-life in adult humans is approximately 4-5 hours. BPA is mostly metabolized to water-soluble BPA-glucuronide, which does not exhibit estrogenic activity like free BPA, and is eliminated from blood by kidneys and excreted via urine. Smaller amounts of BPA result in BPA-sulphate (BPA-S) conjugates (Hines et al., 2017).

Glucuronidation involves the activity of uridine-5-diphospho-glucuronosyltransferases (UGTs). Any abnormalities in UGT functioning can cause an increase in unconjugated BPA levels (Mukhopadhyay et al., 2022). For example, some studies have concluded that PCOS itself can lead to higher BPA levels because of high circulating androgen levels which decrease UGT activity and cause a subsequent decrease in BPA clearance (Kechagias et al., 2020).

<u>BPA as an ERα agonist and ERβ antagonist</u>

BPA is classified as a xenoestrogen that mimics the action of endogenous E2. Due to structural similarities of BPA and E2 (Figure 2), BPA has the ability to bind estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), with a 10-fold higher affinity for ERβ. Although the affinity for ER binding is 10 000 – 100 000-fold weaker than of E2, BPA has been shown to cause similar effects upon binding to these receptors (Vandenberg et al., 2007).

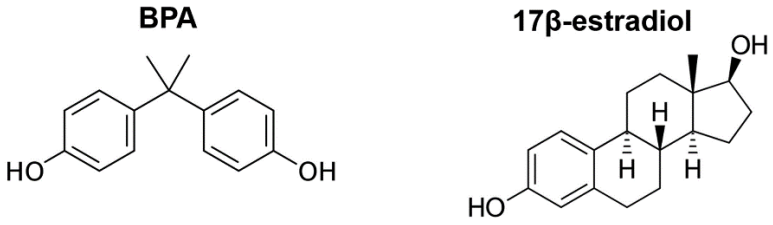


Figure 2. Chemical structure of bisphenol A and estradiol (Harnett et al., 2021).

ERα and ERβ, which are the members of the nuclear receptor superfamily, are located in cytosol and when palmitoylation and binding to caveolin-1 occurs, also located on the plasma membrane. Both types of ER receptors can activate genomic and non-genomic signal transduction pathways (Marino et al., 2012).

ERα non-genomic pathways are activated after E2 binding that results in depalmitoylation and E2:ERα complex dissociation from caveolin-1 and signalling of the complex to ERK/MAPK and Akt. BPA is known to act as an agonist of ERα, causing the ERK/MAPK and Akt phosphorylation and the activation of rapid extra-nuclear pathways. This way, BPA is known to act as a proliferative agent in granulosa cells (Marino et al., 2012).

ERα nuclear pathway is activated, when E2 binding causes receptor dimerization, phosphorylation and nuclear translocation, where the complex binds to estrogen response elements (EREs) causing the activation of gene expression. BPA has been mostly shown to act through this pathway (Marino et al., 2012). ERα is expressed in many tissues including hypothalamus, endometrium and skeletal

muscles. By acting on hypothalamic ERα, BPA can directly affect GnRH (Marino et al., 2012, Sturm & Virant-Klun, 2023). In the follicles, ERα is highly expressed on theca cells (Priya et al., 2021).

ERβ non-genomic pathway involves the binding of ERβ:caveolin-1:p38/MAPK. An increase of this complex at the plasma membrane mediates the activation of p38/MAPK. Upon binding to ERβ, E2 causes the activation of apoptotic pathways. BPA is known to act as ERβ antagonist, preventing p38/MAPK activation and the activity of ERE-containing promoter, and not causing the activation of a pro-apoptotic cascade and has been also associated with an increased risk of cancer (Marino et al., 2012). ERβ is highly expressed in granulosa cells but also in brain, kidney, bone, heart, lungs, intestinal mucosa, endothelial cells and skeletal muscles (Marino et al., 2012, Priya et al., 2021, Sturm & Virant-Klun, 2023).

Nuclear ERβ pathway occurs similarly to ERα, when E2-ERβ binding causes nuclear translocation and activation of ERE-containing genes, although BPA is known as an antagonist for this pathway (Marino et al., 2012).

Effects on the HPO axis

As reproductive function is dependent on HPO axis (Figure 3), any disturbances in hormonal levels that feedback to hypothalamus and pituitary, can result in the disturbance of folliculogenesis. Steroid hormones E2 and P induce kisspeptin expression in hypothalamic neurons, which regulate the release of GnRH that stimulates FSH and LH release from the pituitary (Ziv-Gal & Flaws, 2016).

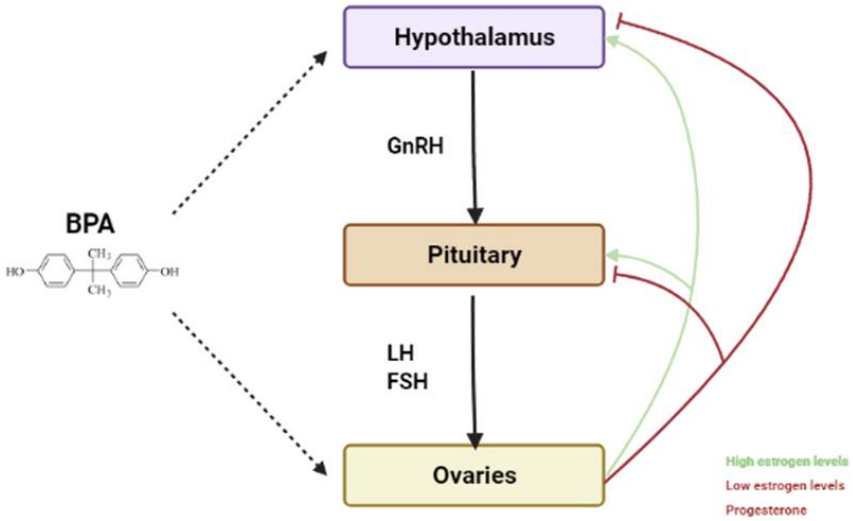


Figure 3. BPA effects on hypothalamic-pituitary-ovarian axis. Bisphenol A (BPA) can cause hormonal imbalances on different levels of hypothalamic-pituitary-ovarian axis, that lead to abnormal secretion of homones involved in folliculogenesis. GnRH – gonadotropin-releasing hormone, LH – luteinizing hormone, FSH – follicle-stimulating hormone (Sturm & Virant-Klun, 2023).

BPA affects the HPO axis by altering hormone production in the ovaries, or by directly altering the expression of kisspeptin, resulting in abnormal FSH and LH secretion (Jozkowiak et al., 2023). It has been shown that women with PCOS have higher GnRH pulse generator activity with its consequent constant LH elevation that impairs folliculogenesis and increases androgen production in theca cells.

A correlation between serum BPA levels and increased testosterone levels has been also found in women with PCOS (Kandaraki et al., 2011). BPA has been also shown to enhance androgen production in rat ovarian theca cells (Zhou et al., 2008).

Effects of BPA on steroidogenesis

Studies of BPA's effects on steroidogenesis have mostly been carried out using immortalized human granulosa cell lines COV434 and KGN. Granulosa cells possess three specific cellular functions: 1) FSH stimulated E2 production, 2) expression of specific biomarkers of apoptosis necessary to induce follicular atresia, and 3) the ability to communicate with other follicular cells (Zhang et al., 2000). COV434 granulosa cells, established from a primary human granulosa tumour (Zhang et al., 2000) and KGN cell line, established from a patient with invasive ovarian GC carcinoma (Nishi et al., 2001), have both retained these three functions.

Ovarian hormone biosynthesis starts in theca cells, where cholesterol is transported to inner mitochondrial membrane by steroidogenic acute regulatory protein (STAR) (Figure 4), where it is converted to pregnenolone by CYP11A1. This is followed by pregnenolone conversion to dehydroepiandrosterone (DHEA) which can diffuse to granulosa cells or be further converted to testosterone by 17β-hydroxysteroid dehydrogenase (17β-HSD), which also diffuses to adjacent granulosa cells for further conversion into E2 by aromatase (CYP19A1). Activation of the enzymes needed for steroidogenesis is triggered by cAMP and PKA activation in response to FSH and LH (Jozkowiak et al., 2023).

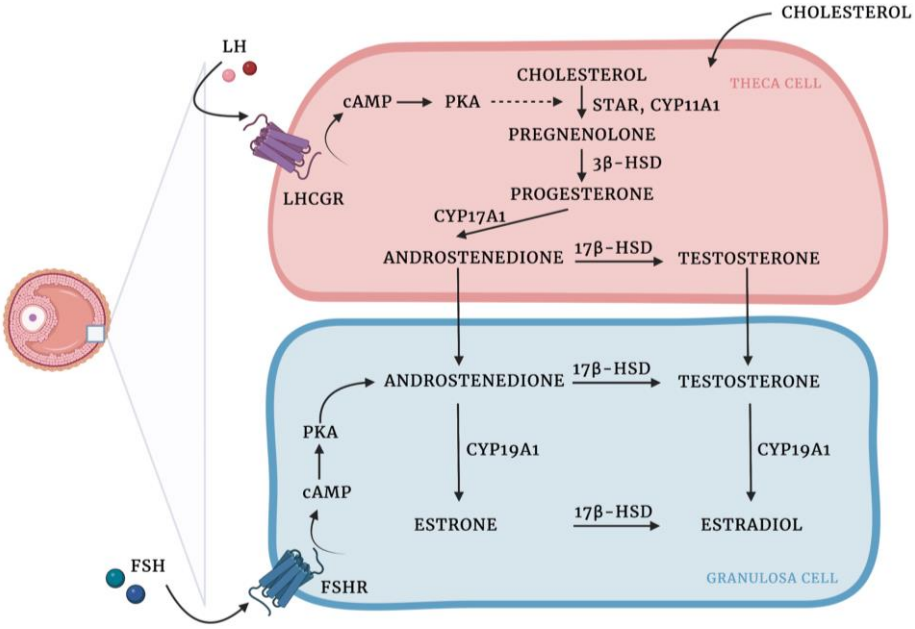


Figure 4. The Two-Cell Two-Gonadotropin System. Steroidogenesis in the antral follicle is carried out by granulosa and theca cells in response to follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Steroidogenesis starts in theca cells, where cholesterol is transported to mitochondria by steroidogenic acute regulatory protein (STAR). After LH binding to its receptor luteinizing hormone/choriogonadotropin receptor (LHCGR), expression of steroidogenic enzymes is increased and androstenedione and testosterone produced in theca cells diffuse to granulosa cells, where they are converted to estrone and estradiol. cAMP – cyclic adenosine monophosphate, PKA – protein kinase A, CYP11A1 – cholesterol sidechain cleavage enzyme, 3β-HSD – 3β-hydroxysteroid dehydrogenase, 17β-HSD – 17β-hydroxysteroid dehydrogenase, CYP19A1 – aromatase (Jozkowiak et al., 2023).

Aromatase expression can be regulated by transcription factors steroidogenic factor-1 (SF-1), liver receptor homolog-1, cAMP response element binding protein (CREB) and GATA binding protein 4 (GATA4). GATA4, SF-1 and CREB are positive regulators while peroxisome proliferator-activated receptor gamma (PPARγ) is a negative regulator of aromatase expression (Fan et al., 2005).

BPA has been shown to inhibit FSH-induced aromatase expression by inducing PPARγ overexpression that mediates the down-regulation of FSH induced IGF-1, SF-1, GATA4 in KGN and human luteinized granulosa cells (Kwintkiewicz et al., 2010, Watanabe et al., 2012), although concentrations used in these studies were higher than environmentally relevant doses. Similar high concentrations were also shown to decrease mRNA levels of *CYP11A1, CYP19A1* and *17*β-*HSD* in human luteinized mural granulosa cells, which might explain the lower levels of E2 after BPA treatment (Mansur et al., 2016). There have also been opposing results, showing E2 production stimulation after environmentally relevant doses of BPA were used on KGN cells. For example, an increase in aromatase expression, mediated by forkhead box L2 transcription factor, was seen after BPA treatment (Liu et al., 2021).

In vivo animal studies, mostly performed on mice and rats, have also revealed the disturbances in steroidogenesis after BPA exposure (Banerjee et al., 2018). Experiment with adult female rats showed a significant downregulation of aromatase and decreased E2 levels in rats treated with environmentally relevant and higher BPA doses. Also, an increase in apoptotic cells during antral stage and after CL formation was shown in both groups, which suggests that BPA exposure can cause the augmentation of follicular atresia and luteal regression during adulthood exposure (Lee et al., 2013). Neonatal exposure to BPA in mice and rats has resulted in inhibited germ cell nest breakdown, decrease in primordial follicles, increase in apoptotic oocytes and an increase in primordial follicle recruitment (Ziv-Gal & Flaws, 2016).

Follicular atresia

Oxidative stress, caused by the increased levels of reactive oxygen species (ROS) due to an imbalance between the production and scavenging of ROS, can cause damage to nucleic acids, proteins and lipids (Zuo et al., 2016). High concentrations of BPA have shown to cause elevated ROS levels and decrease in antioxidant activity of superoxide dismutase, catalase and glutathione synthase and increased nucleic acid damage in KGN and COV434 cell lines (Huang et al., 2020, Mlynarcikova & Scsukova, 2020). BPA-induced KGN cell apoptosis has been shown to occur due to oxidative stress which could also result in increased follicular atresia (Huang et al., 2021). KGN cells treated with environmentally relevant doses of BPA have also showed increased levels of autophagic markers that could also cause increased apoptosis and follicular atresia (Lin et al., 2021).

Follicular atresia has also been associated with decreased levels of a gap junction protein Cx43 at BPA concentrations that have been detected from follicular fluid. Since Cx43 is important for GC-GC communication through all stages of folliculogenesis after primordial follicle activation, a lot of cells could be affected by this (Lin et al., 2021). In addition, a decrease in VEGF-A secretion by COV434 cells has been shown after treatment with physiological doses of BPA (Bujnakova Mlynarcikova & Scsukova, 2021).

Considering these agonistic and antagonistic properties, the effects of BPA at different doses are hard to predict and experiments conducted with various concentrations of BPA have often resulted in non-monotonic dose-response relationships, where the dose-response curve is not just increasing or decreasing. This has been a common feature for many EDCs that makes the determination of safe levels, where exposure to an EDC does not cause any harmful effects, even harder (Acconcia et al., 2015, Lagarde et al., 2015). Also, the fact that exposure to different chemicals can result in additive and synergic effects, the risk assessment is even more complicated. Therefore, it is important to obtain information about the molecular initiating events in different cell types present in the follicle, that take place after the exposure to a chemical or a mixture of chemicals occurs.

1.3. Droplet microfluidics

Microfluidics is the science of manipulating small volumes of liquids ranging from 10^{-9} to 10^{-18} litres, which enables single-cell analysis by compartmentalizing individual cells in droplets, microfluidic wells or traps. In addition to the advantage of studying single cell heterogeneity, microfluidic methods also enable to create conditions that resemble to the environment of cells *in vivo* (Wang et al., 2020).

1.3.1. Monodisperse droplet generation

Monodisperse droplets that are usually in the range of 1 pL – 10 nL and uniform in size can be made using microfluidic chips with different device geometries which enable mixing of two immiscible fluids (Figure 5). Passive methods, that require no external force, are usually based on using microfluidic chips with channel geometries like T-junction, flow-focusing and co-axial focusing geometries. T-junction geometry can be used for introducing a dispersed phase to a continuous phase in a vertical manner. Flow-focusing chip is used to generate droplets by two counter-streaming flows of continuous phase that surround the introduced disperse phase at the cross. Co-axial focusing structure is based on using one channel in the middle of the other channel, where disperse phase flow in the middle capillary is surrounded by continuous phase from the outer channel (Collins et al., 2015, Ou et al., 2021).

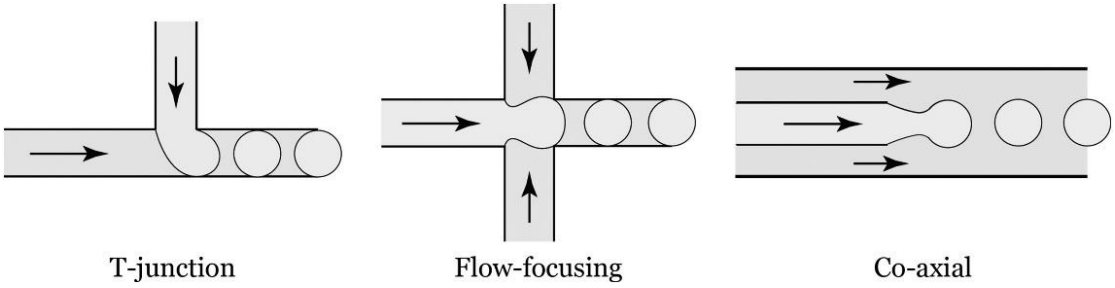


Figure 5. Monodisperse droplets can be made by introducing two immiscible fluids at the T-junction, by using two counter-streaming flows of continuous phase in flow-focusing channels or by using co-axial focusing structure (Ou et al., 2021).

In addition to droplet generation, microfluidic chips can also be used for droplet sorting to recover desired cells, merging to introduce new reagents into droplets and also for splitting the droplets. Droplet analysis can also be carried out using microfluidic chips that enable visualization of different

processes at a single cell level by using fluorescence based or other labelling methods (Chen et al., 2019).

Droplet microfluidics methods have many advantages when compared to standard culture conditions including reduced sample volumes, faster detection of analytes and more efficient working with cells of limited availability. Cell encapsulation in droplets also reduces the risk of cross-contamination and increases the number of technical replicates which enables analysis in a high-throughput manner (Sesen & Whyte, 2020).

All of these advantages have led to droplet microfluidic methods application in various fields. For example, it has been used for antibody and drug screening (Akbari & Pirbodaghi, 2014, Gérard et al., 2020), cell-cell interaction studies (Konry et al., 2013, Siltanen et al., 2017), single-cell RNA sequencing (Karaayvaz et al., 2018), the detection of nucleic acid of pathogens to monitor the dynamics of infections (Gonzalez et al., 2020, Peccia et al., 2020) or for conducting PCR for detecting mutations in rare circulating tumour cells (Ou et al., 2019). Droplets have also been used as potential drug microcarriers to mediate drug distribution in the body by releasing drugs for example in response to pH or temperature changes (Gal et al., 2020).

1.3.2. Cultivating eukaryotic cells in droplets

Droplet microfluidics has been mostly used for isolating single cells and performing single-cell analysis without incubating cells for several days. Longer cultivation in droplets has been used for example in cancer cell studies, where the droplets together with hydrogels and different matrices can be used for cell attachment. Droplets have been used to create conditions similar to tumour microenvironment (TME) since different cell types and conditions in TME can lead to therapy resistance. Therefore, the effect of therapeutic agents on cancer cells should be studied by creating co-cultures of cells that can provide more accurate results than when testing agents only on cancer cells (Sart et al., 2022). Droplets have also been applied for studying different immunological processes. For example, Tiemeijer et al have used droplets for incubating macrophages for two days to study their polarization from pro-inflammatory to anti-inflammatory state (Tiemeijer et al., 2021). Also, single-cell cultivation in droplets has been proposed as a tool for screening mammalian cell factory libraries for production and functional analysis of biopharmaceuticals because the selection of a stable clone, which produces the desired protein with sufficiently high yield, can take up to several months, but by using droplets for single-cell encapsulation, this could be achieved much faster (Rajeswari et al., 2017).

Different types of eukaryotic cells have been shown to survive incubation in droplets for several days. Clausell-Tormos et al showed viability rates higher than 75% after 4 days of incubating encapsulated cells off-chip. The used cell types were Human Embryonic Kidney 293 T cells (HEK293T) and human T lymphocyte cells (Jurkat cells), suggesting also the droplet culture suitability for adherent cells in addition to suspension cells (Clausell-Tormos et al., 2008). Experiments with adherent Chinese Hamster Ovary (CHO) cells have also shown that cells can survive in droplets up to 72 hours with preserving over 90% viability (Rajeswari et al., 2017). Also, monocytic U937 have maintained over 80% survival up to 4 days of incubation (Brouzes et al., 2009). These results suggest that adherent cells could be also studied by using droplet culture system.

1.3.3. Image-based droplet analysis

Imaging-based analysis is the most common method for analysing droplets and their contents. These images are mostly acquired using brightfield and fluorescence microscopes, which allow the imaging of thousands of droplets in a single experiment (Zhu & Fang, 2013). To analyse such large amounts of droplets and their contents, automated image analysis software is needed. Droplet detection and analysis has been mostly conducted by using scripted programs that require special knowledge in programming. The Microfluidics group in our department has examined several free user-friendly software that do not require such programming skills and could be used for droplet experiments instead. Sanka et al have tested the accuracy and user-friendliness of available software ImageJ, QuPath, Ilastik and CellProfiler, and have shown the suitability of CellProfiler for droplet analysis (Bartkova et al., 2020, Sanka et al., 2021).

CellProfiler has been used for measuring various cell features like cell count and size, cell cycle distribution, organelle number, and levels and localization of proteins (Carpenter et al., 2006, Kamentsky et al., 2011). It is used by creating image analysis pipelines by using different modules which process images in some manner. By adjusting the settings in these modules, the resulting pipelines can be used for conducting different experiments. Another user-friendly feature of CellProfiler is the visual feedback about every module, that makes the optimization of the settings for a specific experiment easier (Stirling et al., 2021).

2. Aims of the study

The ovarian follicle is a complex structure containing multiple somatic cell types in addition to the maturing oocyte. While the negative effect of BPA on female infertility has been demonstrated, its mechanisms at physiological concentrations in the ovarian follicle are less clear, considering that the effect may vary depending on the somatic cell population. Hence, it is necessary to set up a model for analysing the effect of BPA (and other EDCs) on individual somatic cells in the follicle. In order to reach this goal, the current thesis aims to search for the possibilities of combining microfluidics, cell culture techniques and image analysis pipelines on a standard ovarian granulosa cell line KGN and test its applicability for single cell exposure to BPA.

More specifically, the aims of the current thesis were the following:

1. Set up a microfluidic system for the short-term single-cell culture of ovarian cell line KGN.
2. Analyse the viability of KGN cells in microfluidic droplet culture.
3. Test the applicability of the established droplet culture system for testing the effect of bisphenol A on KGN viability.
4. Compare the droplet culture system with standard 2D culture.

3. Materials and methods

3.1. Cell culture

In this thesis, KGN cell line was used in all the experiments (Nishi et al., 2001). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Corning, USA) with 4.5 g/L glucose, L-glutamine & Na pyruvate with 10 % fetal bovine serum (Corning, USA), 1 % penicillin and streptomycin (Naxo, Estonia). Cells were grown at 37°C in an environment with 5 % CO_2 content. The cells were split every 3-4 days. Experiments were done when cells had reached 70% confluency.

3.2. Optimizing the dye concentrations of the LIVE/DEAD kit for cell viability analysis

Live KGN cells were washed with phosphate-buffered saline (PBS) three times, trypsinized with 0.05 % Trypsin-EDTA (Corning, USA) and centrifuged at 1000 rpm for 5min. Cell concentration was assessed using Trypan Blue stain 0.4% (Invitrogen) and Countess Automated Cell Counter (Invitrogen, USA).

LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Invitrogen) was used to distinguish live and dead cells. Calcein AM (excitation/emission 494/517nm), used as a live cell marker, is hydrolyzed to green-fluorescent product in living cells and Ethidium homodimer-1 (excitation/emission 528/617nm) results in a red-fluorescent signal after concentrating in the nucleus of cells with compromised membranes. Different dye solutions were made using PBS for both dyes to determine optimal dye concentrations for KGN cell line cells. The following concentrations were tested for both dyes: 10 µM, 4 µM, 2 µM, 0.5 µM, 0.1 µM, 0 µM.

Dead cells were prepared by fixation with 70 % ethanol. Cells were centrifuged at 4°C 1000 rpm for 5 minutes and washed with 100 µl of PBS. 50 000 live and 50 000 dead cells were mixed and 100 µl of stain solution of indicated concentrations was added. Samples were incubated at room temperature in dark for 30 min. Cells were examined using Olympus BX61 fluorescence microscope with a 20X objective (UPlanSApo 20x0.75) and appropriate filters (GFP long pass, GreenEx).

3.3. Poly(dimethylsiloxane) chip fabrication

Solution for making microfluidic chips and moulds was made using 100 g poly(dimethylsiloxane) (PDMS) base (Sylgard 184, US) and 10 g curing agent (Sylgard 184 silicone elastomer curing agent, US). The solution was put in vacuum for 30 minutes to get rid of air bubbles. A chip negative for flow-focusing chip making was made by pouring the solution to aluminium foil and placing a glass chip on top of the solution and was baked at 120°C for 1 hour. The mould was made hydrophobic by placing it inside vacuum holder for 3 hours with 2 1.5 mL Eppendorf tubes that contained 5 µl of 97% 1H, 1H, 2H, 2H-Perfluorooctyltrichlorosilane 97% (Alfa Aesar, USA). The same PDMS base and curing mixture was poured to hydrophobic chip moulds and baked at 120°C for 1 hour. Chip was removed from the mould and inlet/outlet ports for 0.5mmx1.0mm tubes were created using 1.0 mm diameter biopsy pen. Chip surface and glass slide were treated with corona electrode and

pressed together. Channels on the chip were made hydrophobic using Novec 1720 EGC-1720 Novec™ Electronic Grade Coating (Fluorochem) and left drying for 1 hour. Channels were cleaned with methanol and dried before and after droplet generation.

3.4. Monodisperse droplet generation and imaging

KGN cells (Figure 6A) were trypsinized and suspended in culture medium with Calcein AM and ethidium homodimer-1 (EthD-1) with a final concentration of 2 µM and 0.1 µM, respectively. Cascade Blue with a final concentration of 6 µg/mL was also added for subsequent optimal droplet analysis via software. Droplets were generated by using perfluorinated oil (HFE-7500) with 2% concentration of perfluoropolyether (PFPE)−poly (ethylene glycol) (PEG)−PFPE triblock (a kind gift from Professor Piotr Garstecki from the Institute of Physical Chemistry, Polish Academy of Sciences) surfactant as the continuous phase and sample with cells as the dispersed phase. Sample was injected into a PDMS chip (Figure 6B) via tubing (0.5mm x 1mm) using syringe pumps (KF Technology) at a flow rate of 120.0 µl/min for oil with 2 % surfactant and 170 µl/min for sample. Droplets were collected into different test tubes for incubation at 37°C.

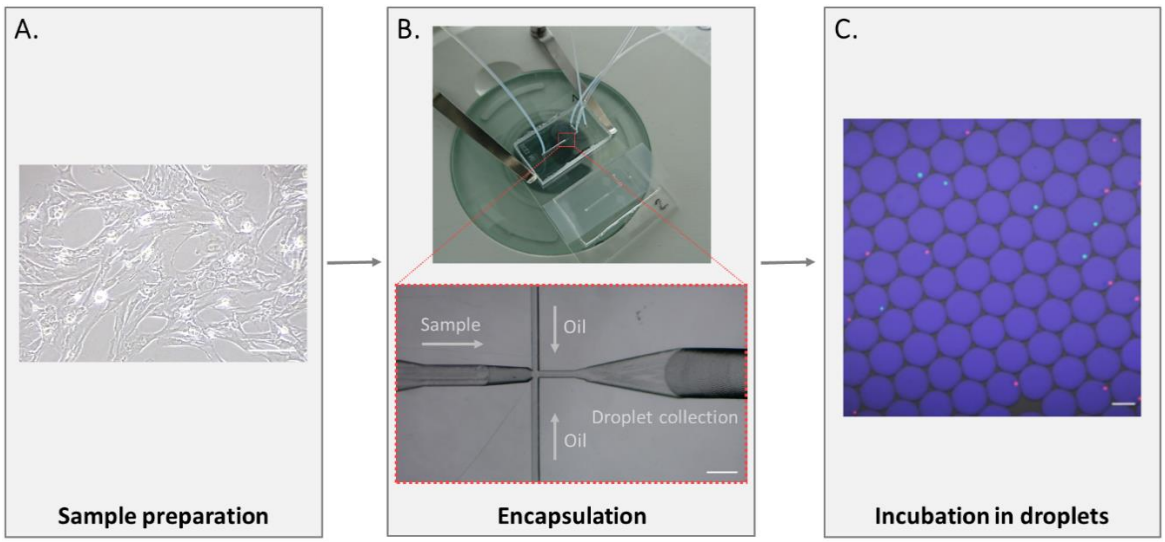


Figure 6. Experimental workflow scheme with illustration. A. Adherent KGN cells are trypsinized and cell suspension is used for droplet generation. Scale bar 60 µm. B. Water-in-oil droplets are made using a flow-focusing microfluidic chip. Scale bar 500 µm. C. Cells are incubated in monodisperse droplets. Scale bar 100 µm.

Monodisperse droplets were imaged using Zeiss LSM 900 confocal microscope (Plan-Apochromat 10x/0.45). 20 µl of droplet sample was pipetted to a Countess imaging slide (Invitrogen). Fluorescence was acquired in three separate channels (Figure 6C), excitation settings were the following: Calcein AM (488nm), Cascade Blue (405nm) and EthD-1 (561nm).

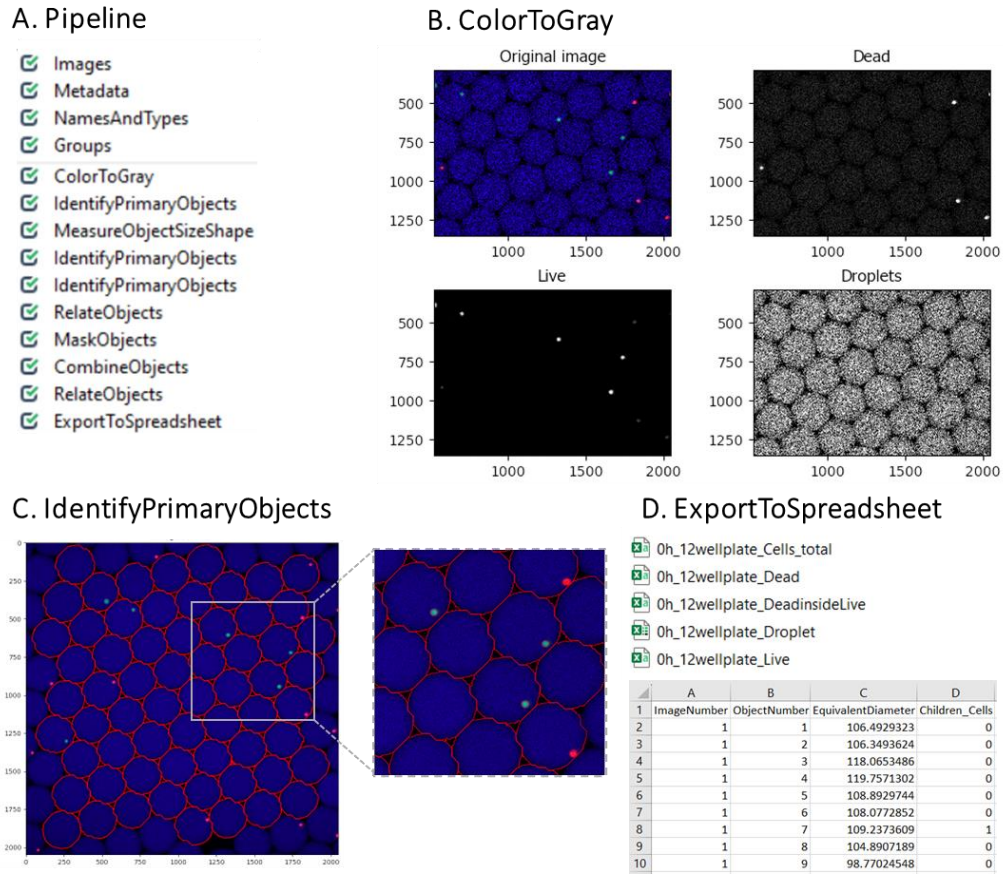
3.5. Droplet incubation in different test-tubes

400 µl of sample was prepared for monodisperse droplet generation for each container.

Four different test tubes were used for droplet incubation:

1) 12-well plate (Cellstar, Greiner Bio-One)
2) 24-well plate (Cellstar, Greiner Bio-One)
3) 5 mL tube (Cellstar, Greiner Bio-One)
4) 50 mL tube (Cellstar, Greiner Bio-One)

Droplets and cell viability were analysed using CellProfiler (vers 4.2.1) image analysis software. Raw Tagged Image File (TIF) format images were imported and analysed using a pipeline constructed for this experiment (Figure 7A).



	A	B	C	D
1	ImageNumber	ObjectNumber	EquivalentDiameter	Children_Cells
2	1	1	106.4929323	0
3	1	2	106.3493624	0
4	1	3	118.0653486	0
5	1	4	119.7571302	0
6	1	5	108.8929744	0
7	1	6	108.0772852	0
8	1	7	109.2373609	1
9	1	8	104.8907189	0
10	1	9	98.77024548	0

Figure 7. Overview of the CellProfiler software analysis pipeline used for cell viability analysis in droplets. A. Pipeline consisting of 14 different modules for identifying cells and droplets. B. ColorToGray module for separating three different fluorescence channels. C. IdentifyPrimaryObjects module used for detecting cells and droplets. D. ExportToSpreadsheet module generates Excel files with cell counts that are used for further cell viability calculations in Excel. This module also generates files with droplet measurements that were used for calculating average droplet diameter. An example of Excel file with droplet measurements is shown below.

The pipeline consisted of 14 modules. *Images* module was used for image input, *Metadata* for metadata extraction, *NamesAndTypes* for assigning names for images and channels and for selecting the image type (Color image). *Groups* module organizes sets of images into groups.

ColorToGray module was used for splitting the channels of a color image (red, green and blue) (Figure 7B) into separate grayscale images for *IdentifyPrimaryObjects* modules (Figure 7C) that identify cells in green and red channels and droplets in blue channel. Droplets and cells were identified based on their diameter. Live cell diameter was set to 10-30 pixels and for dead cells 8-20 pixels since EthD-1 concentrates inside the nucleus. Droplet diameter was set to 60-400 pixels. Objects out of this range and touching the border of the image were discarded. Clumped cells were distinguished based on object intensity and *Propagate* was chosen to draw dividing lines between clumped objects. Clumped droplets were distinguished based on shape. Threshold strategy was set to *Global* and threshold method was set to *Robust Background* for cell detection and *Manual* for droplets*. Threshold smoothing scale* was set to 4 and 1.3488 for live and dead cells, respectively*. Threshold correction factor* was set to 1.5 for both live and dead cells. For droplets, *Manual Threshold* was set to 0.075 and *Threshold smoothing scale* to 2.0.

Since some cells can take up both stains, a module *RelateObjects* was used for counting cells with both stains as dead cells and module *MaskObjects* was used to subtract these from cells that are counted as live cells. *CombineObjects* module was for merging live and dead cells in order to use *RelateObjects* which is used for associating cells with droplets to acquire the cell counts for each droplet. *ExportToSpreadsheet* was used to export Excel files with resulting cell counts and droplet measurements (Figure 7D). MS Excel was also used for further cell viability, average droplet measurement and Poisson distribution analysis. At least 100 cells were analysed for assessing the viability of cells in different containers. Viability score was calculated as the percentage of live cells from the total number of cells (live and dead).

3.6. Droplet collapsing for viability analysis

Droplets without the stains were broken by adding 10% of 1H, 1H, 2H, 2H-tridecafluorooctan-1-ol (Apollo Scientific, UK) and shaking for 5 s. Sample was centrifuged at 6000 rpm for 20 s. Layer of oil was removed and cells in growth medium were centrifuged at 4°C at 1500 rpm for 5 min. Supernatant was removed and 200 µL (at 0h) or 400 µL (at 24h) of dilution of Calcein AM and EthD-1 in DMEM was added to cells and incubated at room temperature for 30 min.

3.7. Cell viability assessment with CellProfiler after collapsing droplets

Images of live and dead cells taken with confocal microscope were also analysed using CellProfiler image analysis software. TIF images were imported and analysed using a pipeline constructed for this experiment (Figure 8A).

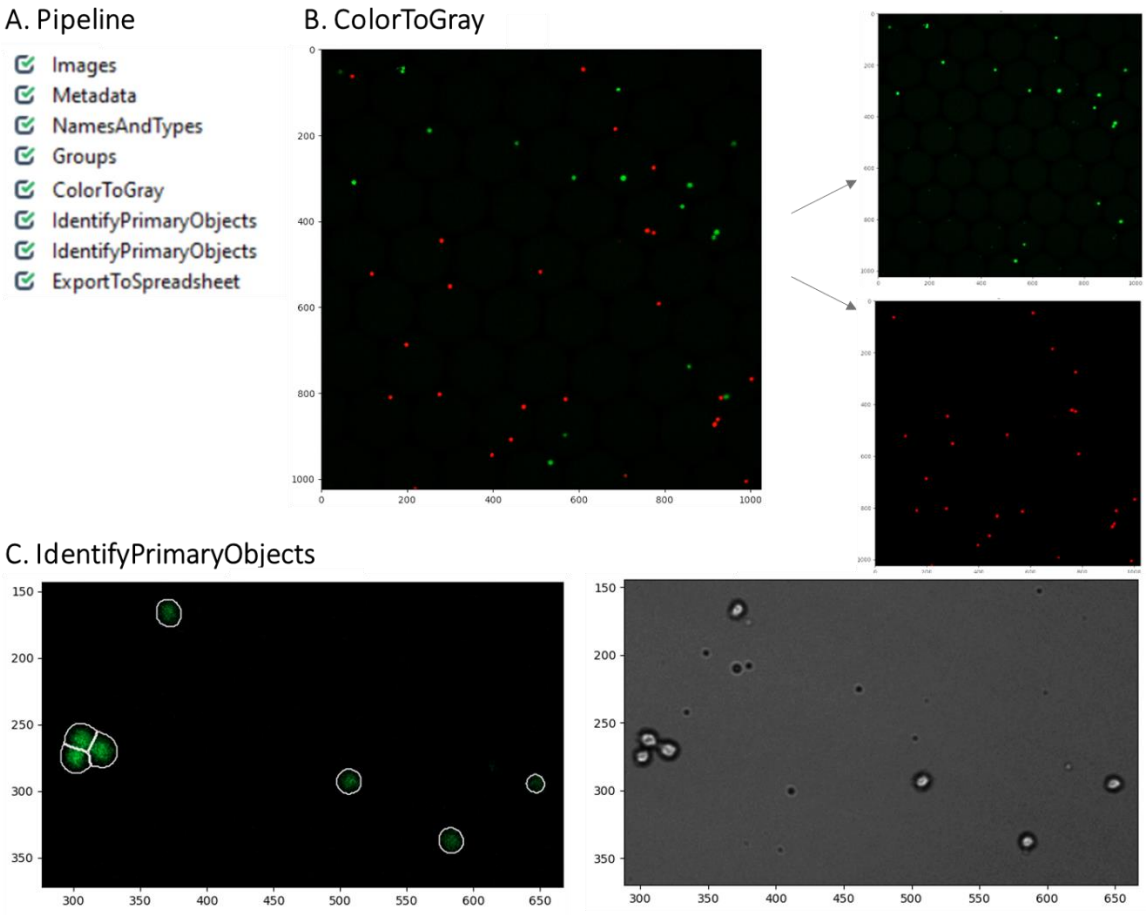


Figure 8. A. CellProfiler pipeline used for scoring dead and live cells. B. Two channels indicating signal from Calcein AM and Ethidium homodimer-1 are split to distinguish dead and live cells. C. Cells are counted based on size, shape and signal intensity.

Firstly, the pipeline separates different channels (red and green) of a colour image (Figure 8B) to identify cells based on their diameter. Live cell diameter was set to 10-30 pixels and for dead cells 8-20 pixels. Objects out of this range and touching the border of the image were discarded. Clumped objects were distinguished based on object intensity and *Propagate* was chosen to draw dividing lines between clumped objects (Figure 8C). Threshold strategy was set to *Global* and threshold method was set to *Robust Background. Threshold smoothing scale* was set to 4 and 1.3488 for live and dead cells respectively. *Threshold correction factor* was set to 1.5 for both live and dead cells. *ExportToSpreadsheet* generated Excel files with live and dead cell counts that were used for viability calculations and statistical tests.

3.8. Bisphenol A treatment in three-dimensional droplet culture

E2 and BPA (Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO) (Panreac AppliChem) were diluted to a concentration of 100 µM in culture medium for droplet stability analysis. 0.1% DMSO in culture medium was used as a negative control. Cascade Blue with a final concentration of 6 µg/mL was added to each sample for visualizing the droplets. Droplets were made and analysed after incubating for 24 hours as described before.

To assess cell survival in droplets with E2 and BPA, KGN cells (1.25 x 10^5 cells/mL) were resuspended in 1000 µL of 50 nM and 100 µM E2 and BPA solutions in culture medium. For negative control, cells were resuspended in 0.1% DMSO dissolved in culture medium. Droplet generation, collapsing and viability analysis was done as described in previous chapters. Viability of cells treated with 0.1% DMSO was considered as 100% and the other viability rates were normalized accordingly.

3.9. Bisphenol A treatment in standard two-dimensional cell culture

KGN cells were seeded in 12-well plates at a density of 1x10^5 cells/well. After 48 hours, cells were washed 3x with PBS and 1 mL of growth medium with 0.1% or 10% DMSO, 50 nM or 100 µM E2 or BPA was added to cells. After incubation for 24 hours, morphological cell changes were observed using Zeiss LSM 900 confocal microscope Plan-Apochromat 10x/0.45 objective.

For cell viability measurements, cells were washed 3x with 500 µL of PBS, trypsinized with 400 µL 0.05 % Trypsin-EDTA (Corning, USA) and collected into a tube for scoring live and dead cells. After centrifugation at 1000 rpm for 5 minutes, cells were resuspended in 100 µL of PBS and 20 µL of the sample was used for counting the cells using Trypan Blue exclusion method and the Countess Automated Cell Counter. Viability of cells treated with 0.1% DMSO was considered as 100% and the other viability rates were normalized accordingly. Live cell counts per mL were also analysed and 0.1% DMSO was considered as 100% and other live cell counts were adjusted accordingly.

3.10. Statistical analysis

The results are represented as mean ± standard deviation. Student's t-test was used for all statistical analyses, $p < 0.05$ were considered statistically significant. All statistical analyses were performed using MS Excel (2021).

4. Results

4.1. Optimal dye concentrations for KGN cell line

LIVE/DEAD viability/cytotoxicity kit was used to distinguish live cells from dead cells. Calcein AM is a fluorogenic esterase substrate that is hydrolyzed to a green-fluorescent product (calcein) in living cells. Green fluorescence is an indicator of cells that have esterase activity as well as an intact membrane to retain the esterase products. Ethidium homodimer-1 is a red-fluorescent nucleic acid stain that passes through compromised membranes of dead cells and stains their nuclei.

Since esterase activity varies between cell types, it is recommended by the manufacturer to determine the optimal dye concentration for a given cell type. Different dye concentrations suggested by the manufacturer were used to determine the optimal concentration for KGN cells. The lowest dye concentration that gave detectable signal in the correct channel, without causing a background noise that would disturb detection in the other channel, was chosen for both dyes. Final concentration of 2 µM Calcein-AM and 0.1 µM EthD-1 was chosen for further experiments (Figure 9).

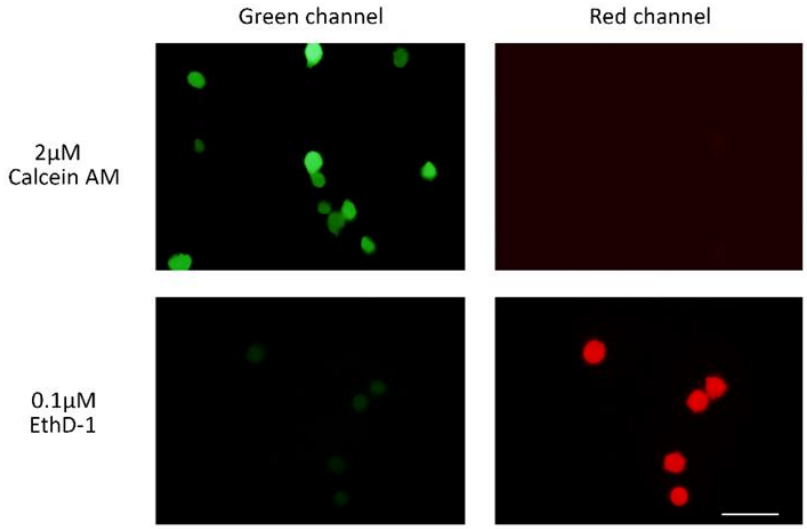


Figure 9. Optimal concentrations of Calcein AM (green) and Ethidium homodimer-1 (red) for detecting the viability of KGN cells. Scale bar 50 µm.

4.2. Single-cell encapsulation efficiency in an experiment with different containers

Cell encapsulation inside droplets was analysed to confirm the suitability of the chosen cell concentration. Cell loading into droplets follows Poisson statistics (Clausell-Tormos et al., 2008). The Poisson distribution for cells is given by equation 1 and 2:

$$P(k, \lambda) = \frac{\lambda^k e^{-\lambda}}{k!}, \qquad \text{(Equation 1)}$$

where k is the number of cells per droplet and λ is the average number of cells per droplet.

$$\lambda = C \cdot V_D \,, \qquad \text{(Equation 2)}$$

where C is the concentration of cells in a solution (cell/mL) and V_D the volume of each droplet (mL).

Thus, λ can be adjusted by controlling the cell density. To ensure that non-empty droplets contain most frequently only one cell, the value of λ should be less than 0.3 (Köster et al., 2008). To calculate the value of λ, at least 700 droplets were analysed for each container (Table 1).

Table 1. Average number of cells per droplet (λ) at a cell density of 1.25 x 10^5 cells/mL calculated for each container, N=1.

Container	λ	Droplets analysed
24-well plate	0.082	1908
12-well plate	0.154	729
5mL tube	0.076	1753
50mL tube	0.231	877

The average encapsulation rate for the cell density of 1,25 x 10^5 cells/mL was 0.117 which corresponds to 89.0% of droplets containing no cells, 10.4% containing one and 0.6% more than one cell. Since λ remained lower than 0.3, the same cell concentration was also chosen for further experiments.

4.3. Determining optimal containers for single cell incubation in droplets

After droplet generation, the cells need to be incubated for assessing their viability over different timepoints. As KGN cells are adhering cells, their viability in non-adhering conditions may be influenced by various physical factors.

The aim of the current experiment was to determine the most suitable container for incubating cells in droplets. Encapsulated cells were incubated in a 12- and 24-well plate and in a 5 mL and 50 mL tube (Figure 10).

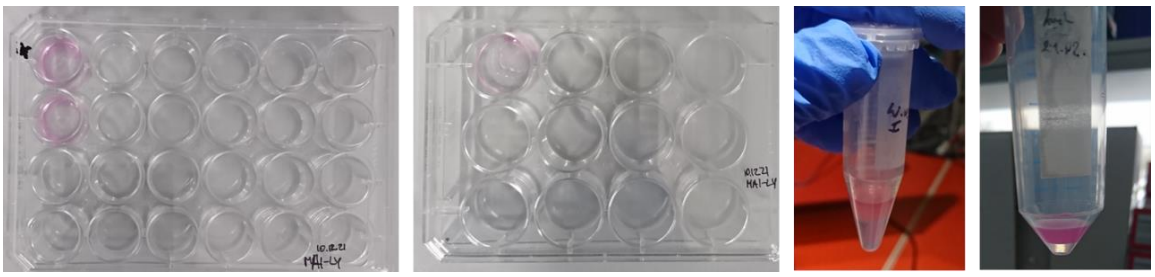


Figure 10. Different test-tubes used for incubating encapsulated cells.

Criteria for container suitability were droplet stability and cell viability, which were analysed at 0h, 24h and 48h. Droplet stability was assessed by comparing average droplet diameter right after droplet generation (0h) to the average diameter at 24h and 48h (Figure 11A).

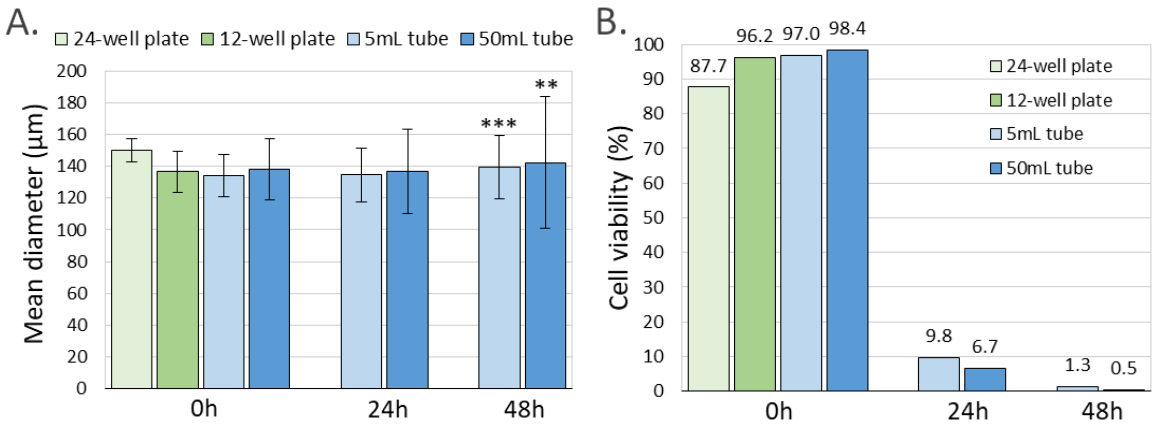


Figure 11. A. Average droplet diameter for each container, N=1. At least 700 droplets were analysed for each container. Student's t-test was used for comparing average droplet diameter at 0h to 24 and 0h to 48 hours (*$p < 0.05$, ***$p < 0.001$). Incubation in 5 mL and 50 mL tubes had no significant effect on average droplet diameter after 24 hours but the droplets lost the uniformity after 48 hours. Average droplet diameter in 24- and 12-well plates is shown only at 0h time-point since sample in the wells had dried out on both plates. **B. Cell viability measurements for each container,** N=1. At least 100 cells were counted for each container. Cell viability decreased drastically in 5 mL and 50 mL tubes by 24 hours and continued to decrease when incubated for 48 hours.

Average droplet diameter at 0h for 24-well plate was 149.95 µm ± 7.10 and for 12-well plate 136.76 µm ± 12.94. After 24 hours of incubation, the sample was dried out on both well plates and no imaging was possible. Thus, the well-plates are not suitable for droplet incubation and were not used in further experiments.

In a 5 mL tube, the average droplet diameter was 134.14 µm ± 13.35 at 0h. The average droplet diameter did not change significantly after 24 hours of incubation, being 134.46 µm ± 16.70, but after 48 hours the average droplet diameter increased significantly ($p < 0.001$) to 139.53 µm ± 19.70.

The average droplet diameter in a 50 mL tube was 138.01 µm ± 19.12 at 0h. The average diameter did not change significantly after incubating for 24 hours and was 136.76 µm ± 26.39 but after 48 hours, it had increased to 142.32 µm ± 41.38 and the change was significant when compared to the average diameter at 0h ($p < 0.05$). This increase in droplet diameter and in standard deviation indicates merging of droplets that occurs after longer incubation (Supplementary figure 1).

Cell viability was also measured (Figure 11B) and at least 100 cells were analysed for each container. At 0 hours, the cell viability was 87.7% in a 24-well plate and 96.2% in a 12-well plate. Since the sample dried out after 24 hours of incubation, no further viability rates were obtained. Cell viability in a 5 mL tube decreased from 97.0% at 0h to only 9.8% after 24 hours. By 48 hours it had decreased to 1.3%. This drastic decrease in cell viability was also observed for 50 mL tube when 98.4% viability had decreased to 6.7% after 24 hours and to 0.5% after 48 hours.

Since the average droplet diameter increased significantly after incubation for 48 hours and the cell viability rates were also very low, the droplets with encapsulated cells were incubated only for 24 hours in further experiments.

In conclusion, 5 mL and 50 mL tubes were suitable containers for droplet incubation for 24 hours regarding droplet stability. However, since the viability rates of KGN cells were very low already at 24 hours in both of these tubes, further optimization of the incubation conditions was needed to

be done. A 5 mL tube was chosen for next experiment since it was easier to work with and would produce less plastic waste than a 50 mL tube.

4.4. Incubation in a 5mL tube with holes in the lid to improve cell viability

Further experiments with 5 mL tube were made to find out whether cell viability is influenced by insufficient bicarbonate buffering of the culture medium. Holes were made in the 5 mL tube cap (Figure 12A) since the cap is tightly on and prevents adequate gas exchange and therefore could be the cause of very low viability measurements.

Droplets were incubated for 24 hours in 5 mL tubes with holes in the lid. Images were taken at 0h and 24h time points. 200 µl of sample with cells was encapsulated for imaging at 0h and 400 µl for imaging at 24h time point. At least 100 cells were counted.

Viability rates at 0h and 24h were 78.8% and 0% respectively. Average droplet diameter at 0h was 147.83 µm ± 19.60 (n=1804). At 24h most of the droplets had collapsed (Figure 12B).

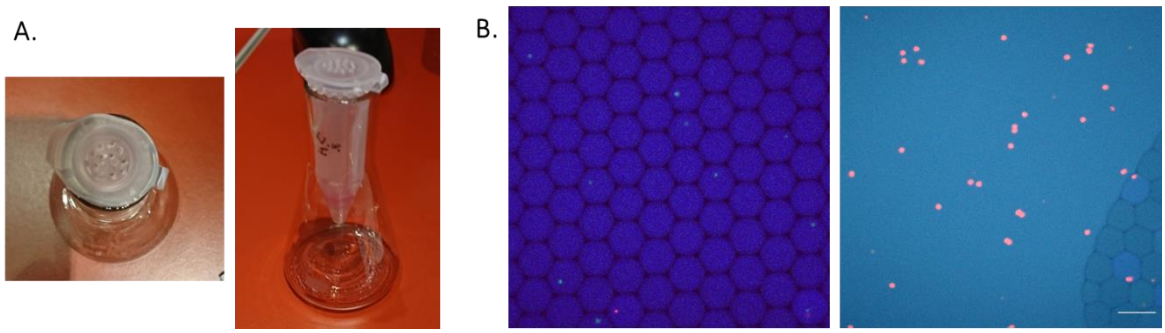


Figure 12. A. A tightly closed 5 mL tube with holes in the lid to provide sufficient gas exchange. B. Droplet quality at 0h (left) and 24h (right) after incubating in 5 mL tube with holes in the lid. Images taken with Zeiss LSM 900 (10X). Scale bar 150 µm.

The holes in a 5 mL tube lid did not contribute to higher viability rate when compared to previous experiment with a 5 mL tube. On the contrary, the viability rate was even lower (Supplementary table 1), and the droplets had merged as well. The collapsing of droplets by 24 hours was likely due to evaporation caused by the holes in the lid of the tube.

To evaluate the effect of droplet collapse on cell viability, an additional experiment with a 5 mL tube with holes in the lid was performed. A 3h time point was added to find out if cells start to die when droplets are still stable. 400 µl of cell sample was used for imaging at 0h. 800 µL of cell sample was used for encapsulation and incubation since an extra time point was added and more sample for imaging was needed.

At 0h, the viability was 93.5% but it had already decreased to 88.4% at 3h and to 35.1% at 24h. Average droplet diameter was 166.60 µm ± 7.89 at 0h and it did not change significantly after 3 hours of incubation ($p > 0.05$) (Supplementary table 1), at least 1000 droplets were analysed. Since the cell viability decreases before droplets collapse, the used stains might affect the viability rate. To evaluate the effect of stains, a 50 mL tube was chosen for next experiment to provide more sufficient gas exchange than a 5 mL tube with a closed cap and to prevent the evaporation of culture medium that was seen when using a 5 mL tube with holes in the lid.

A summary of the results obtained using different containers can be seen in Supplementary table 1.

4.5. Incubation in a 50 mL tube to evaluate the effect of stains on cell viability

The aim of the experiment was to evaluate the effect of stains on cell survival. Cells were incubated with the stains and imaged inside droplets (Figure 13A) or without the stains, but stained and imaged after collapsing the droplets (Figure 13B).

400 µL and 1000 µl of sample was encapsulated for imaging at 0h and 24h, respectively. The increased amount of sample was used for incubation since an extra step of droplet collapsing causes a slight loss in the amount of the sample used for analysing cell viability. In addition, the amount of sample needed to be increased for recovering enough cells for RNA extraction. Droplets were collected into a 50 mL tube with a loosely closed cap for incubation.

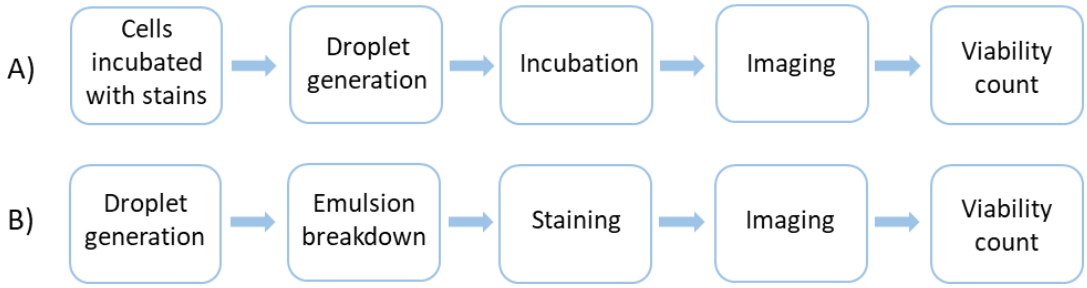


Figure 13. Workflow scheme. A) Cells are incubated with stains and imaged inside droplets. B) Cells are incubated without the stains and stained after breaking the droplets.

Average viability when incubating cells with stains in a 50 mL tube was 95.3% ± 0.2 at 0h (Figure 14) and this decreased significantly to 32.4% ± 5.1 at 24h ($p < 0.05$). Average viability when incubating cells without the stains was 91.9% ± 4.4 at 0h and it decreased to 81.7% ± 2.6 at 24h ($p < 0.05$). Unpaired Student's t-test was used to compare the difference between 24-hour incubation of cells in droplets with and without the stains. The average survival rate at 24h was 2.5-fold higher when incubated without the stains compared to incubation with stains ($p < 0.05$). The experiment confirmed that stains used for viability testing are toxic after hours of exposure. Therefore, cell staining for viability markers must be done after collapsing the droplets.

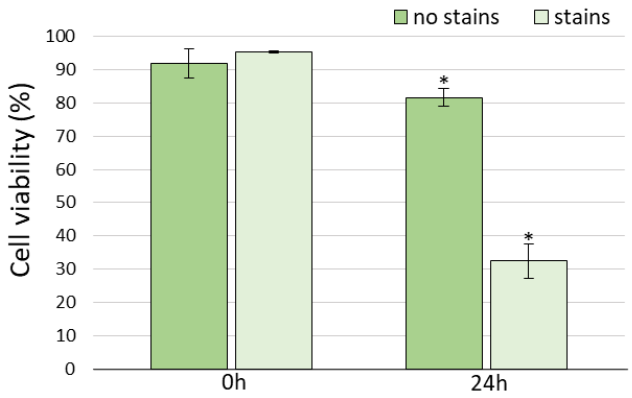


Figure 14. Cell viability measurements in droplets with and without added viability stains. Droplets with cells were incubated in a 50 mL tube. Error bars represent mean ± standard deviation. Cell viability was compared for both conditions between 0h and 24h using two-tailed paired Student's t-test. Cell viability decreased significantly after 24 hours of incubation for both conditions (*$p < 0.05$). Incubation without stains (N=3) resulted in 2.5-fold higher viability ($p < 0.05$) rate than incubation with the stains (N=2).

4.6. Estrogenic compounds estradiol and bisphenol A have no effect on droplet stability

BPA is a xenoestrogen that has been shown to mimic E2 by binding to ERα and ERβ (Vandenberg et al., 2007). In addition, BPA has been shown to alter ovarian steroidogenesis and signalling in the HPO axis, cause oxidative stress, autophagy and apoptosis in granulosa cells, that could result in follicular atresia that has been shown in animal experiments (Sturm & Virant-Klun, 2023).

The aim of this experiment was to study the effect of BPA in single-cell culture. As estrogenic compounds E2 and BPA possess lipophilic properties, the first aim was to test their effect on lipid droplet stability to understand if these compounds are feasible for further cell viability experiments.

Two different concentrations of BPA were chosen to study the effect of this compound on KGN cell viability. Lower concentration of BPA – 50 nM was chosen based on the levels that have been detected in the follicular fluid (Wen et al., 2010, Bellavia et al., 2023). Higher concentration of BPA – 100 µM was chosen based on other studies that have described the induction of apoptosis among other effects at this concentration in KGN cell line (Kwintkiewicz et al., 2010, Huang et al., 2021). E2 concentrations were chosen accordingly.

Only the higher concentrations were chosen to study the effect of these agents on droplet stability. 0.1% DMSO in culture medium was used as a negative control. Droplets were incubated for 24 hours.

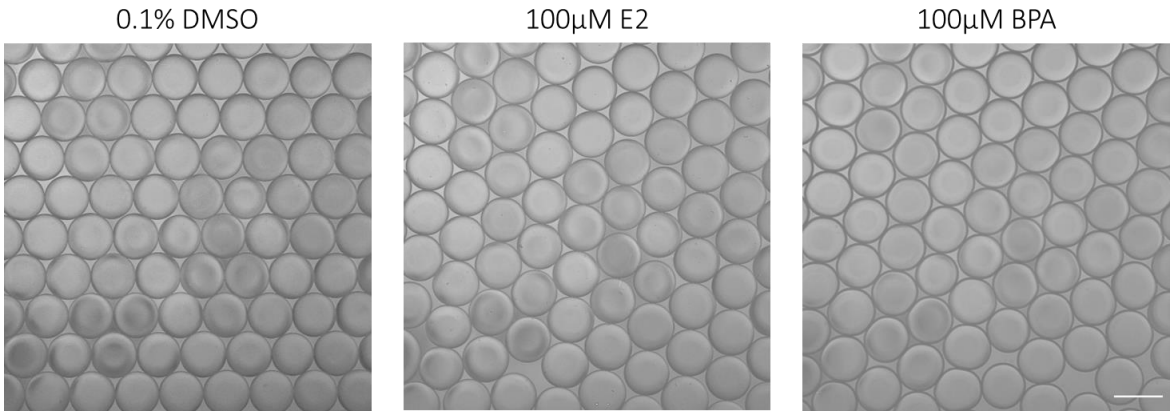


Figure 15. Monodisperse droplets in brightfield after 24h incubation with 0.1 % DMSO, 100 µM estradiol (E2) and 100 µM bisphenol A (BPA) in culture medium. Scale bar 170 µm.

Droplets in all samples remained stable after incubation (Figure 15). Therefore, further experiments with the chosen concentrations were conducted to assess the viability rates when incubating cells with these compounds.

4.7. Effect of estrogenic compounds estradiol and bisphenol A on cell viability in single-cell droplet culture

Granulosa cells were incubated in droplets containing culture medium with E2 or BPA to evaluate the effect of estrogenic compounds on cell viability. Cells were encapsulated and incubated for 24 hours. Cell viability did not change significantly ($p > 0.05$) compared to control when cells were incubated with 50 nM or 100 µM E2 or BPA (Figure 16).

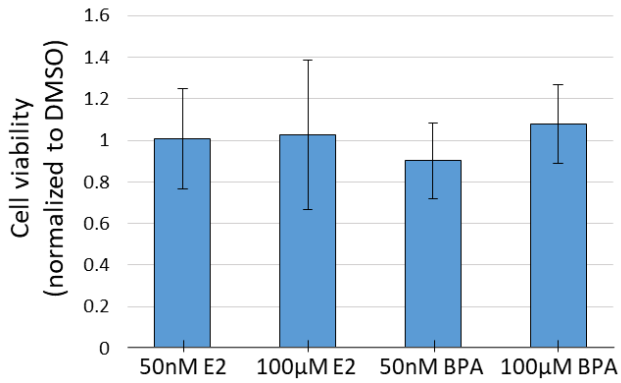


Figure 16. Cell viability measurements in droplets after 24-hour treatment with estradiol (E2) and bisphenol A (BPA). Data is presented as mean ± standard deviation. Cell viability was normalized to 0.1% DMSO control. No significant difference was observed between each treatment and control ($p > 0.05$), N=3.

Average cell viability for 50 nM and 100 µM E2 was 1.01 ± 0.24 and 1.02 ± 0.36, respectively. Treatment with 50 nM and 100 µM BPA resulted in an average viability rate of 0.90 ± 18 and 1.08 ± 0.19, respectively. No significant differences in viability were observed.

4.8. Effect of estrogenic compounds estradiol and bisphenol A on cell viability in 2D cell culture

The effect of E2 and BPA was also examined in 2D cell culture. Cells treated with 50 nM E2 and 50 nM BPA had a similar morphology to 0.1% DMSO control and this long and elongated fibroblast-like shape was more apparent when treated with 100 µM BPA (Figure 17). In contrast, higher concentration of E2 caused more of an epithelial-like shape.

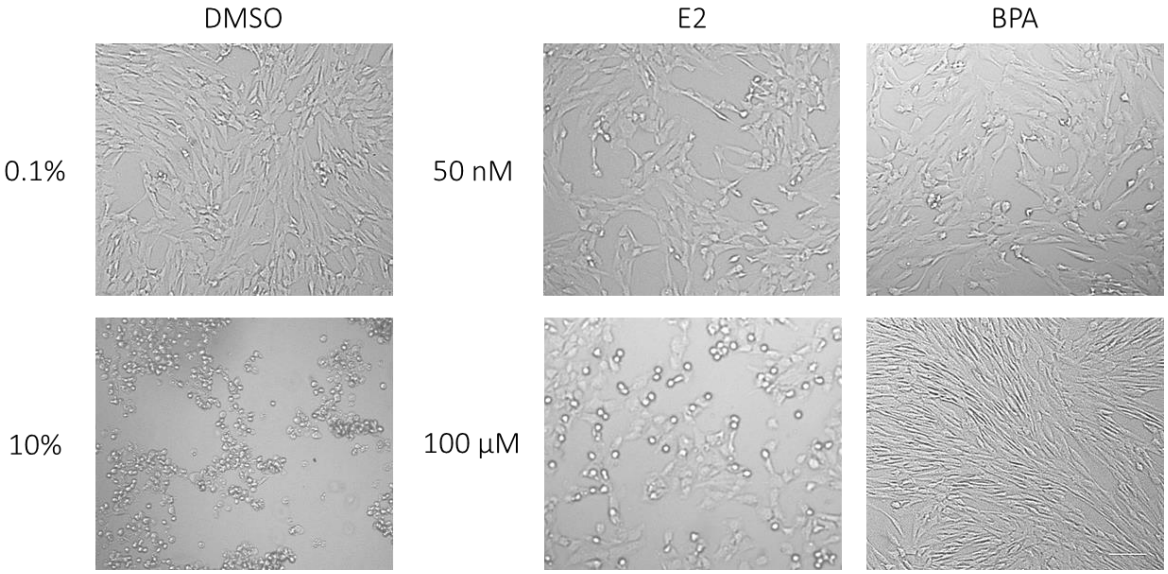


Figure 17. Brightfield images of KGN cell morphology after 24-hour treatment with 0.1% DMSO as a control, 10% DMSO as a positive control for apoptosis, 50 nM and 100 µM estradiol (E2) and bisphenol A (BPA). KGN cells treated with 50 nM E2, 50 nM and 100 µM BPA maintained similar long and elongated fibroblast-like shape as the 0.1% DMSO control group while treatment with 100 µM E2 caused the cells to acquire epithelial-like cell polygonal shape. Cells were examined by Zeiss LSM 900 confocal microscope (10X). Scale bar 100 µm.

Similar to 3D culture, viability rates did not change after 24-hour incubation in 2D culture either (Figure 18A). There was a clear decrease in viability when cells were treated with 10% DMSO ($p < 0.01$) but no change was observed when cells were treated with 50 nM or 100 µM E2 or BPA.

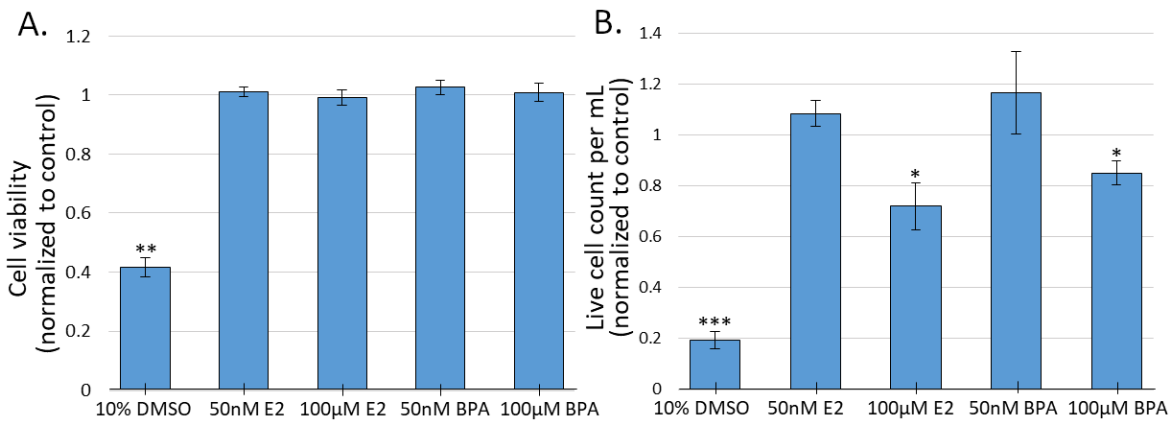


Figure 18. A. Cell viability measurements after 24-hour treatment in 2D cell culture normalized to 0.1% DMSO control. Error bars represent mean proportion of all viable cells ± standard deviation (N=3). Estradiol (E2) and Bisphenol A (BPA) had no effect on cell viability when compared to control. Treatment with 10% DMSO resulted in a statistically significant decrease in cell viability when compared to control. **B. Live cell count per mL after 24-hour treatment in 2D cell culture normalized to 0.1% DMSO control**. Error bars represent mean ± standard deviation (N=3). Treatment with 10% DMSO, 100 µM E2 and 100 µM BPA resulted in a significant decrease in live cell count per mL when compared to control. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

The difference between the groups became clearer when live cell counts per mL were compared, which is an indicator of cell proliferation. DMSO normalized live cell counts show (Figure 18B), that 100 µM E2 and BPA had a negative influence on cell proliferation when compared to control ($p < 0.05$).

Discussion

EDCs have been shown to be associated with many pathologies that can lead to reduced fertility. BPA, one of the most studied EDCs, has been shown to affect the HPO axis, steroidogenesis and granulosa cell proliferation, all of which are important processes for proper follicular development. Since there have been opposing results from experiments with BPA and granulosa cells, and EDCs including BPA have been shown to cause different effects at low and high concentrations, more information about the molecular initiating events in response to BPA or any EDC treatment is needed (Sturm & Virant-Klun, 2023). Droplet microfluidics methods can be used for single-cell analysis to investigate the heterogeneity between different cell types. By creating the same environment for individual cells, these high-throughput methods could enable studying the toxic effects of EDCs using primary cells from humans, in order to acquire human relevant data for more accurate risk assessment of these chemicals.

Droplet microfluidics has been used for single cell cultivation of eukaryotic cells of different origin. For example, incubation of Jurkat and HEK293T cells in a 15 mL centrifuge tube resulted in viability rates higher than 95% after 24 hours of incubation (Clausell-Tormos et al., 2008). Incubation has been also done in a 1 mL plastic syringe, where incubation of CHO cells for 72 hours resulted in viability rates higher than 90% (Rajeswari et al., 2017). Experiments with human monocytic U937 cells, have shown survival rates of 80% after 4 days of incubation in droplets when emulsion incubation was done using 5 mL glass syringes (Brouzes et al., 2009). In this thesis, multiwell-plates and tubes were analysed as possible containers for emulsion incubation. While droplets collapsed in 12- and 24-well plates after 24-hour incubation, 5 mL and a 50 mL tube were considered as suitable containers for droplet incubation, as no significant difference in average droplet diameter was seen in these tubes after 24-hour incubation. Since viability rates of KGN cells were as low as 9.8% and 6.7% at 24 hours in a 5 mL and 50 mL tube, respectively, further optimization of the incubation conditions was needed to be done. After several experiments with these tubes, a 50 mL tube was considered as a suitable container.

Culturing conditions in droplets differ from those in bulk, especially for adherent cells. Therefore, optimal conditions for different cell types have to be determined. For example, droplet size has been shown to affect cell proliferation in droplets (Köster et al., 2008, Rajeswari et al., 2017). When longer incubation of cells in droplets is required, larger droplets have the advantage of avoiding nutrient depletion and providing oxygen availability for cells. On the other hand, when droplets are used for analyte detection and incubation time in droplets is shorter, smaller droplets would be preferred to obtain high concentrations of secreted cellular factors more rapidly (Clausell-Tormos et al., 2008). For example, Rajeswari et al have shown that there is a direct correlation between droplet size to cell division and viability, where larger droplets resulted in higher proliferation and viability rates (Rajeswari et al., 2017). When in the experiment by Rajeswari et al, the decrease in cell viability was seen after 72 hours of incubation, the use of even smaller droplet sizes by Köster et al showed decreased cell survival rates in smaller droplets with only after 3 hours of incubation (Köster et al., 2008). In our experiments, the average droplet diameter was about 10 times larger than KGN cell diameter, which is even higher droplet/cell diameter ratio than in the previously mentioned studies.

To make cell viability analysis faster, stains were initially added to cells before encapsulation which can also have an effect on cell viability during longer incubation. In this thesis, the stains caused 2.5-

fold lower cell viability rates than incubation without the stains ($p < 0.05$). Therefore, staining for viability markers was chosen to be done after emulsion incubation. This resulted in an extra step of droplet collapsing, which also was shown to cause a slight decrease in cell viability, although to a much lesser extent than incubation with stains. In this thesis, KGN cells showed viability rates with an average of 82% after 24-hour incubation in droplets without any added stains. These results are similar, although slightly lower than those described previously for other adherent cell types (Clausell-Tormos et al., 2008, Brouzes et al., 2009, Rajeswari et al., 2017). Therefore, after finding the optimal concentrations of viability stains, confirming the suitability of the chosen cell concentration for single cell encapsulation, finding an optimal container for incubation of the encapsulated cells, and optimizing the staining procedure, an optimal protocol for KGN cell line incubation in droplets was established. Thus, the effect of BPA on KGN cell viability was studied in the next experiments by using this established system.

BPA has been shown to cause an increase in apoptosis, alter steroidogenesis and cause oxidative stress in KGN cells. In this thesis, physiological (50 nM) and supraphysiological (100 µM) concentrations of BPA were used to study the effect of BPA on single cells in droplet culture and in traditional 2D cell culture.

In both, 2D and 3D cell culture, KGN cells treated with BPA and E2 for 24 hours did not show any changes in cell viability rates when compared to control. Cell viability rates are not very informative and no changes in cell viability have also been observed when using primary human cumulus granulosa cells and similar BPA treatment (Pogrmic-Majkic et al., 2019). Therefore, other cell characteristics should be analysed as well. In this thesis, changes in KGN cell morphology were seen in 2D culture, when treatment with 100 µM concentration of BPA resulted in elongated fibroblast-like cells unlike the E2 treated cells and control samples. Also, the analysis of live cell counts revealed a significant decrease in cell proliferation after treatment with 100 µM BPA and 100 µM E2. This also shows that granulosa cells can act similarly to BPA as they do to E2 treatment, demonstrating its ability to act as a xenoestrogen. These results are consistent with a study by Kwintkiewicz et al, where no changes in apoptosis rate were observed but proliferation was significantly inhibited after treating KGN cells with 100 µM BPA (Kwintkiewicz et al., 2010). Unfortunately, the effect of EDCs on cell proliferation is more difficult to study in the single-cell droplet culture and hence, the conventional 2D cell culture demonstrates advantages in the current thesis. However, proliferation of KGN cells could be studied when optimal conditions for incubating cells in droplets for longer than 46 hours were determined, since the doubling time of KGN cells is approximately 46 hours (Nishi et al., 2001). In this thesis, a significant increase was seen in droplet size after 48 hours of incubation, but more experiments would be needed to confirm these results. One possible way of improving droplet quality during longer incubation than 24 hours could be the use of a different oil-surfactant combination.

To confirm the suitability of the established single-cell droplet culture for KGN cell incubation, gene expression analysis of cells incubated using 2D and 3D method should be compared. Since results of BPA treatment in 2D culture showed differences in cell counts and morphology in supraphysiological conditions (100 µM), gene expression analysis could provide insights to changes that take place after BPA treatment at physiological conditions (50 nM). Later, in the future, this droplet culture system should be used for incubating a mixture of different cell types that are

present in the follicle, and further optimization should be done for making this developed method usable for primary cells.

In conclusion, based on the current thesis, 2D and 3D cell culturing methods have their own advantages which should be considered when designing an experiment.

The main advantages of 3D droplet culture include the following:

- Enabling single-cell culture
- Enabling high-throughput single-cell analysis
- Providing more accurate environment for non-adherent cells in the follicle, e.g. leukocytes
- Enabling short-term culture that is suitable for analysing molecular initiating events of EDCs

Main advantages for 2D cell culture include:

- Providing morphological information about cells
- Enabling cell proliferation assessment
- Being a less time-consuming method for culturing adherent cells, e.g. granulosa cells in the follicle

Summary

Endocrine disrupting chemicals (EDCs) can alter hormonal regulation necessary in regulating many biological processes including ovarian folliculogenesis. This process is regulated on many levels and any disruptions can lead to the reduced fertility of a woman since the primordial follicles that form before birth represent a finite pool of the ovarian reserve. Bisphenol A (BPA) that has been associated mostly with the folliculogenesis altering properties, has been shown to cause negative effects in granulosa cells that represent one of the somatic cell types in the follicle. However, there have been no studies that examine the effect of BPA on different cell types in the follicle in a single-cell response.

Single-cell cultivation and analysis can help in investigating cellular heterogeneity. Droplet microfluidics, which enables the compartmentalization of individual cells in nanolitre-sized droplets, has enabled single-cell isolation and analysis in a high-throughput manner. In this thesis, the possibility of combining the ovarian cells with a microfluidic culturing method was tested.

The first aim of this study was to analyse granulosa cell line KGN survival in a 3D single-cell culture system by using droplet microfluidics. The second aim of this study was to examine the established droplet culture system's applicability by testing BPA's effect on KGN cell viability. 2D cell culture system was also used for treating KGN cells with BPA for comparing the results to those from the 3D cell culture.

In this thesis, an optimal protocol for incubating KGN cells in a single-cell culture was established. The results show that normally adherent KGN cells that were incubated for 24 hours in droplets retain high cell viability rates similar to those described in the literature. Testing of the BPA exposure in 3D culture showed that the moderately hydrophobic compound BPA can be tested at physiological but also supraphysiological doses, since droplets incubated with BPA did not collapse by 24 hours. In addition, KGN cell viability rates did not change significantly when treated with BPA neither in 3D nor 2D cell culture. The results from BPA treatment in 2D cell culture show that BPA has a significant effect on cell proliferation and morphology. Therefore, the next step should be gene expression analysis and comparison of the results from cells incubated in 3D and 2D culture, to reveal the cellular response to BPA and the effects of the droplet culture system.

Kokkuvõte

Endokriinsüsteemi häirivad ühendid (*endocrine disrupting chemicals* – EDCs) võivad mõjutada erinevaid hormonaalseid protsesse, mis muuhulgas vastutavad ka munasarja folliikuli kasvu ja arengu eest. Kuna folliikulogeneesi regulatsioon toimub mitmel tasandil, võivad juba üksikud muutused hormonaalsetes radades tekitada kõrvalekaldeid folliikuli arengus ning põhjustada viljakuse vähenemist. Kuivõrd naise munarakkude reserv moodustub juba enne sündi ning elu jooksul ei uuene, siis on EDC-de võimaliku kahjustava mõju uurimine follikulogeneesile väga oluline. Kõige enam uuritud reproduktiivsüsteemi kahjustav EDC on bisfenool A (BPA), mille mõju on näidatud eelkõige granuloosa rakuliinides, mis esindavad vaid üht tüüpi somaatilisi rakke arenevas folliikulis. Teadaolevalt ei ole varem uuritud BPA mõju üksikutele munasarja folliikuli somaatilistele rakkudele.

Üksikute rakkude kultiveerimine võimaldab uurida rakkude heterogeensust ning erinevat vastust uuritavale kemikaalile. Tilga mikrofluidika meetodid võimaldavad eraldada üksikuid rakke tilkadesse ning analüüsida neid suure läbilaskevõimega.

Käesoleva töö eesmärk oli uurida granuloosa rakuliini KGN rakkude elulemust tilga mikrofluidika meetodil loodud üksikraku kultuuris. Lisaks sellele oli eesmärk uurida loodud üksikraku kultuuri kasutamist BPA mõju testimiseks KGN rakkudel. Selle jaoks võrreldi BPA-ga töödeldud rakkude elulemust tilgakultuuris ning mikrotiiterplaatidel.

Magistritöö tulemusena töötati välja meetod, mis võimaldab KGN rakkude inkubeerimist üksikraku kultuuris. Töö tulemused näitavad, et KGN rakke on võimalik inkubeerida tilkades 24 tundi, säilitades seejuures kõrget elulemust sarnaselt kirjanduses leiduvate tulemustega. Lisaks sellele on töös näidatud, et hüdrofoobsete omadustega BPA ei mõjuta tilkade stabiilsust ning selle mõju uurimine tilgakultuuris on võimalik füsioloogilistel aga ka kõrgematel kontsentratsioonidel. Rakkude inkubeerimine BPA-ga ei põhjustanud muutusi rakkude elulemuses ei tilgakultuuris ega ka 2D kultuuris. Lisaks elulemusele uuriti 2D kultuuris kasvatatud rakkude morfoloogiat ja proliferatsiooni. Kuna saadud tulemused näitasid selgelt, et BPA-ga töötlus põhjustab rakkudes erinevusi sarnaselt östradiooli töötlusele, on järgmiseks oluline läbi viia mõlemal meetodil BPA-ga töödeldud rakkude geenieksпressiooni analüüs, mis lisaks BPA mõjule annaks informatsiooni ka tilgakultuuri mõjust rakkudele.

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Supplementary material

Supplementary figure 1. Nonuniform droplets after incubating for 48 hours

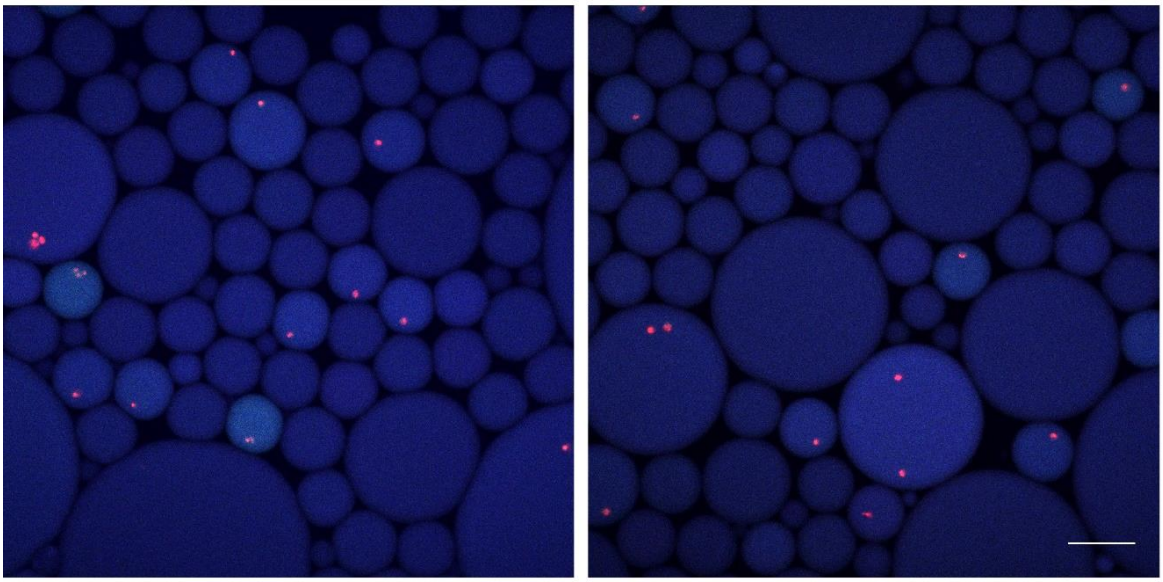

Figure 1. Examples of nonuniform droplets after 48 hours of incubation in a 50mL tube. Images taken with Zeiss LSM 900 (10X). Scale bar 150 µm.

Supplementary table 1. Cell viability and average droplet diameter in different containers

Table 1. Cell viability rates and average droplet diameter when incubating cells with stains in different containers, N=1.

Container	0h		24h		48h	
	Cell viability (%)	Mean droplet diameter (μm)	Cell viability (%)	Mean droplet diameter (μm)	Cell viability (%)	Mean droplet diameter (μm)
24-well plate	87.7	149.95 ± 7.10		-		-
12-well plate	96.2	136.76 ± 12.94		-		-
5mL tube	97	134.14 ± 13.36	9.8	134.46 ± 16.70	1.3	139.53 ± 19.71
50mL tube	98.4	138.01 ± 19.13	6.7	136.76 ± 26.39	0.5	142.32 ± 41.39
	0h		3h		24h	
5mL tube (holes in the lid)	78.8	147.83 ± 19.60		-	0.0	-
5mL tube (holes in the lid)	93.5	166.6 ± 7.89	88.4	166.39 ± 8.62	35.1	-

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