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Original article

Contraceptive activity of 4-(4-hydroxy-3-methyl-hex-5-enyl)-chroman-2,7-diol via inhibiting ovulation in Gonadotropin-primed immature rat model

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ABSTRACT

Gonadotropin-primed immature rats (GPIR) were used to study the anti-ovulatory effect of 4-(4-hydroxy-3-methyl-hex-5-enyl)-chroman-2,7-diol (chroman derivative) isolated from seeds of *Ensete superbum* on ovulation. Present results depicted dose-related anti-ovulatory effect of the chroman derivative at 2 mg/kg bw and 4 mg/kg bw. Chroman derivative showed dose-dependent anti-estrogenic activity on MCF-7 cell line. Attenuation of ovulation was associated with a lowered ovarian estrogen and progesterone levels along with reduced levels of some major cytokines, i.e. TNFα, VEGF, IL-6 and IL-1β, indicating the disrupted cytokinine signaling. Unruptured preovulatory follicles were found in all the ovaries extracted from animals treated with chroman derivative. Our findings demonstrated that chroman derivative has potential contraceptive activity through its anti-estrogenic activity which leads to inhibition in ovulation and attenuated ovarian cytokinine signaling.

1. Introduction

Ovulation, the process by which oocytes are released from the graffian follicle in the ovary, is an inflammatory process and requires proteolytic degradation of the follicle wall, as well as removal of the overlying ovarian tissues accompanied by induction of prostaglandin synthesis [1]. For successful ovulation, follicle rupture has to occur just at the site of the follicle wall facing the ovarian surface, which permits release of the Cumulus-Oocyte Complex (COC) to the periovarian space, while preventing proteolytic damage of the perifollicular tissues at the basolateral follicle sides. Several mutant rodent models have this type of anovulatory phenotype, including animal null for the progesterone receptor [2], cyclooxygenase-II [3], the prostaglandin E_2 receptor [4], which indicate that follicular rupture is a biologically regulated morphological phenomenon, that must occur at a precise and well-regulated cascade of events leading to the transformation of an ovarian follicle into a progesterone producing corpus luteum.

Ensete superbum Cheesm is a stoloniferous stout herb of 3–6 m tall, naturally occurring all around in India, mainly in Western Ghats of India. The flesh of the fruits is given to diabetics under Ayurvedic practice. The ground seeds are also used in ayurveda and certain fractions isolated from the seeds have shown to possess anti-variola and anti-vaccinia properties [5]. Its leaf ash is used for treatment of leucoderma. Leaves smeared with warm coconut oil are kept on head of children for infantile cold along with leaf juice given orally daily in the morning for three days [6]. Earlier study done in our lab reported that chroman derivative 4-(4-hydroxy-3-methyl-hex-5-enyl)-chroman-2,7-diol possesses anti-implantation activity in rats [7] and their histopathological examination suggested that it may have anti-ovulatory activity. Our present work is to evaluate its anti-estrogenic activity and anti-ovulatory activity on Gonadotropin-primed immature rat model and find out the possible mechanism of action of its effect on various important cytokines involved in the ovulation process for its contraceptive ability and use.

2. Materials and methods

2.1. Isolation of standard chroman derivative

Seeds of *Ensete superbum* (Cheesm) were purchased from the local supplier. Seeds were dried in the shade at the temperature of 28 ± 2 °C and were authenticated at Department

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of Botany, by botanist Dr. H.B. Singh, HOD, Dept. of Botany at National Institute of Science Communication and Information Resources (NISCAIR) on 21st September, 2010 reference No. NISCAIR/RHMDC/Consult/2010-11/1536/135, New Delhi, India. Kernels of the dried seeds of *Ensete superbum* (2.5 kg) were extracted with 95% (v/v) ethyl, alcohol in Soxhlet apparatus, a red colored substance was obtained (crude extract, 1.92%) which was further dissolved in water and treated in basic lead, sodium sulphate and calcium carbonate to obtain a yellowish white solid. It was further confirmed through FTIR, NMR, GC-MS and C-NMR that it was 4-(4-hydroxy-3-methyl-hex-5-enyl)-chroman-2,7-diol of molecular formula $C_{16}O_4H_{22}$ as described earlier [8].

2.2. Animals and drugs

Female albino wistar rats (25–26 days), procured from the animal house of Delhi Institute of Pharmaceutical Sciences and Research (DIPSAR), New Delhi, were used. Rats were housed in temperature controlled room at 25 ± 2 °C with a 12-hour light/dark cycle and 57 ± 7% relative humidity under standard hygienic conditions and had free access to fresh tap water and pelleted diet (Amrut rat feed, Pranav agro Ltd., India). The study was approved by the Institutional Animal Ethics Committee (IAEC/DIPSAR/2010-I/02) of DIPSAR, New Delhi, and experiments were carried out in accordance with the guidelines laid down by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

2.3. Gonadotropin-priming and drug treatment

Wistar immature rats were injected s.c. with 10 IU of Equine Chorionic Gonadotropin (eCG) at 17 00 h on 25 or 26 days of age, and 48 h later were injected (s.c.) with 10 IU of hCG [9]. Chroman derivative was administered at dose level 2 mg/kg bw and 4 mg/kg bw per day from eCG priming day till animals were sacrificed (6 per group) at 09 00 h on the day following hCG injection.

2.4. Tissue homogenate, RNA isolation and histopathology

Both the ovaries were recovered surgically and from each rat right ovary were fixed in 10% formalin [10]. Tissues embedded in paraffin blocks were used for histopathological examination. Left ovaries were divided into two equal parts. One part weighed and homogenized in PBS (0.1 g/mL) on ice bath by an ultra turrax. The homogenate obtained was processed as follows: 500 μL were centrifuged at 2000 × g for 10 min at 4 °C and supernatant was stored at −40 °C until TNFα, IL-6, IL-1β, estradiol and progesterone determination. The protein concentration was measured using Bradford protein assay. For expression of VEGF and COX-II total RNA was isolated from the second half of the ovaries with minor modification as per earlier described methods [11] using the trizol reagent. Briefly, tissue was weighed and 500 μL trizol reagent/100 mg tissue weight added. Total RNA was separated from DNA and proteins by adding 250 μL chloroform and precipitated with isopropanol (overnight, 20 °C). The precipitate was washed twice in ethanol, air-dried, and resuspended in 75% diethylpyrocarbonate (DEPC)-treated water and quantified (A260 nm). One microgram of total RNA was reverse-transcribed to cDNA using iScript™ cDNA Synthesis Kit (Bio-RAD Laboratories Inc., CA, USA) in a final volume of 20 μL, according to the manufacturer's instruction.

2.5. Real-time PCR

Primers express software (Version 2.0., Applied Biosystems) was employed for designing and synthesizing primers of quantitative PCR. The VEGF primers were designed to amplify a region common to all VEGF isoforms, so the sense VEGF primer was 5'-CAGCTATTGCCGTCCAATTGA-3' (114-124) and the antisense VEGF primer was 5'-CCAGGGCTTCATCATTGCA-3' (244-226) where a 131-bp (NCBI accession No. AF215726), COX-2 primer was 5'-CCCAATTTGTTGAATCATTT-3' (958-977) and antisense 5'-TCTCATCTCTCTGCTCTGGT-3' (1057-1076) (NCBI accession No. AF207824) a product of 119 bp PCR product was expected. The sense βactin primer was 5'-AGGGAAATCGTGCGTGACAT-3', and the antisense βactin primer was 5'-AACCGCTCATTGCCGATAGT-3' (NCBI accession No. 55574), giving rise to an expected PCR product of 149 bp. Real-time quantitative PCR was performed in the iCycler Thermal Cycler (Bio-RAD Laboratories Inc., Hercules, CA, USA) using SYBR green I detection. The following reaction components were prepared to the indicated end-concentrations: 0.6 μM of each primer, 1X IQ SYBR Green BioRad Supermix (Bio-RAD Laboratories Inc.), 150 ng of cDNA and nuclease free double distilled water were added to a final volume of 25 μL. All samples were performed in duplicate for all genes. The real-time PCR protocol was followed according to the manufacturer's instructions with a heated lid (105 °C), an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of the amplified PCR products was verified by analysis of the melting curve. The relative mRNA level was determined as the PCR cycle number that crosses an arbitrarily placed signal threshold (Ct). The Ct value correlates inversely with the amount of target mRNA in the sample. The relative changes in VEGF, Cox-II with respect to βactin expressions were examined using the ΔΔCt method $\Delta Ct = Ct_{target} - Ct_{\ \beta actin}$. As PCR amplification is an exponential process, a ΔCt difference denotes a shift in regulation by a factor of two (2-ΔΔCt). To validate a real-time PCR, standard curves with $r^2 > 0.95$ and slope values between 3.0 and 3.4 were required. Real-time efficiencies were acquired by amplification of a standardised dilution series and corresponding slopes and PCR efficiencies were calculated using iCycler iQ Real-Time PCR Detection System (Bio-RAD Laboratories Inc.).

2.6. Histological analysis and follicle rupture and ovulation

The right ovaries were serially sectioned (5.0 μm) and stained with hematoxylin and eosin. All sections were examined under the microscope. The total number of cumulus-oocyte complexes (COCs) per ovary were counted as described [12] and number of COCs trapped inside the corpus luteum, released to the ovarian interstitium, retained in the bursal cavity or found in the oviducts, were recorded.

2.7. Cell culture and anti-estrogenic activity

Estrogen receptor-positive human breast cancer MCF-7 cells line is the model used to evaluate anti-estrogenic effect of a New Chemical Entities (NCEs). MCF cell line was obtained from National Centre For Cell Science (NCCS), Pune, and routinely maintained as previously described [13] in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 nM Estradiol (E2), 2 mg/mL insulin, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 4 mM glutamine, and 10% fetal bovine serum. Exponentially growing cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. Experiments were performed in 96-well plates containing phenol red-free DMEM supplemented with 5% CS-FBS. MCF-7 cells were seeded at a plating density of 2×10^3 cells/well in 200 μL of medium and then cultured for 24 h to allow their adhesion to the plate. Two days later, the medium was replaced, and two sets of experiment were performed. Set-I control group cells were exposed to estradiol 1.0 nM and chrome derivative group's cells were exposed with estradiol 1.0 nM at 0.01–100 μg/mL doses of chroman derivative for 4 days. Set-II control group cells were exposed