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Evaluation of biodegradable microspheres containing nomegestrol acetate in a rat model of endometriosis

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ABSTRACT

We assessed the efficacy of biodegradable microspheres (MSs) containing nomegestrol acetate (NOMAC) for treatment of endometriosis in a rat model and investigated its preliminary mechanism of action. Sprague-Dawley rats with surgically implanted endometrial autografts were divided randomly into four groups of thirteen rats each, and subcutaneously injected twice (10 d apart) with either empty MSs or MSs containing nomegestrol acetate (NOMAC-MS; 27–800 mg per kg of rat body weight). Twenty-one days after the first injection, blood and endometriotic tissues were collected and assayed for changes in endometriotic tissue, serum hormone, liver function parameters, and apoptotic protein. No remarkable irritation was observed at the site of injection. NOMAC-MS treatment significantly reduced the volume of the endometrial autografts, decreased serum levels of estradiol, progesterone, triiodothyronine, and alanine aminotransferase, and decreased levels of estrogen receptor alpha protein. Furthermore, NOMAC-MS at the highest dose significantly reduced serum aspartate aminotransferase and endometrial antibody, reduced the Bcl-2/Bax protein ratio, and increased caspase-3 and caspase-9 proteins. There was no pronounced difference observed in alkaline phosphatase, carbohydrate antigen 125, progesterone receptor, or vascular endometrial growth factor receptor 2 (VEGFR2) in any of the tested groups relative to the control. NOMAC-MS significantly changed the expression of apoptotic protein only at the highest dose. Our findings warrant the further investigation of sustained application of steroid hormone via microspheres for the treatment of endometriosis.

1. Introduction

Endometriosis occurs in approximately 6–10% of women during their reproductive years, and is a principle cause of infertility (Bulletti et al., 2010). The disease is estrogen-dependent and characterized by ectopic endometrial-like glands and stroma most often on the pelvic peritoneum, bowel, bladder, uterosacral ligaments, and ovaries. In China, endometriosis therapy usually includes gonadotropin-releasing hormone (GnRH) analog and gestrinone, both of which have unpleasant side effects; GnRH analog is hypoestrogenic, while gestrinone impairs liver function. Therefore, safe and effective medication for long-term use to treat endometriosis is much needed.

Nomegestrol acetate (NOMAC) is a recently developed synthetic steroid hormone that is classified as a pure progestin (Sitruk-Ware, 2006). Unlike the majority of older progestins, NOMAC is derived from 19-nor-progesterone. It has little or no affinity to bind to any steroid receptor other than the progesterone receptor (PR) (Duc et al., 1991), including the androgen and glucocorticoid receptors. Therefore, its pharmacologic profile shows strong progestational activity and no estrogenic activity, and it has potent anti-estrogenic effects on the endometrium (Sitruk-Ware, 2008). In addition, NOMAC has powerful antigonadotropic activity without residual androgenic, glucocorticoid, or anti-mineralocorticoid properties (Lello, 2010).

NOMAC is a component of the daily oral contraceptive Zoely, and can also be used as a progestin-only tablet for the treatment of menopausal syndrome, uterine diseases and menorrhagia, and

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as a component of HRT in combination with estradiol for the relief of menopausal symptoms (Lello, 2010). However, there has yet to be investigated previously specifically for the treatment of endometriosis. In the interest of developing a modality for sustained release of NOMAC in the treatment of endometriosis, we prepared a degradable microsphere by embedding NOMAC into polymer (poly [lactic-co-glycolic acid], or PLGA).

Advantages of excipient biodegradable microspheres include delayed drug action and automatic disintegration. They need not be removed from the site of action after drug delivery, and accumulation of the polymers is avoided. There are already commercialized forms of biodegradable microspheres based on polymer materials for the sustained release of peptide drugs, but microspheres designed specifically for steroid hormone delivery are not available.

In this study, we assessed the effect of degradable microspheres containing NOMAC on the growth of autografts in a rat model of endometriosis and conducted an investigation of its preliminary mechanism of action.

2. Materials and methods

2.1. Materials

NOMAC ($C_{23}H_{30}O_4$, molecular weight 370.48, purity > 98%) was synthesized and provided by Guangdong Medical College (Dongguan, Guangdong Province, China). Microspheres containing 18.6 ± 0.1% NOMAC were prepared by Professor Qinhua Chen. The estradiol (E2) and progesterone ELISA kits were purchased from Demeditec Diagnostics GmbH (Germany). Endometrial antibody (EMAb) and carbohydrate antigen 125 (CA-125) ELISA kits were purchased from Shanghai Zhaorui Biotechnology (China). Rabbit polyclonal Bcl-2 (B-cell lymphoma 2; N-19) antibody and rabbit polyclonal Bax (BCL2-associated X protein; P-19) antibody were purchased from Santa Cruz Biotechnology. (Dallas, TX). Rabbit polyclonal antibody to vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2), rabbit monoclonal antibody to caspase-9 (E23; 35 kD), rabbit monoclonal antibody to caspase-9 (E87; 46 kD), and goat polyclonal secondary antibody to rabbit IgG-H&L (HPR) were purchased from Abcam (Cambridge, MA). An enhanced chemiluminescence (ECL) detection kit for horseradish peroxidase was purchased from Pierce (Rockford, IL).

2.2. Animals

Female Sprague-Dawley rats (weighting 220–240 g) were purchased from Sino-British Experiment Animal (Shanghai, China). The animals were treated in accordance with protocols approved by the Laboratory Animal Ethics Committee at the Shanghai Institute of Planned Parenthood Research. All animals were housed 5 per cage under a 12-h/12-h light/dark cycle with free access to food and tap water. Each animal was weighed once per week and fed with standard chow for rats.

2.3. Model establishment and treatment

The rat model of experimental endometriosis was established by homologous uterine endometrium surgical transplantation, as proposed by Vernon and Wilson (Vernon and Wilson, 1985) with slight modifications. Under sterile surgical conditions, the rats were anesthetized and a small incision was made in the middle of the abdomen. A segment of the left uterine horn was excised and rinsed in saline solution and the muscle and the endometrium were carefully separated. A section of endometrium was cut off and sutured to the inner right wall of the abdomen. The muscle and skin were sutured separately.

The operated animals were allowed a recovery period of three weeks. During this time, all animals were injected subcutaneously with 30 μg/kg benzoic estradiol immediately after the first laparotomy and repeat injection ten days later to promote the growth of the autografts. Three weeks after the recovery period, the rats underwent a second laparotomy to measure three dimensions of the endometriotic autografts, using a caliper (length, width, and height in millimeters). The spherical volume of each ectopic endometrium was calculated using the prolate ellipsoid formula: V, $mm^3 = 0.52 \times A \times B \times C$, where V, A, B, and C denote the volume, width, length, and height, respectively (Uygur et al., 2006). Rats whose endometriotic volume exceeded 25 mm^3 were considered successful models of endometriosis.

Fifty-two model rats with successfully transplanted ectopic endometria were randomly divided into four groups of thirteen rats each based on the volume of the autografts. There was no statistical difference on the volume of the autografts among the groups before the medication treatment (Table 1). Then, group 1 was subcutaneously injected with 800 mg control (empty) microspheres (i.e., without NOMAC) per kg body weight (mg/kg)at the 1st and 11th days after the second laparotomy, respectively, while groups 2, 3 and 4 were injected subcutaneously with 27, 80, and 800 mg NOMAC-impregnated microspheres per kg body weight (mg/kg), respectively, in which the microspheres contained 5, 15 and 150 mg NOMAC per kg microspheres. Both the empty microspheres and the NOMAC-MS were suspended in 0.5% Na carboxymethyl cellulose for this procedure.

Twenty-one days after the NOMAC-MS treatment, all rats were killed by overdose of anesthesia. A third laparotomy was performed. Whether or not skin irritation appeared at the injection site was scrutinized. The sizes of the implants were measured again with the same caliper by the same investigator who was blinded to the treatment groups. The autografts were dissected and fixed immediately in 10% neutral formalin or frozen in liquid nitrogen. Sera were stored at −20 °C until assayed. The rate of growth inhibition was considered to reflect the change in volume of the ectopic endometrium, and calculated as: growth inhibitory rate, $\% = (1 - V_{pre}/V_{post}) \times 100\%$, where V_{pre} denoted the volume of ectopic endometrium before treatment, and V_{post} was the volume of ectopic endometrium after treatment.

2.4. Histological examination

The formalin-fixed endometriotic tissues were embedded in paraffin, sliced into 4-μm thick sections using a rotary microtome, and stained with hematoxylin-eosin (H&E) for histological examination under a light microscope.

2.5. Assays of serum parameters

Serum E2, progesterone, CA-125, and EMAb levels were assayed using ELISA, performed in accordance with the manufacturer's instructions. The absorbance was determined using Biotek Synergy 2 multi-mode microplate readers (Bio-Tek Instruments, Winooski, VT) at 450 nm. Serum levels of triiodothyronine (T3), thyronine (T4), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were detected by chemiluminescence via an ADVIA Centaur immunoassay system (Bayer Healthcare, Leverkusen, Germany).

2.6. Protein isolation and analysis

The preparation of the proteins and the Western blot analysis were performed as in our previous report (Zhu et al., 2007). The