Tubo-ovarian dysplasia in relationship with ovulation induction in rats

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Objective: To assess tubo-ovarian dysplasia via morphologic and immunohistochemical study of rats exposed to ovulation stimulation protocols.
Design: Animal experimental study.
Setting: Academic research hospital.
Animal(s): 72 female Wistar rats divided into three groups.
Intervention(s): Stimulation protocols using follicle-stimulating hormone (FSH) or clomiphene citrate for 3, 6, or 12 cycles, after which the animals were killed.
Main Outcome Measure(s): Ovarian and tubal dysplasia score and immunohistochemical assessment using p53 and Ki67.
Result(s): The ovarian dysplasia score was statistically significantly higher after 12 stimulation cycles in the groups receiving FSH (group B) or clomiphene citrate (group C) compared with control (group A). The tubal dysplasia score was statistically significantly increased after only three stimulation cycles in groups B and C. The Ki67 proliferation marker was statistically significantly expressed in the ovaries from group C, and in the fallopian tubes from groups B and C. P53 was constantly low in all three groups.
Conclusion(s): Ovulation stimulation may induce tubal and ovarian histopathologic and immunohistochemical abnormalities with a dose effect. The role of the fallopian tubes and their interaction with the ovaries require further study. (Fertil Steril® 2013;99:1768–73. ©2013 by American Society for Reproductive Medicine.)
Key Words: Ki67 expression, ovarian dysplasia, ovulation stimulation, p53 expression

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Although many retrospective studies focused on ovarian cancer, the relationship between infertility treatment and cancer remains unclear and controversial. Some investigators have found that women receiving ovarian stimulation are at higher risk of developing cancer, while others have found no correlation between the two (1–6). The two most frequently mentioned risk factors for ovarian neoplasia are hereditary predisposition related with mutations of the BRCA genes and the "incessant ovulation" hypothesis described by Fathalla (7).

Histopathologic study of material from prophylactic oophorectomies performed for a genetic predisposition to ovarian cancer have revealed cytologic and architectural abnormalities considered to be precancerous manifestations. These abnormalities have been termed "dysplasia," by analogy with the preinvasive lesions that have been described for the genital tract (vulva, vagina, cervix, and endometrium) (8–15). Similarly, serous tubal intraepithelial lesions (STIL), a spectrum of epithelial changes ranging from normal appearing tubal epithelium to lesions with cytologic atypia, in the prophylactically removed fallopian tubes of women predisposed to develop ovarian cancer have been described (16, 17).

Several studies have found similar ovarian dysplasia lesions after stimulation of ovulation in infertile patients (18–20), but such tubes never been the subject of STIL investigation in the context of infertility. We performed a morphologic and immunohistochemical study in the rat model of ovaries and tubes that had been exposed to an ovulation stimulation protocol using follicle-stimulating hormone (FSH) and clomiphene citrate.

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MATERIALS AND METHODS

The study took place between November 2010 and May 2011 in collaboration with the Jacques Lisfranc Faculty of Medicine's animal experimental center (PLEXAN unit) in Saint Etienne, and the Saint Etienne University Hospital Centre pathology laboratory. Institutional review board approval was previously obtained from the Auvergne Animal Experiment Ethics Committee (CEMEA Auvergne) in accordance with European regulations applicable to animal experiments (Official Journal of the European Community dated 12–18–1986).

Animals

The study included 72 female Wistar Han rats (aged 70 days, weight 160–185 g, with regular 4-day estrus cycles). The rats were housed in the Jacques Lisfranc Faculty of Medicine at Saint Etienne (PLEXAN unit) in a controlled environment. There were no restrictions on access to food or drink.

Study Groups

The animals were allocated in random fashion to three groups. Group A (n = 24) was the control group receiving normal saline administered by intraperitoneal injections, dosed at 2.5 mL/kg for the first 3 days of the cycle (D1, D2, and D3). Group B (n = 24) was stimulated with clomiphene citrate (Clomid; Aventis) administered by intraperitoneal injections, dosed at 1 mg/kg for the first 2 days of the cycle (D1 and D2), and hCG (Pregnil; Schering-Plough) dosed at 100 IU/kg on the third day of the cycle (D3). Group C (n = 24) was stimulated with urofollitropin or FSH (Fostimon; Genevrier Laboratories) administered by intraperitoneal injections, dosed at 2 IU/kg for the first 2 days of the cycle (D1 and D2) and hCG (Pregnil; Schering-Plough) dosed at 100 IU/kg on the third day of the cycle (D3).

Experimental Protocol

The rat estrus cycle lasts 4 days on average. The treatments were administered during the first 3 days of the cycle (D1, D2, and D3), with a therapeutic interruption on the fourth day (D4). Each animal was weighed on D1 to calculate the doses to be injected during the current cycle. Each group of rats was divided into three subgroups: subgroups A1, B1, and C1 underwent three stimulation cycles; subgroups A2, B2, and C2 underwent six stimulation cycles; and subgroups A3, B3, and C3 underwent 12 stimulation cycles. At the end of the set number of cycles, the animals were killed on D5 by anesthesia via intraperitoneal administration of ketamine (2 mL/kg) followed by a lethal injection of pentobarbital (60 mg/kg). Each animal was then installed on its back; a midline incision was made after careful aseptic preparation of the abdominal region to give access to the pelvic organs. A bilateral salpingo-oophorectomy was performed.

Macroscopic Analysis of Excised Tissues

The rats were weighed before sacrifice to assess their weight gain during the study period. In addition, before embedding the specimens in paraffin, the tubes and ovaries were separated to make the slides easier to read, and the ovaries were measured. The surface index selected was the product of the greatest length by the greatest width, giving a theoretical surface area in square millimeters representing the size of the ovaries. One value only was selected per rat, this being the maximum value obtained after measuring both ovaries.

Histopathologic Analysis

The excised tissues were placed immediately in a 10% formol solution and sent to the pathology laboratory. After embedding the samples in blocks of paraffin (two tubes and two ovaries per block), a microtome was used to obtain sections between 4 and 5 mm thick. The standard staining protocol (hematoxylin and eosin [H&E]) was used for all sections. The slides were read using a photonic microscope by two specialist gynecologic pathologists (M.P. and A.C.) who were blinded to the other's findings. The histopathologic analysis was based on two specific scales for assessment of epithelial ovarian and dysplasia and STIL (20–22).

Eleven cytologic and architectural criteria were used for examination of the ovaries: epithelial pseudo-stratification, epithelial proliferation, surface papillomatosis, nuclear chromatin irregularity, nuclear contour irregularity, cellular pleomorphism, nuclear size (increased or not), presence of inclusion cysts, deep epithelial invaginations, psammoma bodies, and stromal hyperplasia. Seven cytologic and architectural criteria were used to examine the fallopian tubes: epithelial pseudo-stratification, tufting, increased nuclear density, nuclear atypia, loss of nuclear polarity, increased nuclear size, and loss of ciliation.

For each slide, the most representative area for each of the items was allocated a score between 0 and 2 (0: normal; 1: moderately abnormal; 2: severely abnormal). An ovarian dysplasia score and a tubal dysplasia score for each animal were respectively obtained by summing the scores of the 11 items for the ovaries (total range: 0–22) and by summing the scores of the seven items for the tubes (total range: 0–14). In the event of obvious differences between the scores established by each pathologist, a further examination was performed to reach a consensus.

Immunohistochemical Analysis

For the histochemical analysis, the 4–5 mm sections were processed automatically (Benchmark XT Ventana automated slide preparation system) after elimination of paraffin at 75°C. The standard preliminary treatment for antigen unmasking was applied for 60 minutes at 95°C. The monoclonal antibodies used were anti-rat Ki67/MIB5 antibodies (1:20 dilution; DAKO) and antibody anti-p53 (1:80 dilution; DAKO). Incubation took place at 37°C for 48 minutes, and after rinsing, the ultraView universal DAB (Ventana Medical Systems) revelation system was used. The slides were read under the same conditions as described earlier for the morphologic analysis.

To assess the Ki67 immunohistochemical marking, we proposed a scale suitable for preliminary screening of all the slides. For the follicles, marking was assessed according