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Efeito da Atrazina e do Glifosato na função e viabilidade de espermatozoides epididimários

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"A Who's Who of pesticides is therefore of concern to us all. If we are going to live so intimately with these chemicals eating and drinking them, taking them into the very marrow of our bones - we had better know something about their nature and their power." - Rachel Carson, **Silent Spring**

Para concluir esta etapa académica tão importante para mim, a obtenção do grau de mestre em Tecnologias Clinico-Laboratoriais, quero agradecer a todos os que nela estiveram envolvidos, e que a tornaram possível ao longo destes 2 anos.

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O uso intensivo de pesticidas, como atrazina (ATZ) e glifosato (GLY) aumentou exponencialmente, estando associados a efeitos nocivos na saúde humana e animal. Este estudo avaliou os efeitos de ATZ e GLY na viabilidade, motilidade (cauda) e potencial de membrana mitocondrial (ΔΨm) de espermatozoides epididimários bovinos. Amostras isoladas de espermatozoides epididimários (cabeça, corpo e cauda) de 11 touros Holstein-Frísia foram expostos a diferentes concentrações de ATZ (0,1, 1 e 10 μM) e GLY (5, 50 e 360 mg/L), e os parâmetros foram avaliados antes e após 2 e 4 horas de exposição.

Os resultados indicaram que a viabilidade dos espermatozoides não foi afetada pela ATZ até 4 horas, embora tenha havido uma redução na proporção de espermatozoides vivos entre 2 e 4 horas para espermatozoides da cabeça e corpo, mas não da cauda. A motilidade subjetiva não foi afetada pela ATZ até 4 horas, embora um declínio foi observado entre 2 e 4 horas. Para o ΔΨm, observou-se diferenças entre as 2 e as 4 horas de incubação de amostras obtidas da cauda do epidídimo com 1 μM de ATZ.

O GLY não mostrou efeitos na motilidade e apenas espermatozoides da cabeça mostraram uma diminuição na viabilidade entre 2 e 4 horas. O ΔΨm não foi alterado pelas concentrações do GLY em nenhum dos tempos de incubação.

Os resultados sugerem que marcadores convencionais podem não detetar efeitos subtis da ATZ e GLY nos espermatozoides, mas análises de metabolómica e de stress oxidativo podem fornecer uma avaliação mais abrangente e o desenvolvimento de biomarcadores sensíveis.

Palavras-chave: Toxicidade, reprodução, herbicidas, espermatozoides bovinos

The intensive use of pesticides such as atrazine (ATZ) and glyphosate (GLY) has increased exponentially, and they are associated with harmful effects on human and animal health. This study evaluated the effects of ATZ and GLY on the viability, motility (tail) and mitochondrial membrane potential (ΔΨm) of bovine epididymal sperm. Isolated samples of epididymal sperm (head, body and tail) from 11 Holstein-Friesian bulls were exposed to different concentrations of ATZ (0.1, 1 and 10 μM) and GLY (5, 50 and 360 mg/L), and the parameters were assessed before and after 2 and 4 hours of exposure.

The results indicated that sperm viability was not affected by ATZ up to 4 hours, although there was a reduction in the proportion of live sperm between 2 and 4 hours for sperm from the head and body, but not from the tail. Subjective motility was not affected by ATZ up to 4 hours, although a decline was observed between 2 and 4 hours. To ΔΨm, a difference was observed between the 2 and 4 hours of incubation of samples obtained from the cauda epididymis with 1 μM ATZ.

GLY showed no effect on motility and only sperm from the head showed a decrease in viability between 2 and 4 hours. ΔΨm was not altered by GLY concentrations at any of the incubation times.

The results suggest that conventional markers may not detect subtle effects of ATZ and GLY on sperm, but metabolomics and oxidative stress analyses may provide a more comprehensive assessment and the development of sensitive biomarkers.

Keywords: Toxicity, reproduction, herbicides, bovine spermatozoa

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3Rs – Replace, Reduce and Refine
ADI – Acceptable Daily Intake
AMPA - Aminomethylphosphonic acid
ATZ – Atrazine
BHC - β-Hexachlorocyclohexane
CASA – Computer-Assisted Sperm Analysis
DACT - Diamino-chlorotriazine
DDT – Dichlorodiphenyltrichloroethane
DEA – Deethylatrazine
DMSO - Dimethyl sulfoxide
DNA - Deoxyribonucleic acid
ECHA - European Chemicals Agency
EDCs - Endocrine-Disrupting Chemicals
EFSA - European Food Safety Authority
EN - Eosin-Nigrosin
EPA - Environmental Protection Agency
EPSPS - 5-Enolpyruvylshikimate-3-Phosphate Synthase
EtOH – Ethanol
FRAP - Ferric Reducing Ability of Plasma
GLY – Glyphosate
IARC - International Agency for Research on Cancer
JC-1 - 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide
MMP / ΔΨm - Mitochondrial Membrane Potential
MRLs - Maximum Residue Levels
mTALP - Tyrode's Albumin Lactate Pyruvate medium
NMR - Nuclear Magnetic Resonance
PBS – Phosphate-Buffered Saline
SDGs - Sustainable Development Goals
SPZ – Spermatozoa
TCF - Tris–Citrate–Fructose
TEC - Testis–Epididymis Complexes
WHO - World Health Organization

1. Introduction

1.1. Historical context of pesticides

Since the dawn of civilization, humanity has been constantly striving to improve its living conditions, especially about food (1). Throughout history, pests and diseases have been a constant challenge to food security (1,2). To solve this problem, pesticides have been developed to eliminate pests such as insects, rodents, fungi and undesirable plants (weeds), as defined by the World Health Organization (WHO). They are used in public health to eliminate disease vectors, such as mosquitoes, and in agriculture to control pests that damage crops (3). On the negative side, many pesticides are environmentally harmful and known or suspected to be toxic to humans. In fact, according to 2022 data, around 385 million people fall ill every year due to pesticide poisoning (4).

Before the end of the 19th century, pests were mainly controlled with organic compounds, such as sulphur compounds to control insects and mites, one of the first recorded uses of pesticides dating back to around 4,500 years ago (5). Or the use of pyrethrum, obtained from the dried flowers of the chrysanthemum "*Cinerariafolium",* as an insecticide by the Persians in 400 BC (5,6). Subsequently, saw the emergence of synthetic inorganic pesticides. The discovery of synthetic chemicals like Paris Green (copper acetoarsenite) in the 1860s was a key moment. Paris Green was first used to control the Colorado potato beetle and soon became widely used. Another example is the 'Bordeaux mixture', a combination of copper sulphate and lime, which was accidentally discovered in 1882 and successfully used to control downy mildew and potato blight. It is still used as a fungicide today (5–7).

From the beginning of the 20th century, was the dawn of the age of synthetic organic pesticides. Two of the most notable synthetic organic pesticides used were the Dichlorodiphenyltrichloroethane (DDT) and the β-Hexachlorocyclohexane (BHC). These two remarkably effective pesticides against a wide range of insect pests, lack selectivity and are highly toxic and, unfortunately, their extensive use in agriculture and public health led to unforeseen environmental consequences (6).

Currently, a wide range of synthetic products are being developed to provide safer, more selective and effective control for pests and diseases. By one estimate, from 1990 to 2017, the global use of pesticides grew by around 80% (4,6).

The growth of this industry has taken place over several decades for various reasons, such as soil deterioration and the extinction and loss of biodiversity. Climate change, particularly the rise in global temperatures, which, for example, alters the migration patterns of pests and insects that are beneficial to crops (4).

Also, the increased globalization of trade and the motorized transport of people allows pests to spread and establish themselves all over the world, making it necessary to the use of pesticides (5). Consequently, the use of these compounds has generated a major problem related to the resurgence and resistance of pests, which in turn requires the constant development of new pesticides and new resistant cultivars (8).

Pesticides, also known as plant protection products, are extremely diverse in terms of their chemical structures, action and application, but there are difficulties in classifying them due to their wide scope (9,10). Within this wide variety, there are herbicides, fungicides, insecticides, acaricides, plant growth regulators, repellents and others. All these products contain at least one active substance, which can be chemical or microbial, including viruses (9,11).

Pesticides can be divided into two categories: synthetic and natural. On the one hand, biopesticides fall into the natural category and originate from living organisms such as plants, fungi and bacteria. Pesticides of synthetic origin, on the other hand, are man-made chemicals that aren´t found in nature (12). There is a plethora of pesticides of synthetic origin, such as atrazine (ATZ) and glyphosate (GLY), which are among the most widely used herbicides in the world (13,14).

Currently, there are three widespread criteria for classifying pesticides: the mode of entry, the chemical composition and the target that the pesticide aims to eliminate. However, the WHO has chosen to classify pesticides according to their toxicity, distributing them into four levels: extremely hazardous, highly hazardous, moderately hazardous and slightly hazardous (2,12).

1.2. Use, Exposure and Sustainability

Eurostat data indicates that the largest pesticide markets in Europe are France, Italy, Spain and Germany. However, Denmark, for example, stands out for its significant decrease in pesticide use (4).

Today, around 4 million tons of pesticides are used globally, 50% of which are herbicides, 30% insecticides and around 17% fungicides (4).

In Europe, the European Food Safety Authority (EFSA) plays a very important role in regulating the use of pesticides. It is involved in assessing the risks of the active substances used in plant protection products, i.e. analyzing the direct or indirect harmful effects on human, animal and environmental health (9).

Research has been carried out to understand how pesticides behave in the environment, since they are generally not very selective in their destructive action, because the active substances present in them interfere with fundamental metabolic pathways or vital physiological processes common to a great diversity of organisms (8,15).

In the case of the subsoil, pesticide residues end up destabilizing the diversity of microorganisms present in it, such as bacteria, fungi and the subterranean fauna itself, which are essential for keeping the soil healthy (4).

In the case of humans, the occupational exposure (production, transport and application of these compounds), living in areas with high levels of pesticide residues, as well as the circulation and accumulation of these pesticides in the food chain, has been a growing source of public concern (16).

Consequently, exposure to pesticides is potentially associated with health risks. Experimental and epidemiological evidence between pesticide exposure and the incidence of various human diseases, such as neurodegenerative, respiratory, metabolic and reproductive, has justified this concern in the community (17,18).

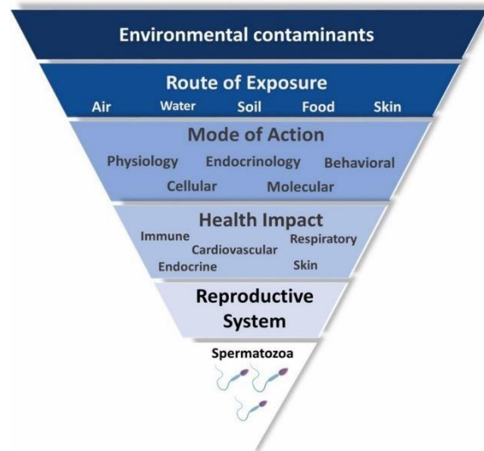


Figure 1.1 - Flow chart summarizing the impact of environmental contaminants on spermatozoa. Environmental contaminants accumulate in the air, water, soil and food. Humans and animals are routinely exposed to these contaminants which have different actions and outcomes which in turn affect sperm quality. Adapted from (19).

Currently, several chemical compounds, especially pesticides, have been identified as endocrine-disrupting chemicals (EDCs). These are external agents that interfere with natural processes related to the synthesis, secretion, transport, binding, action or elimination of hormones, as defined by the Environmental Protection Agency (EPA) (20).

One of the pesticides classified as an EDC is atrazine, but its mechanism of action has not been well characterized (21,22). There have already been several studies on the action of ATZ on hormonal imbalance, such as the relationship with tumors in the mammary gland in rodents, blocking ovulation in immature rats treated with gonadotrophin, delayed gonadal development (gonadal dysgenesis), testicular oogenesis (hermaphroditism) in amphibians, in mice it impairs glycolysis and the glycolytic capacity of Sertoli cells and consequently impairs spermatogenesis (23–26).

In addition to atrazine, another ubiquitous micropollutant is glyphosate, which after three decades of research, including in vivo, in vitro and epidemiological approaches, there is still no clear consensus on its effect on the human endocrine system. However, reports indicate that it fulfils at least 8 of the 10 main characteristics of an endocrine disruptor, according to a guideline of ten key characteristics of EDC proposed in the expert consensus statement published in 2020 (27,28). Despite this, growing evidence from in vitro and in vivo studies in animal models indicates its potential disruption of normal endocrine function (29–31).

However, pesticides are not only a problem for human health, but they also cause serious damage to animal health. This occurs either through the consumption of

contaminated water and feed, the use of veterinary medicines or even exposure in public parks and homes (32). Some studies have shown that there is an association between exposure to pesticides and disease, as in the case of dogs, breast cancer, bladder cancer and lymphomas have been reported (33–36). In rural areas, the pesticides most likely contaminate the air, vegetation and water in the region, and the livestock end up inhaling and/or consuming them (37). Previous studies have shown that animals intended for consumption, such as cattle, tend to bioaccumulate pesticide residues, making meat and milk potential sources of contamination for humans (38–42).

In this context, the European Commission, in close co-operation with EFSA, has established Maximum Residue Levels (MRLs), which indicate the highest levels of pesticide residues legally permitted in food or feed when the pesticide is applied correctly. This guideline aims to establish a fixed value for the amounts of residues found in food so that they are safe for consumers and are as low as possible (9,43).

In addition to this measure, in 2015 all United Nations member states approved a set of common goals, known as the Sustainable Development Goals (SDGs), which propose a sustainable global action plan until 2030 (44). In total, there are 17 goals, two of which relate to the use of pesticides, namely goals 6 and 12. Goal 6 aims to improve water quality by 2030 by reducing pollution and minimizing the dumping of chemicals. Goal 12, meanwhile, aimed to achieve proper environmental management of chemicals and all waste by 2020 (45) Recently, the European Green Deal, in line with the Farm to Fork and Zero Pollution strategies, proposes halving the use of pesticides, eradicating soil pollution and achieving at least 25% organic farmland in Europe by 2030 (46,47).

Animal models are used to carry out numerous scientific research studies in various fields. However, there is growing ethical concern about the welfare of these animals.

In this respect, in 1959, Russell and Burch introduced three fundamental principles of bioethics known as the "3Rs - Replace, Reduce and Refine" (48). The aim is to direct scientific research towards finding alternatives to animal testing, such as *in vitro, ex vivo* or *in silico tests* (Replace), to maximize the amount of information obtained with as few animals as possible (Reduce) and to adopt methods that minimize the suffering and stress of these animals (Refine). However, it is also crucial to promote animal welfare when its use is unavoidable (48)(49).

Research into reproductive toxicology is a complex field in the sense that it involves multiple organs and tissues that interact with and depend on the endocrine system, in

addition to the potentially different modes of action of toxicants (50).

However, the use of female or male gametes makes it possible to carry out these tests in accordance with the principles of the 3R's (50). Obtaining gametes, particularly male gametes, directly from the animal is a common practice in many animal reproduction and biological research studies (51–53). In this context, testis-epididymis complexes are collected from animals through elective orchiectomies or after slaughter in abattoirs. Thus, although these procedures are invasive, they are not carried out for the sole purpose of research, but rather for other purposes, and so the use of these biological samples can be an opportunity for further studies (54–56).

Research has shown that bovine sperm collected post-mortem can be successfully used in techniques such as artificial insemination and in vitro fertilization (57). In addition, these sperm have also been used in cryopreservation studies, toxicity tests and evaluation of seminal quality (58,59). This *in vitro* exposure model offers a dual and complementary approach in toxicology studies. On the one hand, it makes it possible to assess the rate of exposure to these compounds in rural regions, acting as a sentinel species for humans and other animals that share the same environment. On the other hand, it also makes it possible to analyses the effects of these chemicals, specifically in the context of the bull's male fertility, offering insights into how they can affect its reproductive capacity (60,61).

In this sense, bovine sperm collected *post-mortem* in slaughterhouses represents a valuable source of biological material for various applications in research and veterinary practice. However, it is important to carry out additional studies to optimize the methods for collecting, storing and using these sperm, thus guaranteeing consistent and reliable results (49).

1.3. Male Reproductive System – Bovine

The reproductive system of the bull comprises the testicles, secondary sex organs (epididymis, vas deferens and penis) and three accessory sex glands (seminal vesicles, prostate and bulbourethral gland) (62).

The spermatogenesis is a complex biological process that takes place in the male reproductive system and varies in duration according to the species, such as one month in mice and around sixty days in bulls (63). It is well established that testicular

spermatozoa (SPZ) are immature and only develop the capacity for motility and fertilization during their transit through the epididymis. This organ is comprised of a long, convoluted tubule that connects the efferent ducts of the testis to the vas deferens. The epididymis has three main anatomical regions: the head (or caput), the corpus, and the tail (or cauda) (figure 1.2). Sperm maturation occurs during epididymal transit by the interaction of sperm cells with the unique luminal environment of each epididymal region. Thus, sperm formed in the testicles travel along the epididymis three parts, starting in the head, then the body and finally the tail, where they are stored until they are ejaculated (62,64,65). In figure 1.2 the three anatomic regions of the epididymis are depicted along with descriptions of sperm maturation characteristics and the expected number of sperm present by region.

During the maturation process in the epididymis, SPZ is susceptible to environmental influences, including exposure to various chemical compounds present in the environment, such as pesticides (62,66). However, the direct effects of pesticides on the later stages of spermatogenesis, particularly on sperm stored in the epididymis, are not well investigated. In addition, epididymal sperm can be considered as a better cellular model in toxicological studies than ejaculate sperm, because the seminal plasma, derived from the accessory glands contains many stimulators of glycolysis, the citric acid cycle and oxidative phosphorylation, so that the absence of prior stimulation facilitates in vitro manipulation and more concise results in the evaluation in question (67,68).

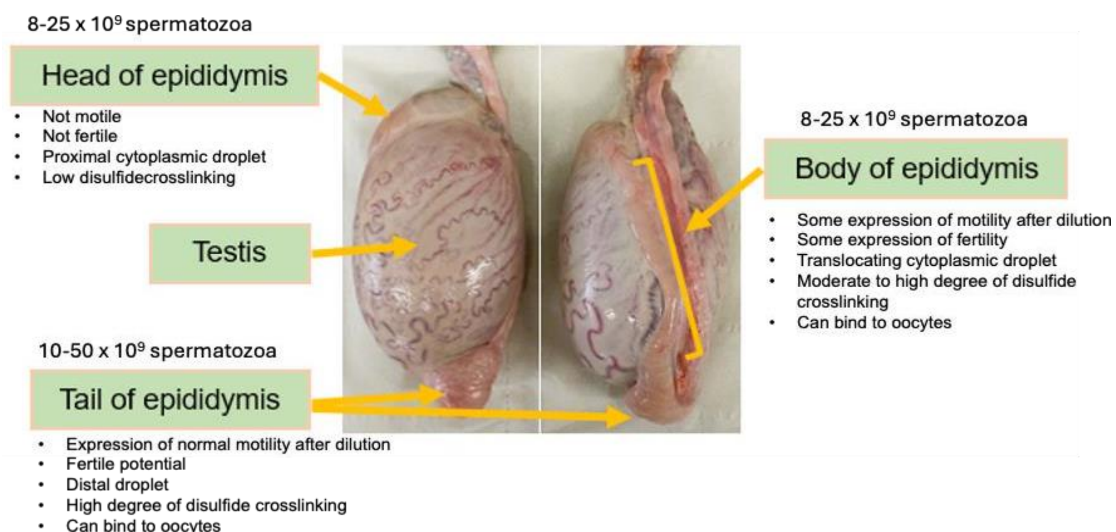


Figure 1.2 - Bovine Testicular-epididymal complexes with indication of the three anatomic regions of the epididymis, and with descriptions of sperm maturation characteristics by region of epididymis.

2. Herbicides

2.1. Atrazine

Atrazine (ATZ; IUPAC name: 6-Chloro-N2-ethyl-N4-(propan-2-yl)-1,3,5-triazine-2,4-diamine) is an herbicide introduced at the end of the 1950s (14,69). It belongs to the category of triazines, acting on the electron chain during photosynthesis, specifically in photosystem II, reducing biomass production and inducing oxidative stress, making it possible to selectively control weeds and broadleaf grasses of various crops such as maize, sugar cane and soya (14,70–72).

However, ATZ gives rise to metabolites by hydrolysis and/or dealkylation in the environment, such as deethylatrazine (DEA) and diamino-chlorotriazine (DACT), which are as toxic or even more so than ATZ itself (73).

Despite being an effective, low-cost and flexible herbicide, it has a high average lifespan, ranging from 13 to 261 days, due to its slow biodegradation. In addition, it has high persistence and mobility in soils, and moderate water solubility (33 mg/L a 25 °C) (14, 72-77).

Because of these characteristics, ATZ presents a high risk of contaminating natural resources such as groundwater and surface water through leaching and surface runoff, respectively (78). With its regular use, contamination effects have begun to appear in the environment, especially in surface and groundwater, such as rainwater and seawater, and even in ice. In addition to these, it has already been detected in atmospheric dispersion, particularly in fog and air (69,79–81).

Recently, the presence of ATZ was detected in groundwater in western Germany, even though it has been banned there since 1991. Even after its ban throughout Europe since 2003, this population continues to encounter this compound (69,71,82). However, formulations containing ATZ continue to be produced for export in some European member states, such as the United Kingdom, Spain, Portugal and Ireland (82). Given the indiscriminate use of this pesticide in the United States, South America and China, several studies have been carried out to understand the potential threat it poses to human health and ecosystems (69,72).

There are various ways of encountering this compound, whether through groundwater

as the main source of drinking water, or through contaminated food. As a lipophilic herbicide, ATZ has already been detected in basic foods for human consumption, namely foods rich in fat such as fish, meat and bovine milk. In addition, occupational exposure represents another significant route, with exposure a thousand times greater than residential exposure, in humans. Exposure through inhalation of air containing particles of this herbicide is also possible, although less common (72,82).

Some organizations have established a maximum permitted level of ATZ in drinking water of 0.1 µg/L (European Union), 2 µg/L (WHO), 3 µg/L (EPA) (69,71,83,84).

A simple way to detect the presence of ATZ in the body is through urine and, according to Barr et al, the percentage distribution of ATZ metabolites in human urine varies with the extent of exposure (i.e., high or low acute exposures or environmental exposures) (85). This statement is supported by a study carried out with several non-agricultural and agricultural families in the United States, which found that urinary levels ranged from 0.00062 µg/L to 68 µg/L, respectively (86).

Regarding the study of ATZ toxicity, the literature has focused mainly on the endocrine and reproductive systems (87). In humans, it has been linked to premature births, birth defects, fetal growth restriction, carcinogenic potential, menstrual cycle irregularities, delayed menopause and its presence in body fluids (sperm, follicles and cervical mucus) and breast milk (69,88–96). In animal models, there have been adverse effects on pubertal development in rats, neurotoxicity during embryogenesis and changes in methylation in zebrafish, prenatal exposure and/or during lactation induces immunomodulation in the offspring in rodents and post-implantation embryo loss in sheep (97–101).

2.1.1. Effects on male reproductive health in mammals

Research into the effects of ATZ on male reproductive function has advanced significantly over the years, both *in vitro* and *in vivo.*

In humans, a decrease in semen quality, male genital malformations (such as hypospadias) and a reduction in serum testosterone in post-pubertal males have been reported (69; 102; 103; 104). As a way of assessing exposure to ATZ in agricultural areas where it is commonly used, a case-control study carried out in the USA concluded that fertile American men living in these regions show a reduction in semen quality, namely

concentration and motility, compared to men living in urban regions (105).

However, several studies on mice have been carried out demonstrating the potential negative impact of ATZ on the male reproductive system. An *in vivo* study reported that high doses of ATZ (over 31 days) led to a significant increase in estrone and estradiol in the serum of rats (106). Another study showed that exposure to ATZ, starting *in utero,* causes a shortening (demasculinization) of penile structures and increases the incidence of hypospadias in mice (107). Reductions in testosterone 2α-hydroxylase and estradiol 2-hydroxylase were also observed in rats, along with decreases in the number of testicular and epididymal sperm, sperm motility and an increase in dead and abnormal sperm after exposure to ATZ (108; 109). *In vivo* exposure *in* rats caused a depletion of the antioxidant defense system in the testes and epididymis, indicating the induction of oxidative stress (109). In addition, another in vivo study in the same species found a reduction in the epididymal migration rate of sperm, induction of histopathological changes in the testes, a reduction in testicular protein concentrations and a reduction in the total number and motility of SPZ (110).

Chernyak S et al. exposed porcine SPZ to increasing concentrations of ATZ (0, 20, 40, 60, 100 µM) and found a decrease in capacitation and a significant increase in spontaneous acrosome reaction and progesterone-induced acrosome reaction at all concentrations (111). *In* this species, it was also found *in vitro* that sperm viability and progressive motility were strongly affected after exposure to ATZ at concentrations of 100 and 500 µM (112).

In vivo, exposure of domestic male goats to relatively low doses of ATZ (15 mg ATZ/kg body weight) affected morphology, viability, ΔΨm (increased in epididymal SPZ, but not in ejaculate SPZ) and the lipid constitution of the sperm membrane (63).

2.2. Glyphosate

Glyphosate (GLY; IUPAC name: *N-(phosphonomethyl)glycine*) was first synthesized by chemist Henry Martin in 1950 from a derivative of the amino acid glycine, but its phytotoxic activity was only identified two decades later by John Franz, a chemist at Monsanto® (USA). He developed Roundup, the first glyphosate-based herbicide, which was formulated with various adjuvants added to increase its cytotoxic properties (113 – 116).

Glyphosate is a biocide that has been applied before planting traditional agricultural crops and after planting transgenic crops resistant to this compound. It is also used as a desiccant when harvesting cereals, in the urban and industrial environment on around 100 species of weeds, and in the aquatic environment to eliminate invasive species (117–119). Due to the indiscriminate and negligent use of glyphosate, the areas treated with this herbicide have increased rapidly, which has promoted the emergence of resistant weeds. However, they are expected to continue to expand, mainly due to the introduction of transgenic crops resistant to glyphosate, representing almost 90% of agricultural land worldwide (28,118,120,121).

From the point of view of the mechanism of action, GLY was patented as a pesticide with disruptive activity and lethal effects on a broad and non-selective spectrum of plants with active photosynthesis, making it the most widely used herbicide worldwide (119). Glyphosate acts by inhibiting the shikimate pathway - more specifically, by inhibiting 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a pathway present in various microorganisms, parasites and plants and absent in mammals and other vertebrates (122,123). By inhibiting EPSPS, the synthesis of tyrosine, tryptophan and phenylalanine, which are essential amino acids for plant growth, is blocked, killing the plants within 1 to 3 weeks (120,122,123).

Despite being a pesticide that is resistant to degradation, some microorganisms can carry out this task, either in living plants or in the soil, giving rise to its main metabolite, aminomethylphosphonic acid (AMPA), which in turn is degraded in chemical products used in industry, water treatment and household detergents (118,123). However, this chemical and its metabolites have been detected in various environmental matrices, such as soil, air, surface and groundwater, open water reservoirs and food products (122,124).

Exposure of humans to GLY occurs through food, such as meat, fruit, honey, vegetables and infant formulae, but also through inhalation of air or dust, consumption of drinking water and occupational exposure (27,123,125–130). According to Flachowsky et al, cattle in the US consume feed consisting of 95% ingredients that include genetically modified crops such as corn and soya, and cattle outside the US consume feed consisting of 70-90% of these compounds (131,132).

Studies based on the oral administration of GLY in rats were able to assess the toxicokinetic of this herbicide and it was shown to be slowly and poorly absorbed by the gastrointestinal system, however based on tissue distribution, organs such as bone,

kidney and liver are considered targets (133,134).

The acceptable daily intake (ADI) of GLY is 1 mg/kg body weight in the USA and 0.5 mg/kg body weight in Europe (EFSA) (123,135). However, the latter has stated that in the case of daily exposure of operators the value is 0.1 mg/kg body weight (136).

Similarly, to ATZ, GLY can also be measured through urine, and in this sense, studies have revealed the presence of lower amounts of GLY in the urine of people living in urban areas compared to those living near agricultural regions (126,137). To the impact of GLY on humans, its ability to cross the placenta in pregnant women has been reported, as has its presence in serum during labor, in umbilical cord samples and in the urine of pregnant women living in rural and urban areas (137–139).

Glyphosate has been the focus of discussion and restrictions in several countries since 2015, when it was declared "probably carcinogenic to humans (Group 2A)" by the International Agency for Research on Cancer (IARC) (5,125,138). However, some independent experts reviewed the IARC's assessment and reported that it is "unlikely to pose a carcinogenic risk to humans" (140). In 2017, the European Union selected the European Chemicals Agency (ECHA) to investigate its use and concluded that "the scientific evidence currently available does not fulfil the criteria for classifying GLY as carcinogenic, mutagenic or toxic to reproduction" (141).

In Europe, however, GLY approval was renewed for a further 10 years at the end of 2023 (142). Luxembourg is currently the only European country that has banned the use of plant protection products containing the herbicide GLY (4).

2.2.1. Effects on male reproductive health in mammals

The intensive use of GLY as a risk to reproductive health was, and still is, a controversial issue. Gary M. Williams et al. evaluated the safety and risk of GLY and concluded that it did not result in adverse effects on reproduction or endocrine systems in humans and other mammals (143).

However, many studies have been carried out to really understand the impact, particularly on the male reproductive system, on human and animal health. In humans, Anifandis G. et al, incubated SPZ with 1 µg/mL of Roundup (corresponding to a GLY concentration of 0.36 µg/mL) for 1 hour and found that it led to a drop in progressive motility and depletion of mitochondrial activity (145).

Using mice as an experimental model, it has been shown *in vitro* that Roundup induces a notable cytotoxic effect on all testicular cells after incubation at 0.1% (corresponding to 360 µg/mL of glyphosate) for 24 hours (146). However, an *in vivo* study found that exposing rats to 5 µg/mL of Roundup for 8 days increased the proportion of morphologically abnormal sperm and altered nuclear integrity (147). A meta-analysis revealed that exposure to GLY decreased sperm concentration in mice and rats, indicating adverse effects on reproductive parameters (148).

In addition, studies have been carried out on other mammalian species, notably male pigs, which after *in vivo* exposure to GLY formulations have been shown to affect testicular weight, severely impairing testicular growth performance and inducing reproductive toxicity. To evaluate the toxicity of GLY *in vitro* (0, 5, 25, 50, 100 and 360 µg/mL) and observed a decrease in sperm motility (total and progressive), viability and ΔΨm, but only at the highest concentration (360 µg/mL) after 1 and 3 hours of incubation. In this study, concentrations equivalent to those of GLY were also evaluated, but using Roundup, and it proved to be more toxic and dose-dependent than the previous one, but both do not seem to alter DNA integrity (149).

Rabbits have already been used in this area of study, where it was observed that glyphosate, depending on the dose, caused a significant increase in abnormal and dead sperm. This suggests that the impact on semen quality can be attributed to the direct cytotoxic effect of GLY on spermatogenesis, and/or indirectly through the hypothalamic-pituitary-testicular axis, which regulates reproductive efficiency (150).

At the endocrine level, studies on equine testes have found that both GLY and Roundup have an inhibitory effect on the activity of aromatase, which is responsible for the irreversible conversion of androgens into estrogens and have proved that they interact with the active site of the purified enzyme (151).

The increasing use of pesticides such as ATZ and GLY are still widely used in many parts of the world. At the same time, there are growing problems with human and animal fertility. Several of the above studies clearly demonstrate that there is a relationship between increased exposure to these phytochemicals and decreased sperm quality. However, the direct effects of ATZ and GLY on the later stages of spermatogenesis, particularly on sperm stored in the epididymis, are not understood. Understanding these effects is essential for developing strategies to mitigate the risks posed by them.

3. Objectives

Atrazine and glyphosate are still widely used all over the world, especially in areas with agricultural fields and animal husbandry, such as bulls. Thus, it is likely that these animals may suffer significant health impacts, particularly reproductive, resulting in fertility problems.

In this context, the general objective of the present work was to test *in vitro* how ATZ and GLY affect the function and viability of bovine epididymal spermatozoa. In addition, to test whether SPZ at different stages of maturation in the epididymis have varying sensitivity to these pesticides.

With a view to minimizing the use of animals in toxicity tests, this study adopted a non-animal approach by employing bovine spermatozoa.

The following specific objectives can also be described for this study:

- Analysis of viability and motility (only the sperm from the cauda of the epididymis).
- Evaluation of mitochondrial membrane potential.

4. Materials and Methods

4.1. Chemicals and Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise specified. Atrazine (ATZ; SC- 210846; purity: 98.9%) and Glyphosate (GLY; SC-211568D; purity: 96.7%) were purchased from Santa Cruz Biotecnology (Dallas,TX).

Tris–citrate–fructose solution (TCF) (3.025 Tris, 1.7 citric Acid, 1.25 fructose, 0.1 g streptomycin, 0.06 benzyl penicillin, to 100 ml distilled water) was used for sperm collection and incubation and was prepared according to Rota et al (155).

4.2. Epididymal sperm collection and evaluation

4.2.1. Sperm collection

Excised testis–epididymis complexes (TEC) from 11 Holstein-Friesian bulls aged 12–24 months were collected at a commercial slaughterhouse (Direção Geral de Alimentação e Veterinária permission number, N.12.006.UDER). All animals were healthy and did not exhibit any gonad macroscopic pathology. Of the 11 TEC, 6 pairs were used for the ATZ assay and 5 pairs for the GLY assay.

Immediately after collection, each pair of TEC was refrigerated (5ºC), transported in a styrofoam box to the laboratory where it was kept at 5ºC until processing. Approximately 24 hours later, the scrotum, subcutaneous tissue and the tunica vaginalis were removed and the epididymis was isolated from the testis and cleaned with phosphate-buffered saline (PBS; Sigma-Aldrich P4417; SLCL7026). Subsequently, the epididymal head, body and tail were carefully dissected to enable sperm collection from each individual compartment (figure 4.1) according to the protocol described by Turri et al (153) with some modifications.

Briefly, the retrograde flushing method was employed to obtain sperm from the epididymal tail, as this approach allows for the sample to be obtained more rapidly and with reduced contamination. The cauda epididymis and a fraction of the ductus deferens

were isolated from the rest of the epididymis by cutting between the body and the tail of the epididymis with a scalpel. Afterwards, the lumen of the ductus deferens was cannulated with a needle attached to a syringe filled with TCF medium (37ºC), and then the SPZ were washed in a retrograde direction from the ductus deferens through the cauda of the epididymis.

To obtain sperm from the head and body of the epididymis, the float-up technique was used. In separate petri dish, the head and body of the epididymis were sliced with a scalpel, starting from the middle of each structure to a more marginal region in order to avoid as much as possible contamination with sperm from the contiguous epididymal compartments. To collect the sperm, TCF medium (37ºC) was added to the area where an incision had been made, and the medium was aspirated with the help of a micropipette, where the sperm were mixed. This procedure was carried out several times in order to obtain at least the minimum required amount of sperm to perform each experiment. For ATZ and GLY experiments a minimum of $300x10^6$ and $240x10^6$ sperm were used, for each compartment, respectively.

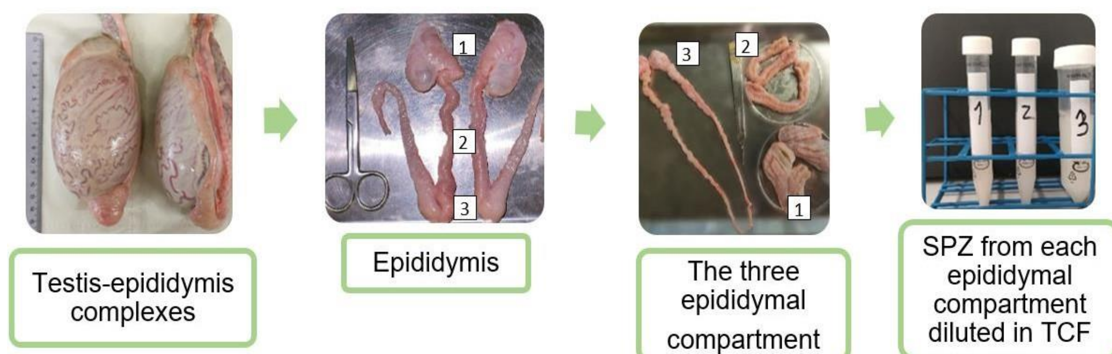


Figure 4.1 - Epididymal sperm collection from each epididymal compartment head, body and tail, 1,2 and 3, respectively.

Immediately after collection, volume of each sample was determined and all samples were evaluated for sperm concentration, total subjective motility (epididymal tail), viability and ΔΨm, according to methods described below.

4.2.2. Sperm concentration assessment

Sperm concentration was determined using a Neubauer counting chamber (MARIENFELD; 0640010). A coverslip was put on the Neubauer chamber central area

and then, approximately 20 µL of the sperm dilution (1:100 or 1:200) in distilled water, was used to fill in each of the Neubauer chambers. Both chambers were observed under a light microscope (Motic; BA210E, magnification 400x).

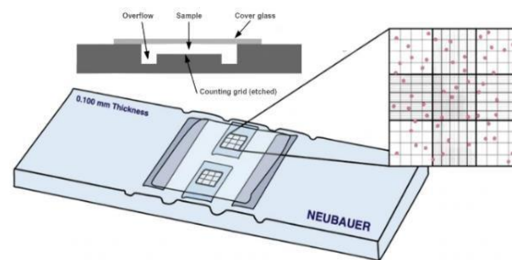


Figure 4.2 - Neubauer chamber used for sperm counting.

After determining sperm concentration, the total number of sperm present in each sample was calculated by multiplying the concentration with the respective volume. Subsequently, for each sample a total of $300x10^6$ sperm were pipetted into a new tube and diluted in TCF medium to obtain a final concentration of $30x10^6$ spz/ml, in a final volume of 10ml. These $30x10^6$ spz/ml sperm suspension solutions were kept at 37ºC until use for the *in vitro* ATZ or GLY exposure assays.

4.2.3. Total subjective motility assessment (tail)

Total subjective motility was assessed only in samples obtained from the epididymal tail. For that, a drop of 10 µL of the sperm suspension was placed in a slide kept at 37°C and covered with a cover slip maintained at the same temperature, and five fields were assessed in a light microscope (Motic; BA210E, magnification 400x). This analysis was carried out by the same user and the same criteria were applied to all the samples. Results are expressed as percentage of total subjective motile SPZ.

4.2.4. Viability assessment

Sperm viability was assessed using eosin-nigrosin (EN) stained slides smears. For that, 10 µL of sperm sample was gently mixed with 10uL of EN, and then spread across a microscope slide and allowed to dry on a heated plate (37ºC). Then, using a light microscope (Motic; BA210E, magnification 1000x), a total of 100 SPZ were counted per slide in continuous random fields, noting the 'dead' (stained) and 'live' (not stained) sperm (figure 4.3). The results were expressed as a percentage of life sperm.

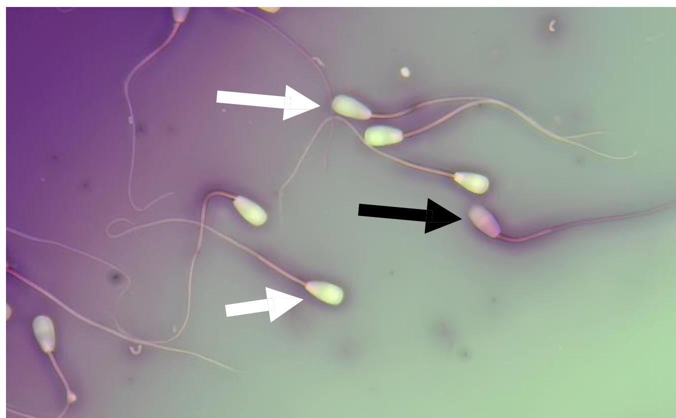


Figure 4.3 - Spermatozoa stained with eosin-nigrosin observed under a light microscope (x1000). White arrows: live sperm; Black arrow: dead sperm.

4.2.5. Fluorometric assessment of mitochondrial membrane potential (MMP) – ΔΨm

Mitochondrial membrane potential (ΔΨm) was evaluated using fluorimetric probe 5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide fluorescent probe (JC-1; ENZOBiochem, New York, NY, USA) as previously described by Carrageta et al, (157). To prepare the JC-1 reagent, a stock JC-1 dye solution (5 mg/ml in DMSO) was mixed with Pluronic® F-127 (InvitrogenTM, cat. no. P6867) solution, 10% (w/v) and filtered PBS solution (pH 7.4).

For this analysis, a total of $3x10^6$ sperm per sample were transferred to an eppendorf containing 500ul of PBS, previously warmed to 37ºC, and gently mix. Then samples were centrifuged for 5 minutes at 500 x g and the supernatant was carefully removed and discarded. The sperm pellet was immediately resuspended in 0,75 ml of JC-1 working solution and incubated for 30 min at 37°C, in the dark. As a positive control for the assay, a sample with the same sperm concentration was incubated in a 20% DMSO solution. After incubation, samples were washed twice by resuspend in 1ml of PBS, centrifuged (5 minutes at 500 x g) and gently discarding the supernatant. After discarding the last supernatant, sperm pellets were resuspended in 750 µl of PBS and gently mixed by pipetting. Subsequently 200 µl of each sample were transferred to a 96-well black microplates (FluoroNunc™ F96-MicroWell™ plate, with lid, sterile). Experiments were performed in triplicate per plate. Fluorescence analysis (JC-1 monomers - 485/530 nm and J-aggregates - 535/390 nm, Ex/Em respectively) was performed using the multi-well fluorescence plate reader Synergy™ H1 multi-mode (BioTek, Winooski, VT, USA,

wavelength range 250-700 nm). The average JC-1 aggregates J/monomer ratio was calculated as an indicator of MMP.

The monomeric form of JC-1 only accumulates in depolarized mitochondria with low MMP, emitting green fluorescence. In contrast, in mitochondria with high MMP, JC-1 forms aggregates, which emit red-orange fluorescence (154) (figure 4.4).

Figure 4.4 - JC-1 imaging in bull spermatozoa. Hoechst 33342 was used to stain the nuclei. (1) Monomers (green) or (2) J-aggregates (red) observed in the mitochondria of bull spermatozoa. (3) Merged images of both monomers and J-aggregates observed in the mitochondria of bull spermatozoa.

4.2.6. In vitro assays with Atrazine

Three stock solutions of ATZ (1000, 10000 and 100000 μM) were prepared in pure dimethyl sulphoxide (DMSO; Sigma-Aldrich; CAS number 67-68-5 C2H60S; lot # RNBH8641), and then frozen at -18°C. For each experiment, ATZ stock solutions (1000, 10000 and 100000 μM) were thawed to prepared 1, 10 and 100 μM working solutions, by adding TCF medium. All solutions were warmed to 37ºC.

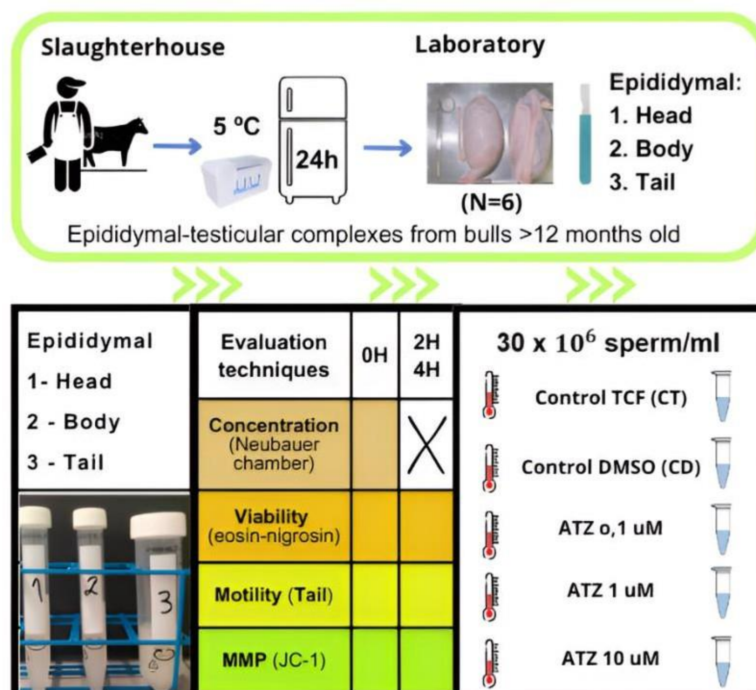


Figure 4.5 - Scheme of the ATZ experimental design. Abbreviations: ATZ - atrazine; CT - tris-citrate-fructose control; CD - dimethyl sulphoxide control; MMP - mitochondrial membrane potential.

The scheme of the ATZ experimental design is depicted in figure 4.5. Six pairs of epididymis were used to assess the effect of ATZ on epididymal sperm. For each experiment, three sets of five eppendorfs were prepared, one for each epididymal compartment (head, body, tail). In each set of eppendorfs, two were used as controls, and the remaining were used for the three ATZ concentrations groups 0.1, 1 and 10 µM. These concentrations are environmentally relevant and were based on studies with bulls (58; 59; 155; 156; 174). One of the controls consisted of only TCF medium and the other of a 0,01% DMSO diluted in TCF medium, corresponding to the maximum DMSO concentration in the experimental groups. In all experimental groups, $30x10^6$ sperm/ml were used for incubation with or without ATZ at 37°C for 2 hours and 4 hours.

After incubation for 2 and 4 hours, sperm parameters (total subjective motility (epididymal tail), viability and MMP) were reevaluated as described above.

4.2.7. In vitro assays with Glyphosate

Three GLY working solutions (50, 500 and 3600 mg/L) were prepared once by dissolving

directly in TCF medium, and then frozen at -18ºC. At the beginning of each experiment, aliquots of GLY working solutions (50; 500 and 3600 mg/L) were thawed and warmed to 37ºC.

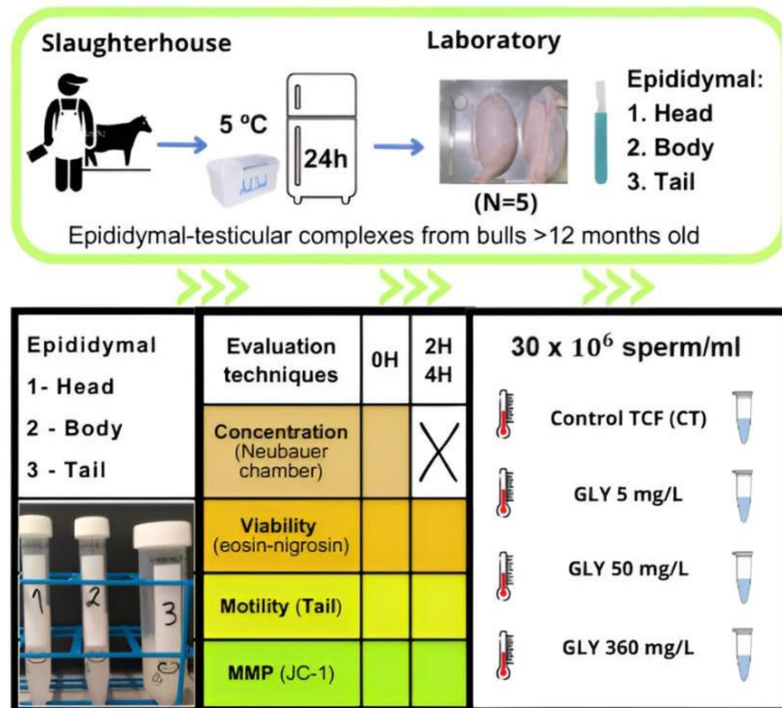


Figure 4.6 - Scheme of the GLY experimental design. Abbreviations: GLY - glyphosate; CT - tris-citrate-fructose control; MMP - mitochondrial membrane potential.

The scheme of the GLY experimental design is depicted in figure 4.6. Five pairs of epididymis were used to assess the effect of GLY on epididymal sperm. For each experiment, three sets of four eppendorfs were prepared, one for each epididymal compartment (head, body, tail). In each set of eppendorfs, one was used as control that consisted of only TCF medium, and the remaining were used for the three GLY concentrations groups 5, 50 and 360 mg/L. The concentrations used are biologically relevant and were selected based on data from studies carried out on humans and other animals (146, 149, 173, 175). In all experimental groups, $30x10^6$ sperm/ml were used for incubation with or without GLY at 37°C for 2 hours and 4 hours.

After incubation for 2 and 4 hours, sperm parameters (total subjective motility (epididymal tail), viability and MMP) were reevaluated as described above.

4.3. Statistical analysis

Epididymal sperm viability and motility data were analyzed using Statistica software (v. 14.1, Cloud Software Group, Inc. (2023) (157). An ANOVA model using pesticide concentration, time and their interaction as fixed effects was performed. Where differences were observed, post-hoc comparisons were made using the Scheffé test. Normality and homoscedasticity of the data were tested using the Shapiro-Wilk and Bartlett tests, respectively. Data are expressed as mean ± SD of percentages.

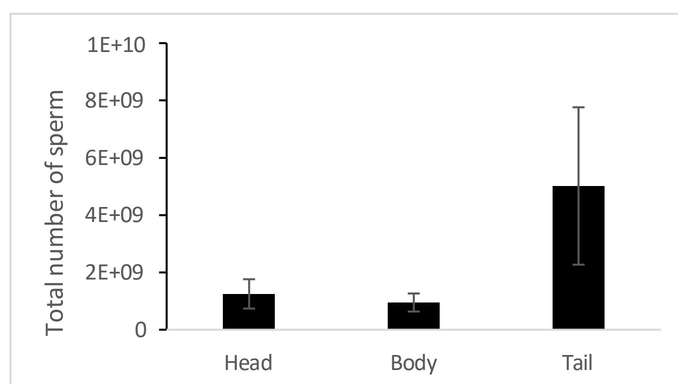
For all the remaining data the GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA) was used. Regarding the MMP data, all values in the fluorometric assessment for experiments were analyzed fold variation to the control (for ATZ control DMSO and for GLY control TCF) before analysis.

For all analyses, $P<0.05$ was considered significant.

5. Results

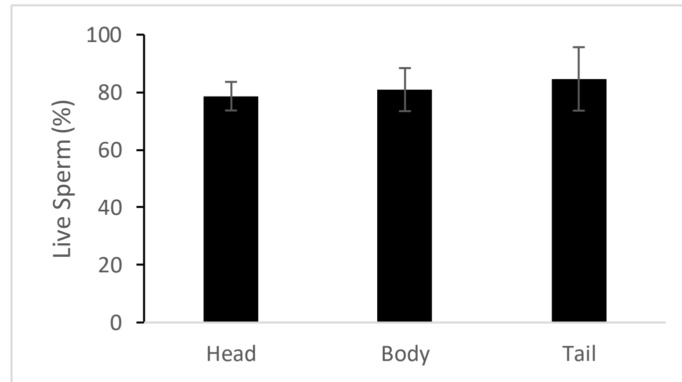
5.1. Epididymal sperm at collection

Overall, the mean total number of SPZ obtained from the epididymal head, body and tail of the 11 bulls was $1.2 \times 10^9 \pm 5.11 \times 10^8$, $9.50 \times 10^8 \pm 3.15 \times 10^8$ and $5.02 \times 10^9 \pm 2.75 \times 10^9$, respectively, and is shown in graphic 5.1. No differences were observed ($p>0.05$) between bulls used for the ATZ ($1{,}25 \times 10^9 \pm 5{,}96 \times 10^8$; $8{,}70 \times 10^8 \pm 3{,}23 \times 10^8$ and $4{,}38 \times 10^9 \pm 2{,}15 \times 10^9$, respectively; N=6) and the GLY ($1{,}25 \times 10^9 \pm 4{,}58 \times 10^8$; $1{,}05 \times 10^9 \pm 3{,}10 \times 10^8$ and $5{,}78 \times 10^9 \pm 3{,}43 \times 10^9$; respectively, N=5) experiments regarding the mean total number of sperm obtained from each epididymal compartment.



Graphic 5.1 - Mean total number of sperm collected from the different compartments of the epididymis (N=11 bulls). Data is presented as mean ± SD.

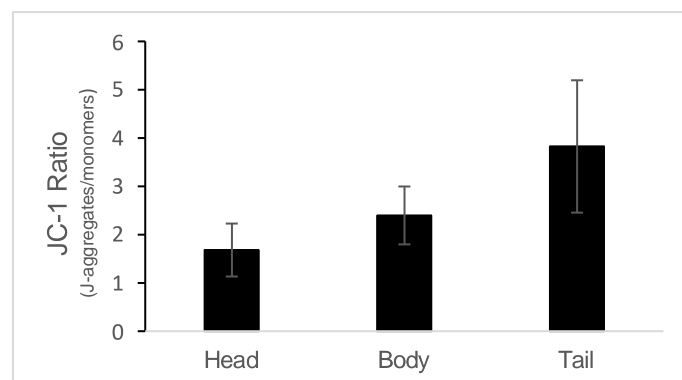
Regarding the viability of the SPZ collected from the epididymal head, body and tail, the mean percentages of live SPZ for the 11 bulls were 79 ± 5%, 81 ± 7% and 85 ± 11%, respectively, as shown in graphic 5.2. No differences ($p>0.05$) were observed between bull samples used for the ATZ (78 ± 6%; 78 ± 8% and 80 ± 12%; respectively, N=6) and the GLY (79 ± 4%; 85 ± 6% and 91 ± 5%; respectively, N=5) experiments regarding the percentage of live SPZ in each epididymal compartment.



Graphic 5.2 - Percentage of live epididymal spermatozoa immediately after collection from each epididymal compartment (N=11 bulls). Data is presented as mean ± SD.

The mean total subjective motility, only assessed for samples obtained from the epididymal tail, was 62 ± 17% (N= 11 bulls) and no differences (p>0.05) were observed between samples used for the ATZ (67 ± 19%; N=6) and GLY (56 ± 15%; N=5) experiments regarding this parameter.

Mitochondrial membrane potential (MMP) - ΔΨm of SPZ immediately after collection (graphic 5.3), for the 11 bulls, was lower in samples obtained from the epididymal head and body (1.70 ± 0.54 and 2.39 ± 0.60 J-aggregates/monomers, respectively) than from the tail of the epididymis (3.83 ± 1.37 J-aggregates/monomers), which is consistent with the increased maturation status of the sperm along the epididymis. Similar to the other sperm parameters described above, no differences (p>0.05) were observed between bull samples used for the ATZ (1,56 ± 0,34; 2,61 ± 0,71 and 3,60 ± 1,03 J-aggregates/monomers; respectively N=6) and the GLY (1,83 ± 0,75; 2,14 ± 0,36 and 4,1 ± 1,79 J-aggregates/monomers; N=5) experiments with regard to MMP at the time of collection.



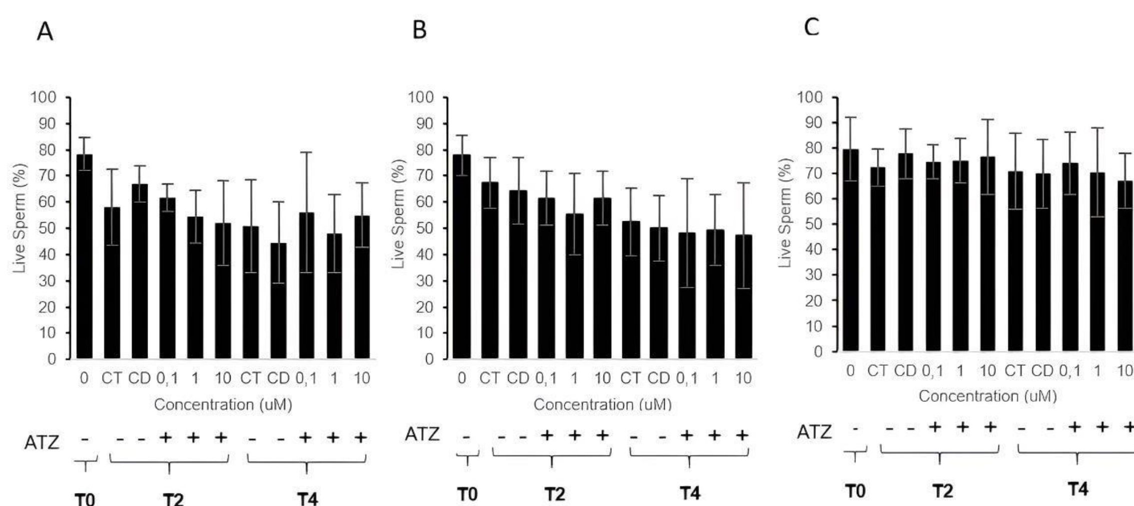
Graphic 5.3 - Mitochondrial membrane potential (MMP) - ΔΨm of spermatozoa immediately after collection from each epididymal compartment, as determined by JC-1. The data is presented as the average proportion of sperm stained red (high potential)/green (low potential).

5.2. Effects of Atrazine on bovine epididymal sperm

5.2.1. Sperm Viability and Total motility (tail)

Regarding sperm viability and total motility (tail), no interaction ($p>0.05$) was detected between time and treatment groups (CT, CD, 0,1, 1 and 10) in any of the epididymal compartments in the ANOVA model.

The maximum concentrations of the solvent used as a vehicle (0.01% DMSO) had no significant effect on any of the parameters examined when tested as a solvent effect control.



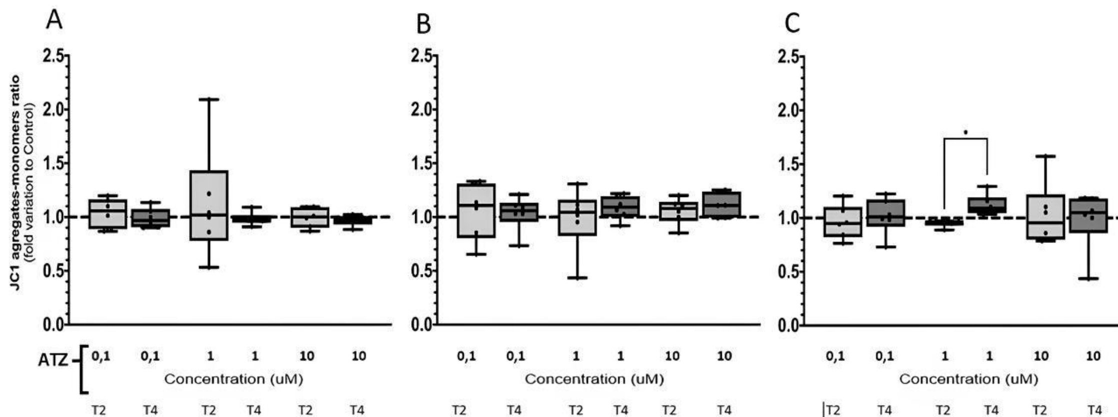
Graphic 5.4 - Effect of ATZ on epididymal sperm viability. Spermatozoa were isolated from epididymis compartments (head (A), body (B) and tail (C)) and incubated for 4 h (T0; T2 and T4) with 0.1, 1 or 10 µM ATZ. Data are presented as the mean ± SD. Abbreviations: CT (TCF- tris-citrate-fructose control); CD (DMSO-Dimethyl sulfoxide control).

In the three sperm compartments, no differences ($p>0.05$) were observed in the percentage of live SPZ between the experimental groups (CT; CD; 0,1; 1 and 10) (graphic 5.4). However, time (2 and 4 h) had a different effect on sperm viability depending on the epididymal compartment from which the sperm was obtained. Specifically, sperm viability from samples obtained from the epididymal head and body

was significantly higher (p<0.05) at 2 hours (59 ± 6%) and (62 ± 4%), respectively than at 4 hours (51 ± 5%) and (49 ± 2%), respectively, whereas no difference (p>0.05) was observed for the percentage of live sperm after 2 and 4 hours of incubation (75 ± 2% and 70 ± 2%, respectively) in epididymal tail samples.

Total sperm motility, evaluated only for epididymal tail samples, did not differ between the experimental groups (CT; CD; 0,1; 1 and 10). However, a significant decrease in total motility was observed between 2 and 4 hours of incubation (26 ± 5 and 20 ± 12%, respectively).

5.2.2. Mitochondrial Membrane Potential



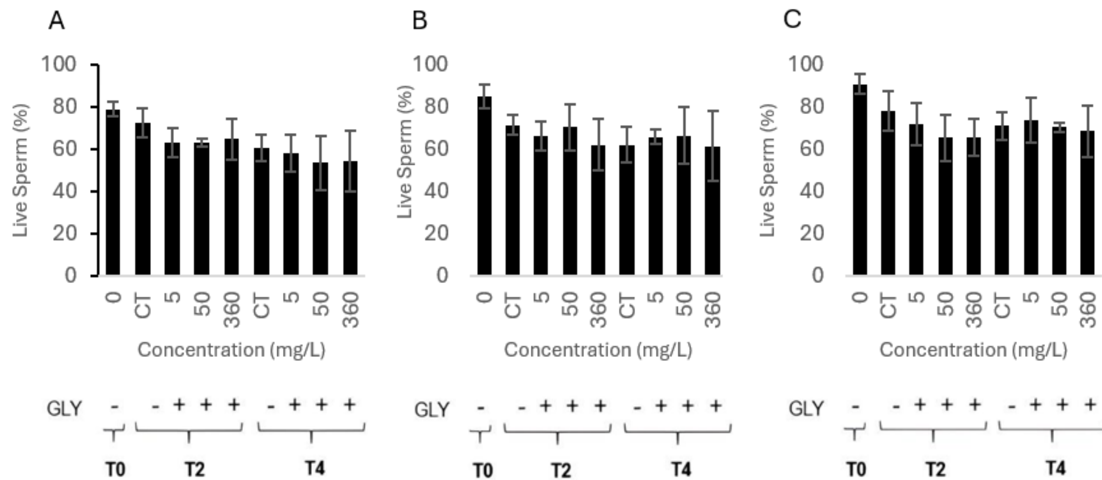
Graphic 5.5 - Mitochondrial membrane potential on sperm isolated from the epididymis exposure to atrazine (ATZ), as determined by JC-1. Spermatozoa were isolated from epididymis compartments (head (A), body (B) and tail (C)) and incubated for 2 and 4 h (T2; T4) with 0.1, 1 or 10 µM ATZ. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles 1.5 IQR).

Concerning the impact of different ATZ concentrations on the ΔΨm of sperm obtained from the different epididymal compartments no significant effect was observed (p>0.05). Still, a difference (p<0.05) between ATZ 1 from samples obtained from the epididymal tail at 2 and 4 hours of incubation was seen (0,95 ± 0,03 and 1,12 ± 0,1 J-aggregates/monomers, respectively) (graphic 5.5).

5.3. Effects of Glyphosate on bovine epididymal sperm

5.3.1. Sperm Viability and Total motility (tail)

In relation to sperm viability and total motility (tail), no interaction (p>0.05) was detected between time and the experimental groups (CT, 5, 50 and 360) in the ANOVA model in any of the epididymal compartments.

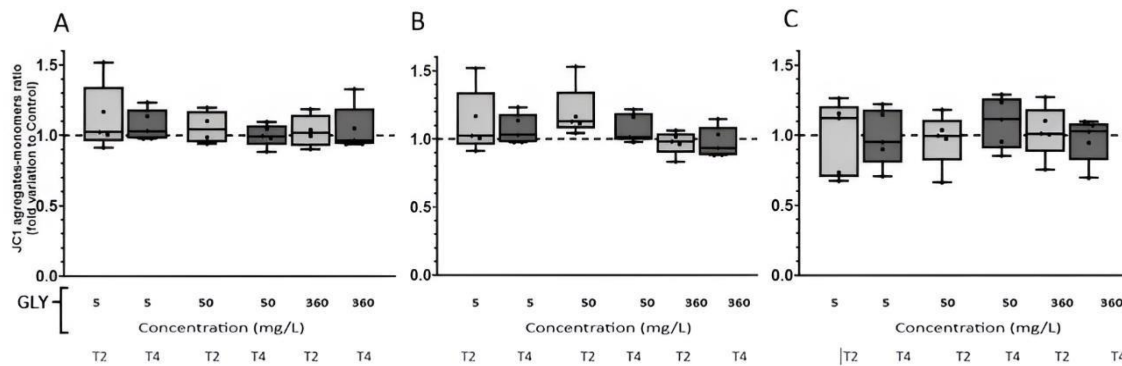


Graphic 5.6 - Effect of GLY on epididymal sperm viability. Spermatozoa were isolated from epididymis compartments (head (A), body (B) and tail (C)) (T0) and incubated for 2 and 4 h (T2; T4) without (CT) or with 5, 50 or 360 mg/L GLY dissolved in TCF. Data are presented as the mean ± SD. Abbreviations: CT (TCF-tris-citrate-fructose control).

In the three epididymal compartment, no differences (p>0.05) in the percentage of live sperm between experimental groups (CT, 5, 50 and 360) were observed (graphic 5.6). However, time (2 and 4 h) had a different effect on sperm viability depending on the epididymal compartment from where sperm cells were obtained. Specifically, sperm viability from samples obtained from the epididymal head was significantly higher (p<0.05) at 2 hours (66 ± 4%) than at 4 hours (57 ± 3%), while no difference (p>0.05) was observed for the percentage of live sperm after 2 and 4 hours of incubation in epididymal body (68 ± 4% and 64 ± 3%, respectively) and tail (70 ± 6% and 71 ± 2%, respectively) sperm samples.

Total sperm motility, evaluated only for epididymal tail samples, did not differ between experimental groups (CT, 5, 50 and 360). Nevertheless, a significant reduction in total motility was observed between 2 and 4 hours of incubation (23 ± 3 and 13 ± 3%, respectively).

5.3.2. Mitochondrial Membrane Potential



Graphic 5.7 - Mitochondrial membrane potential on sperm isolated from the epididymis exposure to glyphosate, as determined by JC-1. Spermatozoa were isolated from epididymis compartments (head (A), body (B) and tail (C)) and incubated for 2h and 4 h (T2; T4) with 5, 50 or 360 mg/L GLY. Results are expressed as Tukey's boxplot (median, 25th to 75th percentiles 1.5 IQR).

Concerning the impact of different GLY concentrations on the ΔΨm of sperm obtained from the different epididymal compartments after incubation for 2 and 4 hours, no significant effect was observed (p>0.05) (graphic 5.7).

6. Discussion

Although the adverse health and environmental effects of pesticides are well known, their use continues to increase worldwide. Atrazine and glyphosate are prominent examples of herbicides that are widely used on a wide variety of crops and have become ubiquitous in the environment. Despite its effectiveness in controlling weeds, ATZ is persistent in water, even in areas where it hasn't been used for many years. This raises environmental and health concerns, as ATZ is classified as an endocrine disruptor. GLY is often used indiscriminately and negligently and has been detected at high concentrations in various environmental matrices, such as soil, air and water (4,120).

The aim of this study was to assess the effects of ATZ and GLY individually on total subjective motility, viability and ΔΨm in bovine epididymal cells. To investigate the impact of these herbicides on the reproductive capacity of bulls, whose seminal quality is crucial for fertility and productivity, an in vitro methodology was employed, thus avoiding the use of animal experimentation. There has been a notable increase in interest in reproductive studies on the adverse effects of these phytochemicals. However, the use of epididymal sperm remains a relatively under-explored area. These cells are highly susceptible to external influences, rendering them sensitive indicators of the effects of ATZ and GLY (62;66).

Recent research into animal and human sperm physiology highlights the growing importance of mitochondria as a crucial biomarker for assessing sperm health and fertility (158,159). ΔΨm is crucial for assessing the function of mitochondria, reflecting their energetic state. In humans, a directly proportional relationship was observed between impaired mitochondrial function and decreased sperm motility (144,160,161). Espinoza et al found a correlation between ΔΨm and sperm motility and viability (158). It seems that the mitochondria of sperm from the head of the epididymis are inactive, unlike those of sperm from the tail of the epididymis, which are polarized. This is associated with the passage of sperm along the epididymis and the activation of mitochondria (162).

As expected, a much higher number of SPZ were obtained from the tail of the epididymis, followed by the head and finally the body of the epididymis, in the total of 11 bulls. In general, this distribution of the number of SPZ collected from the head, body and tail of the epididymis follows what has been previously described by several studies in bovine species, which reported that the cauda epididymis contained 46%, the corpus 18% and

the caput 36% of the total SPZ counted in the epididymis (163). The cauda epididymis is the major sperm storage organ, sperm numbers within this segment are indicative of sperm reserves immediately available for ejaculation.
The viability of SPZ and the success of fertilization depend on the integrity and correct functioning of their plasma membranes. These membranes ensure a balance between the sperm's internal and external environment, and damage to the plasma membrane disrupts this, leading to a deterioration in the sperm's health, a reduction in its ability to fertilize and, ultimately, cell death. Several other studies have reported on the epididymal sperm viability of the bull, using the same technique as in this study, and in general the values of the percentage of live sperm are similar to those obtained in this study (164–166). In addition, the percentage of live sperm collected from each epididymal compartment, of the 11 bulls, were very similar.

Evaluation of sperm motility is an essential parameter, as it measures the sperm's ability to move efficiently and in the right direction to fertilize the oocyte. However, because immature SPZ from the head and body of the epididymis are immotile or present very limited ability to move after dilution, sperm motility was only evaluated for epididymal tail spermatozoa in this research. In the bovine species, some studies have been carried out evaluating the motility of SPZ from the tail of the epididymis, and in general the values are like those obtained in our study (164; 166). It is important to note that the decrease in motility over time is expected, since SPZ, especially mammalian SPZ, are highly sensitive to ex vivo conditions and require exogenous substrates, such as glycolysable sugars, to maintain intracellular energy, cellular components and support motility. Thus, in this study, TCF medium was used, which contains fructose, which is found in seminal plasma and is considered an important source of energy for mammalian SPZ.

In addition, sperm motility is directly related to the transformation and spending of energy produced in the mitochondria. As such, is has been reported that immature SPZ from the caput epididymis, that are immotile, displayed a low $\Delta\Psi m$ (MMP) whereas mature SPZ from the caudal epididymis, present motility after dilution, actively maintain a high MMP. Similarly, in this study, $\Delta\Psi m$ of SPZ immediately after collection for the 11 bulls, was lower in samples obtained from the epididymal head and body than from the tail of the epididymis which is consistent with the increased maturation status of the sperm along the epididymis (167).

Of the 11 TECs used for collection and evaluation of epididymal SPZ, 6 pairs were used for the ATZ assay and 5 pairs for the GLY assay, but no differences were found between

the groups of TEC use for each pesticide with respect to sperm motility (tail), viability and ΔΨm, thus allowing for a more uniform sample.

6.1. Effects of Atrazine on bovine epididymal sperm

Atrazine frequently contaminates soil, groundwater, rivers and lakes, and has been detected between 0.1 and 600 µg/L for water samples and from 1.0 to 700 µg/kg for soil samples (168). This study used three different concentrations of ATZ (0.1, 1 and 10 µM) considered to be environmentally relevant.

The data obtained indicated that the viability of SPZ isolated from the various epididymal compartments was not affected by the incubation with 0.1, 1 and 10 µM ATZ for up to 4 hours. However, regardless of ATZ concentration, a reduction in the proportion of live SPZ was noted between 2 and 4 hours of incubation for SPZ isolated from the epididymal head and body, but not from the epididymal tail. According to Komsky-Elbaz et al, incubation of bovine epididymal sperm, with identical ATZ concentrations to those used in our study, resulted in a higher proportion of dead sperm from the epididymal body when exposed to 10 µM ATZ for a period of 2 hours than in the control group (without solvent). Moreover, in the same study, sperm from the epididymal tail presented a higher proportion of dead sperm for all ATZ concentrations, after 2 hours of incubation, than the control group. However, no differences in this parameter were observed for sperm obtained from the epididymal head after 2 hours incubation neither for samples from any epididymal compartment after 4 hours (59). The results from the two studies cannot be directly compared as there are significant differences in the methodology employed in each. In our study, TCF was employed as the medium for sperm incubation, with DMSO used as the solvent for ATZ (in accordance with the supplier's recommendations). In contrast, the study by Komsky-Elbaz et al. utilized mTALP medium and absolute EtOH for the same purposes. Moreover, different techniques were employed to assess sperm viability, namely the EN staining and the propidium iodide fluorescent probe, respectively. These techniques have been shown to present different abilities to identify non-viable SPZ. As such, the observed discrepancies in the results may also be attributed to these methodological differences. In another study, this parameter was assessed in capacitated sperm from boar ejaculate after exposure to ATZ (0; 8; 20 and 40 uM) for 5 hours, and they found that viability decreased linearly with increasing ATZ concentration (111). Also, in this species, fresh semen was exposed to high

concentrations of ATZ (0, 50, 100 and 500 µM) for 1 hour and there was no correlation between sperm viability and exposure to the different concentrations (112). Considering all these studies, including the present one, one factor that could potentially be related to these differences seems to be the time of exposure to ATZ.

The current study found no effect of ATZ (0,1, 1, 10 µM) on total subjective sperm motility following incubation of samples from the cauda epididymis for up to four hours. However, a notable decline in this parameter was observed between the two tested incubation periods (2 h vs. 4 h). Nowadays, most studies that assess sperm motility use computer-assisted sperm analysis (CASA), as it is a more objective approach than the traditional subjective motility assessment used in this study. In addition, CASA also provides a battery of kinematic quantitative parameters that characterize sperm cell motility in greater detail than just its motility and progressivity. This could be particularly beneficial for the assessment of epididymal sperm samples, which typically exhibit lower progressive motility than ejaculate sperm. In this context, a previously mentioned study used this methodology but for ejaculate sperm and found that ATZ at concentrations of 100 and 500 uM strongly affected the progressive motility of boar sperm (112).

Although the relationship between ATZ and the mitochondrial respiratory chain in sperm is not well studied, it seems to be affected by this herbicide particularly in relation to the observed reduction in motility following exposure to this compound (112). In our study, a difference was observed between the 2 and 4 hours of incubation of samples obtained from the cauda epididymis with 1 µM ATZ. However, the existing data is somewhat contradictory, with some studies showing that high concentrations of ATZ (100 µM) are required to induce mitochondrial dysfunction, while others have shown that lower concentrations, such as 1 µM ATZ for 4 hours incubation, are sufficient in bovine epididymal SPZ, a finding supported by our study (59,170). A study carried out with the zebrafish model exposed to 2, 10 and 100 µg/L of ATZ for 11 days found that it decreased both the motility and mitochondrial functionality of the sperm at all concentrations (171).

Feeding male goats 15 mg ATZ/kg body weight daily for 6 months led to altered morphology and impaired viability. In addition, the $\Delta\Psi m$ of both ejaculated and epididymal sperm was altered, as there were changes in the lipid composition of the membrane (19).

A study that exposed mouse Sertoli cells to ATZ (0.3, 3, 30, 300 or 3000 µg/L), after 24 hours observed no changes in $\Delta\Psi m$ at all concentrations tested (26).

6.2. Effects of Glyphosate on bovine epididymal sperm

The GLY values selected for this study are based on relevant environmental concentrations, i.e. in water, 5153 ug/L of this herbicide has been detected (130). However, it is known that the concentrations used in agriculture are much higher (146).

As was the case with ATZ in this study, GLY did not seem to affect the epididymal sperm under the tested conditions. In addition, only sperm samples obtained from the head of the epididymis showed a significant decrease in the percentage of live sperm between 2 and 4 hours of incubation. Likewise, total subjective motility (epididymal tail) only showed a significant decrease over the exposure time.

To our best knowledge, the present study is the first to assess the effects of GLY on viability, motility and ΔΨm in sperm from the epididymis of bulls. Many studies available in the literature test Roundup, which is a commercial formulation made up of GLY and adjuvants that increase the effectiveness of this active compound, and Roundup has already been found to be more toxic than pure GLY itself (149).

Exposure of ejaculated cryopreserved bull sperm to 0.72, 7.2 and 360 mg/L of RoundUp (in equivalent concentrations of glyphosate) significantly affected sperm viability after 30 minutes of incubation with the two highest concentrations (7.2 and 360 mg/L) and in all of three concentrations, after 90 minutes. In the same study, sperm total motility, showed decreased after 30 minutes only in the highest concentration, although this also occurred after 90 minutes with exposure to 7.2 mg/L (172). These results are discrepant from those obtained in the present study, even though the same species was used in both studies. However, differences in susceptibility to membrane damaged between cryopreserved ejaculated sperm and fresh epididymal sperm might explain these discrepancies. In other species, such as humans, Anifandis et al, found a lower progressive motility in fresh ejaculated sperm exposed to 0.36 mg/L of GLY after 1h of incubation than in the control sample without GLY (144). The first study, which tested the effects of 0, 5, 25, 50, 100 and 360 ug/ml of GLY in wild boar ejaculate, found a significant decrease in viability, total motility and mitochondrial activity after 1h and 3h of incubation with 360ug/ml of GLY (149). The results in these studies are also in contrast to the present study where no significant changes in those parameters were observed. This discrepancy may be due to a species-specific difference to GLY exposure and/or may

also be attributable to the different procedures for assessing sperm motility, for example, using a CASA system in the study with bull sperm (171).

ΔΨm was also assessed in sperm from the three compartments of the epididymis. However, compared to the control, there were no significant differences between the doses of GLY in any of the incubation time. In the boar study mentioned above, mitochondrial activity after 1 h of incubation with Roundup at concentrations ≥ 25 µg/mL of GLY-equivalent concentration, was significantly reduced ($p < 0.05$). In addition, exposure to Roundup corresponding to ≥ 50 or more µg/mL of GLY-equivalent decreased the percentage of spermatozoa with high MMP after 3 h of incubation (149). According to the only in vitro experiment conducted so far, incubating human sperm with 1 mg/L of Roundup (corresponding to a GLY concentration of 0.36 µg/L) for 1 h causes a depletion in mitochondrial activity (145).

7. Conclusions

In recent years, the harmful effects of ATZ and GLY on various cell models have become a growing concern. These chemicals, ubiquitously present in the environment and in food, have been associated with various adverse outcomes in human and animal health. Despite their known reproductive toxicities, little is known about their specific impact on epididymal SPZ. This study seeks to address this critical knowledge gap by investigating the effects of environmentally relevant concentrations of ATZ and GLY on viability and motility, as well as on $\Delta\Psi m$.

In this study, we showed that exposure to different concentrations of ATZ or GLY did not interfere with viability or total subjective motility, only variations over time were observed. However, $\Delta\Psi m$ seems to be compromised in sperm from the cauda epididymis when exposed to 1 uM ATZ from 2h to 4h. Contrary, no changes in $\Delta\Psi m$ were observed for any GLY group.

These findings suggest that the conventional markers may not be sufficient to detect subtle or early effects of ATZ or GLY on epididymal spermatozoa. To address this limitation, additional markers are being studied to provide a more comprehensive assessment of ATZ or GLY effects. Specifically, metabolomic analysis uses NMR to reveal alterations in biochemical pathways and identify potential biomarkers of early ATZ or GLY toxicity. Furthermore, oxidative stress levels induced by ATZ or GLY exposure may be pivotal as it is a critical factor in sperm health. This approach will enhance our understanding of ATZ's or GLY's impact on reproductive health and contribute to the development of more sensitive and specific biomarkers for assessing environmental toxicants in agricultural settings.

8. Limitations and future goals

The research has some limitations that should be considered in future studies.

The small number of samples and the dependence on slaughterhouses may explain the large variation observed in some analyses.

A longer investigation would make it possible to complete other planned analyses, such as metabolic analysis by NMR, assessment of oxidative stress by SLOT-BLOT and antioxidant potential by FRAP. Further studies are needed to elucidate the underlying biochemical mechanisms to understand how these herbicides damage sperm.

In the case of the herbicide ATZ, its rapid metabolization in the body results in several metabolites, which suggests that the effect of these metabolites cannot be excluded. It is therefore essential to carry out future studies with a greater number of samples, also focusing on the impact of specific metabolites, such as DACT, when assessing the impact on spermatozoa.

In addition, research assessing the energy metabolism of bovine sperm could make a significant contribution to understanding the possible causes of reduced sperm quality and fertilization failures related to these metabolic pathways. This could, in turn, improve existing biotechnologies, such as artificial insemination, resulting in higher fertility rates.

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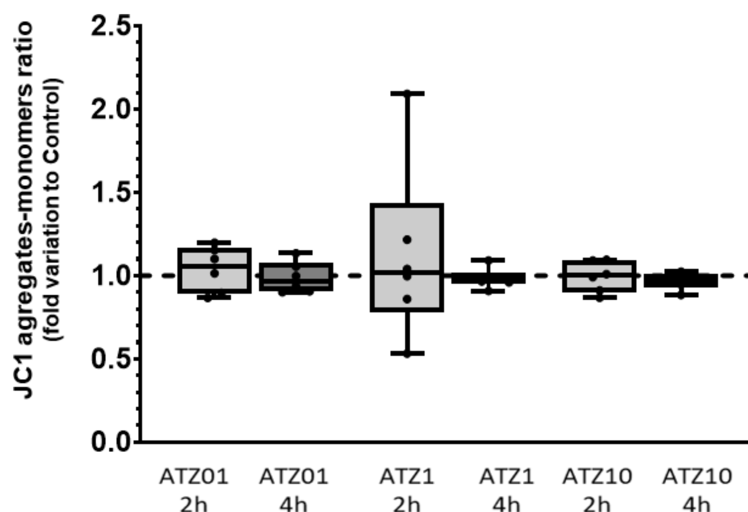
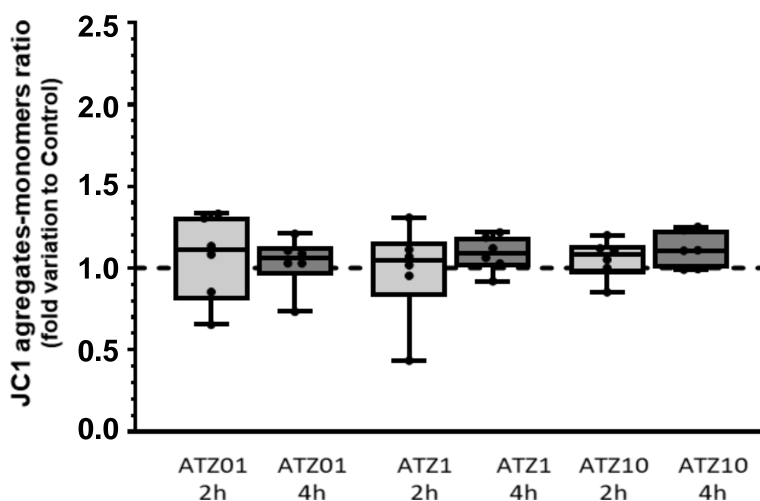
10. Attachments

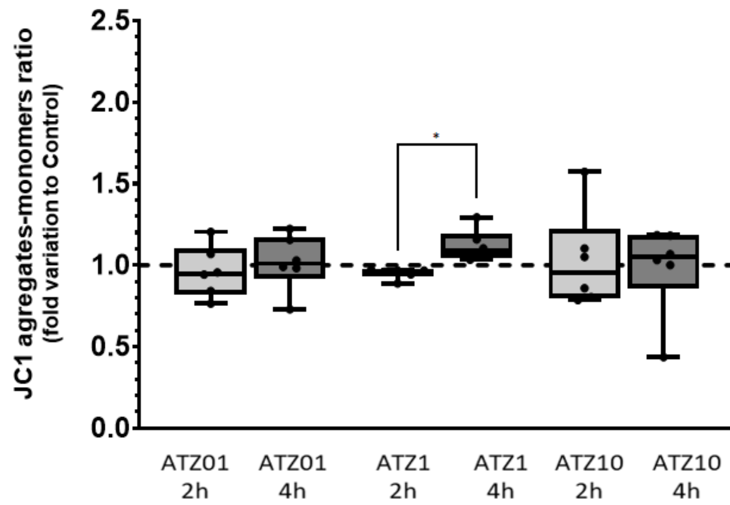
APPENDIX I - Graphs of mitochondrial membrane potential results in the three compartments of the epididymis after exposure to atrazine and glyphosate.



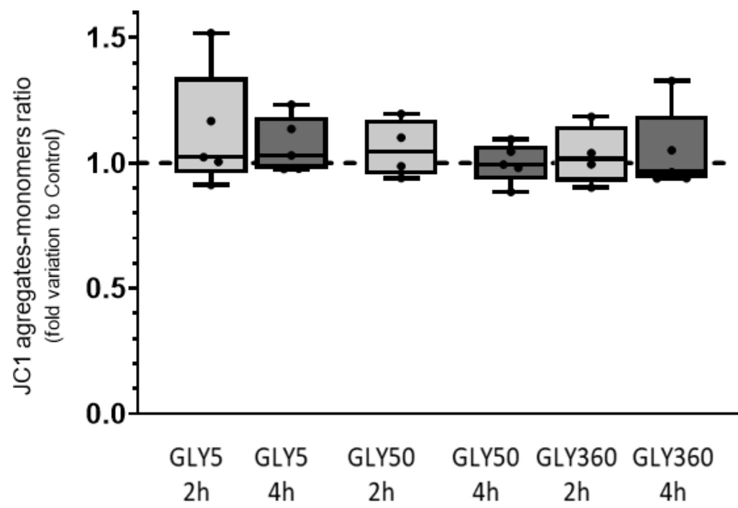
Graphic 5.5. Mitochondrial membrane potential on sperm isolated from the epididymis exposure to atrazine (ATZ), spermatozoa were isolated from head of epididymis.



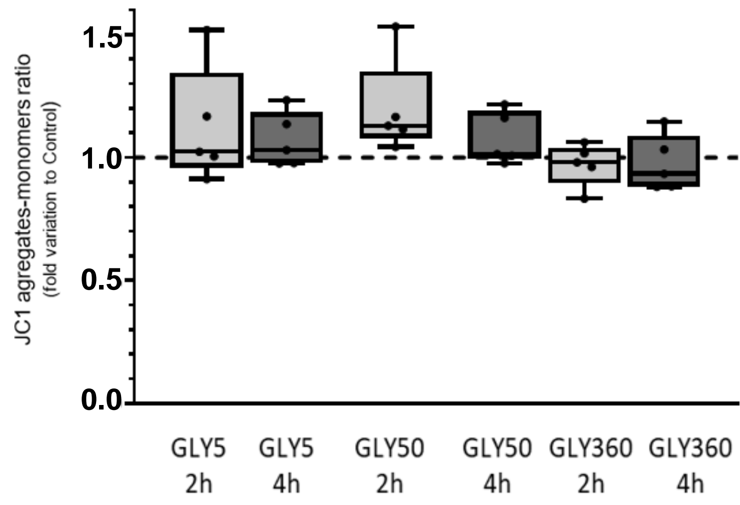
Graphic 5.5. Mitochondrial membrane potential on sperm isolated from the epididymis exposure to atrazine (ATZ), spermatozoa were isolated from body of epididymis.



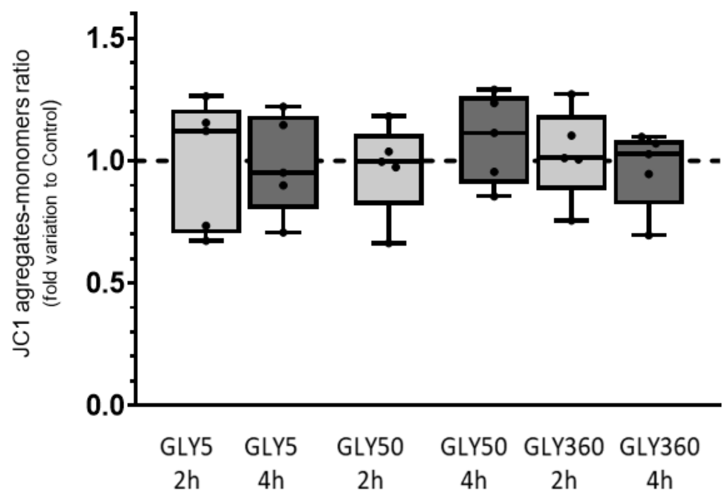
Graphic 5.5. Mitochondrial membrane potential on sperm isolated from the epididymis exposure to atrazine (ATZ), spermatozoa were isolated from tail of epididymis.



Graphic 5.7. Mitochondrial membrane potential on sperm isolated from the epididymis exposure to glyphosate (GLY), spermatozoa were isolated from head of epididymis



Graphic 5.7. Mitochondrial membrane potential on sperm isolated from the epididymis exposure to glyphosate (GLY), spermatozoa were isolated from body of epididymis.



Graphic 5.7. Mitochondrial membrane potential on sperm isolated from the epididymis exposure to glyphosate (GLY), spermatozoa were isolated from tail of epididymis.

APPENDIX II - Authorization to collect biological material - testicle-epididymis complexes

Assunto: Pedido de autorização para recolha de SPOA no IZICAR para fins educativos e investigação - ICBAS-UP

Exmos. Senhores,

Encarrega-me a Senhora Diretora de Serviços de Alimentação e Veterinária da Região Norte, Mestre Elsa Machado de informar:
em resposta ao solicitado no V. email de 11/03/2024, relativamente ao pedido de autorização para a recolha, transporte e utilização de subprodutos animais de bovino de categoria 3, **durante o ano de 2024**, provenientes da unidade de abate Izicar-Fábrica de Produtos Porcinos, Lda., com NCV N 04, sito na Travessa das Regadas, n.º 184 4770-459 Vila Nova de Famalicão, nomeadamente:

- 20 úteros (4 recolhas entre Julho/Setembro e Novembro/Dezembro) - Instituto de Ciências Biomédicas Abel Salazar (N.12.006.UDER) - fins educativos;
- ovários (até 30/recolha) e testículos (2 pares) uma vez por semana - Centro Clínico e de Investigação Veterinária de Vairão (N128064SPA) - fins de investigação;

informa-se V.ª Ex.ª, que ao abrigo do disposto no Artigo 17.º do Regulamento (CE) n.º 1069/2009 de 21 de outubro, é autorizado o manuseamento e utilização de subprodutos animais da categoria 3, destinados a fins educativos e de investigação desde que, para garante do controlo dos riscos para a saúde pública e animal, sejam cumpridas as seguintes condições:

- O operador dos subprodutos animais para fins de investigação deve tomar todas as medidas necessárias para evitar a propagação de doenças transmissíveis aos seres humanos ou aos animais durante o manuseamento das matérias sob a sua responsabilidade, sobretudo através da aplicação de boas práticas de laboratório;
- É proibida qualquer utilização subsequente dos subprodutos animais para outros fins que não o exame no âmbito das atividades autorizadas;
- O transporte até ao destino final deve ser efetuado em embalagem, veículo ou

contentor adequado para o efeito e identificados com a menção «Destinados à investigação e ao diagnóstico»;

- Durante o transporte, desde o local de origem até ao destino final no território nacional, os subprodutos animais devem ser acompanhados da guia de acompanhamento de subprodutos animais, modelo 376/DGAV, de acordo com os pontos 1 e 2 do Despacho n.º 8442/2017 de 26 de setembro;
- Obrigação de eliminar os subprodutos animais ou produtos derivados com segurança;
- A menos que sejam conservadas para efeitos de referência, as amostras para diagnóstico e investigação, e quaisquer produtos derivados da utilização dessas amostras, devem ser eliminados:
  - Como resíduos, por incineração ou coincineração;
  - No caso dos subprodutos animais ou produtos derivados referidos no artigo 8.º, alínea a), subalínea iv), no artigo 8.º, alínea c) e alínea d), no artigo 9.º e no artigo 10.º do Regulamento (CE) n.º 1069/2009 que fazem parte de culturas de células, kits de laboratório ou amostras de laboratório, através de um tratamento em condições que são pelo menos equivalentes ao método validado para autoclaves a vapor[1] e subsequente eliminação como resíduos ou águas residuais, em conformidade com a legislação pertinente da União;
  - Por esterilização sob pressão e subsequente eliminação ou utilização, em conformidade com os artigos 12.º, 13.º e 14.º do Regulamento (CE) n.º 1069/2009.

- O utilizador deve proceder a um registo datado dos subprodutos animais utilizados, que deve especificar a descrição das matérias, espécie animal, categoria, quantidade, data, local de origem, nome do expedidor, nome do utilizador e método de eliminação das amostras e de quaisquer produtos derivados.

Mais se informa que, nos termos do disposto na alínea a), n.º 1, Artigo 23.º do Regulamento (CE) n.º 1069/2009 de 21 de outubro, foi atribuído ao Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto, com sede social em Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto o número de registo N.12.006.UDER, como utilizador de subprodutos animais da categoria 3 para fins de Fins de Diagnóstico, Educativos e Investigação.
Ainda, nos termos do disposto na alínea a), n.º 1, Artigo 23.º do Regulamento (CE) n.º 1069/2009 de 21 de outubro, informa-se que foi atribuído ao Centro Clínico e de

Investigação Veterinária de Vairão (CCIVV)- ICBAS, UP, com sede social na Rua da Braziela, nº 100 4485-144 Vila do Conde o número de registo N128064SPA, como utilizador de subprodutos animais da categoria 3 para Fins de Diagnóstico, Educativos e Investigação.
O operador deverá facultar informações atualizadas de quaisquer alterações significativas à atividade ou encerramento, de acordo com o ponto 2 do artigo 23.º do regulamento comunitário supracitado.

Recomenda-se ainda, uma vez que se trata de recolhas efetuadas ao longo do ano, a disponibilização da presente autorização a qualquer elemento do Corpo de Inspeção Sanitária que a solicite.

[1] CEN TC/102 - Esterilizadores para fins médicos - EN 285:2006 + A2:2009 - Esterilização – Esterilizadores a vapor – Grandes esterilizadores; referência publicada no JO C 293 de 2.12.2009, p. 39.

GD 31492/24-V

Cumprimentos,
Marta Cunha
Médica Veterinária
Técnica Superior da Direção de Serviços de Alimentação e Veterinária da Região Norte

APPENDIX III - Authorization from the Ethics Council of the Lisbon School of Health Technologies

REFERÊNCIA INTERNA DO PROJETO: CE-ESTeSL-Nº 64-2023– Adriana Andrade Pereira

TÍTULO DO PROJETO: Efeito da Atrazina e do Glifosato na função e viabilidade de espermatozoides epididimários de cão

TIPO de Projeto/Estudo: Dissertação mestrado

INVESTIGADOR: Adriana Andrade Pereira

ORIENTADOR(ES): Edna Ribeiro e Maria da Graça Cunha Antunes Lopes

EQUIPA: Marco Aurélio Gouveia Alves

INSTITUIÇÃO PROMOTORA: ESTeSL-IPL

INSTITUIÇÃO(ÕES) ENVOLVIDA(S): ESTeSL-IPL; ICBAS - Universidade do Porto; Universidade de Aveiro

SUBMISSÃO do PROJETO: 28 outubro 2023

RESPOSTA CE-ESTeSL: 27 novembro 2023

RESPOSTA: 1 dezembro 2023

Exma. Senhora Professora Dra. Edna Ribeiro

Exma. Senhora Professora Dra. Maria da Graça Cunha Antunes Lopes Exma. Senhora Dra. Adriana Andrade Pereira

Após os esclarecimentos, a Comissão de Ética da Escola Superior de Tecnologia da Saúde de Lisboa (CE-ESTeSL) aprovou por unanimidade a emissão de parecer favorável.

O presente parecer tem em consideração a versão submetida do projeto e demais documentação enviada. Eventuais alterações nestes documentos determinam a necessidade de revisão do presente parecer. Lembramos que todos os estudos que envolvem a autorização dos participantes e a recolha de amostras e dados

anonimizados e/ou codificados têm de cumprir com o estabelecido no Regulamento Geral sobre a Proteção de Dados de 27 de abril de 2016. Por último, solicita-se que, ao abrigo do artº 19 da Lei 21/2014 de 16 de abril e do disposto no nº 23 da atual versão da Declaração de Helsínquia, seja dado conhecimento à CE-ESTeSL do relatório final, com as conclusões do estudo, bem como de eventuais alterações ao protocolo de investigação e demais informações tidas por relevantes.

Aproveitamos ainda para desejar o maior sucesso no desenvolvimento deste trabalho.

Com os melhores cumprimentos,
Rute Borrego