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Direct infiltration effects of dimethyl sulfoxide into ovarian bursas on spontaneous ovulation and the estrous cycle of the rat: antagonism on the type 2 dopaminergic receptor

Efectos de la infiltración directa de dimetil sulfóxido dentro de las bursas ováricas sobre la ovulación espontánea y el ciclo estral de la rata: antagonismo del receptor dopaminérgico tipo 2

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Abstract

We studied effects of microinjection (MI) into ovarian burses (OB) of infiltering agent dimethyl sulfoxide (DMSO) on number of ova shed (NOS) and estrous cycle (EC) of adult female rats with fourth day regular estrous cycles (REC), and we compared these effects with a MI of 100µg sulpiride solution in DMSO. Between 13:00-14:00h of different days of EC, CER groups received 20µL of pure DMSO into both OB; the sham groups received MI with 20µL distillated water (H_2O) into each OB. All rats with MI performed and an intact cyclic rat group were autopsied in the morning of next estrous vaginal shown (EVS). Both DMSO and H_2O MI does not modify the EC duration and NOS. Other groups of cyclic rats received a MI with 20µL sulpiride solution into each OB and were autopsied at next EVS. Just the sulpiride MI performed on diestrous-1 day delayed 24h the next EVS, but that don't occur in other sulpiride groups. The NOS were not modified in all experimental group. The DMSO direct administration in ovarian tissue does not affect gonadal primordial functions and its use is recommended like an infiltrating agent of non-polar drugs.

Spontaneous ovulation, Oestrous cycle, Dimethyl sulfoxide, Ovarian dopaminergic system, Sulpiride

Resumen

Se estudiaron los efectos de la microinyección (MI) dentro de las bursas ováricas (BO) del agente infiltrante dimetilsulfóxido (DMSO) sobre el número de ovocitos liberados (NOL) y el ciclo estral (CE) de ratas adultas con ciclo estral regular (CER) de 4 días y se compararon estos efectos de la MI con 100µg de sulpirida disuelto en DMSO. Entre las 13:00-14:00h de cada día del CE, grupos de animales con CER recibieron MI con 20µL de DMSO puro dentro de cada BO; los grupos testigo recibieron 20µL de agua destilada (H_2O). Todos los animales con MI y un grupo de animales cíclicos intactos se sacrificaron al estro vaginal observado (EVO). La MI del DMSO o H_2O no modificó la duración del CE ni el NOL. Otros grupos de animales con CER recibieron una MI con 20µL de sulpirida en cada BO y se sacrificaron al EVO. La MI con sulpirida en el diestro-1 retrasó el EVO 24 horas, pero no ocurrió en los otros grupos con sulpirida. No hubo cambios en el NOL entre los grupos. El DMSO administrado directamente sobre el tejido ovárico no afecta las funciones primordiales de la gónada y se recomienda su uso como agente infiltrante.

Ovulación espontánea, Ciclo estral, Dimetil sulfóxido, Sistema dopaminérgico del ovario, Sulpirida

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Introduction

Biological membranes are characterized by their extreme selectivity, so that several solutes cannot pass through them freely and even fail to interact with their lipids and proteins (Alberts et al. 2008).

Due to their polar properties, many experimental drugs and those in certified clinical use have difficulty in performing their actions at the membrane level, which forces the increase of doses and prolonged waiting times for these drugs to have the expected actions and effects (Brunton *et al*., 2012). On the other hand, it is known that the extracellular matrix plays a preponderant role in the mechanisms of recognition and biological immunity (Alberts *et al*., 2008). According to the above, how can the problem of rapid action of an active drug to solve a health problem be solved? The vehicle used as a means of transporting the drug can be a solution.

Dimethyl sulfoxide (DMSO; $(CH_3)2SO$) is a colorless liquid, has been used as an organic and industrial solvent, organ and tissue cryopreservative (Pegg, 2007), as a drug in Veterinary and Human Medicine (Parkin *et al*., 1997; Pope and Oliver, 1966) and, in the last twenty years, in the innovation of technologies in Molecular and Cell Biology (Chakrabarti and Schutt, 2001).

DMSO was discovered in 1866 by the Russian scientist Alexander Saytzeff. It is obtained as a by-product during the processing of wood pulp for paper manufacturing (Shirley *et al*., 1978).

The great potential of DMSO as an infiltration agent postulates it as an excellent choice for the experimental use of an array of active drugs whose water solubility is low. For example, dopamine antagonists are very insoluble in water and their use in in vivo systems makes it difficult to interpret experimental data when they are used as tools in the study of their effects on biological systems.
There is a growing interest in analyzing the functional role of DMSO for its amphipathic properties and its effects when interacting directly on the extracellular matrix in tissues where it is deposited as an infiltrative agent.

However, there is ambiguous and contradictory information on the supposedly toxic effects of DMSO, and therefore in the present work the biological effects in vivo of the solvent applied as an infiltrating agent in the ovarian tissue of the adult female rat were analyzed. Its direct effects when applied in living tissues were analyzed by looking for signs of cytotoxicity by means of conventional histological techniques of bright field microscopy and recording the duration of the estrous cycle, as well as the number of gametes released in the adult rat model, as indicators of the functional alteration of the gonad. On the other hand, its efficacy will be tested when used as an infiltrating agent of a specific and selective dopaminergic antagonist of the dopamine type 2 receptor, sulpiride.

Functional structure of the ovary

The ovaries of the rat are oval-shaped paired structures that vary in appearance and size depending on the stage of the reproductive cycle. The microscopic anatomy of the rodent ovary is shown in Figure 1.

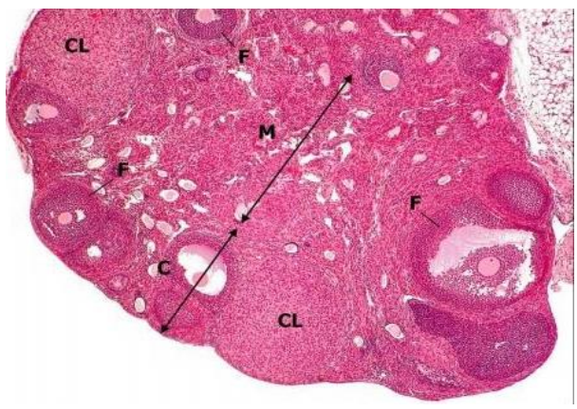

Figure 1 Microscopic anatomy of a normal rodent ovary (mouse, 40X). The cortex (C) contains numerous follicles (F) at various stages of maturation and corpora lutea (CL). The medulla (M), which is not always present in histological sections, contains lymphatic vessels, nerves and blood vessels
Source: (Taken from Cartwright and Moreland, 2008)

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Covering the surface of the ovary is a single layer of modified peritoneal mesothelium, the ovarian epithelium (OSE) and continues with the broad ligament (mesovarium) that provides support for the organ. The OSE of the ovary can vary in type from squamous to cuboidal, columnar to pseudostratified epithelium; this regional variation in the surface morphology of the OSE is accompanied with the cyclic changes that occur within the underlying ovarian parenchyma during the estrous cycle (Erickson, 1995; Figures 2 and 3).

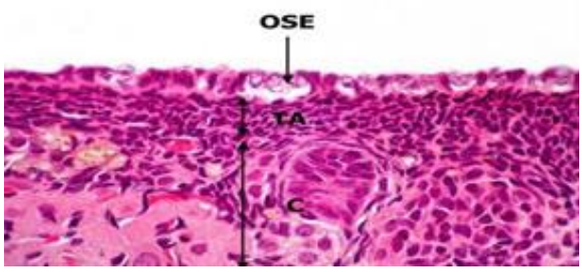

Figura 2 Ovarian superficial epithelium (OSE) - columnar type epithelium. Tunica albuginea (TA). Cortex (C) (rat, 400X)
Source: (Cartwright y Moreland, 2008)

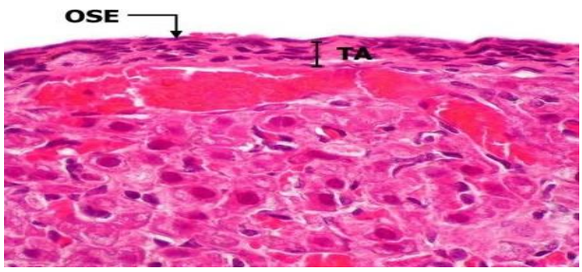

Figure 3 Ovarian surface epithelium (OSE) - squamous epithelium. Tunica albuginea (TA) (rat, 400X)
Source: (Cartwright and Moreland, 2008)

Three distinct zones can be distinguished in the mammalian ovary: 1) the cortex, which is the most dominant zone and contains the follicles at different stages of maturation and the corpora lutea, between the follicles are the supporting connective tissue and stromal cells. 2) the medulla, which contains a rich vascular network and connective tissue; and 3) the hilum, where the ovarian artery and vein, lymphatic vessels, nerve terminals and interstitial cells are found (Erickson, 1995; Sanchez-Criado, 1999; Yao and Barh, 1999) (Figure 1).

The ovary is lined by a single layer of epithelial cells: the germinal epithelium. The adult ovary consists of three different functional units: 1) the interstitial tissue, 2) the follicles and 3) the corpora lutea. These structures are in constant transformation. Thus, while some of them regress (e.g., atresic follicles become part of the stroma), others may form (the follicle after ovulation becomes a corpus luteum) (Erickson, 1995).

Future interstitial cells are found in the hilum, which are derived from mesenchymal cells in the stroma of the ovary. Interstitial cells synthesize and produce androgens which are very important in the regulation of a number of fundamental reproductive processes and are therefore of great physiological relevance (Erickson, 1995).

Four classes of interstitial cells are found in the ovarian stroma and are classified by their characteristics and position in the ovary as primary, thecal, secondary and hilar. The thecal interstitial cells originate from the stroma and migrate toward the basement membrane of the follicle where they are arranged in bands to form the inner and outer thecae. This migration toward the basement membrane takes place when the oocyte is maturing, and the follicle contains two or three layers of granulosa cells. After ovulation, the thecal cells are transformed into thecal luteal cells of the corpus luteum. The thecal-interstitial cells of the atretic follicles with antrum become part of the interstitial gland. In contrast, the cells surrounding the preantral follicles in which cell differentiation has not occurred and which enter atresia do not form part of the interstitial gland because they do not possess LH receptors. The same is true for thecal cells of preovulatory follicles that enter atresia and will not be part of the interstitial gland either. Interstitial thecal cells have receptors for LH, prolactin, adrenocorticotropin (ACTH), noradrenaline, GnRH and estrogens (Dominguez, 1993).

When the follicles fail to release their oocytes and consequently become atretic, the thecal interstitial cells are transformed into secondary interstitial cells that maintain steroidogenic activity and are innervated by adrenergic terminals. The interstitial cells synthesize and secrete testosterone in response to LH stimulation (Erickson, 1995).

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The ovarian follicle is the anatomical and functional unit of the ovary. Most follicles are located mainly in the periphery of the cortex, immediately below the tunica albuginea. The follicle consists of an oocyte, which is surrounded by granulosa cells that in turn form the corona radiata cells (cumulus oophorus), follicular antrum, granulosa cells, basal lamina, inner theca cells, theca-interstitial cells, connective tissue, outer theca (Greenwald and Terranova 1988; Erickson, 1995; Fawcett, 1995; Van Voorhis, 1999; Zhang, 1999) (Figure 4).

The corpus luteum is a transient endocrine gland, formed by follicular cells that remain in the ovarian tissue after ovulation (Juengel et al. 1999). The formation of the corpus luteum is initiated by a series of morphological and biochemical changes in the cells of the inner theca and granulosa membrane of the preovulatory follicle. This is called luteinization and occurs as a result of an increase in blood LH levels associated with the preovulatory surge of this hormone. A more complete description of the morphological changes associated with luteinization in the rat is that of Anderson and Little (1985), who state that after ovulation and follicular elimination, the follicle wall collapses and the granulosa cell layer is arranged in folds. The basal lamina that previously separated the granulosa cells and the inner theca is ruptured, where there is some extravasation of blood from the capillaries of the outer theca resulting in the formation of a central clot that invades the cavity of the ruptured follicle, the growth of these new vessels appears to be due to an angiogenic factor that must be secreted soon after rupture of the follicle (Figure 4).

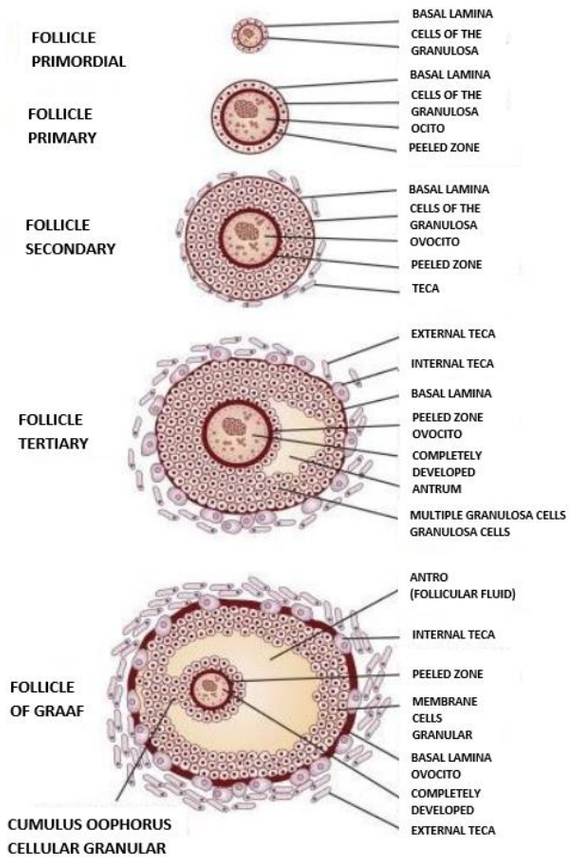

Figure 4 Diagram showing the development and different stages of ovarian follicle growth; throughout follicular development, the main compartments undergo gradual changes characterized by the proliferation of granulosa cells and theca cells, as well as the appearance of the antrum; At the onset of development, the oocyte leaves its prophase I dictyotene state and continues its differentiation until the completion of the second meiotic division if and only if it is fertilized by the spermatozoon
Source: (Taken from Bulun and Adashi, 2003)

Granulosa and inner theca cells undergo cytological changes. They enlarge and accumulate lipids and transform into pale polygonal cells, called luteal cells. In the rat, the number of junctional spaces between granulosa cells increases as the follicle matures, but decreases just before ovulation (Juengel et al. 1999; Niswender and Nett, 1988).

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The number of corpora lutea present in the ovary depends on the number of ovulations which, in turn, varies according to the species. After ovulation, the transformation of the follicle into a corpus luteum is mainly due to hypertrophy of the luteal cells and increased vascularization. Since the luteal cells are derived from the granulosa and inner theca cells, two types of luteal cells can be distinguished: those in the interior, which constitute the major part of the luteal tissue and are larger cells, and those in the periphery, which are smaller cells. Large luteal cells (granulosa-luteal cells, type II cells) are polyhedral and contain all the elements of steroid-secreting cells (mitochondria, smooth endoplasmic reticulum and secretory granules); on the contrary, small luteal cells are irregular and derived from theca cells (type I cell), contain ribosomes attached to the endoplasmic reticulum and do not present secretory granules (Niswender,1988).

Stral cycle

Much of what is known about spontaneous ovulation is based on knowledge of the estrous cycle of the rat. The rat cycle is unique in its brevity. The periodicity of light plays an important role in the length of the stages of the estrous cycle. The word estrus is a Latin adaptation of the Greek word oistros meaning: accessory, stinging or frenzy. This term was used by Heape, to describe the special period of the female's sexual desire and is distinguished from the male's cycle (Freeman, 1988).

The estrous cycle of the rat lasts 4 to 5 days and is regulated by endogenous factors, particularly by the interaction of the hypothalamic-pituitary-ovarian axis and can be influenced by exogenous factors such as light, temperature and chemicals sensed by the olfactory epithelium (Freeman, 1988; Kilen and Schwartz, 1999).

From the analysis of the changes of the vaginal epithelium, the estrous cycle is divided into four phases diestrous-1, diestrous-2, proestrus and estrus, below are the main changes of the vaginal epithelium in relation to the hormonal environment. The estrous cycle comprises a given period of time and is divided into the following phases: Estrus-1. This phase has a duration of 6 to 8 hours.

During diestrous-1 the plasma concentration of LH, FSH, estrogens and progesterone are basal. Follicles in all stages of growth are observed in the ovary. Mating and copulation are not allowed. Progesterone secretion by the corpus luteum and estradiol secretion by the follicles inhibit gonadotropin secretion. Vascularization and motility of the uterus are decreased. Vaginal smear shows leukocyte infiltration along with some cornified cells. Recently formed corpora lutea are the main source of progesterone secretion. During this day, functional regression of the corpus luteum begins as long as there has been no copulation, which in some rodents stimulates the release of prolactin (luteal-trophic hormone). Estradiol secretion by the growing follicles continues to increase (Freeman, 1988; Kilen and Schwartz, 1999).

Diestro-2. This phase lasts 55 to 57 h. Like the diestro-1 phase, ovarian steroids inhibit basal secretion of gonadotropins, which in turn maintain follicular growth. Plasma estrogen concentrations begin to increase in the afternoon of this day as a result of stimulation of the enzyme aromatase by FSH. FSH also stimulates mitotic division of the granulosa cells which results in the growth and differentiation of the follicles that will ovulate in this cycle. The corpus luteum continues to regress. The uterus is small, anemic and non-contractile (Freeman, 1988; Kilen and Schwartz, 1999).

Proestrus. The proestrus phase lasts 12 to 14 hours. The follicles have reached the stage of preovulatory follicles and secrete large amounts of estradiol. This hormone now exerts a stimulating effect (positive feedback) on the secretion of gonadotropins. On the morning of this day, plasma estrogen concentrations rise sharply, reach a maximum (preovulatory estrogen peak), and then fall sharply, which stimulates the activity of noradrenergic neurons in the hypothalamus, causing preovulatory GnRH secretion. This event stimulates preovulatory FSH and LH release, but FSH release occurs slightly earlier than preovulatory LH discharge. In the afternoon of the same day, maximum concentrations of these hormones are reached. The peak in plasma LH concentration stimulates the synthesis of plasminogen in the ovary, which initiates the cellular mechanisms that lead to the rupture of the follicle wall so that the oocyte can be expelled hours later.

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The interstitial gland secretes progesterone, which promotes ovulation. LH induces ovulation and luteinization of the remains of the follicle that released its oocyte. The uterus, by the action of estradiol, becomes extremely contractile and nucleated epithelial cells appear in the vagina. Copulation is accepted only in late proestrus, with the onset of the dark phase. FSH secretion on the morning of estrus, which is due to decreased ovarian secretion of inhibin, stimulates follicular growth (Freeman, 1988; Kilen and Schwartz, 1999; Sanchez-Criado, 1999).

Estrus. The estrous phase lasts from 25 to 27 hours. On this day, the estrus period occurs, in which the female shows patterns of sexual behavior. Approach, mating and copulation are accepted. As ovulation occurs in the early morning of this day, the eggs are in the oviduct. The postovulatory follicle begins to structure itself as a corpus luteum while a new series of primary follicles begin to develop. Numerous mitoses are found in the vaginal mucosa, displacing the more superficial layers (squamous and cornified epithelium), which are exfoliated into the lumen of the vagina. The presence of these cells in the vaginal smear is indicative of estrus and a sign of probable ovulation (Freeman, 1988; Kilen and Schwartz, 1999; Sanchez-Criado, 1999). The plasma concentration of progesterone increases on the day of estrus, while estrogens and LH are basal, except for FSH because in the morning of this day of the cycle a second peak in plasma FSH concentration occurs, although of lesser magnitude than in the afternoon of proestrus, whose role is to recruit the follicles that will ovulate in the following cycle (Freeman, 1988; Figure 5).

FSH binds to its respective receptor on the cell membrane and stimulates estrogen synthesis by the granulosa cells. The hormone-receptor complex acts on the adenylate cyclase enzyme system, induces an increase in cAMP and stimulates the synthesis and activity of the enzyme aromatase, which transforms androgens to estrogens (Freeman, 1988).

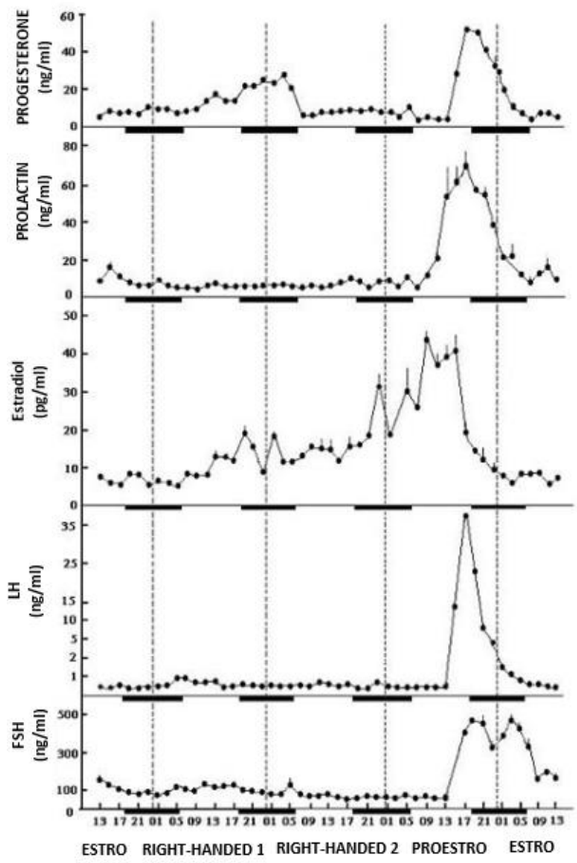

Figure 5 Plasma concentrations of progesterone, prolactin, estradiol, LH (Luteinizing Hormone) and FSH (Follicle Stimulating Hormone) obtained at two-hour intervals during the four days of the estrous cycle of the rat. Each point represents the mean ± E.E.M. concentration of the hormones. The black bars represent the dark phase from 18:00 to 06:00 hours the following day
Source: (Smith et al. 1975; cited by Freeman, 2006).

LH regulates estrogen synthesis by its effects on androgen production by thecal cells and by stimulating the synthesis and aromatase enzyme activity of granulosa cells that have previously been stimulated by FSH (Freeman, 1988). Prolactin inhibits the aromatase activity of granulosa cells and blocks the effects of FSH on the cells themselves resulting in decreased estrogen production. It also acts on the theca cells where it blocks androgen synthesis by inhibiting the formation of cAMP and enzymes that induce the cleavage of the cholesterol side chain.

Estrogen secretion by the follicle is regulated by FSH, LH, prolactin and other factors and hormones whose effects are coupled to those of FSH and LH. Some of these factors and hormones are: GnRH, oxytocin, epidermal growth factor, vasopressin, estrogens, adrenal corticosteroids and prostaglandin (Freeman, 1988).

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Hypothalamus-Pituitary-Ovary Axis

Of all the elements involved in the regulation of ovarian function, the hypothalamus is the site of control and integration of all the nervous and humoral signals coming from the CNS itself, the pituitary, the ovaries and the uterus. In contrast to other endocrine systems, the hypothalamic-pituitary-ovarian axis involves hypothalamic hormones (GnRH), various trophic adenohypophyseal hormones (LH, FSH, prolactin), ovarian hormones (estrogens, androgens and progesterone), peptide hormones and local factors and hormones produced by the uterus (prostaglandins, endogenous opioids and neurotransmitters). Gonadotropin secretion depends on the action of GnRH on the adenohypophysis (Fink, 1999). The output signal from the pituitary is, consequently, the pulsatile secretion of LH and FSH. This pituitary secretion is inhibited (negative feedback) by ovarian hormones (estradiol and progesterone) (Everett, 1988).

The ovary is a cyclic functioning gland, which implies that its components are ephemeral. The input signal is gonadotropins on the one hand, local factors on the other hand, and in some cases substances from the uterus (Speroff et al. 1999). FSH stimulates the growth and maturation of ovarian follicles, while LH causes follicle rupture under the influence of basal concentrations of FSH and LH, the granulosa cells secrete estrogens and at the time of follicular rupture undergo structural and biochemical transformations resulting in the formation of the corpus luteum (Niswender and Nett, 1988).

The output signal, independently of the mature ovum, includes ovarian steroids and inhibin which in addition to their peripheral actions regulate the secretion of GnRH, LH and FSH. While FSH acts only on granulosa cells, LH has multiple sites of action in the ovary: thecal, interstitial, granulosa and luteal cells.

FSH and LH are glycoproteins consisting of two different chains: α-subunit of 96 residues and β-subunit of 114 amino acid residues; and both subunits are linked by non-covalent bonds.

The α and β subunits are synthesized from precursors that undergo posttranslational maturation that includes the addition and modification of oligosaccharides; this glycosylation occurs in both the α and β subunits. It has been postulated that each stage of the maturation process represents a potential locus for physiological control of hormone formation and secretion (Counis, 1999; Fink, 1988).

Gonadotropins are synthesized in the adenohypophysis by specialized cells, the "gonadotropes". They are capable of producing one or several hormones. The synthesis and release of gonadotropins are stimulated by GnRH, a decapeptide secreted in a pulsatile manner by neurons located in different regions of the hypothalamus (Childs, 1999). Estrogens also stimulate the secretion of gonadotropins, particularly LH, through their effects on the pituitary gland and on neurons that regulate GnRH release (Fink, 1988; Blake, 1999; Weick et al. 1971).

Although GnRH stimulates the release of both gonadotropins, there is evidence that FSH and LH secretion is differential and involves other specific hypothalamic factors that stimulate and inhibit FSH secretion (Dominguez, 1993).

FSH and LH secretion is regulated by feedback mechanisms involving the CNS, the adenohypophysis and the gonads. In both the female and the male, basal secretion of gonadotropins is maintained throughout most of their reproductive life, however, in the female this basal secretion is interrupted by a massive release of gonadotropins that occurs periodically and precisely in the form of an intricate positive feedback cascade, which is induced and promoted by ovarian steroids. In both the female and the male, gonadotropin secretion occurs in a pulsatile manner. In the female, LH and FSH secretion is inhibited by estrogens (Fink, 1988; Aguilar, 1992).

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The secretion of gonadotropins is regulated by several neurotransmitter systems, the most studied being the catecholaminergic system (Vijayan and McCaan, 1978), in particular noradrenaline as a regulator of GnRH secretion and, therefore, of FSH and LH. From results obtained by pharmacological studies, it can be presumed that the participation of the various neurotransmitter systems in the regulation of gonadotropin secretion varies during the estrous cycle (Dominguez, 1993).

Like LH, FSH is released in response to GnRH stimulus and shows a maximal release when plasma concentrations of GnRH are increased in the portal-hypothalamic-pituitary system. In the rat, FSH release rates remain relatively high even after the drop in preovulatory LH secretion (Fink, 1988).

Preovulatory FSH and LH secretion is the result of two simultaneous events: 1) the increase in the frequency and amplitude of the secretory pulses of GnRH-ergic neurons in the portal-hypothalamic-pituitary system and, 2) the progressive increase in the sensitivity of the gonadotropes to this hormone (Childs, 1999; Fink, 1988).

The cascade of events leading to preovulatory LH release is initiated by increased secretion of 17 β-estradiol (Fink, 1988). Increased estradiol concentrations allow the expression of a neural signal for LH release: preovulatory GnRH secretion, as well as the progressive increase in the gonadotrope response to GnRH (Childs, 1999; Fink, 1988).

In the rat, preovulatory secretion of FSH occurs approximately 11 hours after that of LH. It has been suggested that this lag between the two preovulatory peaks may be the result of the following factors: that there is a hormone that specifically stimulates FSH secretion; that steroid hormones, inhibin or other factors differentially regulate the response of the gonadotropes to GnRH; or that LH secretion from the adenohypophysis is continuously exposed to GnRH, while FSH is triggered by but then independent of GnRH (Fink, 1988).

Another adenohypophyseal hormone important in the regulation of the estrous cycle is prolactin. Prolactin has a single polypeptide chain of 198 amino acids 198 amino acids, is synthesized as a prohormone and upon release undergoes proteolytic cleavage, thus forming the molecular structure normally found in plasma. Electrophoresis and chromatographic analyses of prolactin show that this hormone is not a single molecule, but a family composed of four molecular moieties, which are encoded by different DNA clones (Devesa and Tresguerres, 1992; Freeman, 1988; Nagy et al. 1999).

In the rat, estradiol stimulates prolactin release by acting directly on the adenohypophysis and indirectly by acting on target neurons in the brain (Freeman, 1988). Prolactin plays a fundamental role in the development of the mammary gland and the initiation and maintenance of lactation. It also modulates gonadotropin secretion from the anterior pituitary and thus gonadal function in both sexes (Devesa et al. 1992; Nagy et al. 1999; Weiner et al. 1988).

During the estrous cycle of the rat, plasma prolactin concentrations are low relative to those during the evening and night of proestrus, which correlates with the estrogen secretion profiles of the preovulatory follicles during this period (Freeman, 1988).

Prolactin is able to regulate GnRH synthesis and secretion. The control of prolactin secretion is established by a short feedback mechanism similar to what has been described for gonadotropins (Weiner et al. 1988).

Regulation of prolactin secretion is provided by two fundamental types of hypothalamic stimulatory (prolactin releasing factors or PRF's) and inhibitory (dopamine; prolactin inhibitory factors or PIF's) substances. Prolactin directly stimulates hypothalamic neurons producing PIF, which spills into the portal plexus and reaches the anterior pituitary, inhibiting its secretion. If prolactin levels decrease, PIF also decreases and prolactin biosynthesis and secretion by the pituitary is restored. The main PIF of prolactin secretion is dopamine (Freeman, 1988; Devesa et al. 1992; Nagy, 1999).

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As for FSH and LH, prolactin secretion occurs in a pulsatile manner, which depends on the mode of secretion of dopamine that acts as a PIF; this dopamine originates from the tuberoinfundibular system (A14) located in the medial basal hypothalamus. High-affinity dopamine stereospecific receptors have been detected in lactotropes and their density varies during the estrous cycle. Drugs that increase hypothalamic dopamine levels decrease circulating prolactin levels, as do drugs with dopamine agonist effect (e.g., bromocriptine) and conversely all dopamine antagonist drugs (e.g., haloperidol) produce an increase in circulating levels (Freeman, 1988; Devesa et al. 1992; Nagy et al. 1999).

Ovulation

One of the processes that culminates follicular growth and differentiation is the expulsion of the mature oocyte and is called ovulation (Domínguez, 1993).

Ovulation is considered to be the result of a localized inflammatory process, since before the oocyte is expelled there is edema in the internal theca and cell death and an increase in prostaglandins in the area. For ovulation to occur, several changes in the follicle wall and in the relationships between the granulosa and thecal cells are necessary. During the last stage of follicular growth and differentiation there is the disappearance of the desmosomes present in the granulosa and thecal cells, as well as the degradation of the collagen fibers, caused by fibrinolysin synthesized by the granulosa cells. This enzyme is activated by plasminogen, a product of the granulosa cells. The disappearance of desmosomes and granulosa cell junctions is a consequence of the decrease in estrogen concentration in the follicular liquor, produced immediately before ovulation, contains more water than that formed before and appears to be secreted at a higher rate since after the LH peak the capacity for estrogen synthesis by the granulosa cells decreases rapidly, while that of progesterone increases (Dominguez et al. 1991; Dominguez, 1993; Espey and Lipner, 1994).

Ovulation depends on a complex succession of endocrine phenomena involving the hypothalamus, pituitary and ovary. The central control of the cycle resides in the arcuate region of the basal medial hypothalamus. Neurons in this region have pulsatile GnRH release activity, which is transported by vessels of the portal-hypothalamic-pituitary system to the anterior lobe of the pituitary (Dominguez et al. 1991; Dominguez, 1993).

The regulation of ovulation is the result of a set of neuroendocrine events that modulate the growth and differentiation of ovarian follicles. These involve gonadotropins: (FSH) stimulates the development, maturation and differentiation of the ovarian follicles and (LH) causes the rupture of the mature follicle and the release of the oocyte (Fink, 1988). Also involved are hormones secreted by the ovary, particularly estrogens; hormones from the adrenal and thymus glands; trophic and energy-regulating hormones such as thyroid-secreted hormones and growth hormone; and classical and peptidergic neurotransmitters that reach the ovary via nerves or are synthesized in the ovary (Cruz et al. 1992; Dominguez et al. 1991; Espey and Lipner, 1994).

Background

Many drugs and various substances used as pharmacological tools in biological tests and bioassays that have proven physiological and physiotherapeutic activity present problems in their administration, particularly due to their insolubility. They frequently must be administered in an irritating or toxic vehicle. DMSO is an appropriate solvent for this class of water-insoluble agents and also presents biological properties not yet studied that could make it a suitable solvent for infiltrating drugs that act directly at the level of the extracellular matrix.

Why is DMSO, which is significantly less toxic and can be used as a replacement for other solvents, either pure or in mixtures of safe solvents, apparently underutilized? Perhaps the usefulness of DMSO has not been fully communicated to those in positions capable of making decisions about commercial solvent selection, or perhaps it is due to "biosafety myths" that have surrounded DMSO for several years (Borgioli, 2007; Vignes, 2000).

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Higher doses, more concentrated solutions, a higher frequency of exposure, or a longer exposure period may produce one or more of the toxic effects observed in some laboratory and clinical studies (Smith et al. 1983).

Tolerance limits for DMSO, however, have not been established for all exposure conditions. In addition, there are scientific papers and reports that show data recommending its therapeutic use in domestic animals and even in humans.

Physical and chemical properties of DMSO

DMSO is an amphipathic molecule with a highly polar domain and two apolar methyl groups, making it soluble in both aqueous and organic media (Santos et al. 2002).

DMSO dissolves various organic substances, including carbohydrates, polymers and peptides, as well as inorganic salts and gases. They generally do not have any serious biological effects, therefore, DMSO-water solutions of organic compounds can be used in various bioassays (Balakin et al. 2006).

DMSO is a polar, strongly hygroscopic solvent that shows solubility in water and lipids. Application of this substance to the skin results in hyperhydration of the stratum corneum with a subsequent increase in permeability. While it has no known effects on plasma membranes, it can cause changes in keratin filaments, its in vivo effects are short-lived (Brisson, 1974).

Most of the physiological properties of DMSO appear to be related to its penetration properties, its potential to inhibit or stimulate enzymes, and act as a free radical scavenger, among others. These properties are largely based on the chemical characteristics of DMSO, including its affinity for hydrogen bonds, affinity for water, ability to exchange water between cell membranes, and ability to react with organic molecules (Wexler et al. 2005).

Because of its property to rapidly cross the epidermis and cell membranes, DMSO has been proposed as an excellent carrier of drugs or poisons, which could represent multiple advantages for testing the effects of insoluble or hydrophobic drugs, in addition it has been reported that DMSO is less toxic than other amphipathic solvents used in the pharmaceutical and service industry, such as dimethylformamide, dimethylacetamide, N-methyl-2-pyrrolidone, among others (Baker, 1968).

Potential uses of DMSO in biology and experimental biomedicine

Numerous laboratory studies have documented the primary pharmacological actions of DMSO, including cell membrane penetration, effects on connective tissue, anti-inflammatory action, analgesia, diuresis, enhancement or reduction of the efficacy of other drugs, cholinesterase inhibition, vasodilatation, muscle relaxation, antagonism to platelet aggregation, among others (Jacob and Herschler, 1986).

DMSO is one of the most common solvents for in vivo administration of various water-insoluble substances. It is unclear how the response to a particular substance is altered by DMSO, but it is generally believed to act as a penetrant carrier of substances across membranes at all levels of biological organization.

The most important properties of DMSO as a penetrating carrier involve the ability to change the conformation of proteins and to replace water (Rammier and Zaffaroni, 1967).

DMSO is readily absorbed dermally and orally in animals and humans and enhances the absorption of many chemicals by these routes, DMSO at high concentrations is better absorbed than in dilutions in combination with water (Wexler et al. 2005). There are four main variables that influence the penetration of a solute through any membrane: 1) the diffusion coefficient across the membrane, 2) the concentration of the agent in the vehicle, 3) the partition coefficient between the membrane and the vehicle, and 4) the thickness of the membrane barrier.

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Penetrating agents are designed to affect one or more of these variables without causing permanent structural or chemical modification of the physiological barrier. Altering membrane thickness is less practical for drug delivery (it is difficult to conceive of non-toxic agents that could reversibly decrease stratum corneum thickness), so most penetrating agents, including DMSO, are believed to reversibly alter principles 1 through 3. There is some evidence to suggest that DMSO can increase diffusion through the stratum corneum by disrupting barrier function.

Some evidence suggests that DMSO may increase diffusion through the stratum corneum by disruption of barrier function, this likely occurs through aprotic interactions with intercellular lipids, as well as may include reversible distortion of lipid heads resulting in a more permeable packing arrangement.

In addition to the above, it is known to have an effect on less soluble agents in combination with a variety of vehicles as it favors increased penetration by transporting a higher concentration of the substance across the membrane barrier (Capriotti and Capriotti, 2012).

DMSO is commonly used in veterinary medicine as a salve, alone or in combination with other ingredients, in the latter case DMSO is used as a solvent to carry the other ingredients across the skin. In addition, in horses DMSO is used intravenously without combination or in combination with other drugs for the treatment of increased intracranial pressure and/or cerebral edema (Schleining and Reinertson, 2007a, 2007b).

Numerous studies have shown that DMSO increases skin permeability in humans and animals (Astley and Levine, 1976; Baker, 1968; Malten and Den Arend, 1978; Mitryukovskii, 1970; Scheuplein and Ross, 1970; Sweeney et al. 1966;). The concentration of DMSO determines the degree of change in permeability and solvent removal on partial or complete recovery (Astley and Levine, 1976).

DMSO has been used in various therapeutic situations in humans. In 1978 it was approved by the US Food and Drug Administration (FDA) for use in the treatment of interstitial cystitis by intravesical instillation (Parkin et al. 1997). Its effects do not appear to be related to a release of histamine from mast cells (Stout et al. 1995). It has been used successfully in dermatological (Burgess et al. 1998; Hsieh et al. 1987; Wong and Lin 1988;), urinary (McCammon et al. 1998), pulmonary (Iwasaki et al. 1994), rheumatoid and renal (Iwasaki et al. 1994) treatments. 1994), rheumatic and renal (Morassi et al. 1989), amyloidosis, (Salim, 1991; Salim, 1992a; Salim, 1992b; Salim, 1992c), DMSO crosses the blood-brain barrier (Broadwell et al. 1982) and has been effective in the treatment of traumatic brain edema (Ikeda and Long, 1990). It has also been used in the treatment of musculoskeletal disorders (Rosenstein et al. 1999; Zuckner et al. 1967) lung adenocarcinoma (Goto et al. 1996), rheumatological diseases (Abdullaeva and Shakimova, 1989; Murav'ev, 1986), chronic prostatitis (Shirley et al. 1978), dermatological diseases (Bertelli et al. 1995; Guerrey et al. 1999; Swanson, 1985) and as a topical analgesic (Kingery, 1997). Additionally, it has been suggested for the treatment of Alzheimer's disease (Regelson and Harkins, 1997).

Toxicology of DMSO

In addition to all of the above mentioned for pharmacological applications in the treatment of different pathologies, it is worth mentioning that several adverse systemic side effects have also been reported for the use of DMSO, such as nausea, vomiting (Davis et al. 1990), diarrhea (O'Donnell et al. 1981), hemolysis (Samoszuk et al. 1983), anaphylactic reactions manifesting with skin rash, flushing and occasionally bronchospasm (Berenson et al. 1987; Stroncek et al. 1991), renal failure (Smith et al. 1987), diastolic and systolic hypertension (Hameroff et al. 1983), bradycardia, heart block (Rapoport et al. 1991; Shlafer et al. 1976; Styler et al. 1992); rarely, pulmonary edema or cardiac arrest (Baum et al. 1992; Pegg and Kem, 1960).

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Other side effects of DMSO include: garlic-smelling breath and taste in the mouth due to pulmonary excretion of a small percentage of DMSO as dimethyl sulfide (Jacob and Herschler, 1983); its topical application, although well tolerated, may cause mild temporary local burning (Bertelli et al. 1995), skin rashes and pruritus (Swanson, 1985). A case of sulphohemoglobinemia has been reported after dermal application of DMSO in the treatment of interstitial cystitis with fatigue, cyanosis and dyspnea with mild exercise (Burgess et al. 1998).

The best documented side effect of DMSO treatment is intravascular hemolysis after intravenous infusion of 40% or greater solution that may result in urinary excretion of hemoglobin (Waller et al. 1983). Despite transient dose-dependent hemolysis and presenting hemoglobinuria, no alteration in renal function has been reported (Muther and Bennett, 1980).

Serum hyperosmolality has also been described in the control of increased intracranial pressure with intravenously administered DMSO (Wolf and Simon 1983); the same effect was also observed in in vitro studies in human blood (Santos et al. 2002); however, DMSO penetrates the cell membrane and causes an increase in osmolarity inside and outside the cell, preventing any significant hemolysis due to the formation of an osmotic gradient (Franco et al. 1983). One of the most important questions about any medicinal therapy is safety. Adverse reactions of DMSO are relatively mild and may occur in relation to its concentration and mode of administration (Jacob and De la Torre, 2009).

The acute toxicity of DMSO is low in animals. The LD50 in humans is 1800 mg/kg in skin and 606 mg/kg intravenously. The oral LD50 in the rat ranges from 14.5 to 28 g/kg and the dermal LD50 above 40 g/kg, intraperitoneal and intravenous LD50 in mice, rats and dogs exceeds 15 g/kg. It has been shown that acute lethal doses in experimental animals can produce tachypnea, restlessness, coma, hyperthermia, and sudden death, and can also cause death after several days due to renal failure (Jacob and Herschler. 1983).

DMSO is an experimental teratogen and also causes other reproductive effects in experimental animals (Wexler et al. 2005), however, so far there is no history that can explain the influence of DMSO in action as a teratogenic agent due to the diversity in the experimental procedures employed and the types of abnormalities that these studies indicate (Smith et al. 1983).

There is controversy about the apparent toxic role of DMSO in the rat, which is known to have teratogenic effects when administered during the first week of gestation inducing fetal abnormalities.

However, DMSO is not considered directly embryotoxic and has been shown to be a successful cryoprotectant for mammalian semen and embryos (Pegg, 2007).

Research has reported teratogenic effects in several species of experimental animals. Caujolle et al. (1967) observed developmental abnormalities including: malformations of limbs, beak, eyes and coelosomia in chicken embryos injected with a 50% DMSO solution. Other authors described anomalies such as exencephaly, microphthalmia, fused ribs and cleft lip in hamster embryos whose mothers were treated with an injection of 2.5 g/kg or more of DMSO on the 8th day of gestation (Ferm, 1966; Marín-Padilla, 1966; Staples and Pecharo, 1973;).

Both Caujolle et al. (1967) and Staples and Pecharo (1973) found evidence of abnormal embryos (anencephaly, malformed limbs, and celosomia) in mice injected intraperitoneally with 5 g/kg/day or more of DMSO during the second week of gestation. Juma and Staples (1967) reported increased resorptions in embryos of rats treated with 10.25 g/kg/day of 90% DMSO on days 8-10 of gestation, but no teratogenic effects. Caujolle (1967) observed an increase in developmental abnormalities when DMSO (5-10 g/kg) was administered daily from the 6th to the 12th day of gestation.

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Caujolle, (1967) documented that daily oral or subcutaneous doses of 4 to 5 g/kg of DMSO administered to rabbits on days 6 to 14 of gestation did not cause embryonic mortality or teratogenicity; and according to Staples and Pecharo, (1973) embryonic mortality increases with subcutaneous doses of 3 g/kg on days 8 to 11 but has no teratogenic effects. Several studies have been carried out with the aim of recognizing embryonic and fetal teratogenic abnormalities in rodents (Table 1).

Dose/ concentration	Route of administration	Effects	Reference
5-10 g/kg/d del 6º-12º día de gestación (50% DMSO)	Oral, Ip	Malformations of the nervous system, extremities, jaw, celosomia and edema.	Caujolle, 1967
10.25 g/kg/d from the 8th-10th day of gestation (90% DMSO)	Sc	Decreased number of live offspring, increased resorptions, no serious malformations.	Juma and Staples, 1967
10.25 g/kg/d from 8th-10th day of gestation (90% DMSO)	Ip	Not teratogenic.	Staples and Pecharo, 1973

Table 1 Teratogenic and embryotoxic effects in the rat
Source: (Smith et al. 1983)

Some teratological studies suggest that DMSO is not a teratogen in mammals when administered orally and dermally at doses that do not produce overt maternal toxicity, DMSO is not a teratogen at low doses, regardless of the route of administration.

Effects of the use of DMSO as a vehicle for teratogenic agents

The teratogenic effects caused by a single injection of the antimalarial agent pyrimethamine or the antitumor agent 6-mercaptopurine in rats on the 13th day of gestation were reduced by pretreatment with DMSO (Barilyak et al. 1978). However, in another study DMSO was not involved in the teratogenic action of pyrimethamine (Anderson and Morse, 1966). Injections of the insecticide dieldrin (days 6-14) into pregnant mice resulted in maternal and fetal toxicity which was increased when DMSO was used as a vehicle (Dix, 1977). Embryonic and fetal toxicity induced by the fungal metabolite (secalonic acid) was reduced when DMSO was used as a vehicle (Reddy et al. 1981).

Fetal mortality and abnormalities in hamsters treated with the insecticide thiram or disulfiram orally on the 7th or 8th day of gestation were increased when used together with DMSO (Robens, 1959).

In a study by Lauder and Salam (1972), seven substances with teratogenic capacity were evaluated in chick embryos to determine the effects that DMSO might have on development and mortality. The chemicals tested on four-day-old embryos were: 3-acetylpyridine, 6-aminonicotinamide, bidrin, sulfanilamide, 3-amino-1, 2, 4-triazole, physostigmine, and nicotine. DMSO had no effect on embryo mortality, however, there was an increase in teratogenic effects when used in conjunction with sulfanilamide, a decrease when used together with 3-acetylpyridine, 6-aminonicotinamide, 3-amino-1, 2, 4-triazole, and no change in combination with physostigmine and nicotine. Unfortunately, there are no precedents that can explain the influence of DMSO in action as a teratogenic agent because of the diversity in the experimental procedures employed and the types of abnormalities that these studies indicate (Smith et al. 1983).

Dopamine and dopaminergic receptors

Catecholamines are characterized by a catechol group: a benzene ring with two hydroxyl groups to which an amine group is attached. Catecholamines include dopamine (DA), norepinephrine (noradrenaline) and epinephrine (adrenaline). All catecholamines are synthesized from the amino acid L-Tyrosine. They are synthesized mainly in the brain, in the adrenal medulla and in some sympathetic nerve fibers (Tresguerres et al. 2008). Dopamine is synthesized by specific neurons that have only the first two enzymes of the biosynthesis pathway: tyrosine hydroxylase and L-Dopa-decarboxylase that act sequentially for the exclusive production of dopamine: dopaminergic neurons, which, after chemical or electrical stimulation, release dopamine at the synapse (García-Sevilla and Meana, 1988).

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Catecholamines play a key role in nutrient metabolism and body heat generation, stimulating not only oxygen consumption but also the consumption of fuels such as glucose and free fatty acids, thus generating heat. They stimulate glycogenolysis and the degradation of triglycerides to free fatty acids (lipolysis). They also play a role in regulating the secretion of multiple hormones. For example, as indicated above, dopamine inhibits prolactin secretion, but norepinephrine stimulates GnRH secretion and epinephrine inhibits insulin secretion by the beta cells of the islets of Langerhans of the pancreas (Garcia-Sevilla and Meana, 1988).

Dopamine is a neurotransmitter widely distributed in the central nervous system and some peripheral areas including the cardiovascular system and the renal system. In the brain, dopamine is involved in the control of movement, cognition, emotion, memory, reward and the mechanism of regulation of prolactin secretion by the pituitary gland. Several neurodegenerative diseases and psychiatric disorders have been linked to alterations in dopaminergic transmission (Hoffmann and Lefkowitz, 1996). The physiological effects of dopamine are mediated by membrane proteins that serve as receptors for specific chemical signals, termed dopaminergic receptors (DRRs), which have widespread expression throughout the mammalian brain (Rangel-Barajas et al. 2015).

According to Rangel-Barajas et al. (2015), DDRs belong to the G-protein-coupled receptor family. There are five mammalian receptor subtypes that are divided into two families according to their structure and biological response. The RDA1 family includes RDA1 and RDA5 receptors, the RDA2 family consists of RDA2, RDA3 and RDA4. RDA1 subtypes are positively coupled to adenylate cyclase (AC) to induce intracellular accumulation of 3,5 adenine monophosphatidyl cyclase (cAMP) and cAMP-dependent protein kinase (PKA) activation. In contrast, RDA2 is negatively coupled to CA, as a result of its activation, cAMP accumulation decreases and modulation of PKA activity and its effectors occurs. The activation of this receptor is also associated with other signaling pathways and may even act differently depending on the brain area or physiological conditions (Cooper et al. 1995).

Dopaminergic receptors and ovulation

Some years ago, the importance of the ovarian dopaminergic system has been demonstrated, confirming the observations of Dominguez and coworkers (1987). In that study, it was shown that in adult rats on day 1 of the estrous cycle, the dopaminergic receptor (DOR) system is indispensable for ovulation to occur on the expected day of estrus. The role of dopaminergic receptors in the rat ovary was recently reevaluated when three different dopaminergic antagonists: nonspecific antagonist haloperidol, type 2 receptor antagonist sulpiride (RDA2), and type 1 receptor antagonist SCH23390 (RDA1) were administered into the ovarian bursa at different times throughout the estrous cycle. Interestingly, sulpiride and haloperidol behaved similarly in "blocking" ovulation when administered on the night of estrus, all of estrus 1 and the morning of estrus-2, in contrast to SCH23390, which had effects only on the morning and evening of estrus-1. Increased tyrosine hydroxylase (TH) expression could also be shown in haloperidol administered animals, which could indicate an increase in dopamine synthesis, while expression of dopamine and cAMP-regulated phosphoprotein (DARPP-32) similarly increased, proteins that are activated by the interaction of dopamine and RDA1 (Venegas et al. 2015).

Dopamine has been found in the follicular fluid of human preovulatory follicles in high concentrations and in granulosa cell culture of human follicles has shown the presence of four of the five RDA subtypes, with the type 3 receptor being absent. In the rat ovary, type I and type II receptor mRNA was found in corpora lutea and interstitial tissue while in ovarian follicles its presence is moderate (Rey-Ares et al. 2007). The ovary also has the DA reuptake system, in human granulosa cells the dopamine transporter (DAT), catechol-O-methyltransferase (COMT), monoamine oxidase A and B, in addition to vesicular monoamine transporter 2 (VMAT2), while in Wistar and Sprague-Dawley rat strains it was shown that they express both DAT and VMAT (Greiner et al. 2008; Saller et al. 2014).

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Dopamine antagonists: sulpiride

Dopamine antagonists play an important role as a treatment against various psychiatric and neurological conditions, but also as a research tool, to understand the origin and development of these diseases or to create and improve therapies involving dopamine (Bahena-Trujillo et al. 2000).

Sulpiride: (RS)-N-[(1-ethylpyrrolidin-2-yl)-methyl]-2-methoxy-5-sulfamoylbenzamide, a neuroleptic and antipsychotic of the benzamine class, is used in the treatment of a wide range of psychotic disorders. It is a selective postsynaptic dopaminergic antagonist of RDA2 family receptors and does not produce extrapyramidal side effects like other benzamines in clinical use (Martin et al. 1996).

The effect of sulpiride occurs on limbic structures; it does not interact significantly with receptors of other neurotransmitters. It appears as an almost white crystalline powder, insoluble in water, slightly soluble in alcohol and methyl chloride and can be dissolved in mineral acids and alkaline hydroxides (Palomo, 1991).

Justification of the work

Due to the insolubility of dopamine antagonists and, in many cases, their low affinity to their receptors, this makes it difficult to use some dopamine antagonists in different bioassays, in this aspect DMSO can be very useful to infiltrate this type of poorly soluble agents. The biological effects of DMSO applied in the ovarian tissue of the adult female rat are unknown. Many drugs and pharmacological tools with proven physiological and physiotherapeutic activity present problems in their administration due to their insolubility and often must be administered in an irritating or toxic vehicle. DMSO is a solvent that has demonstrated great capacity to dissolve water-insoluble agents and also presents biological properties that have not yet been studied, which could make it a suitable solvent to infiltrate drugs that act directly at the level of the extracellular matrix.

For this reason, in the present work we analyzed the direct effects of DMSO when applied to live tissue, looking for signs of cytotoxicity by means of conventional histology with bright field microscopy, in addition to analyzing the effects that may alter the secretion of gonadotropins or affect ovulation, recording the duration of the estrous cycle, as well as the number of oocytes released in the adult rat model. On the other hand, its efficacy was tested by using it as a vehicle for a type 2 dopaminergic antagonist such as sulpiride, whose activity has been proven on these parameters, and comparing the effects of DMSO with similar studies in which other vehicles have been used for the administration of the antagonist, such as distilled water.

Methodology

Sixty-eight adult female rats of the CII-ZV strain, aged 90-120 days and with a body weight of 200-250 g, maintained under controlled lighting conditions (14 h light/10 h darkness; lights from 05:00 h and with free access to water and balanced feed. In order to monitor the phases of the reproductive cycle, the estrous cycle was recorded by means of vaginal smears taken daily between 09:00 and 10:00 h. Once the animals presented three consecutive four-day cycles (diestrous-1, diestrous-2, proestrus and estrus; cyclic rats) they were assigned to the different experimental groups. All experiments were in accordance with NOM-062-ZOO-1999.

Evaluation of DMSO cytotoxicity on spontaneous ovulation and estrous cycle length

Between 13:00-14:00 of Diestro-1, Diestro-2, Proestrus or Estro, groups of 6 cyclic animals were formed and sedated with isoflurane vapors and submitted to a dorso-bilateral laparotomy at the level of the inguinal region in order to exteriorize the ovaries and perform a microinjection inside each ovarian bursa with 20 μL of 100% DMSO.

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Similarly, the control groups received the microinjection with distilled water solution. In all animals, the recording of the estrous cycle was resumed the following day Animals were sedated with isoflurane vapors and sacrificed between 09:00 and 10:00h on the morning of the next vaginal estrus (observed vaginal estrus) the duration of the estrous cycle was recorded in each group, ovaries and oviducts were dissected where signs of ovulation were looked for and the number of released oocytes was counted. The ovaries of the animals were processed for brightfield histology, embedded in kerosene blocks, cut in microtome at 10 µm thickness (Gaviño et al. 1992) and stained with the hematoxylin-eosin technique (Luna, 1975). Signs of inflammation or necrosis in the ovarian tissues were recorded according to the methodology proposed by Yoshida et al (2009).

Analysis of the effect of specific dopamine receptor antagonism using DMSO as the infiltrating vehicle

Other groups of 8 and 4 cyclic animals respectively were distributed in experimental groups to perform the microinjection into the ovarian bursae of a dopaminergic receptor type 2 antagonist: sulpiride, using DMSO as a vehicle. Between 13:00-14:00 of diestrous-1, diestrous-2, proestrus and estrus, groups of cyclic animals were formed and sedated with isoflurane vapors and submitted to dorso-bilateral laparotomy at the level of the inguinal region to exteriorize the ovaries and perform a microinjection inside each ovarian bursa with 20 µL of sulpiride solution (5µg/µL) in 100% DMSO. As an absolute control group, 8 intact cyclic animals (Intact Control Group), sacrificed on the morning of vaginal estrus, were used. Similar to the previous experiment, in all these animals, the recording of the estrous cycle was resumed the following day The animals were sedated with isoflurane vapors and sacrificed between 09:00 and 10:00h on the morning of the next vaginal estrus (observed vaginal estrus) the duration of the estrous cycle was recorded in each group, the ovaries and oviducts were dissected where signs of ovulation were looked for and the number of oocytes released was counted. The ovaries of the animals were also processed for brightfield histology to record signs of inflammation or necrosis in the ovarian tissues.

Statistical analysis

The duration of the estrous cycle and the number of oocytes released were analyzed by Kruskall-Wallis test followed by Dunn's multiple comparisons test. Signs of tissue alteration or cytotoxicity in ovarian tissues were recorded in contingency tables and analyzed with Fisher's Exact Probability test, as appropriate. Differences equal to or less than $p<0.05$ were considered statistically significant.

Results

The body weight between the control groups and the groups treated with DMSO or sulpiride was similar (Intact Control (n=8): 218±5 grams vs Distilled Water (n=16): 247±4 grams vs DMSO (n=24): 235±4 grams vs Sulpiride (n=20): 235±4, ns). For such reason, ovarian and uterine weights were expressed in mg/100g body weight.

	H2O (n=4)		DMSO (n=6)		Sulpirida(n=4)	
	OI	OD	OI	OD	OI	OD
E	12.0±0.8	12.1±0.8	12.1±1.1	12.0±1.2	12.3±1.3	12.0±1.3
D1	10.2±0.3	10.4±0.5	12.0±0.7	12.4±0.8	11.1±0.6	12.3±0.6
D2	10.7±0.6	11.2±0.8	13.2±0.5	12.7±0.9	11.2±1.1	12.6±0.7
P	13.2±1.5	15.2±0.6	16.5±1.6	14.9±1.9	12.3±1.9	13.9±1.4
	Ovarian Mass					
	H2O (n=4)		DMSO (n=6)		Sulpirida (n=4)	
E	24.1±0.6		24.2±2.1		24.3±3.2	
D1	20.5±0.4		24.3±1.4		23.4±1.0	
D2	21.7±1.4		25.9±1.4		23.8±0.8	
P	28.4±1.6		31.4±3.4		26.3±3.2	
	Weight of the Uterus					
	H2O (n=4)		DMSO (n=6)		Sulpirida (n=4)	
E	187±16		168±18		175±29	
D1	173±10		181±21		187±16	
D2	163±4		179±10		191±29	
P	195±18		180±6		174±9	

Table 2 Relative weight of ovaries (mg/100 g body weight) ± s.e.m. and relative weight of uterus (mg/100 g body weight) ± s.e.m. in animals with regular four-day estrous cycle (cyclic) that received a 20 µL microinjection of: DMSO, Distilled Water (H2O) or 100 µg Sulpiride into the ovarian bursae at 13:00h on the day of Estrus (E), Diestrous-1 (D1), Diestrous 2 (D2) or Proestrus (P). The animals were sacrificed on the morning of the next observed vaginal estrus. LA: Left Ovary; RH: Right Ovary

No significant differences wer found in the weight of the ovaries or uterus between the different experimental groups (Ovarian mass):

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Intact Control (n=8): 26.3±1.6 mg vs Distilled Water (n=16): 23.3±0.8 mg vs DMSO (n=24): 26.5±1.2 mg vs Sulpiride (n=20): 24.2±0. 9, ns // Uterus: Intact Control (n=8): 163±7 mg vs Distilled Water (n=16): 180±7 mg vs DMSO (n=24): 177±7 mg vs Sulpiride (n=20): 183±10, ns) (Table 1).

Analysis of estrous cycle length between the groups treated with distilled water and DMSO did not show any alteration nor in the number of oocytes released (Table 2).

	Duration of the Stral Cycle		No. of Ocytes Released	
	H2O (n=4)	DMSO (n=6)	H2O (n=4)	DMSO (n=6)
Estro	4.0±0.0	4.0±0.0	13.5±0.9	12.2±1.4
Right-handed1	4.0±0.0	4.0±0.0	14.8±1.0	12.0±0.9
Diestro2	4.0±0.0	4.0±0.0	12.8±0.9	12.0±0.6
Proestrus	4.0±0.0	4.0±0.0	12.8±0.6	11.8±1.0
			13.4±0.4 (n=16)	12.0±0.5 (n=24)

Table 3 Duration of the estrous cycle (days)±e.e.m. and number of total oocytes released±e.e.m. in cyclic animals that received a microinjection of 20 µL DMSO or Distilled Water (H2O) into the ovarian bursae at 13:00h on one of the days of the estrous cycle. Animals were sacrificed on the morning of the next observed vaginal estrus

However, treatment with sulpiride induced a delay in the presence of vaginal estrus of almost 24 hours, but did not modify the number of oocytes released in this group with respect to the control group treated with DMSO or distilled water (Tables 3 and 4).

	Duration of the Stral Cycle DMSO + Sulpirida	No. of Ocytes Released DMSO + Sulpirida
Estro	4.0±0.0 (n=4)	11.5±0.5 (n=4)
Diestro-1	4.8±0.2* (n=8)	10.6±0.7 (n=8)
Diestro-2	4.0±0.0 (n=4)	11.0±0.4 (n=4)
Proestro	4.0±0.0 (n=4)	11.2±0.7 (n=4)
		11.0±0.3 (n=20)

Table 4 Duration of the estrous cycle (days)±e.i.m. and number of oocytes released±e.i.m. in cyclic animals that received a microinjection of 20 µL DMSO+100 µg sulpiride into the ovarian bursae at 13:00h on one of the days of the estrous cycle. The animals were sacrificed on the morning of the next observed vaginal estrus

	Distilled water (n=4)		DMSO (n=6)	
	NOL OI	NOL OD	NOL OI	NOL OD
Estro	7.3±0.5 (n=4)	6.3±0.9 (n=4)	6.0±0.7 (n=6)	6.0±1.2 (n=6)
Diestro-1	8.2±0.8 (n=4)	6.8±0.5 (n=4)	5.6±0.4 (n=6)	6.5±0.6 (n=6)
Diestro-2	6.3±0.5 (n=4)	6.5±0.5 (n=4)	5.8±0.3 (n=6)	6.5±0.5 (n=6)
Proestro	6.8±0.9 (n=4)	6.0±0.4 (n=4)	6.3±0.7 (n=6)	5.5±0.6 (n=6)
	DMSO + Sulpiride			
	OI		OD	
Estro	6.0±0.4 (n=4)		5.7±0.5 (n=4)	
Diestro-1	5.1±0.6 (n=8)		5.2±0.3 (n=8)	
Diestro-2	5.7±0.5 (n=4)		5.2±0.5 (n=4)	
Proestro	5.5±0.3 (n=4)		5.7±0.7 (n=4)	

Table 4 Number of oocytes released (NOL)±e.e.m. from the left ovary (LO) and right ovary (RO) of each of the cyclic groups of animals that received a microinjection of 20 µL of Distilled Water, DMSO or DMSO+100 µg sulpiride into each ovarian bursa at 13:00h of Estrus, Oestrus-1, Oestrus.2 or Proestrus. Animals were sacrificed on the morning of the next observed vaginal estrus

No significant alterations were observed in the histology of the main compartments of the ovarian cortex. The follicles and corpora lutea showed a normal appearance, with no signs of necrosis or other alteration compromising their function. Microinjection of DMSO showed some signs of vascular distention in the medullary zone of the ovary that are similar to the effects of microinjection with distilled water, 0.9% saline or 0.1% ascorbic acid solution (images of these last two vehicles were obtained from another similar study) (Figures 10 and 11).

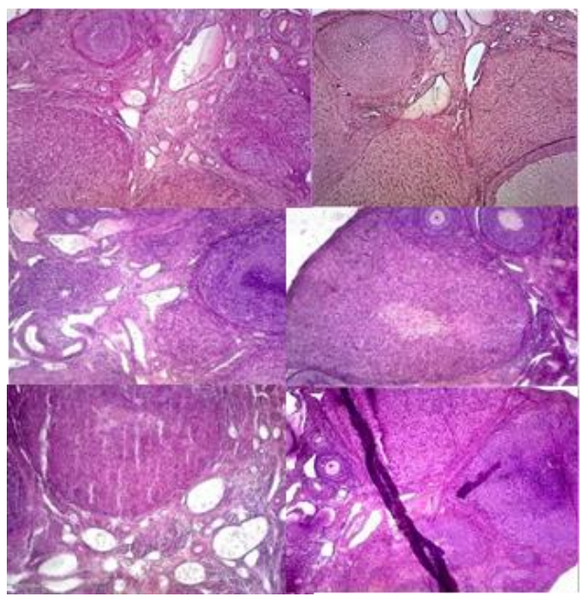

Figure 5 Images showing the effect of MI with 20 µL 100% DMSO; 20 µL SHAM (H2O) on the ovarian tissue of the rat that received treatment on the different days of the estrous cycle. Apparently, cortical tissues showed no signs of structural alteration; MO: ovarian medulla; VL: lymphatic vessel; A: arteriole; CL: corpus luteum; F: follicle; D1: diestrus 1; D2: diestrus 2; E: estrus.

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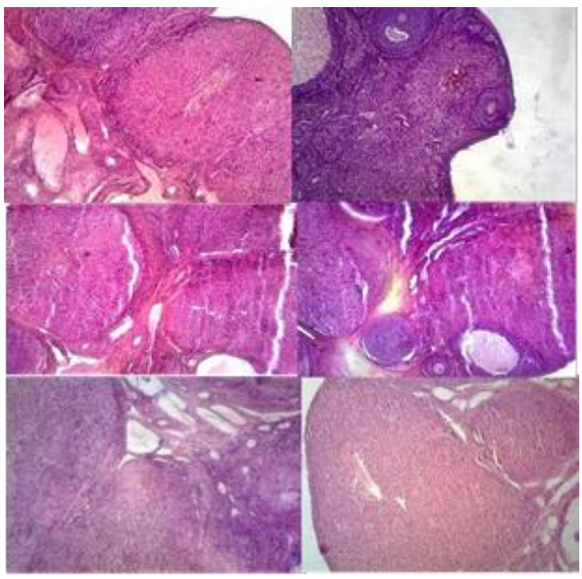

Figure 6 Images showing the effect of MI with 20 µL of 100% DMSO; 20 µL of 100% DMSO + sulpiride and 20 µL of Ascorbic Acid + sulpiride on the ovarian tissue of the rat that received treatment on the different days of the estrous cycle of the adult rat. Apparently, cortical tissues showed no signs of structural alteration; MO: ovarian medulla; VL: lymphatic vessel; A: arteriole; CL: corpus luteum; F: follicle; D1: diestrous 1; P: proestrus; E: estrus; S: sulpiride.

Discussion

The results of the present study show that DMSO did not induce significant changes in the cortical structure of the ovaries.

Apparently, infiltration of the solvent through the ovarian bursae did not have cytotoxic effects that compromised the primary functions of the ovary.

DMSO applied in therapies against inflammation (Dujovny et al. 1983; Parkin et al. 1997), viral (Aliaga et al. 1992) or bacterial (Pottz et al. 1967) analgesic (Gaspar et al. 2012; Kingery, 1997) and as a cryopreservative (Pegg, 2007) has shown fairly safe effects. Our results when applied directly to an organ within the body cavity show that its effects are easily controlled by the natural mechanisms of detoxification and elimination of waste substances, where the lymphatic system participates. As is known, the lymphatic systems comprise a one-way transport system for fluids and proteins by collecting them from the interstitial space and returning them to the blood circulation (Swartz, 2001).

It is a remarkable fact that the surgical technique and the application of DMSO administered directly to the ovarian tissue did not modify gonadotropin secretion or affect the primary functions of the gonads. That is, the rat organism rapidly readjusted to its substantive functions. The apparent non-alteration of ovarian functions is a reflection of the correct incidence of sex steroids in the higher centers controlling the secretion of GnRH and gonadotropins.

Based on the above, the use of DMSO as an infiltrative agent could be widely recommended to analyze the physiological or pharmacological role of experimental chemicals. In the present work, it was used as a vehicle for the infiltration of the dopaminergic antagonist sulpiride, and its use induced the same effects as those observed in other works when ascorbic acid 0.1% was used as a vehicle (Moonlighting). 0.1% as a vehicle (Letras Luna, 2016; Venegas et al., 2015; González-Quiroz, personal communication) and it is speculated that it would have similar effects to vehicles used to administer other dopamine antagonists, for example, alcohol-water mixtures, saline or distilled water (González et al. 2016.; Guzmán et al. 2018; Venegas et al. 2015; Venegas et al. 2017).

In order to use it as a harmless vehicle, it is clear that there is a lack of complementary studies that deepen the knowledge of the biosafety margin with which it can be used. For example, its effects could be compared with respect to other conventional vehicles supported with immunohistochemical techniques to analyze the presence of apoptosis indicators, expression of key proteins in the intracellular signaling cascades that lead to the biosynthesis of gonadotropin receptors, dopamine receptors, factors intimately linked to angiogenesis, to mention a few. Likewise, with simple molecular biology techniques, a comparative analysis of the eventual damage or alteration of the gene expression of these proteins could be carried out.

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There are multiple alternatives for scientific work in the field of Reproductive Biology and Experimental Reproductive Endocrinology where this infiltrating agent could be used. In our case, further analysis of ovarian functions and its organs (corpus luteum and ovarian follicle) or key tissues (theca cells, granulosa cells, oocyte or luteal cells), would require the use of an innocuous vehicle, which allows the solubilization of liposoluble substances and, most importantly, in minimum concentrations that produce clear physiological effects and not artificial ones. DMSO could be used as an effective vehicle to study the effects of bioactive drugs at lower doses and even shorter reaction times. According to novel information on the functions performed by the different anatomical-functional compartments of the ovary, it would be interesting to test its effects as an infiltrative agent and the production of reactive oxygen species and their indicators of oxidative stress, since these substances are known to be involved in ovulation, but also in various ovarian pathologies.

On the other hand, the effects of dopaminergic antagonism in ovarian tissue using DMSO as a vehicle reproduced previous findings on the alteration of neuroendocrine and endocrine signals leading to spontaneous ovulation. Thus, Venegas et al. (2015) studied the effects of microinjection of sulpiride and other dopamine antagonists through the ovarian bursae on spontaneous ovulation in rats with regular four-day estrous cycle and their results showed an estrous lag with a net delay of one day, which is consistent with our data, even compared to the hemicastered animal model (Letras et al. 2016).

The causes of vaginal estrus delay can be explained by alteration or disruption of sex steroids acting at the level of the central nervous system and hypothalamus. Somehow, in animals with dopaminergic receptor type 2 antagonism, the life of the corpus luteum is maintained twenty-four hours longer than expected, i.e., progesterone maintains its inhibitory effects on the centers leading to phasic discharge of GnRH and consequently, the preovulatory discharge of gonadotropins is withheld until the system accumulates enough estrogens to exert their stimulatory effects on the phasic secretion of GnRH and until that time, the ovary will be in a position to release oocytes when the preovulatory peaks of gonadotropins occur.

Venegas and collaborators (2015) demonstrated that GnRH injection in the afternoon of the expected proestrus induces ovulation in 100% in animals with dopaminergic antagonism, although in the model of the present study we did not verify this.

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Conclusions

- The results of the present study suggest that DMSO administered directly on ovarian tissue does not affect the main ovarian functions, estimated by the duration of the estrous cycle (indicator of gonadotropin and sex steroid secretion) or spontaneous ovulation (number of oocytes released).

- The administration of DMSO as a vehicle for sulpiride induced effects similar to those observed in other studies where other vehicles have been used; therefore, its use as an infiltrative agent would be advisable.

Perspectives

Based on the findings of the present study on the safety margin of DMSO as an infiltrative vehicle for the experimental use of dopamine antagonists, and perhaps other drugs, which due to their chemical nature are poorly soluble in aqueous solutions, further experimental trials are needed to promote their use.

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It is very likely that their use as a vehicle will facilitate handling, calculate and/or economize reagents, use biologically optimal doses to analyze the effect of chemical substances administered in living tissues or organisms, and prevent the installation of experimental artifices that could mask the true effects of pharmacological manipulations in experimental models.

In particular, our working group is interested in deepening the knowledge of the functional role that ovarian and hypothalamic dopamine plays both in the function of the gonad and at other levels of the hypothalamus-adenohypophysis-ovary axis. Therefore, it would be interesting to conduct an in-depth study on the acute or long-term effects that occur when DMSO comes into contact with the tissues of the ovarian cortex or in the brain of the rat, which form an essential part of the regulation of reproductive function. The function of dopamine in rat testicular tissue has also begun to be explored, since they have dopamine receptors and apparently play an important role in testicular function. In this context, the possible uses of DMSO as an infiltrating vehicle open a wide panorama for different studies in the field of Reproductive Neuroendocrinology.

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