UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

Cytotoxicity Assessment of Mancozeb 80% (commercially aviable) on Human Neuroblastoma Cell Line.

Trabajo de integración curricular presentado como requisito para la obtención del título de Ingeniera Biomédica.

Autor:

Cuadros Buenaventura Evelin Geoconda

Tutor:

Ramirez Cando Lenin, Ph.D.

Urcuquí, Diciembre 2022.

AUTORÍA

Yo, **Evelin Geoconda Cuadros Buenaventura**, con cédula de identidad **131667711-9**, declaro que las ideas, juicios, valoraciones, interpretaciones, consultas bibliográficas, definiciones y conceptualizaciones expuestas en el presente trabajo; así cómo, los procedimientos y herramientas utilizadas en la investigación, son de absoluta responsabilidad de el/la autora (a) del trabajo de integración curricular. Así mismo, me acojo a los reglamentos internos de la Universidad de Investigación de Tecnología Experimental Yachay.

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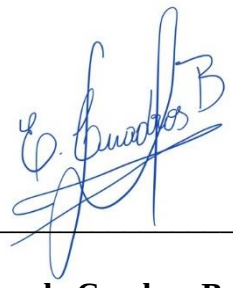

Evelin Geoconda Cuadros Buenaventura
C.I.: 131667711-9

DEDICATORIA

Cuando empecé a realizar este trabajo, no podía dejar de pensar en todas las personas que se dedican a la agricultura aquí, en mi país. Esto me ayudó mucho a llevar a cabo esta investigación con mucho compromiso y cariño. Por tal motivo, me gustaría dedicar esta tesis a todos/as los/as agricultores/ras de Ecuador, quienes pese a no tener el reconocimiento merecido, son un pilar importante para nuestro país.

Por supuesto, también dedico este trabajo con todo mi corazón a mi papi y mami, a quienes les debo todo. A mis hermanas Vale y Tina, y a mis bebés perrunos Cuqui y Muñe.

AGRADECIMIENTO

Agradezco infinitamente a mi Tutor, profesor Lenin Ramirez, y al profesor Santiago Ballaz por darme la oportunidad de trabajar en este gran proyecto e introducirme al mundo del trabajo en un laboratorio. Gracias por acompañarme durante este proceso y compartir conmigo de su experiencia y conocimiento.

A mis primeros compañeros de lab Marcelo y Fernando V., quienes fueron mi guía en el laboratorio las primeras semanas.

A Angie, Ronny, Nayely, Paula y Geomara, por acompañarme y brindarme su ayuda durante el desarrollo de los experimentos.

A Paola, Fernando G., y Katy, por permitirme el acceso a el lab todos los días y estar pendiente de lo materiales, reactivos y equipos que necesitaba.

A mis amigos Maya, Nico y Raúl, quienes han estado siempre desde que los conocí. Gracias por hacer de mis días en la U más bonitos y divertidos, y por echarme porras y acompañarme cada vez que lo necesité. Conocerlos ha sido de las casualidades más bonitas de mi vida.

A mi amiguis Judith, por estar pendiente de mi proceso y recordarme que con esfuerzo y perseverancia puedo lograr todo lo que me proponga.

Finalmente, quiero agradecer con todo mi corazón a mi familia.

Gracias papi y mami por brindarme la mejor educación que pudieron, por ser mi soporte y darme su amor incondicional. Gracias por no limitarme, y apoyarme en todos los proyectos en los que me he involucrado.

Gracias a mis hermanas Vale y Tina quienes siempre han creído en mí y han apoyado y acompañado cada uno de mis sueños.

A Jonatan, por estar presto a ayudarme en lo que necesité.

A mis bebés perrunos por haber sido mi apoyo emocional durante todo este tiempo, acompañarme en mis noches de desvelo y levantarme en las mañanas de la mejor manera.

RESUMEN

Mancozeb es el nombre común dado al Etilenbis(ditiocarbamato) de manganeso (polimérico) con sal de zinc. Este compuesto es utilizado como fungicida, el cual, debido a su actividad multisitio es uno de los agroquímicos más usados en todo el mundo. Pese a todas sus ventajas, el uso del mancozeb ha sido cuestionado debido a los posibles efectos adversos que este producto podría tener sobre la salud humana. En efecto, el mancozeb ha sido vinculado con la disrupción del sistema endocrino y problemas de reproducción y desarrollo. Además, en los últimos años, un creciente número de evidencia propone al mancozeb como un agente neurotóxico. Por esta razón, el objetivo de este trabajo es evaluar la citotoxicidad del mancozeb en las células SH-SY5Y, una línea de células humanas derivadas de neuroblastoma. Los resultados mostraron que el mancozeb a concentraciones de 0.5 - 10 µM afecta notablemente la morfología de las células SH-SY5Y. Específicamente, el mancozeb produjo la reducción del número y longitud de neuritas por células de forma dosis dependiente. Además, se observó el incremento en la concentración de especies reactivas de oxigeno por efecto del mancozeb. Finalmente, a concentraciones de 5 - 80 µM el mancozeb redujo considerablemente la viabilidad de las células SH-SY5Y. Basados en estos resultados, se concluye que el mancozeb tiene efectos citotóxicos en las células SH-SY5Y.

Palabras clave: *Mancozeb, fungicida, neurotoxicidad, células SH-SY5Y, ROS, viabilidad celular.*

ABSTRACT

Mancozeb is the common name given to manganese ethylenebis(dithiocarbamate) (polymeric) with zinc salt. This compound is used as a fungicide, and due to its multisite activity, it is one of the most used agrochemicals worldwide. However, despite all its advantages, the use of macozeb has been questioned due to the adverse effects that this product might have on human health. Indeed, mancozeb has been linked to endocrine system disruption and reproductive and developmental problems. Furthermore, in recent years, a growing body of evidence proposes mancozeb as a neurotoxic agent. For this reason, this work aims to evaluate the cytotoxicity of mancozeb in SH-SY5Y cells, a human neuroblastoma cell. The results showed that mancozeb at concentrations of 0.5 - 10 µM markedly affected the morphology of SH-SY5Y cells. Specifically, mancozeb caused a reduction in the number and length of neurites per cell in a dose-dependent manner. In addition, the increase in the concentration of reactive oxygen species due to the effect of mancozeb was observed. Finally, at concentrations of 5-80 µM, mancozeb considerably reduced the viability of SH-SY5Y cells. Based on these results, it is concluded that mancozeb has cytotoxic effects on SH-SY5Y cells.

Keywords: *Mancozeb, fungicide, neurotoxicity, SH-SY5Y cells, ROS, cell viability.*

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1. INTRODUCTION

Mancozeb (MCZ) is the common name given to the manganese ethylenebis (dithiocarbamate) (polymeric) complex with zinc salt (IUPAC) (1). This compound is known to be part of the group of dithiocarbamate pesticides. Specifically, MCZ is used as a non-systemic fungicide (surface acting) in different crops which include fruits, vegetables, ornamental plants and sod. The primary function of MCZ, like other fungicides, is to prevent the loss or reduction of crop yields due to fungal diseases (2).

According to its mode of action, MCZ is classified by the Fungicide Resistance Action Committee (FRAC) as a type M agrochemical, meaning it has multi-site activity (3). Undoubtedly, this is one of the most attractive characteristics of this fungicide. The multi-site activity of MCZ makes it a broad-spectrum strategic product that prevents its target organisms from creating resistance since it interferes with different stages of critical metabolic processes (citrate cycle) of fungi (2,4). For this reason, MCZ is also widely used in co-formulations with other fungicides (systemic and single-site inhibitors) as an anti-resistance tool (2,5,6). Furthermore, being a broad-spectrum and low-cost agrochemical, MCZ has the flexibility to be used on different crops and for different fungal diseases. In fact, this fungicide is approved to be used in more than 70 crops and for approximately 400 types of plant fungal diseases (2).

Together, these characteristics make MCZ a highly demanded product in the agricultural sector, and the statistics reflect this fact. Since its introduction to the market in 1962 (2), MCZ has been on the list of the best-selling pesticides worldwide. By 2022, MCZ sales are projected to reach USD 847.2 million (being part of the world's top 5 most used fungicides). Also, it is estimated that by 2028 its market size will be USD 1029.4 million, reaching a Compound Annual Growth Rate (CAGR) of 3.3% during that period (7,8).

Despite all its advantages, MCZ has been a controversial agrochemical due to the possible adverse effects it may have on human health (9). An extensive number of *in vitro,* animal, occupational and epidemiological studies have attributed to MCZ the ability to produce toxic effects on the liver, kidneys, and the immune system, as well as being a carcinogen agent (10–15). However, studies have focused mainly on its toxicity over the thyroid gland, the reproductive system since they seem to be its main target (16,17).

Nonetheless, the hypothesis that MCZ might be a neurotoxic substance has become more popular in recent years. Structural similarities between MCZ and maneb (MB), a fungicide used to develop animal and cell models of Parkinson's disease (PD) (18), in addition to some occupational and epidemiological studies showing a positive correlation between MCZ exposure and different neurological problems (19–21), have contributed to adding neurotoxicity to the list of adverse effects that MCZ may have on human health.

1.1. Problem Statement

The use of crop protection chemicals in Ecuador is an important factor in its economy since the agricultural sector contributes 8% of its total annual production (22). Furthermore, according to 2018 data from the Food and Agriculture Organization of the United Nations (FAO), Ecuador ranks tenth among the countries that use the most pesticides worldwide (23). Also, it is the second country in the world that uses the largest amount of pesticide per cropland, and the group of fungicides and bactericides seem to be the most used agrochemicals in this territory (Figure 1) (23,24).

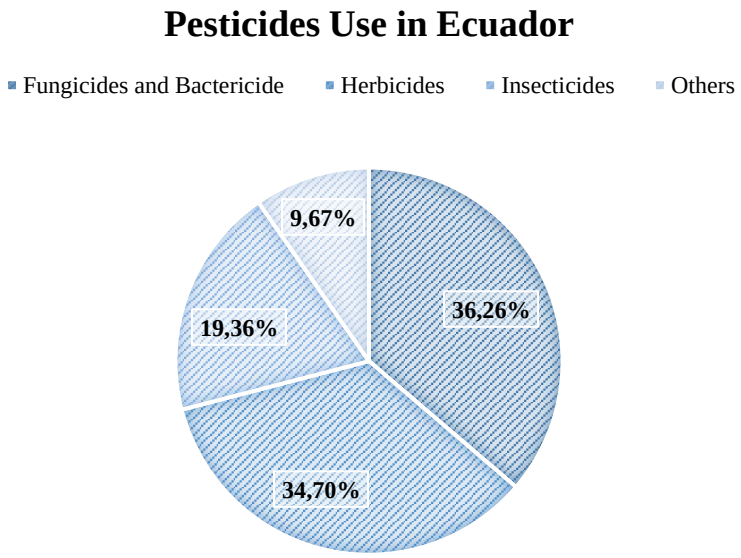

Figure 1: Pesticides use in Ecuador according their target organisms. This graph was made based on 2020 FAOSTAT data.

Until this work was redacted, no official data on the sales and use of MCZ in Ecuador were found. However, in document G/SPS/GEN/1808 which was made in response to the

notification about the non-renewal of the approval of the active substance MCZ by the European Union, the sanitary and phytosanitary measures committee of Ecuador together with that of Colombia, explicitly express in their comments that MCZ "...is essential to control pests in a wide variety of crops, including bananas, the main export product of Colombia and Ecuador to the European Union " (25,26). Specifically, in banana crops, which are among the largest crops in the Ecuadorian territory (230,000 hectares), the use of MCZ is essential for the control of Black Sigatoka (a disease caused by the *Mycosphaerella fijiensis* fungus) since the sale of chlorothalonil, which was the main substance to control this pest, was recently prohibited (26,27). In addition, MCZ is commonly used in other transitory crops with extensive territorial occupation in Ecuador, such as potatoes and rice (28). Considering this information, it is inferred that Ecuador is one of the biggest consumers of MCZ in the region. Based on this, the safety of this fungicide should be guaranteed.

However, accordingly to a considerable number of studies, MCZ may have neuronal-related (among others) adverse effects. A study performed in a population located near banana plantations in Costa Rica, where MCZ is commonly administered by aerial spraying over these crops, showed a positive correlation between prenatal exposure to MCZ and manganese, and neurodevelopmental problems (cognitive abilities in girls and social-emotional development in boys and girls) in 1-year-old infants (20). Similar results were shown by a study conducted in Thailand with a sample of 855 children (413 control and 442 exposed to pesticides) between 9 and 42 months. They found a positive association between prenatal and postnatal exposure to MCZ and a suggested developmental delay (19). Furthermore, in Uganda, MCZ exposure was associated with lower semantic verbal fluency in smallholder farmers (21).

For this reason, it is important to evaluate the neurotoxicity of MCZ since the agricultural sector employs approximately 2.2 million people (12% of the population) (22). This group includes people who are in direct contact with all kinds of agrochemicals, including MCZ, and in many cases, they do not use adequate protective equipment (Figure 2) (29). In addition, the misuse of these agrochemicals (application of excessive or insufficient doses and/or the application of the wrong product according to the requirements of the crops) is very frequent in Ecuador and other countries of the Andean region (30). Together, these situations make people, who are occupationally exposed to

agrochemicals, more susceptible to poisoning or long-term effects that this type of substance could be causing to their health.

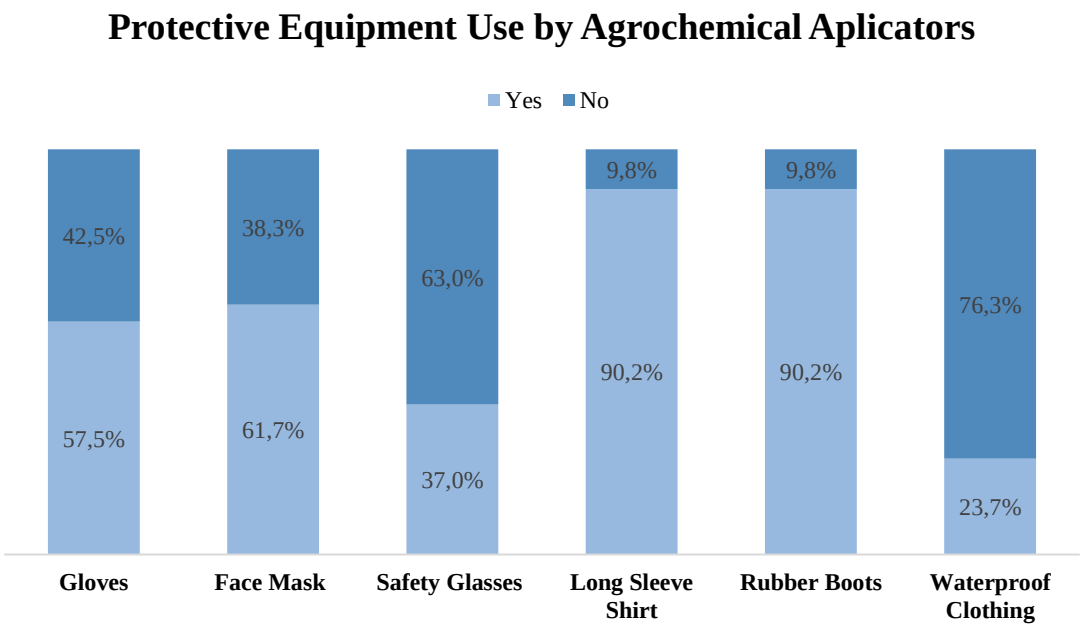

Figure 2*:* Agricultures who use protective elements when applying pesticides, by type of protective element. This graph was made based on the data obtained from INEC.

1.2. Objectives

1.2.1. General Objective

- To evaluate the cytotoxicity of a commercial formulation of Mancozeb 80% in SH-SY5Y cells, a human neuroblastoma cell line, through cell morphology, ROS production and cell viability (trypan blue and MTT) studies.

1.2.2. Specific Objectives

- To treat SH-SY5Y cells with multiple concentrations of Mancozeb 80%.
- To observe and evaluate the morphological changes of the SH-SY5Y cells when exposed to the treatments.
- To observe if the commercial formulation of Mancozeb 80% induce a ROS overload.
- To determine the viability of SH-SY5Y cells after being exposed to the treatments.

2. LITERATURE REVIEW

2.1. Dithiocarbamates pesticides

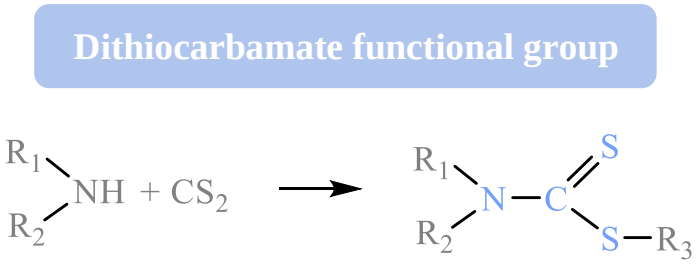

Figure 7: Formation of the dithiocarbamate functional group.

Dithiocarbamates (DTCs) are a group of sulfur-containing carbamate chemicals with pesticidal action. They are mainly non-systemic and non-specific in their mode of action (31,32). DTCs are the combination between an amine (aliphatic or aromatic) and a carbon disulfide (CS_2) (Figure 3) which results in the formation of the NC(S)SH group that is essential for its biocidal action (33). Currently, there is a wide variety of DTC pesticides which have been derived by dissociation of H attached to S and replaced by metals (Zn, Mn) or other radicals (34). This group of pesticides are considered to have high biological activity due to their metal-chelating capacity and their affinity with enzymes that contain the sulfhydryl group (35). According to its chemical structure, this group is divided into propylenebis (dithiocarbamates), ethylenebis (dithiocarbamates) (EBDCs) and dimethyl (dithiocarbamates) (Figure 4) (31). This review will focus on the EBDCs, a subgroup to which the MCZ belongs.

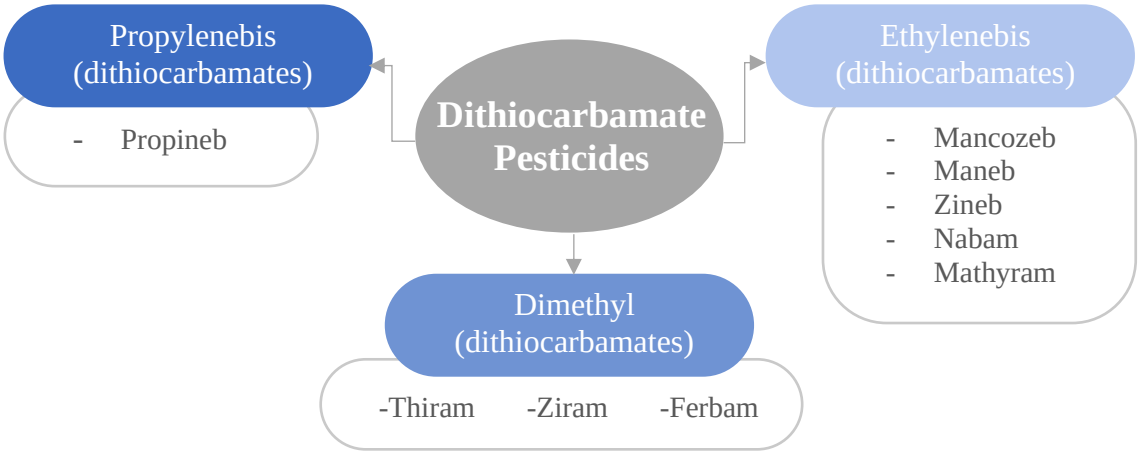

Figure 13: Classification of dithiocarbamate pesticides.

2.1.1. Ethylenebis (dithiocarbamate) fungicides

(The MCZ will not be taken into account as it will be reviewed in detail in the following sections)

EBDC fungicides are organometallic compounds poorly soluble in water; also, they are unstable in humid environments, in the presence of oxygen, and in biological systems (32). The main characteristic shared by these compounds is their three most common degradation products: ethylenebisisothiocyanate (EBIS), carbon disulfide (CS_2), and ethylene thiourea (ETU) (36). Among these, ETU is the one that is produced in greater proportion at the moment of the EBDCs molecular breakdown (32).

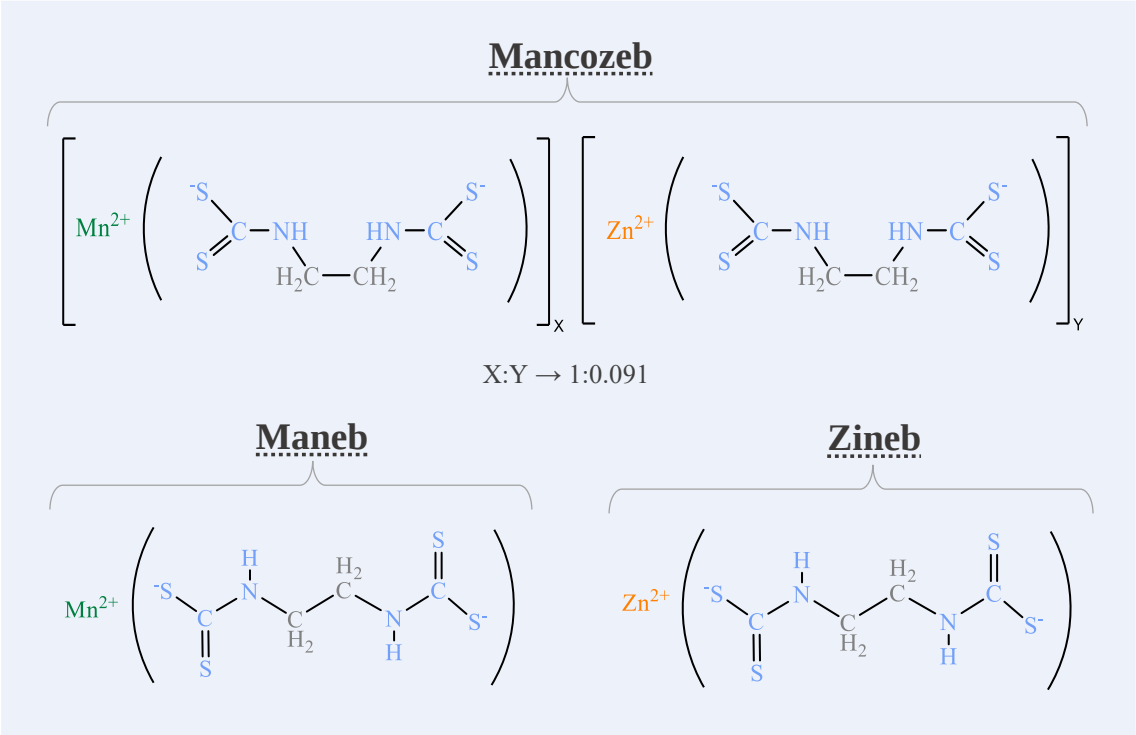

Figure 14: Chemical structure of the principal EBDCs: Mancozeb, Maneb and Zineb. These structures were based on Compound Summary provided by PubChem®.

2.1.1.1. ETU-related toxicity

EBDCs are widely used chemicals in the agricultural sector. Their popularity is due to their high effectiveness in controlling fungal pests and their advantages related to their characteristic multi-site activity (31). At the same time, EBDCs are considered of major human health concern, primarily due to their main metabolite ETU (32,36). ETU is thought to be responsible for the toxicity that EBDCs may have on humans, other mammals, and non-target organisms. Indeed, studies have shown that ETU might produce

thyroid and developmental toxicity, as well as, it seems to be a substance with carcinogenic properties (Table 1) (32). However, despite having the same degradation metabolite, each EBDC is known to have different characteristic toxic outcomes. This means that they produce not only ETU-related toxicity but also toxicity elicited by EBDCs may be linked to each compound by themselves or due to its metallic part (37,38).

2.1.1.2. Non-ETU-related toxicity

Elevated reactive oxygen species (ROS) production is postulated to be the convergence pathway for EBDCs toxicity. Studies performed in fibroblastic cells (V79) showed that part of the mechanism of the high load of ROS produced by MB occurs through the increase of superoxide dismutase 2 (SOD2) but not SOD1 and SOD3 (39). In contrast, zineb (ZB) produces a decrease in catalase (CAT) activity (39). The group of enzymes SOD, CAT, as well as glutathione peroxidase (GP) are part of the natural enzymatic defense of cells against oxidative stress (40). SODs catalyze the conversion of superoxide anion ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2), which is subsequently converted into water (H_2O) and oxygen (O_2) by the action of CAT and GP (40). Therefore, the overexpression of SOD2 produced by MB is considered a defense mechanism against the increase in free radicals potentiated by this fungicide. As a consequence, H_2O_2 might increase in concentration, and if there is no adequate response by CAT and GP, the cells would be exposed to the detrimental effects of oxidative stress (i.e. protein, lipid and nucleic acids damage and cell death) (39,40). On the other hand, the decrease in CAT activity produced by ZB would also be due to an imbalance in the cellular redox environment. This imbalance is probably due to the enhancement in ROS production due to ZB exposure, as has been observed in studies performed on human keratinocytes (HaCaT) cells (41). However, the initial mechanism of initiation of this ROS production chain remains unclear.

Elevated levels of ROS are closely related to neurotoxicity, one of the major concerns of EBDCs (42). MB in particular, whose metallic component is Mn, a potential neurotoxin, is perhaps the most linked of the EBDCs to neuron-related toxicity (43). Epidemiological studies have shown that environmental exposure to MB contributes to the development of PD, the second most common neurodegenerative disease that selectively affects dopaminergic (DAergic) neurons of the substantia nigra (SN) (44–46).

Toxicity	Characteristics of the study	Results	Reference
Thyroid	**Organisms:** Sprague Dawley-derived rats. **Treatment:** ETU at 0, 1, 5, 25, 125 and 625 ppm up to 90 days.	**Rats that received the treatment of 625 ppm of ETU showed:** - ↓ serum T-3 and T-4. - ↑ serum TSH (this was also showed by 125 ppm treated rats). - ↓ iodide uptake by the thyroid. **Rats that received the treatment of 25 ppm of ETU showed:** - Thyroid hyperplasia.	(47)
Developmental toxicity and teratogenicity	**Organisms:** Wistar-imamichi rat embryos from days 11 to 13 of gestation. **Treatment:** Embryos were culture for 48 in medium from 0 to 300 µg/mL of ETU.	- Malformation in the head, tails, limbs and face were found in all embryos exposed to 150 and 300 µg/mL of ETU. - Histological studies showed thinner neuroepithelium in head in ETU treated embryos. - Inhibition of the differentiation of midbrain cells into neurons in embryos exposed from 30 and 600 µg/mL of ETU	(48)
Carcinogenicity	**Organisms:** F344/N rats and B6C3F1 mice. **Treatment:** Perinatal and/or adult dietary exposure of rats for a period of 2 years to ETU from 9 to 90 ppm (perinatal) and from 25 to 250 ppm (adult). Perinatal and/or adult dietary exposure of mice for a period of 2 years to ETU from 33 to 330 ppm (perinatal) and from 100 to 1000 ppm (adult).	**Perinatal exposure of ETU:** - No carcinogenic to rats or mice. **Adult and perinatal/adult exposure to ETU:** - Carcinogenic in rats and mice (follicular cell adenomas and carcinomas. - Thyroid gland was the main target of ETU carcinogenicity in rats and mice. - Liver and pituitary gland showed ETU carcinogenic effects in mice.	(49)

Table 1: *In vivo* studies of ETU toxicity in rats and mice.

Fungicide	Characteristics of the study	Results	Reference
Zineb	**Cells:** Human keratinocyte (HaCaT) cells. **Treatment:** 1-40 µg/mL of ZB for 24 hours.	- ↓ cell viability. - Induce apoptosis in a dose-dependent manner via the Bax/Bcl2 and caspase pathway. - ↓ Bax and caspase 3 and decrease levels of Bcl2.	(41)
	Cells: Chinese hamster ovary (CHO) cells. **Treatment:** 0.1-100 µg/mL of ZB or Azurro (ZB 70%) for 24 hours.	- Considerable delay in cell-cycle progression (5-25 µg/mL). - ↓ mitotic activity in a dose-dependent manner (1-25 µg/mL). - Cytotoxicity in doses higher than 50 µg/mL. - DNA damage in a dose-dependent manner (25-100 µg/mL).	(50)
	Cells: Human Neuroblastoma (SH-SY5Y) cells with catecholaminergic phenotype. **Treatment:** Exposure to ZB from 50–400 µM or to a mixture of ZB and endosulfan at 100µM each for 16 hours.	- Apoptotic cell death in a dose-dependent manner for ZB alone and the mixture of ZB and endosulfam. - Cells morphology were significantly affected: they showed more circular shape and a visible reduction in neurite extension (ZB at 100 µM). - Apoptotic bodies formation, cell shrinkage and cell blebbing, and a decreased adhesion to flask were observed in ZB and endosulfan treated cells. - DNA fragmentation was observed in mixture of ZB and endosulfan 10 hours treated cells.	(51)
Maneb	**Cells:** Human neuroblastoma SK-N-AS cells. **Treatment:** Exposure to 50µM of MB for 48h**.**	- Decreased mitochondria respiration, altered lactate dynamics and metabolic stress. - Acute alteration of central carbon metabolism. - Alteration of thiol redox status. - Cellular glucose preferentially metabolized through the pentose-phosphate pathway. - Acute disruption of glycolysis.	(37)
	Cells: Rat pheochromocytoma (PC12) cells. **Treatment:** Exposure of cells to 20µM of MB for 8 hours.	- Upregulation of the RTP801 protein (DNA damge response) was shown in 20µM MB exposed cells for 8 hours.	(52)
	Cells: PC12 cells. **Treatment:** Exposure of cells to 1 µg/mL of MB for 2 hours.	- Apoptotic cell death. - Aggregation of alpha synuclein. - Alteration of mitochondria membrane potential.	(53)

Table 2: *In vitro* studies of EBDCs toxicity.

Fungicide	Characteristics of the study	Results	Reference
Zineb	**Organism:** New Zealand White Rabbit. **Treatment:** Rabbits were fed with a diet containing 0, 0.3 or 0.6% of ZB for 90 days.	- ↓ hemoglobin concentration, hematocrit, erythrocyte and weight gain. - ↓ thyroid hormones in a dose-dependent manner. - ↑ serum concentration of cholesterol and triglycerides. - ↓ hepatic lipid concentration (0.6% treatment). - Enlargement of the thyroid gland in a dose-dependent manner.	(54)
	Organism: Wistar SPF rats. **Treatment:** Rats were fed with a diet containing 5, 50 and 500 ppb of ZB for 5 days.	- ↓ T4 levels (50 ppb). - ↓ blood glucose in a dose-unrelated manner.	(55)
Maneb	**Organism:** Male Swiss mice. **Treatment:** Mice were exposed to a combination of 0.3 mg/kg paraquat and 1.0 mg/kg MB from postnatal day 5 to 19.	- Neither motor-related parameters nor induction of mortality. - ↓ tyrosine hydroxylase and dopamine transporter positive neurons in the SN pars compacta and striatum. **Re-exposure of mice at 3 month of age with 10 mh/kg of paraquat and 30 mg/kg de MB for 6 weeks (twice a week):** - Motor deficit - ↓ tyrosine hydroxylase and dopamine transporter positive neurons in the SN pars compacta.	(56)
	Organism: Swiss adult female mice. **Treatment:** Intrapritoneal treatment with MB at doses of 1/8, 1/6, 1/4 and 1/2 of its lethal dose (LD_{50} 1500mg/kg body weight) for seven days.	- Disruption in total with blood cells and platelets. - ↓ plasmatic levels of ferrozine. - ↑ malondialdehyde, lipid hydroperoxides and H_2O_2. - ↓ CAT and GP activity. - Nephrotoxicity signs (↑ plasma urea, albumin levels, lactate dehydrogenase activity and ↓ uric acid). - Degradation of nucleic acid. - Renal injuries.	(57)
	Organism: Sprague-Dawley male rats. **Treatment:** Intraperitoneal injection of MB for 9 or 18 days at doses of 4 mg/kg.	- No liver function alteration. - ↓ basal level of plasma testosterone.	(58)

Table 3: *In vivo* studies of EBDCs toxicity.

(Continued) **Table 3:** *In vivo* studies of EBDCs toxicity.

Fungicide	Characteristics of the study	Results	Reference
	Organism: Common and green toad tadpoles at the development stage 21. **Treatment:** Tadpoles were exposed to MB for 120 hours from 0-5 mg/L.	**Common and green toad tadpoles:** - Visceral oedema and tail deformation. - Liver necrosis, pronephric tubule deformations, somite deteriorations and visceral oedema (for common toad tadpoles at concentrations ≥ 0.1 mg/L and for green toad tadpoles at concentrations ≥ 0.01 mg/L)	(59)
Maneb	**Organism:** Zebrafish embryos at 6 hours post fertilization. **Treatment:** The embryos and larvae were exposed at 0.1, 0.5, 1.0 and 10.0 µM of MB for 96 hours.	- The LC_{50} value at 96 hours was established at 4.29 µM. - ↑ mortality and notochord deformity, as well as ↓ hatching rate, all of them in a dose-dependent manner after the 72 hours of exposure at doses of 1.0 and 10.0 µM. - ↓ total body length with treatment of 1.0 µM. - ↓ basal oxygen consumption after 24 hours of exposure to 10.0 µM. - Larvae hypoactivity was notice after 7 days of exposure to 0.5 and 1.0 µM of MB.	(60)

This epidemiological correlation is supported by studies carried out in cells and animals. Liu and colleagues recently showed that exposing SH-SY5Y cells to MB at concentrations of 0, 0.1, 5, and 10 mg/L reduced their viability in a dose-dependent manner (61). Furthermore, they showed that A53T transgenic mice exposed to 60 mg/kg MB developed PD-like motor symptoms (61). In addition, MB is used as a co-treatment of paraquat (herbicide) to generate mouse PD models (62,63). These models exhibit motor and non-motor symptoms of PD, selective loss of DAergic neurons, aggregation of alpha-synuclein (an important hallmark of PD) and dopamine depletion in the striatum (64). The mechanism of MB neurotoxicity is not clear, but due to its selectivity with the nigrostriatal system, it is assumed that it is capable of crossing the blood-brain barrier (18). Furthermore, omics studies have suggested the involvement of MB in mitochondria impairment and oxidative stress. It would also cause interference in the phenylalanine and tryptophan metabolism pathways, dopaminergic synapse and synaptic vesicle cycle (61).

In addition to neurotoxicity, EBDCs are related to other adverse outcomes (Table 2 and Table 3).

2.2. Physical and chemical characteristics of mancozeb

MCZ is an organometallic compound commercially available as a grayish-yellow wettable powder (WP) with a slight hydrogen sulfide odor (65). Due to its low vapor pressure (9.8×10^{-8} mm Hg at 25 °C), it is an unlikely product of volatility, but it can persist in the air for up to 72 hours after application due to air-borne particles or spray drifts (66). In addition, like its relative EBDCs, MCZ is not soluble in water or most organic solvents (65). This characteristic, in addition to its low environmental persistence, makes MCZ a substance with moderate mobility in the soil, which reduces the environmental impact it could have (66). However, MCZ is a rapidly degrading substance (with a half-life of less than 2 days in soil and water) in the presence of light, heat, and moisture (66,67). Its main degradation products are ETU, EBIS and ethylene urea (EU) (67). The metabolites and their ratio will result according to various factors of the medium in which the MCZ degrades, including pH, light, and temperature (67). For example, López-Fernandez and colleagues showed that a basic environment would favor the decomposition of MCZ to ETU regardless of the presence of light (except at temperatures

of 25 and 29 ºC in the presence of light). In addition, under temperature conditions close to 39 ºC, the ETU derived from MCZ reaches maximum concentrations (67).

ETU is, in general, the most common degradation product of MCZ. Unlike it, ETU has excellent stability and high solubility in water, which makes it a product with a higher risk of environmental contamination and spreading rate (67,68). It is of significant importance since, as discussed in previous sections, ETU is one of the major human health concerns related to MCZ (32).

At the molecular level, the active substance MCZ is composed of an organic part, which consists of the EBDC functional group, and a metallic part made up of Mn and Zn in a ratio of 1:0.091, as shown in Figure 5 (69). These Metallic ions are pretty unstable in the molecular configuration of the MCZ, so in the process of degradation, they are rapidly dissociated, especially with increasing temperature (70).

2.3. Use and mode of action of mancozeb

For its use, the MCZ WP is mixed with water and commonly applied in the field through manual or mechanical sprayers, allowing the agrochemical spread over the entire crop extension. The indications for the use of MCZ at 80% cover a range between 150 - 240 gr dissolved in 100 L of water, with a wetting rate of no less than 150 L/ha and no more than 3000 L/ha. In addition, the frequency of application of this product varies between 2 - 3 applications per season. However, more specific doses and frequencies are indicated according to the crop, the pest to be attacked and the environmental conditions (71).

Furthermore, it is important to mention that MCZ is used as a preventive treatment for inhibiting fungal spores germination. Therefore, the use of MCZ is not effective once the disease has begun to affect the crops. This is because, being a non-systemic fungicide, MCZ does not penetrate plant tissues. This characteristic makes MCZ a product with high crop safety since it is not likely to produce phytotoxicity (2).

On the other hand, the fungicidal mode of action of MCZ is based on the decomposition of its molecule. As reviewed in the previous section, one of the main hydrolysis products of the MCZ molecule is EBIS, which is converted to ethylene bisisothiocyanate (EBI) by the action of UV light. Both thiocyanate molecules have a

high affinity for fungal cell proteins that contain the sulfhydryl group (2). Also, due to their multi-site action, the exact mechanisms and pathways in which these molecules interfere within the fungal cells have not been defined. Even so, it is estimated that EBIS and EBI toxic effect is the result of the disruption of at least six biochemical processes that take place in the cytoplasm and mitochondria of these cells because of their interaction with proteins that contain the sulfhydryl group (2). Part of this group of proteins are recognized as essential in the process of cellular respiration (72,73).

2.4. Exposure to mancozeb: ways and biological limits

In general, exposure to all types of agrochemicals occurs through three main routes: through the ingestion of contaminated food or products, through the inhalation of particles present in the air, and through absorption of the substance by the skin (74).

In particular, the essential use of MCZ in a wide variety of agricultural products for daily consumption is directly related to the passive exposure of the general population to this agrochemical and its metabolites (75). Studies in animals related to the pharmacokinetics and metabolism of MCZ and other EBDCs have shown a rapid absorption of the orally administered dose and it is estimated that most of the dose is excreted before 24 hours through feces and urine (76).

According to the latest renewal of the approval of MCZ and ETU within the framework of the EU, under Regulation (EC) No 1107/2009, the values of acute reference dose (ARfD) and acceptable daily intake (ADI), which are derived only from oral exposure, have been re-evaluated and lowered compared to the last report (77). Currently, the ARfD and ADI values are established at 0.15 mg/kg body weight (bw) and 0.023 mg/kg bw per day, respectively for MCZ, and for ETU these values correspond to 0.01 mg/kg bw for ARfD and 0.002 mg/kg bw per day for ADI (77). These values are considered low compared to other agrochemicals, which implies a greater risk for consumers of agricultural products sprayed with MCZ (78). Indeed, several studies have reported the presence of MCZ and/or ETU in fruits and vegetables even after they have been washed, canned, frozen, or processed for juice production (32). In foods such as tomatoes, carrots, spinach and apples, residues of MCZ and ETU were found in a range of 0.1 - 61.9 mg/kg and 0.01 - 0.71 mg/kg, respectively (32). Depending on the volume

and frequency of consumption, these values could exceed the minimum values of ADI. However, according to recent EFSA data, chronic exposure to MCZ through diet barely reaches 19% of the ADI (79).

On the other hand, there is a part of the population who is actively exposed to MCZ. This group mainly includes applicators of MCZ to crops, harvesters, people directly involved in the manufacture of MCZ, and people living around crop fields. For this group of people, exposure to MCZ through respiratory and dermal routes are the most relevant (76). According to the EFSA, MCZ is a substance that has low acute toxicity by inhalation and dermal routes due to its low established LC_{50} values in animals. These values correspond to > 2000 mg/kg bw for inhalation exposure and > 5 mg/L (4 hours) for dermal exposure (75).

One of the most comparable indicators of exposure via inhalation is the no-observed adverse effect level (NOAEL). This value for MCZ is 79 mg/m^3 (76); however, these calculations are still based on data obtained from animal experiments. In addition, until this document was written, no studies were found that evaluated and compared these available values with the actual exposure by the inhalation route of MCZ in people occupationally exposed to this agrochemical.

On the other hand, in the case of dermal exposure, the acceptable operator exposure level (AOEL) is available, which for MCZ has a value of 0.0025 mg/kg bw per day (80). A study in vineyards workers, who were responsible for MCZ application, revealed an estimated absorbed dose per worker via the skin of 0.0012–0.168 mg per day (8 hours of work). Considering the average weight of a person as 70 kg, the upper limit of these estimates is comparable to the MCZ AOEL (0.0025 mg x 70 kg = 0.175 mg per day) (80). These estimates were based on the assumption that only 1.5% of MCZ deposited on the skin of workers is absorbed (80).

In general terms, these data suggest that despite the widespread use of MCZ and the general population's exposure to this fungicide, this substance, if used correctly, would not constitute a risk factor for human health. However, a large body of evidence suggests that MCZ might affect human health. This will be reviewed in the next section.

2.5. Non-neuronal related mancozeb toxicity

In recent years, the use of MCZ has been questioned due to the possible adverse effects of this fungicide and its primary metabolite ETU on human health. Indeed, being an agrochemical of primary use worldwide, its safety has been extensively investigated. Currently, the evidence collected through studies in different cell lines, primary cultures, and animals, as well as epidemiological and occupational studies, has allowed to generate an extensive list of negative outcomes MCZ might have on human health.

The endocrine system is probably the most significant target of MCZ and ETU. The thyroid gland, in particular, is one of the principal tissue in which MCZ accumulation has been observed after exposure (16,81). Indeed, several animal studies have shown the disruption of various thyroid-related processes due to MCZ exposure (16). For example, disturbance in the secretion and/or biosynthesis pathways of thyroid hormones has been reported in several studies (16). Furthermore, a T3 and T4 concentration reduction and an increase in TSH concentration are usually observed following MCZ exposure (82,83). In addition, other effects related to other parts of the endocrine system have also been described. These include decreased testosterone levels (due to alterations in the morphology of the testicles) and structural and functional disruptions related to the ovaries and female fertility (16).

In spite of these and other evidence, Skalny and colleagues reviewed the hypothesis that the endocrine disruption produced by MCZ and ETU could be an indirect effect of the neurotoxicity of this fungicide affecting the hypothalamus (16). This idea is proposed due to the pivotal role of this structure on the hypothalamus-pituitary-glands axis, which is the center of the endocrine system function (84).

On the other hand, the reproductive system is another of the main targets of the MCZ (17). In an in vitro study carried out in mouse granulosa cells, ultrastructural effects produced at the level of the nuclear membrane and genetic material by exposure to MCZ were observed (85). The granulosa cells are associated with the development of the oocytes, which is why their condition may have implications in the generation of the ovule. Indeed, a dose-dependent degenerative effect of MCZ was observed on buffalo oocytes (cells that give rise to the ovule) during in vitro maturation (86).

In addition, in vivo studies have shown a decrease in oocyte quality and development capacity related to MCZ exposure (87). Other effects, such as the quantity and quality of follicles and the weight of the ovaries, have been attributed to the toxic effects of MCZ (87,88).

Although endocrine and reproductive disruption are the best-known detrimental effects of MCZ on human health, immunomodulatory effects that could trigger immune diseases or health disruption have also been attributed to this agrochemical (89). Finally, MCZ has been observed to be toxic at the renal and hepatic levels, as well as to a potent carcinogenic agent (14).

2.6. Neuronal related mancozeb toxicity

Based on the growing epidemiological evidence that links exposure to pesticides with the incidence of PD, neurodevelopmental problems and cognitive problems, the neurotoxicity of MCZ has become relevant as a research topic in the last decade (20,90,91). Consequently, the neurotoxicity of MCZ has been assessed in various primary cultures, *in vitro* and animal models.

In mesencephalic cells extracted from rat embryos, it was observed that MCZ (10 - 120 µM) affected cell viability in a dose-dependent manner selectively to DAergic and GABAergic mesencephalic populations (38). This study also showed that at non-toxic concentrations of MCZ, cells exhibited decreased ATP levels, increased ROS concentration, mild mitochondrial uncoupling, and inhibition of NADH-linked stage 3 respiration (38,92).

Similar results regarding increased ROS levels and toxicity were observed in hippocampal astrocytic cells obtained from newly born male pups from Sprague-Dawley rats (93). However, a lower [MCZ] range (5 - 20 uM) was used in this study. In addition, the antioxidant butylated hydroxytoluene (BHT) was shown to have a protective effect against MCZ toxicity in astrocytic cells (93).

Analogously, the neurotoxicity of MCZ has also been evaluated in models such as *Caenorhabditis elegans* (*C. elegans*). This model is highly used in neurotoxicology due to its well-characterized nervous system and its structural and functional resemblance to

the human nervous system (94). Brody and colleagues found that 24 hours of exposure of nematodes to MCZ in a concentration range of 0 - 1.5% (v/v) has the outcome of selective DAergic neuron damage, preceded by behavioral damage (95). In agreement with that, a study published after the one mentioned above reported similar results regarding the selective toxicity of MCZ, this time not only to DAergic neurons but also to GABAergic neurons (96).

Another reliable model in toxicology studies is *Drosophila melanogaster* (*d. melanogaster*). Toxicological assessment of MCZ in this model resulted in mitochondria impairment evidenced by decreased oxygen consumption and bioenergetic rate, as well as inhibition of complex I and II of the electron transport chain (97). This is why it is suggested that the mechanism of toxicity executed by MCZ in this model is the decrease in ATP, which is essential for many cellular processes.

Cyprinus carpio (carp) and *Danio rerio* (zebrafish) were also used as models for the evaluation of MCZ neurotoxicity. Indeed, exposure of zebrafish embryos to doses of 5 - 20 µg/L of MCZ negatively affects spontaneous movements, escape response and behavior, and swimming ability (98). Furthermore, Costa-Silva and colleagues used similar doses in carp specimens. They observed elevated ROS and antioxidant enzyme levels and decreased levels of CAT and SOD enzymes (99).

These results suggest that the toxicity of MCZ may be due to mitochondrial dysfunction, which in turn is related to elevated levels of ROS that together with selective cell degeneration, are part of the hallmarks of some neurodegenerative diseases such as PD (100).

Other outcomes after exposure to MCZ have been evidenced through *in vitro* and *in vivo* experimentation. In primary cell cultures from mice cerebellar cortex, MCZ produced synaptic disruption, neuroinflammation, and increased excitatory neurotransmitter release (101). These results were comparable to those reported by Morales-Ovalles and colleagues. They subchronically exposed mice to MCZ doses of 30 and 90 mg/kg via intraperitoneal injection 3 times per week for 6 weeks (102). The most relevant result of this study was the increase in the excitatory/inhibitory ratio in the hypothalamus of mice exposed to MCZ. However, similar to the previously described study, the researchers also found associated with MCZ exposure, signs of

neuroinflammation, as well as elevated levels of apoptosis and demyelination in hypothalamic secretory cells (102).

2.6.1. The metallic part of mancozeb: Mn, is involved in its neurotoxicity

Mn is a ubiquitous, essential metal in maintaining the adequate physiology of living organisms. Generally, humans and animals acquire the minimum requirements for Mn through diet. However, despite its intrinsic presence in the body, Mn homeostasis must be maintained within biological systems. This is because the overdose or insufficiency of this metal could trigger detrimental effects in an organism, especially those related to neurotoxicity (103).

Interestingly, when there is an overdose of Mn concentration in the brain, this metal is stored in specific areas, which include the basal ganglia, SN, striatum and palladium (103). The increased Mn concentration in the brain could cause motor symptoms similar to PD, cognitive impairment, learning deficit, decreased neurite length and neurodegeneration (103). Also, these alterations of Mn homeostasis in the brain usually result from overexposure to this metal from various sources such as diet, contaminated water, the environment, or occupational exposure (103). In fact, the use of fungicides that contain Mn, as is the case of MCZ, constitutes a risk factor for overexposure to Mn (99).

In vitro studies in astrocytic cells have shown that after exposure to MCZ, the intracellular concentration of Mn increases (93). Indeed, Mn but not Zn (both metal components of MCZ) is essential for the potentiating effect that MCZ has on $KCNQ_2$ potassium channels. Because of the central role of $KCNQ_2$ potassium channels in neuronal excitability, its MCZ-mediated potentiation activity has been postulated as one of the mechanisms of MCZ toxicity in the nervous system (104).

In addition, it has been observed that Mn and the EBDCs moiety, but not Zn, are required components for ROS generation stimulated by MCZ exposure of primary cultures of mesencephalic cells (92). These results are in accordance with the implication of Mn in MCZ-mediated neurotoxicity.

In retinoic acid SH-SY5Y differentiated cells, exposure to Mn (II) chloride or Mn (II) citrate at 876 µM and 945 µM, respectively, reduced cell viability to 50% (105). The

authors state that the probable cause of its toxic effect on cells is the disruption of protein metabolism. The mechanism of Mn (II) chloride specifically affects the metabolism of amino acids, which leads to problems in protein synthesis (105). On the other hand, Mn (II) citrate inhibits the E3 ubiquitin ligases-target protein degradation pathway, which would trigger the increase of damaged and unfolded proteins (105). All this together could affect cell homeostasis, leading to cell death. Interestingly, 84% of the pathways involved in Mn (II) cytotoxicity in SH-SY5Y cells are related to neurodegenerative diseases (105).

2.7. SH-SY5Y neuroblastoma cell line

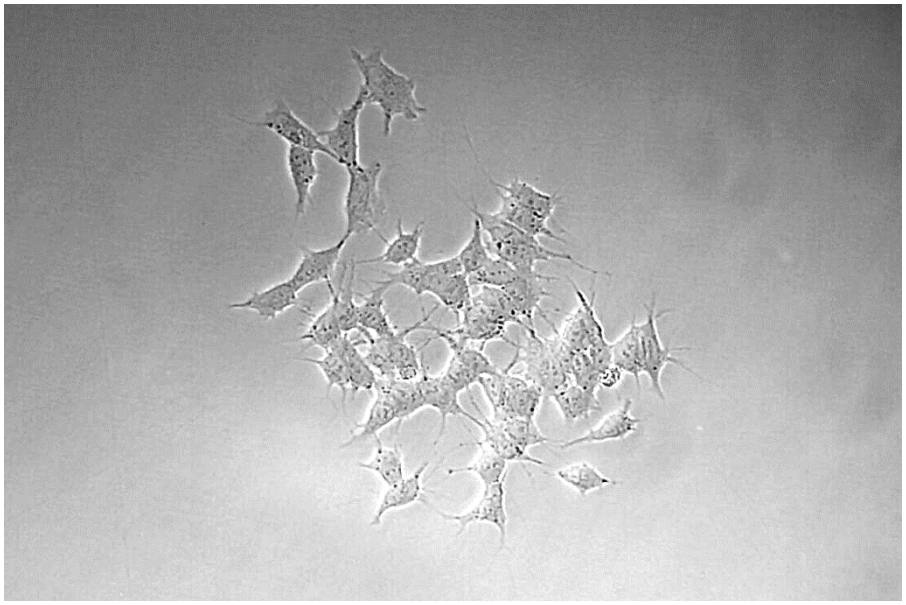

Figure 19: Undifferentiated SH-SY5Y cells observed at 20X magnification trough inverted microscopy.

SK-N-SH cells were isolated in 1970 from a metastatic bone marrow tumor in a 4-year-old girl with neuroblastoma. Three successive clones of this cell line gave rise to a new neuroblastoma cell line established as SH-SY5Y (106).

SH-SY5Y are adherent cells which, in their undifferentiated state, most appear as neuroblast-like cells with a characteristic polygonal-shaped cell body and truncated neuritic processes (Figure 6). However, in cultures of these cells, it is common to observe a small percentage of floating cells and epithelial-like cells, both of which are viable cells (107,108).

In addition, unless these cells go through a cell differentiation process, SH-SY5Y cells do not have the biochemical, functional or morphological characteristics of a fully mature neuron. However, undifferentiated SH-SY5Y are positive for tyrosine hydroxylase (TH) and dopamine-β-hydroxylase characteristic of catecholaminergic neurons. Furthermore, undifferentiated SH-SY5Y cells may provide a good model to evaluate the effects of a large number of neurotoxins, such as pesticides, in immature nervous systems (107).

Currently, SH-SY5Y is easily found as a commercial standardized human cell line. This model of cells is widely used in the field of neuroscientific research due to several of its advantages. These include no ethical concerns and rapid proliferation. In addition, to being human cells, they express specific human proteins that are not present in primary cultures (108).

3. METHODOLOGY

3.1. Chemicals

A commercial formulation of Mancozeb 80% wettable powder (WP) was used to prepare the treatments in this study. The composition of this product consists of 800g/kg of MCZ and excipients sufficient for 1kg. This fungicide was purchased at a local store of agricultural products in the city of Ibarra - Ecuador.

3.2. Culture medium

Dulbecco's Modification of Eagle's Medium (DMEM) was used as a cell culture medium. Due to availability, Gibco™ DMEM/F-12 + GlutaMAX™ was used for morphology and trypan blue experiments, and DMEM/F-12 Ham Sigma-Aldrich was used for MTT and oxidative stress experiments. No difference in cell growth was observed between these two brands of DMEM. As a supplement to the culture medium, Fetal Bovine Serum (FBS) and penicillin/streptomycin were also needed.

For all experiments, the culture medium was prepared on the basis of DMEM supplemented with 10% (v/v) of FBS and 1% (v/v) penicillin/streptomycin. This mixture was filtered on Thermo Scientific™ disposable filter units with PES membranes with a pore size of 0.1 µm. The culture medium was stored at 8 ºC. For use, the medium was heated to 25 °C.

3.3. SH-SY5Y cell line culture

An aliquot of SH-SY5Y human neuroblastoma cells, that was preserved in liquid nitrogen (-196 ºC), was kindly provided by Dr. Javier Sáez de Castresana from Universidad de Navarra (Spain). For cell recovering from the freezing process, 3 passages were performed before running the experiments. These cells were were grown in t75 Nunc™ EasYFlask™ Cell Culture Flasks containing 15 mL of culture medium. The flasks were incubated at 37.0 °C and 5% CO_2. In order to maintain the cell culture, the medium was refreshed every 2-3 days. Each time cell reached 80 - 90% of confluence, a

passage would be performed. To be harvested, cells were first washed with PBS, prepared as indicated in Table 4, and then incubated for 5 minutes at 37 ºC with 2.5 mL of Gibco™ Cell Dissociation Buffer Enzyme-Free PBS-based to detach the cells from the flask surface. Finally, the cells suspended in the dissociation buffer were neutralized with 2.5 mL of culture medium. Then the entire content was collected in a 15 mL falcon tube, centrifuged at 5,000 rpm for 5 minutes, and re-suspended in 2-3 mL of culture medium. Finally, cells were counted in a Neubauer chamber, and diluted in culture medium according to the needs of the experiments.

Component	Concentration (mM)
NaCl	138
KCl	3
Na_2HPO_4	8.1
KH_2PO_4	8.5
Distilled water	--

Table 4: Salts composition and proportions for PBS preparation.

3.4. Treatments preparation

In order to reach the required concentration of MCZ for each treatment, the calculations were based on the molecular weight of MCZ in its monomeric form (541.1 gr/mol). Because the concentrations of the treatments corresponded to that of the active substance MCZ, not that of the formulation, [MCZ] in the commercial product was considered. Furthermore, each concentration of MCZ was prepared just before treating the cells.

For the treatments, a stock solution was prepared by mixing commercial product in distilled and autoclaved water. Then, the dilutions were made in the culture medium according to the concentrations required for the experiments. The concentrations tested in the experiments performed in this study covered a range of 0.5 - 80 µM. These concentrations were defined based on pilot experiments performed prior to those reported in this work.

3.5. Cell morphology characterization

Cells were seeded in 24-well plates (4 wells per concentration) at a density of 5 x 10^3 cells/well with 400 µL of culture medium. Each concentration was tested on a different plate to avoid cross-contamination, as observed in previous experiments. After 48 hours, culture medium was removed from each well and a fresh culture medium contained the treatments of MCZ at 0, 0.5, 1, 2.5, 5 and 10 µM were applied at each well containing cells. For the analysis, 3 random pictures of each well were obtained through the Motic inverted microscope 24, 72 and 120 hours after the treatments were applied. These images were processed through the free software ImageJ. The cells were counted, and neurites were traced through the NeuronJ plugin.

3.6. Trypan blue exclusion test

The trypan blue exclusion test is used to determine the percentage of viable cells in a cell population. This method consists of blue staining of dead cells due to the penetration of trypan blue dye into the cells due to damage to their plasma membrane. Therefore, those stained cells correspond to non-viable cells (109).

In order to perform this experiment, cells at a density of 5 x 10^3 cell/well were seeded in 24-well plates with 400 µL of culture medium. To avoid interference between each treatment, one plate was used for each concentration. After 48 hours, the medium from each well was removed, and MCZ treatments at 0, 0.5, 1, 2.5, 5 and 10 µM were immediately applied to each well containing cells. Cell viability was analyzed at 24, 72 and 120 hours after treatments. To stain dead cells, 400 µL of 0.4% trypan blue was added to each well and incubated for 10 minutes. The content of each well was removed. Finally, blue cells and clear cells were counted in the images obtained through the Motic inverted microscope.

3.7. MTT assay

The MTT is an assay that evaluates cell viability through a purple color profile. This color is given by the reduction of yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-

2,5- diphenyltetrazolium bromide (MTT) to formazan crystals that, when dissolved in DMSO, will result in a purple colored solution. The intensity of this color, which is the measurable indicator of cell viability, will be given by the amount of functional NAD(P) H-dependent oxidoreductase enzymes in the culture. These enzymes will be provided by the viable cells and are responsible for reducing MTT to formazan. For this reason, the greater the cell viability, the greater the amount of NAD(P)H- dependent oxidoreductase enzymes available. Consequently, a more significant amount of MTT molecules will be transformed into formazan, and therefore a higher color intensity will be obtained (110).

For this experiment, cells were seeded at a density of 6 x 10^4 cells/well in 96-well microplates with 200 µL of culture medium. As in the previous experiments, treatments were tested on separate plates for each concentration. After 48 hours of incubation at 37 ºC, the cells having reached 90-100% confluence in each well, MCZ treatments were applied at concentrations of 0, 5, 10, 20, 40 and 80 µM. After either 24, 72 or 120 hours, treatments were removed, and each well was washed with PBS in order to eliminate MCZ residues since the color of MCZ could interfere with the absorbance measurement. The cells were then incubated for 2 hours at 37 ºC with 100 µL of medium containing 10% (v/v) of MTT from Roche's Cell Proliferation Kit I (MTT). The MTT was handled with the biosafety cabinet light off to ensure the reagent's integrity. Next, the content of each well was removed, and 100 µL of DMSO was added to each well to dissolve the purple formazan crystals. Posteriorly, absorbance of the dilution was measured in the Rayto RT-2100C Microplate Reader spectrophotometer at a wavelength of 630 nm.

3.8. Oxidative stress detection

To detect the oxidative stress produced by the MCZ treatments, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was used. This is a green fluorescent probe which label ROS, predominantly H_2O_2, in live cells samples. The fluorescence emitted by the DCF-tagged ROS must be observed through using the green channel on a fluorescence microscope (111).

For this experiment, two sterile coverslips were placed in the middle of a petri dish (one petri dish for each [MCZ]). Cells were seeded in the Petri dish at a density of 3 x 10^6 cells/ Petri dish with 4 mL of medium. After 24 hours of incubation, cells were attached

to the coverslips, the medium was removed from Petri dishes, and MCZ treatments were applied at concentrations of 5, 10, 20, 30 and 40 µM. After 24 hours, the medium containing treatments was removed, and the Petri dishes were washed with PBS. Subsequently, it was incubated for 30 minutes at 37 ºC with culture medium containing 10 Mm of DCF at 0.1% (v/v) (dissolved in DMSO). Finally, the culture medium containing DCF was removed from the petri dishes and the coverslips containing the cells were treated with 10% (v/v) formaldehyde in PBS and incubated for 10 minutes on ice. The remains of formaldehyde were washed with PBS, and the coverslips were carefully removed from the Petri dishes and left to dry inside the biosafety cabinet. Once the coverslips were dry, they were placed on the slides with a drop of Entellan™. This protocol was performed by keeping the lights of the biosafety cabinet off to preserve the integrity of the DCF. To obtain the images, the green channel (band pass filter of 546/14 nm) of a Leica fluorescence microscope was used.

Images obtained from this experiment were processed through ImagenJ free software to obtain the relative fluorescence per cell area.

3.9. Data analysis

The data obtained from the count of neurites per cell (n), the length of neurites per cell (µm), and the count of dead cells (%) from the trypan blue exclusion test, were analyzed through the one-way analysis of variance test (one-way ANOVA) and Dunnett's test was performed as a post-hoc test. These analyzes were run in the free software Rstudio.

To analyze the absorbance data obtained from the MTT assay, it was necessary to get the real absorbance values by subtracting the absorbance value of the blank (DMSO) from those provided by the spectrophotometer. Subsequently, the real absorbance values were calculated in percentages respect to the real absorbance of the control. With these data, through Origin software (Learning Edition), a scatter graph was plotted from which a dose-response curve was adjusted by the Logistic Sigmoidal Type 1 model of the origin software represented by the formula:

$$y = \frac{\alpha}{1 + e^{-k(x - x_c)}}$$

where $\boldsymbol{\alpha}$ represents the amplitude of the function, $\boldsymbol{x_c}$ the center of the function and $\boldsymbol{k}$ represents the coefficient which acts as a scale parameter on $\boldsymbol{x}$, influencing the growth rate. This model was applied to the results obtained at 24, 72 and 120 hours of exposure to the treatments based on the parameters values indicated in Table 5.

Finally, the data obtained from the ROS detection experiments were treated similarly to that of the MTT. The relative intensity was plotted on a scatter graph with respect to the applied doses of MCZ, and based on this, a 4th order polynomial response curve was fitted, represented by

$$y = A_0 + A_1 x + A_2 x^2 + A_3 x^3 + A_4 x^4$$

This curve was constructed based on the parameters contained in Table 6 in the Origin (Learning Edition) software.

Parameters	**24 hours**	**72 hours**	**120 hours**
	Value of parameter ± SD		
$\boldsymbol{\alpha}$	117.6715 ± 28.2844	237.3859 ± 181.8322	129.1702 ± 21.0346
$\boldsymbol{x_c}$	22.2824 ± 10.8616	-3.0054 ± 11.6062	7.9906 ± 1.9784
$\boldsymbol{k}$	-0.0515 ± 0.0138	-0.1152 ± 0.0306	-0.1972 ± 0.0421

Table 5: Summary table of parameter values for modeling of dose-response curve at 24, 72 y 120 hours based on cell viability (data obtained by MTT assay).

Parameter	**Values ± SD**
$\mathbf{A_0}$	0.1330 ± 0.0994
$\mathbf{A_1}$	-0.0233 ± 0.0435
$\mathbf{A_2}$	0.0109 ± 0.0053
$\mathbf{A_3}$	-5.1105E-4 ± 2.1785E-4
$\mathbf{A_4}$	6.4471E-6 ± 2.7777E-6

Table 6: Summary table of parameter values for modeling of dose-response curve based on relative intensity (data obtained by ROS detection experiments).

4. RESULTS

4.1. Mancozeb affects the morphology of SH-SY5Y cells

The morphology of SH-SY5Y cells was evaluated after being treated with different [MCZ] (0.5, 1, 2.5, 5 and 10 µM). At first sight, an acute effect (24 hours) on the body shape of treated cells was noticeable. The characteristic polygonal-shaped body of SH-SY5Y cells was lost as [MCZ] increased. As can be seen in Figure 8 C-E, the surviving cells gradually acquired a more circular shape. This morphological change may be related to the decrease in the number and length of neurites per cell in a dose-dependent manner (Figure 7). The variance analysis of these results showed a statistically significant (p-value $<2 \times 10^{-16}$) difference between MCZ different concentrations for both number and length of neurite per cell (Table 7 and Table 9). Interestingly, despite the significant loss of neurites, most cells remained attached to the flask surface, even those fully circular. In addition, it should be noted that cells treated at concentrations of 2.5, 5 and 10 µM did not show a significant difference between them in the number and length of neurites per cell due to the floor effect observed in these measures.

Chronic exposure (72 and 120 hours) of SH-SY5Y cells to MCZ showed similar results concerning the morphological characteristics evaluated in this study. However, in most of the [MCZ] no significant difference was observed between acute and chronic MCZ exposure regarding the number and length of neurites per cell (Figure 9, Table 8 and Table 10).

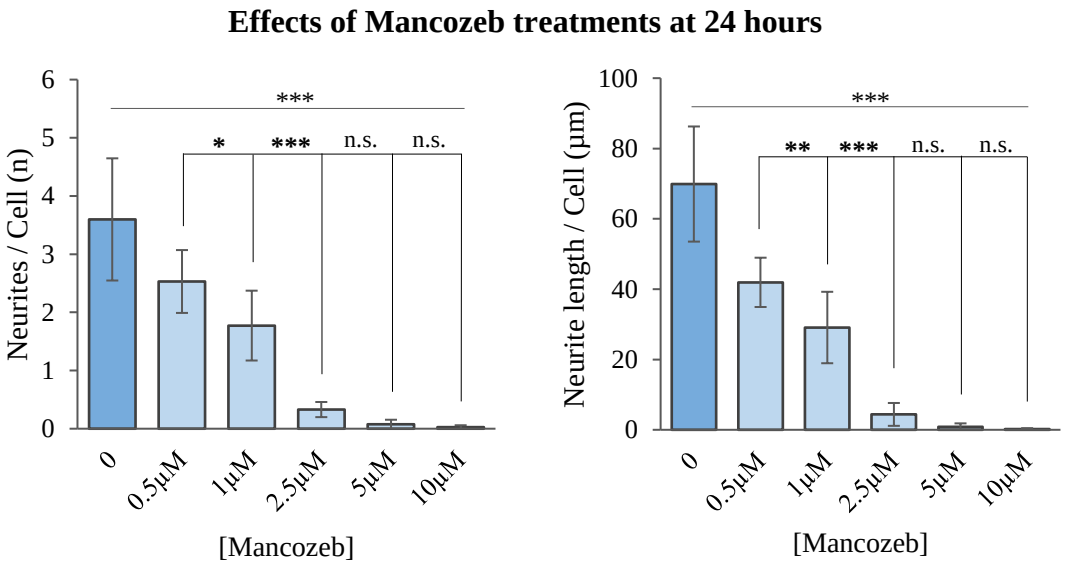

Figure 25: Effect of MCZ at different concentration over the number and length of neurites per cell.

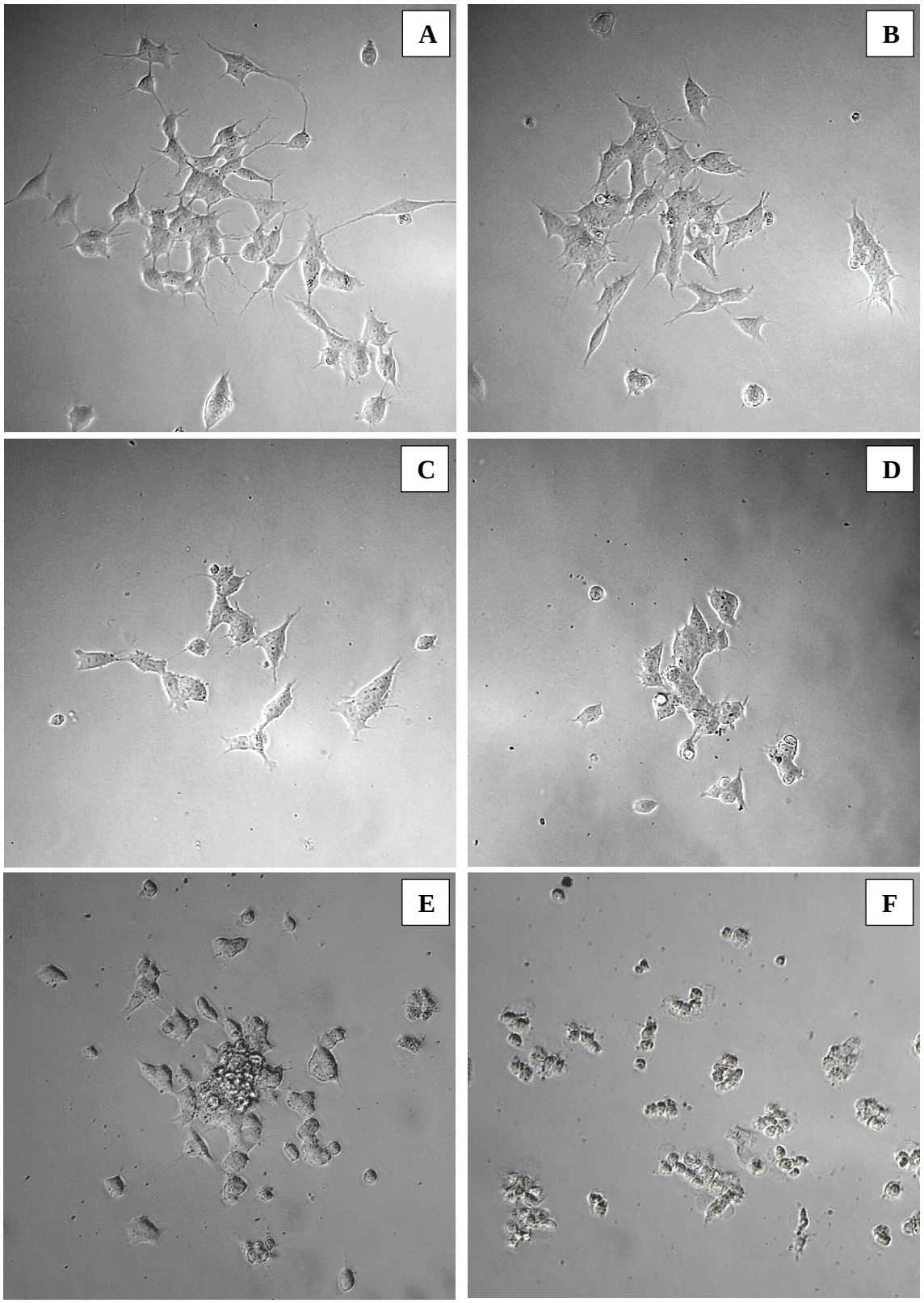

Figure 26: Images obtained from inverted microscopy at 20X magnification of SH-SY5Y cells exposed at 0 (A), 0.5 (B), 1 (C), 2.5 (D), 5 (E) and 10 (F) µM of MCZ.

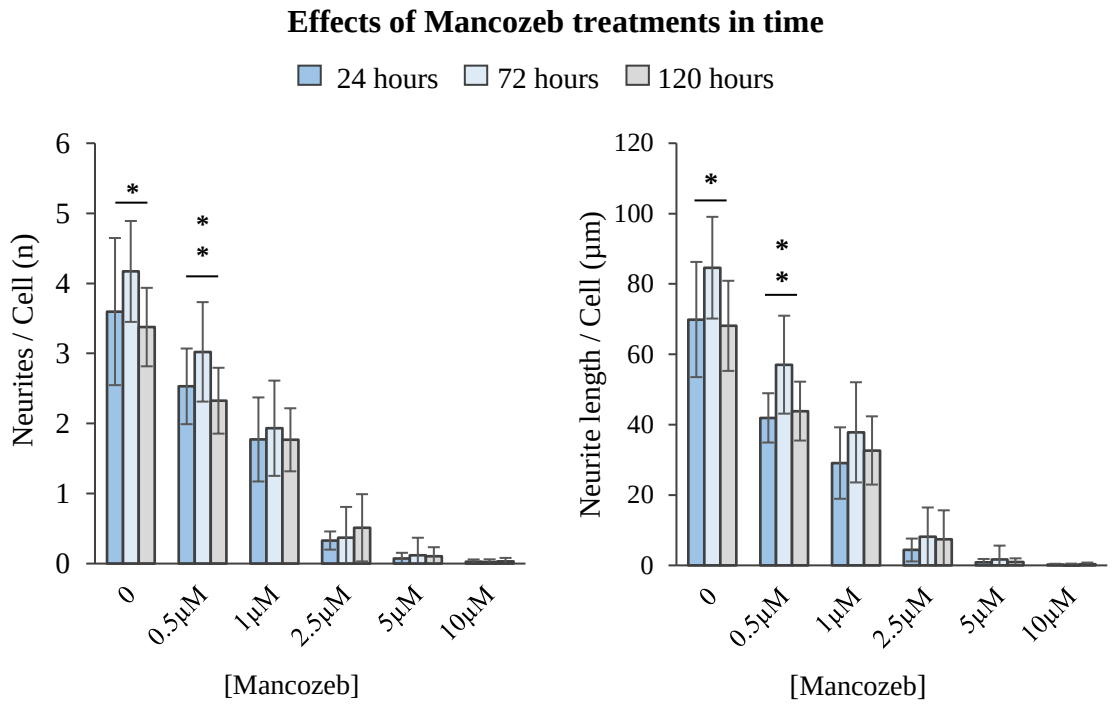

Figure 32: Effects of 0 - 10 µM of MCZ doses on the number and length of neurites per cell

Time of treatment	df	Sum Sq	Mean Sq	F value	P-value	Significance
24 Hours	5	121.26	24.25	81.91	<2E-16	***
72 Hours	5	172.00	34.41	119.40	<2E-16	***
120 Hours	5	101.46	20.29	123.50	<2E-16	***

Table 7: Variance analysis of results obtained from the number of neurites per cell between [MCZ] at 24, 72 y 120 hours of treatment.

[Mancozeb]	df	Sum Sq	Mean Sq	F value	P-value	Significance
0	2	5.003	2.501	3.892	0.0314	*
0.5µM	2	3.912	1.956	5.813	0.00735	**
1µM	2	0.113	0.056	0.166	0.848	n.s.
2.5µM	2	0.196	0.098	0.664	0.522	n.s.
5µM	2	0.011	0.006	0.198	0.821	n.s.
10µM	2	0.001	0.000	0.187	0.831	n.s.

Table 8: Variance analysis of results obtained from the number of neurites per cell between 24, 72 and 120 hours of exposure to MCZ at different [MCZ].

Time of treatment	df	Sum Sq	Mean Sq	F value	P-value	Significance
24 Hours	5	43323.0	8665.0	120.40	<2E-16	***
72 Hours	5	65249.0	13050.0	113.40	<2E-16	***
120 Hours	5	41419.0	8284.0	125.20	<2E-16	***

Table 9: Variance analysis of results obtained from the length of neurites per cell between [MCZ] at 24, 72 y 120 hours of treatment.

[Mancozeb]	df	Sum Sq	Mean Sq	F value	P-value	Significance
0	2	1808.000	904.100	4.229	0.0241	*
0.5µM	2	1491.000	745.600	7.145	0.0029	**
1µM	2	424.000	212.000	1.590	0.221	n.s.
2.5µM	2	89.100	44.540	0.911	0.413	n.s.
5µM	2	4.150	2.074	0.348	0.709	n.s.
10µM	2	0.102	0.051	0.383	0.685	n.s.

Table 10: Variance analysis of results obtained from the length of neurites per cell between 24, 72 and 120 hours of exposure to MCZ at different [MCZ].

4.2. Mancozeb induce elevated ROS levels in SH-SY5Y cells.

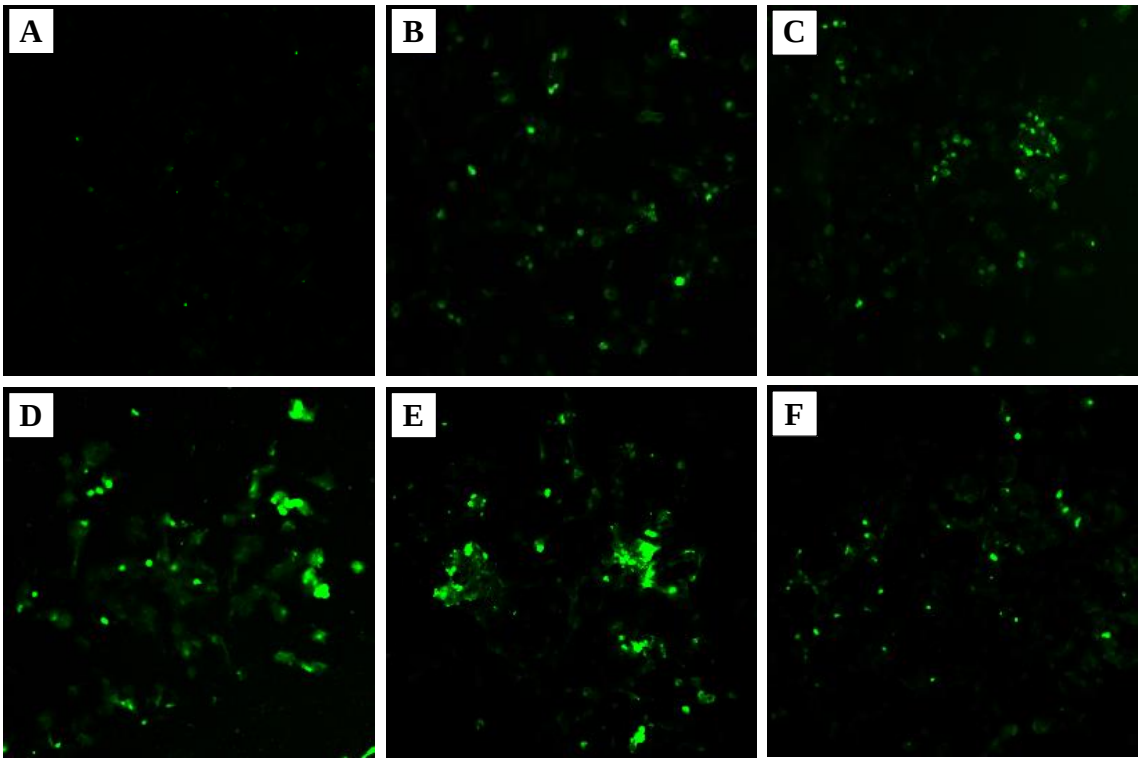

Figure 33: MCZ exposed DCF-SH-SY5Y labeled cells at 0 (control) (A), 5 (B), 10 (C), 20 (D), 30 (E) y 40 µM (F). The fluorescent images were obtained using the green channel of a fluorescence microscope at 20X magnification.

SH-SY5Y were exposed for 24 hours to different doses of MCZ (5, 10, 20, 30 and 40 µM), which have been shown to induce high levels of ROS in primary cultures of mesencephalic cells. As shown in Figure 10, the increased fluorescence emitted by labelled DCF-ROS is evident in cells exposed to MCZ compared to control. In addition, Figure 11 shows the fitted 4th-grade polynomial curve as the dose-dependent behavioral ROS model. These results suggest that ROS levels (represented by the relative intensity) modulated by MCZ in SH-SY5Y cells have biphasic behavior as the dose of this substance increases. First, an increase in ROS is observed as dose-dependent until it reaches a maximum point in ROS level observed in cells exposed at MCZ at 20 µM. The second phase is the decrease in ROS levels, which follows an inverse relationship with the dose of MCZ. Altogether, these results showed the increment of ROS levels influenced by MCZ in SH-SY5Y cells that moderately follow a biphasic model given by a 4th-grade polynomial curve in the [MCZ] range of 5 - 40 µM.

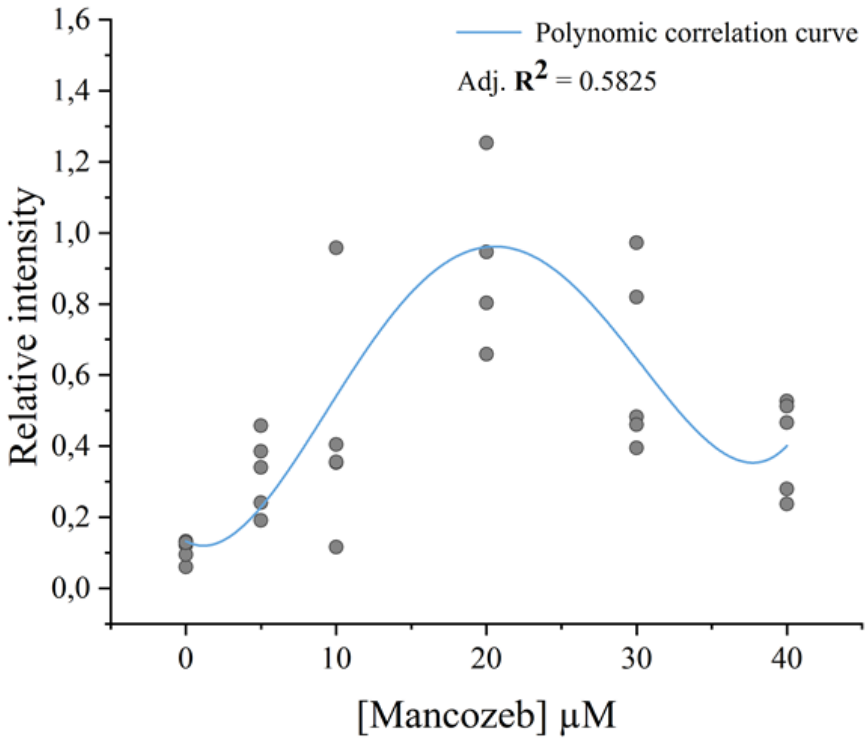

Figure 38: Relative intensity of fluorescence measure in a [MCZ] function represented in a scatter plot with an adjusted 4th grade polynomial response curve.

4.3. Mancozeb affects the viability of SH-SY5Y cells

SH-SY5Y cells were exposed to a wide range of [MCZ] ranging from 0.5 µM to 80 µM. The lethality of MCZ in the cells that received the lowest [MCZ] (0.5, 1, 2.5, 5 and 10 µM) was evaluated through the dye (trypan blue) exclusion test, while the effect over cell viability of higher [MCZ] (5, 10, 20, 40 and 80 µM) was tested through the MTT assay.

The results obtained from the trypan blue test are shown in Figure 12, where the percentage of dead cells (inverse relationship to cell viability) is represented at 24, 72 and 120 hours of exposure to MCZ. In all cases, a progressive increase in the percentage of dead cells was observed. However, despite the significant difference between the means for each case (p-value $<2^{-16}$) (Table 11), the results obtained at 24 and 72 hours showed no significant difference between the percentages of cell death in the cultures treated at concentrations of 5 and 10 µM. At 120 hours of treatment, the percentage of cell death was very similar in the cultures treated at concentrations of 2.5, 5 and 10 µM of MCZ. In both cases, the difference between the percentages of dead cells was insignificant because almost the entire population was dead cells. Furthermore, after 24 hours of treatment, no relevant difference was observed between the percentage of dead cells in the cultures treated at 0.5 and 1 µM of MCZ. However, this difference was much more evident in the cultures after 72 and 120 hours of treatment, attributing to MCZ not only acute but also chronic toxic effects.

Furthermore, it is interesting to mention that in the cultures treated with MCZ at 2.5 µM for 24 h, high variability was observed in the percentage of cell death (min: 32.43% and max: 100%) and as shown in Table 13, chronic effects of MCZ are only significant in cells treated at 2.5 y 1 µM. At 72 and 120 hours of exposure, this high variability was observed in the cultures treated at 1 µM of MCZ due to the chronic effect of MCZ on SH-SY5Y. This variability can be interpreted as a critical [MCZ] concentration affecting cell viability.

On the other hand, the MCZ-induced decrease in dose-dependent viability of SH-SY5Y was also observed through the results of the MTT assay. These results have been represented in dose-response curves constructed from the Origin software's Logistic Sigmoidal function type 1. This model was chosen due to its good fit to the results obtained in the MTT assays.

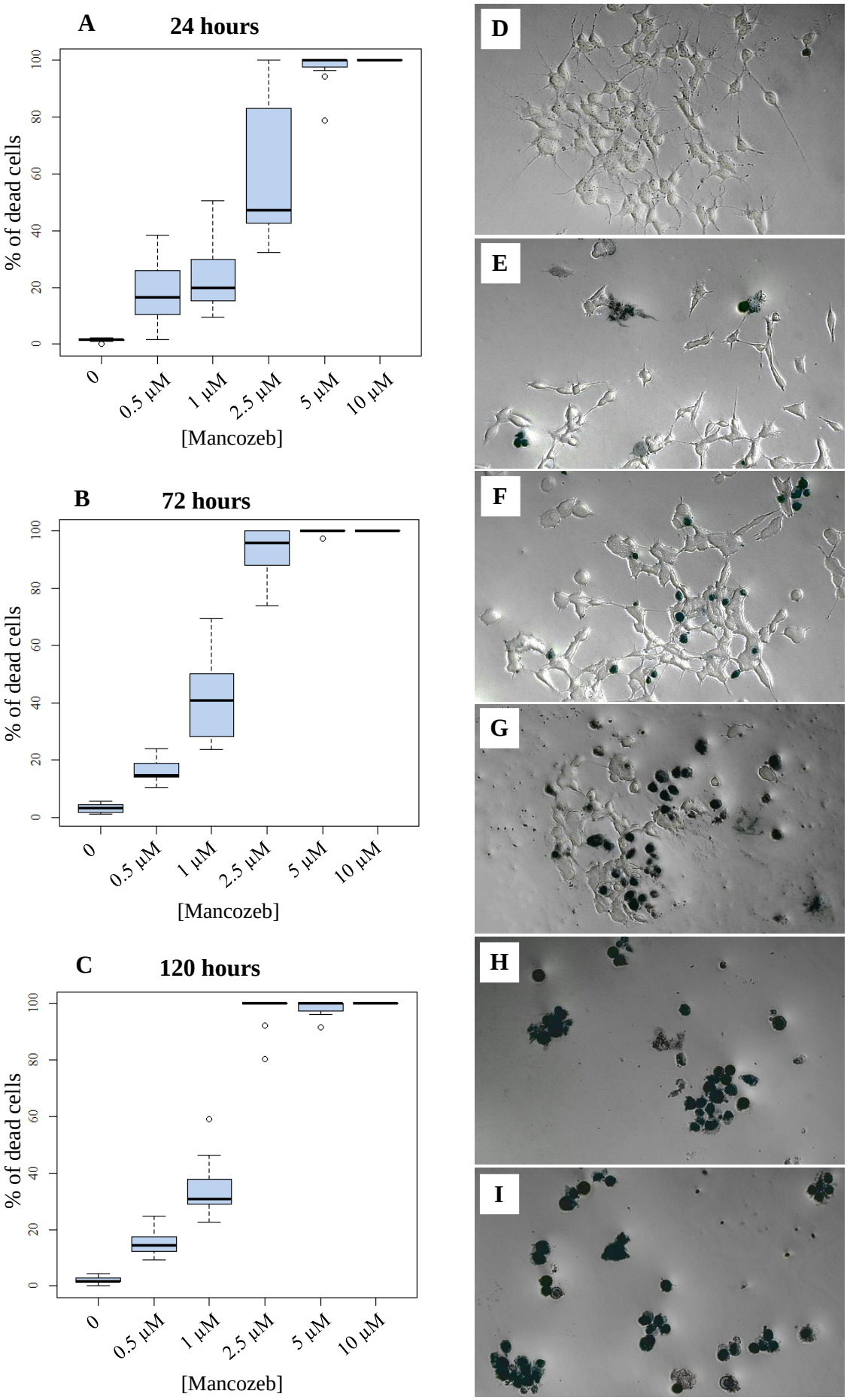

Figure 43: Data representation of toxicity of MCZ, illustrated by the percentage of dead cells at 24 (A), 72(B) and 120 (C) hours of treatments exposure. On the right side are shown images of trypan blue treated SH-SY5Y exposed for 24 hours to MCZ at concentrations of 0 (D), 0.5 (E), 1 (F), 2.5 (G), 5 (H) and 10 (I) µM obtained from inverted microscope at 20X magnification.

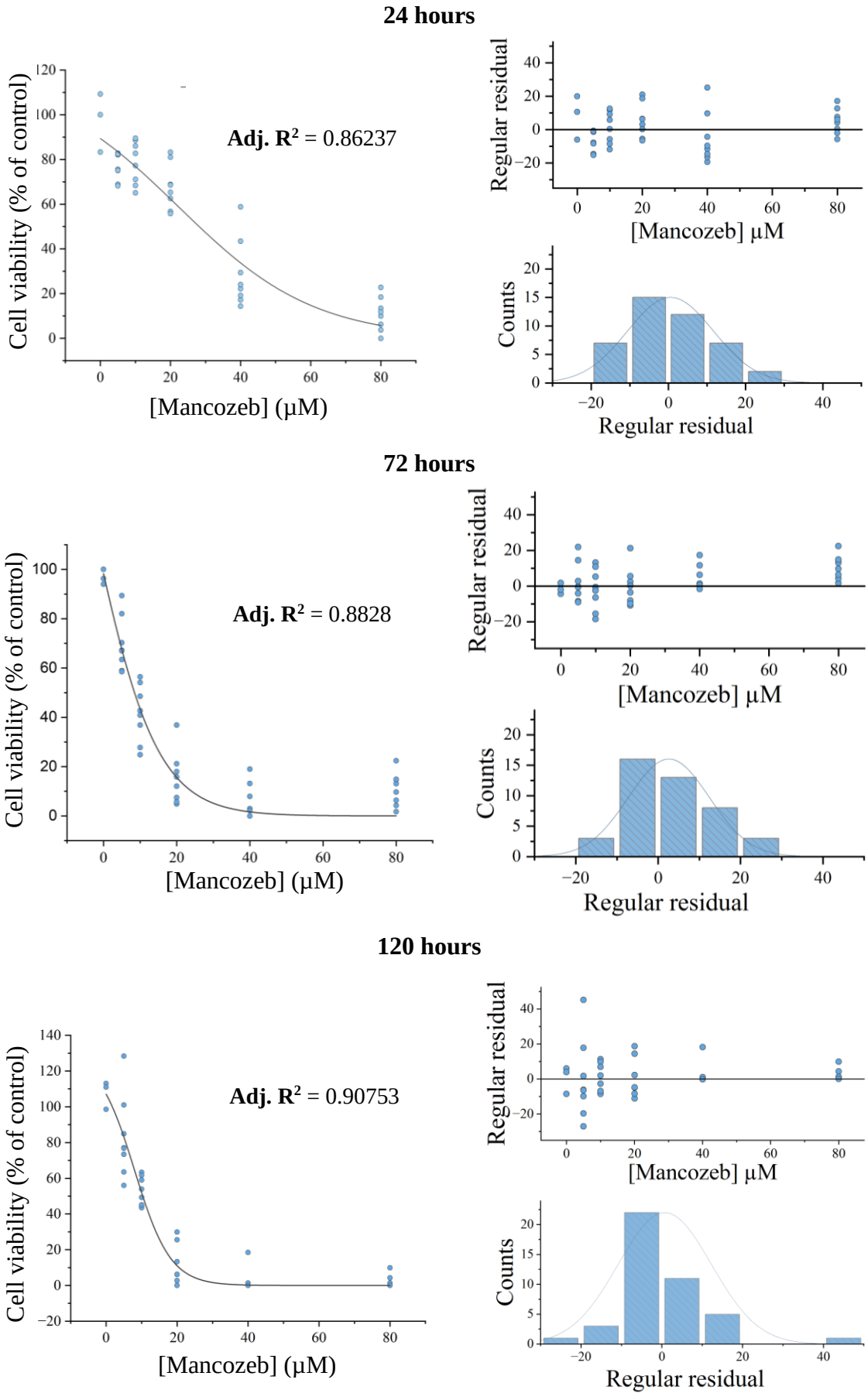

Figure 47: Logistic sigmoidal modeling of cell viability (% of control) respect to MCZ exposure doses at 24, 72 and 120 hours of cells exposure to treatments.

Time of tratment	df	Sum Sq	Mean Sq	F value	P-value	Significance
24 Hours	5	98121.00	19624.00	117.20	<2E-16	***
72 Hours	5	106975.00	21395.00	357.10	<2E-16	***
120 Hours	5	114958.00	22992.00	790.50	<2E-16	***

Table 11: Variance analysis of results obtained from percentage of dead cells between [MCZ] at 24, 72 y 120 hours of treatment.

[Mancozeb]	df	Sum Sq	Mean Sq	F value	P-value	Significance
0	2	14.21	7.105	4.858	0.0149	*
0.5µM	2	70.90	35.450	0.591	0.56	n.s.
1µM	2	1698.00	849.200	5.049	0.0129	*
2.5µM	2	7384.00	3.692.000	13.820	5.58E-05	***
5µM	2	36.60	18.310	1.150	0.33	n.s.
10µM	2	0.00	0.000	1.000	0.38	n.s.

Table 12: Variance analysis of results obtained from percentage of dead cells between 24, 72 and 120 hours of exposure to MCZ at different [MCZ].

Time of treatment	df	Sum Sq	Mean Sq	F value	P-value	Significance
24 Hours	5	37889	7578	53.900	<2E-16	***
72 Hours	5	46133	9227	100.300	<2E-16	***
120 Hours	5	50456	10091	68.520	<2E-16	***

Table 13: Variance analysis of results obtained from MTT cell viability data between [MCZ] at 24, 72 y 120 hours of treatment.

[Mancozeb]	df	Sum Sq	Mean Sq	F value	P-value	Significance
0	2	328	164.100	0.790	0.467	n.s.
5µM	2	225	112.500	0.583	0.567	n.s.
10µM	2	5589	2794.600	31.490	4.78E-07	***
20µM	2	14737	7368.000	56.620	3.47E-09	***
40µM	2	2295	1147.700	11.500	0.000424	***
80µM	2	380	190.400	4.680	0.0208	*

Table 14: Variance analysis of results obtained from MTT cell viability data between 24, 72 and 120 hours of exposure to MCZ at different [MCZ].

Figure 13 (A-C) shows dose-response curves based on MTT results, plotted in % relative to control. Notably, the adjusted R square values are above 0.7 in the 3 cases, indicating a high correlation level between de model prediction values and the real values.

In addition, in Figure 13 (D-G), it is observed that the distribution of the residuals for the three cases is considerably adjusted to a normal distribution with its center at 0. That indicates that most of the actual data is close to the model. In addition, as is shown in Table 13, cell viability at 24, 72 and 120 hours was significantly different between MCZ treatments. Also in Table 14, it is possible to observe that MCZ exerts a chronic effect over SH-SY5Y cell viability after the treatment of MCZ 5 µM.

5. DISCUSION

Since it was commercially available, MCZ has been an indispensable fungicide in controlling a wide variety of plant diseases caused by fungi. Its multi-site activity is one of its main advantages, making it a powerful anti-resistance tool (2). Due to its prevalent use in agriculture worldwide, the safety of this fungicide has been extensively investigated. As mentioned in previous sections of this work, MCZ is widely linked to endocrine system disruption and reproductive toxicity (16,17). However, information on its neurotoxicity remains limited. In fact, until the moment this work was written, no neurotoxicological study carried out on human cells was found. For this reason, this work intends to contribute to filling this gap.

As shown in the results section, under low concentrations (0.5, 1, 2.5 and 5 µM), MCZ affects SH-SY5Y cell morphology (section 4.1). The loss of its polygonal-shaped soma and the significant reduction in the number and length of neurites was quite evident in the cell morphology analysis performed in this study. Similar results were reported in the same cell line due to exposure to ZB, a Zn-containing EBDCs (51). The researchers of this study not only reported a reduction in the length and number of neurites, they also observed other signs of toxicity that MCZ exposure may not have over these cells. These include the appearance of cell blebbing, cell shrinkage, apoptotic body formation, and decreased adhesion to the flask (51). However, it is highlightable that the evaluated range of fungicide concentrations in the mentioned study was broader (50-400 µM) compared to those used in the morphology experiments reported in this work. On the other hand, no studies of the toxicity of MCZ were found in the SH-SY5Y cell line, but in primary cultures of mouse mesencephalic cells does. The thyroxine hydroxylase-positive cells of these cultures shown a reduction in the number of neurites due to the effect of MCZ exposure (38). However, these effects were observed at higher concentrations (10-120 µM) compared to those used in this experiments. This difference in the effective dose for the morphological alteration of the cells, could reveal a greater sensitivity to MCZ of SH-SY5Y cells when compared with mouse mesencephalic cells.

Nontheless, the morphological alteration of the cells was not the only toxic trait caused by MCZ to SH-SY5Y cells. Through DCF probe, it was observed that this agrochemical produces an increase in the concentration of ROS in human neuroblastoma cells. Indeed, Domico and colleagues hypothesize that the increase in intracellular ROS could be an indirect consequence of MCZ toxicity (92). This observation was made

because they found that in mesencephalic cells at concentrations of 15 and 30 µM of MCZ, alterations in the electron transport chain and respiration inhibition, respectively, were produced (92). Consequently, elevated levels of ROS might be produced. Additionally, it is well known that, despite the physiological role of ROS, an imbalance in the levels of these species can lead to the activation of apoptotic pathways (112). Therefore, it can be inferred that one of the mechanisms of MCZ neurotoxicity is mediated by mitochondrial disruption.

In addition, this study evaluated cell viability of SH-SY5Y cultures exposed to low (0.5, 1, 2.5, 5, and 10 µM) and high (5, 10, 20, 40, and 80 µM) concentrations of MCZ using the dye exclusion test and the MTT assay, respectively. Interestingly, cell density was found to have a regulatory effect on MCZ cytotoxicity. MCZ treatments at concentrations of 5 and 10 µM had completely different effects on cell viability when evaluated by the trypan blue test or the MTT assay. A significant reduction (total in 10 µM treatments) in the cell viability of the cells treated at 5 and 10 µM compared to the control was observed in the results of the trypan blue test (Annex, Table 4). In contrast, the cell viability of the cultures treated at these same concentrations was minimally affected according to the results obtained from the MTT assay. It is important to mention that, due to the high cell density requirements for performing the MTT assay, the cell density for these experiments was approximately 60-fold higher compared to the trypan blue experiments. Therefore, it is hypothesized that cell density in SH-SY5Y cultures may affect the outcomes of neurotoxin assessments, probably due to the need of these cells for cell-cell communication to proliferate, since when these cells are plated highly separately, as in the case of cell morphology analysis and trypan blue experiments, cell proliferation has been reduced due to the lack of cell-cell communication (108). However, regardless of this, both cell viability experiments show a strong influence of MCZ in the dose-dependent reduction of cell viability.

In the same way, based on the data obtained from the MTT assay, it was possible to find a model, in this case sigmoidal, that was well adjusted to the data obtained at 24, 72 and 120 hours. In all three cases, the adjusted R-squared values are greater than 0.7, which indicates a good fit of the model to the data obtained. Comparing these curves, the chronic effect of MCZ on SH-SY5Y cells can be clearly observed. This is reflected in the pronounced change in the curve response (represented by the slope of the curve) of 72 and 120 hours (compared with the 24 hours curve) of exposure to MCZ, especially in the

dose range corresponding to 10 - 40 µM (Figure 13 (A-C)). This means that the longer the exposure time to MCZ, the greater the toxic effects at lowest concentration. Unfortunately, no studies have been found to date of the chronic effects of MCZ in other cellular or animal models.

Interestingly, the MCZ-induced toxicity outcomes observed in the experiments performed in this work have been dercribed as distintives features (at cellular level) of neurodegenerative diseases such as PD (113). Additionally, according to the literature, the toxicity of MCZ and PD can trigger the activation of the same cellular defense mechanisms. In fact, the transcription factor Nrf2, a protective protein against oxidative stress damage, mitochondrial disfunction and neuroinflammation, have been associate with the pathogenesis of PD, and was found to be increased in the brains of carp exposed to MCZ (99,114). Furthermore, some studies in animal models have shown motor symptoms and selective neurodegeneration after MCZ exposure (95,96,98).

Besides, as reviewed in section 2.6.1, some of these effects are related to the neurotoxicity associated with Mn, one of the metallic components of the MCZ molecule. For example, Mn (II) citrate inhibits the E3 ubiquitin ligases-target protein degradation pathway, which would trigger the increase of damaged and unfolded proteins (105). Comparable with this in the context of some neurodegenerative diseases, the function of some E3 ubiquitin ligases has been altered (115). Indeed, Parkin, one of the E3 ubiquitin ligases, is widely related to PD and the control of mitochondrial ROS homeostasis (116).

Finally, despite the fact that the *in-vitro* studies performed in this work show that MCZ in one of its commercial formulations might be a neurotoxic agent, there are many unknowns about the real effects of MCZ on human neuronal health. As discussed in section 2.4. official entities have established safe exposure limits for the use of MCZ depending on its time and type of exposure (oral, inhalation and skin absorption). However, the information about the systemic distribution according to the type of exposure of MCZ is very limited, especially when is about to the nervous system. Therefore, it becomes challenging to explore the relationship between the dose and the neurotoxic effect that MCZ might have on humans. However, the results of *in-vitro* studies, such as those carried out in this work, together with *in-vivo* and epidemiological studies, have allowed unknowns to be generated about the use of MCZ and its possible neurotoxicity, thus opening a line of research of great importance.

6. CONCLUSION

The results of this study strongly suggest that MCZ has cytotoxic effects on SH-SY5Y cells. This was reflected in the decrease in cell viability after MCZ exposure in a dose-dependent manner. In addition, disruptive effects were observed at the morphological level at very low MCZ concentrations. These results are comparable with the limited literature currently available regarding the neurotoxicity of MCZ. Finally, it is important to mention that since it is a widely used product in agricultural production, the neurotoxic effects of MCZ must be more deeply investigated in order to take the corresponding precautions.

7. RECOMMENDATIONS

Given the lack of conclusive evidence of the neurotoxic effects that MCZ could have on humans, it is recommended that preventive measures be taken, especially in agricultural countries, such as Ecuador, where a large part of the population is directly exposed to this agrochemical. These measures may be based on education on the proper use of agrochemicals and the socialization of the importance of the proper use of personal protective equipment.

Although MCZ has been on the market for a long time, studies on the neurotoxicity of this substance remain limited. Therefore, it is recommended to generate new epidemiological studies to determine whether or not there is a relationship between occupational exposure to MCZ and neurotoxicity. In addition, it is important to explore the systemic distribution of MCZ in the nervous system through *in-vivo* studies as well as molecular neurotoxical mechanisms of this agrochemical.

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9. ANNEX

24 hours					
Comparison	**diff**	**lwr.ci**	**upr.ci**	**P - value**	**Significance**
0.5µM - 0	-1.067	-1.667	-0.468	0.00012	***
1µM - 0	-1.826	-2.425	-1.226	6.90E-11	***
2.5µM - 0	-3.268	-3.867	-2.668	<2E-16	***
5µM - 0	-3.523	-4.122	-2.924	<2E-16	***
10µM - 0	-3.568	-4.167	-2.968	<2E-16	***

Annex, Table 1: Dunnett test for the results obtained from the analysis of the number of neurites per cell at 24 hours of MCZ treatment.

72 hours					
Comparison	**diff**	**lwr.ci**	**upr.ci**	**P - value**	**Significance**
0.5µM - 0	-1.154	-1.746	-0.562	1.80E-05	***
1µM - 0	-2.396	-2.988	-1.805	1.90E-15	***
2.5µM - 0	-3.826	-4.417	-3.234	<2E-16	***
5µM - 0	-4.269	-4.861	-3.677	<2E-16	***
10µM - 0	-4.171	-4.763	-3.580	<2E-16	***

Annex, Table 2: Dunnett test for the results obtained from the analysis of the number of neurites per cell at 72 hours of MCZ treatment.

120 hours					
Comparison	**diff**	**lwr.ci**	**upr.ci**	**P - value**	**Significance**
0.5µM - 0	-1.051	-1.497	-0.605	3.90E-07	***
1µM - 0	-1.609	-2.055	-1.163	3.50E-14	***
2.5µM - 0	-2.865	-3.311	-2.419	<2E-16	***
5µM - 0	-3.272	-3.718	-2.826	<2E-16	***
10µM - 0	-3.345	-3.791	-2.898	<2E-16	***

Annex, Table 3: Dunnett test for the results obtained from the analysis of the number of neurites per cell at 120 hours of MCZ treatment.

24 hours					
Comparison	**diff**	**lwr.ci**	**upr.ci**	**P - value**	**Significance**
0.5µM - 0	-27.959	-37.307	-18.611	1.80E-10	***
1µM - 0	-40.798	-50.146	-31.450	<2E-16	***
2.5µM - 0	-65.514	-74.862	-56.166	<2E-16	***
5µM - 0	-69.043	-78.391	-59.695	<2E-16	***
10µM - 0	-69.657	-79.005	-60.309	<2E-16	***

Annex, Table 4: Dunnett test for the results obtained from the analysis of neurite length per cell at 24 hours of MCZ treatment.

72 hours					
Comparison	**diff**	**lwr.ci**	**upr.ci**	**P - value**	**Significance**
0.5µM - 0	-27.562	-39.365	-15.758	2.70E-07	***
1µM - 0	-46.792	-58.595	-34.989	3.30E-15	***
2.5µM - 0	-76.568	-88.260	-64.654	<2E-16	***
5µM - 0	-84.440	-96.243	-72.636	<2E-16	***
10µM - 0	-82.973	-94.777	-71.170	<2E-16	***

Annex, Table 5: Dunnett test for the results obtained from the analysis of neurite length per cell at 72 hours of MCZ treatment.

120 hours					
Comparison	**diff**	**lwr.ci**	**upr.ci**	**P - value**	**Significance**
0.5µM - 0	-24.238	-33.187	-15.290	5.90E-09	***
1µM - 0	-35.423	-44.372	-26.475	9.80E-15	***
2.5µM - 0	-60.637	-69.585	-51.688	<2E-16	***
5µM - 0	-67.148	-76.096	-58.199	<2E-16	***
10µM - 0	-67.780	-76.729	-58.832	<2E-16	***

Annex, Table 6: Dunnett test for the results obtained from the analysis of neurite length per cell at 120 hours of MCZ treatment.

24 hours					
Comparison	**diff**	**lwr.ci**	**upr.ci**	**P - value**	**Significance**
0.5µM - 0	17.145	2.909	31.381	0.01258	*
1µM - 0	22.393	8.157	36.629	6.90E-04	***
2.5µM - 0	62.045	47.809	76.281	<2E-16	***
5µM - 0	95.667	81.431	109.903	<2E-16	***
10µM - 0	98.487	84.251	112.723	<2E-16	***

Annex, Table 7: Dunnett test for the results obtained from the dye exclusion test (percentage of dead cells) at 24 hours of MCZ treatment.

72 hours					
Comparison	**diff**	**lwr.ci**	**upr.ci**	**P - value**	**Significance**
0.5µM - 0	13.193	4.667	21.720	8.90E-04	***
1µM - 0	38.146	29.619	46.672	<2E-16	***
2.5µM - 0	89.352	80.826	97.879	<2E-16	***
5µM - 0	96.660	88.134	105.187	<2E-16	***
10µM - 0	96.899	88.373	105.426	<2E-16	***

Annex, Table 8: Dunnett test for the results obtained from the dye exclusion test (percentage of dead cells) at 72 hours of MCZ treatment.

120 hours					
Comparison	**diff**	**lwr.ci**	**upr.ci**	**P - value**	**Significance**
0.5µM - 0	13.048	7.111	18.985	1.80E-06	***
1µM - 0	32.958	27.021	38.895	<2E-16	***
2.5µM - 0	95.430	89.493	101.367	<2E-16	***
5µM - 0	96.344	90.407	102.281	<2E-16	***
10µM - 0	97.912	91.975	103.849	<2E-16	***

Annex, Table 9: Dunnett test for the results obtained from the dye exclusion test (percentage of dead cells) at 120 hours of MCZ treatment.

24 hours					
Comparison	**diff**	**lwr.ci**	**upr.ci**	**P - value**	**Significance**
5µM - 0	-4.427	-19.925	11.071	0.913	n.s.
10µM - 0	-5.419	-20.918	10.079	0.828	n.s.
20µM - 0	-16.927	-32.425	-1.429	0.028	*
40µM - 0	-56.724	-72.222	-41.226	9.80E-13	***
80µM - 0	-72.323	-87.811	-56.814	<2E-16	***

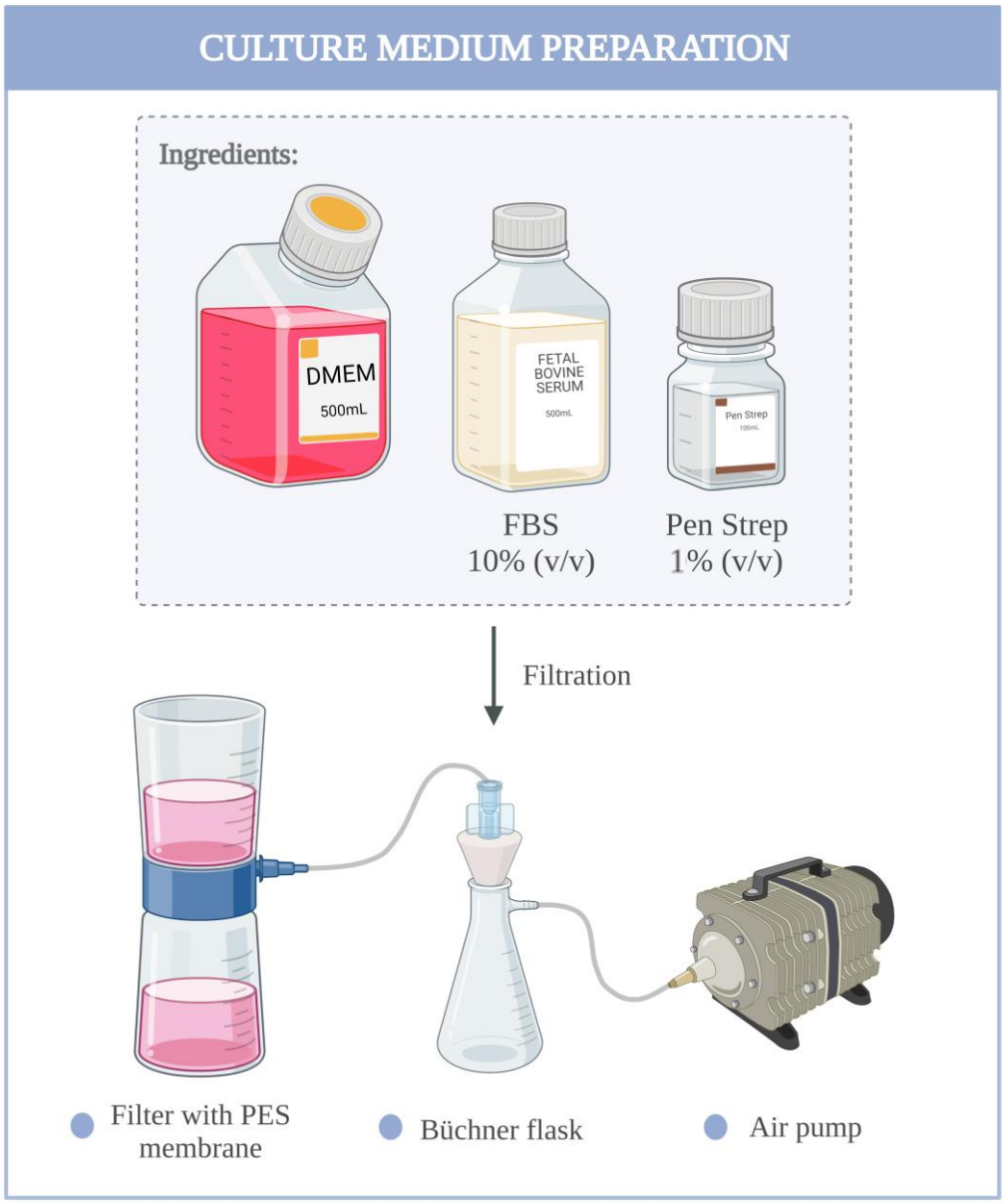
Annex, Table 10: Dunnett test for the results obtained from MTT cell viability (% of control) at 24 hours of MCZ treatment.

72 hours					
Comparison	**diff**	**lwr.ci**	**upr.ci**	**P - value**	**Significance**
5µM - 0	-18.002	-30.539	-5.465	0.002	**
10µM - 0	-46.076	-58.613	-33.539	1.00E-12	***
20µM - 0	-72.338	-84.875	-59.801	<2E-16	***
40µM - 0	-76.701	-89.239	-64.164	<2E-16	***
80µM - 0	-81.929	-94.466	-69.392	<2E-16	***

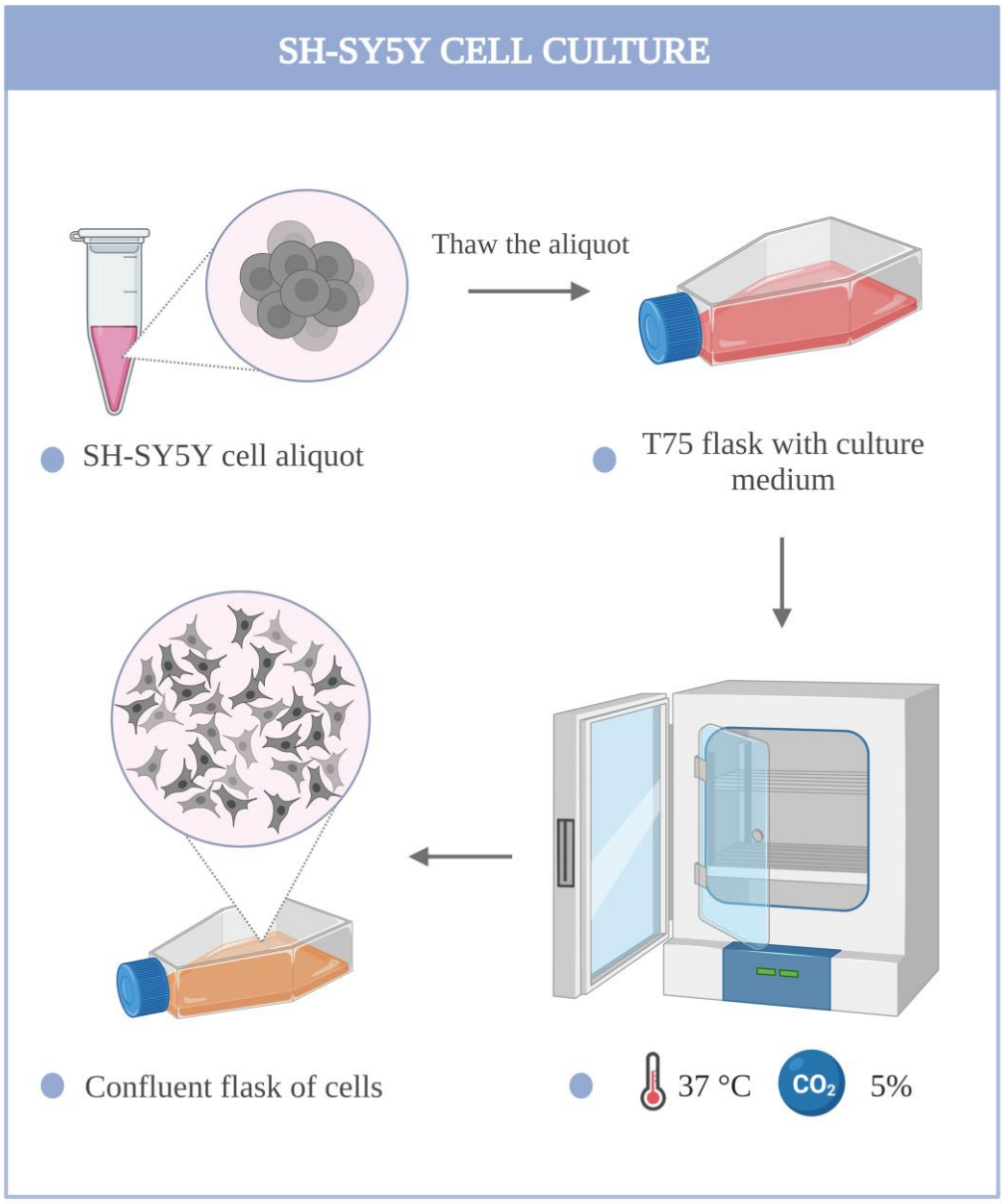
Annex, Table 11: Dunnett test for the results obtained from MTT cell viability (% of control) at 72 hours of MCZ treatment.

120 hours					
Comparison	**diff**	**lwr.ci**	**upr.ci**	**P - value**	**Significance**
5µM - 0	-5.624	-21.489	10.240	0.82	n.s.
10µM - 0	-32.240	-48.104	-16.376	1.30E-05	***
20µM - 0	-69.449	-85.313	-53.584	2.20E-16	***
40µM - 0	-76.008	-91.872	-60.144	<2E-16	***
80µM - 0	-77.017	-92.881	-61.152	<2E-16	***

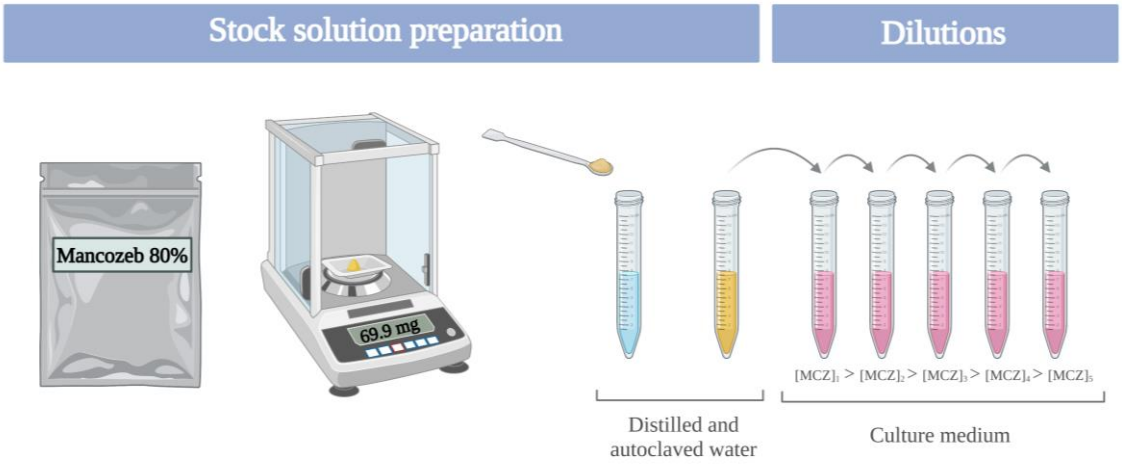
Annex, Table 12: Dunnett test for the results obtained from MTT cell viability (% of control) at 120 hours of MCZ treatment.



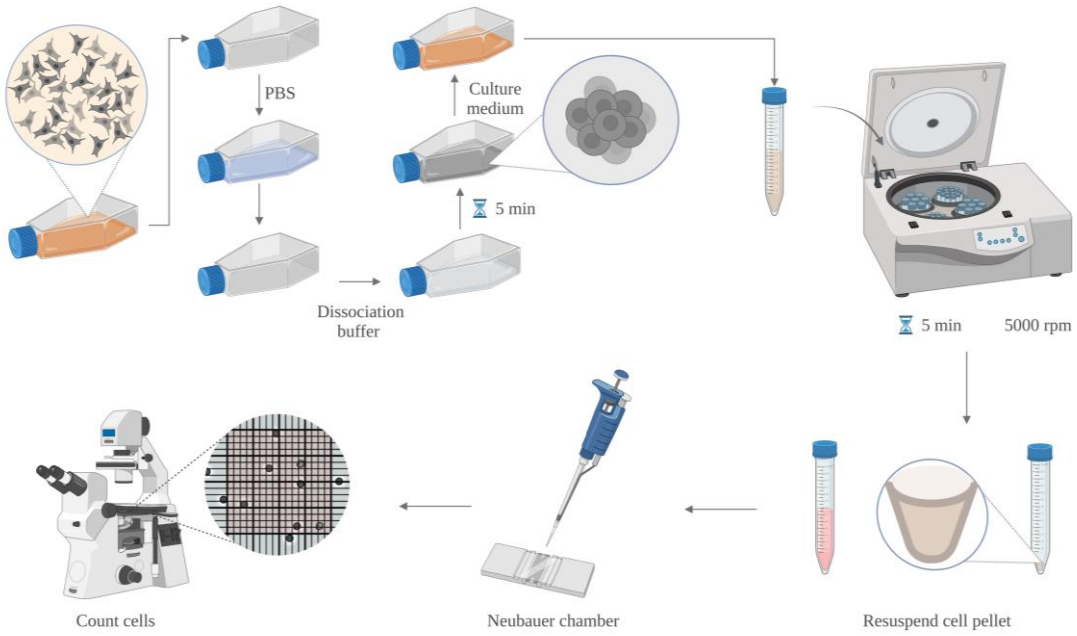
Annex, Figure 1: Culture medium preparation protocol.



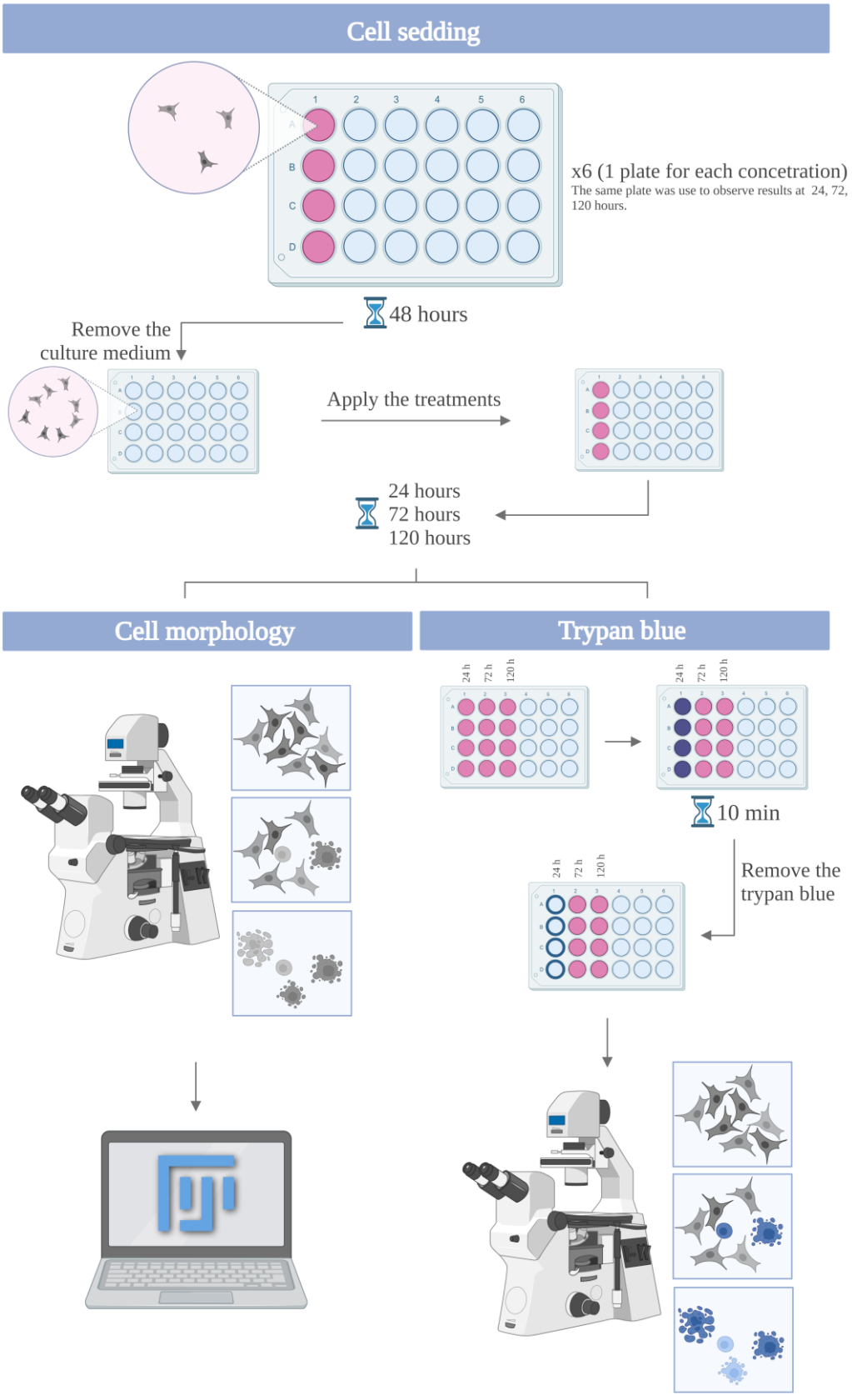
Annex, Figure 2: SH-SY5Y cell culture protocol.



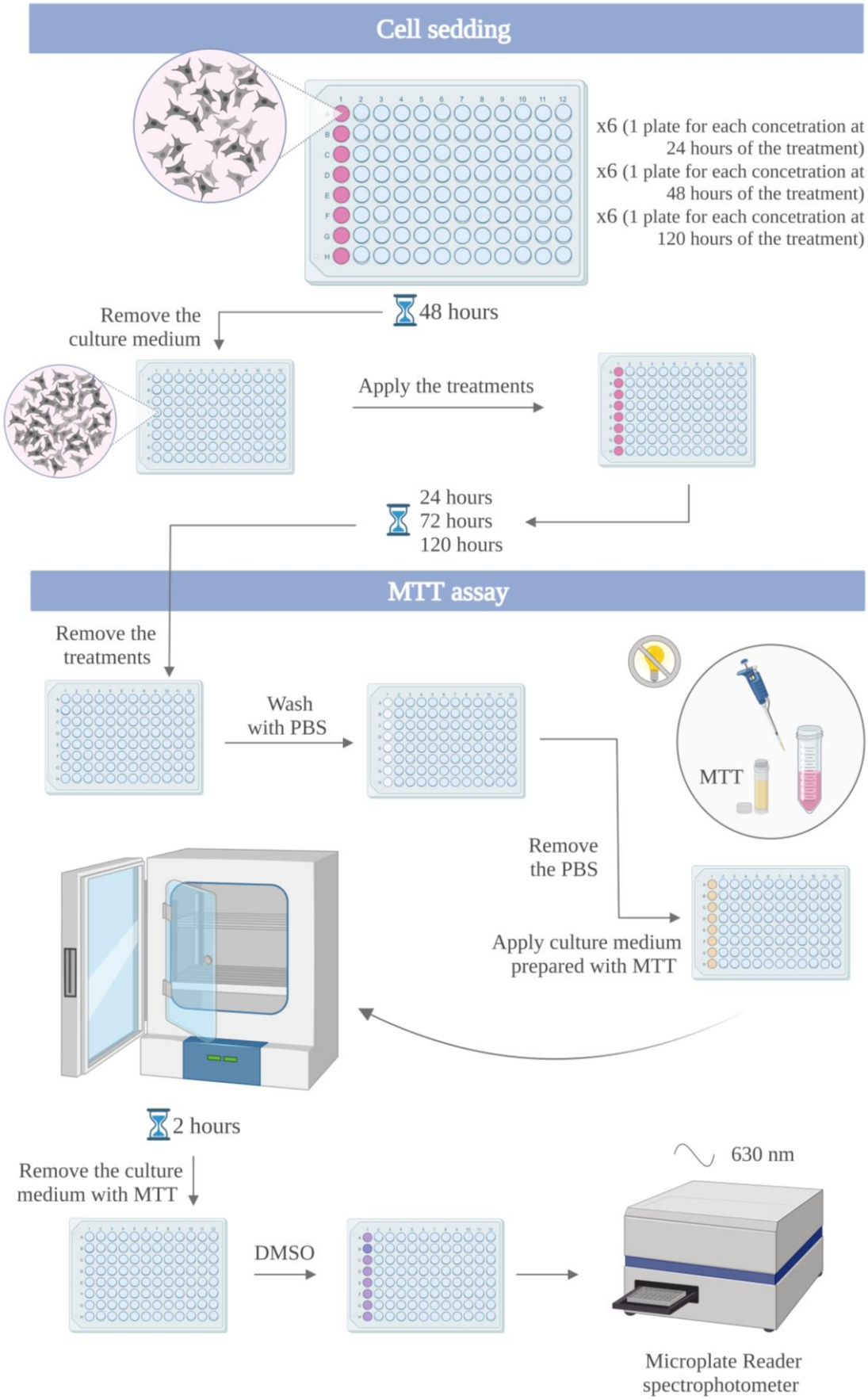
Annex, Figure 3: Treatments preparation protocol.



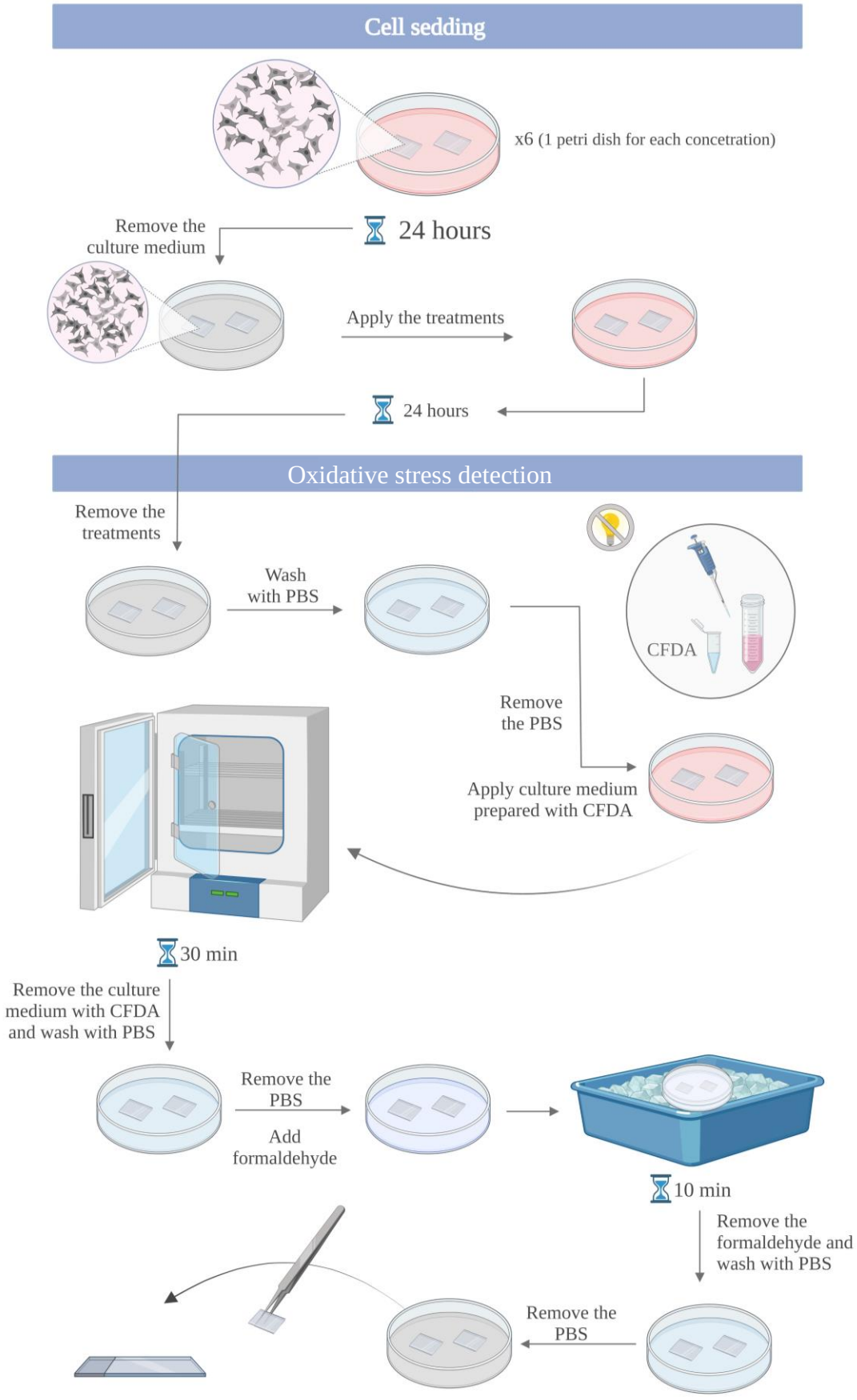
Annex, Figure 4: Cell harvesting and cell count protocol.



Annex, Figure 5: Cell morphology analysis and trypan blue exclusion test protocol.



Annex, Figure 6: MTT assay protocol.



Annex, Figure 7: ROS detection protocol.