Lectin Histochemical Study of Rat Reproductive Tissues Treated with Ether Fraction of *Anethum graveolens* L. Extracts

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ABSTRACT

Introduction: Nowadays, there is special interest in finding a new contraceptive drug with high safety and fewer side effects. Investigations have shown that dill (*Anethum graveolens* L.) can be used either as a menstrual cycle regulator or contraceptive. Since cell surface terminal sugars have crucial roles in oocyte maturation, fertilization and implantation, in this study we investigated the effects of ether fraction of dill seed extracts on ovarian and uterine cell surface glycoconjugates.

Methods: Adult Wistar female rats were divided into 6 groups: control, sham, low (0.5 g/kg) and high (5 g/kg) doses of ether fraction of dill seed aqueous extract and low (0.045 g/kg) and high (0.45 g/kg) doses of ether fraction of dill seed ethanol extracts and treated for 10 days. Histological sections of uterus and ovary were prepared and stained by Con A, DBA, SBA, UEA, and PNA lectins. Intensity of reactions of uterine and glycoconjugates of ovarian cell surfaces was measured by Image-Java software.

Results: The intensity of reactions of endometrial epithelial cells (P=0.01 and P =0.00) and myometrial cells (P=0.02 and P=0.03) after Con A and DBA staining were significantly lower in the high-dose aqueous extract treated group than in the control group. Corpus luteum granulosa cell reactions in low doses of aqueous extract (P =0.01) and corpus luteum granulosa cell and oocytes in high doses of aqueous extract (P =0.02 and P =0.04) were decreased compared to the control group.

Conclusion: Ether fraction of dill extracts, especially in the form of aqueous extract, could affect cell surface terminal sugar.

Key Words:
Dill Extracts,
Ether Fraction,
Glycoconjugate,
Ovary, Uterus.

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1. Introduction

A wide range of hormonal and non-hormonal contraception is used for planning and limiting births not only in developing countries, but also in many least-developed countries of the world. Many women in reproductive age use contraception. However, given the side effects of these contraceptive drugs, a great deal of research is devoted to making safe and effective contraception and novel products, leading to the recent introduction of new implants, contraceptive vaginal rings, transdermal patches and newer combinations of oral contraceptives [1].

According to the traditional medicine of Iran, *Anethum graveolens* L. belongs to the family Umbelliferae, is available in local markets and widely used for its antihyperlipidemic and antihypercholesterolemic effects [2] , such as increasing mother's milk production, promoting menstruation and reducing menstrual irregularity and pain [3-5].

The effective components of an herbal plant could determine its different fractions' preparation. In previous studies, we investigated the effects of different fractions of *Anethum graveolens* on diverse parameters of female reproduction, such as uterine and ovarian histological examination, duration of estrous cycle, progesterone concentration and the ultrastructure of corpus luteum granulosa cells [6-9]. *Anethum graveolens* causes histological changes in different organelles of granulosa lutein cells and significantly increases the duration of the estrous cycle and progesterone concentration.

Ether fraction of dill seed aqueous and ethanol extracts reduces the thickness of endometrium and myometrium and the diameter of granulosa cells of corpus luteum without any histological changes in these structures [9]. We assume that other mechanisms could be involved in the infertility potential of this herb. Glycoconjugates have vast diversity and an important role in the female reproductive system, such as oocyte maturation, fertilization and embryo implantation [10-14] , so it is probable that any deterioration or synthesis defects or any changes in the quantity or quality of cell surface glycoconjugates in different parts of the female reproductive system lead to infertility. Given these circumstances, in the present study we investigated glycoconjugate-related oocyte and uterine changes that respectively are involved in sperm recognition and embryonic implantation. For this purpose, we performed a histochemical technique using a panel of various lectins with different carbohydrate affinities that are highly sensitive to the detection of glycoconjugates of the cell surface and extracellular matrix.

2. Materials and Methods

Extracts and Fraction Preparation

Dill seeds were purchased from a commercial source in Shiraz (Fars Province, southwest of Iran) . The identity of the seeds was confirmed by the Biology Department of Shiraz University. A voucher specimen (1015) was kept at the herbarium of the Department of Biology, Shiraz University. The seeds were then powdered and 100 g of the powder and 300 ml of 80% ethanol (for ethanol extract) , as well as 100 g of the powder and 300 ml of distilled water (for aqueous extract) , were percolated for 24 hours. Subsequently, the mixtures were filtered and concentrated under reduced pressure using a rotary evaporator. The yields (w/w) of the aqueous and ethanol extracts were 8.2% (g/g) and 4.5% (g/g) solid residue, respectively. In the subsequent course, 20 g of the aqueous extract and 40 g of the ethanol extract were independently rinsed three times with ether at room temperature. The oily liquids were evaporated to dryness under vacuum conditions. The yield (w/w) of ether fractions of the aqueous extract was 2.5% (g/g) , while the yield (w/w) of ether fractions of ethanol extract was 70% (g/g) .

Animal Grouping

Adult Wistar female rats (10-12 weeks old, 160-220g) were randomly drawn from the stock colony in the animal house of the Razi Institute of Shiraz, and were housed individually in polypropylene cages under standard housing conditions (controlled atmosphere with 12:12 hour light/dark cycles and an ambient temperature of 22-24 °C) . The rats were acclimatized for two week before the experiment. The rats were maintained on commercial pelleted diet (Javaneh Khorasan Company, Mashhad, Iran) and had free access to food and tap water. All procedures with animals were conducted strictly in accordance with approved guidelines of the National Institutes of Health (NIH) [15] , which were followed at all times, with maximum care taken to minimize animal suffering; in addition, the number of rats was kept at a minimum. Animals were weighed before and after the experiment. In order to select normal estrous cycles female rats, vaginal smears were examined daily. Female rats with regular estrous cycles were randomly divided into 5 groups (7 rats in each) . Control group (CON) received 1 ml distilled water. The experimental groups

received either a low (0.5 g/kg) or a high (5 g/kg) dose of ether fractions of the aqueous extract (LDAE and HDAE, respectively) , and a low (0.045 g/kg) or a high (0.45 g/kg) dose of ether fractions of the ethanol extract (LDEE and HDEE, respectively) . Ether fraction was dissolved in 1 ml distilled water and administered daily for 10 days (2 regular estrous cycles) .

Lectin Histochemistry

The left ovary and the uterus of each rat were removed surgically and fixed in 10% buffered formalin, dehydrated through ascending grades of ethanol (70%, 90%, and 95%, v/v) , cleaned in xylene, and embedded in paraffin wax (melting point 56 ˚C) . Serial sections were cut using a rotary microtome (Zeiss, Germany) at 7 microns thickness. Then, ovarian and endometrial sections were placed on poly-l-lysine coated slides, deparaffinized by keeping at 60 ˚C for one hour, rehydrated through graded series of ethanol grades (100%, 90%, and 70%, v/v) and washed in PBS (phosphate buffered saline) solution for 30 min.

Afterwards, in order to block the endogenous peroxidase activity, the sections were incubated in 1% H2O2 in methanol for 15 min and washed in PBS solution for 30 min.

Then, each specimen was incubated with different kinds of lectin (Sigma, USA) included in Table 1 separately at a final concentration of 10 mg/ml for 2 h at room temperature. Since Con A lectin is not conjugated to peroxidase, at this stage Con A lectin were incubated in H2O2 for 45 min. After washing, the binding sites were visualized by incubating the sections in 100ml PBS containing 200 ml H2O2 and 0.03gr DAB for 10 min. The Sections were washed between each incubation. Finally, the sections were washed with water, counterstained with alcian blue (0.5%) for 30-45 min, dehydrated through ascending grades of ethanol (70%, 90%, and 95%, v/v) , cleaned in xylene and mounted. Photographs were taken with a digital camera. The intensity of the reaction to each lectin of corpus luteum granulosa cell, follicular granulosa cells, oocyte, and epithelial cells of endometrium and myometrium was assessed with Image-Java software (Oracle, USA) .

Data Analysis

The data were expressed as one-way ANOVA (analysis of variance) , followed by Scheffé's test. Statistical analysis was done with SPSS v. 11.5 software (SAS Institute, USA) . $P<0.05$ was considered statistically significant.

3.Results

Histochemical study of the ovary and uterus of female rats treated with ether fraction of dill seed aqueous and ethanol extracts revealed no significant staining intensity in different compartments of ovary and myometrial smooth muscles after Con A staining in treated female rats compared to the control group (Table 2) . However, in the uterus, the intensity of the reaction of cell surfaces of endometrial epithelial cells (P=0.01) and myometrium (P=0.02) of HDAE-treated group was significantly lower than in the control group (Table 2) . After initial investigation, we observed that PNA lectin is not capable of detecting glycoconjugates in the uterus, so we applied this lectin only in ovarian tissue. PNA intensity reaction showed a significant decrease in corpus luteum granulosa cells of the LDAE-treated group (P=0.01) and the HDAE-treated group (P=0.02) . After PNA, cell surface terminal sugar of oocytes or zona pellucida of HDAE-treated group (P=0.04) reacted with lower intensity than the control group (Figures 1 and 4) .

Cell surface terminal sugar that react with DBA lectin were decreased in the endometrium (P=0.00) and myometrium (P=0.03) of the HDAE-treated group

Table 1. Lectins used and their carbohydrate specificity

LECTIN DESIGNATION/ABBREVIATION	Carbohydrate Binding Sugar
Con A (Canavalia ensiformes)	α-D-Mannose
DBA (Dolichus biflorus agglutinin)	α-N-acetylgalactoseamine
SBA (Soybean agglutinin)	β-N-acetylgalactoseamine
UEA (Ulexe uropaeus agglutinin)	α-Fucose
PNA (Peanut agglutinin)	Galactose-N-acetylgalactosamine

Table 2. Intensity of reactions of uterine layer (pixel) with Con A and DBA lectins in rats treated with ether fractions of dill seed aqueous and ethanol extracts. Values showed as means ± S.D.

	Lectin	CON	LDAE	LDEE	HDAE	HDEE
Myometrium	DBA	62.07±5.91	56.36±5.60	72.42±6.50	47.43±2.72*	67.16±6.70
	Con A	69.79±10.37	79.13±8.29	78.58±4.34	53.02±2.34*	75.92±5.67
	SBA	74.02±11.72	72.01±4.34	72.73±8.38	68.54±4.79	68.50±7.19
	UEA	71.13±7.96	69.91±3.76	66.52±7.28	75.33±7.38	72.56±3.45
Oocyte	DBA	59.12±10.03	60.38±13.62	73.34±13.39	55.23±10.36	63.19±10.79
	Con A	75.13±4.95	73.23±7.91	70.17±14.33	68.40±13.50	74.77±12.49
	SBA	66.74±3.92	64.39±2.11	63.88±5.49	64.75±4.18	66.43±1.57
	UEA	73.74±7.42	65.36±10.35	73.11±3.24	62.36±12.59	67.57±3.44
	PNA	78.59±6.11	60.63±7.53	59.07±12.18	50.39±7.35*	64.27±12.15
Connective tissue	DBA	78.71±7.03	79.63±13.86	74.92±11.82	79.27±5.26	80.37±11.52
	Con A	60.56±4.86	61.72±7.08	55.78±2.30	62.70±3.93	64.29±11.52
	SBA	64.63±2.49	69.33±6.37	72.38±4.17	71.45±5.45	66.27±5.29
	UEA	68.51±4.64	63.67±4.24	66.39±4.53	61.69±9.19	69.66±4.05
	PNA	74.88±13.62	63.60±18.55	60.10±9.04	69.54±8.55	62.77±8.15

* Show significant differences compared with the control group (P<0.05)

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CON: control; LDAE: low dose of aqueous extract; LDEE: low dose of ethanol extract; HDAE: high dose of aqueous extract; HDEE: high dose of ethanol extract

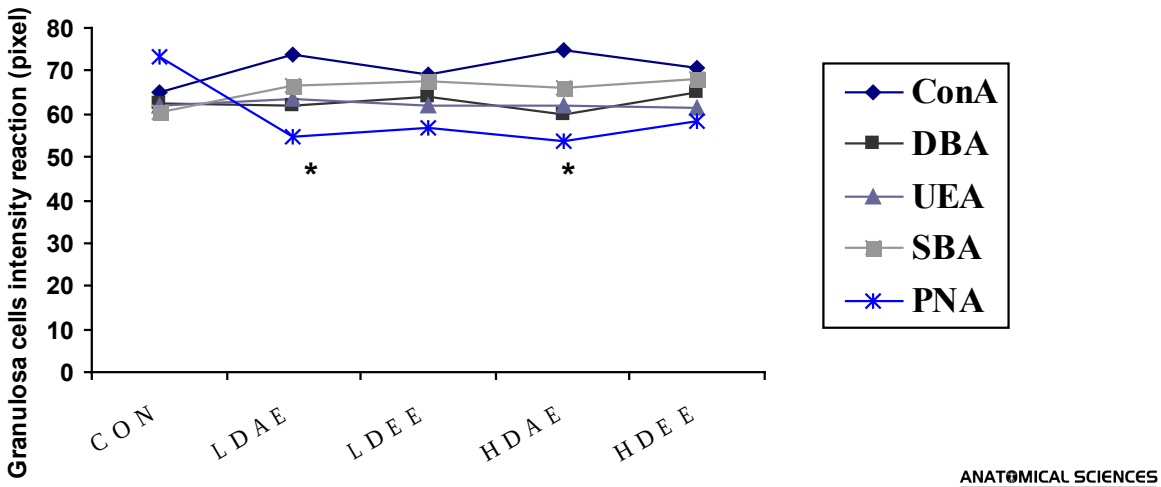

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Figure 1. Intensity of reactions of corpus luteum granulosa cells (pixel) with different lectins in rats treated with ether fractions of dill seed aqueous and ethanol extracts. CON: control; LDAE: low dose of aqueous extract; LDEE: low dose of ethanol extract; HDAE: high dose of aqueous extract; HDEE: high dose of ethanol extract.

compared to the control group (Table 2; Figures 2 and 3) , while no difference was observed between different compartments of ovary between the control and experimental groups (Table 2) . Staining by UEA and SBA lectin showed no significant reaction in different compartments of ovary and uterus in treated female rats compared to the control group (Table 2) .

4. Discussion

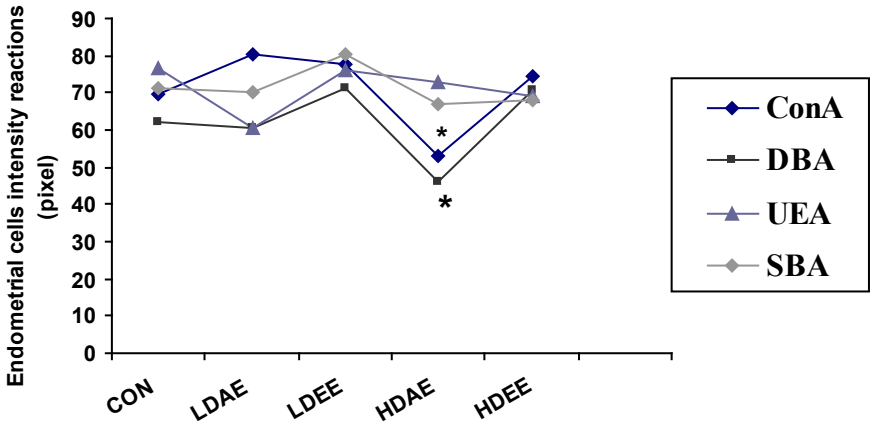

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Figure 2. Intensity of reactions of epithelial cells of endometrium (pixel) with different lectins in rats treated with ether fractions of dill seed aqueous and ethanol extracts. CON: control; LDAE: low dose of aqueous extract; LDEE: low dose of ethanol extract; HDAE: high dose of aqueous extract; HDEE: high dose of ethanol extract.

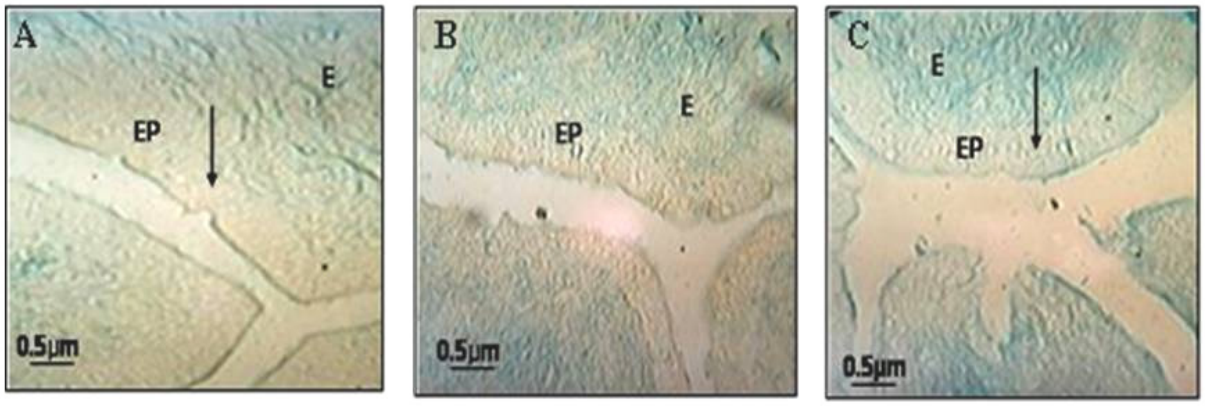

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Figure 3. Intensity reactivity of endometrium in ether fractions of high dose of dill seed aqueous (HDAE) treated group after staining with Con A and DBA lectins. A) Con A staining, B) Control group and C) DBA staining. Arrow shows significant decrease in staining intensity in connective tissue (E) and epithelium (EP) .

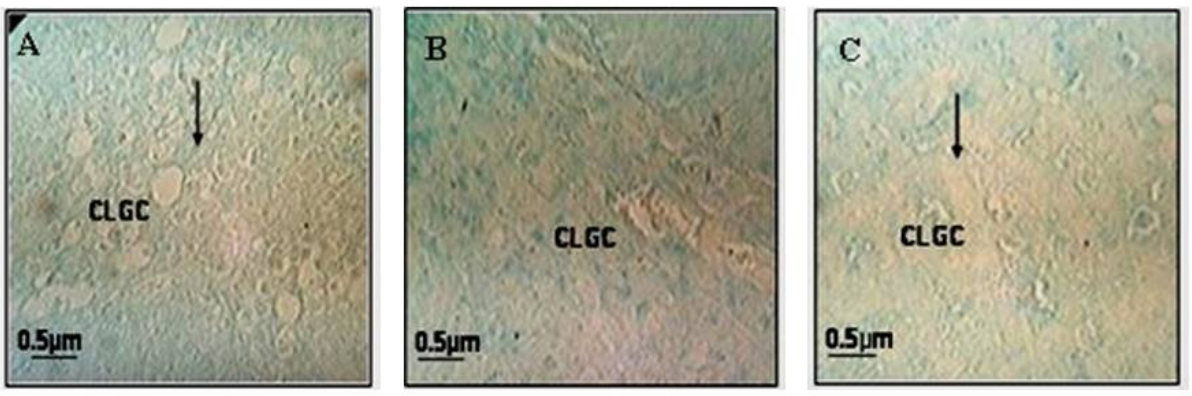

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Figure 4. Intensity reactivity of corpus luteum granulosa cells (pixel) in ether fractions of high (HDAE) and low dose (LDAE) of dill seed aqueous treated groups after staining with PNA. A) HDAE-treated group, B) Control group and C) LDAE-treated group. Arrow shows significant decrease in staining intensity of corpus luteum granulosa cells (CLGC) .

It is well documented that glycoconjugate residues on the cell surface have critical function in various stages of reproduction from gamete maturation, fertilization and folliculogenesis to embryo implantation, and also in regular changes of uterus and ovary during the reproductive cycle [10-14]. Since previous studies showed effects of dill extract on male and female reproduction properties [6-9, 16, 17] , in this study we evaluated any possible rearrangement of glycoconjugate in different compartments of ovary and uterus of female rats treated with ether fraction of low and high doses of aqueous and alcoholic extract using a panel of lectins to probe glycoconjugates expression and clarify precisely the mechanism of dill seed on the reproductive system.

Con A lectin detects α-D-mannose, one of the carbohydrates that regulates the shape and function of intercellular adhesion molecules [18]. It is located on the surface of the zona pellucida, which binds to sperm's receptors [19]. In addition, the expression of this glycoconjugate has been observed on apical surface of endometrial epithelial cells of monkey, which reacted at the time of embryo implantation [20].

DBA lectin specifically binds to terminal α-N-acetyl galactosamine. It is suggested that distribution of this carbohydrate is controlled by galactosyl transferase, which is affected by hormone alterations during proliferative and secretory phases of menstrual cycle [21]. Galactose has a critical role in various reproductive activities such as sperm-oocyte interaction, compaction of the embryo, sperm maturation, preparation and establishment of a receptive endometrium and the attachment of the blastocyst to the epithelium of the uterus at the time of embryo implantation [22, 22]. Since studies have shown that overall, glycoproteins on the cell surface of the endometrium quantitatively are increased at the time of implantation, and are involved in the intercellular recognition and adhesion of the embryo to receptive luminal epithelium [24] , the attenuated level of Con A and DBA staining intensity after exposure to the ether fraction of HDAE as compared to the control group might affect feto-maternal cell recognition and adhesion during the implantation process.

UEA and SBA lectins bind to α-fucose and β-N acetyl galactose amine, respectively, and in spite of the importance of these glycoconjugates in normal development and implantation, these lectin-reactive sites did not show obvious alterations in ovary and uterus.

In PNA staining, it was shown that staining intensity of luteinized granulosa cells of corpus luteum in the LDAE- and HDAE-treated group and oocytes and granulose cells of HDAE-treated group is significantly lower than in the control group. Since this lectin specifically binds to terminal N-acetylgalactosamine, we concluded that ether fraction of dill seed aqueous and ethanol extracts decreases and changes the distribution of this glycoconjugate in the mentioned ovarian compartments at different doses.

N-acetylgalactosamine on the surface of zona pellucida contributes in inducing the acrosomal reaction and in binding the sperm to oocyte, and also has crucial function in cleavage and formation of morula. Its presence on cell membrane of blastomeres and trophoblasts is necessary for interaction of these cells with other cells, and especially for endometrial epithelium [25]. The expression pattern of PNA-reactive glycoconjugates that were modified after exposure to ether fraction of high dose of aqueous extract of dill seed may affect sperm-oocyte and trophoblast-endometrial epithelium interaction and finally induce fertilization or implantation failure.

5.Conclusion

Ether fractions of dill seed aqueous and ethanol either induce some modifications in glycoconjugate distribution on the oocyte which interfere with fertilization, or lead to incomplete glycosylation in the endometrium that result in implantation defect.

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Conflicts of Interest

The authors declared no conflicts of interest.

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