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Prenatal Exposure to Hydroalcoholic Extract of *Foeniculum vulgare* Mill. Seeds Alters *DMRT1* and *Inhibin* Gene Expression and Sexually Dimorphic Nuclei Development in *Gallus gallus domesticus*

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ABSTRACT

The present study aimed to investigate the effects of prenatal exposure to hydroalcoholic extract of *Foeniculum vulgare* Mill. seeds on the expression of sex-related genes and structural alterations of sexually dimorphic nuclei in *Gallus gallus domesticus*. Forty-six fertilized chicken eggs were randomly assigned to control and experimental groups. On embryonic day 2 (E2), eggs were windowed under sterile conditions, and on embryonic day 12 (E12), experimental groups received a single intra-amniotic injection of F. vulgare extract (140 mg/kg), whereas control groups received physiological saline. After hatching, 38 chicks were included in molecular and histological analyses. The hypothalamus, pituitary gland, and gonads were collected for RNA extraction, cDNA synthesis, and quantitative real-time PCR analysis of *DMRT1* and *Inhibin* gene expression. Histological evaluation of hypothalamic sexually dimorphic nuclei was performed using toluidine blue staining and stereological analysis. Prenatal administration of F. vulgare extract significantly decreased *DMRT1* expression in male experimental chicks compared with male controls, while *Inhibin* expression showed significant alterations in treated groups. Histological examination revealed changes in the size, neuronal density, and organization of sexually dimorphic nuclei in the hypothalamus, particularly in male chicks. These findings indicate that prenatal exposure to phytoestrogen-rich *F. vulgare* extract may interfere with endocrine regulation and neural sexual differentiation in chickens. Further studies are required to clarify the molecular mechanisms involving estrogen signaling pathways and long-term reproductive and behavioral consequences.

Keywords: Chicken, *DMRT1*, *Foeniculum vulgare* Mill, Phytoestrogen, Sexually dimorphic nuclei

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INTRODUCTION

Foeniculum vulgare Mill. (fennel) is an aromatic perennial medicinal plant belonging to the Apiaceae family and is widely distributed in Mediterranean regions, Western Asia, North Africa, and many temperate areas of the world. This plant is characterized by hollow stems, finely divided leaves, yellow compound umbels, and aromatic fruits commonly known as fennel seeds. Due to its adaptability to different climatic conditions, fennel is cultivated commercially in several countries, including India, Egypt, Turkey, Iran, and Mediterranean countries, where it is used as a culinary spice, medicinal plant, and source of essential oils for pharmaceutical and food industries [1,2]. In Iran, fennel is traditionally cultivated in several regions with suitable semi-arid climates, and its seeds have long been used in traditional medicine for digestive, reproductive, and hormonal disorders [3].

Phytochemical investigations have demonstrated that F. vulgare contains a wide range of secondary metabolites, including essential oils, phenolic compounds, flavonoids, coumarins, terpenoids, fatty acids, sterols, and tannins [1,4]. The essential oil fraction represents one of the most biologically active components of fennel, with trans-

anethole considered its major constituent, accounting for approximately 50–80% of the volatile compounds depending on genetic and environmental conditions [2,5]. Other important compounds include estragole, fenchone, limonene, α-pinene, and phenolic derivatives. Among these compounds, trans-anethole has attracted particular scientific attention because of its structural similarity to endogenous estrogen and its potential interaction with estrogen receptors. Therefore, fennel is considered a plant containing phytoestrogenic compounds that may influence endocrine regulation and hormone-dependent physiological processes [6,7].

Numerous pharmacological studies have demonstrated that *F. vulgare* possesses antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, antidiabetic, neuroprotective, and reproductive regulatory properties [1,4,8]. In human studies, fennel preparations have been investigated for the management of dysmenorrhea, menopausal symptoms, lactation stimulation, gastrointestinal disorders, and metabolic abnormalities [3,9]. In animal models, fennel extracts have been reported to influence reproductive tissues, ovarian function, estrogen receptor expression, and endocrine-related pathways. Recent studies have suggested that prenatal or early-life exposure to fennel-derived phytoestrogens may modify estrogen-dependent signaling pathways and epigenetic regulation in reproductive organs, emphasizing the importance of evaluating its developmental effects [10].

Sexual differentiation of the brain and reproductive system in birds is controlled by a complex interaction between genetic mechanisms and steroid hormones. In chickens, sex determination is primarily regulated by genetic factors located on sex chromosomes, and *DMRT1* (Doublesex and Mab-3 Related Transcription Factor 1), located on the Z chromosome, is recognized as one of the most important regulatory genes involved in male gonadal differentiation [11,12]. Increased expression of *DMRT1* during embryonic development promotes testicular differentiation, whereas reduction of its activity may interfere with normal male developmental pathways [13]. In addition, *Inhibin*, a member of the transforming growth factor-beta (TGF-β) superfamily, plays an important role in reproductive regulation through modulation of follicle-stimulating hormone (FSH) secretion and communication within the hypothalamic-pituitary-gonadal (HPG) axis [14].

The hypothalamus represents a critical neural center involved in endocrine regulation, reproductive behavior, and sexual differentiation of the brain. Sexually dimorphic nuclei (SDN) within hypothalamic regions undergo organizational changes during embryonic development under the influence of steroid hormones, particularly estrogens [15,16]. Phytoestrogenic compounds may mimic or modify endogenous estrogen activity and consequently affect neuronal development, gene expression, and reproductive-related behaviors. Although the biological effects of fennel have been extensively studied in relation to general health and reproductive physiology, limited information is available regarding its effects on embryonic brain sexual differentiation and the molecular regulation of sex-related genes in avian species.

Therefore, the present study was designed to investigate the effects of prenatal administration of hydroalcoholic extract of *Foeniculum vulgare* Mill. seeds on *DMRT1* and *Inhibin* gene expression in the hypothalamus, pituitary gland, and gonads, as well as structural alterations of sexually dimorphic nuclei in newly hatched chickens. We hypothesized that exposure to fennel extract during embryonic development may alter estrogen-sensitive pathways involved in neuroendocrine regulation and sexual differentiation.

MATERIALS AND METHODS

Study Design and Ethical Approval

This experimental study was conducted during 2023–2024 at the Department of Biology, Faculty of Basic Sciences, Islamic Azad University, Mashhad Branch, Iran. The experimental protocol was reviewed and approved by the Research Ethics Committee of Islamic Azad University, Mashhad Branch (Ethics Code: IR.IAU.MSHD.REC.1401.032). All procedures involving fertilized chicken eggs and newly hatched chicks were performed according to institutional guidelines for animal research and ethical principles for the use of experimental animals [17].

Preparation of Hydroalcoholic Extract of *Foeniculum vulgare* Seeds

Mature seeds of *Foeniculum vulgare* Mill. were collected from Gorgan Province, Iran, and botanically authenticated by the Herbarium of Ferdowsi University of Mashhad (Voucher No. 11455). The collected seeds were washed, shade-dried at room temperature, and powdered using an electric grinder.

The hydroalcoholic extract was prepared using the Soxhlet continuous hot extraction method, which is considered an effective technique for extracting bioactive secondary metabolites, including phenolic compounds, flavonoids, and other polar phytochemicals from medicinal plants [18]. Briefly, 70% ethanol (ethanol: distilled water, 70:30 v/v) was used as the extraction solvent. The powdered fennel seeds were extracted continuously for 8 h using a Soxhlet apparatus. The obtained extract was concentrated under reduced pressure using a rotary evaporator at 40 °C to remove the solvent. The dried extract was stored in sterile amber containers at −20 °C until experimental administration.

The extraction procedure was performed based on previously described protocols with minor modifications [18,19].

Experimental Animals and Egg Incubation

A total of 46 fertilized eggs of Gallus gallus domesticus were obtained from Toos Poultry Company, Mashhad, Iran. Eggs with similar weight, normal shell structure, and no visible abnormalities were selected and randomly allocated into experimental groups.

The eggs were incubated under controlled conditions at 37.8 ± 0.2 °C and 65 ± 5% relative humidity with automatic rotation until hatching. The experimental groups were established according to treatment and sex:

Male control group (♂ Control): n = 10
Female control group (♀ Control): n = 10
Male experimental group (♂ Experimental): n = 9
Female experimental group (♀ Experimental): n = 9

Thus, 38 chicks were successfully hatched and included in the final molecular and histological analyses. The reduction from the initial 46 fertilized eggs to 38 successfully hatched chicks was attributable to unsuccessful hatching during embryonic development.

Egg Windowing and Administration of *Foeniculum vulgare* Extract

On embryonic day 2 (E2), a small window was created at the blunt end of each egg under sterile conditions to permit access to and

monitoring of the developing embryo. Following confirmation of embryo viability, the opening was temporarily sealed with sterile parafilm, and incubation was continued.

On embryonic day 12 (E12), the egg window was reopened, and the experimental groups received a single intra-amniotic administration of hydroalcoholic extract of F. vulgare seeds at a dose of 140 mg/kgusing a sterile Hamilton microsyringe. Control groups received an equivalent volume of sterile physiological saline.

Following injection, the eggshell opening was immediately sealed with sterile melted paraffin, and incubation continued until hatching. The dose of 140 mg/kg was selected based on previous experimental evidence indicating biological activity of F. vulgare preparations at comparable exposure levels [19,20]. The selected dose was intended to provide a biologically active exposure under the experimental conditions without producing overt embryonic toxicity.

Chick Collection and Tissue Sampling

Among the 46 fertilized eggs initially included in the experiment, 38 chicks hatched successfully and were included in the final molecular and histological analyses.

After hatching, chicks were examined and sex was determined based on gonadal morphology. Animals were subsequently categorized into male and female groups.

For molecular analysis, samples were collected from the:

- hypothalamus;
- pituitary gland; and
- gonads (testis in males and ovary in females).

Tissues intended for gene-expression analysis were collected under RNase-free conditions, rapidly frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

For histological analysis, hypothalamic tissues containing the regions of interest were collected and immediately fixed in 10% neutral buffered formalin.

RNA Extraction and Quantitative Real-Time PCR (RT-qPCR)

Total RNA was extracted separately from hypothalamic, pituitary, and gonadal tissues using the Pars Toos RNA Extraction Kit according to the manufacturer's instructions. The concentration and purity of extracted RNA were assessed using spectrophotometric analysis. Complementary DNA (cDNA) was synthesized using the Dena Zist cDNA Synthesis Kit according to the manufacturer's protocol.

Quantitative real-time PCR (RT-qPCR) was performed to determine the relative expression levels of *DMRT1,* and *inhibin* was used as the internal reference gene. Gene-specific primers were used for amplification, and their sequences are presented in Table 1.

Each sample was analyzed in three technical replicates, and the mean Ct value obtained from the technical replicates was used for subsequent statistical analysis. No-template controls (NTCs) were included to monitor potential contamination and nonspecific amplification.

The RT-qPCR amplification protocol consisted of an initial denaturation at 95 °C for 5 min, followed by 40 amplification cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. A melting-curve analysis was subsequently performed to evaluate amplification specificity.

The presence of a single melting-curve peak was considered evidence of specific amplification. Relative gene expression was calculated using the comparative $2^{-}\Delta\Delta Ct$ method described by Livak and Schmittgen [21].

Table 1 Primer Sequences of Genes

Gene	Sense	Anti-sense
DMRT1	CCCCTGAGCCAGTTGTCA	ATCCGTCCCTCTGGTGGT
INHIBIN	CGGGAACTGTGCCGAAGG	GTTGGGCACCGTCTCGTAC

Histological Examination of Hypothalamic Sexually Dimorphic Nuclei

Fixed hypothalamic tissues were processed using standard histological procedures. Briefly, tissues were dehydrated through a graded ethanol series, cleared in xylene, embedded in paraffin, and serially sectioned at a thickness of 7 µm.

Sections were stained with Toluidine Blue for visualization of neuronal structures and examined using a Zeiss light microscope.

Histological assessment focused on hypothalamic regions containing sexually dimorphic nuclei (SDN).

Stereological analysis was performed using an unbiased approach based on previously described stereological principles [22]. Representative microscopic images were obtained from the experimental groups under standardized imaging conditions.

Parameter

Male Control (n=10)

Male Experimental (n=9)

Female Control (n=10)

Female Experimental (n=9)

Table 2 Quantitative stereological assessment of hypothalamic sexually dimorphic nuclei

SDN volume (µm³)	Total neuronal number	Neuronal density (cells/mm³)	
0.112 ± 0.082	23	55.01 ± 378.26	Male Control
0.050 ± 0.054	18	48.29 ± 373.23	Male Experimental
-	-	-	Female Control
-	-	-	Female Experimental

***** Include this parameter only if neuronal area was measured.

Table note: Values are presented as mean ± SE. Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different superscript letters indicate statistically significant differences among groups ($P < 0.01$).

RESULTS

A total of 46 fertilized eggs were included in the experiment. Among them, 38 chicks hatched successfully and were used for molecular and histological analyses. No gross developmental abnormalities or

embryonic mortality unrelated to treatment were observed in the control groups.

DMRT1 Gene Expression in Gonadal Tissue

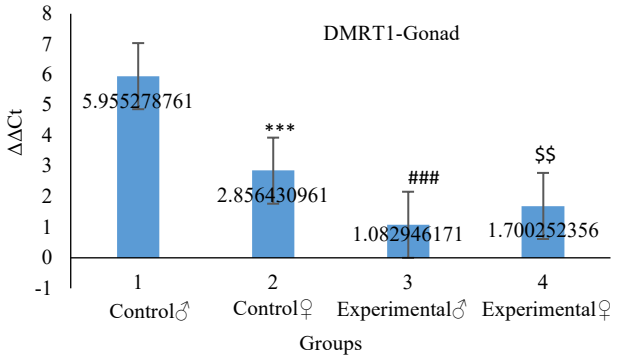


Fig. 1 *DMRT1* gene expression in Gonad in different groups.
Control (♂): Male control samples that received only physiological serum. Control (♀): Female control samples that received only physiological serum. Experimental (♂): Male experimental samples that received the extract at a dose of 140 mg/kg. Experimental (♀): Female experimental samples that received the extract at a dose of 140 mg/kg. *** Comparison of female control with male control (P<0.001). ### Comparison of Male experimental with Male control (P<0.001). $$ Comparison of Female experimental with Female control (P<0.050).

Figure 1 shows the relative expression of the *DMRT1* gene in the gonadal tissue of control and experimental male and female chicks following prenatal administration of *Foeniculum vulgare* hydroalcoholic extract (140 mg/kg). Data are expressed as Mean ± SE. Control groups received physiological saline, whereas experimental groups received the extract. Statistical significance is indicated as: ***P < 0.001 compared with the male control group and ###P < 0.001 compared with the corresponding control group.

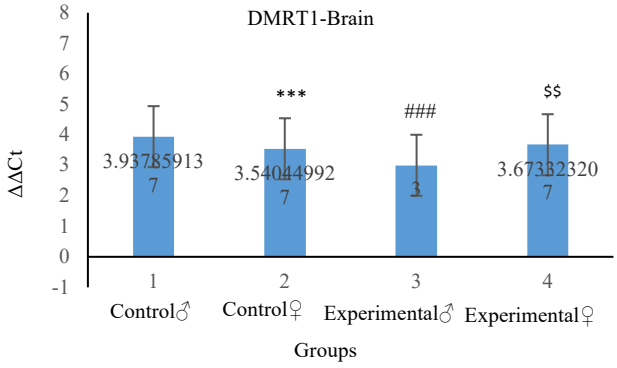


Fig. 2 *DMRT1* gene expression in Brain in different groups.
Control (♂): Male control samples that received only physiological serum. Control (♀): Female control samples that received only physiological serum. Experimental (♂): Male experimental samples that received the extract at a dose of 140 mg/kg. Experimental (♀): Female experimental samples that received the extract at a dose of 140 mg/kg. *** Comparison of female control with male control (P<0.050). ### Comparison of Male experimental with Male control (P<0.001). $$ Comparison of Female experimental with Female control (P<0.050).

As shown in figure 1, the expression of *DMRT1* in the gonadal tissue differed significantly among the experimental groups. The female control group showed significantly lower *DMRT1* expression than the male control group (P < 0.001). Administration of *Foeniculum vulgare* extract resulted in a significant reduction in DMRT1 expression in the male experimental group compared with the male control group (P < 0.001). A moderate reduction was also observed in the female experimental group compared with the female control group (P < 0.050).

These findings indicate that prenatal exposure to F. vulgare extract may modify the molecular regulation of a sex-related developmental gene, particularly during male embryonic development.

DMRT1 Gene Expression in the Hypothalamus

Figure 2 displays the relative expression of the *DMRT1* gene in the hypothalamus of control and experimental chicks. Data are presented as Mean ± SE. Statistical significance is shown as $$P < 0.050 and ***P < 0.001.

As illustrated in figure 2, *DMRT1* expression in the hypothalamus was significantly lower in female control chicks than in male controls (P < 0.050). Prenatal exposure to *Foeniculum vulgare* extract significantly decreased hypothalamic *DMRT1* expression in the male experimental group compared with the male control group (P < 0.001). No statistically significant difference was observed between the female control and female experimental groups (P > 0.050).

Inhibin Gene Expression in the Pituitary Gland

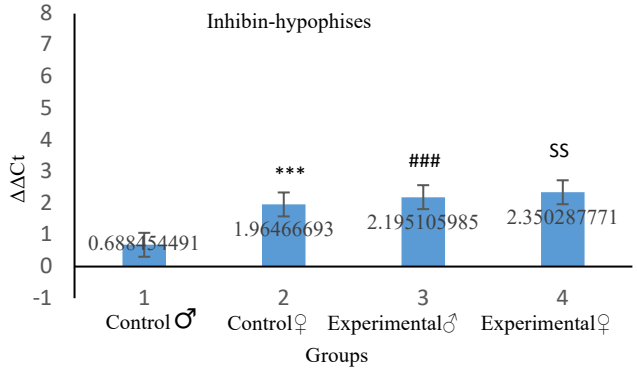


Fig. 3 *Inhibin* gene expression in hypophises in different groups.
Control (♂): Male control samples that received only physiological serum. Control (♀): Female control samples that received only physiological serum. Experimental (♂): Male experimental samples that received the extract at a dose of 140 mg/kg. Experimental (♀): Female experimental samples that received the extract at a dose of 140 mg/kg. *** Comparison of female control with male control (P<0.001). ### Comparison of Male experimental with Male control (P<0.001). $$ Comparison of Female experimental with Female control (P<0.050).

Figure 3 indicates the relative expression of the *Inhibin* gene in the pituitary gland of control and experimental chicks. Data are expressed as Mean ± SE. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test.

As presented in figure 3, *Inhibin* expression in the pituitary gland was significantly higher in female control chicks than in male controls (P < 0.001). Following treatment with *Foeniculum vulgare* extract, *Inhibin* expression increased significantly in both male and female experimental groups compared with their respective control groups (P < 0.001 and P < 0.050, respectively).

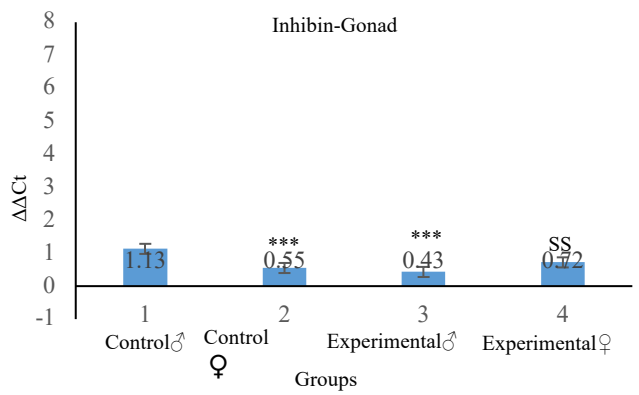


Fig. 4 *Inhibin* gene expression in Gonad in different groups.
Control (♂): Male control samples that received only physiological serum. Control (♀): Female control samples that received only physiological serum. Experimental (♂): Male experimental samples that received the extract at a dose of 140 mg/kg. Experimental (♀): Female experimental samples that received the extract at a dose of 140 mg/kg. *** Comparison of female control with male control (P<0.001). ### Comparison of Male experimental with Male control (P<0.001). $$ Comparison of Female experimental with Female control (P<0.050).

Figure 4 exhibits the relative expression of the *Inhibin* gene in the gonadal tissue of control and experimental chicks. Data are expressed as Mean ± SE. Significant differences were considered at $P < 0.050$. As shown in figure 4, the female control group exhibited significantly lower *Inhibin* expression than the male control group ($P < 0.001$). In contrast, the female experimental group showed significantly increased expression compared with the female control group ($P < 0.050$). A significant decrease in *Inhibin* expression was also observed in the male experimental group compared with the male control group ($P < 0.001$).

Histological Findings

Figure 5 presents representative photomicrographs of the hypothalamus obtained from one-day-old control and experimental chicks (Magnification: ×50). The experimental groups exhibited marked structural alterations in the hypothalamic region following prenatal exposure to *Foeniculum vulgare* extract.

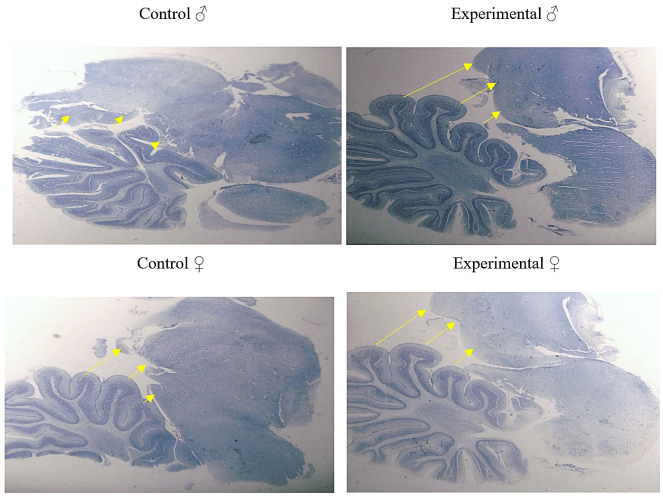


Fig. 5 Longitudinal section of one-day-old chick brain. 50X

Histological examination of the hypothalamus (Fig. 5) demonstrated obvious morphological changes in chicks exposed to *Foeniculum vulgare Mill*. Enlargement and structural distortion of the hypothalamic region were evident in both experimental groups compared with the controls. Tissue organization appeared less compact, and anatomical boundaries between adjacent hypothalamic structures became less distinct.

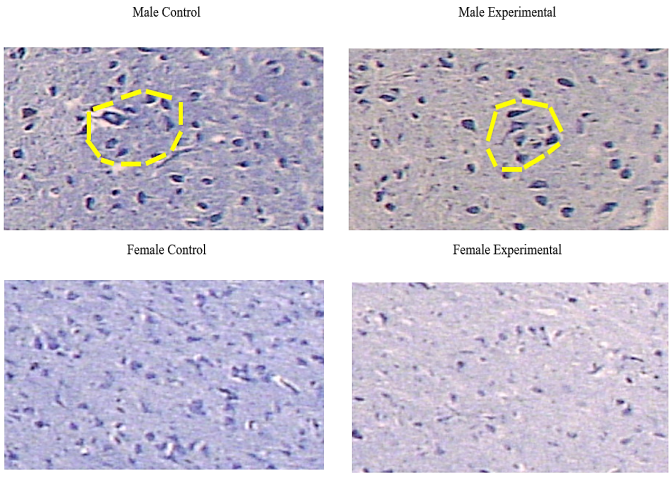


Fig. 6 Section of one-day-old chick brain .400X

Figure 6 illustrates the representative histological sections of the sexually dimorphic nuclei (SDN) of the hypothalamus in one-day-old chicks stained with Toluidine Blue (Magnification: ×400).

Microscopic evaluation (Fig. 6) demonstrated that prenatal exposure to *Foeniculum vulgare* hydroalcoholic extract induced marked alterations in the sexually dimorphic nuclei of the hypothalamus. In the male experimental group, the SDN exhibited a noticeable reduction in nuclear size, decreased neuronal density, and disruption of normal cellular organization compared with the male control group. Morphological changes in female chicks were less pronounced but showed mild alterations in tissue architecture. These histological observations were consistent with the molecular findings, suggesting that prenatal exposure to the phytoestrogen-rich extract influenced sexual differentiation of the hypothalamus.

DISCUSSION

The present study provides experimental evidence that prenatal exposure to hydroalcoholic extract of *Foeniculum vulgare* seeds can influence molecular and neuroanatomical characteristics associated with sexual differentiation in developing chickens. The observed alterations in DMRT1 and INHIBIN expression, together with changes in the structural characteristics of hypothalamic sexually dimorphic nuclei, suggest that prenatal exposure to fennel-derived bioactive compounds may affect developmental endocrine and neural pathways.

The alteration in *DMRT1* expression observed in male experimental chicks is of particular interest because *DMRT1* is a major Z-linked regulator of male gonadal differentiation in chickens. Previous studies have demonstrated that appropriate expression of *DMRT1* during embryonic development is required for normal male developmental pathways [11–13]. Therefore, changes in *DMRT1*

expression following prenatal exposure to F. vulgare extract may reflect an interaction between plant-derived bioactive compounds and developmental regulatory mechanisms.

The changes observed in *inhibin* expression may also indicate modulation of reproductive endocrine pathways. Because inhibin participates in the regulation of FSH and gonadal feedback within the HPG axis, altered expression may reflect changes in endocrine signaling during development. However, gene-expression changes alone cannot establish functional disruption of the HPG axis, and further hormonal and functional studies are required.

The histological findings provide additional evidence that prenatal exposure may influence the developing hypothalamus. Sexually dimorphic nuclei are developmentally sensitive neural structures whose organization can be influenced by endocrine signals. The observed changes in SDN volume and neuronal parameters may therefore represent neurodevelopmental responses to altered endocrine signaling during embryogenesis.

Importantly, the present findings should not be interpreted as evidence of a change in the genetic sex of the embryos or chicks. Rather, they indicate alterations in sex-related molecular expression and neuroanatomical characteristics associated with sexual differentiation. This distinction is particularly important because genetic sex determination and sexual differentiation are related but biologically distinct processes.

The possible involvement of estrogen-responsive signaling is biologically plausible because fennel contains several bioactive compounds that have been investigated for endocrine activity. Nevertheless, the present study did not directly measure estrogen receptor activation or downstream estrogen-responsive signaling. Therefore, the proposed estrogen-related mechanism remains a hypothesis requiring experimental confirmation.

The findings should also be interpreted within the limitations of the experimental design. The study used a relatively limited number of successfully hatched chicks, and the extract contained multiple phytochemical constituents whose individual contributions cannot be distinguished. In addition, only selected genes and neuroanatomical parameters were investigated. Future studies should therefore incorporate dose-response experiments, direct hormonal measurements, estrogen receptor-related analyses, broader gene-expression profiling, and longer-term reproductive and behavioral assessments.

CONCLUSION

The present study demonstrated that prenatal exposure to hydroalcoholic extract of *Foeniculum vulgare* seeds was associated with alterations in DMRT1 and INHIBIN gene expression and changes in the structural characteristics of hypothalamic sexually dimorphic nuclei in developing chickens.

These findings suggest that prenatal exposure to F. vulgare extract may alter sex-related gene expression and neuroanatomical pathways associated with sexual differentiation. However, these findings should not be interpreted as evidence that fennel extract changes the genetic or phenotypic sex of developing chickens.

Importantly, the present study was conducted exclusively in an avian developmental model. Therefore, the findings cannot be directly extrapolated to human pregnancy or used to establish the safety or risk of fennel consumption in pregnant women. Differences in species, developmental biology, dose, route of exposure, extract composition, and endocrine physiology must be considered.

Further studies using appropriate mammalian models, dose-response designs, hormonal measurements, estrogen-responsive signaling analyses, and long-term reproductive and behavioral assessments are required to clarify the biological significance of the observed effects and their potential relevance to other species.

Consent for Publication

Not applicable.

Data Availability Statement

Not Applicable.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Authors' Contributions

E.D.M. conducted the experiments, collected and analyzed the data, and prepared the initial draft of the manuscript. G.V. supervised the research and contributed to the study design, interpretation of the results, and critical revision of the manuscript. M.T. contributed to the supervision of the research, interpretation of the results, and critical revision of the manuscript. V.H. contributed to the study design, interpretation of the results, and critical revision of the manuscript. All authors read and approved the final version of the manuscript.

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