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The GnRH analogue triptorelin confers ovarian radio-protection to adult female rats

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ABSTRACT

There is a controversy regarding the effects of the analogues of the gonadotrophin-releasing hormone (GnRH) in radiotherapy. This has led us to study the possible radio-protection of the ovarian function of a GnRH agonist analogue (GnRHa), triptorelin, in adult, female rats (*Rattus norvegicus* sp.).

The effects of the X-irradiation on the oocytes of ovarian primordial follicles, with and without GnRHa treatment, were compared, directly in the female rats (F_0) with reproductive parameters, and in the somatic cells of the resulting foetuses (F_1) with cytogenetical parameters. In order to do this, the ovaries and uteri from 82 females were extracted for the reproductive analysis and 236 foetuses were obtained for cytogenetical analysis.

The cytogenetical study was based on the data from 22,151 metaphases analysed. The cytogenetical parameters analysed to assess the existence of chromosomal instability were the number of aberrant metaphases (2234) and the number (2854) and type of structural chromosomal aberrations, including gaps and breaks. Concerning the reproductive analysis of the ovaries and the uteri, the parameters analysed were the number of *corpora lutea*, implantations, implantation losses and foetuses.

Triptorelin confers radio-protection of the ovaries in front of chromosomal instability, which is different, with respect to the single and fractioned dose. The cytogenetical analysis shows a general decrease in most of the parameters of the triptorelin-treated groups, with respect to their controls, and some of these differences were considered to be statistically significant.

The reproductive analysis indicates that there is also radio-protection by the agonist, although minor to the cytogenetical one. Only some of the analysed parameters show a statistically significant decrease in the triptorelin-treated groups.

1. Introduction

In the last several years, a great number of people has suffered from cancer disease, but survived thanks to radiotherapy. The secondary effects of this treatment include different degrees of gonadal dysfunction and prevent these people from returning to a totally normal life. In accordance with that, a good knowledge of the effects of ionising radiation upon the female gonadal function and the possibility of treatment or prevention of the ovarian lesion could lessen some of the consequences of these therapies in cancer patients.

When the adult female mammals are irradiated, the ovaries are full of follicles in different stages; the majority of them are primordial follicles (PFs). The PFs are non-growing follicles and they are morphologically constituted by a small-sized oocyte, arrested in the first meiotic prophase (in dyctiotene), wrapped by a single layer of non-proliferative epithelium. There is a controversy, with respect to the renovation of the mammalian female germ line [1–3] but, at the moment, it is considered that the pool of PFs decreases progressively along the life of the female mammal because of folliculogenesis (growth and development of the PFs) and degeneration processes. Follicular recruitment, independent of gonadotrophins, starts around birth and continues until the end of the reproductive life of the female. At puberty, follicle selection, gonadotrophin-dependent, is established in every estrous cycle.

The gonadotrophin-releasing hormone (GnRH) is a peptidic hormone synthesised in the hypothalamic neurons and it is an essential component of the hypothalamic–hypophysal–gonadal

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axis. Its prime function is the neuro-hormonal control of reproduction, as it stimulates the synthesis and release of the hypophysary gonadotrophins (Gn): the follicle-stimulating hormone (FSH) and the luteinising hormone (LH). These hormones promote the development of the gonadal functions: the production and release of the sexual steroids and gametogenesis. Throughout the early years of life, GnRH has a pulsating baseline-level secretion, which strengthens at the prepubertal period. However, if this GnRH secretion is continuous, as seen in some pathologies, the effect of the hormone is opposite to its natural function and the capacity of secretion of Gn is suppressed [4].

This dual potential of GnRH has been seized and analogue molecules have been designed. The two types of GnRH analogues (agonists (GnRHas) and antagonists) have been thoroughly studied in male and female rodents [5–8] and in primates including humans [4,9]. Unlike the antagonists, the GnRHas, such as triptorelin (D-Trp^6-LHRH), preserve the dual effect of the original GnRH molecule and so they can be applied in both GnRH-like functions. The inhibition of the secretion of Gn, achieved after a continued period of exposure to these molecules [8], will be regained when the treatment is interrupted.

Studies concerning radiotherapy and GnRHas are scarce and refer to clinical, histological, morphological or pharmacological data and, none of them analyse cytogenetical markers. In males (human and animal models), the majority of these studies indicate the existence of radio-protection [10–13]. On the other hand, in females (primates including human and mainly rodents) the results obtained are controversial [14–18], and conclude positively or negatively, depending on the analogue studied, the species analysed and the dose and type of irradiation.

In this context, an experiment with female *Rattus norvegicus* as the animal model was designed to study the possible radioprotection of the ovarian function of the GnRH analogue (GnRHa) triptorelin. According to this, the effects of the X-irradiation on the oocytes of PFs with and without GnRHa treatment were compared, directly in the female rats (F_0) with reproductive parameters, and in the somatic cells of the resulting foetuses (F_1) with cytogenetical parameters.

2. Materials and methods

The diagram showing the experimental design is in Fig. 1.

2.1. Females, foetuses, cell cultures and chromosomal preparations

In order to study the effects of the GnRH analogue triptorelin (GnRHa), a total of 83 *R. norvegicus* (RNO) adult, female Sprague–Dawley-strain rats ($2n = 42$, *Servicio de Animales de Laboratorio*, *Universidad de Murcia* and *Servei d'Estabulari*, *Universitat Autònoma de Barcelona*, *Spain*) were analysed.

An experiment with six different groups of treatment was designed: a non-treated group (C), an X-irradiated one with a dose of 5 Gray (Gy) (C + 5), an X-irradiated one with a dose of 12 Gy (C + 12); a GnRHa-treated group (A); a GnRHa-treated and subsequently 5-Gy-irradiated group (A + 5) and a GnRHa-treated and subsequently 12-Gy-irradiated group (A + 12), as shown in Fig. 1.

After 45 days of irradiation, all of the females cohabitated for two weeks with adult, Sprague–Dawley males of proved fertility. A monitoring of the successful matings was performed by the observation of the presence of the dropped copulation plug or of vaginal sperm every morning to determine Day 0 of pregnancy.

After 14–16 days of pregnancy, all of the 83 females were sacrificed. The uteri and ovaries from 82 were extracted for the reproductive analysis. Foetuses from 65 female rats were obtained for the cytogenetical analysis.

A total of 236 foetuses were analysed with a mean of three to four foetuses per rat (Fig. 1 and Table 1). Forty-two of these corresponded to non-X-irradiated females (C and A) and 194 of them came from X-irradiated females (C + 5, C + 12, A + 5 and A + 12). A total of 82 uteri and 164 ovaries were also analysed, corresponding to the pregnant females of all of the groups.

Primary cultures of fibroblasts were established for each foetus. The primary fibroblasts were obtained from the whole foetuses. These primary cultures lasted from 4 to 7 days (short cultures) in order to obtain no differentiation in specific tissue cells and no genetic modification. A single subculture was performed followed by the harvest of the cells on the next day. Chromosomal preparations were obtained according to standard procedures.

Table 1
Females, foetuses, metaphases analysed and aberrant metaphases of the cytogenetical analysis.

	C	C + 5	C + 12	A	A + 5	A + 12	Totals
Females	7	14	14	4	11	15	65
Foetuses	26	45	50	16	40	59	236
Total metaphases	2694	4419	4833	1602	3959	4644	22151
Aberrant metaphases	204	598	528	91	311	502	2234

C: control group; C + 5: 5-Gy-treated group; C + 12: 12-Gy-treated group; A: GnRH-analogue-treated group; A + 5: GnRH-analogue and 5-Gy-treated group; A + 12: GnRH-analogue and 12-Gy-treated group.

2.1.1. Hormonal treatment

The GnRHa triptorelin (Decapeptyl®, Ipsen) was administered for three times (Days 0, 28 and 56) to three groups of treatment: A, A + 5, A + 12 (Fig. 1). The dosage was of 0.05 ml per each 100 g of weight (0.094 mg/100 g), and it was inoculated into the leg muscle.

2.1.2. X-irradiation

Previously anaesthesised (Thalamonal, Roche; 0.1 cm^3/100 g), 54 females were X-irradiated in the abdominal region (5 cm × 5 cm) at two different dosages: 25 were irradiated with a single dose of 5 Gray (Gy) for 2.57 min (C + 5 and A + 5) on Day 84, and 29 were irradiated with a fractioned dose of 12 Gy (C + 12 and A + 12) for 4 days at a dose of 3 Gy for 1.54 min per day, on Days 84–87. The X-irradiation was conducted in the Unit of Cobaltotherapy at the *Servicio de Radiología*, *Hospital Universitario Vírgen de la Arrixaca*, *Murcia*, *Spain*.

At the moment of irradiation of the adult females, theoretically, the ovaries were full of follicles in different stages. The long period of recovery (45 days) permitted that the germ cells ready for fertilisation at mating were the ones from those X-irradiated primordial follicles. Therefore, this experiment allowed for the analysis of the results of the X-irradiation in the oocytes I in primordial follicles of the mothers and their consequences in the F_1.

2.2. Cytogenetical analysis

The detection of breakpoints was performed by analysing metaphases by sequential staining: conventional staining (with Leishman's stain) for the detection of chromosomal aberrations, followed by the sequential G-banding technique (with Wright's stain) for the precise localisation of breakpoints (Fig. 2). Metaphases were analysed with an Olympus AX70 optic microscope, and digital images were obtained using a coupled camera with CytoVision software (*Applied Imaging, Inc.*).

The parameters analysed to assess the existence of chromosomal instability were the number of aberrant metaphases and the number and types of gaps and breaks and of structural chromosomal aberrations. Chromosomal aberrations included two groups of aberrations: gaps and breaks and structural aberrations. Gaps and breaks included chromatid gaps (meaning a gap in one chromatid of the chromosome (chtg)), chromosome gaps (meaning a gap including both chromatids (chsg)), chromatid breaks (meaning different types of breakages in one chromatid of a chromosome (chtb)) and chromosome breaks (meaning different types of breakages including both chromatids (chsb)). The structural aberrations included (a) stable aberrations (translocations (t), fusions (f), inversions (inv), insertions (ins), marker chromosomes (mar), chromatid deletions (cht del) and chromosome deletions (chs del)); (b) unstable aberrations (acentric fragments with only a chromatid (ace cht) and with two chromatids (ace chs), reorganisations between two or more chromosomes (re), ring chromosomes (r) and dicentric chromosomes (dic)), and (c) other aberrations (derivative and odd chromosomes). Furthermore, aberrant metaphases were classified into six groups depending on the number of aberrations contained. The frequencies of all parameters obtained, with respect to total and aberrant metaphases, and with respect to total aberrations, include aberrant metaphases per total metaphases (AM/TM), total aberrations per total metaphases (Ab/TM) and total aberrations per aberrant metaphases (Ab/AM).

The rat ideogram and G-band nomenclature were established according to Levan [19] with a resolution of 238 bands per haploid genome. Roughly 100 cells per rat foetus were analysed.

2.3. Reproductive analysis

An examination of the uteri and ovaries of each pregnant female was performed. The uteri were analysed macroscopically and the ovaries were examined under a binocular magnifying glass with a coupled cold light, in both cases without fixation. The uteri were analysed by counting the foetuses and the post-implantation losses of every pregnant female, while the *corpora lutea* in each of the ovaries were recorded. The implantations (foetuses + post-implantation losses) and the pre-implantation losses (*corpora lutea*—implantations) were also calculated. The means per group of