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Review

The contribution of lower vertebrate animal models in human reproduction research

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ABSTRACT

Many advances have been carried out on the estrogens, GnRH and endocannabinoid system that have impact in the reproductive field. Indeed, estrogens, the generally accepted female hormones, have performed an unsuspected role in male sexual functions thanks to studies on non-mammalian vertebrates. Similarly, these animal models have provided important contributions to the identification of several GnRH ligand and receptor variants and their possible involvement in sexual behavior and gonadal function regulation. Moreover, the use of non-mammalian animal models has contributed to a better comprehension about the endocannabinoid system action in several mammalian reproductive events. We wish to highlight here how non-mammalian vertebrate animal model research contributes to advancements with implications on human health as well as providing a phylogenetic perspective on the evolution of reproductive systems in vertebrates.

1. Introduction

The resolution of a scientific problem needs to satisfy two main requirements: to prefigure an experimental approach and to accurately choose the animal model. In this regard, in 1887, Griffini tried to study partial testis regeneration in mammals; however, the difficulties found in this matter brought him to select a new experimental model, amphibian testis – as a non-mammalian vertebrate model system that is exemplary for its morphological peculiarities [77]. Accordingly, in 1998 Hogben, in his autobiography, asserted: "In many fields of experimental biology, advance in the understanding of a function takes place on a wider front and at a greater tempo if one can find the animal uniquely fitted for study" [88].

Although the simple reproductive anatomy of non-mammalian vertebrates [157] makes them a suitable model for endocrine studies, it is worth noting their high complexity, primarily, in reproductive behavior. Indeed, amphibians show a rich repertoire of vocalizations in intraspecies communication to coordinate courtship and male-male dominance [213]. Similarly, fish change their integumentary hues during courtship and for mutual communications among individuals of the same species [69].

Among several aspects of reproductive activity faced in recent years by many researches worldwide, non-mammalian vertebrates have exerted an important role in achieving outstanding advancements with respect to the regulatory role of several endocrine/paracrine/autocrine bioregulators. In our opinion, male estrogens, gonadotropin releasing hormone (GnRH) and endocannabinoid system regulatory role discovered in non-mammalian vertebrate animal models has contributed to a great extent to the knowledge of mammalian reproductive physiology with obvious implications on human health.

2. What have we learned from non-mammalian vertebrates?

2.1. Estrogens

The name of estrogen, the primary female sex hormone, comes from *estrus/oistros* (period of fertility for female mammals) and *gen/gonos*, which means to generate. Traditionally, testosterone and estrogen were considered male only and female only hormones, respectively [211]. However, at the beginning of 1930's, it was reported that the developmental exposure to high doses of estrogens could induce malformation of the male reproductive tract in mammals [216], thus suggesting that estrogens might regulate male reproduction. In 1934 the isolation of estrogens in the urine of stallion suggested the conversion of testosterone into the female hormone, thus considering testosterone as a pro-hormone for estrogens [221]. This discovery opened a new field of

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comparative endocrinology research focusing on the biological significance of estrogens in male reproduction.

Estrogenic hormones, such as 17β-estradiol, have been initially isolated in mammalian testis [9]. However, we are indebted to Giovanni Chieffi [25] for demonstrating that also in non-mammalian vertebrates, such as in the elasmobranch (*Scynlliorhinus stellaris*) testis, 17β-estradiol is present as well. This observation filled an evolutionary gap and set the stage for using non-mammalian animal models for understanding the male reproductive physiology of mammals. Accordingly, the use of non-mammalian vertebrate animal models has given insight into the mechanisms underlying spermatogenesis [18]. Several advantages can be ascribed to the use of these models. In particular, seasonal breeder species allow for the study of different stages of germ cells appearing progressively during the annual sexual cycle [162]. In all vertebrates, the testis is organized into germinal and interstitial compartment. In cyclostomes, elasmobranches, teleosts and amphibians, germ cells are structured into coordinated group of cells at a synchronous stage of development, thus permitting an easy recognition of germ cell associations and facilitating the analysis of processes underlying spermatogenesis of *in vivo* models [19]. Conversely, in mammals the maturation of germ cells starts from the periphery of the seminiferous tubule, where spermatogonia (SPG) are located, and proceeds toward the center of the tubule [63]. Consequently, the highly complex organization of the mammalian testis makes difficult to study discrete stages of germ cell progression *in vivo* and requires the development of *in vitro* systems to address this issue [167,94].

Although the testis is basically under gonadotropin control, intriguing local mechanisms of regulation involving autocrine and paracrine mediators produced by different testicular compartments also exist [158]. In this respect, we will focus on estrogen effects, no longer considered female hormone.

In vertebrates, 17β-estradiol is locally produced in the testis by Leydig [99] and germ cells [85], and regulates the hypothalamus-pituitary activity [179], acting through both central and local (intratesticular) mechanisms [157]. Indeed, in the male frog, *Rana esculenta*, 17β-estradiol inhibits androgen production. This hormone peaks in early spring and remains at significantly high concentrations until nearly summer [59], in association with both testosterone and 5α-dihydrotestosterone decrease during this time of the year [60]. In this period of the annual reproductive cycle, the frog testis is also characterized by a strong mitogenic activity of primary SPG [163]. Consistently, this estrogenic activity has been confirmed by *in vitro* treatment of *R. esculenta* testis. This effect is efficiently counteracted by tamoxifen, thus supporting the existence of intratesticular (autocrine/paracrine) regulation [134]. The involvement of c-fos activation by 17β-estradiol has also been demonstrated [28,29,30,31]. Similar to anurans, 17β-estradiol increases type A SPG number [135,172] in eels (*Anguilla japonica*) and roe deer (*Capreolus capreolus*), respectively, thus validating the hypothesis that estrogens are involved in seasonal regulation of spermatogenesis [172]. Since environmental estrogens may block the mitogenic effect of endogenous estrogens, this may have a detrimental effect on male reproductive development and may be related to the reported low sperm count in humans exposed to xenoestrogens [181,200]. Similarly to non-mammalian vertebrates, 17β-estradiol, as paracrine growth factor, increases gonocyte number in mammalian species [107], where it enhances also spermatogonial proliferation, by stimulating replicative DNA synthesis [206]. Altogether these findings indicate an evolutionary conserved regulation of spermatogonial number by estrogens.

We are also indebted to non-mammalian vertebrates for the discovery of membrane-associated steroid receptors. Indeed, progesterone receptors were firstly detected in *Xenopus laevis* oocytes [92]. With respect to estrogens, a specific receptor (ERα) was firstly detected in the 1969 [45] and, in turn, cloned in the 1980s [207]. Historically, ERα belongs to ligand-activated nuclear receptor family (Fig. 1A). In detail, this receptor is a transcription factor, generally located in the cytoplasm and translocating to the nucleus upon ligand binding [115]. In the nucleus, ER can either directly bind to estrogens consensus target DNA sequence or interact with other nuclear proteins, thereby regulating gene expression [144]. This mechanism has been called genomic or nuclear-initiated estrogen response. However, rapid effects (within seconds to minute after ligand binding) of estrogens have also been described since 1975 and named non-genomic or membrane-initiated estrogen response [160,127]. Nevertheless, a precise distinction between genomic and non-genomic response does not exist yet, because there are many examples of membrane-initiated signals that, through signalling cascades, result in transcriptional events [141].

In 1975, the first evidence of estrogen binding receptors in the epididymal epithelium of immature rabbits was reported, thus suggesting some roles in male reproductive tissues [41].

Soon after, estrogen binding component, having physicochemical properties comparable to mammalian ERα [1], has been demonstrated in the testis of the urodele amphibian, *Necturus maculosus* [113], and spiny dogfish, *Squalus acanthias* [17]. Interestingly, taking advantage of topographically segregation of germ and steroidogenic cells within the testis, ERs have been detected in Leydig cells [113] and SPG [17] of an amphibian and an elasmobranch, respectively. Noteworthy, these investigations on non-mammalian vertebrates suggest that estrogens may somehow be involved in regulating Leydig and germ cells differentiation. In this respect, it has been reported that estrogens inhibit both fetal Leydig cells development [42] and proliferation of precursor Leydig cells [219] in mammals. Moreover, recent findings on human testis show that 17β-estradiol blocks the *in vitro* induced apoptosis of round spermatids, thus suggesting a survival role in human testis, whereas the lack of 17β-estradiol promotes apoptosis with a resulting failure in elongated spermatid differentiation [154].

Later on, using ^{3}H-17β-estradiol for auto-radiographic localization, estradiol-positive tissues in the male mouse reproductive tract have been identified. In particular, a strong labelling has been

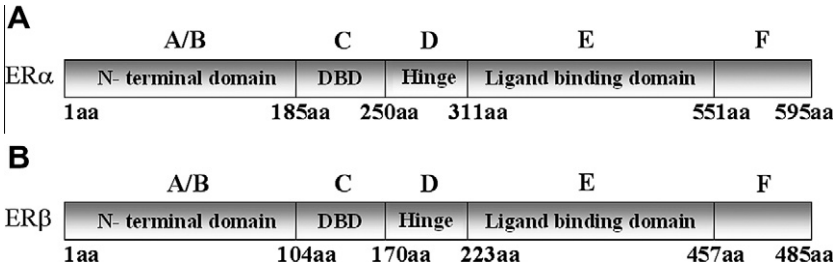

Fig. 1. (A and B) Structure of human ERα and ERβ proteins. The functional domains A–F are shown. In detail, the N-terminal domain (A/B domain), the DNA binding domain (DBD) (C domain), the hinge region (D domain), the ligand-binding domain (E domain), and the C-terminal region (F domain) are indicated. Numbers indicate aminoacid positions.