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Impacto de tiodicarbe na espermatogénese em células murinas de Leydig: um estudo in vitro

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Impact of thiodicarb in spermatogenesis murine Leydig cells: An In Vitro Study

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica do Doutor Pedro F. Oliveira, Professor Auxiliar do Departamento de Química da Universidade de Aveiro e da Doutora Maria de Lourdes Gomes Pereira, Professora Associada com Agregação do Departamento de Ciências Médicas.

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palavras-chave Administração de tiodicarbe; Infertilidade masculina; Células de Leydig BLTK-1; Citotoxicidade;Apoptose; Produção de androgénios

resumo A infertilidade masculina constitui um problema de saúde crescente que afeta aproximadamente 6% dos homens adultos, contribuindo para até 50% dos casos de infertilidade. Enquanto uma parte significativa da infertilidade masculina permanece inexplicada, destacando a necessidade de investigações abrangentes, existem inúmeros fatores conhecidos por contribuir para a infertilidade masculina, incluindo condições genéticas, anomalias congénitas, distúrbios endócrinos, causas obstrutivas, infeções, problemas vasculares, condições neoplásicas, fatores ambientais, e estilo de vida. Os carbamatos, um grupo de compostos que inclui o tiodicarbe, foram identificados entre esses fatores ambientais, exibindo efeitos adversos na fertilidade masculina ao alterar as funções das células de Leydig e dos espermatozoides. Vários estudos também demonstraram alterações substanciais nos tecidos testiculares após a exposição aos carbamatos, enfatizando ainda mais sua toxicidade reprodutiva. Este estudo investiga o impacto do tiodicarbe, um composto com potenciais efeitos significativos na fertilidade masculina, com foco na sua influência na função das células de Leydig e na produção de androgénios. Utilizámos as células de Leydig BLTK-1 como nosso modelo para investigar essa possível ligação. Inicialmente, examinamos as repercussões do aumento das concentrações de tiodicarbe (até 500 µM) na proliferação celular e na viabilidade. Resultados mostraram que concentrações mais altas de tiodicarbe, especialmente a 500 µM, provocam efeitos citotóxicos, levando à diminuição da proliferação e da viabilidade das células de Leydig. Em seguida, exploramos se o tiodicarbe desencadeia a morte celular nas células de Leydig, revelando uma atividade elevada da caspase 3, mostrando seu potencial como indutor de apoptose para este sistema celular. Curiosamente, o mecanismo pelo qual o tiodicarbe promove a apoptose parece se desviar da via BAX/BCL-2 associada à apoptose intrínseca. Notavelmente, a exposição das células de Leydig ao tiodicarbe a uma concentração de 500 µM resulta numa redução significativa na produção de androgénios, uma função fundamental dessas células. Essas descobertas destacam a necessidade imperativa de pesquisas mais aprofundadas para desvendar a toxicidade reprodutiva do tiodicarbe e suas amplas implicações para a fertilidade masculina, saúde pública e diretrizes regulatórias que governam o uso de tais compostos de carbamato em diversas aplicações e indústrias. Além disso, compreender as intrincadas vias celulares afetadas pelo tiodicarbe abrirá caminho para intervenções direcionadas e o desenvolvimento de medidas preventivas para mitigar os seus potenciais efeitos deletérios na saúde reprodutiva masculina.

keywords

Administration of Thiodicarb; Male infertility; BLTK-1 Leydig cells; Cytotoxicity; Apoptosis; Androgen production.

abstract

Male infertility constitutes a growing health issue affecting approximately 6% of adult males, contributing to up to 50% of infertility cases. While a notable portion of male infertility remains unexplained, underscoring the need for comprehensive investigations, there is a myriad of factors known to contribute to male infertility, encompassing genetic conditions, congenital anomalies, endocrine disorders, obstructive causes, infections, vascular issues, neoplastic conditions, lifestyle, and environmental factors. Carbamates, a group of compounds including thiodicarb, have been placed among those environmental factors, exhibiting adverse effects on male fertility by altering Leydig cell and spermatozoa functions. Several studies have also illustrated substantial testicular tissue alterations following exposure to carbamates, further emphasizing their reproductive toxicity. This study investigates the impact of thiodicarb, a carbamate compound with potentially significant effects on male fertility, focusing on its impact on Leydig cell function and androgen production. We employ BLTK-1 Leydig cells as our model to investigate this eventual link. Initially, we examined the repercussions of increasing thiodicarb concentrations (up to 500 µM) on cellular proliferation and viability. Our findings showed that higher thiodicarb concentrations, particularly at 500 µM, elicit cytotoxic effects, leading to decreased Leydig cell proliferation and viability. We then explored whether thiodicarb triggers cell death in Leydig cells, revealing an elevated caspase 3 activity, showing its potential as an apoptosis inducer for this cellular system. Intriguingly, the mechanism through which thiodicarb promotes apoptosis appears to deviate from the BAX/BCL-2 pathway associated with intrinsic apoptosis. Remarkably, the exposure of Leydig cells to thiodicarb at a concentration of 500 µM results in a significant reduction in androgen production, a key function of these cells. These findings underscore the imperative need for further in-depth research to unravel the reproductive toxicity of thiodicarb and its far-reaching implications for male fertility, public health, and regulatory guidelines governing the use of such carbamate compounds in various applications and industries. Additionally, understanding the intricate cellular pathways impacted by thiodicarb will pave the way for targeted interventions and the development of preventive measures to mitigate its potential deleterious effects on male reproductive health.

Index

List of Figures

List of Tables

1. INTRODUCTION

1.1. Testicular anatomy and histology

Mammalian testes are two intricate organs. The septum, which are fibrous inner extensions of the tunica albuginea, separating them into compartments known as testicular lobules (Figure 1) (Saladin, 2007). A human testis has 250 to 300 lobules, each of which includes one to four seminiferous tubules that are tightly coiled (Hoehn, 2007). The testicular functional units known as seminiferous tubules (Cheng, 2010) house Sertoli cells (SCs) and germ cells at various phases of their development. Contractile myoid cells surround each seminiferous tubule, facilitating the passage of mature sperm and testicular fluids through the tubules (Hoehn, 2007). All of the blood and lymphatic channels required for the passage of hormones and nutrients into and out of the testes are present in the interstitial spaces between the seminiferous tubules (O'donnell, 2001). The seminiferous tubules join together to form the rete testis, a network of channels from which 15 to 20 ductuli efferentes carry spermatozoa to the epididymis, the initial portion of the ductus deferens (Hoehn, 2007).

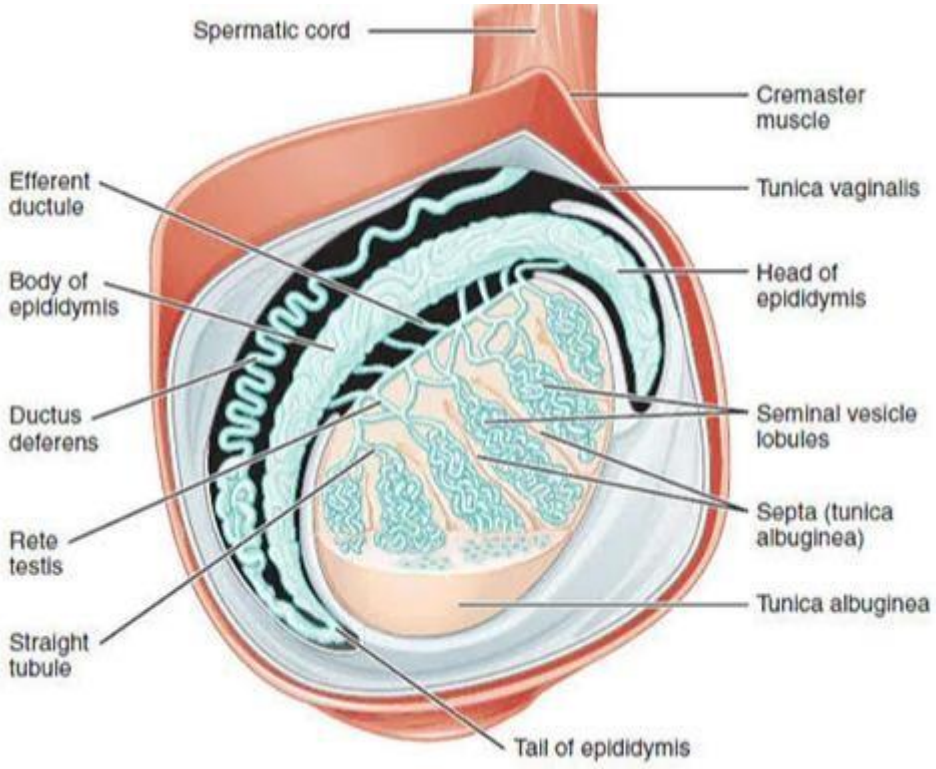

Figure 1: Representation of the anatomical structure of the mammalian testis and epididymis. Adapted from (Dave Holson, MD; Josh Palka, 2022)

The testis is enveloped by a dual-layered covering comprising the outermost layer called the tunica vaginalis and the inner layer known as the tunica albuginea. Projections originating from the tunica albuginea, referred to as septa, partition the testis into lobules, housing the seminiferous tubules. These seminiferous tubules converge toward the rete testis, forming a connection with the efferent ducts. The head of the epididymis establishes its link with the testis through a network of several efferent ducts.

The main tasks of the testes are the production of haploid sperm and the synthesis of steroid hormones, particularly testosterone (Hermo, 2010). The hypothalamic-pituitary-testicular (HPT) axis, which controls the spermatogenic process by interacting with the hypothalamus, pituitary, and testes, is the primary hormonal regulatory mechanism of these two functions (Schulz & Miura, 2002). Gonadotropin-releasing hormone (GnRH) is released by the hypothalamus into the hypophyseal-portal circulation, stimulating the anterior pituitary's gonadotrophic cells to secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Walker & Cheng, 2005). Whilst FSH acts on SCs, LH acts on the testosterone producing Leydig cells (LCs) (Griswold, 1998). Nevertheless, the management of spermatogenesis is mediated by a series of intricate local interactions between the numerous testicular cell types, including the Leydig, germ, peritubular, and SCs (Lambtt, 1993).

Spermatogenesis, which produces haploid, elongated spermatids, is the process by which immature germ cells divide, differentiate, and go through meiosis. Germ cells and SCs are strongly associated during this process that takes place in the seminiferous tubules, while LCs are outside the basal lamina, providing testosterone, the androgen responsible for supporting spermatogenesis (Walker, 2011). SCs are crucial for the expression of a male phenotype, the growth of functional testicles, and are essential for spermatogenesis (Griswold, 1998). SCs are found in the seminiferous tubules, extending upward from the basement membrane to the lumen and engage in direct contact with the germ cells' development (Figure 2). The blood-testis barrier (BTB) is made up of tight junctional complexes formed by adjacent SCs. These complexes separate the seminiferous epithelium into two compartments: the basal compartment, which contains spermatogonia and spermatocytes, and the apical (or adluminal) compartment, which contains spermatozoa, round spermatids, elongated spermatids, in different meiotic stages (Mruk & Cheng, 2004).

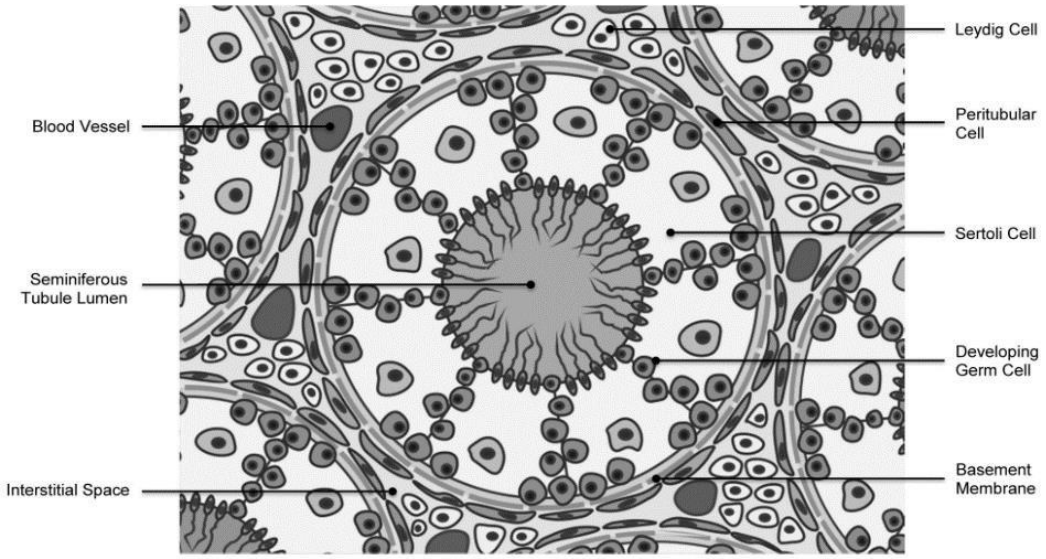

Figure 2: Representation of a cross-sectional cut of the testis. Adapted from (Rato et al., 2012)

The blood-testis barrier (BTB) serves as a physical partition separating blood vessels from the inner lumen of seminiferous tubules. It is formed by tight connections established among Sertoli cells (SCs). Beyond the BTB lies the basal compartment, where spermatogonial renewal occurs, while within the BTB, the apical compartment hosts meiosis, spermiogenesis, and spermiation. The interstitial space houses blood vessels and Leydig cells, responsible for testosterone production upon luteinizing hormone (LH) stimulation. The cytoplasmic extensions enveloping developing germ cells play a vital role in providing structural support through microtubular filaments found in SC cytoplasm. This architectural configuration is dynamic and varies with the seminiferous tubules' developmental stage. Additionally, SCs maintain control over the internal microenvironment within the seminiferous tubule. External to the basement membrane, one finds several layers of modified myofibroblastic cells known as peritubular cells. These cells are responsible for the irregular contractions observed in seminiferous tubules, which help propel the fluid secreted by SCs. The intricate organization outlined above underscores the significance of establishing a functional and regulated BTB. Such an arrangement is crucial in creating an optimal environment for the unhindered development of fully functional spermatozoa.

1.2. Leydig Cell – Structure and Steroidogenic Function

Depending on the species, LCs make up between 2-37% of the testicular volume (Loh & Gemmell, 1980) being that in humans there are roughly 700 x 10^6 of these cells in the testes (Mori & Christensen, 1980). When described at the light microscopic level, LCs have a rounded cytoplasmic profile (Kaler & Neaves, 1978). At the electron microscopic level, these cells are normally characterized by a ovoid or round nucleus, along with numerous mitochondria, an abundance of smooth endoplasmic reticulum (SER), sporadic patches of rough endoplasmic reticulum, and lipid droplets (Loh & Gemmell, 1980).

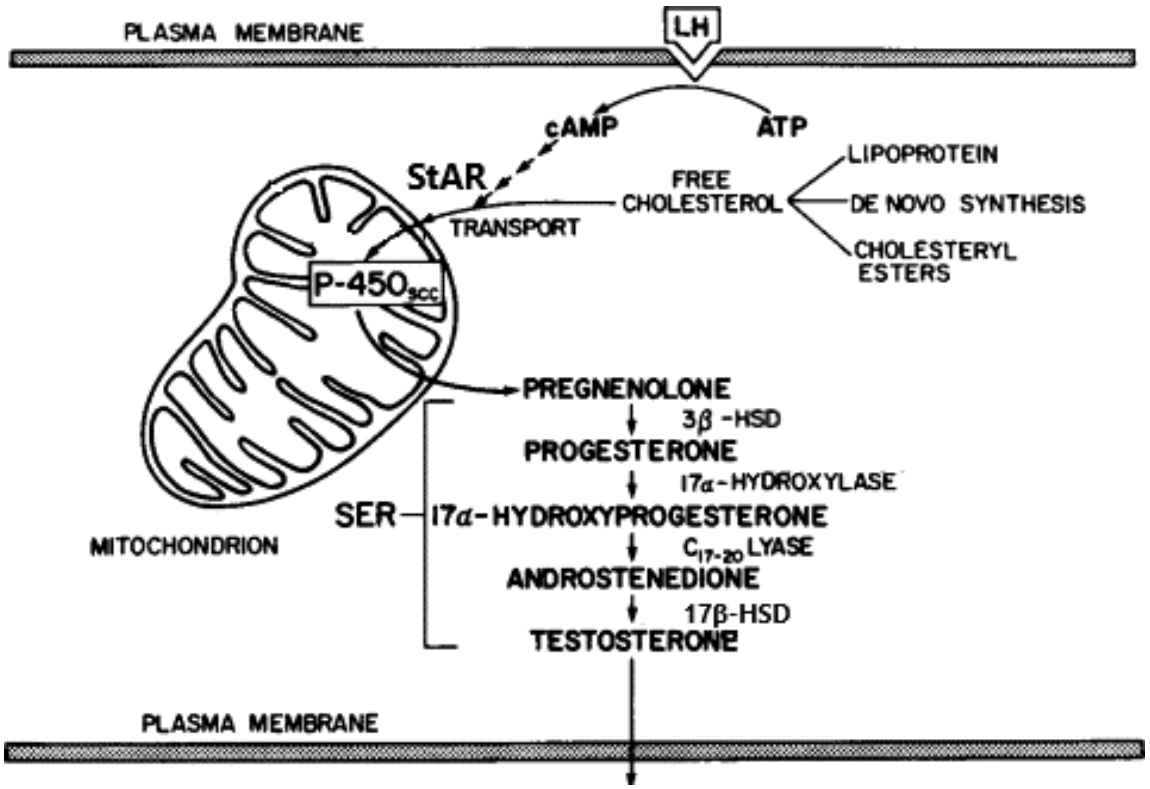

Figure 3: Testosterone biosynthetic pathways in Leydig cells. Adapted from (A. Payne, 1985).

Initially, cholesterol can be synthesized or transported from the bloodstream from low-density lipoproteins (LDL) and high-density lipoproteins (HDL) into the cell via the LDL receptor (LDL-R) or the Scavenger receptor class B type 1 (SR-BI) receptor, respectively. Subsequently, StAR protein facilitates the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. The activity of StAR is regulated through the binding of luteinizing hormone (LH) to its receptor (LHR). Within the inner mitochondrial membrane, cholesterol undergoes conversion into pregnenolone, catalyzed by the enzyme P450scc. Pregnenolone is then transported to the smooth endoplasmic reticulum situated in the cytoplasm. It isat this point that pregnenolone is further transformed into progesterone by the action of 3b-HSD. Subsequently, progesterone undergoes successive conversions into 17α-hydroxyprogesterone and androstenedione, facilitated by the enzyme CYP17a. Conversion of androstenedione into testosterone is then accomplished by the enzyme 17β-HSD.

LCs are the primary source of testosterone in males (Hall, 1979), and they produce it through steroidogenesis. This process is composed by multiple steps, in which cholesterol is converted into steroid hormones (Fenfen, 2017). The three sources of cholesterol are the blood plasma, cholesterol esters stored in lipid droplets, and *de novo* production from acetate (Brown & Goldstein, 1976). For the creation of testosterone in Leydig cells,

endogenous cholesterol production is the primary source. Nevertheless, increased extracellular cholesterol uptake from low-density lipoproteins (LDL) and high-density lipoproteins (HDL) using the LDL receptor and Scavenger receptor class B type 1 (SR-BI) surface receptors (respectively) can satisfy increased cholesterol demand (Eacker et al., 2008). At the outer mitochondrial membrane, the steroidogenic acute regulatory protein (StAR) and the translocator protein (TSPO) are recruited and activated to begin the translocation of cytoplasmic cholesterol to the inner mitochondrial membrane (Stocco, 2001). The CYP11A1 (or P450scc) enzyme converts cholesterol to pregnenolone once it has enters the mitochondria (A. H. Payne & Hales, 2004). Once pregnenolone is produced, it separates from CYP11A1 active site to leave the mitochondria via diffusion and proceeds to the smooth endoplasmic reticulum (SER), where three enzymatic reactions will take place involving CYP17A1, HSD17D3 and HSD3B (Simard et al., 2005), completing the synthesis of testosterone.

Leydig cells' capacity to synthesize the genes encoding the steroidogenic enzymes is mostly controlled by the LH, which triggers the cyclic adenosine monophosphate (cAMP)/Protein kinase A (PKA) signalling pathway. The activation of PKA and adenylate cyclase (conversion of ATP to cAMP) occurs as a result of the LH binding to its G-protein-coupled membrane receptor (LHCGR). Substrates for PKA involve the StAR, which is in charge of carrying cholesterol to the inside of the mitochondria, and transcription factors essential for the production of genes involved in steroidogenesis. In addition to the cAMP/PKA pathway, it has been indicated that other signalling pathways, including the calcium/calmodulin-dependent protein kinase (CAMK), mitogen-activated protein kinase (MAPK), Janus kinase/signal transducer and activator of transcription proteins (JAK/STAT), and protein kinase C (PKC), influence steroidogenesis in testicular Leydig cells (Evaul & Hammes, 2008).

Controlling the proliferation, development, differentiation, and/or death of these cells is thus one way to regulate the synthesis of testosterone. Because pubertal masculinization and fetal development are linked to the emergence of morphologically identifiable, steroidogenically active LCs, it appears that this regulatory technique is employed frequently (Ewing, 1980). The loss of steroidogenically active LCs and/or a reduction in the number of these cells may be the cause of the fall in testosterone concentration in the peripheral blood of males (Kaler & Neaves, 1978).

1.3. Programmed Cell Death – Apoptosis

The two-stage cell death process known as apoptosis involves the formation of tiny, spherical apoptotic bodies, which are then phagocytosed and broken down. Separation from neighbouring cells, nuclear condensation, membrane blebbing, fragmentation and cell shrinkage, as well as the development and release of apoptotic bodies are all distinctive morphological characteristics of apoptotic cells. These apoptotic bodies that have been released are then degraded by lysosomes and phagocytosed by other cells (Nössing & Ryan, 2023). Apoptosis helps maintain tissue homeostasis by eliminating unnecessary or damaged cells, and its dysregulation can contribute to a wide range of human conditions such as neurodegenerative diseases, ischemic damage, autoimmune disorders, and various types of cancer (Elmore, 2007). Depending on the source of the perturbations that cause cell death, apoptosis can be initiated through one of two distinct routes: extrinsic apoptosis pathway, driven by the activation of death receptors or dependence receptors, and intrinsic apoptosis pathway, caused by intracellular stresses (Galluzzi et al., 2018).

Once extracellular ligands such tumour necrosis factor (TNF), TNF-related apoptosis-inducing ligand (TRAIL), or Fas ligand (Fas-L or CD95-L) connect to the extracellular domain of their respective death receptors, extrinsic apoptosis is triggered. Fas-L or TRAIL binding conducts receptor trimerization and exposes the cell to death domain (DD), which serves as a binding motif for the Fas-associated death domain (FADD). The death-inducing signalling complex (DISC) forms when this adaptor protein brings in procaspase-8. Once activated, DISC promotes procaspase-8 catalytic cleavage, caspase-8 dimerization, and executioner caspase activation (Dickens et al., 2012).

The B-cell lymphoma 2 protein family (BCL-2) regulates intrinsic apoptosis. The BCL-2 protein family, which consists of anti- and pro-apoptotic members, can become unbalanced as a result of a number of intracellular events (such as DNA damage, lack of cytokines, or excessive amounts of ROS). Their differences can be seen in the number of BCL-2 homology domains (BH). Pro-apoptotic proteins like BAX only have the BH1-BH3 domains, whereas anti-apoptotic proteins like BCL-2 have the domains BH1-4. Anti-apoptotic proteins attach to and deactivate their pro-apoptotic counterparts in healthy cells. Due to apoptotic stressors, the amount of BCL-2 proteins can be reduced, or the production of a third member of the BCL-2 family—the BH3-only proteins—can take place (Czabotar et al., 2014). By attaching to BCL-2, these pro-apoptotic proteins can counteract its' anti-apoptotic activities. As a result, BAX (effector proteins of intrinsic apoptosis (Wei et al., 2001)) inhibition is no more, causing it to

homo-oligomerize and insert themselves into the outer mitochondrial membrane, inducing mitochondrial outer membrane permeabilization (MOMP) (Tait & Green, 2010). Multiple mitochondrial intermembrane proteins are released into the cytosol as a result of the membrane breakdown. Cytochrome-c, amongst other released proteins, is the primary activator apoptosis downstream of MOMP. The leaked cytochrome-c binds to apoptotic protease-activating factor-1 (APAF-1), forming the apoptosome (Cain et al., 2002). This complex triggers caspase-9, which eventually causes executioner caspases and apoptosis to be activated (Dickens et al., 2012).

The climatic point of both intrinsic and extrinsic apoptosis pathways is the activation of executioners like caspase-3. Numerous target proteins are broken down by these cysteine-dependent aspartate-directed proteases (caspases), which then induce apoptotic cell death (Julien & Wells, 2017). The apoptotic bodies are then phagocytosed, attracting phagocytes through signals including modified membrane lipids (lysophosphatidylcholine (Lauber et al., 2003) and sphingosine-1-phosphate (Gude et al., 2008)), chemokines (Truman et al., 2008) and nucleotides (ATP and UTP).

1.4. Carbamates linked to male infertility

Increasing levels of infertility have been reported, being present in both developed and developing nations (Sengupta et al., 2017) (Levine et al., 2017). Data from World Bank shows that the fertility rate dropped significantly, being that births per woman reached a total of 5.3 in 1963, and since then it's been declining to births per woman as low as 2.3. (Leaver, 2016) Mentions that 30% of fertility problems are caused by the man, and 90% of these cases are due to low sperm count, quality, or both. Male infertility is a complication that can occur due to many factors, such as congenital, acquired and idiopathic (Agarwal et al., 2021). The latter factor includes environmental or occupational exposure to toxins, such as pesticides.

Pesticides are chemical compounds that are used to eradicate undesired organisms such as insects, rodents, fungi, and killing weeds (WHO, 2020), to increase food production and aid in the processing, storing, transporting, or marketing of the food and agricultural products (Mehrpour et al., 2014). These chemicals were already being used 4500 years ago by Sumerians, utilizing sulphur compounds to control mites and insects (Unsworth, 2010), and over the years many other forms of pesticides were discovered and used, such as DDT (Dichlorodiphenyltrichloroethane), Nobel Prize winning discovery, that was able to effectively

kill insects and control vector diseases such as malaria and yellow fever. However, it was later found that, following high doses of exposure, symptoms could include vomiting, tremors, and seizures, and studies on laboratory animals showed negative effects in the liver and reproduction (cdc, 2021). Adding on this, numerous studies have shown a link between pesticide exposure and an increased risk of chronic illnesses like cancer, diabetes, Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS), as well as birth defects (Mostafalou & Abdollahi, 2013).

There are a number of classes of pesticides, one of them being carbamates. In comparison to other pesticides, carbamates have one of the highest varieties of subtypes (among them, carbendazim, carbaryl, carbofuran, aminocarb, thiodicarb, and mancozeb). Each substance has a specific use (for example, as a herbicide, fungicide, or insecticide), properties, and negative effects (King & Aaron, 2015). Regardless of these distinctions, the compounds within the carbamate family collectively possess the characteristic of acting as inhibitors for acetylcholinesterase (AChE), achieved through carbamylation. Additionally, they function as endocrine-disrupting chemicals (EDCs), thereby impacting the hypothalamic–pituitary–testicular (HPT) axis, potentially leading to various reproductive issues (Gupta et al., 2017).

The endocrine system consists of a series of glands distributed throughout the body. These glands generate and release hormones, which play a pivotal role in regulating a diverse array of physiological processes. These include respiration, metabolism, reproduction, sensory perception, movement, sexual maturation, and growth. The principal glands responsible for hormone production include the hypothalamus, pituitary, parathyroid, pancreas, thyroid, adrenal, pineal, ovaries, and testis. Each of these glands produces specific hormones or responds to hormonal signals, thereby governing bodily homeostasis. Numerous factors have the potential to impact the performance of endocrine organs. These factors encompass the effects of aging, specific diseases, stress, environmental influences, and genetic predispositions (Liu et al., 2019). EDCs are capable of functioning in a manner similar to that of hormones by attaching to their receptors and either blocking or stimulating the corresponding cascade and the HPT axis. EDCs can also change the levels of hormones by promoting or discouraging the availability, synthesis, or the breakdown of those hormones (Combarnous, 2017).

According to research, exposure to carbamates, such as thiodicarb, may have adverse effects on male fertility. Rats exposed to thiodicarb showed multiple organ damage, including alterations in hepatic, renal, and splenic tissues, indicating a systemic impact (Dias et al., 2013). Furthermore, the possibility that carbamates may impair steroidogenesis and

spermatogenesis is raised by their capacity to interfere with signalling pathways, including those mediated by acetylcholine or kisspeptin (Moreira et al., 2022).

Table 1. Effects of in vivo carbamates exposure on male reproductive function.
Adapted from (Moreira et al., 2022).

Reference	Formulation	Dose	Duration of Treatment	Administration	Animal Model	Main Findings
Liu et al.	Carbendazim	0.1, 1, and 10 mg/kg	5 weeks	Oral gavage	ICR mice (n = 30 per group)	Decreased sperm concentration and motility Impaired spermatogenesis Decreased estrogen signaling Alterations in histone and DNA methylation
Salem et al.	Carbendazim	100 mg/kg	8 weeks	Oral gavage	Swiss albino rats (n = 10 per group)	Decreased sperm concentration, motility, and viability Increased percentage of morphological abnormal sperm Decreased concentrations of serum testosterone, gonadotropins, and inhibin B Increased oxidative stress Alterations on the seminiferous tubules structure
Elsharkawy et al.	Mancozeb	100 mg/kg	12 weeks	Oral gavage (twice per week)	White New Zealand rabbit (*Oryctolagus cuniculus*) (n = 9 per group)	Decreased concentrations of serum testosterone and gonadotropins Decreased sperm viability Increased percentage of morphological abnormal sperm Disruption of the germinal epithelium Vacuolization of Leydig cells
Mohanty et al.	Mancozeb	0.14 and 0.28 mg/day	30 days	Oral (mixed with food)	Red Avadavat (*Amandava amandava*) (n = 9 per group)	Decreased concentrations of serum testosterone and gonadotropins Impaired gonadal development Altered hypothalamic expression of GnRH Disruption of the HPT axis
Meng et al.	Methomyl	0.2, 2, 20, and 200 µg/L	30 days	Dissolved in water	Nile tilapia (*Oreochromis niloticus*) (n = 30 per tank, n = 3 per condition)	Altered expression of HPT-related genes in the hypothalamus, pituitary, and testis at 20 and 200 µg/L The effects of 200 µg/L were considered irreversible
Yue et al.	Semicarbazide	1, 10, and 100 µg/L	130 days	Dissolved in water	Japanese flounder (*Paralichthys olivaceus*) (n = 6 per group)	Decreased expression of genes involved in steroidogenesis Decreased concentration of serum testosterone and estradiol Disruption of the HPT axis Alterations in the kiss/gpr54 system and GABA synthesis
Guo et al.	Ziram	2 and 4 mg/kg/day	4 weeks	Oral gavage	Sprague–Dawley rats (n = 6 per group)	Decreased concentrations of serum testosterone and FSH Decreased Leydig cell number

The existing body of literature addressing the impact of carbamates on male reproductive function remains limited. Among the limited number of studies found, the majority are in vivo investigations conducted using rodent models. A study conducted by (Liu et al., 2019) showed that carbendazim exposure (during 5 weeks) in mice promoted a significant decrease in sperm motility in all concentrations and significant decrease in sperm concentration when exposed to 10 mg/kg. Along with this, main findings also include impaired spermatogenesis, decrease in estrogen signalling, and alterations in histone and DNA methylation. Another study also investigated carbendazim, through an 8 week exposure, showing a significant decrease in sperm concentration, motility and viability in Swiss albino rats compared to the control group, as well as an increase in morphological abnormal sperm, decrease in concentrations of serum testosterone, gonadotropins, and inhibin B (Salem et al., 2021). Additionally, results showed an increase in oxidative stress, and alterations on the seminiferous tubules structure was detected. Elsharkawy et al's study, white new Zealand rabbits were exposed to mancozeb (100 mg/kg) for 12 weeks, and main findings showed a decrease in serum testosterone and gonadotropins concentrations, and sperm viability. An increase in morphological abnormal sperm was observed, germinal epithelium was disrupted and Leydig cells suffered vacuolization (Elsharkawy et al., 2019). Mohanty et al also investigated the effects of mancozeb, in lower concentrations (0.14 and 0.28mg/day for 30 days) in red avadavats (Mohanty et al., 2017). Main findings include a decrease in concentrations of serum testosterone and gonadotropins, impaired gonadal development, altered hypothalamic expression of GnRH, and disruption of the HPT axis. In (Meng et al., 2016)'s study, Nile tilapia were exposed to methomyl (0.2, 2, 20 and 200 µg/L for 30 days) and results showed an altered expression of HPT-related genes in the hypothalamus, pituitary, and testis at the concentrations of 20 and 200 µg/L. In (Yue et al., 2018)'s study, Japanese flounder were exposed to 1, 10 and 100 µg/L of semicarbazide for 130 days, resulting in a decrease in expression of genes involved in steroidogenesis, and concentration of serum testosterone and estradiol. Findings also include disruption of the HPT axis, alterations in the kiss/gpr54 system and GABA synthesis. Guo et al's study conducted an exposure of ziram (2 and 4mg/kg/day for 4 weeks) to Sprague-Dawley rats. Results showed a decrease in concentrations of serum testosterone and FSH, as well as a decrease in leydig cells (Guo et al., 2017).

While the present findings provide insights into the potential impacts of carbamates on male reproductive function, the molecular mechanisms behind these effects have yet to be fully elucidated. This knowledge gap persists primarily because a significant portion of these investigations is conducted in vivo.

2. OBJECTIVES

Thiodicarb, a pesticide employed in agriculture for pest control, places agricultural workers in direct exposure to it. However, thiodicarb implications extend further, potentially infiltrating the broader environment and establishing pathways for indirect exposure among the general populace. This prompts a pertinent question: Could thiodicarb widespread use and subsequent exposure serve as a contributing factor to male infertility? The intricate interplay between thiodicarb and the intricate mechanisms of human reproductive health invites careful examination.

To investigate the effects of thiodicarb exposure, we are cultivating murine Leydig cells and subjecting them to increasing thiodicarb concentrations. These cells will undergo cytotoxicity tests, allowing us to assess their viability and health. Additionally, we will analyze the expression of proteins related to apoptosis, providing insights into potential cellular responses. This multifaceted approach aims to better understand the impact of thiodicarb on Leydig cells and its implications for male fertility, human safety, and health regulation.

3. MATERIALS AND METHODS

3.1. Chemicals

BLTK-1 Leydig Cells were granted by investigator Nafis Rahman, MD, PhD Faculty of Medicine, Institute of Biomedicine, University of Turku Pen-Strep Gibco. Dulbecco's Modified Eagle Medium, Ham's Nutrient Mixture F12 (DMEM: Ham's F12), Ethylene Diamine Bovine Serum Albumin (BSA), Fetal Bovine Serum (FBS), trypsin-ethylenediaminetetraacetic acid (EDTA) and Amphotericin B were all purchased from Sigma–Aldrich (St. Louis, MO, USA). Sulforhodamine B (SRB) was purchased from Biotium (Hayward, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). LDH CitoxTM Assay Kit was obtained from BioLegend® (San Diego, CA, USA). Gentamicin was obtained from lonza (Basel, Switerzland). RIPAS Lysis Extraction Reagent and BCA Protein Assay Kit were obtained from Thermo Scientific (Whalthan, MA, USA). WesternBrightTM ECL substrate was purchased from Advansta (Menlo Park, CA, USA). Anti-Rabbit igG 1:1000 was obtained from Sigma-Aldrich (Germany). Protease inhibitor cocktail was purchased on Bimake. BCA Protein Assay Kit was bought from Thermo Fischer Scientific, (Waltham, MA, USA). Trans-Blot® Turbo™ System, low fluorescence polyvinylidene difluoride (PVDF) membrane and 12.5% polyacrylamide gel from TGX Stain-Free™ FastCast™ Acrylamide Kit were all bought from Bio-Rad (Hemel Hempstead, UK). Thiodicarb was purchased from Sigma–Aldrich (Germany) in powder form and dissolved in pure ethanol.

3.2. BLTK-1 Leydig cell culture

In short, the cells were cultured in T75 flasks (VWR collection, Amadora, Portugal) at 37ºC, 5% CO_2 and maintained in Leydig culture medium (DMEM:Ham's F12 1:1, supplemented with 10% heat inactivated fetal bovine serum (FBS), 50 µm/mL gentamicin, 1% Penicillin-Streptomycin, 1% Amphotericin B, 15 mM HEPES, ph 7,4). BLTK-1 cells are murine Leydig cells with entirely functioning steroidogenic pathway that generates low basal amounts of testosterone (T) and expresses all required steroidogenic enzymes, such as Star, Cyp11a1, Cyp17a1, Hsd3b1, Hsd17b3, and Srd5a1 (Forgacs et al., 2012).

3.3. Experimental Groups

BLTK-1 Leydig cells were kept in the same conditions throughout their growth, being washed thoroughly with phosphate buffered saline (PBS) solution every two days and replenished with 10 mL of Sertoli culture medium. When these cells reached at least 70% confluence, the medium was removed and substituted with the treatment medium (Leydig culture medium with 5% FBS instead of 10%, supplemented with increasing concentrations of thiodicarb). To evaluate the dose-response, the cells were exposed to 0.05, 0.5, 5, 50 and 500 µM of thiodicarb. Control conditions were set with cells being exposed only to 1% pure ethanol (same concentration of pure ethanol that cells that suffered thiodicarb treatment were exposed to). After 24 hours of exposure, the cells were used for cytotoxic assays or collected for protein extraction.

3.4. Evaluation of cytotoxic profile of thiodicarb on murine Leydig cells

The cells were seeded in 24 and 48-wells plates until reaching a confluence of at least 70%. Afterwards, the culture medium was aspirated and replaced with Leydig culture medium (with 5% FBS) supplemented with the chosen concentrations of thiodicarb and maintained at 37º C, with 5% CO2 humified atmosphere for 24 hours. To evaluate the cytotoxicity of thiodicarb on murine Leydig cells, we used three different assays: Sulforhodamine B (SRB) assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, and Lactate Dehydrogenase (LDH) release assay.

3.4.1. SRB cytotoxicity assay

SRB is a pink-colored aminoxanthene dye that binds to basic amino-acid residues when the environment is acidic. Due to its direct correlation with the quantity of dye removed from labelled cells, this approach enables the determination of cell mass (Vichai & Kirtikara, 2006), allowing to compare cell proliferation when treated with thiodicarb and assess its' cytotoxicity.

In short, cells were cultured in a 24-well plate and treated with the chosen concentrations of thiodicarb. After a 24-hour exposure, the treatment medium was removed, and the cells were washed with phosphate-buffered saline (PBS). Afterwards, cells were fixed for at least 1 hour at -20°C using a solution of 1% acetic acid in 99% methanol. This was followed by an hour at 37°C incubation with a solution of SRB at 0.05% prepared with 1%

acetic acid in water. The bound dye was removed from the cells using a 10 mM Tris solution (pH 10) after being washed with 1% acetic acid in water. Then, 100 µL of this solution were transferred to a 96-well plate. The optical density of the solution was then measured at 490 nm using the BioTek Synergy HT (BioTek, Winooski, VT, U.S.A). Resulting values were divided by the mean of the control group and expressed in fold variation versus the control group.

3.4.2. MTT cytotoxicity assay

MTT assay is used to gauge metabolic cell viability by the evaluation of mitochondrial viability. The formation of formazan from tetrazolium makes this possible. This compound is water-soluble, but dehydrogenases found in the mitochondria transform it into insoluble crystals (formazan). Therefore, a higher conversion rate of formazan from tetrazolium will indicate increased viability, which may be determined by spectrometry by seeing the color change from yellow (MTT) to purple (formazan crystals).

After the cells were seeded and exposed to the chosen concentrations of thiodicarb in a 48-well plate, the treatment medium was removed and replaced with 500 µL Leydig culture medium plus 50 µL of MTT solution at 5 mg/mL and cells were incubated for 3 h at 37º C in the dark. Then, the medium was removed and 250 µL of dimethyl sulfoxide (DMSO) were added to each well, to dissolve the formazan crystals formed inside the cells. Afterwards, 100 µL of the DMSO solution from each well were transferred to a 96 wells plate and a blank was made with DMSO only. The absorbance of each sample was measured at 570 nm, which has been determined to be the best wavelength for detecting the amount of solubilized formazan, and 650 nm, which serves as a reference to detect the baseline and is also regarded as the assay blank. The absorbance was measured at 570 nm and 655 nm through a BioTek Synergy HT (BioTek, Winooski, VT, U.S.A). Resulting values were divided by the mean of the control group and expressed in fold variation versus the control group.

3.4.3. LDH cytotoxicity assay

The LDH release assay can assess the integrity of the plasma membrane. LDH is an intracytoplasmic enzyme, and if it is found in extracellular media, the plasmatic membrane has likely been ruptured. The LDH-Cytox™ Assay Kit was utilized to quantify LDH in the extracellular medium, following the manufacturer's instructions.

In brief, BLTK-1 Leydig cells were incubated in a 24-well plate with the different concentrations of thiodicarb for 24 hours. Following this, 50 µL of the extracellular medium were transferred to 96-well plates plus 50 µL of Reaction Mix were added. The plates were then incubated for 30 minutes at room temperature in the dark. The reaction was halted using the STOP Solution at the conclusion of the incubation, and BioTek Synergy HT (BioTek, Winooski, VT, U.S.A) was used to measure the absorbance at 490 nm of each sample. Resulting values were divided by the mean of the control group and expressed in fold variation versus the control group.

3.5. Protein extraction and quantification

Using a lysis buffer (PBS 1x, 1% NP-40, 0.5 % Sodium deoxycholate and 0.1% SDS 10%), supplemented with 1% of proteases inhibitors cocktail and 10 mM of sodium orthovanadate, the total protein from BLTK-1 Leydig cells was extracted. The cells were maintained on ice for 20 minutes. The resultant suspension was then centrifuged for 20 minutes at 14000.*g*, discarding the pellet afterwards. Total protein concentration was determined using PierceTM BCA Protein Assay Kit according to manufacturer's instructions. Calibration curve was calculated by using different concentrations of BSA as the standards for protein quantification. Optical densities of samples were determined at 560 nm.

3.6. Western Blot

Western Blot is a technique utilized for the identification of a specific protein within a complex mixture, in this case being BAX and BCL-2. The method involves several key steps. Initially, protein samples (25 µg) were mixed with sample buffer (1.5% Tris, 20% glycerol, 4.1 % SDS, β-mercaptoethanol, 0.02 % bromophenol blue, pH 6.8) and denatured for 10 minutes at 75ºC. These proteins were then separated by weight through electrophoresis on 12% polyacrylamide gels for approximately 90 minutes at 100 V. Subsequently, the proteins were transferred from the gel to a previously activated polyvinylidene difluoride membrane, in a Mini Trans-Blot® cell (Bio-Rad, Hemel Hempstead, UK) with a 7-minute application of 1.3 mA and 25 V. Detection of total protein was accomplished using Bio-Rad QuemiDoc XRS and quantified using Image Lab™ software (BioRad, Hemel Hempstead, UK). The membrane was then blocked for 90 minutes using a 5% non-fat milk solution of tris-buffered saline solution at

room temperature. Further steps involve incubating the membranes overnight at 4ºC with the primary antibody anti-BCL-2 (1:2000, Merck Millipore, Burlington, MA, USA) or anti- BAX (1:5000, Merck Millipore, Burlington, MA, USA), followed by an incubation with HRP-bound anti-rabbit igG (1:1000) during 1 hour at room temperature. Chemiluminescence levels were determined out using WesternBright™ ECL Chemiluminescent HRP Substrate (Advansta, Menlo Park, CA, USA), and band densities were quantified through standard procedures using Image Lab™. Band density was normalized to the total protein loaded for each sample and results are presented after being divided by the control group values.

3.7. Caspase-3 activity

Activity of caspase 3 was assessed using a spectrophotometric method, involving the measurement of the cleavage of the respective substrate. To start the process, protein samples (25 µg) were incubated alongside the assay buffer (25 mM HEPES at pH 7.5, 0.1% CHAPS, 10% sucrose, and 10 mM DTT) and 100 µM of the caspase-3 substrate (ac-DEVD-pNA). This mixture was allowed to incubate for a period of 2 hours at a temperature of 37ºC. The enzymatic activity analogous to caspase-3 was determined by detecting the resultant p-nitroaniline chromophore, measured at 405 nm utilizing a spectrophotometer, post the cleavage of the labelled substrate. The technique was standardized by creating a calibration curve with known concentrations of p-nitroaniline. The calculation of Caspase-3 activity was calculated and normalizing against the control group.

3.8. Androstenedione production assay

BLTK-1 and other LC lines lack functional 17β-HSD3, preventing direct testosterone measurement under basal conditions (Engeli et al., 2018). To assess steroidogenesis, we used a competitive ELISA kit to detect androstenedione, the intermediate androgen converted to testosterone by 17β-HSD3 and exported by these cells (FineTest, USA). To facilitate quantification, we concentrated 1 mL of BLTK1 culture medium exposed to CrPic3 and PA for 24 hours using a SpeedVac, resuspending it in 200 µL of DMEM: HAM's F12.

For androstenedione quantification, we followed the manufacturer's instructions. Briefly, we washed the assay plate twice and added 50 µL of concentrated BLTK1 culture medium to each well. We also added 50 µL of biotin-labelled antibody working solution and

incubated it for 45 minutes at 37°C. Afterward, we removed the well contents, washing each well three times. Then, we added 100 µL of HRP-streptavidin conjugate to each well, incubating the plate for 30 minutes at 37°C. After washing the wells five times, we added 90 µL of TMB (3,3',5,5'-Tetramethylbenzidine) substrate to the wells, incubating for 15 minutes, and measured optical density at 450nm. The calibration curve used different androstenedione concentrations as standards for quantification (10, 5, 2.52, 1.25, 0.625, 0.312, and 0.156 ng/ml). Androstenedione concentrations were calculated from obtained absorbances using CurveExpert software (FineTest, USA), dividing the results by the respective dilution factor, and expressed in ng/ml.

3.9. Statistical analysis

Statistical significance within the experimental groups was evaluated through a one-way analysis of variance (ANOVA), and subsequent multiple comparisons were conducted using the Fisher Least Significant Difference (LSD) method. All experimental data are shown as mean ± SEM. The statistical analysis was conducted employing GraphPad Prism 8 (GraphPad software, San Diego, CA, USA). To enhance data integrity, potential outliers were eliminated using the ROUT method with a threshold q value of 1%. Results were deemed statistically significant when the p-value was less than 0.05.

4. RESULTS

4.1. Thiodicarb exposure alters cellular proliferation and metabolic viability in BLTK-1 Leydig cells

We utilized the SRB assay, a popular method for analysing proliferation in cell cultures, to assess the effects of thiodicarb exposure on BLTK-1 Leydig cell proliferation. Because SRB binds stoichiometrically to proteins, it is possible to extrapolate to measure cell proliferation because the amount of bound dye is directly related to cell mass. The results showed differences on cell proliferation when we compared the cells from the groups exposed to thiodicarb in concentrations up to 50 µM to those of the control group (Figure 4). We only observed a significant decrease in the proliferation of cells exposed to 500 µM (0.71±0.06 – fold variation to control) of thiodicarb, which exhibited lower proliferation when compared to the cells that were not exposed to thiodicarb, compared to 0.05 µM (1.08±0.03 – fold variation

to control), to 0.5 µM (1.10±0.04 – fold variation to control), to 5 µM (0.99±0.06 – fold variation to control) and to 50 µM (1.07±0.06 – fold variation to control).

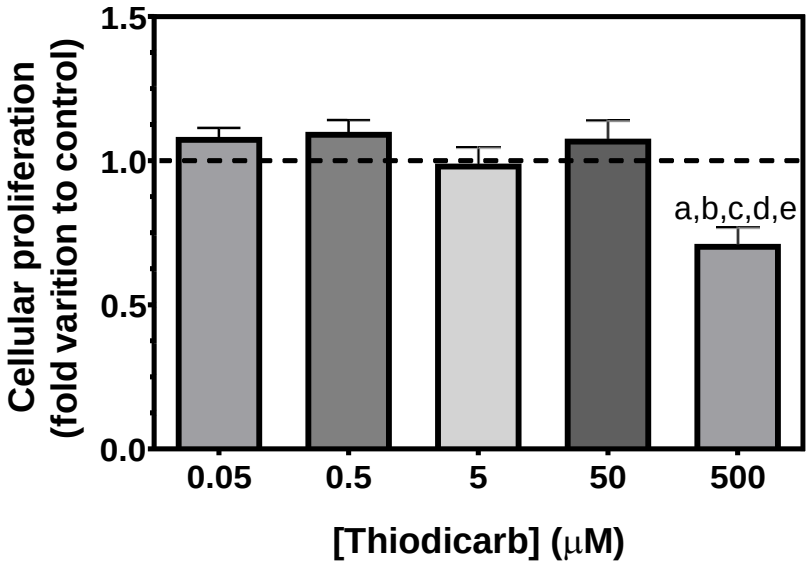

Figure 4: Evaluation of cell proliferation on BLTK-1 Leydig cells after exposure to thiodicarb.

Results are expressed as mean ± SEM (n=8 for each condition). Significantly different results (P< 0.05) are indicated as: a – relative to control group, b – relative to 0.05 µM, c – relative to 0.5 µM, d – relative to 5 µM, e – relative to 50 µM. Dashed line is the control group.

The impact of thiodicarb on the cellular viability of LCs was evaluated using the MTT assay, which measures cell metabolic activity by determining functioning of mitochondrial oxidases. Data indicates that exposure to concentrations of 50 µM (0.91±0.05 – fold variation to control) affect the metabolic viability of BLTK-1 Leydig cells, when compared to control group and 0.05 µM (0.99±0.04 – fold variation to control). Cells exposed to 500 µM (0.51±0.05 – fold variation to control) also show a decrease in cellular viability when compared to LCs from the control group and 0.05 µM, as well as those exposed to 0.5 µM (0.95±0.05 – fold variation to control), 5 µM (0.94±0.05 – fold variation to control), and 50 µM (Figure 5).

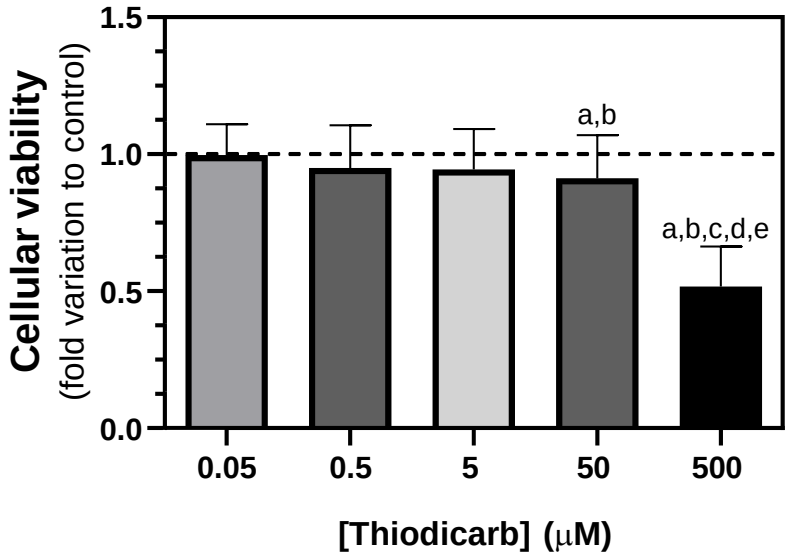

Figure 5: Evaluation of metabolic activity on BLTK-1 Leydig cells after exposure to thiodicarb.

Results are expressed as mean ± SEM (n=8 for each condition). Significantly different results ($P < 0.05$) are indicated as: a - relative to control group, b - relative to 0.05 µM, c - relative to 0.5 µM, d – relative to 5 µM, e – relative to 50 µM. Dashed line is the control group.

4.2. Exposure to thiodicarb promoted an increase in LDH release in BLTK-1 Leydig cells

LDH, an enzyme present in the cellular cytoplasm, exhibits elevated levels in the extracellular medium when cellular viability is compromised. Results showed a significant increase in LDH release in cells exposed to 500 µM (1.80±0.05 – fold variation to control) when compared to control group, 0.05 µM (1.02±0.05 – fold variation to control), 0.5 µM (0.99±0.05 – fold variation to control), 5 µM (1.04±0.05 – fold variation to control) and 50 µM (0.95±0.05 – fold variation to control) (Figure 6).

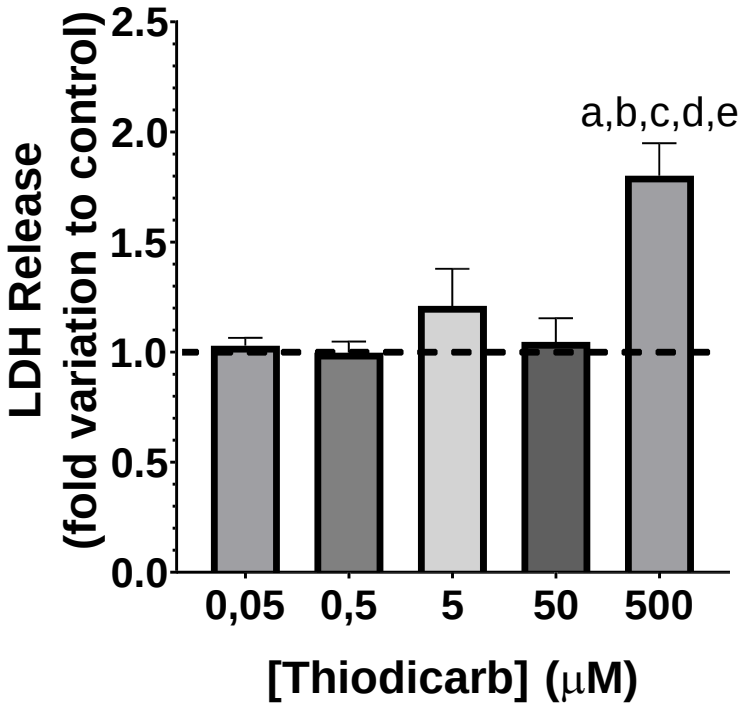

Figure 6: Evaluation of LDH release on BLTK-1 Leydig cells after exposure to thiodicarb.

Results are expressed as mean ± SEM (n=8 for each condition). Significantly different results (P< 0.05) are indicated as: a - relative to control group, b - relative to 0.05 µM, c - relative to 0.5 µM, d – relative to 5 µM, e – relative to 50 µM. Dashed line is the control group.

4.3. Thiodicarb exposure results in the activation of caspase-3 in BLTK-1 Leydig cells

To investigate the impact of thiodicarb in apoptotic events in BLTK-1 Leydig cells, an analysis of both the BAX/BCL-2 protein ratio and the activity of caspase-3 was conducted. BCL-2 is a member of the BCL-2 protein family, known for its regulatory role in apoptosis, predominantly displaying an anti-apoptotic characteristic. Conversely, BAX is a part of a protein family that shares structural similarities with BCL-2, but exhibits a pro-apoptotic profile. No significant results were found in BAX and BCL-2 abundance, nor in the BAX/BCL-2 protein ratio (Figure 7).

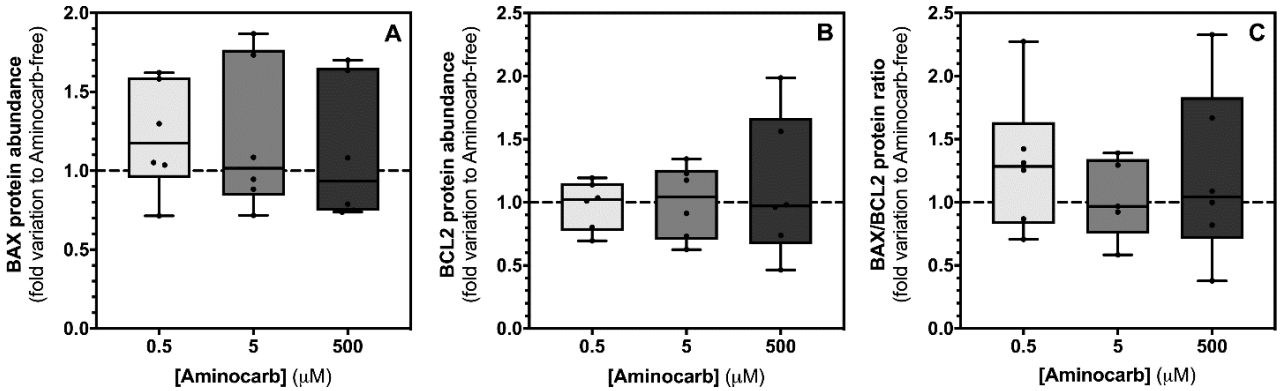

Figure 7: Evaluation of the ratio of BAX/BCL-2 on BLTK-1 Leydig cells after exposure to thiodicarb.

Results are expressed as mean ± SEM (n=6 for each condition). Dashed line is the control group.

Caspase-3 is a component of a conserved family of cysteine proteases involved in apoptosis, which is a natural process necessary for maintaining the normal function of an entire organism. Results showed an increase in caspase-3 activity the higher the concentration of thiodicarb was, being statistically significant at 500 µM (5.72±2.46 – fold variation to control) when compared to cells that were not exposed to thiodicarb (1.00±0.18). Concentrations of 0.5 µM (2.33±0.66 – fold variation to control) and 5 µM (5.16±1.57– fold variation to control) showed no significant differences in caspase 3 activity. (Figure 8).

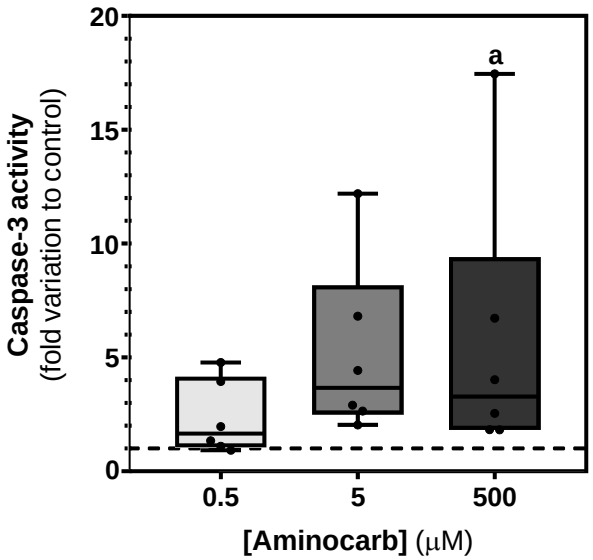

Figure 8: Evaluation of the caspase-3 activity on BLTK-1 Leydig cells after exposure to thiodicarb.

Results are expressed as mean ± SEM (n=6 for each condition). Significantly different results ($P < 0.05$) are indicated as: a - relative to control group. Dashed line is the control group.

4.4. Thiodicarb exposure altered the production of androgens in BLTK-1 Leydig cells

To investigate the impact of thiodicarb on the production of androgens by BLTK-1 Leydig cells, we evaluated the secretion of androstenedione using a competitive ELISA assay. Results showed a significant decrease in androstenedione production in LCs exposed to 500 µM (1.45±0.12 – fold variation to control) when compared to the control group (2.48±0.58). Concentrations of 0.5 µM (1.85±0.40 – fold variation to control) and 5 µM (1.96±0.20 – fold variation to control) showed no significant difference in androstenedione production (Figure 9).

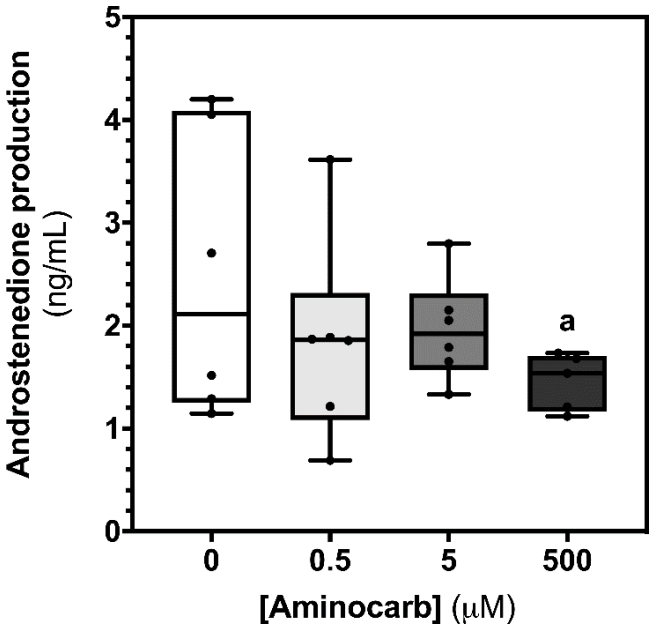

Figure 9: Androstenedione production by BLTK-1 Leydig cells after exposure to thiodicarb.

The results are expressed in as mean ± SEM (n=6 for each condition) and presented as ng/mL. Significantly different results ($p<0.05$) are indicated as: a. relative to control.

5. DISCUSSION

The global sperm count has undergone a 50% reduction in the last five decades, and the rate of this decline has more than doubled since the year 2000 (Szabó et al., 2023). This concerning pattern has caught the attention of the scientific community, sparking heightened concern. There is a pressing need to comprehensively comprehend and tackle the factors contributing to the decline in male fertility. Approximately 6% of adult males are estimated to face infertility, with male factors contributing up to 50% of infertility cases. Male infertility is associated with compromised semen quality and can stem from diverse factors, encompassing genetic conditions (like Klinefelter's syndrome), congenital issues (such as cryptorchidism), endocrine disorders (like hypogonadism), obstructive causes (like vasectomy), infections (like chlamydia), vascular issues (like varicocele), neoplastic conditions (such as testicular carcinoma), as well as lifestyle and environmental factors such as heat, drug usage, exposure to pesticides, and irradiation. Sexual dysfunction linked to erection and ejaculation also contributes to male infertility. In a considerable proportion of cases (40–75%), male infertility is considered idiopathic, lacking a discernible cause (Assidi, 2022)(Amalia Yunia Rahmawati, 2023).

Carbamates have shown that they are capable of impairing male fertility through altering Leydig cell function and/or damage spermatozoa (Sengupta & Banerjee, 2014). Almasiova et al showed that, exposing adult rabbits to a carbamate pesticide, resulted in testicular tissue exhibited notable alterations, including the detachment of developing sex cells, the presence of vacuoles within Sertoli cells, and among different spermatogenic cells. The interstitial Leydig cells demonstrated reduced size, characterized by shrunken nuclei and a distinctly vacuolated dark cytoplasm. The extent of these changes directly correlated with the duration of the experiment. Detailed ultrastructural analysis identified diverse cellular abnormalities within the germinal epithelium and the interstitial Leydig cells (Almasiova et al., 2012).

While carbamates' reproductive toxicity has been studied, thiodicarb, a carbamate pesticide, has not been thoroughly investigated concerning its potential impact on male fertility. To address this gap in knowledge, we conducted a study on murine Leydig cells, specifically the BLTK-1 Leydig cells, exposing them to thiodicarb. Our findings revealed that the Leydig cells exposed to a concentration of 500 µM of thiodicarb exhibited decreased cellular proliferation and viability, indicating cytotoxic effects. In contrast, cells exposed to lower concentrations (0.05, 0.5, and 5 µM) showed no significant alterations in cellular proliferation,

viability, or cellular membrane damage, as measured by LDH release. Cells exposed to 50 µM showed a decrease in cellular viability but exhibited no changes in proliferation or LDH release. These results align with a study by Lu and Liu (2023), which exposed TM3 Leydig cells to methomyl, another carbamate, and observed increased cytotoxicity with higher concentrations over the same time period (Lu & Liu, 2023).

Following the observation of a cytotoxic effect of thiodicarb on BLTK-1 Leydig cells, we assessed the possible effect of this carbamate as an apoptosis inducer for these cells. We started by examining the activity of caspase 3, a crucial mediator of apoptosis (Porter & Jänicke, 1999). Carbamate pesticides, such as thiram, have been shown to increase the intracellular level of active caspase 3, indicating their potential to induce apoptosis in Leydig cells (Li et al., 2015). Our results showed that in cells exposed to 500 µM, caspase 3 activity was significantly higher when compared to the control group. These results are in agreement with the cytotoxic results seen in LCs exposed to 500 µM of thiodicarb, which as said showed a decline in proliferation and viability, and an increase in caspase 3 activity (indicating that this concentration induces apoptosis).

In face of these results, we investigated which pathway(s) of apoptosis thiodicarb might be promoting. It was reported that ziram, a carbamate, induced immature Leydig cells´ apoptosis by upregulating BAX and downregulating BCL-2 (Guo et al., 2017), proteins that indulge in the intrinsic apoptosis pathway. We chose to investigate if thiodicarb would affect this pathway, searching for imbalances in the expression levels of BAX and BCL-2, and BAX/BCL-2 ratio within the exposed cells. The results supported that thiodicarb does not affect this pathway in BLTK-1 Leydig cells, meaning that it could promote cellular apoptosis through a distinct pathway.

Finally, we evaluated whether thiodicarb exposure could alter androgen production, specifically the secretion of androstenedione. Many previous studies have reported that carbamate exposure, in vivo, impacts testosterone production and the expression of steroidogenesis-related genes. In our study, we observed that BLTK-1 Leydig cells exposed to 500 µM of thiodicarb exhibited decreased androgen production. This finding is in line with research by Lu and Liu, which found that TM3 Leydig Cells exposed to methomyl had downregulated testosterone production, showing the impact of carbamates on testosterone production and steroidogenesis-related genes (Lu & Liu, 2023).

6. CONCLUSION

In conclusion, the alarming decline in global sperm count over the past five decades, which has accelerated in recent years, has raised significant concerns within the scientific community. Male infertility is a multifaceted issue with various factors contributing to its prevalence. Genetic conditions, congenital issues, endocrine disorders, obstructive causes, infections, vascular issues, neoplastic conditions, and lifestyle and environmental factors all play a role in compromised male fertility. Additionally, sexual dysfunction is a contributing factor, with a substantial proportion of cases deemed idiopathic. Studies have shown that carbamates, a group of compounds including thiodicarb, can impair male fertility by altering Leydig cell function and causing damage to spermatozoa.

In our study, we explored the impact of thiodicarb on male fertility, specifically its effects on androgen production. Overall, our findings showed deleterious effects of higher concentrations of this pesticide on both Leydig cells' survival and androgen production, hinting on the potential reproductive toxicity of thiodicarb, a relatively unexplored carbamate pesticide, and its impact on in vivo Leydig cells and androgen production. They further underscore the importance of more research into the effects of this compound on male fertility and the need for increased awareness of the potential risks associated with carbamate exposure. As male fertility continues to face challenges, understanding and addressing the factors contributing to this decline becomes increasingly crucial for public health and reproductive well-being.

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