The Role of *Zingiber officinale* (Ginger) Extract in the Kiss1 Gene Expression in the Ovary and Blood of Wistar Rats

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Abstract

Background: Ginger (*Zingiber officinale*) is one of the most commonly consumed dietary condiments and herbal supplements in the world.

Aim: This study investigated the effect of methanolic extract of *Zingiber officinale* (MEZO) on the expression level of the Kiss1 gene in the blood and ovary of Wistar rats.

Methods: Fifteen (15) Wistar rats with an average weight of 147g were randomly divided into three (3) groups (A-C). Group A was given no treatment and served as the normal control group. Groups B and C received only oral administration of 200 mg/kg and 400 mg/kg of MEZO respectively. MEZO was administered once a day for 21 days.

Results: Group C showed a significantly reduced relative intensity of Kiss1 gene expression in the blood compared to the control ($p = 0.006$). There is also a significant increase between the expression intensity observed in the blood compared to the ovary in each experimental group except in group C (group A = CI: 0.55 - 0.94, $p = 0.013$; group B = CI: 0.69 - 0.86, $p = 0.001$; group C = CI: -0.09 - 0.73, $p = 0.076$). However, there is no significant correlation (between the Kiss1 gene relative expression intensity and reproductive hormones. There is no significant difference in the hormonal levels, oxidative stress, and relative ovarian weights across the experimental groups ($p > 0.05$).

Conclusion: High dose of MEZO showed the potentials of causing the downregulation of Kiss1 gene expression in the blood without any corresponding change in the reproductive hormonal levels.

Keywords: *Zingiber officinale*; Kiss1; Ovary; Blood; Oxidative Stress; Histology; Hormones; Gene Expression; Ginger; Kisspeptin

Introduction

Ginger (*Zingiber officinale*) is an herbaceous tropical plant commonly utilized for medicinal and culinary purposes [1]. The rhizomes (roots) of ginger contains numerous bioactive components such as 6-gingerol, 1, 4'-hydroxy, 3'-methoxyphenyl and 5-hydroxy, 3-decanoate [Jolad., *et al.* 2004]. Gingerol is the primary pungent ingredient of ginger and is considered to be responsible for some of the therapeutic properties of the plant [Jolad., *et al.* 2004]. Extracts of ginger have been found to possess anti-cancer [2], anti-inflammatory [3], antioxidant [4,5], anti-viral [6] and gonado-protective properties [7].

Kiss1 gene is a tumor metastasis suppressor gene that encodes a family of peptides known as kisspeptins. While originally described for its role in the suppression of tumor progression and metastasis [8], the Kiss1 gene has since been known to play a key regulatory role in reproduction and sexual maturity [9]. The Kiss1 gene and its receptor Kiss1R are primarily expressed in the hypothalamic neurons where their protein products operate as key stimulatory agents for the release of the Gonadotropin-releasing hormone [10]. In addition to being expressed in the hypothalamus, the Kiss1 gene and Kiss1R are highly expressed in the pituitary gland, adipose tissue [11], ovary [12], testis [13], liver [14], amygdala [15], uterus and trophoblast cells [16]. The dysregulation or inactivation of the Kiss1 gene has been shown to disrupt reproductive processes [17]; mutations of the Kiss1/Kiss1R system have been linked to the onset of precocious puberty and isolated hypogonadotropic hypogonadism [18]. Also, changes in the expression of the Kiss1 gene have been associated with the onset of different reproductive pathologies including Polycystic ovarian syndrome (PCOS) [19]. A recent study found the level of kisspeptin to be significantly higher in women with PCOS [20], indicating a possible role of the Kiss1 gene in the pathogenesis of the disease. Although the expression of the Kiss1 gene is mainly regulated by endogenous sex steroids [15], exogenous compounds and chemicals have been demonstrated to alter the level of Kiss1 gene expression. Studies by [21] reported a down-regulation in the expression of the Kiss1 gene following the administration of dietary soybean isoflavones. The down-regulation of the Kiss1 gene by soybean isoflavones was able to reduce the level of gonadotropin-releasing hormone and luteinizing hormone, as well as induce delayed puberty. This present study evaluated the effects of the methanolic extract of ginger

on the expression of Kiss1 gene in the blood and ovary of Wistar rats and assessed for corresponding changes in reproductive hormones, ovarian tissue and its oxidant status.

Materials and Methods

Study setting

This experimental study was carried out in the research laboratory of the Department of Anatomy, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, College of Health Science, Nnewi Campus, Anambra State, and lasted for about 3 months.

Plant collection, identification and extraction

The aerial part of the plant was harvested from Nnewi, Anambra state. The botanical identification and authentication were carried out in the Department of Pharmacognosy and Traditional Medicine, College of Pharmacy, Nnamdi Azikiwe University, Agulu Campus, Anambra State, Nigeria with identification number PCG/474/A/024. The plant calyces were shade-dried and ground. 1000g of the powdered plant sample was used for methanolic extraction as described by [22]. The filtrate (extract) was then stored in the refrigerator at 4°C. The extract was made up to solution at varying doses per mL on each day of administration and given according to body weight and group treatment doses.

Animal procurement, care and handling

Fifteen (15) female Wistar rats were procured from the animal house of College of Health Sciences, Nnamdi Azikiwe University, Okofia Nnewi Campus and acclimatized for two (2) weeks (to exclude any intercurrent infection) under standard housing condition (ventilated room with 12/12-hour light/dark cycle at $24 \pm 2^\circ\text{C}$). The rats were fed *ad libitum* with water and standard rat chow throughout the experimental period. Animal health status was monitored throughout the experiment according to the federation of European Laboratory Animal Science Associations (FELASA) guidelines.

Experimental design

Fifteen (15) rats with an average weight of 147g were randomly divided into three (3) groups (A-C). Group A was given no treatment and served as the normal control group. Groups B and C received oral administration of 200 mg/kg and 400 mg/kg of methanolic extract of *Zingiber officinale* (MEZO) respectively. The extract was administered once a day for 21 days.

Animal sacrifice and sample collection

The animals were fasted overnight after the last day of MEZO administration and anesthetized using chloroform. 2 mL of blood was collected from the animals by ocular puncture using capillary tubes into two different sample tubes. One was collected into a plain tube for hormonal assay, and the other into an RNA protector-containing plain tubes for Kiss1 gene analysis. The animals were sacrificed after blood collection, and the ovarian tissues were harvested, weighed and divided into three parts. One was fixed in a 10% formal saline for histological processing and analysis. The second part was homogenized and used for oxidative status analysis. The last part was stored in an RNA protector-containing plain tube before gene analysis.

Hormonal assay

The blood was allowed to clot and centrifuged at 5,000 rpm for 10 minutes within one hour after collection. The serum was extracted and used for hormonal assay. AccuBind enzyme-linked immunosorbent assay (ELISA) microwells for Estradiol (EST), Follicle-stimulating hormone (FSH), and Luteinizing hormone (LH) purchased from Calbiotech Inc. (catalog number: E5380s), Bioassay technology laboratory China (catalog number: EO182Ra), Bioassay technology laboratory (catalog number EO179Ra) respectively were used for the assay. All analyses were carried out following the accompanying ELISA kit protocol for each parameter.

Antioxidants quantification

The oxidant status was determined in the ovarian tissue by quantifying the Superoxide Dismutase (SOD), Glutathione (GSH), and Catalase (CAT) levels in the ovarian tissue samples using the tissue homogenate as described in our earlier studies [23].

Kiss1 RNA extraction

Total RNA was extracted using the Zymo Research (ZR) whole-blood RNA MiniPrep according to ZR specification. A 600 µl volume red blood cell lysis buffers were added to 200 µl volume of ribonucleic acid-guard (RNAguard) stored whole blood sample in an RNase-free tube and mixed by inverting. The mixture was incubated for 5 minutes at 25°C and centrifuge at $\geq 12,000 \times g$ for 1 minute. The supernatant was removed. A 600 µl volume of blood RNA buffer was added to the cell pellet and mixed properly. The resultant mixture was transferred into the Zymo-Spin IIC column in a collection tube and centrifuged at $\geq 12,000 \times g$ for 2 minutes. The column was transferred into a new collection tube. A 400 µl volume

of RNA pre-wash buffer was added to the column and centrifuged at $\geq 12,000 \times g$ for 30 seconds. The column was transferred into an RNase free tube. 100 µl RNA recovery buffer was added to the Zymo spin IIC column and centrifuged at $\geq 12,000 \times g$ for 30 seconds. A 100 µl volume ethanol (100%) was added to the flow-through in the RNase free tube and mixed by pipetting. The mixture was transferred into the Zymo spin IC column in a collection tube and centrifuged at $\geq 12,000 \times g$ for 30 seconds. A 400 µl volume of the RNA prep buffer was added to the column and centrifuged at $\geq 12,000 \times g$ for 1 minute, the flow-through was discarded. An 800 µl volume of the RNA wash buffer was added to the column and centrifuged at $\geq 12,000 \times g$ for 1 minute, the flow-through was discarded. The wash step was repeated with 400 µl volume of RNA wash buffer. The Zymo-spin IC column was centrifuged in an empty collection tube at $\geq 12,000 \times g$ for 2 minutes. It was then transferred into an RNase free tube. Total RNA was eluted by added 80 µl volume of DNase/RNase free water directly to the column matrix and centrifuged at $10,000 \times g$ for 30 seconds. A 70 µl volume of the Total RNA extracted was transferred into an RNA stable tube supplied by Biomatrix (catalog number 93221-001) for storage of Total RNA at room temperature while 10 µl was used for quality control check on the total RNA extracted.

RNA detection

One gram of agarose powder was weighed and poured into 100 mL of Tris EDTA buffer in a Pyrex conical flask. It was heated using a microwave at 100°C for 5 minutes. It was allowed to cool to 56°C and 6 µl volume of ethidium bromide was added to 100 ml of the gel mixture. The gel was poured into the electrophoresis chamber and allowed to solidify. A 3 µl volume of loading dye was added to 7 µl volume of the total RNA from each sample, the molecular marker was loaded in the first lane, followed by the samples. Electrophoresis was performed at 90 volts for 30 minutes. The gel was removed and viewed on the UV transilluminator; the picture of the gel was taken.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

The extracted total RNA was retro-transcribed and amplified using One Taq one-Step RT-PCR kit (catalog number NEB E5315S) by New England BioLabs incorporation according to the manufacturer's specification. Selected primers were used to target lymphocyte genes using MJ research Peltier thermal cycler polymerase chain reaction machine. The PCR was performed in a 50 µl volume reaction mixture containing 25 µl volume of one Taq one-step reac-

tion master mix (2x), 2 µl volume of One Taq one-step enzyme mix (2x), 2 µl volume of each gene-specific forward primer (10 µM), 2 µl volume of each gene-specific reverse primer (10 µM), 9 µl volume of nuclease-free water and 10 µl volume of the RNA template was added. Negative control samples for the RT-PCR consisted of a mixture to which all reagents added except RNA. The PCR was started immediately as follows: Reverse transcriptase at 48°C for 30 seconds, initial denaturation at 94°C for 1 minute, denaturation at 94°C for 15 seconds, annealing at Tm-5°C (the lowest melting temperature of each set of Kiss1 gene) for 30 seconds, extension at 68°C for 1 minute, denaturation step for 39 cycles, final extension at 68°C for 5 minutes and final holding at 4°C. The Kiss1 gene nucleotide sequence (5'-3') for the primers are as follows: forward primer - CTACGACTCCTTGTGCTTTG, and reverse primer - TGATCTTCACTGTAGTTGGTGG.

Electrophoresis

5 µl of the amplified PCR products and DNA ladder were analyzed on 1% agarose gel containing ethidium bromide in 1X Tris EDTA buffer. One percent agarose gel was prepared by dissolving 1.0g of LE Agarose powder in 100 ml volume of Tris Borate EDTA Buffer. The mixture was then heated in a microwave at 1000 C for 5 minutes and allowed to cool to 56°C and 6 µl volume of ethidium bromide was added to it. The agarose gel was poured into the electrophoresis chambers with gel comb and allowed to solidify. Electrophoresis was performed at 90 volts for 30 minutes with the Edvotek tetra source electrophoresis machine, Bethesda, USA. After electrophoresis, the Kiss1 gene was visualized with the Wealtec Dolphin-Doc UV transilluminator and photographed.

Kiss1 gene expression

ImageJ 1.53a software was used to calculate the absolute intensity of expression from the electrophoresis generated gel images across all the experimental groups in both the blood and ovary. ImageJ generates the absolute intensity (derived by mean value multiplied by the pixel value or percent for each band) of each band. The absolute intensity is an integrated measure of the intensity and size of the band. The relative intensity was calculated by dividing the absolute intensity of each sample band by the absolute intensity of the standard.

Tissue processing

The tissue samples were trimmed down to a size of about 3 mm x 3 mm thick for an easy study of sections under the microscope and fixed in 10% formalin. After fixation, dehydration of the fixed tissues was done in ascending grades of alcohol 50%, 70%, 95%, and 100%, and cleared in xylene. Staining was done with hematoxylin and eosin (H&E) and mounted using DPX, after which, the sections were viewed under the light microscope. Photomicrographs

of these sections were obtained using the Leica DM 750 digital photomicroscope.

Statistical analysis

The data were analyzed using IBM statistical package for social science (SPSS) for Windows, version 23 (IBM Corporation, Armonk, New York, USA). One-way analysis of variance (ANOVA), post hoc LSD, student's t-test, and Pearson's correlation analysis was used to test for significance in changes seen in the variables across groups. Tables and figures were used for the representation of data, and values were considered significant at $p < 0.05$.

Ethical statement

This study was approved by the Research Ethics Committee of Anatomy Department, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi Campus. The experimental procedures of this study complied with ARRIVE guidelines, National Institutes of Health (NIH) guidelines and National Health Research ethics committee of Nigeria (NHREC) guidelines for the care and use of laboratory animals. Animal health status was monitored throughout the experiment according to the federation of European Laboratory Animal Science Associations (FELASA) guidelines. No informed consent was required for this study.

Results

The effect of MEZO on the bodyweight

Table 1 shows no change in the average body weight of rats in groups B and C when the pre and post-administration body weights were compared ($p = 0.086, 0.189$). However, the control group showed a significant increase in the post-administration body weight when compared to the weight before administration ($p = 0.004$).

Groups		Body Weight (g) Mean $\pm$ SD	p-value
A (Control)	Pre- administration	116.0 $\pm$ 5.48	0.004*
	Post-administration	138.00 $\pm$ 4.47	
B (MEZO 200)	Pre- administration	122.00 $\pm$ 13.04	0.086
	Post-administration	144.00 $\pm$ 16.73	
C (MEZO 400)	Pre- administration	128.00 $\pm$ 17.89	0.189
	Post-administration	148.00 $\pm$ 17.89	

Table 1: The effect of MEZO on the bodyweight of adult female Wistar rats.

Data were analyzed using the Students' dependent t-test. Values were expressed as mean $\pm$ standard deviation (SD). *: Means values significant at $p < 0.05$.

The Effect of MEZO on the relative ovarian weight

There was no significant difference in the relative ovarian weights of the experimental groups ($p = 0.105$).

Groups	Relative weight (MEAN $\pm$ SD)	p-value
A (Control)	0.04 $\pm$ 0.01	-
B (MEZO 200)	0.03 $\pm$ 0.01	
C (MEZO 400)	0.05 $\pm$ 0.02	
		0.105

Table 2: The effect of MEZO on the relative ovarian weight of adult Wistar rats.

Data were analyzed using One-way ANOVA; values were presented as mean $\pm$ standard deviation (SD) and data were considered significant at $*p < 0.05$.

The Effect of MEZO on the reproductive hormonal levels

The FSH, LH and EST levels did not change significantly in all the treated groups when compared to the control ($p < 0.05$).

Hormones	Groups	MEAN $\pm$ SD	p-value
LH (IU/L)	A	3.90 $\pm$ 0.33	0.387
	B	4.10 $\pm$ 0.35	
	C	7.35 $\pm$ 5.98	
FSH (μ lu/ml)	A	5.90 $\pm$ 1.00	0.130
	B	6.60 $\pm$ 0.87	
	C	5.05 $\pm$ 0.78	
EST (pg/ml)	A	22.20 $\pm$ 4.71	0.624
	B	19.80 $\pm$ 4.02	
	C	21.80 $\pm$ 3.49	

Table 3: The effect of MEZO on LH, FSH, and EST levels in female Wistar rats.

Data were analyzed using One-way ANOVA, values were presented as mean $\pm$ standard deviation and data were considered significant at $*p < 0.05$.

The effect of MEZO on the antioxidant levels in the ovary

No significant changes were seen in the levels of SOD, GSH, and CAT across all the test groups when compared to the control ($p < 0.05$).

Histological findings

Figure 1 represents the histological features of the ovarian tissues in various study groups. The MEZO-treated groups showed normal ovarian histology with no sign of necrosis or toxicity.

Antioxidants	Groups	MEAN $\pm$ SD	p-value
SOD (μ mol/ml/min/mg pro)	A	10.50 $\pm$ 8.20	0.579
	B	10.10 $\pm$ 8.77	
	C	17.30 $\pm$ 2.26	
GSH (μ mol/ml/mg pro)	A	11.31 $\pm$ 5.77	0.776
	B	13.73 $\pm$ 10.48	
	C	8.44 $\pm$ 3.00	
CAT (μ mol/ml/min/mg pro)	A	66.84 $\pm$ 21.00	0.851
	B	53.68 $\pm$ 26.12	
	C	62.67 $\pm$ 32.42	

Table 4: The oxidant status of the Wistar rat ovary after administration of MEZO.

Data were analyzed using One-way ANOVA, values were presented as mean $\pm$ standard deviation and data were considered significant at $*p < 0.05$.

Figure 1: Plate 1 represents the histological section of the ovary of rat administered only distilled water for 21 days. Plate 2 represents the histological section of the ovary of a rat administered 200 mg/kg MEZO for 21 days. No sign of abnormality is present.

Plate 3 represents the histological section of the ovary of a rat administered 400 mg/kg MEZO for 21 days. The section shows follicles at varying stages of development and corpora lutea (indicating ovulation). No abnormalities are seen.

Kiss1 gene expression in the ovary and blood

PCR band images (Figure 2) showed the detection of the Kiss1 gene in both the blood and ovary of Wistar rats in all the test groups at different intensities. Figure 2A and 2B represents the PCR amplicon bands images of Kiss1 gene expression in the ovary (A) and the blood (B).

There is a significant difference in the expression levels in the blood ($p = 0.009$) unlike the ovary ($p = 0.184$) across treatment groups when compared to their control groups. Group C showed a significantly reduced relative intensity of expression in the blood compared to the control ($p = 0.006$). There is also a significant difference between the relative expression intensity observed in the blood compared to the ovary in each experimental group except in group C (A = CI: 0.55 - 0.94, $p = 0.013$; B = CI: 0.69 - 0.86, $p = 0.001$; C = CI: -0.09 - 0.73, $p = 0.076$) (Figure 2C).

Correlation between Kiss1 gene expression and reproductive hormonal levels

There is no significant correlation between the relative intensity of Kiss 1 expression variables and the reproductive hormone levels ($p > 0.05$) (Table 5).

Test Variables		LH	FSH	EST
RIB	Pearson Correlation	-.274	-.781	.399
	Sig. (2-tailed)	.656	.119	.434
RIO	Pearson Correlation	.664	.698	.065
	Sig. (2-tailed)	.222	.190	.902

Table 5: Correlation between the relative intensity of Kiss1 gene expression and serum hormonal levels.

RIB: Relative expression intensity in the blood; RIO: Relative Expression Intensity in the Ovary; LH: Luteinizing Hormone; FSH: Follicle-Stimulating Hormone; EST: Estradiol; Values are significant at $p < 0.05$.

Discussion

In this present study, no significant change was observed in the body weight of animals in groups B and C when the pre and post-administration bodyweights were compared ($p = 0.086$ and 0.189). This is as opposed to the normal control group (group A) which showed a significant increase in their body weight at the end of the study period ($p = 0.004$). This finding disagrees with the studies of Abdulrazaq, *et al.* [24], which reported a significant increase in body weight following the administration of 500 mg/kg *Zingiber officinale* extract. This study outcome variation could be as a result of the higher dose of 500 mg/kg used in their study.

Treatment with MEZO showed a dose-dependent effect on the expression level of the Kiss1 gene in the blood. 400 mg/kg of MEZO caused a significant reduction in the relative intensity of KISS1 gene expression in the blood when compared to the control ($p = 0.006$), while treatment with 200 mg/kg did not cause any significant change.

Interestingly, however, the observed down-regulation of Kiss1 gene in the blood did not cause any significant hormonal change, as hormonal analysis revealed no significant alteration in the level of serum LH, FSH, and EST in all MEZO-treated groups when compared to the control group (Table 3). Based on this finding, it could be suggested that the local expression of the Kiss1 gene in the blood may not be directly involved in the regulation of local reproductive activities in the ovary. Previous studies on the effect of ovarian-expressed Kiss1 gene reported a direct stimulatory effect on progesterone secretion [25], as well as a positive and negative correlation with LH and FSH [19]. The hormonal parameters observed in this study appeared to be consistent with the expression of ovarian Kiss1, as no significant change was observed in the expression of Kiss1 gene in the ovary of all experimental animals. The effect of ginger observed in this study is comparable to the findings of Afzali and Ghalehkandi [26], which reported no significant change in the levels of LH, FSH, and testosterone following the oral administration of 100, 200, and 300 mg/kg ginger for four weeks.

The general expression of Kiss1 gene was found to be higher in the blood than in the ovary as the relative intensity of Kiss1 gene expression was significantly higher in the blood when compared to the ovary in each experimental group, except group C (group A = CI: 0.55 - 0.94, $p = 0.013$; group B = CI: 0.69 - 0.86, $p = 0.001$; group C = CI: -0.09 - 0.73, $p = 0.076$) (Table 5). This finding shows a differential level of Kiss1 gene expression in different body tissues and

may offer an alternative explanation on why an apparent increase in the expression of the Kiss1 gene in the blood failed to cause any change in the hormonal levels.

Furthermore, correlation studies found no significant correlation between the relative intensity of Kiss1 gene expression and the reproductive hormones ($p > 0.05$) (Table 5).

The antioxidant properties of ginger have been validated by previous works [27-29]. Active ingredients contained in ginger like shogaols have been shown to enhance the antioxidant defense system of the body [4]. However, our results showed no significant change in the antioxidative parameters investigated - SOD, GSH and CAT – after the administration of MEZO across all the treated groups (Table 4). This validates the histopathological findings which showed no sign of abnormality or tissue damage in the ovarian tissues (Figure 1).

Conclusion

A high dose of MEZO may cause the down-regulation of the Kiss1 gene in the blood, without causing any corresponding change in the reproductive hormonal levels. Further studies on the specific effect of blood-derived Kiss1 gene expression on the reproductive activities is recommended to understand the relationship between locally expressed Kiss1 gene in the blood and reproductive functions.

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Conflict of Interest

The authors have no conflict of interest to declare.

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