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Ethylene Glycol Monomethyl Ether (EGME) Disturbs the Gonadotropic Hormones and the Biological Quality of Semen in Rabbit, *Oryctolagus cuniculus*

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DN proposal of the idea of the research, planned and cunducted it, perfromed the formal analysis and wrote the manuscript. SD, WH and LF conducted the experiments, treated the animals, performed lab tests and helped in writing the manuscript.

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ABSTRACT

Glycol ethers (GEs) are amphiphilic solvents widely used for industrial or domestic applications. Their toxic effects on the reproduction and development of mammals have been well studied. This family presents important structural differences leading to various behaviors in terms of their metabolism and their toxic effects. This work aims to study the impact of ethylene glycol monomethyl ether (EGME) on the gonadotropic axis and semen biology in the rabbit, *Oryctolagus cuniculus*. Thirty adult male rabbits were divided into three groups (n = 10): a control group and 2 groups treated with EGME. The solvent was applied at two increasing doses: 50 ppm and 150 ppm for five successive weeks, orally. The level of the circulating gonadotropin hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH), the level of testosterone, the biological characteristics of the sperm, the weight and the histopathology of the testes were studied. The results obtained show a significant dose-dependent decrease in LH, FSH and testosterone in the treated groups compared to control. The administered solvent caused severe deterioration in sperm quality, decreased sperm concentration, mobility and vitality, with increased sperm morphological malformations. The treated individuals show hypogonadism and severe tissue damage, especially in group treated with 150ppm dose.

INTRODUCTION

Glycol ethers (GE) are chemicals that have been used for decades due to their remarkable properties. miscibility in water and fats, low volatility and odor, very slightly harmful during conventional short-term toxicological tests. GE have multiple industrial uses (as solvents, preservatives, tensioning agents, coalescing agents, vehicles of additives), here are present in a very wide range of consumer products (Fastier *et al*., 2005). There are two subgroups: the ethylene series (E) and the propylene series (P). In the E series, EGME is the most studied and serves as a reference molecule. Some of these have been classified as reprotoxic. The substitutes of glycol ethers of the P series, and E series are considered less dangerous or have been replaced by other products (INRS, 2011).

The toxicity of EGME is manifested over a broad spectrum of the human and animal body. Several studies have shown harmful effects of the product on hepatic, renal, hematological and reproductive function.

The reprotoxicity includes harmful effects on sexual maturation, production and transport of gametes in adulthood, sexual behavior, gestation, and other dependent functions of the reproductive system (Malik and Gupta, 2013). EGME is one of the recognized testicular toxins, a large number of studies have been able to describe the testicular effects and the mechanism of action). The specificity of the testicular effect of EGME means that it can be used in experimental models *in vivo* and *in vitro* to obtain germline depletion and thus study the paracrine interactions between the different cellular components of the testis (Matsuyama *et al*., 2018). All the toxicological studies carried out *in vivo* in laboratory animals consistently show the deleterious consequences of EGME (and its acetate) on testicular function, which may lead to impaired fertility (Adeyemo-Salami *et al*., 2018).

In humans, European regulations have defined

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criteria for determining whether or not chemicals are dangerous. Seven glycol ethers have been classified as "toxic for reproduction" and are labeled with the following phrases "may impair fertility" and / or "may cause harm to the unborn child" (Garlantézec *et al*., 2020). Only five of them are marketed. These are glycol ethers derived from ethylene glycol (Series E) namely ethylene glycol ethyl ether (EGEE), ethylene glycol methyl ether (EGME), their acetates (EGEEA, EGMEA) and diethylene glycol dimethyl ether (DEGDME).

The most toxic glycol ethers, containing 0.5% or more of substances classified as reprotoxic, have been prohibited for sale to consumers since 1994. However, not all glycol ethers are currently prohibited, particularly in the workplace. However, since 2001, the most dangerous glycol ethers have been the subject of specific regulations in the workplace with the obligation of substitution by a non-dangerous or less dangerous product, the organization of reinforced medical monitoring of workers with exposure traceability and a ban on exposing pregnant or breastfeeding women to agents toxic for reproduction (Nisse *et al*., 2017).

In this context, this work aims to assess endocrine disruption of reproductive function and alteration of sperm quality following exposure to EGME in the local rabbit, *Oryctolagus cuniculus*.

MATERIALS AND METHODS

Thirty adult male rabbits were divided into three groups, fed and raised in a pet store: control group received water and a healthy diet; group D1: treated with 50 ppm dose of EGME solvent and group D2: treated with 150 ppm dose of EGME solvent.

The animals used in this study are adult male rabbits of the species *Oryctolagus cuniculus*. They are subjected to a period of adaptation to laboratory conditions (10 days) at room temperature (25°C) and a photoperiod of 12h/ 12h.

The solvent used is part of the "E" category of glycol ethers.

The treatment was carried out orally by administering 1 ml of the product every 24 h for five successive weeks.

At the end of the treatment, the rabbits of the 3 groups were fasted for 15 h and then were sacrificed (by decapitation). The blood was immediately collected and centrifuged to get the plasma which was stored at -20°C for estimation of luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone.

After dissection, the testes are carefully removed, stripped of their adipose tissue, rinsed in 0.9% sodium chloride solution, and then weighed. Sperms were collected after a small incision at the tail of epididymis, diluted in physiological saline (NaCl 0.9%) in order to study the biological characteristics of the sperm according to the methods of WHO (2010).

Determination of gonadotropin hormones (LH and FSH)

The blood was collected in heparin tubes, centrifuged at 5000 rpm for 10 min and the plasma was collected in eppendorf tubes stored at -20ºC. Radioimmunological (RIA) assay was used for estimating LH and FSH using UCB-Bioproducts-Belgium kits (Established Reference Hormones: Ovine FSH-B-5 and LH-B-5). The sensitivity of the LH and FSH assays was 100 pg/ml. The rate expression was determined using a calibration curve (Steyn *et al*., 2013).

Determination of testosterone

The assay of the male sex hormone was carried out by applying the electrochemiluminescence test (ECLIA) using Elecsys-testosterone kit (Ref 1177606) which is suitable for immunological assays on an Elecsys 1010 device. The test uses a competition principle using a specific antibody directed against testosterone (Fritz *et al*., 2008). The results were obtained using a calibration curve. A reference curve was stored in the reagent barcode and readjusted for the analyzer used by a two-point calibration.

Study of the biological characteristics of sperm

CASA system (Computer-Aided Sperm Analysis: Hamilton Thorne (HT) CEROS II Clinical): an automated system for calculating the quantity and quality of sperm (Tomlinson and Naeem, 2018) was used to quantify the concentration (total number), mobility and vitality of sperm, as well as the rate of morphological malformations of sperm. The measurement methods are recommended by WHO (2010).

Histopathological study of the testis

After sacrificing the rabbits, the samples of testis (1 to 2 cm^2 and a thickness of 1.5mm) were immediately fixed in 10% formalin for 12 h. The samples are then dehydrated at least 12 h passing the tissue through ethanol baths of increasing concentrations (70%, 80%, 90% and 100%), and then embedded in liquid paraffin. Thin sections (4 to 5 µm) were cut and then stained with HES (Hematéine/ Eosin / Saffron). The stained sections are mounted between slide and coverslip with a synthetic resin and were observed at magnification x 40 under the optical microscope.

Statistical analysis

The results are expressed as the mean ± standard deviation (X ± SD). The statistical processing of the data and the comparison of results was carried out using the

software "Minitab: Data Analysis, Statistical and Process Improvement Tools" by the application analysis of variance with 2 classification criteria (ANOVA).

RESULTS

Gonadotropin hormone and testosterone levels

Our results show that rabbits treated with EGME have low levels of LH and FSH hormones and testosterone compared to the control group. This decrease is highly significant ($p<0.01$) between the controls and the group treated with 50 and 150 ppm dose, and significant between the control and the D1 group, the effect of which appears to be dose-dependent (Table I).

Table I. Effect of EGME on LH, FSH and testosterone levels in blood of rabbits. a: difference between controls and D1. b: difference between controls and D2. * p<0.05, ** p<0.01.

Groups	Control	Concentrartion of EGME		Stat
		50 ppm (D1)	150 ppm (D2)	
LH (UI/L)	8.64±1.02	5.02±0.80	1.01±0.04	a*, b**
FSH (UI/L)	12.81±1.11	8.68±0.12	2.31±0.01	a*, b**
Testosterone (ng/ml)	11.64±2.02	6.41±0.92	2.39±0.09	a**, b**, c*

Biological characteristics of spermatozoa

All biological characteristics of sperm were altered in individuals treated with EGME. The sperm concentration and motility marked a very highly significant decrease ($p<0.001$) between control and treated groups, as well as a significant decrease ($p<0.05$) between the D1 group and the D2 group. The vitality of spermatozoa decreases significantly ($p<0.05$) between control and D1 group, and very highly significantly ($p<0.001$) between control and D2 group (Table II).

Table III shows that administration of EGME has caused the development of several types of morphological malformations in sperm, on the head (A), the middle part (B) and the flagellum (C). The rates of malformations show a significant increase ($p<0.05$) in the D1 group compared to the control, and a highly significant increase ($p<0.01$) in the D2 group.

Testicular weight

We recorded a significant ($p<0.05$) decrease in testicular weight in D1 group (50ppm) compared to control, and highly significant ($p<0.01$) in the group treated with the 150ppm dose (Table IV).

Table II. Effect of EGME on the concentration, mobility and vitality of sperums of rabbit. a: difference between controls and D1. b: difference between controls and D2. c: difference between D1 and D2. * p <0.05, *** p <0.001.

Groups	Control	Concentrartion of EGME		Stat
		50 ppm (D1)	150 ppm (D2)	
Concentration ($x10^6$/ml)	476.84± 80.17	269.31± 32.22	113.52± 6.06	a***, b***, c*
Mobility (%)	86.91± 11.02	22.32± 1.02	10.05± 0.88	a***, b***, c*
Vitality : living cells (%)	69.87± 9.24	41.83± 5.22	32.17± 4.48	a*, b***

Table III. Effect of EGME on the morphological malformations of rabbit spermatozoa. a: difference between controls and D1. b: difference between controls and D2. * p <0.05, ** p <0.01.

Groups	Control	Concentrartion of EGME		Stat
		50 ppm (D1)	150 ppm (D2)	
Type A (%)	19.54±2.20	32.15±6.31	53.77±9.70	a*, b**, c*
Type B (%)	21.85±3.91	39.67±5.73	69.52±9.49	a*, b**, c*
Type C (%)	18.47±2.24	29.36±4.37	63.79±8.84	a*, b**

Table IV. Effect of EGME on the testicular weight of rabbit. a: difference between controls and D1. b: difference between controls and D2. * p <0.05, ** p <0.01.

Groups	Control	Concentrartion of EGME		Stat
		50 ppm (D1)	150 ppm (D2)	
Testicular weight (g)	5.45±0.97	3.55±0.42	1.95±0.03	a*, b**

Histopathology of testis

The histopathological study makes it possible to visualize the testicular alterations. The section of the testis from the control group shows a normal architecture with tightly cylindrical seminiferous tubules (ST) and weak interstitial spaces (IS), the lumen (L) of each tube is almost occupied by the mature sperm mass (Spz) (Fig. 1A).

In contrast, in rabbits treated with EGME (50ppm), the seminiferous tubules lost their cylindrical shape, and the volume of the sperm mass decreased, as well as partial atrophy of the interstitial tissue (Fig. 1B).

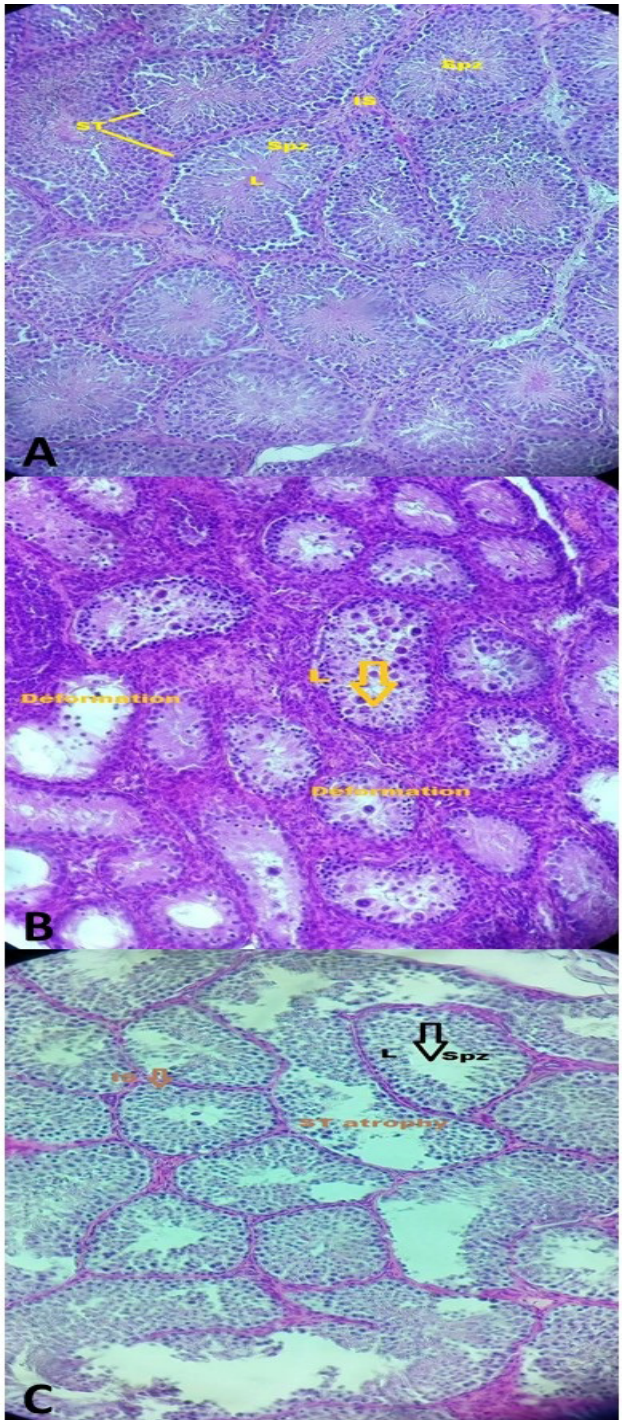

Fig. 1. Effect of different concentrations of EGME on the histological structure of testes of *Oryctologus cuniculus*. A, control; B, 50ppm treatment; C, 150ppm treatment. Magnification: 40X. Stain: HES.

In group D2 (150ppm), the histological changes seem to be more severe: the action of the product is very clear, a decrease in the lumen of the seminiferous tubes, an atrophy of the interstitial tissue between the seminiferous tubes (absence of Leydig cells), which means the total absence of spermatogenesis and spermiogenesis (Fig. 1C).

DISCUSSION

Industries that manufacture, process and use GEs can emit them into the air and release them into surface or groundwater. In 1992, in United States, approximately 1,700 tonnes of EGME were released to environmental media (Eckel *et al*., 1996). These GEs were found in landfill sites as the usual pollutants, and subsequently they would represent a dangerous source of toxicity in humans and animals.

According to the results of several works, the main toxic effects of GEs, at repeated doses, are hematological, testicular, and teratogenic, as well as other systemic effects (hepatic, renal, neurological) (Cadena *et al*., 2018). EGME exerts its toxicity through its acid metabolites (methoxyacetic acid, MAA) and even more aldehydes (methoxyaldehyde, MALD). These are able to enter the nucleus of cells and alter the structure and function of the genome governing cell growth and development (Somade *et al*., 2020).

According to our results, exposure of rabbits to doses of 50ppm and 150ppm orally induced a decrease in gonadotropic hormones LH and FSH, as well as the male sex hormone (testosterone). These effects are accompanied by decrease in testicular weight, concentration, mobility, vitality of spermatozoa as well as increase in morphological malformations of spermatozoa.

Knowing that LH and FSH represent the pituitary factors responsible for male and female fertility, the highly toxic metabolites of EGME are able to act on specific receptors for hypothalamic GnRH (Gonadotropin-Releasing Hormone) in the anterior pituitary and cause disturbances in the synthesis of pituitary LH and FSH. In the same axis, these metabolites can block LH receptors in Leydig cells and inhibit the production of testosterone, on the other hand, there can be an effect on the structure and function of Sertoli cells by the decrease the amount of testosterone transporter proteins (Semet *et al*., 2017). The circulating testosterone must then reach the seminiferous tubes within the testes to trigger germ cell division and ensure spermatogenesis.

This toxicity appears to be strongly dependent on the metabolism of EGME since pretreatment with an alcohol dehydrogenase inhibitor of rats exposed to EGME prevents testicular effects suggesting a role of alkoxy acids in the

genesis of testicular lesions. MAA, the main metabolite of EGME, also reproduces the effects of the parent substance in rats (atrophy of seminiferous tubules, specific and preferential damage to pachytene spermatocytes) (Masoudi *et al*., 2016).

These testicular effects are mainly linked to hypogonadotropic hypogonadism, i.e. a decrease in the synthesis of sex hormones due to a decrease in the secretion of gonadotropic hormones LH and FSH (Slama *et al*., 2016). This decrease in the synthesis of sex hormones leads to an alteration in sperm production. Hypogonadotropic hypogonadism should be considered in the presence of azoospermia or oligospermia (Young, 2016).

Several international studies have confirmed the reprotoxic effect of GEs, and have shown that the testes are target organs for toxic products released into the environment (Zama *et al*., 2016; Khosravi *et al*., 2019).

In the same context and histopathologically, exposure to GEs can cause deleterious effects on the structure and function of the seminiferous tubes. These effects are specific, they are the cells of the germ line (spermatocytes at the pachytene stage) who are the target. Meiosis-blocking germline involvement can lead to arrest of spermatogenesis (Somade *et al*., 2017).

For low doses or short-term exposures, lesions are reversible. For prolonged exposure or at high doses, the lesions can affect the stem cells (spermatogonia) and in addition the Leydig cells, in this case, the arrest of spermatogenesis can be permanent and the testis atrophy (Yoon *et al*., 2003; Vachhrajani, 2005).

Other studies have proposed the hypothesis of the phenomenon of apoptosis (cell death) of germ cells spermatocytes or spermatids to explain the reduction in total number of spermatozoa (Matsuyama *et al*., 2018), which is why in groups treated with EGME we noticed the presence of some cellular debris in the lumen of seminiferous tubes.

Animals exposed to the 150 ppm dose showed a high decrease in sperm mobility, same result recorded in workers of a building painting factory (Arafa *et al*., 2018), this decrease is due to the effect of EGME (its metabolites) on the structure and function of flagellum and the intermediate piece inducing malformations which make the flagellum more fragile and incapable of growing sperm. This effect can also be explained by the action of membrane lipid peroxidation induced by the toxic product (Jansen *et al*., 2004; Kido and Namiki, 2000), EGME can also affect the number of mitochondria responsible for producing the energy necessary for the movement of spermatozoa. Damage to the spermatids (elongated nucleus) leads to a reduction in sperm mobility by acting on the spermiogenesis phase (transformation of spermatids into mature spermatozoa), and by also altering Sertoli cells: feeder cells of sperm cells (Adedara *et al*., 2013).

Regarding the malformations observed at the sperm head following exposure to EGME, they can be explained by the cytotoxicity of the solvent at the time of spermiogenesis during which the head, the intermediate part and the flagellum are formed, it can induce an imbalance during the accumulation of Tubulin and Dynein fibers (Adeyemo and Farombi, 2018).

The MAA, but not EGME, induces depletion of pachytene spermatocytes. MALD generates the same effects but seems more effective than MAA. These substances could, by reducing the production of lactate in Sertoli cells, reduce the energy intake of the germ line. However, the decrease in the production of lactate in the Sertoli cell is a usual manifestation of the action of many testicular toxins and therefore nonspecific. For others, alkoxyacids could intervene in the synthesis of DNA and RNA of germ line. Alkoxyacids (in particular MAA) inhibit the DNA synthesis of cells in culture of mouse embryos, this inhibition is attenuated by the addition of simple molecules such as sarcosine, glucose or glycine. These physiological compounds prevent EGME toxicity *in vivo*. An explanation would be that MAA would compete with the transfer of mono-carbon residues (methyl, formyl), from these simple molecules, to purine and pyrimidine bases. This would result in a base deficit which could lead to blockage of cell divisions. In the case of pachytene spermatocytes, RNA synthesis would be affected (Aitken *et al*., 2015).

The study of the genotoxicity of GEs raises a recent concern of testicular atrophy effects in mice. The question asked was that of the potential carcinogenicity of these substances. In the absence of results from epidemiological studies and long-term experiments in animals, it was appropriate as a first approach to assess the mutagenic and genotoxic potentials of these substances using cell models validated for this purpose (Alamo *et al*., 2019). In the absence of mutagenic effects of ethylene glycol ethers on models in the classics, research has been directed towards the study of their metabolites.

CONCLUSION

Most chemicals intended for use in industry have toxic effects on parameters indicative of male fertility in humans and animals. Ethylene glycol monomethyl ether (EGME) as a study example exerts its toxicity through acidic metabolites and even more aldehydes. This work demonstrated the reprotoxic effect of glycol ether (EGME) on the endocrine and cellular function of testes and on the structure and function of seminiferous tubules

inducing cases of infertility. The germ cell line, mature sperm, Leydig cells, Sertoli cells represent a main target of EGME metabolites. It remains useful to investigate in perspective the cellular and molecular mode of action on the hypothalamic-pituitary and cellular axes.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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