Regulation of Male Fertility by the Opioid System

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Endogenous opioid peptides are substances involved in cell communication. They are present in various organs and tissues of the male and female reproductive tract, suggesting that they may regulate some of the processes involved in reproductive function. In fact, the opioid system that operates as a multi-messenger system can participate in the regulation of reproductive physiology at multiple levels, for example, at the levels of the central nervous system, at the testes level and at sperm level. A better understanding of the implication of the opioid system in reproductive processes may contribute to clarifying the etiology of many cases of infertility and the effect of opiate abuse on fertility. Indeed, a novel biochemical tool for the diagnosis and treatment of male infertility could be based upon components of the opioid system. The presence of the opioid system in sperm cells also represents a novel opportunity for reproductive management, for either enhancing the probability of fertilization or reducing it through the development of novel targeted contraceptives.

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THE OPIOID SYSTEM

The opioid system is a biological communication system for which activity is mediated by the so-called endogenous opioid peptides (EOPs). These peptides are synthesized from the processing of their precursors, which are encoded by three different genes: *pro-enkephalin* (*PENK*), *pro-opiomelanocortin* (*POMC*) and *pro-dynorphin* (*PDYN*) (1). Each PENK molecule contains seven EOP amino acid sequences and mainly produces met- and leuenkephalin (2). The POMC precursor, which in part contains the β-endorphin peptide, is a precursor of adrenocorticotropin hormone (ACTH), α- and β-melanotropin (MSH), and α-, β- and γ-lipotropin (LPH), among others (3). Each molecule of PDYN contains three sequences of leuenkephalin and other larger peptides such as neo-endorphins and A and B dynorphins (2) (Figure 1).

EOPs exert their action through opioid receptors. Opioid receptors are G-protein–coupled receptors that are monomeric and exhibit seven transmembrane domains. There are three principal types of opioid receptors: the delta-opioid receptor (DOR), the mu-opioid receptor (MOR) and the kappa-opioid receptor (KOR) (4–7). More recently, the orphanin 1 (ORL1) receptor (also known as the nociceptin receptor) was discovered and found to share a large homology with opioid receptors (8,9) (Table 1).

EOPs exhibit different affinities for these opioid receptors. Met- and leuenkephalin are considered to be endogenous ligands for DOR, since they have higher affinity for DOR. Nevertheless, they are capable of binding with lower affinity to the MOR. β-Endorphins bind to DOR and MOR with similar affinity, whereas dynorphins preferentially bind to KOR (10). Nociceptin, which is derived from the processing of the precursor pro-nociceptin (PNOC) (11), has been reported to be an endogenous ligand of the ORL1 receptor and has very weak activity at other opioid receptors (12). MOR exhibit high affinity for morphine, whereas KOR have preferential affinity for ketocyclazocine. Concerning antagonist selectivity, naloxone behaves as an antagonist at all opioid receptors, with highest affinity for MOR (10) (Table 1).

Opioid receptors are known to couple to G_0 and G_i proteins, since signaling by these receptors is effectively blocked by pertussis toxin. Opioid signaling pathways involve the following: (a) inhibition of the adenylate cyclase enzyme (13), (b) inactivation of voltage-dependent calcium channels (14,15), (c) activation of potassium channels (16), (d) mobilization of intracellular Ca^{2+} reserves through the activation of phospholipase Cβ (17,18) and (e) stimulation of MAP kinase pathways (19).

The most well-known physiological effect associated with EOPs is their efficacy in pain reduction or analgesia, although their effect on a variety of other physiological functions has become apparent in recent years (20). In particular, evidence of the widespread presence of opioid peptides and receptors in different organs and tissues of the male reproductive system indicates that EOPs likely

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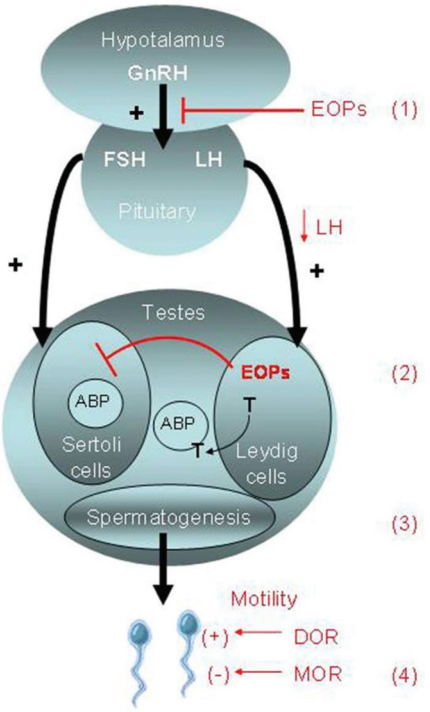

Figure 1. Control of male reproductive function by the opioid system at multiple levels. (1) At the level of the CNS, EOPs inhibit the secretion of GnRH, thereby suppressing the release of LH from the pituitary. (2) At the testes level, EOPs are synthesized mainly in Leydig cells after LH stimulation, and they exert an inhibitory effect on Sertoli cells. In particular, EOPs can regulate the levels of testosterone (T) indirectly, inhibiting the production of ABP that is stimulated by FSH in Sertoli cells. (3) Genes encoding opioid peptide precursors are differentially expressed in germ cells, and somatic cells of the testes and their transcripts are not efficiently translated in spermatogenic germ cells. (4) In spegrmatozoa, the opioid system regulates sperm motility in a distinct manner by the activation of distinct receptors.

participate in the regulation of male reproductive function.

HORMONAL CONTROL OF REPRODUCTIVE FUNCTION BY THE OPIOID SYSTEM

Sterility is a problem that affects a large percentage of couples of reproductive age. It is estimated that in 40–45% of cases, the principal cause of sterility resides in the male partner. Anatomical defects, genetic diseases and injuries, as well as testicular sperm and hormonal dysfunction are major causes of male infertility.

The opioid system is one of a host of neuroendocrine products (dopamine, arginine vasopressin [AVP], corticotropin-releasing hormone [CRH], cholecystokinin [CCK] and more) that are involved in the control of releasing gonadotropin-releasing hormone (GnRH) and thus the sex hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both FSH and LH are secreted by the anterior pituitary and act directly on the testes to stimulate the somatic cells that contribute to spermatogenesis. The synthesis and release of these hormones is controlled by GnRH, which is secreted by the hypothalamus. The FSH receptor is expressed exclusively in Sertoli cells (21). In contrast, the LH receptor is expressed mainly in Leydig cells but is also mildly expressed in spermatogenic cells (22,23). The main function of FSH is to stimulate the proliferation of Sertoli cells during puberty, whereas LH regulates the synthesis of testosterone in the adult testes (24). Administration of morphine is associated with a suppression of LH release, whereas opioid antagonists such as naloxone and naltrexone produce increased serum levels of LH in humans and many animal species (25,26). The opioid-induced inhibition of LH release is mediated by a hypothalamic action because, on the one hand, opioid receptors have been described in purified gonadotrophs and, on the other hand, *in vivo* treatment with GnRH antagonists completely blocked the release of GnRH induced by naloxone (27–29). Therefore, opioids generally decrease not only LH but also testosterone and estradiol, which have effects on testicular function (30,31). In fact, opioid abuse primarily leads to hypogonadism and includes decreased libido and erectile dysfunction in men and infertility (32). Administration of opioid antagonists such as naltrexone can improve symptoms of hypogonadism and improved erectile function, although the antagonist did not increase testosterone or LH levels, suggesting regulation at the central rather than the peripheral level (33).

These studies clearly indicate that EOPs can regulate reproductive function by inhibiting the secretion of GnRH at the level of the central nervous system (CNS) (26,28,34) (Figure 2).

Table 1. Profiles of affinity of opioid ligands on the basis of binding studies.

Ligands	K_i (nmol/L) δ	μ	κ	ORL_1
(Met^5)-enkephalin	0.9	9.5	4440	—
(Leu^5)-enkephalin	3	20	835	—
β-Endorphin	2.7	2	57	—
Dynorphin A	3.2	0.7	0.12	110
Dynorphin B	4.4	3.4	9	—
α-Neoendorphin	0.57	1.3	0.2	—
Endomorphin	>500	0.67	>500	—
Nociceptin	380	133	267	0.1

REGULATION OF TESTICULAR FUNCTION BY THE OPIOID SYSTEM

Numerous studies have demonstrated the presence of EOPs in different testicular cell types (35–37). In addition, binding studies have revealed the presence of all three types of opioid receptors (DOR, MOR and KOR) in rat testes. Moreover, the genes encoding the opioid precursors *PENK*, *PDYN* and *POMC* have also been found to be expressed in the testes of the rat and mouse (38,39), providing further evidence that the opioid system may play a role in regulating testicular function.

Spermatogenesis consists of a series of processes involving the proliferation and differentiation of developing germ cells into sperm. In addition to being regu-

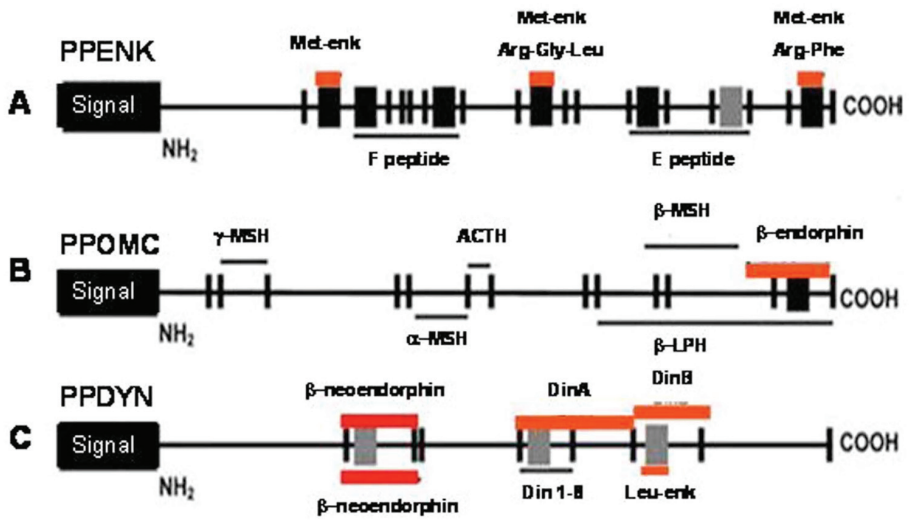

Figure 2. Opioid peptide precursor proteins: PENK (A), POMC (B) and PDYN (C). Opioid peptides are shown in red.

lated by endocrine mechanisms of the hypothalamic-pituitary axis, spermatogenesis is controlled by paracrine and autocrine processes that take place between different cell types present in the testicles.

Sertoli cells provide the structural and metabolic support that is necessary for the differentiation of germ cells. Dysfunctional FSH receptors lead to a decrease in the size of the testicles due to a decrease in the number of Sertoli cells (40). Dysfunctions that occur during spermatogenesis can lead to pathologies such as oligozoospermia and teratozoospermia, although males may nevertheless be fertile (41).

Leydig cells are responsible for the production of testicular testosterone. Testosterone and its metabolites (dihydrosterone and estradiol) are male sex hormones that regulate reproductive function, since spermatogenesis depends on the presence of an adequate level of intratesticular testosterone (42). The concentration of testosterone in the seminiferous tubule is 100 times higher than in peripheral blood (43). These high intratesticular testosterone levels are achieved thanks to the LH-induced production of testosterone around the tubules in the Leydig cells and to the activity of androgen-binding protein (ABP), which is synthesized in Sertoli cells and which transports testosterone into the lumen of the seminiferous tubule (44).

As previously mentioned, binding studies found all three types of opioid receptors (DOR, MOR and KOR) in the rat testis, although subsequent higher-resolution localization studies found that these receptors are exclusively expressed by Sertoli cells (45). With regard to EOPs, the genes encoding the opioid precursors *PENK*, *POMC* and *PDYN* were found to be expressed in the rat and mouse testes (38,39,46), supporting the idea that testicular function could be regulated through local synthesis of EOPs. The finding that transgenic mice who overexpress PENK in the testes have impaired fertility, morphologically abnormal testicles and low sperm motility has provided further evidence suggesting that the opioid system participates in an important way in the regulation of testicular function (47).

In rats and mice, the *PENK* gene is differentially expressed in germ cells and somatic cells of the testes (48). Testicular germ cells express a large 1.7-kb transcript that is expressed under the control of a specific promoter (49), whereas Leydig cells, Sertoli cells and peritubular cells express a smaller 1.45-kb mRNA (39,48–50). Both transcripts contain an intact coding sequence and differ in the 5′ noncoding sequence (48). Polysome expression profiles indicate that the *PENK* transcript, which is specifically expressed in germ cells, is not translated efficiently, whereas the PENK transcript expressed by Leydig cells is efficiently translated. This result would explain why the concentration of PENK products detected in rat testes homogenates is relatively low (<2 pmol/g), compared with the levels of mRNA detected for the *PENK* gene (39). Moreover, in Sertoli cells exposed to FSH *in vitro*, the expression of the *PENK* gene, as well as levels of metenkephalin, were found to be upregulated (46), suggesting that not only Leydig cells, but also Sertoli cells, contribute to the *de novo* synthesis of EOPs in the testes. On the other hand, it was suggested that germ cells may also regulate levels of EOPs during spermatogenesis by controlling the expression of *PENK* in the surrounding cells; however, the underlying mechanism has not yet been characterized (51).

POMC gene expression was localized to Leydig cells (38) and to germ cells (46) by *in situ* hybridization. In the mouse testis, the *POMC* transcript has two different sizes. Leydig cells and some stages of germ cells expressed a long *POMC* transcript of about 675–750 nucleotides, but pachytene spermatozoa expressed a shorter *POMC* transcript. As occurs with *PENK*, the *POMC* transcript is efficiently transcribed in Leydig cells, so that it is likely to be responsible for synthesizing POMC-derived peptides in the testis. However, the small *POMC* transcript in pachytene spermatozoa was found to be inefficiently transcribed during spermatogenesis (46). These findings are consistent with the detection of β-endorphin immunoreactivity in Leydig cells in many species (37). The synthesis of β-endorphin is controlled by LH secreted by the pituitary in adult rats (52) and also by corticotropin-releasing factor (CRF). Leydig cells in culture exposed to CRF also produce higher levels of β-endorphin, and this effect is completely reversed in the presence of an α-helix CRF antagonist, confirming its specificity of action (53). CRF regulates the function of Leydig cells in an autocrine manner

(54), since the levels of CRF increased in Leydig cells after LH treatment, and these cells also specifically expressed the corresponding receptor (55,56).

Thus, EOPs are mainly synthesized *de novo* by Leydig cells in the testis, although EOPs have no effect on testosterone production in cultured Leydig cells or in rat testicular tissue after human chorionic gonadotropin (hCG) treatment (37,57). However, intratesticular administration to rats of the opioid antagonist naloxone and nalmefene was found to decrease basal secretion of testosterone and reduce levels of serum testosterone (58). Binding studies have not detected opioid receptors in Leydig cells (45), which suggests that EOPs indirectly regulate the secretion of testosterone.

On the other hand, β-endorphins synthesized by Leydig cells produce an inhibitory paracrine effect on Sertoli cell function (58,59). Specifically, β-endorphins inhibit the production of ABP stimulated by FSH in Sertoli cells, and this inhibition is reversed in the presence of naloxone (36). ABP can regulate intratubular testosterone levels since, as mentioned before, it is responsible for testosterone transport into the lumen of the seminiferous tubule (44). Consequently, β-endorphin is involved in the control of the precise levels of testosterone that are required for proper spermatogenesis. Furthermore, β-endorphins have been reported to inhibit the proliferation and differentiation of Sertoli cells stimulated by FSH in the testes (60) and to alter the proliferative dynamics of Sertoli cells and enhance the levels of ABP after treatment of the testes of newborn rats with the opioid antagonist nalmefene (58). Taken together, these findings suggest that EOPs inhibit the development of Sertoli cells and thus may contribute to maintaining the quiescent sexual state of the testes before puberty (61).

Dynorphin A and B immunoreactivity was detected in testicular extracts using chromatography (62). The *PDYN* transcript in rat testes is smaller than that described in brain, as a result of alternative splicing of mRNA, but the same final protein is transcribed. Polysome expression profiles indicate that the mRNA of testicular *PDYN* is translated as efficiently as the larger brain transcript (48). This precursor is expressed mainly in Sertoli cells. Because PDYN products have been immunolocalized to Sertoli cells (63) and to the interstitial compartment of the rat testis, it was suggested that dynorphins secreted by Sertoli cells may regulate the function of these cells in an autocrine manner (62).

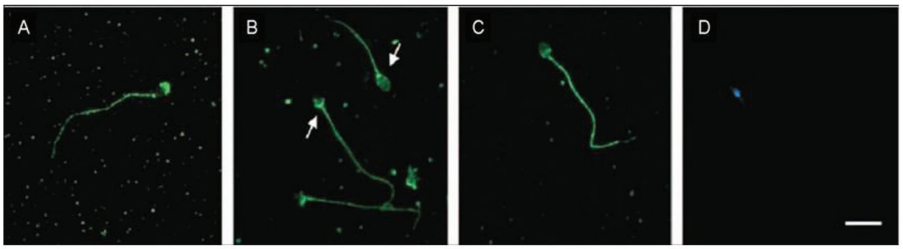

Figure 3. Immunofluorescence analysis of opioid receptors in human sperm cells. Distribution of DOR (A), MOR (B) and KOR (C) in spermatozoa is shown. DNA staining was done with Hoechst 33342 (D). Scale bar = 10 μm (modified from ref. 64).

In summary, EOPs are present in different cells of the male gonads and are likely to intervene in the mechanisms that regulate spermatogenesis. Opioid precursors are expressed differentially in somatic and germ cells of the testes, indicating that EOPs may regulate testicular function locally by *de novo* synthesis. In particular, LH and CRF stimulate the production of EOPs in Leydig cells, and these opioid peptides inhibit the role of Sertoli cells in a paracrine manner (Figure 2).

REGULATION OF SPERM FERTILITY BY THE OPIOID SYSTEM

The presence of three types of opioid receptors (MOR, DOR and KOR) on human sperm membranes (64,65) (Figure 3) indicates that EOPs may contribute to the regulation of human reproductive function by means of a direct effect on sperm. In addition to these opioid receptors, leuenkephalin, metenkephalin and β-endorphin immunoreactivity has been detected in spermatozoa (66; Subirán, unpublished data, 2011), and immunoreactivity to a variety of PENK products was found in the acrosome region (67). Expression of the opioid peptide precursors PENK, PDYN and POMC was also detected by immunohistochemistry in different subcellular regions of human sperm (Subirán, unpublished data, 2011). EOPs such as enkephalins and endorphins are present in seminal fluid, and their concentration is between 6 and 12 times higher than that detected in blood plasma (68). EOP levels are controlled by enzymatic degradation. The two enzymes that specifically degrade enkephalins are aminopeptidase N (APN) and endopeptidase neutral N 24.11 (NEP) (Figure 3). These enzymes are present in both sperm and seminal fractions (69), and their activity in semen is particularly high with respect to other body tissues (70). Interestingly, APN activity levels were found to be altered in semen from subfertile patients, suggesting that this enzyme may play an important role in male fertility (71).

Although the three types of opioid receptors are present in human spermatozoa, these receptors show differential expression and localization patterns. *MOR* and *KOR* transcripts have been detected in mature sperm by reverse transcription–polymerase chain reaction (RT-PCR), whereas DOR mRNA was absent (64). Selective RNA degradation was reported in human semen, in which specific RNA populations appear to be protected. This result points to the existence of a stable population of RNAs that have previously been synthesized during spermatogenesis (72), since sperm are tran-

scriptionally and translationally inactive. Although the function of RNA in spermatozoa is currently unknown, the following functions for this RNA have been suggested: (a) to facilitate chromatin packing; (b) a role in gene imprinting; (c) to be translated into proteins by mitochondrial polysomes; (d) involvement in the activation of the zygote during early development; and (e) to establish spatial cues in the developing embryo (73,74). However, the three types of opioid receptors (DOR, MOR and KOR) are present in the membranes of sperm cells (64), suggesting that opioid receptors may be expressed during spermatogenesis, although opioid receptors are found exclusively in Sertoli cells in rats (45). Future experiments should focus on characterizing the expression and activation of opioid receptors during spermatogenesis and the function of opioid receptor transcripts in mature sperm.

Sperm motility is an essential feature for reproductive success, since both the journey that the sperm has to make to reach the oocyte and the penetration of the extracellular matrix required for fertilization depends on sperm movement. As a result, sperm motility is considered to be one of the key functions that control reproduction (75). The opioid system could be involved in the control of sperm movement, since asthenozoospermia (reduced motility) is a common pathology found in the sperm of opiate drug addicts (76), and asthenozoospermic patients show reduced levels of metenkephalin in seminal plasma (77).

EOPs can regulate sperm motility, but paradoxical results have been reported. On the one hand, high concentrations of enkephalins and β-endorphins were found to decrease sperm motility (66). Furthermore, synthetic analogs of metenkephalin [D-Ala2, MePhe4, Met(o)5-ol]enkephalin (DAMME)] decreased sperm motility, without affecting sperm viability (78). On the other hand, low concentrations of enkephalins appear to be necessary to maintain sperm motility (77), whereas other studies did not find any effect of metenkephalin on motility (79). Other studies suggested that an adequate level of enkephalins may be necessary to maintain sperm motility and that the effect of these peptides on sperm motility is concentration dependent. Hence, enkephalin-degrading enzymes that participate in regulating the levels of EOP in seminal fluid may affect sperm motility, since the inhibition of both enzymes was found to attenuate the natural, time-dependent decrease in sperm motility. This effect, moreover, was antagonized by naloxone, indicating that the regulation of sperm motility by these enzymes is specifically mediated by an opioid receptor pathway (69).

Experiments carried out with agonists and antagonists of opioid receptors suggest that EOPs have a biphasic effect on sperm motility. On the one hand, sperm motility decreased after morphine (an MOR agonist) treatment, and on the other hand, naltrindole (a DOR antagonist) inhibited sperm motility also (64,80). The inhibitory effect of naltrindole could be due to the displacement of endogenous enkephalin from the delta receptor, which may be necessary to maintain motility. However, the inhibitory action of higher doses of enkephalins may be the result of their effect on MOR (64). This result is also consistent with the effects of the different doses of the opioid antagonist naloxone on sperm motility, where progressive motility was significantly reduced after incubation with high doses of naloxone, whereas it increased significantly after low doses of naloxone (65). Thus, the opioid system can regulate sperm motility in a variety of ways by the activation of distinct receptors (Table 2 and Figure 2). However, recent studies carried out with rats have shown that oxymorphone does not affect sperm count, motility or reproductive organ weights in males (81). The latter finding may be due to a compensatory effect of DOR and MOR, since oxymorphone displays almost equal binding affinity at both receptors (82).

Ejaculated sperm cells are immature and infertile and must undergo many

Table 2. Effect of the opioid system on sperm motility, capacitation and acrosome reaction.

	Motility	Capacitation	Acrosome reaction	References
EOP				
↑(ENK)	Decrease	—	—	66
↓(ENK)	Necessary	—	—	77
β-Endorphin	Decrease	—	—	68
DAMME	Decrease	—	Decrease	78
Opioid receptor agonist				
DPDPE	Unaffected	—	—	64
	Decrease	Unaffected	Decrease	80
Morphine	Decrease	—	—	64
U-50488	Unaffected	—	—	64
Opioid receptor antagonists				
Naltrindol	Decrease		—	64,80
		Increase	Increase	80
↑(Nlx)	Decrease	—	—	65
↓(Nlx)	Increase	Increase	Unaffected	65
nor-Bin	Unaffected	—	—	64
Drugs				
Heroin	Decrease	—	—	76
Methadone	Decrease	—	—	76

ENK: enkephalin; DAMME: D-Ala2, MePhe4, Met(o)5-ol)enkephalin; DPDPE: (d-Pen2,d-Pen5)-enkephalin (DOR agonist); morphine (MOR agonist); U-50488 (KOR agonist); naltrindol (DOR antagonist); Nlx: naloxone (Universal opioid receptor antagonist with higher affinity for MOR); nor-Bin: nor-Binaltorphimine (KOR antagonist).

modifications to become fertilization competent. These sperm modifications include different processes such as activation of sperm motility, capacitation and hyperactivation. Capacitation is a process that involves sperm membrane reorganization (83), whereas hyperactivation involves changes to the motility pattern. Hyperactive motility is characterized by an increase in the amplitude and asymmetry of flagellar beat, which leads to sudden changes in the direction of travel (84). Only capacitated and hyperactivated sperm can join to and penetrate the zona pellucida of the oocyte; thus, both processes are essential for spermatozoa fertility (85). The union of sperm cells to the oocyte induces multiple signals that induce the sperm acrosome reaction, which involves sperm fusion to the oolemma (plasma membrane of the oocyte) and oocyte penetration (83). Mammalian spermatozoa become capacitated and hyperactivated in the female oviduct (86). It is believed that both processes are due to signals originating in the oviduct and that substances released by the egg would chemotactically attract the sperm toward the egg (83,86). The concentration of metenkephalins and β-endorphins was found to vary along the different parts of the cow reproductive tract (87,88), raising the possibility that the mechanisms regulating these processes in humans may involve EOPs. Nevertheless, the role of the opioid system in capacitation, hyperactivation and acrosome reaction is poorly understood. *In vitro* studies of horse sperm have found that naloxone can induce the capacitation of sperm cells without affecting the acrosome reaction (65). Moreover, the DOR antagonist naltrindol was found to induce capacitation and acrosome reaction, whereas the corresponding agonist DPDPE ([d-Pen2,d-Pen5]-enkephalin) inhibited the acrosome reaction without affecting capacitation (80). Furthermore, other studies have shown that DAMME, a synthetic analog of metenkephalin, inhibits the spontaneous acrosome reaction in a dose-dependent manner (78) (Table 2). To date, no *in vivo* studies have been performed in this regard. Therefore, future experiments will need to characterize the participation of the opioid system in these processes.

CLOSING REMARKS

Here, we have briefly reviewed current knowledge about the role of the opioid system in male fertility. The activity of the opioid signaling system is mediated by endogenous opioid peptides. The extensive distribution of opioid peptides and receptors in different organs and in tissues of the male reproductive system indicates that the opioid system operates as a multi-messenger system in the CNS and testes and exerts direct effects on spermatozoa.

In summary, at the level of the CNS, EOPs regulate reproductive function by inhibiting the secretion of GnRH, thereby suppressing the release of LH and sex hormonal steroids such as testosterone and estradiol. Because of the shortcomings of currently available methods of male contraception, opioid system may contribute to develop additional nonhormonal male contraceptive since currently available methods require the administration of exogenous testosterone (89). In the testis, EOPs are mainly synthesized *de novo* by Leydig cells and in Sertili cells and appear to be able to inhibit Sertoli cell function in an autocrine and paracrine manner. However, opioid precursors are expressed differentially in somatic and germ cells, and the role of EOPs in germline sperm cells is still unknown. Finally, the detection in sperm cells of EOPs, specific enzymes for their degradation and opioid receptors suggests that the opioid system may contribute to sperm fertility, and EOPs may be used as a biochemical tool for the diagnosis and treatment of the human male fertility (90). These findings open up a novel area of therapeutic exploitation of the treatment of male infertility.

Interestingly, different EOPs are also present in the cow reproductive tract (87,88). Recent studies have demonstrated that MOR is expressed in the bovine (91), human (92), canine (93) and equine (94) cumulus–oocyte complex (COC) (95). MOR, by inducing an increase in intracellular calcium, has been shown to participate in the cumulus–oocyte coupled signaling associated with oocyte maturation (94,95). In the mare, a seasonal breeder, MOR was found to be expressed with higher intensity in anoestrous specimens (96). However, at the moment, the role of the opioid system in female fertility is still largely unknown. A thorough understanding of how the opioid system participates in the regulation of reproduction will also require a better understanding of the implication of this system in female reproductive processes.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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