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In Vitro Mutagenicity Test using Canine Intestinal Organoids

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Abstract

Intestinal organoids could offer a significant advantage for genotoxicity studies compared to traditional cell lines like Chinese Hamster Ovary (CHO) cells due to their higher cellular diversity and more accurate representation of the intestinal environment. As the intestine is a primary site for drug and toxin exposure, using organoids derived from intestinal tissue allows for a more physiologically relevant model. These organoids contain multiple cell types, including enterocytes, goblet cells, and stem cells, which better mimic the complex interactions and responses to genotoxic agents than the more homogenous CHO cells. The main goal of genotoxicity studies of intestinal organoids could be a more reliable model for assessing the genotoxic potential of substances in a context that closely mirrors *in vivo* conditions. In this study, we carried out a comparative study of intestinal canine organoids and Chinese Hamster Ovary (CHO) cells as models to estimate the mutagenicity potential. The chemotherapeutical drug cyclophosphamide (CPA) was used on both organoids and CHO cells to compare their ability of cell systems to detect the drug mutagenicity potential. CHO cells, widely used in biological research due to their rapid growth, ease of genetic manipulation, were used as a reference model. CPA is extensively employed as a positive control in genotoxicity testing, particularly in the micronucleus (MN) assay. This study aimed to explore intestinal organoid's ability to induce micronuclei formation, a marker of chromosomal damage. This study proposes for the first time the establishing the micronuclei (MN) assay using intestinal organoid. For comparison and validation of the results, we also used CHO cells, which treated with CPA as the positive control. CPA as an alkylating agent, is known to affect DNA and cause chromosomal damage. Its mechanism of action involves DNA alkylation, leading to cross-linking of DNA strands, mispairing, and disruption of cellular processes, particularly during cell division. CPA's impact on the spindle apparatus during metaphase increases chromosomal damage, potentially affecting cell viability. Through a micronucleus assay using chromosome staining and fluorescence microscopy, we found that canine intestinal organoids were able to detect the genotoxic effect of CPA via micronucleus test. These findings suggest that CPA

induces genotoxic effects in both models. This study highlights the potential of canine intestinal organoids as a reliable, cost-effective, and ethical alternative model system for studying food-drug-gut interactions, drug absorption, and genotoxicity. It offers a promising alternative to animal testing in drug development and toxicology research, paving the way for new opportunities in preclinical research, especially in veterinary medicine, food development, and food-drug allergy testing.

Keywords: Intestinal organoids, genotoxicity studies, Chinese Hamster Ovary (CHO) cells, mutagenicity potential, cyclophosphamide (CPA), micronucleus (MN) assay, chromosomal damage, canine organoids, food-drug-gut interactions, drug absorption, alternative model systems, preclinical research, veterinary medicine, toxicology research, DNA alkylation, spindle apparatus, fluorescence microscopy.

Zusammenfassung

Intestinale Organoide könnten für Genotoxizitätsstudien im Vergleich zu traditionellen Zelllinien wie den Ovarialzellen des Chinesischen Hamsters (CHO-Zellen) einen erheblichen Vorteil bieten, da sie eine höhere zelluläre Vielfalt und eine genauere Darstellung der intestinalen Umgebung aufweisen. Da der Darm ein Hauptort für die Exposition gegenüber Medikamenten und Toxinen ist, ermöglicht die Verwendung von aus Darmgewebe abgeleiteten Organoiden ein physiologisch relevanteres Modell. Diese Organoide enthalten verschiedene Zelltypen, einschließlich Enterozyten, Becherzellen und Stammzellen, die komplexe Interaktionen und Reaktionen auf genotoxische Agenzien besser nachahmen als die homogeneren CHO-Zellen. Das Hauptziel von Genotoxizitätsstudien mit intestinalen Organoiden könnte ein zuverlässigeres Modell zur Bewertung des genotoxischen Potenzials von Substanzen in einem Kontext sein, der in vivo-Bedingungen näher kommt. In dieser Studie führten wir eine vergleichende Untersuchung von intestinalen Organoiden des Hundes und CHO-Zellen durch, um das Mutagenitätspotenzial abzuschätzen. Das chemotherapeutische Medikament Cyclophosphamid (CPA) wurde auf beiden Modellen angewendet, um ihre Fähigkeit zu vergleichen, das mutagene Potenzial des Medikaments zu erkennen. CHO-Zellen, die aufgrund ihres schnellen Wachstums und ihrer einfachen genetischen Manipulierbarkeit häufig in der biologischen Forschung verwendet werden, dienten als Referenzmodell. CPA wird häufig als positiver Kontrollstoff in Genotoxizitätstests, insbesondere im Mikronukleus-Test (MN-Test), eingesetzt. Ziel dieser Studie war es, die Fähigkeit intestinaler Organoide zur Induktion von Mikronuklei, einem Marker für chromosomale Schäden, zu untersuchen. Diese Studie schlägt erstmals die Etablierung des Mikronukleus-Tests mit intestinalen Organoiden vor. Zum Vergleich und zur Validierung der Ergebnisse verwendeten wir ebenfalls CHO-Zellen, wobei ein Medikament als positive Kontrolle eingesetzt wurde. CPA, ein alkylierendes Mittel, ist dafür bekannt, DNA zu beeinflussen und chromosomale Schäden zu verursachen. Sein Wirkmechanismus umfasst die Alkylierung der DNA, was zur Quervernetzung von DNA-Strängen, Fehlpaarungen und Störungen der zellulären Prozesse, insbesondere während der Zellteilung, führt. Die Auswirkungen von CPA auf den Spindelapparat während der Metaphase erhöhen das Risiko chromosomaler Schäden, was möglicherweise die

Zellviabilität beeinträchtigt. Durch einen Mikronukleus-Test unter Verwendung von Chromosomenfärbung und Fluoreszenzmikroskopie stellten wir fest, dass intestinale Organoide des Hundes in der Lage waren, die genotoxische Wirkung von CPA mittels Mikronukleus-Test zu erkennen. Diese Ergebnisse deuten darauf hin, dass CPA in beiden Modellen genotoxische Effekte hervorruft. Diese Studie hebt das Potenzial von intestinalen Organoiden des Hundes als zuverlässiges, kostengünstiges und ethisches alternatives Modellsystem zur Untersuchung von Wechselwirkungen zwischen Nahrung, Medikamenten und Darm, der Arzneimittelabsorption und der Genotoxizität hervor. Sie bietet eine vielversprechende Alternative zu Tierversuchen in der Arzneimittelentwicklung und der Toxikologieforschung und ebnet den Weg für neue Möglichkeiten in der präklinischen Forschung, insbesondere in der Veterinärmedizin, der Lebensmittelentwicklung und der Prüfung von Lebensmittel- und Arzneimittelallergien.

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1. Introduction

1.1. Gastrointestinal (GI) disorders

Gastrointestinal (GI) disorders including inflammatory bowel disease (IBD), infection-related GI conditions, and cancer, significantly harm human health and place a substantial financial strain on healthcare systems. Infectious diarrhea ranks as the second leading cause of death among young children (Kim et al., 2014), and GI cancers are the third most common cancer type globally, with one million new cases diagnosed annually (Bray et al., 2018). Potentially fatal GI diseases also impact livestock and companion animals, often involving enterotoxic bacteria and enteropathogenic viruses in the onset and progression of these conditions (Gallagher et al., 2020). Despite significant efforts to develop new treatments for human and veterinary GI diseases, mortality rates remain high. This is primarily because the translation of biomedical research into clinical practice is hindered by the absence of epithelial models that accurately replicate the organ and mirror the diseases in patients (Albani et al., 2010; Albani & Prakken, 2009). Initial efforts to cultivate the intestinal epithelium *in vitro* were generally short-lived. For instance, explanted biopsies and epithelial cells typically disintegrated after 72 hours and two weeks, respectively (Browning & Trier, 1969; Kedinger et al., 1987; Evans et al., 1992).

1.2. Culture system and organoids

To create culture systems capable of sustaining long-lived homogeneous cell populations, tissue explants were either grown within collagen gels or placed on 3T3 feeder layers at an air-liquid interface (Ootani et al., 2009; Wang et al., 2015). However, the need of growth factor-secreting (myo)fibroblasts, which are essential for extended cultivation in these systems, introduced variability between experiments. A significant advancement in addressing these challenges was the development of three-dimensional (3D) murine intestinal organoids, generated from adult intestinal stem cells (T. Sato et al., 2009a). Organoids are *in vitro* organ-like structures that mimic key features of real organs. In recent decades, significant advancements have been made in developing three-dimensional (3D) culture systems that generate organoids from stem cells. These

3D organoid models represent a major technological leap, effectively bridging the gap between traditional two-dimensional (2D) *in vitro* models and *in vivo* animal studies. They offer an unparalleled opportunity to explore the complexities of veterinary diseases, including their origins, prevention, treatment, and potential future organ replacement strategies. The shift from traditional monolayer cell cultures to three-dimensional (3D) systems has been the most significant innovation in *in vitro* studies over the past decade. Among the most notable advancements in 3D models is the development of organoids, often referred to as "mini organs on a dish," which represents a cutting-edge technology. Recently, there have been a growing number of research publications utilizing organoid models to study diseases of veterinary relevance. However, compared to medical research, the potential of organoids in veterinary medicine remains underexplored. The term "organoid," meaning "resembling an organ," was first used in 1946 when researchers described a cystic teratoma. Teratomas originate from pluripotent stem cells (PSCs) of the germ line and form various organ-like structures, such as skin, nerve, intestine, bone, and tooth, by recapitulating processes of cell segregation and fate determination. Today, "organoid" is widely understood to refer to a 3D cluster of organ-specific cell types. Similar to teratomas, organoids arise from stem cells or organ progenitors (e.g., intestinal crypts) and self-organize through processes resembling those in *in vivo* development. This self-organization is a key feature that distinguishes organoids from two-dimensional cultures.

1.3. Generating organoids

The critical steps in generating an organoid involve the initial proliferation of stem and precursor cells in a suitable environment. A defining characteristic of organoids is their derivation from stem cells, which sets them apart from tissue explants that originate from organotypic cultures of cells or small tissue segments. The stem cells used include embryonic stem cells (iESCs), induced pluripotent stem cells (iPSCs), or organ-specific adult stem cells (ASCs). ESCs are derived from early-stage embryos, while iPSCs are adult somatic cells that have been genetically reprogrammed to exhibit properties like ESCs. ASCs are typically sourced from "mature" or adult tissues but can also be obtained from juvenile or even advanced embryonic stages. Cancer stem cells (CSCs), a

type of ASC, can also generate 3D tumoroids in appropriate conditions. Organoids derived from ASCs are intrinsically programmed with location-specific functions, making them more "adult-like" than those derived from ESCs, which retain tissue-associated mesenchymal cells (**Figure 1**) (Lancaster & Knoblich, 2014; E. Smith et al. 1946 ; Resau et al., 1991; Pain, 2021; Fatehullah et al., 2016; Tatullo et al., 2020; Kar et al., 2021).

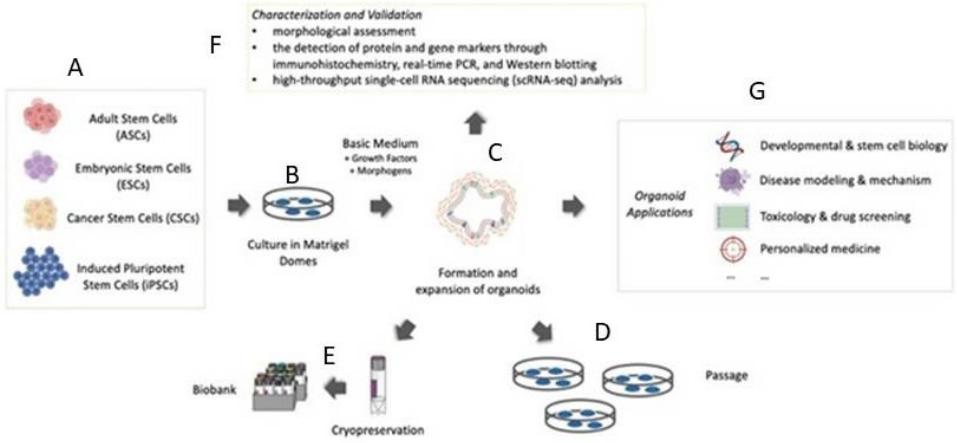


Figure 1. This figure provides a schematic representation of the process of generating, validating, and using organoids for various research applications. **A:** Stem Cell Sources, **B:** Culture in Matrigel Domes, **C:** Formation and Expansion of Organoids, **D:** Passage, **E:** Cryopreservation and Biobank, **F:** Characterization and Validation, **G:** Organoid Applications. (Created with BioRender.com).

Three-dimensional (3D) cell culture techniques refer to any method of culturing cells that recreates their natural 3D structure and facilitates cell-to-cell and cell-to-matrix interactions *in vitro*. When multiple cell types are cultured together, this is often called co-culture. Under the right culture conditions and with suitable scaffolding, stem cells can develop into miniature organ models known as organoids or spheroids, which replicate the cell types, structures, and functions of real organs (Koledova, 2017; Sumbal et al., 2020; Sato et al., 2009b; Augustyniak et al., 2019). Some viewpoints offer a stricter definition, suggesting that spheroids are typically scaffold-free, simple aggregates of cells originating from a single cell type (K. Sato et al., 2021; Nath & Devi, 2016). In contrast, a more precise definition of "organoid" has been proposed, which requires three key criteria to be met: first, an organoid must consist of more than one cell type found in the organ it models; second, it should replicate specific functions of that organ; and third, the cells within the organoid should exhibit a spatial organization that resemble actual organs. Due to their greater

complexity and closer resemblance to the organs they model, organoids are increasingly favored by researchers for disease modeling, drug discovery, and personalized medicine. On the other hand, in cancer research, spheroids generated from cancer cell lines or tumor fragments are often preferred because, as simple clusters of cancer stem cells, they effectively mimic the key structural and functional characteristics of solid tumors (Fang & Eglen, 2017; Nunes et al., 2018).

1.4. Enteroids, colonoids and intestinal organoids

Confusion has arisen in the terminology used for gastrointestinal organoids, especially with terms like "enteroids," "colonoids," and "intestinal organoids." Typically, "enteroids" refer to organoids derived from isolated intestinal crypts or intestinal stem cells, while "colonoids" are the equivalent structures derived from the colon. The term "intestinal organoids" is often used to encompass both enteroids and colonoids, though some researchers use "intestinal organoids" and "enteroids" interchangeably, without distinguishing between them and colonoids. In some research contexts, "organoids" specifically refer to cultures containing both epithelial and mesenchymal components, whereas "enteroids" are used to describe 3D structures composed solely of epithelial cells. Additionally, the term "intestinal organoids" is sometimes reserved for 3D structures derived from inducible pluripotent stem cells. The term "organoids" is used broadly to include all these complex, multicellular 3D systems, whether they originate from stem cells or tumor cells. Several other specific terms are also common in organoid research.

1.5. Assembloids and tumoroids

"Assembloids" refer to organoids created through the spatial fusion and functional integration of multiple cell types. "Tumoroids" are organoids that mimic tumors. "Mammospheres" describe aggregates of mammary epithelial stem cells but can also refer to clusters derived from breast cancer cells or cell lines (Zachos et al., 2016; Kratochvil et al., 2019; Vogt, 2021; Xu et al., 2022; Grimshaw et., 2008).

1.6. Advances in intestinal organoid cultivation and differentiation

Organoids housed long-lasting stem cells that developed into the key cell types found in the murine small intestine, including paneth cells, enterocytes, enteroendocrine cells, and goblet cells. The creation of this advanced *in vitro* model was made possible by using a laminin-rich extracellular matrix (Matrigel) and growth media enriched with epithelial growth factor (EGF), Noggin, and R-spondin, as thoroughly reviewed by Date and Sato (Date & Sato, 2015). To cultivate human intestinal organoids, the growth medium needed to be enriched with Wnt3a, gastrin, a p38-MAPK inhibitor, nicotinamide, and an ALK4/5/7 inhibitor. This combination prevented differentiation into goblet and enteroendocrine cells, keeping the enterocytes in an immature state (T. Sato et al., 2011). Removing nicotinamide and the p38-MAPK inhibitor triggered new differentiation, which reduced the lifespan of the intestinal stem cell population, turning this system into an endpoint assay. Advancements in human 3D intestinal organoid models were made using microscaffolds that replicate the size and spacing of crypts in transwell assays (T. Sato et al., 2011). Differentiation was achieved using a growth factor gradient system, where the lower wells contained an expansion medium that supported intestinal stem cells, and the upper wells received a differentiation medium. This encouraged migrating cells to differentiate into goblet cells, enteroendocrine cells, and enterocytes, simulating the crypt/villus axis. However, this method is not suitable for high-throughput screening, as it requires significant time, labor, and complicates downstream analysis. An alternative approach, focusing on receptor tyrosine kinase signaling between the crypt base and its surrounding environment, successfully avoided the need for nicotinamide and p38-MAPK inhibitors. This was accomplished by using two ligands—insulin-like growth factor 1 (IGF1) and basic fibroblast growth factor (FGF2) which supported stem cell growth and promoted simultaneous differentiation into goblet and enteroendocrine cells, better mimicking natural epithelial tissue with less effort (Kruitwagen et al., 2017; Derricott et al., 2019; Hamilton et al., 2018; Pierzchalska et al., 2012).

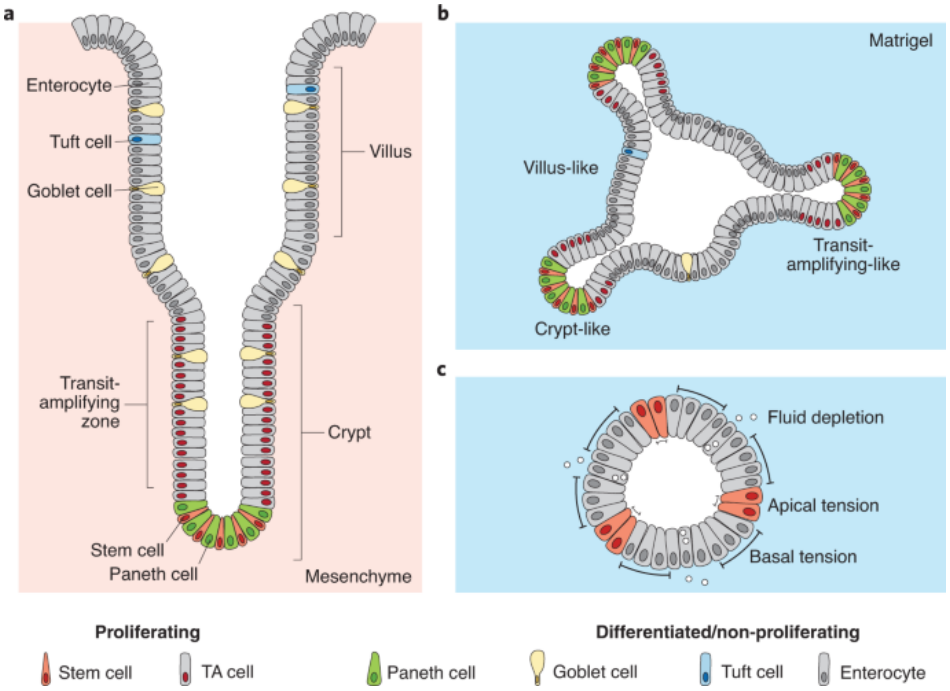


Figure 2: This figure illustrates the structure and cellular organization of intestinal crypt-villus units and the formation of intestinal organoids in a 3D matrix. **a**: Shows the structure of the intestinal crypt-villus axis, highlighting different cell types such as stem cells at the base and differentiated cells like enterocytes, goblet cells, and tuft cells along the villus. Stem cells are located in a specialized niche at the base of these crypts. As the daughter cells divide, they move upward through the transit-amplifying zone, eventually becoming non-dividing, differentiated cells within the villi. **b**: Illustrates a 3D organoid model grown in Matrigel, with crypt-like and villus-like regions mimicking the in vivo organization of intestinal cells. Stem cells in 3D intestinal organoids organize into structures that mimic the intestine, with differentiated cells in villus-like areas, stem cells in crypt-like zones, and proliferating cells in transit-amplifying-like regions. **c**: Displays a cross-sectional view of an organoid, showing the distribution of various cell types and indicating mechanical forces like apical and basal tension acting on the organoid structure. The shape of the crypt is influenced by tension gradients caused by apical contraction of the stem cells and basal contraction of the proliferating cells. Invagination also depends on the removal of fluid from the lumen, driven by transport processes across enterocytes (Nelson et al., 2021).

1.7. Organoid models for infections and chronic inflammatory diseases

Organoid models could offer insights into cellular responses to infections, overcoming limitations of traditional *in vitro* intestinal models. In particular, they address challenges faced with porcine enteropathogenic coronaviruses (Li et al., 2019; Luo et al., 2020; Y. Li et al., 2020), feline infectious peritonitis virus (FIPV) (Tekes & Thiel, 2016) rabbit hemorrhagic disease virus (RHDV) (Kardia et al., 2021; Kardia et al., 2023) and *Lawsonia intracellularis* (Resende et al., n.d.), which exhibit poor viral propagation or bacterial infection in standard 2D models. Organoids can be also a model for chronic inflammatory disease like inflammatory bowel disease (IBD). Inflammatory bowel disease (IBD) refers to a collection of chronic, idiopathic gastrointestinal disorders that

cause severe and debilitating inflammation in the digestive tract. In dogs, like IBD in humans, canine IBD (cIBD) is a complex condition influenced by genetic factors, changes in gut microbiota, and immune system abnormalities affecting the intestinal lining (Kopper et al., 2021). The canine model stands out among the large animal models utilized in researching various chronic human conditions, due to its significant relevance. This relevance is rooted in the environmental and genetic parallels between dogs and humans, as well as similarities in gut anatomy, physiology, pathology, and the composition of gut microbiota (Kararli, 1995; Cerquetella et al., 2010a; Coelho et al., 2018). Canine intestinal organoids, in particular, have been extensively developed and characterized, making them a valuable tool for both veterinary and translational research (Kramer et al., n.d.-a; Chandra et al., 2019a; Sahoo et al., 2022; Kopper et al., 2021b; Ambrosini et al., 2020; Gabriel et al., 2022; Gabriel, Zdyrski, Sahoo, et al., 2022; Sahoo et al., 2023a).

The canine intestinal organoid-derived monolayer not only supports epithelial differentiation but also provides tissue interface, displaying features such as polarization, lineage-specific differentiation, tight junction formation, permeability, and the expression of vital efflux pumps. Creating organoids from intestinal tissues or biopsies taken from dogs with chronic IBD (cIBD) leads to a valuable approach for studying disease mechanisms, characteristics, and potential treatments without the need to sacrifice living animals. Two studies have successfully generated intestinal organoids from dogs with IBD. However, histological analysis and transmission electron microscopy revealed no significant differences between organoids derived from IBD-affected and healthy dogs. Additionally, RNA in situ hybridization (ISH) probing of the EP4 prostaglandin receptor (EP4R), which plays a role in IBD pathogenesis and serves as a treatment target, showed no notable differences, potentially due to the organoids consisting of a single epithelial lineage. Despite this, it is recognized that organoids accurately retain the genetic features of the original tissue, even after multiple passages, allowing for the detailed study of transcriptomic profiles in intestinal organoids from IBD-affected dogs following lipopolysaccharide (LPS) stimulation. LPS treatment led to a decrease in the expression of several cancer-related genes and revealed contrasting patterns of gene expression related to anion transport, transcription and translation,

apoptosis, and adaptive immune response regulation between IBD enteroids and colonoids. The organoid model for cIBD presents new insights into gene expression profiles and, when combined with co-culture systems incorporating other cell types, such as immune cells, and intestinal microbiota, could play a crucial role in drug screening and the discovery of effective treatments for this complex disease in both humans and animals (Ambrosini et al., 2020; Chandra et al., 2019a; Sahoo et al., 2022).

1.8. 3D canine intestinal organoid model for GI disease research

In recent years, organoid technology has also begun to be applied in veterinary research, although on a smaller scale Current animal organoid systems mostly consist of stem and undifferentiated cells that require species-specific media tailored for proper differentiation. Interestingly, dogs and their owners share similar environments, diets, and exposure to carcinogens, resulting in similar gastrointestinal (GI) diseases, including GI cancer, infections, and inflammatory bowel disease (IBD) (Cerquetella et al., 2010b). This makes canine patients a valuable natural model for studying human GI disorders, especially since most dog breeds exhibit reduced genetic variation (Shearin & Ostrander, 2010). To fully leverage canine intestinal organoids for accurate organ modeling, it is essential to strike a balance between stem cell self-renewal and differentiation, creating a physiologically relevant system.

In this context, a new culture system for canine intestinal organoids was presented, based on recent work by Fujii *et al*. (Fujii et al., 2018). This system effectively supports the long-term growth of stem cells derived from the duodenum, jejunum, and colon, while also promoting their differentiation into secretory lineage cells. Rodent models, particularly mice, have been widely employed in the study of gastrointestinal (GI) diseases due to their cost efficiency, ethical suitability, and the relative ease of utilizing genetic engineering techniques. However, despite their prevalent use in biomedical research, the relevance of mouse studies to human disease remains a subject of debate. Moreover, mice and other rodents frequently fall short in accurately reflecting

human conditions and drug responses in terms of toxicity and efficacy (Beumer & Clevers, 2021; Chandra et al., 2019; Perlman, 2016; Ziegler et al., 2016; Wong et al., 2016, Mochel et al., 2017).

1.9. Advances in organoid models for cancer research in humans and animals

In cancer research, organoids have become promising models that closely mimic human cancers. Recently, many effective and reliable methods have been developed to create human organoids from various types of tumors, including those originating in the lungs, breast, stomach, liver, pancreas, kidneys, urinary bladder, and prostate. However, while veterinary oncology has seen fewer studies on cancer organoids, the number of such investigations is growing. Organoid models of animal cancers, which can be developed from a small population of stem cells, have advanced our understanding of the molecular characteristics and tumor behavior in animals. These models also serve as early-stage platforms for drug screening (Xu et al., 2022b; Xu et al., 2022b; Elshafae et al., 2020; Packeiser et al., 2020; Simmons et al., 2014; Thudi et al., 2011). For example, research on canine medullary thyroid carcinoma is limited, and treatment results have been disappointing, highlighting the need for a canine organoid model to find optimal therapeutic strategies. Organoids can also be created from small cell samples, such as urine-derived stem cells, which have been successfully used to study canine prostate and urinary tract cancers. With the adoption of organoid culture systems in cancer research, the availability of primary samples is not a significant challenge (Scheemaeker et al., 2023; Elbadawy et al., 2019; Abugomaa et al., 2020; Elbadawy et al., 2021).

Monolayer cultures derived from organoids, which maintain their differentiated traits, have been utilized for investigating veterinary tumors. In research focusing on canine bladder cancer, these monolayer cultures exhibited rapid growth and comparable responsiveness to anti-cancer treatments. When these monolayer organoid cells were injected into immunocompromised mice, they formed tumors with histopathological features like urothelial carcinoma. More recently, the same veterinary research team has developed direct monolayer cancer organoid models using tissue samples from dogs and cats (Abugomaa et al., 2022).

1.10. Stem cell-derived intestinal organoids

Intestinal organoids derived from stem cells which mimic the structure, cell composition, and some functions of the small intestine. These organoids grow in special media that replicate the stem cell niche signaling pathways found in the body, which is important for their development. This media supports stem cell activity, promoting their growth, expansion, and eventual differentiation. Organoids can be generated from two main types of stem cells: (1) pluripotent stem cells (PSCs), which include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) involved in early organ development; and (2) adult stem cells (ASCs), which are specific to organs and play a key role in maintaining and regenerating mature tissues. PSCs, like ESCs and iPSCs, have unlimited potential for expansion, which is essential for their discovery and use. ASCs, on the other hand, can also form organoids when cultured with the right matrix and growth factors, despite being previously thought to have limited proliferation capacity *in vitro* (Artegiani & Clevers, 2018; McCauley & Wells, 2017; Clevers, 2016).

1.11. Pluripotent stem cell-derived intestinal organoids

Since the creation of pluripotent stem cell (PSC) lines, researchers have been able to develop various differentiated cell types with the help of developmental biology (Chen et al., 2014; Cherry & Daley, 2012). To date, human intestinal organoids have been derived from both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), while mouse intestinal organoids have been generated from ESCs. Signals like WNT and fibroblast growth factor (FGF) play a crucial role in transforming the endoderm into the mid/hindgut. By applying WNT3A and FGF4 to human PSCs treated with activin, researchers were able to culture 3D mid/hindgut spheroids from 2D epithelial cell layers. These spheroids were then embedded in Matrigel and further grown with R-spondin-1, EGF, and noggin, leading to their differentiation into intestinal organoids. After 1–3 months of cultivation, these organoids form a polarized intestinal epithelium, developing villus-

like structures and crypt-like regions that include all major intestinal cell types (Múnera & Wells, 2017; McCracken et al., 2014; Spence et al., 2010; Tsakmaki et al., 2017).

1.12. Adult stem cell-derived intestinal organoids

Unlike organoids developed from pluripotent stem cells (PSCs), adult stem cells (ASCs) can be stimulated to form 3D organoids by replicating the conditions of the intestinal stem cell niche necessary for tissue renewal. In 2009, a culture system was developed that enabled the long-term cultivation and expansion of epithelial structures resembling villi from single sorted Lgr5+ stem cells or purified intestinal crypts (T. Sato et al., 2009c; Sugimoto & Sato, 2017). These cells were embedded in laminin-rich Matrigel and grown in a serum-free medium containing R-spondin-1, EGF, and the BMP inhibitor Noggin (T. Sato et al., 2010). Within days, this setup led to the formation of cystic epithelial structures with a central lumen, a process known as "crypt-like budding." Over time, these structures evolved into multiple budding crypts with normal distribution of cell types. The resulting 3D organoids exhibit high polarization, with enterocyte brush borders forming the luminal surface and basolateral sides facing outward. These organoids are highly stable, growing continuously every 5–7 days for years without significant genetic or phenotypic changes (Bigorgne et al., 2014; T. Sato & Clevers, 2013).

1.13. Factors for developing and differentiating organoids

The growth and differentiation of crypt stem cells are regulated by four main signaling pathways. The WNT pathway is important for promoting stem cell proliferation and survival. The NOTCH pathway helps maintain the undifferentiated state of proliferating cells. EGF signaling is important for stimulating stem cell mitosis. Lastly, the BMP signaling pathway plays an active role in organoid development, with BMP inhibition being essential for creating an optimal environment for crypt differentiation (Clevers, 2013).

Lgr5 and CD44 are key markers for intestinal stem cells and are crucial for crypt organoid development and maintenance. Lgr5 marks stem cells at the base of intestinal crypts, which self-renew and differentiate into various epithelial cells. In crypt organoids, Lgr5-positive cells drive growth, supported by Wnt signaling. CD44, expressed alongside Lgr5, aids in cell adhesion, migration, and niche maintenance, enhancing stem cell function. Together, Lgr5 and CD44 enable crypt organoids to mimic intestinal epithelium, making them important for studying intestinal biology and diseases. Besides the stem cells located at the base of the crypts, there has been ongoing speculation about the existence of alternative intestinal stem cells situated at the +4 position relative to the crypt base. These cells, known as label-retaining cells (LRCs), are characterized by their slow cell cycle and quiescence (G. Colozza et al., 2022; Potten et al., 2002; N. Li & Clevers, 2010), their ability to retain labeled DNA reflects their slow cycling nature compared to the more active crypt base stem cells.

The canonical Wnt signaling pathway, mediated by β-catenin, is important for the development and maintenance of intestinal tissues, including in canine intestinal organoids. It regulates cell proliferation, differentiation, and tissue homeostasis, playing a key role in maintaining the stem cell niche and supporting organoid structure. Disruptions in this pathway can change differentiation and compromised tissue integrity. Similarly, the Bone Morphogenetic Protein (BMP) signaling pathway is essential for intestinal development and organoid formation. BMPs regulate cell growth, differentiation, and apoptosis, influencing the formation of villi and crypt structures within the organoid. Disruptions in BMP signaling can result in abnormal tissue formation and function.

The intestinal epithelium excels at preserving the consistent size and structure of crypts despite undergoing full tissue renewal every 4–5 days. When this balance is disrupted, it can lead to serious conditions such as inflammatory bowel disease and gastrointestinal cancers. Research aims to find how the processes of crypt cell proliferation and differentiation are controlled. The regulation of these processes is influenced by two key signaling pathways: WNT and bone morphogenetic protein (BMP). WNT signaling supports the renewal and proliferation of stem

cells, whereas BMP signaling drives cell differentiation. These pathways are mediated by various sources within the intestinal tissue. WNT is released by myofibroblasts in the surrounding mesenchyme and paneth cells located at the base of the epithelial crypts. On the other hand, BMP may be secreted by differentiated epithelial cells. More understanding of these signaling mechanisms is crucial for managing the balance within crypts and addressing associated intestinal diseases (Crosnier et al., 2006; Maloy & Powrie, 2011; Auclair et al., 2007; Batts et al., 2006).

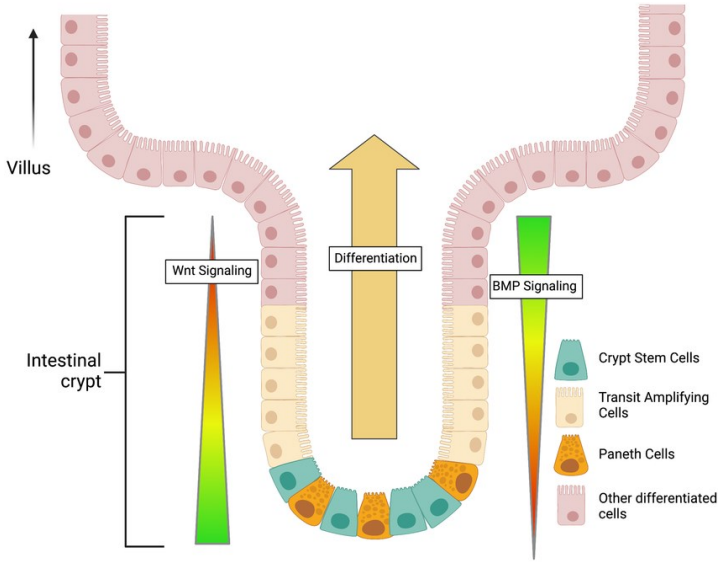


Figure 3: This figure illustrates the regulatory mechanisms of Wnt and BMP signaling in the intestinal crypt, which controls the balance between stem cell maintenance and cell differentiation. Wnt signaling, strongest at the crypt base, maintains stem cells, promoting cell stemness, while BMP signaling, highest near the top, drives cell differentiation. As crypt stem cells transition into transit amplifying cells, they move upward toward the villus, where they fully differentiate. Paneth cells remain at the crypt base, playing a key role in regulating the crypt environment. The balance between these two signaling pathways ensures proper intestinal tissue maintenance and regeneration (Eggington et al., 2022).

1.14. Canine intestinal organoids for toxicity and drug screening

Gastrointestinal (GI) toxicity is a serious side effect of chemotherapy which limits the dosage, patients can tolerate. This condition manifests as diarrhea, dehydration, and ulceration, which heighten the risk of infection, partly due to damage or loss of crypt and villi structures in the small intestine. In oncology therapies continue to be developed, evaluating their potential GI toxicity becomes increasingly important. The mouse small intestinal organoid model, introduced by Sato et al. in 2009, has been instrumental in studying of intestinal stem cells and the genes that regulate them. This model has attracted significant interest from the pharmaceutical industry as a screening

tool before moving to *in vivo* studies. Recently, these methods were extended to canine tissue, where canine organoids were successfully established and assessed their effectiveness in comparison to other species. The culture conditions for intestinal organoids were designed to replicate the stem cell niche, facilitating cell differentiation and proliferation. The canine organoids displayed all expected intestinal lineages, and their epithelial structure closely matched what is observed *in vivo*.

Canine intestinal organoids have become an important *in vitro* tool for exploring gastrointestinal diseases and drug effects. These three-dimensional cultures mimic the key physiological and molecular characteristics of the intestinal environment. Organoids derived from different parts of the canine intestine can be cultured long-term while maintaining their cellular diversity and ability to differentiate. They have proven effective as a preclinical screening method for evaluating gastrointestinal toxicity of cancer drugs, with results correlating well with in vivo findings. Additionally, these organoids can be adapted into two-dimensional monolayer systems to assess drug permeability, providing a species-specific alternative to conventional cell lines like Caco-2 (it is the name of a specific human cell line derived from a colorectal carcinoma). Canine intestinal organoids offer a valuable platform for investigating both naturally occurring intestinal diseases in dogs and humans, as well as for toxicology studies and analyzing host-pathogen interactions, thus bridging the gap between animal research and human studies (Chandra et al., 2019d; Kramer et al., n.d.; Sahoo et al., 2023).

1.15. Cyclophosphamide effects on canine intestinal organoids and clinical impact

Cyclophosphamide (CPA) is a chemotherapy drug and immunosuppressant commonly used in the treatment of various cancers and autoimmune diseases. It belongs to the class of alkylating agents, which means it interferes with the DNA of rapidly dividing cells, inhibiting their growth and causing cell death. Cyclophosphamide is among the earliest anticancer agents. It was first identified in 1958 and began being used in cancer treatment in 1959 (Brock & Wilmanns, 1958). This chemotherapy drug commonly used for treating ovarian, breast, and hematological cancers, as

well as some autoimmune disorders. However, its use is linked to reproductive issues, including reproductive failure and premature ovarian insufficiency. The exact mechanism by which CPA or its major metabolite, acrolein, impacts female fertility is not fully understood, though it is believed to involve excessive production of reactive oxygen species (ROS) (Jeelani et al., 2017).

The drug is used in various conditioning treatments before bone marrow transplants for blood cancers such as acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS), as well as for aplastic anemia. Beyond its role in cancer therapy, it is also prescribed for severe cases of autoimmune and immune-mediated conditions like lupus nephritis, Wegener's granulomatosis, and multiple sclerosis. Key side effects include low white blood cell counts (leukopenia), low platelet counts (thrombocytopenia), anemia, and toxicity to the heart and bladder. To prevent hemorrhagic cystitis, MESNA (sodium 2-sulfanylethanesulfonate) is given prior to cyclophosphamide to neutralize the toxic byproduct acrolein in the urine. Additionally, the drug can cause kidney, heart, and liver toxicity, with these effects being highly dependent on the dose. Cardiotoxicity can be a limiting factor in high-dose treatment regimens (G W Santos et al., 1972).

This harmful agent to cells has been found to trigger chromosomal irregularities in numerous experiments. Research on mice revealed that it led to structural changes in chromosomes, such as translocations, in sperm cells when given at various points during sperm development. The impact of the drug was detected during both the meiotic and premeiotic phases, with different levels of sensitivity to the treatment (Francesca et al., 1983). In individuals with rheumatoid arthritis and scleroderma, extended use of cyclophosphamide notably elevated the incidence of severe chromosomal abnormalities, such as reduced chromosome numbers and chromosomal breaks (Tolchin et al., 1974). The drug's effects on chromosomes were noted in cells at metaphase, indicating its potential to disrupt normal cell division. These observations emphasize the need to take into account cyclophosphamide's genotoxic properties in both research and clinical contexts, especially concerning how it is metabolized in the body (Torkelson et al., 1975). The active form of cyclophosphamide, phosphoramide mustard, generates a highly reactive cyclic aziridinium ion that can interact with guanine and cytidine in DNA. This reactivity leads to the formation of both

interstrand and intrastrand cross-links. Consequently, cyclophosphamide's effects are independent of the cell cycle. Nonetheless, like other alkylating agents, it is particularly effective against rapidly dividing cells. Despite its long history of clinical use, new treatment approaches such as metronomic and high-dose therapies have been developed. Currently, innovative treatment regimens are being explored to address immunosuppression in advanced cancer cases (J. Y. Wang et al., 1993; Fleer & Brendel, 1981; L M Van Putten et al., 1972).

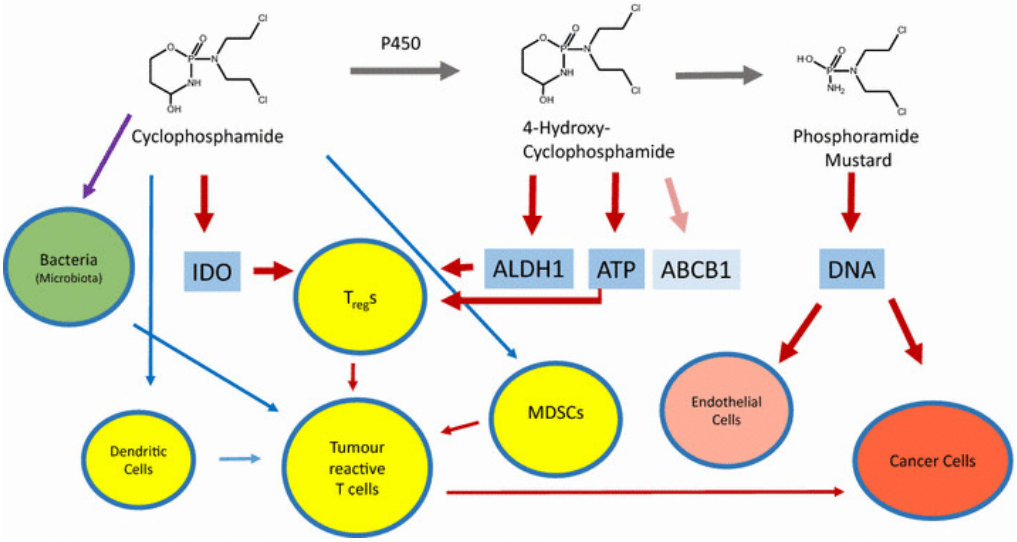


Figure 4: This diagram illustrates the metabolic process of cyclophosphamide (CPA) and its impact on both immune and cancer cells. CPA is broken down by P450 enzymes into 4-Hydroxy-Cyclophosphamide, which is then converted into Phosphoramide Mustard, a compound that damages the DNA of cancer cells. The gut microbiota plays a role in activating dendritic cells, which in turn influence the immune response. Additionally, CPA reduces the number of regulatory T cells (Tregs), allowing tumor-reactive T cells to attack cancer cells. Myeloid-Derived Suppressor Cells (MDSCs) and endothelial cells also participate in this process. However, resistance mechanisms involving ALDH1, ATP, and ABCB1 limit the drug's effectiveness. In summary, CPA affects both cancer and immune cells but is challenged by resistance mechanisms (Ahlmann & Hempel, 2016).

Cyclophosphamide can be administered either orally or intravenously. When taken orally, it is absorbed very efficiently. To produce its cytotoxic effects, cyclophosphamide must be bioactivated. This activation is carried out by cytochrome P450 enzymes, primarily CYP 2B6 and 3A4, as well as CYP 2A6, 2C8, 2C9, 2C19, and 3A5. These enzymes oxidize cyclophosphamide into 4-hydroxy-cyclophosphamide, which exists in equilibrium with aldophosphamide (S M Yule et al., 1995). Both forms can penetrate cells and are converted into phosphoramide mustard, which disrupts DNA by forming cross-links (Phosphoramide mustard is a cytotoxic compound that forms

as a metabolite of the chemotherapy drug cyclophosphamide). When aldophosphamide is broken down, it also produces acrolein, which accumulates in the bladder and can cause severe toxicity. To counteract this, MESNA (sodium 2-sulfanylethanesulfonate) is given prior to cyclophosphamide to neutralize acrolein in the bladder by reacting with its vinyl group.

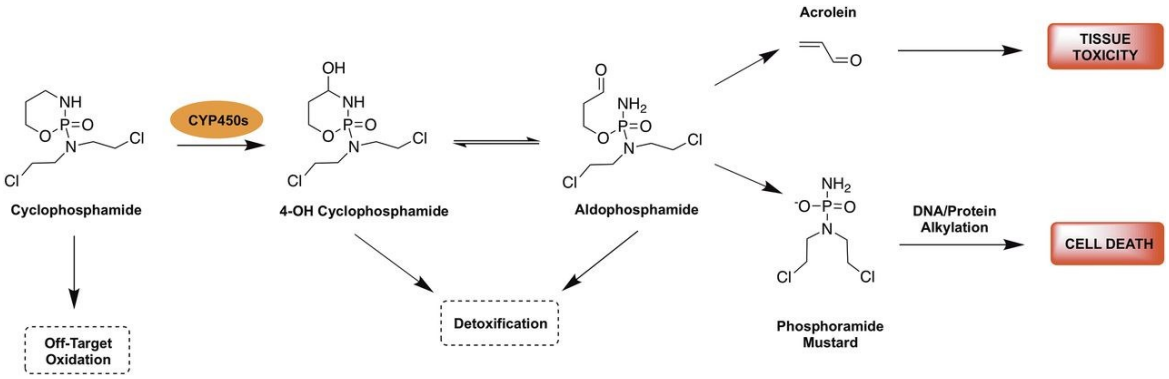


Figure 5: This figure shows the metabolic activation of cyclophosphamide (CPA) and its effects. CPA is initially metabolized by CYP450 enzymes into 4-Hydroxy-Cyclophosphamide, which is then converted into Aldophosphamide. Aldophosphamide can either be detoxified or further processed into Phosphoramide Mustard, a key metabolite that causes DNA and protein alkylation, leading to cell death. Additionally, Aldophosphamide produces Acrolein, a toxic byproduct associated with tissue damage. This illustrates how CPA exerts its therapeutic effect through Phosphoramide Mustard while also causing potential tissue toxicity through Acrolein (Ramirez et al., 2019).

The side-chain oxidation of cyclophosphamide to dechloroethyl-cyclophosphamide is mediated by cytochrome P450, with the CYP 3A4 isoenzyme playing a major role and CYP 2B6 also contributing. Since both the activation and inactivation of cyclophosphamide are driven largely by the same enzyme, attempts to improve the drug's therapeutic index by using P450 inhibitors have not been successful. Additionally, the by-product chloroacetaldehyde has demonstrated cytotoxic effects in laboratory studies and may be a factor in cyclophosphamide's neurotoxicity. Another method of drug inactivation involves its breakdown by glutathione, facilitated by GSTM1, GSTP1, and GSTT1. Variations in the genes encoding these enzymes may influence the drug's toxicity and effectiveness (Brüggemann et al., 1997; Sood et al., 1996; Audemard-Verger et al., 2016). Both laboratory and clinical studies suggest that the activation of cyclophosphamide to 4-hydroxy-cyclophosphamide reaches a saturation point, while the side-chain oxidation process for inactivation does not. This observation is also supported by pharmacokinetic modeling studies (Bohnenstengel et al., 1996; De Jonge et al., 2005).

In contrast, research on microsomal activation in organoids aims to understand how cyclophosphamide is metabolized and activated within a more complex tissue context. This approach helps elucidate the roles of different enzymes in drug activation and the formation of active metabolites. For example, liver-derived organoids can be used to investigate how cyclophosphamide is activated by cytochrome P450 enzymes and how this affects drug efficacy and safety. However, there is an important gap in studies involving cyclophosphamide and organoids, particularly with canine intestinal organoids.

1.16. MRP2, drug transport in intestinal organoids, and efflux transporters

In vitro models of the small intestine (Intestinal Organoids) are crucial for studying drug transport mechanisms. Drug transport is linked to the processes of drug absorption, distribution, and elimination, making it a key factor in the discovery and development of new medications (Zhang et al., 2021; A. P. Li, 2001; Y. Li et al., 2019). MRP2 (Multidrug Resistance-associated Protein 2) can influence the pharmacokinetics (absorption, distribution, metabolism, and excretion) of drugs. Certain drugs may inhibit or induce the transporter, potentially leading to changes in drug levels and efficacy. MRP2 is a key efflux transporter expressed on the apical surface of intestinal cells. It actively transports drugs, toxins, and metabolites out of the cells, which can limit the intracellular concentration of drugs and have a significant role in drug resistance.

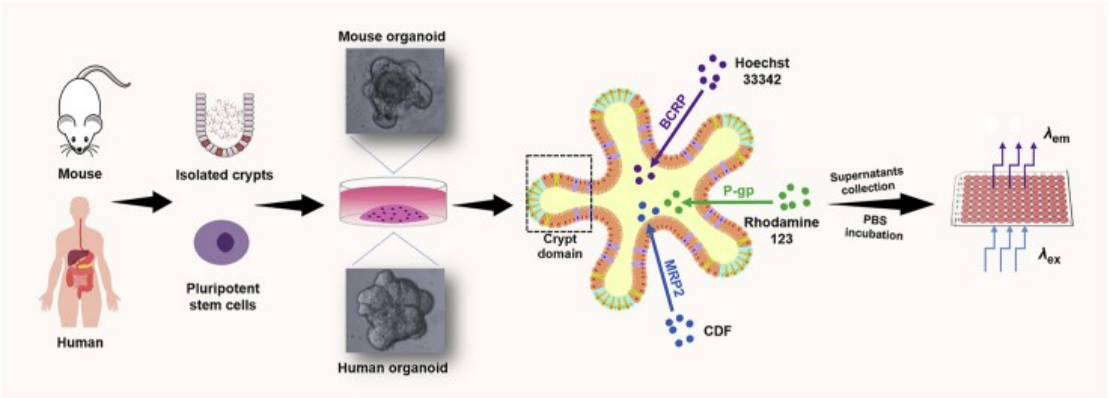


Figure 6: This figure depicts the process of using organoids derived from mouse intestinal crypts and human pluripotent stem cells to study transporter activity. Mouse and human organoids are generated and sectioned to expose the crypt domain. Various fluorescent dyes and substrates, such as Hoechst 33342, Rhodamine 123, and CDF, are applied to the organoids to assess the activity of transporters like P-glycoprotein (P-gp) and MRP2. The supernatants are collected after PBS incubation, and the emitted fluorescence is measured to evaluate transporter function, providing insights into how drugs and substances are processed by intestinal cells (Y. Zhang et al., 2021b)

MRP2, an essential efflux transporter involved in drug-drug interactions (DDI), has garnered increasing attention. MRP2 is a transmembrane protein composed of 1,545 amino acids. It is found in the apical membranes of various tissues, including the small intestine, liver, kidneys, brain, and placenta. Research has highlighted the association between MRP2 and several cancers, such as gastric, colon, breast, and lung cancer (Kamata et al., 2008; Durmus et al., 2019; Hjorth et al., 2019 ; Sun et al., 2010; Sandusky et al., 2002). In studies using mouse intestinal 3D organoids, MRP2 was detected on the inner surface of these organoids, like P-gp and BCRP. P-gp (P-glycoprotein) and BCRP (Breast Cancer Resistance Protein) are crucial efflux transporters that play significant roles in drug transport and multidrug resistance.

The mRNA expression levels of MRP2 were consistent across the proximal, middle, and distal sections of the small intestine. For drug efflux transport studies, MK-571 and probenecid were used as inhibitors, while 5(6)-carboxy-2′,7′-dichlorofluorescein (CDF) served as the MRP2 substrate. Both MK-571 and probenecid notably reduced CDF accumulation in the 3D organoids. These findings indicate that the 3D intestinal organoid model is effective for studying MRP2-mediated drug transport (L. Zhang et al., 2019). MK-571 is an important inhibitor used primarily to study the function of multidrug resistance-associated proteins (MRPs), particularly MRP2. It works by blocking the activity of MRP2 and other related transporters, thereby helping researchers investigate the role of these transport proteins in drug efflux and resistance. In research settings, MK-571 is used to assess how transporters like MRP2 influence drug absorption, distribution, and elimination.

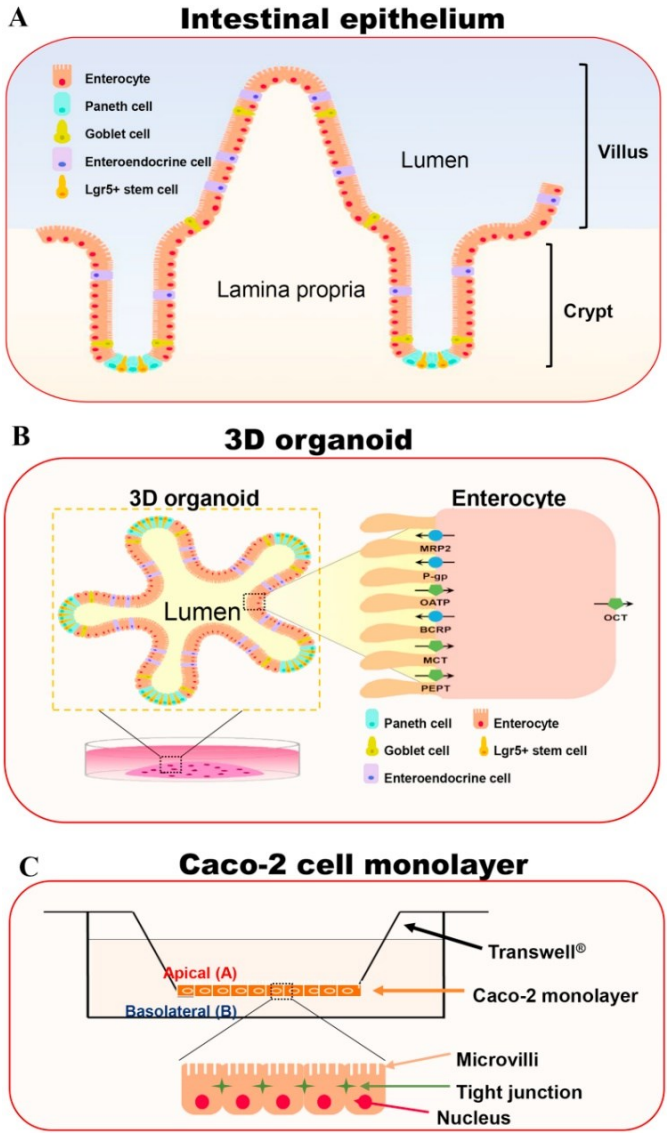


Figure 7: This figure compares three models used to study intestinal epithelium: intestinal epithelium, 3D organoids, and Caco-2 (Cancer coli-2) cell monolayers. **Panel A** represents the intestinal epithelium, showing different cell types, including enterocytes, Paneth cells, goblet cells, enteroendocrine cells, and Lgr5+ stem cells. The structure is divided into villi (absorptive regions) and crypts (stem cell niches), with the lumen and lamina propria highlighted. **Panel B** shows a 3D organoid model, a miniaturized version of the intestinal epithelium. The organoid consists of a lumen surrounded by the same cell types as the epithelium. It shows key transporters expressed in enterocytes, such as MRP2, P-gp, BCRP, and others, used to study drug transport and absorption in a more physiologically relevant system. **Panel C** shows the Caco-2 cell monolayer, a widely used *in vitro* model to study drug absorption. Caco-2 cells are cultured on a Transwell® system, forming a monolayer with distinct apical and basolateral sides. The model includes microvilli, tight junctions, and a nucleus, allowing for the study of intestinal permeability and transporter activity. Each model provides valuable insights into the structure and function of the intestinal barrier, with 3D organoids offering a more complex structure compared to Caco-2 monolayers (Y. Zhang et al., 2021c).

2. Aim of the thesis

In this study, we aim to use canine intestinal organoids as a model to evaluate the genotoxicity of cyclophosphamide (CPA) and assess how well they mimic *in vivo* and find more realistic and better tests to determine genotoxic effect of drugs and chemicals, which are present in medications, food and environment in general. We will compare the genotoxic effects observed in canine intestinal organoids with traditional 2D cultures of CHO cells and develop a reliable model for genotoxicity testing relevant to both veterinary and comparative studies. While human and rodent organoids have been widely studied, little research exists on genotoxicity testing using

canine intestinal organoids, providing an opportunity to investigate their response to genotoxic agents like CPA. We hypothesize that canine organoids will effectively model genotoxic damage, correlating with results from traditional assays and *in vivo* studies, contributing to improved risk assessment for veterinary drug safety. For these aims, we will use the micronucleus (MN) assay as a proof of principle to establish this system using canine intestinal organoids. The study focuses on assessing CPA-induced genotoxicity using the MN assay, where both organoids and CHO cells are treated with 0.5 µl of 50 µg/ml CPA for 24 hours. We expect to detect approximately 50-100 micronuclei in 1,000 cells from both models. DAPI staining and fluorescence microscopy will be used to visualize micronuclei, confirming DNA damage in CHO and organoid cells.

3. Materials and Methods

3.1. Cell culture

3.1.1. Cell line cCO35

The intestinal crypts from canine cell lines used in this study were originally extracted previously through biopsies from the colon of healthy canine at Small Animal Department of Veterinary Medicine University of Vienna, following the protocols outlined by Kramer *et al*. (Kramer et al., 2020).

3.1.2. *In Vitro* growth conditions

Organoids were grown in 24-well plates (Biologix Group Limited) at 37°C in a CO2 incubator with 5% humidity, using refined medium. This medium was made up of 37% basal medium (Advanced DMEM/F12 with 2mM GlutaMAX and 10mM HEPES), 1x B27 (ThermoFisher Scientific), 1mM Acetylcysteine (ThermoFisher Scientific), 500 nM A8301 (Bio-Techne Ltd.), 10 nM Gastrin (Sigma-Aldrich), 100 ng/ml mNoggin, 50 ng/ml HGF (PeproTech), 100 ng/ml IGF1 (PeproTech), 50 ng/ml FGF2 (PeproTech), 10% Rspondin 1, and 50% Wnt3a. The growth medium

was replaced every 2–3 days, depending on the density and size of the organoids. Passaging was done through mechanical dissociation or trypsinization.

DMEM Medium was prepared using Dulbecco's Modified Eagle Medium (DMEM, Gibco, Germany), supplemented with 50 ml fetal bovine serum (Fetal Bovine Serum, Gibco, Origin: Brazil) and 5 µl of Penicillin-Streptomycin (PenStrep) antibiotic solution per 500 ml of DMEM. The final concentration of PenStrep in the medium was 100 U/ml of penicillin and 100 µg/ml of streptomycin. The prepared medium was stored at 4°C until use.

Table 1: List of components of refined medium

Component	Stock concentration	Amount for 50 ml	Final Concentration
Basal Medium	1X	18,4 ml	
B27	50X	1 ml	1x
n-Acetylcysteine	500 mM	100 µl	1 mM
A8301	500 µM	50 µl	500 nM
Gastrin	10 µM	50 µl	10 nM
HGF	50 µg/ml	50 µl	50 ng/ml
hlGF1	100 µg/ml	50 µl	100 ng/ml
hFGF2	100 µg/ml	25 µl	50 ng/ml
mNoggin	50 µg/ml	100 µl	100 ng/ml
Wnt3a c.m.	100 %	25 ml	50 %
R-spo c.m.	100 %	5 ml	10 %
Gentamycin	10 mg/ml	100 µl	
Primocin		100 µl	

3.1.3. Cultivation of organoid cells (cCO35)

Organoids were passaged every 3–4 days, they were removed from the Matrigel and mechanically split into smaller fragments (using SurPhob 1000 µl filter pipette tips) capable of reassembling into new organoids. These fragments were then resuspended in medium and transferred to a falcon tube. The organoids were further broken down into smaller clusters by repeatedly pipetting the solution against the wall of the falcon tube using a flame-polished pasteur pipette. Alternatively, organoids were separated through trypsinization. To activate the enzyme, trypsin was pre-warmed briefly at 37°C. It was then added to the cell pellet and vigorously shaken and incubated into a water bath. Once the organoids were dissociated to the desired level, trypsin activity was halted by adding culture medium. After splitting, the solution was centrifuged at 80g for 5 minutes at 8°C.

The supernatant was discarded, and basal medium was added to the organoid pellet according to the number of wells needed. To each well, 50 µl droplets of the organoid-Matrigel solution (BD Biosciences), mixed in a 1:1 ratio, were seeded. The inverted plate was incubated at 37°C for 10 minutes to allow the Matrigel to polymerize. Finally, an appropriate volume of refined medium (500 µl) was added to each well to completely cover the droplets.

3.1.4. Cultivation of CHO cells (CHO-Z 7p)

CHO cells were passaged every 3–4 days upon reaching 80-90% confluence. The DMEM medium was first removed, and the cells were washed with 5 ml DPBS (Dulbecco's Phosphate-Buffered Saline, Sigma Aldrich, USA). To detach the cells, 500 µl of prewarmed 0.05% Trypsin-EDTA (Gibco, Germany) was added, and the flask (T25) was incubated for 2-3 minutes until detachment occurred. The trypsin was then neutralized by adding 3.5 ml of prewarmed DMEM medium. The cells were resuspended to a final volume of 4 ml, 3 ml of which was removed. Subsequently, 6 ml of prewarmed DMEM medium was added to the remaining 1 ml of cells, and the cells were replated at a 1:4 ratio into a fresh T25 flask.

3.2. CHO cell banking

3.2.1. Defrosting of CHO cells

The following materials were required for defrosting CHO cells: a T75 flask, a 37°C water bath, ice, and DMEM (1x) + GlutaMax (Dulbecco’s Modified Eagle Medium, Ref: 10567-014, Gibco) supplemented with PenStrep (Penicillin Streptomycin, Ref. 15140-122, Gibco) and FBS (Ref. A5209401-Gibco). One vial of frozen CHO cells was retrieved using an ice box with dry ice. The DMEM medium was pre-warmed in a 50 ml falcon tube in the water bath. The thawing process was conducted in less than one minute to avoid cell damage.

CHO cells were rapidly thawed in a 37°C water bath and immediately transferred to a prepared T75 flask containing 16 ml of pre-warmed culture medium (DMEM with 10% FBS and 1% PenStrep). The cells were gently resuspended in the medium, effectively diluting the DMSO from the frozen mixture to prevent damage to the CHO cells. The flask was incubated at 37°C in a humidified environment with 5% CO2. After 24 hours, the medium was replaced to support cell recovery and promote growth.

3.2.2. Freezing of CHO cells

The following materials were required for freezing CHO cells: 2 ml cryovials, 15 ml falcon tubes, a 37°C water bath, and freezing medium (Dulbecco's Modified Eagle Medium with high glucose content, supplemented with 10% serum and 10% DMSO). DMEM medium (Ref: 41965-039-Gibco), supplemented with PenStrep (Penicillin Streptomycin, Ref. 15140-122, Gibco) and FBS (Ref. A5209401-Gibco), along with prewarmed DPBS (Dulbecco's Phosphate-Buffered Saline, Sigma Aldrich, USA), were also prepared.

The DMEM medium was removed from CHO cells, and the cells, which had reached 70-80% confluence, were washed twice with 5 ml of prewarmed DPBS. To detach the cells, 2 ml of prewarmed 0.05% Trypsin-EDTA (Gibco, Germany) was added to the T75 flask, and the flask was incubated for 2-3 minutes until the cells detached. The trypsin was then neutralized by adding 6 ml of prewarmed DMEM medium. The cells were resuspended in a final volume of 8 ml, transferred to a 15 ml falcon tube, and centrifuged at 400 g for 5 minutes at room temperature. The supernatant was discarded, and the pellet was placed on ice. 5 ml of DMEM medium were added to resuspend the pellet, and a cell viability test was carried out before freezing.

For viability test 50 µl cells were mixed with 2.5 µl Solution 18, AO/DAPI (AO 80 ug/ml, DAPI 40 ug/ml), 10 µl from the mixture used for viability test. Cell viability test shows the number of live cells in 1 ml, for example $1.48x10^6$: 1,480,000 cells in 1 ml, that means in 5 ml we had 7.4 $x10^6$ (7,400,000). After making sure that more than 90% of cells are alive, 500 µl from 5 ml cells

were added to a T25 flask with 6.5 ml DMEM medium for further culture, and the rest of 4.5 ml cells (around 6,700,000 cells) were used for freezing. To obtain 2,000,000 cells in each 2 ml freezing cryovial, three cryovials were prepared and labelled properly. The cell suspension (4.5 ml) was centrifuged at 1500g for 5 minutes, the supernatant was discarded, and 3 ml of DMEM was added to the pellet and resuspended. Then, 3 ml of the cell suspension and 3 ml of freezing medium (2,700 µl DMEM + 300 µl DMSO) were mixed in a 50:50 ratio. The total volume of 6 ml was divided equally, with 2 ml of the cell, and freezing medium mixture added to each cryovial. The freezing medium was kept on ice before being added to the cells. The cryovials were placed in a controlled-rate freezing container at -80°C for 24 hours. Subsequently, the vials were transferred to liquid nitrogen for long-term storage.

3.3. Organoid banking

3.3.1. Defrosting of organoids (cCO35)

Basal medium (40 ml) were prewarmed in a 37°C water bath. To revive frozen organoids, 2 ml cryovials were transferred from -80°C on dry ice and rapidly thawed in a 37°C water bath. Once thawed, 1 ml of prewarmed basal medium was added to the cryovial and mixed thoroughly. Four ml of prewarmed basal medium were prepared in a 15 ml Falcon tube, and the contents of the cryovial were transferred to the falcon tube. The mixture was centrifuged at 400 g for 5 minutes at 8°C. The supernatant was discarded, and 4 ml of basal medium was added to the pellet, which was resuspended and centrifuged again using the same settings, to ensure removal of DMSO from the freezing medium. The organoid pellet was then resuspended in fresh Matrigel and plated in a 24-well plate. 50 µl of the organoid-Matrigel mixture, prepared in a 1:1 ratio, were seeded into each well. The plate was inverted and incubated at 37°C for 10 minutes to allow the Matrigel to polymerize. Afterward, an appropriate volume of refined medium was added to each well to fully cover the droplets.

Table 2: Basal medium

Component	Stock	Amount	Final Concentration
Adv. DMEM/F12		500 ml	
Glutamax	100x	5 ml	1x
HEPES	1 M	5 ml	10 mM
Antibiotics (P/S)	100x	5 ml	1x

3.3.2. Freezing of organoids (cCO35)

The following materials were required for freezing organoid cells: five 2 ml cryovials, 15 ml falcon tubes, a 37°C water bath, freezing medium (90% Basal medium with FBS and 10% DMSO), DPBS (Dulbecco's Phosphate-Buffered Saline, Sigma Aldrich, USA), and SurPhob 1000 µl filter pipette tips. The organoids were first mechanically dissociated into small fragments (using SurPhob 1000 µl filter pipette tips) and collected from the Geltrex. The fragments were transferred to a 15 ml falcon tube and centrifuged at 80g for 5 minutes. After centrifugation, the supernatant was discarded, and the pellet was washed with cold DPBS. The cells were then centrifuged again under the same conditions, the supernatant discarded, and the pellet was kept on ice. To further dissociate the pellet into single cells, 500 µl of 0.05% Trypsin-EDTA (Gibco, Germany) was added to the pellet and resuspended in the falcon tube, which was then incubated in a 37°C water bath for 5–10 minutes. During this time, the cells were monitored under a light microscope to confirm single-cell dissociation. Once the organoids were dissociated into single cells, to neutralize the trypsin, 4 ml of cold basal medium was added, and the cells were centrifuged at 400g for 5 minutes at 8°C. The supernatant was discarded, and the pellet was resuspended in 5 ml of cold basal medium for a viability test.

For viability test 50 µl cells were mixed with 2.5 µl Solution 18, AO/DAPI (AO 80 ug/ml, DAPI 40 ug/ml), 10 µl from the mixture used for viability test. After making sure that more than 90% of cells are alive, cells were centrifuged at 1500 g for 5 to get pellet (pellet kept on ice). cell viability test shows the number of live cells in 1 ml, for example $1x10^6$: 1,000,000 cells in 1 ml, that means

in 5 ml we had 5 x10^6 (5,000,000). To obtain 1,000,000 cells in each 2 ml freezing cryovial, five cryovials were prepared and labeled properly. 5 ml of basal medium was added to the pellet and resuspended. Then, 5 ml of the cell suspension and 5 ml of freezing medium (90% Basal medium with FBS and 10% DMSO) were mixed in a 50:50 ratio. The total volume of 10 ml was divided equally, with 2 ml of the cell and freezing medium mixture added to each cryovial. The freezing medium was kept on ice before being added to the cells. The cryovials were placed in a controlled-rate freezing container at -80°C for 24 hours. Subsequently, the vials were transferred to liquid nitrogen for long-term storage.

3.4. Micronucleus test on organoid cells (cCO35) and CHO cells

3.4.1. Seeding organoid cells into a 24-well cell culture plate

The organoids were removed from the Matrigel and mechanically broken into smaller fragments (using SurPhob 1000 µl filter pipette tips), capable of reassembling into new organoids, and resuspended in medium before being transferred to a falcon tube. The organoids were further fragmented into smaller clusters by repeatedly pipetting the solution against the wall of the falcon tube using a flame-polished Pasteur pipette. The cell fragments were centrifuged at 80g for 5 minutes at 8°C. After centrifugation, the supernatant was discarded, and the pellet was washed with cold DPBS to remove apoptotic cells and undifferentiated stem cells from intestinal canine organoids (cCO35). The cells were centrifuged again under the same conditions, and the supernatant (DPBS) was discarded while the pellet was kept on ice.

Next, 500 µl of 0.05% Trypsin-EDTA (Gibco, Germany) was added to the pellet and resuspended in the falcon tube, which was then incubated in a 37°C water bath for 5–10 minutes. During this period, the cells were monitored under a light microscope to ensure single-cell dissociation. Once dissociation into single cells was achieved, 4 ml of cold basal medium was added to neutralize the trypsin. The cells were centrifuged at 400g for 5 minutes at 8°C, after which the supernatant was discarded, and the pellet was resuspended in 100 µl of cold basal medium and 100 µl Geltrex. Four

wells of a 24-well cell culture plate were used (2 wells for CPA, and 2 wells for DMSO). In each well, 50 µl droplets of the organoid-Matrigel solution (BD Biosciences), mixed in a 1:1 ratio, were seeded. The plate was then inverted and incubated at 37°C for 10 minutes to allow the Matrigel to polymerize. Finally, 500 µl of prewarmed refined medium was added to each well to completely cover the droplets, and the plate was placed in a CO2 incubator at 37°C.

3.4.2. Seeding CHO cells into a 24-well cell culture plate

The DMEM medium was removed from CHO cells in a T75 flask, and the cells which had reached 70-80% confluence, were washed twice with 5 ml of prewarmed DPBS. To detach the cells, 2 ml of prewarmed 0.05% Trypsin-EDTA (Gibco, Germany) was added to the T75 flask, and the flask was incubated for 2-3 minutes until the cells detached. The trypsin was then neutralized by adding 6 ml of prewarmed DMEM medium. The cells were resuspended in a final volume of 8 ml, transferred to a 15 ml falcon tube, and centrifuged at 400 g for 5 minutes at room temperature. The supernatant was discarded, and the pellet was placed on ice. 5 ml of DMEM medium were added to resuspend the pellet, and a cell viability test was carried out before seeding the CHO cells. A total of 14,000 cells per well were needed, and 10 wells were used for seeding CHO cells. Five wells for CPA and five wells for DMSO.

For viability test 50 µl cells were mixed with 2.5 µl Solution 18, AO/DAPI (AO 80 ug/ml, DAPI 40 ug/ml), 10 µl from the mixture used for viability test.

Table 3: Viability test for CHO cells before seeding and CPA treatment.

Viability (%)	97.1
Live (cells/m)	$3.11x10^6$
Dead (cells/ml)	$9.21x10^4$
Total (cells/ml)	$3.20x10^6$

The measurement was 3,200,000 cells per 1 ml, which means in 5 ml there were 16,000,000 cells. To obtain 14,000 cells per well, we needed to take 4.3 µl from the 5 ml cell suspension for each well, along with 500 µl of prewarmed DMEM medium (for each well). We kept both CHO cells

and canine intestinal organoids (cCO35) in a CO_2 incubator at 37 °C for about 2 days until the CHO cells and organoids reached 50% confluence for drug testing.

3.4.3. Drug testing for organoid cells (cCO35) and CHO cells

After approximately 2 days, the confluences of both cell types were checked by light microscopy and found to be 50%. Therefore, 0.5 µl of 50 µg/ml Cyclophosphamide (CPA) was added to CHO cells (positive control) and cCO35 cells on a 24-well culture plate, while 0.5 µl of DMSO was added to wells of CHO cells and cCO35 cells as a control (DMSO).

3.4.4. Viability test on CHO cells and organoid cells (cCO35) after drug testing

For the viability test, CHO cells which treated with the toxin (CPA) and DMSO, as well as organoids which treated with the toxin and DMSO separately, were collected from the wells into a 15 ml falcon tube. The organoid cells were mechanically dissociated using pipette tips (SurPhob 1000 µl filter pipette tips) and transferred to a 15 ml falcon tube. They were then centrifuged at 80 g for 5 minutes at 8°C. The supernatant was discarded, and the pellet was collected and placed on ice. Next, 500 µl of 0.05% Trypsin-EDTA (Gibco, Germany) was added to the pellet, which was resuspended in the falcon tube and incubated in a 37°C water bath for 5–10 minutes. During this period, the cells were monitored under a light microscope to ensure single-cell dissociation. Once single-cell dissociation was achieved, 4 ml of cold basal medium was added to neutralize the trypsin. The cells were then centrifuged at 400 g for 5 minutes at 8°C. Afterward, the supernatant was discarded, and the pellet was resuspended in 5 ml of basal medium for the viability test. This process was done separately for organoids treated with the toxin and those treated with the control (DMSO). For viability test 50 µl cells were mixed with 2.5 µl Solution 18, AO/DAPI (AO 80 ug/ml, DAPI 40 ug/ml), 10 µl from the mixture used for viability test. For the CHO cells, first, the DMEM medium was removed, and 500 µl of 0.05% Trypsin-EDTA (Gibco, Germany) was added to each well of CHO cells. The culture plate was then placed in a 37 °C incubator for 2-3 minutes.

Afterward, the cells from the toxin wells (5 wells) and the DMSO wells (5 wells) were separately collected into two 15 ml falcon tubes. The trypsin was neutralized by adding 6 ml of prewarmed DMEM medium. The cells were resuspended and centrifuged at 400 g for 5 minutes at room temperature. The supernatant was discarded, and the pellet was placed on ice. Next, 5 ml of DMEM medium was added to resuspend the pellet, and a cell viability test was carried out. For the viability test, 50 µl of cells were mixed with 2.5 µl of Solution 18, AO/DAPI (AO 80 µg/ml, DAPI 40 µg/ml), and 10 µl from the mixture was used.

3.4.5. Fixation of CHO cells and organoid cells (cCO35)

After the viability test for the four different cell types (CHO cells with toxin, CHO cells with DMSO, organoids with toxin, and organoids with DMSO), all four 15 ml falcon tubes were centrifuged at 100 g for 10 minutes at 10°C to collect the pellets. Four pellets were kept on ice. Next, each pellet was washed with 1 ml of cold DPBS and centrifuged using the same settings. The supernatant was removed as much as possible, and the pellets were kept on ice. The fixative solution was prepared as a 1:3 (v/v) mixture of acetic acid and methanol under a fume hood: 10 ml of acetic acid was mixed with 30 ml of methanol. A total of 40 ml of fixative solution was kept in a 50 ml falcon tube on ice. 1 ml of the cold fixative solution was added drop by drop to each pellet in the 15 ml falcon tubes using a glass pasteur pipette, and the pellets were resuspended with 4 new glass pipettes (for each pellet). The cells are now fixed.

3.4.6. Preparation of microscope slides and fluorescence microscopy

Blank microscope slides and covers were carefully washed with tap water and ddH_2O, then cleaned, dried, and labeled properly. We positioned the microscope slide at a 45-degree angle, using one glass pasteur pipette for each cell type. With this pipette, 3 or 4 drops of fixed cells were placed onto the labeled microscope slide, allowing the cells to settle and dry. After the cells dried on the slide, 10 µl of Solution 18, AO/DAPI (AO 80 µg/ml, DAPI 40 µg/ml), was added to the

center of the slide, and the cover was carefully placed on top. The microscope slides were then observed using a Leica CTR advanced inverted fluorescence microscope.

3.4.7. Statistical analysis

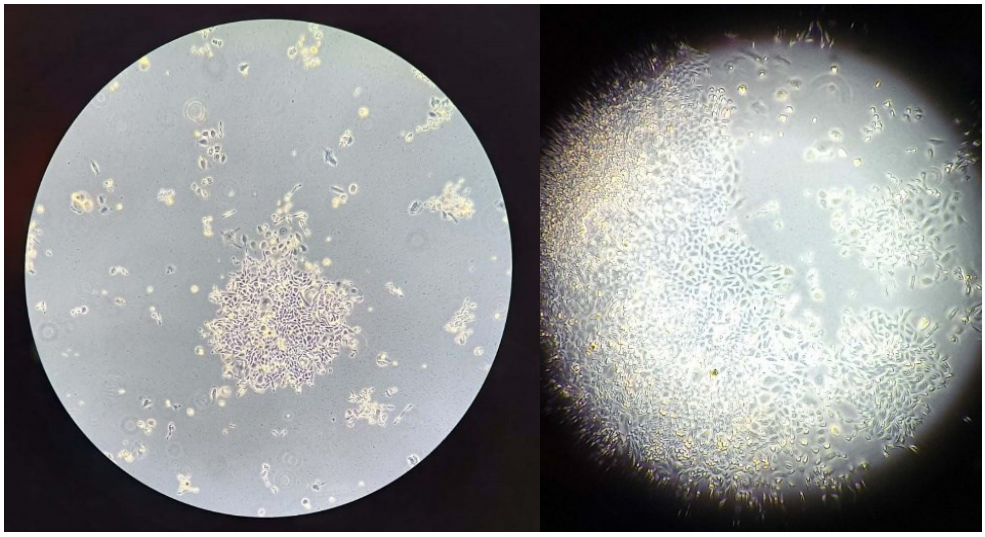
Statistical analysis was performed using R software. For pairwise comparisons, the Student's t-test was applied. Dose-response relationships were analysed using the drc package, enabling non-linear regression modelling to determine dose-response curves.

4. Results

4.1. CHO cell and canine intestinal organoid cell culture

4.1.1. CHO cell culture results

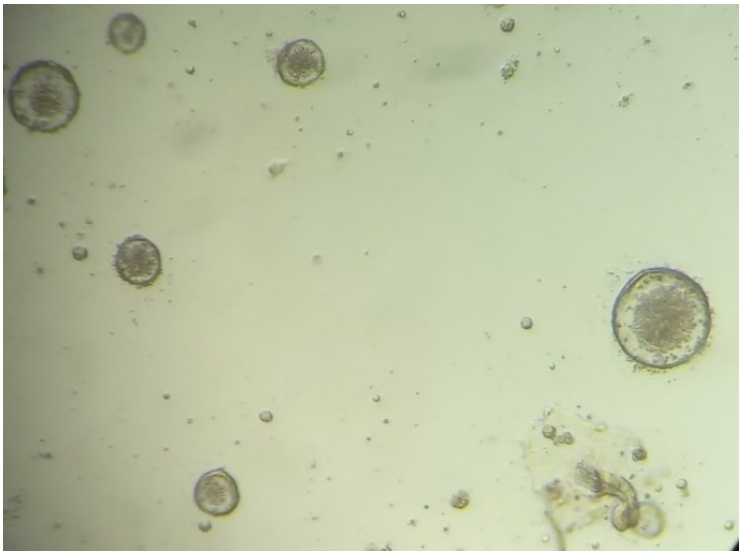
Culturing CHO cells was easier and faster than culturing organoids, at **Figure 8** the morphology and confluent of CHO cells is shown, highlighting their typical epithelial-like appearance. The cells appear uniformly spread and exhibit the expected cobblestone-like morphology, indicative of optimal culture conditions and proper maintenance of cell quality. Boundaries and uniform distribution further confirm the cells' viability under the applied culture conditions.



Figures 8: CHO cells at 25% (left) and 70% (right) confluence, respectively. Observed under bright field microscopy. The magnification is 200X.

4.1.2. Canine intestinal organoid culture results

Figure 9 presents the structure and morphology of canine intestinal organoids in Geltrex. The organoids have a characteristic three-dimensional (3D) architecture, with well-defined, confirm the organoids' structural integrity and quality. The translucent, multi-lobed structure, visible in the image, indicates successful differentiation of intestinal organoids, which are important for replicating *in vivo*-like intestinal functions. However, culturing the organoids was complicated and challenging compared to culturing CHO cells.



Figures 9: Canine intestinal organoids in Geltrex, observed under bright field microscopy. The magnification is 200X. The organoids exhibit a three-dimensional, multi-lobed structure, indicating successful differentiation and mimicking *in vivo* like intestinal functions.

4.2. Cell viability testing before and after drug treatment

To evaluate the health of CHO cells and canine intestinal organoids prior to and after drug testing, cell viability assays were performed. Before the administration of cyclophosphamide (CPA), the viability of CHO cells and organoid cells was assessed and found to be over 90%. This high level of viability indicated that both cell cultures were in good condition and suitable for further

experimental procedures. Following treatment with 0.5 µl of 50 µg/ml CPA for 24 hours, a second viability test was conducted to determine the cytotoxic effects of the drug. Once again, viability for both CHO cells and organoids remained above 90%, confirming that CPA did not induce significant cytotoxicity in either cell type.

This result was expected and desired, as the purpose of the study was to examine the genotoxic effects of CPA, not its cytotoxicity. The fact that the viability remained high after drug treatment indicates that the cells were not damaged or killed by the CPA, allowing for an accurate assessment of its genotoxic effects, such as the formation of micronuclei, without interference from cell death. This aligns with the goal of the study, which was to focus on CPA's role in inducing genotoxicity through disruption of cell division rather than cytotoxic effects.

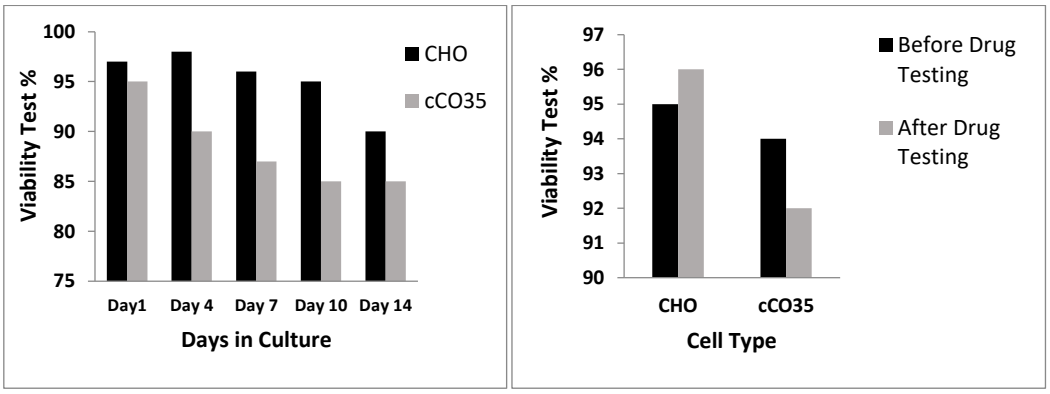


Figure 10: The plot on the left side shows the cell viability of CHO cells compared to organoids throughout a culture period of 14 days, highlighting the differences in growth stability and viability over time. The varying responses to culture conditions underscore the importance of monitoring cellular health in both cell types for experimental relevance. The graph on the right side indicates that after drug testing with CPA (50 µg/ml), both CHO cells and organoids maintain viability percentages above 90%. This suggests that drug testing is not cytotoxic at this dose, as both cell types retain significant viability post-exposure, which is necessary for a good assessment of genotoxicity.

4.3. Confluency of CHO cells and organoids before drug testing

Before the administration of cyclophosphamide (CPA) for genotoxicity testing, the confluency of CHO cells was maintained at around 50% or even lower, and confluency of canine intestinal organoid was mostly not higher than 50%-60%. This was necessary due to the mechanosensory

properties of cells. At higher confluency, cells sense mechanical signals through cell junctions, which trigger contact inhibition. This inhibition signals cause the cells to stop dividing, as they become tightly packed and begin to stabilize (except in cancer cells). However, for the genotoxicity test with CPA, it was essential that cells remained in an active dividing state, particularly during the metaphase stage of the cell cycle. This is because CPA exerts its genotoxic effects by disrupting microtubule dynamics during cell division, leading to the formation of micronuclei. Micronuclei, which are small, extranuclear bodies, arise due to chromosomal fragments or whole chromosomes being improperly segregated during mitosis. These micronuclei are a hallmark of genotoxic damage and are measured in the micronucleus test to assess CPA's impact.

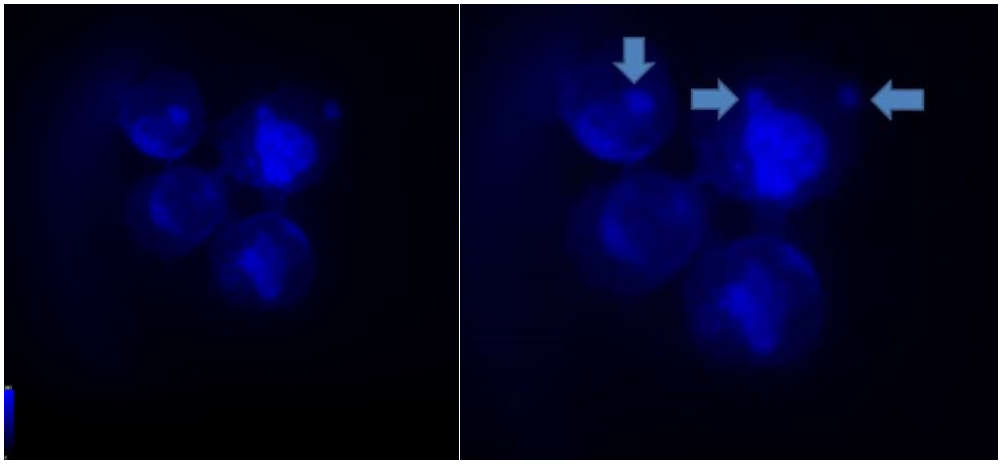
By maintaining lower confluency, we can be sure that a high number of cells remained in the proliferative phase, which was crucial for observing the genotoxic effects of CPA. If the confluency had been too high, the cells would have largely quit the cell cycle, limiting the number of cells with mitosis, and thus reducing the opportunity to see micronuclei formation. Therefore, maintaining lower confluency was important for ensuring optimal conditions for the genotoxicity assessment.

4.4. Micronucleus detection in CHO cells

4.4.1. Fluorescence microscopy of CHO cells treated with CPA

Figures 11 present Chinese Hamster Ovary (CHO) cells treated with 0.5 µl of 50 µg/ml cyclophosphamide (CPA) for 24 hours. The cells were stained with DAPI, a fluorescent dye that selectively binds to DNA, enabling the visualization of nuclei and micronuclei. The images reveal the presence of micronuclei as distinct, smaller fluorescent bodies located outside the main nucleus, which indicates chromosomal damage and confirms the genotoxicity caused by CPA treatment. To assess the presence of micronuclei, five times monitoring was performed using fluorescence microscopy. For each replicate, 1,000 cells were analyzed on newly prepared microscopy slides. Across all replicates, an average of 50 micronuclei observed. This confirms the

results' reproducibility and drug's genotoxic effects. This result is related to the expected outcomes for the CPA, which disrupts microtubule function and induces chromosomal fragmentation. The presence of micronuclei, including those budding from the nucleus but not fully separated, highlights the efficacy of CPA in causing mitotic damage.



Figures 11: CHO cells treated with 0.5 µl of 50 µg/ml CPA after 24 hours, showing micronuclei. Observed under fluorescence microscopy. The magnification is 1000X.

4.4.2. Fluorescence microscopy of CHO cells treated with DMSO (Control)

To assess the presence of micronuclei at CHO cells after treating with 0.5 µl DMSO, which served as the control group, five times monitoring were performed using fluorescence microscopy. For each replicate, 1,000 cells were analyzed on newly prepared microscopy slides, DAPI staining used to visualize the nuclei. Across all replicates, an average of 5 micronuclei were observed, as for NT control, an average of 3 MN were observed. For NT controls, only cells were used, meaning CHO cells or organoids, without CPA or DMSO.

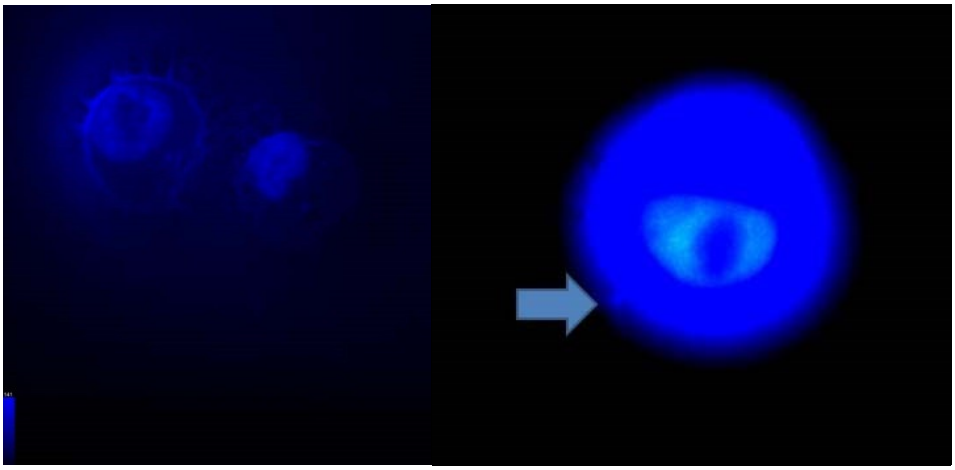


Figure 12: CHO cells treated with 0.5 µl of DMSO after 24 hours, without micronulei (left), with micronulei (right). Observed under fluorescence microscopy. The magnification is 1000X.

4.5. Micronucleus detection in canine intestinal organoids

4.5.1. Fluorescence microscopy of organoids (cCO35) treated with CPA

Figures 13 presents canine intestinal organoids treated with 0.5 µl of 50 µg/ml cyclophosphamide (CPA) for 24 hours. The organoid cells were stained with DAPI, a fluorescent dye that selectively binds to DNA, enabling the visualization of nuclei and micronuclei. The images reveal the presence of micronuclei as distinct, smaller fluorescent bodies located outside the main nucleus, indicating chromosomal damage and confirming the genotoxicity caused by CPA treatment. To assess the presence of micronuclei, five times monitoring were performed using fluorescence microscopy. For each replicate, 1,000 cells were analyzed on newly prepared microscopy slides. Across all replicates, an average of 51 micronuclei observed. This confirms the reproducibility and reliability of these findings. Micronuclei were successfully observed in CPA treated canine intestinal organoids, validating the genotoxic effects of the drug. This result is consistent with the expected outcomes of CPA, which disrupts microtubule function and induces chromosomal fragmentation.

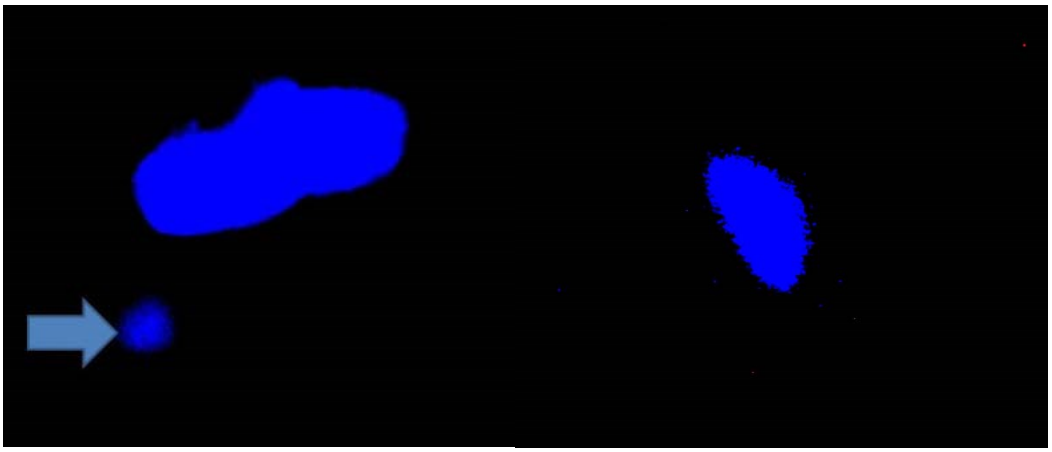


Figure 13: Organoids treated with 0.5 µl of 50 µg/ml CPA after 24 hours, with micronulei (left), without micronulei (right). Observed under fluorescence microscopy. The magnification is 1000X.

4.5.2. Fluorescence microscopy of organoids (cCO35) treated with DMSO

To analyse the presence of micronuclei at canine intestinal organoids after treating with 0.5 µl DMSO for 24 hours, which served as the control group, five time monitoring were performed using fluorescence microscopy. For each replicate, 1,000 cells from the organoids were analysed on newly prepared microscopy slides, with DAPI staining used to visualize the nuclei. Across all replicates, an average of 2 micronuclei were observed, as for NT, an average of 3 MN were observed.

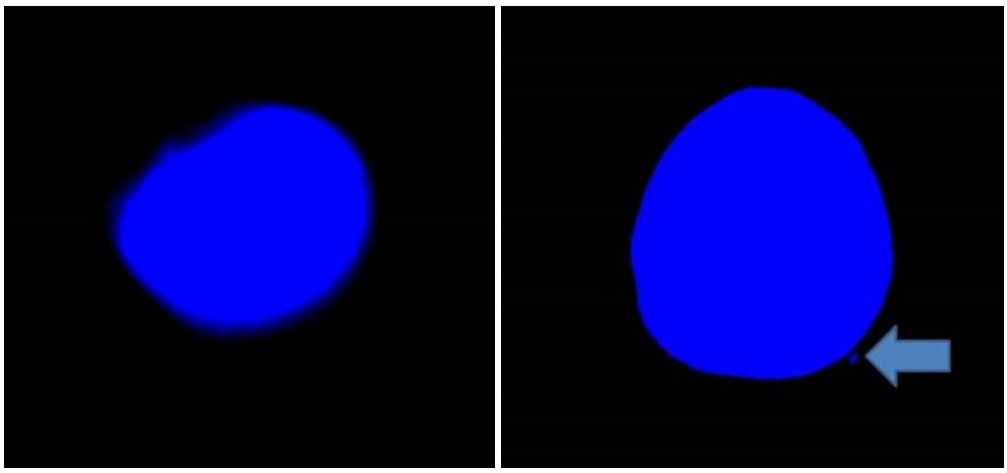


Figure 14: Organoid cells treated with 0.5 µl of DMSO after 24 hours, without micronulei (left), with micronulei (right). Observed under fluorescence microscopy. The magnification is 1000X.

It is well known that cyclophosphamide (CPA) is a chemotherapeutic and immunosuppressive drug that belongs to the class of alkylating agents. These agents work by transferring alkyl groups to DNA, resulting in damage and disruption of its normal functions. The genotoxicity of CPA is primarily driven by its role as a DNA-alkylating agent. Once metabolized in cells, CPA produces an active metabolite called phosphoramide mustard, which transfers alkyl groups to DNA. This alkylation causing cross-links both within and between DNA strands. The alkylation of DNA by CPA leads to two types of cross-links, 1) Intra-strand cross-links which occur when two nucleotides on the same DNA strand are chemically linked, distorting the DNA structure. 2) Inter-strand cross-links form between guanine bases on opposite DNA strands, preventing the normal separation of DNA during replication and transcription.

These cross-links cause the DNA to form loops, which interfere with DNA replication and transcription. This effect is particularly damaging during cell division, as it compromises the structural integrity of chromosomal DNA, leading to chromosomal fragmentation. This disruption is observed in both canine intestinal organoids and CHO cells. Additionally, alkylation and cross-linking by CPA often lead to double-strand breaks (DSBs). When a cross-linked or alkylated DNA strand cannot be repaired, the DNA replication process breaks down, resulting in a DSB. DSBs are among the most severe forms of DNA damage, and if they are not properly repaired, they lead to chromosome missegregation during mitosis. These breaks lead to formation of micronuclei, small extra-nuclear bodies that contain chromosomal fragments.

In my study, micronuclei were observed in approximately 5% of cells (CHO cells and organoids cells cCO35) when treated with 0.5 µl of 50 µg/ml CPA for 24 hours, demonstrating the substantial chromosomal damage induced by the drug. This effect was observed across five replicates in both canine intestinal organoids and CHO cells. The presence of micronuclei in both models directly reflects the chromosomal damage caused by CPA's alkylation of DNA. These micronuclei not only serve as markers of genotoxicity but also show the failure of cellular DNA repair mechanisms, demonstrating that CPA has stressed the cells' ability to repair chromosomal damage.

In canine intestinal organoids, the formation of micronuclei shows CPA's genotoxic effects on the epithelial cells lining the gut, a tissue characterized by rapid cell turnover and a high rate of division. These cells are particularly vulnerable to DNA damage from CPA, which specifically targets dividing cells. In CHO cells, widely used in cytogenetic studies, the number of micronuclei shows CPA's genotoxic effects on mammalian cells. CHO cells serve as an effective model for studying drug-induced chromosomal damage, and the formation of micronuclei in this cell line provides a reliable indicator of CPA's ability to disrupt mitosis and induce chromosomal fragmentation.

Table 4: The table shows that organoid cells and CHO cells were treated for 24 hours with 0.5 µl of 50 µg/ml cyclophosphamide and 0.5 µl DMSO. The number of micronuclei was counted after the MN assay, using fluorescence microscopy.

Replica	MN/1000 CHO (NT)	MN/1000 CHO (DMSO)	MN/1000, CHO (CPA) 50 µg/ml
1	2	3	63
2	7	5	49
3	0	6	48
4	3	2	35
5	5	11	57
Replica	**MN/1000 cCO35 (NT)**	**MN/1000 cCO35 (DMSO)**	**MN/1000, cCO35 (CPA), 50 µg/ml**
1	6	0	54
2	2	3	37
3	0	5	57
4	0	0	68
5	9	2	39

We found presence of micronuclei in similar frequencies in both canine intestinal organoids and CHO cells treated with CPA. These results shows not only the drug's potent genotoxic effects in both systems, caused primarily by the alkylation of DNA, but the ability of intestinal organoid to detect this kind of DNA damage. These findings indicate that intestinal organoids could be a sensitive and reliable system to detect DNA damage. Since CPA must first be metabolized by

cytochrome P450 enzymes to form 4-hydroxycyclophosphamide, which is then converted into phosphoramide mustard (the active form of CPA), an alkylating agent that damages DNA and creates MN, this suggests that cytochrome P450 enzymes are present in both canine intestinal organoids and CHO cells. In intestinal organoids, these enzymes may be secreted by enterocyte cells, but in CHO cells, it remains unclear which specific cells produce this enzyme.

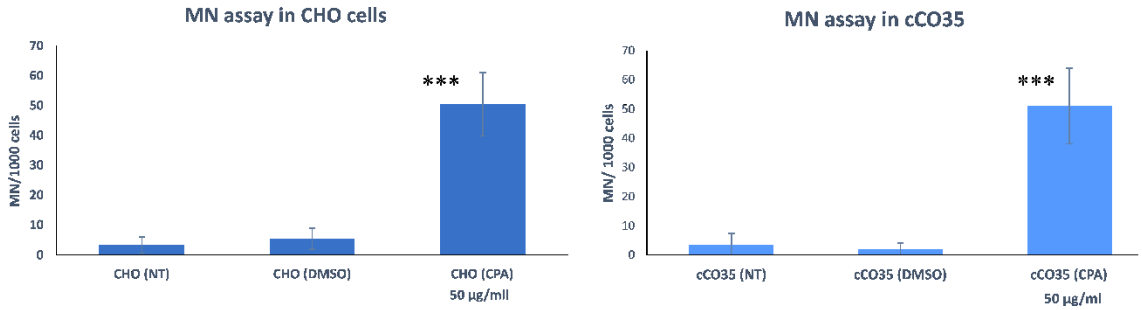


Figure 15: This bar graph represents the number of micronuclei observed every 1000 cells of CHO cells and canine intestinal organoids treated with 0.5 µl of 50 µg/ml CPA (toxin), DMSO and NT (negative control). The y-axis shows the number of micronuclei (averaged from 5 sets of 1000 cells for each cell type), while the x-axis represents the different treatment. Treatment with CPA increases micronuclei formation in both cCO35 and CHO cell types compared to treatment with DMSO. It suggests that the CPA induces genotoxicity in both cCO35 and CHO cells, as evidenced by the substantial increase in micronuclei formation compared to the DMSO and NT control. The asterisks denote a *p*-value below 0.0001 for cells treated with CPA compared to both controls.

The basal level of micronuclei formation in the DMSO and NT treated groups (**Figure 15)** confirms that the increase observed in the CPA-treated groups is specifically caused by the toxin and not by other experimental factors such as handling or solvent exposure. This indicates genotoxic effect of CPA on these cell types, making canine intestinal organoids, reliable tool for assessing DNA damage in similar experimental setups.

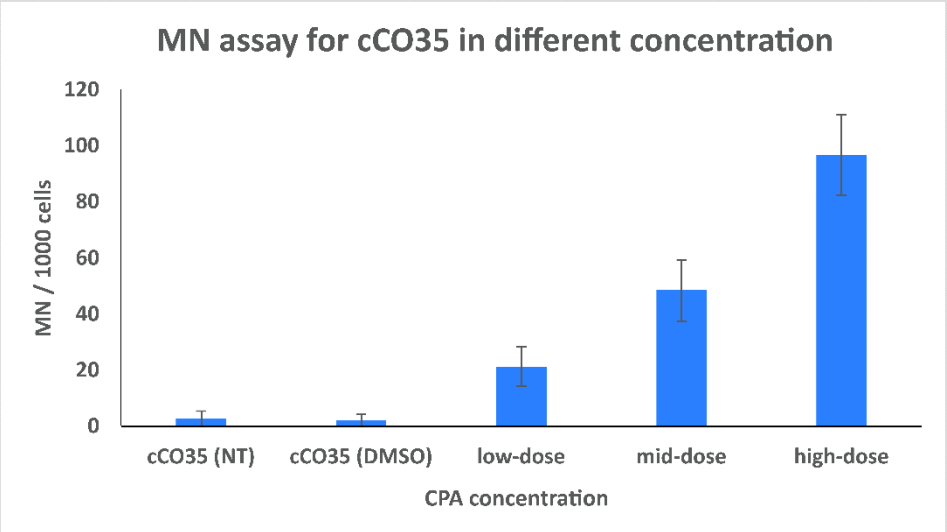


Figure 16: plots showing that increasing the concentration of CPA leads to an increase in the formation of micronuclei in cCO35 cells. **Low dose**: 25 µg/ml, **mid-dose**: 50 µg/ml, and **high dose**: 75 µg/ml.

The previous results suggest that the increasing concentration of CPA also increases the frequency of micronucleus pointing to a clear dose-response effect, which is a fundamental feature to ensure the good functionality of cellular systems to detect mutagenicity *in vitro*. To analyse the dose-response relationship we utilized the drc package (version 4.2.2) in the R statistical environment (R Core Team, 2022). The drc package provides functions for fitting a variety of dose-response models. Specifically, we employed a four-parameter log-logistic model (LL.4) commonly used for sigmoidal dose-response data.

Dose-response data were fitted using the function drc, with the response variable modelled as a function of the dose. Repeated dose-response measurements were specified to account for repeated observations. Model fit and parameter estimates were evaluated using summary statistics, and goodness-of-fit was assessed by visual inspection of residuals and predicted versus observed values. The result of the model fit can is represented in the **figure 17**.

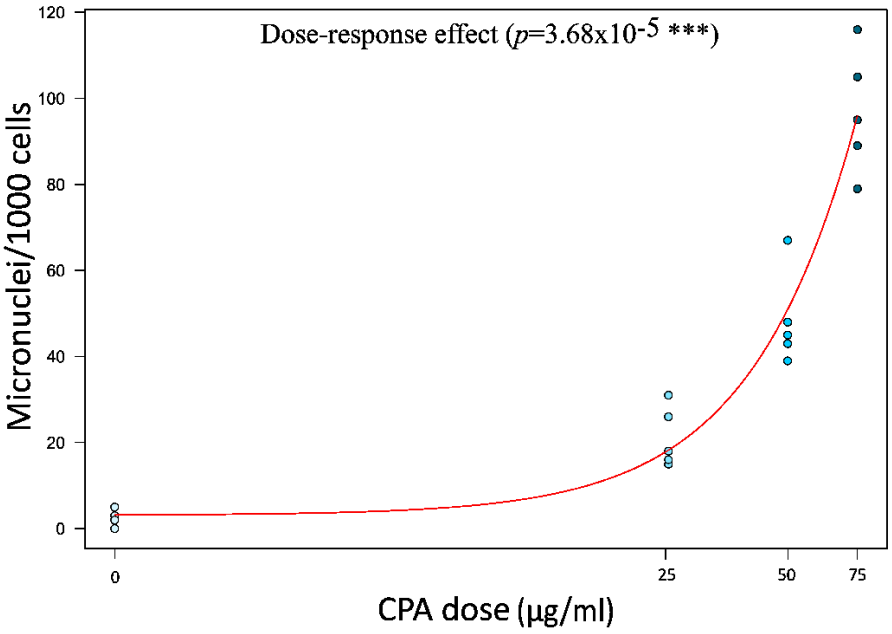


Figure 17: Dose-response assessment fitted via function drc, and model fit on the function of the dose. Five dose-response repeated measurements were to generate the fitting and measure the consistency of the observations that indicate a significant dose-effect response.

5. Discussion

One critical aspect of genotoxicity testing is the evaluation of a drug's cytotoxicity, which refers to its potential to damage or kill cells. It is essential to ensure that the drug concentration used during testing does not reach excessively toxic levels, as high concentrations can cause cell death. By using a concentration that preserves cell viability, researchers can more accurately determine whether observed DNA damage results from the drug's genotoxic properties or is simply a consequence of widespread cellular toxicity. On the other hand one significant challenge during the study was maintaining the quality and confluency of the canine intestinal organoids (cCO35), particularly after repeated passaging. After approximately 20 passages, the organoids began to show signs of deterioration, with compromised structural integrity and reduced ability to reach the confluency levels required for effective drug testing (**Figure 18**). Additionally, the culture contained undifferentiated stem cells that failed to differentiate into organoids, reducing essential

nutrients from the DMEM medium and preventing organoid growth. These undifferentiated cells were removed after dissociation of the organoids, followed by washing with DPBS and centrifugation.

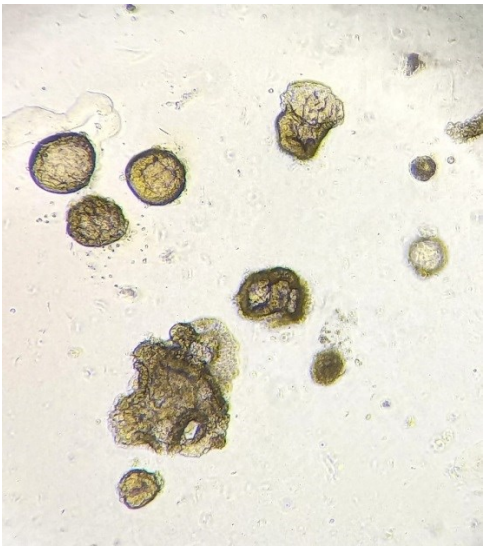


Figure 18: Organoid (cCO35) cell culture after the 20th cell passage. Observed under bright field microscopy. The magnification is 200X.

Stem cells that remain undifferentiated in organoid cultures are probably the result of suboptimal differentiation conditions. Incomplete differentiation may stem from disruptions in cell signaling pathways such as Wnt, Notch, or TGF-β. By adjusting factors like growth conditions, extracellular matrix (ECM) composition, or cell density, it may be possible to guide these stem cells toward differentiation. However, in this study, removing undifferentiated cells was necessary to preserve the quality of the organoid cultures.

A key area for further research is the role of efflux transporters, such as MRP2, P-gp and BCRP, in drug resistance within canine intestinal organoids. Specifically, it is important to investigate whether these transporters significantly affect drug absorption by actively pumping drugs back into the intestinal lumen, thereby reducing the bioavailability and efficacy of chemotherapeutic agents like CPA. Although CHO cells also express ABC transporters like canine intestinal organoids, the role of these efflux mechanisms in drug resistance, particularly in the context of canine organoids, remains unclear. Since the MN assay indicated that both CHO cells and intestinal organoid cells had around 5% micronuclei after CPA treatment, which is relatively low, we cannot attribute this directly to ABC (ATP-binding cassette) transporters. The low level of micronuclei may instead be due to the activity of detoxification enzymes in enterocytes, such as CYP3A4, CYP2C9, or ALDH1. Understanding this

relationship could help clarify whether these enzymes contribute to variability in drug response and resistance across different cell models, influencing the design of future therapeutic strategies.

In conclusion, this study highlights the value of canine intestinal organoids as a promising model for drug testing, providing comparable genotoxicity results to the traditional 2D CHO cell model. However, challenges related to organoid culture quality and confluency, particularly after repeated passaging, must be addressed to ensure reliable and reproducible results. Additionally, further investigation into the influence of efflux transporters on drug resistance could provide important insights into optimizing the use of organoids in pharmaceutical research.

6. Conclusions

This study demonstrated that canine intestinal organoids (cCO35) are a promising alternative to traditional 2D cell cultures, such as CHO cells, for evaluating the genotoxic effects of chemotherapeutic agents like cyclophosphamide (CPA) and other chemical compounds which are present in medication or in food. Both models exhibited similar levels of chromosomal damage, as evidenced by the formation of approximately 50 micronuclei for cells which treated with CPA, underscoring the genotoxicity of the drug. Despite the presence of ABC transporters in both cell types, no significant drug resistance was observed.

However, maintaining the quality and confluency of the organoids over extended passaging posed notable challenges. After approximately 20 passages, the structural integrity of the organoids began to deteriorate, and the presence of undifferentiated stem cells hindered their ability to achieve optimal confluency for drug testing. This limitation highlights the need for improved methods in organoid culture, particularly for long-term studies. Future research could focus on quantifying and comparing ABC transporter levels in both CHO cells and intestinal organoids, and ensuring optimal confluency and cellular conditions in organoids for effective genotoxicity testing, exploring alternative methods or models to assess genotoxicity more effectively in organoid systems, and addressing these factors could yield valuable insights into the mechanisms of drug resistance and enhance the accuracy of genotoxicity assessments in organoid models.

The formation of micronuclei in both canine intestinal organoids and CHO cells treated with CPA related to our hypothesis, and clearly demonstrates the drug's potent genotoxic effects, attributed to DNA alkylation, DNA loop formation, and double-strand breaks. These forms of DNA damage disrupt proper chromosome segregation during mitosis, leading to the development of micronuclei, which serve as hallmarks of chromosomal instability. Findings in two different cellular models improve the reliability of CPA's genotoxicity assessment. Furthermore, understanding CPA's mechanism of action

leads to therapeutic applications in cancer treatment, as well as its cytotoxic effects on healthy tissues, particularly those with high rates of cell turnover, such as the intestinal epithelium.

In summary, canine intestinal organoids offer a viable and ethical alternative to animal models in research, providing comparable genotoxicity results to CHO cells. Nevertheless, future studies could focus on optimizing organoid culture conditions and further investigating the role of efflux transporters in drug response, which could enhance the reliability of this model for pharmaceutical and toxicological applications.

7. References

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