Original Research Article

ADENO-HYPOPHYSEAL CONSEQUENCE OF UTERINE FIBROID AND THE EFFECTS OF GINGER EXTRACT ON THE MONOSODIUM GLUTAMATE-INDUCED TUMOR

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Author's contribution:

This work was carried out in collaboration between all authors. Authors Desalu and Olanrewaju designed, wrote the protocol, supervised the study, performed the statistical analysis, and wrote the first draft of the manuscript. Authors Olanrewaju and Oribamise managed the analyses of the study. Author Oribamise managed data collection, the literature searches, and laboratory work. All authors read and approved the final manuscript.

Abstract:

Background: Uterine fibroids, also referred to as uterine myomas, leiomyomas, myomatas, or simply fibroid are benign soft-tissue tumors that arise from uterine smooth muscle tissue (myometrium) [1,2]. They have been described numerously to be hormone-dependent [3] and uterine structure-damaging [4], but the present study reports the implication of this soft-tissue tumor to the functioning and eventual anatomical change in the pituitary gland and the potential role of ginger extract in reversing the damages.

Aim: To understand uterine fibroids at the level of the pituitary gland, while studying the effects that aqueous extract of ginger will play in the Monosodium Glutamate-induced uterine fibroid

Study design: Experimental

Place and Duration of Study: Department of Anatomy, Benjamin S. Carson School of Medicine, Babcock University, Ilishan-Remo, Ogun State, Nigeria between January 2017 and May 2017

Methodology: Acclimatization lasted for 10 days following procurement, after which oral administration of Monosodium Glutamate (MSG) and Aqueous ginger extract ensued to determine the prophylactic, protective and curative effects of ginger on MSG-induced uterine fibroid in adult female wistar rats. Administration lasted for 50 days, after which the experimental animals were sacrificed via cervical dislocation, blood samples were collected for Luteinizing Hormone (LH) and Follicular

Stimulating Hormone (FSH) level determination and the pituitary gland was fixed in 10% formal saline for histological analysis.

Results: Results showed that MSG-induced uterine fibroid had abnormal effects on the pituitary gland histology and in the level of Luteinizing and Follicular Stimulating Hormones, while ginger extract reversed this effect.

Conclusion: Ginger has fibroid-preventing and fibroid-reducing properties at the level of the pituitary gland. The results of this study may contribute greatly to knowledge and may offer a non-invasive therapy of treating women with fibroids.

1. INTRODUCTION

Uterine fibroids are non-malignant tumors of the uterus. Uterine fibroids occur in up to 80% of reproductive-age women, causing significant morbidity in up to 30% of women [5,6,7]. Ethnic origin has been considered one of the known risk factors for uterine leiomyoma and African-American women are at a higher risk of fibroid, as many as 50% have fibroids of a significant size. One study found that between 80% and 90% of African American women and 70% of white women will develop fibroids by age 50 [8]. Among U.S. women ages 25 to 44, about 30% have symptoms of fibroids [9] Affected African American women are more likely to have multiple fibroids [10]. Some studies suggest that black women who are obese and who have high blood pressure are more likely to have fibroids [11]. Also, uterine fibroids are the most common indication for hysterectomy in women of child-bearing age and therefore, are a major health issue in the public: It is the leading indication for hysterectomies in women of reproductive age [12]. Of the 600,000 hysterectomies performed annually in the U.S, one-third are due to fibroids. In the United States, uterine fibroids are a common reason for surgical removal of the uterus [13]. Studies relating to these showed that every year, fibroids lead to more than 200,000 hysterectomies [14], with yearly cost estimates of $5.9-34.4 billion [15]. Hence, it is of utmost importance that natural, non-surgical therapies such as ginger be investigated for the cure of the global health hazard. Also, according to their location, uterine fibroids are classified as submucosal, intramural or subserosal [1].

They have been considered as growths with unknown aetiology [16], but depend mostly on estrogen hormone which has cranial control from pituitary hormones including LH and FSH.

The pituitary gland is a pea-sized gland, also known as the master endocrine gland. It measures 12 × 8 mm and weight approximately 500 mg [17]

The hypothalamus and pituitary gland (also referred to as the master gland) regulate the reproductive hormones. The hypothalamus releases the GnRH which, in turn, stimulates the pituitary gland to produce FSH and LH. At the command of FSH and LH, estrogen and progesterone are released from the ovaries. The pituitary gland (hypophysis) is anatomically divided into the anterior and posterior lobes (adenohypophysis and neurohypophysis respectively). The anterior lobe is considered in this study. GnRH-I is the classic hypothalamic hormone responsible for

the regulation, synthesis, and secretion of the pituitary gonadotropins FSH and LH [18]. GnRH-I acts on the anterior pituitary leading to the synthesis and storage of gonadotropins, movement of the gonadotropins from the reserve pool to a readily released point, and finally the secretion of gonadotropins. For this action to take place appropriately, pulstaile GnRH release is necessary [19,20]. Continuous GnRH secretion will lead to the suppression of FSH and LH release as well as suppression of FSH and LH gene transcription by the anterior pituitary (Belchetz et al., 1978; Haisenleder, 1991) . This is the basis of use of GnRH agonists such as Lupron for the suppression gonadotropin secretion. The pulse frequency of GnRH will vary depending on the menstrual cycle phase. LH pulse frequency is used to indicate GnRH pulse secretion.

MSG: Monosodium Glutamate (MSG) is a salt of glutamate, synthesized from L-glutamic acids; it is also used as a flavour enhancer in foods [23]. It is important to note that various processed and prepared foods such as traditional seasonings sauce and certain restaurant foods contain significant levels of free glutamate (as MSG), both from natural sources and from added monosodium glutamate [24,25]. Various studies have reported the toxicity of MSG in humans and experimental animals [26,27,28]. Also, monosodium glutamate has been used to induce uterine fibroids in experimental animals [29,30].
Ginger (Zingiber officinale Roscoe) is one of the most commonly consumed dietary condiments in the world [31], a commonly used spice in the world found to have medicinal precedence [32], reported to have anti-inflammatory [33], anti-oxidant [34], and anti-tumor effects [35].

2. METHODOLOGY

After receiving ethical approval from Babcock University Health Research Ethics Committee (BUHREC), Forty nine (49) healthy, non-pregnant adult female wistar rats (Rattus novergicus) weighing between 190-230g were obtained from, housed, and cared for at the Babcock University animal house, Babcock University, Ilishan-Remo, where a good housing measure was observed as recommended by the Animal Research Review Panel [36]. MSG, which was used to induce uterine fibroid, was obtained from a major seasoning shop at Agric Bus-stop, Ikorodu, Lagos State, dissolved in distilled water to produce the desired concentration of 900mg/kg body weight and stored in the refrigerator below 4ºC. The median lethal dose of MSG is 15,000mg/kg [37]. Ginger extract was used as a prophylactic, protective and curative agent in the present study. As stated earlier, ginger is one of the most commonly consumed dietary condiments in the world [31]. The horizontal stem from which the roots grow, the rhizome, is the main portion of ginger that is consumed. Fresh ginger rhizomes were purchased from Mile 12 market in Lagos directly from Kaduna State Ginger Processing Company in Kaduna, Nigeria. They were cut into thin slices, washed in distilled water and ground in distilled water for 1 minute using a kitchen blender (Gray NutriBullet 12-Piece High-Speed Blender, with a High-torque power

base and 600-watt motor). The ground mixture was filtered through a fine cotton cloth and the aqueous extracts was administered orally to the animals in various grades (i.e., 500mg/kg, 900mg/kg, 1,700mg/kg) as instructed by the treatment regimen in Table 1.0. After the animals were allowed to acclimatize for a period of 10 days with free access to feed and water, administration began.

Table 1.0: Table showing treatment regimen design for control and experimental groups

GROUP	Animals	Treatment schedule	Rationale
Group A	7	A placebo of water	Control group
Group B	7	Animals were treated orally with 900mg/kg body weight of MSG only for 25 consecutive days	(Negative control group) To induce Uterine fibroid
Group C	7	Animals receiving 900mg/kg body weight of ginger extract only for 25 consecutive days	(Positive control group) A placebo for ginger extract
Group D	7	Animals receiving 900mg/kg body weight of ginger extract orally for 25 days, followed by oral administration 900mg/kg of MSG for 25 consecutive days	Prophylactic group
Group E	7	Animals receiving oral administration of 900mg/kg of MSG + 900mg/kg body weight of ginger extract for 25 consecutive days	Protective group
Group F	7	Animals receiving oral administration of 900mg/kg of MSG for 25 consecutive days, followed by oral administration of low dose ginger (500mg/kg) extract for 25 consecutive days	(Curative group 1) To reverse uterine fibroid induced by MSG
Group G	7	Animals receiving oral administration of 900mg/kg of MSG for 25 consecutive days, followed by oral administration of high dose ginger (1,700mg/kg) extract for 25 consecutive days	(Curative group 2) To reverse uterine fibroid induced by MSG

For each of the animals in groups A, F and G, blood tests were carried out on the 25^{th} day to confirm the presence of fibroids before administration of ginger extract. These were done using the Accubind ELISA kit (2013) to test for the levels of estradiol and progesterone in the serum of the respective animals. The animals in

Groups F and G showed abnormally high levels of serum estradiol and progesterone in contrast to those of the control group. As earlier stated, it is also important to note that Obochi *et al*. (2009), Zia *et al.* (2012) and Koffour *et al*. in 2013, amongst other authors have carried out biochemical assays on serum estradiol, serum progesterone as these hormones have been reported to be notable markers for uterine fibroid.

The day after the last administration, the experimental animals were weighed, blood was also collected from their orbit using a micro-hematocrit capillary tube, the animals were weighed and then sacrificed by cervical dislocation. The portion of brain tissue near the pituitary gland was excised from animals in each group and fixed in 10% formal saline for histological demonstration.

Procedure for Haematoxylin and Eosin (H&E) for general histoarchitecture of the Pituitary glands

1. The harvested tissue samples were immersion-fixed in 10% formal saline at room temperature
2. The tissues were then dehydrated in ascending grades of alcohol (70%, 95%, 100%, and 100%)
3. The tissues were cleared with two changes of xylene for one hour, 30 minutes each
4. They were then transferred into two changes of molten paraffin wax I and II for one and half hour each and wax- III for overnight in an oven at 65^{o}C for infiltration.
5. A microtome was used to section tissue blocks at a thickness of 6µm. The resulting strips of sections were then gently lowered into the surface of a warm water bath at 40℃.
6. The floated sections were mounted on egg albumin-coated microscopic slides, and put in an oven maintained at 60℃ for 30 minutes to fix the tissue firmly on the slide.
7. The slides were dewaxed with two changes of xylene and hydrated with decreasing alcohol concentration and then immersed in water for 5 minutes.
8. The sectioned tissues were then stained with Ehrlich's hematoxylin and counter stained with Eosin.
9. Tissues were then washed in tap water and dehydrated by rinsing in increasing concentration of alcohol and then xylene-I. They were then placed in xylene-II until mounting on the glass slides
10. Finally, a drop of mountant DPX (A mixture of Distyrene, a Plasticizer, and Xylene) was placed on top of the sections and the cover slip was applied.

SERUM FSH and SERUM LH [using Elabscience ELISA kit (2015)]

After blood was collected from the animals through ocular puncture and transferred into plain red-covered, clot-activator sample bottles, the blood was allowed to clot and then centrifuged at 3000rpm for 15 minutes to separate serum from the cells.

Serum samples were then refrigerated at -7°C for 4 days (this is so as to prevent loss of bioactivity and contamination)

1. All reagents and samples were brought to room temperature before use; the samples were centrifuged after thawing before and the reagents were mixed thoroughly by gentle swirling before pipetting as foaming was avoided. The samples and standards were assayed in duplicate.
2. 100µL of standard, blank, or sample per well was added according to the assigned well. The blank well was added with Reference Standard & Sample diluent and solutions were added to the bottom of micro ELISA plate well, and inside wall touching and foaming were avoided. After mixing, the plate was covered with the sealer and incubated for 90 minutes at 37℃.
3. The liquid of each well was removed, after which 100µL of Biotinylated Detection Ab working solution was added immediately to each well after which they were covered with the Plate sealer. The plate was tapped gently to ensure thorough mixing and incubation ensued for 1 hour at 37℃.
4. Each well was then aspirated and washed by filing with approximately 350ul, this was repeated three times, (complete removal of liquid was ensured at each step). After the last wash, remaining wash buffer was removed by aspirating. The plate was then inverted and patted against thick clean absorbent paper.
5. 100µL of Avidin-Horseradish Peroxidase (HRP) Conjugate working solution was added to each well, covered with the plate sealer, incubated for 30 minutes at 37℃ and the wash process was repeated for five times as conducted earlier
6. 90µL of Substrate Solution was also added to each well and covered with a new plate sealer and Incubated for about 15 minutes at 37℃, while being protected from light. The reaction time of 30 minutes was shortened with observance of colour change. With appearance of apparent gradient in the standard wells, the reaction was terminated.
7. 50µLof Stop Solution was added to each well in the same order the substrate solution was added, and an immediate colour change to yellow was observed.
8. The optical density (OD value) of each well was determined simultaneously using a micro-plate reader set to 450 nm.

Calculation of results

Cholesterol conc. of unknown (ng/ml) = $\frac{\text{Cholesterol conc. of } \Delta\text{control} \times \text{450 of unknown}}{\Delta \text{ 450 of control}}$

Precautions:

- It was ensured that Substrate Reagent wasn't kept at -20℃
- Exposure of reagents to strong light was avoided in the process of incubation and storage

- It was also ensured that all the taps of reagents were tightened to prevent evaporation and microbial contamination.
- Hemolysis was avoided during serum aspiration
- The micro-plate reader was opened in advance, the instruments preheated, and the testing parameters set.

Statistical Analysis

Bar graphs were obtained by the software Graph Pad Prism for Windows version 5 (GraphPad Software, San Diego, CA, USA) and subjected to One-Way Analysis of Variance (ANOVA) with Newman-Keuls's *post hoc* test. $P \leq 0.05$ was considered statistically significant in all analysis.

3. RESULTS

3.1. Results from fibroid confirmation

3.1.1. Serum estradiol level:

Group A: Control group

Group F: MSG → Ginger low dose

Group G: MSG → Ginger high dose

Results from biochemical confirmation of uterine fibroid before commencement of ginger extract showed in Fig 1.0 that estradiol levels of groups F (25.28+1.414) and G (26.23±1.403) were significantly higher than that of the group A (7.677±0.4707)

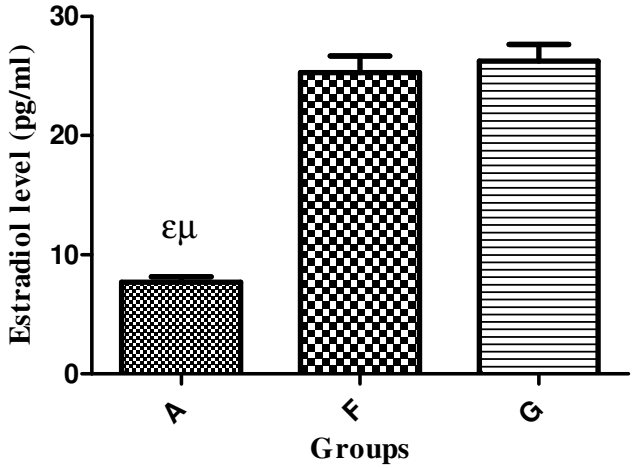

Fig 1.0. Bar graph showing estradiol levels (pg/ml) across groups A, F, G

ε= P<0.05 when compared with Group F

μ=P<0.05 when compared with Group G

3.1.2. Serum progesterone level:

Group A: Control group

Group F: MSG → Ginger low dose

Group G: MSG → Ginger high dose

Results from biochemical confirmation of uterine fibroid before commencement of ginger extract showed in Fig 1.1 that estradiol levels of groups F (12.45±0.6867) and G (12.71±0.7123) were significantly higher than that of the group A (6.390±0.3635)

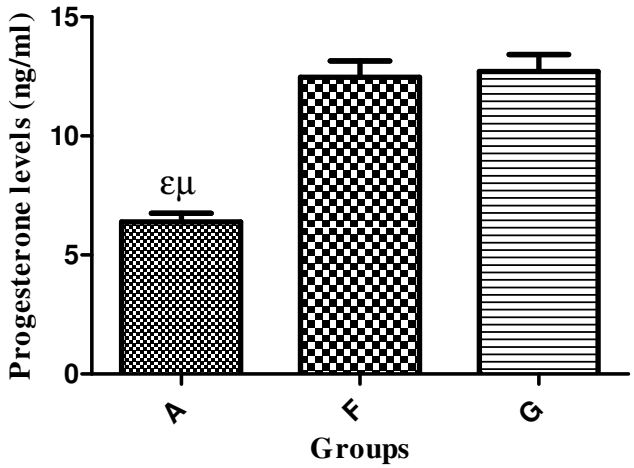

Fig 1.1. Bar graph showing progesterone levels (ng/ml) across groups A, E, F

ε= P<0.05 when compared with Group E
μ=P<0.05 when compared with Group F

HISTOLOGICAL ANALYSIS

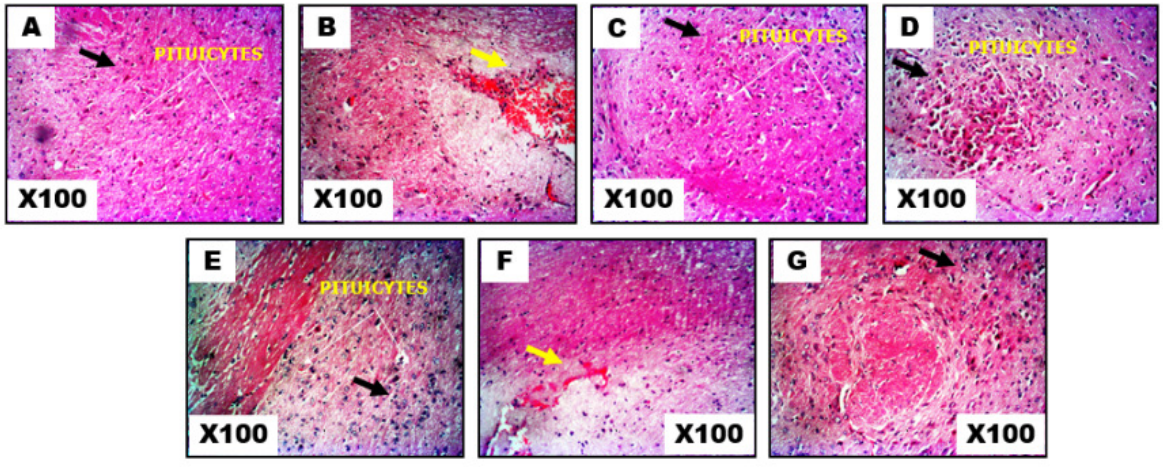

Plate 1: Photomicrographs showing panoramic views of hypophyseal general histomorphological presentations in Adult female Wistar rats across the various groups A-G. H&E stain (*x100*).

Luteinizing Hormone (LH) level

Group A: Control
Group B: MSG only
Group C: Ginger only
Group D: Ginger → MSG
Group E: Ginger + MSG
Group F: MSG → Ginger (Low dose)
Group G: MSG → Ginger (High dose)

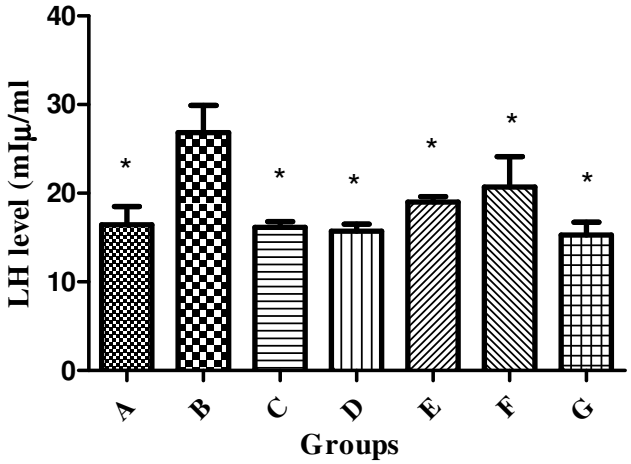

Fig 1.2. Bar graph showing LH levels (mIμ/ml) of control and experimental groups
**=P<0.05 when compared with Group B*
As shown in Fig 1.2., there was a statistically significant increase in the LH level of group B (26.84±3.088) in comparison with that of group A (16.44±2.067). Also, when compared to group B, there was a significant decrease in the LH levels of groups D (15.72±0.8120), E (19.01±0.6106), F (20.71±3.410), G (15.30±1.458), as these groups held no statistical significant difference when compared to group A (16.44±2.067). Also, in comparison with group A, Group C (16.17±0.6548) held no significant statistical difference.

It is also depicted in Fig 1.2. that group B (26.84±3.088) had the highest LH level while group G (15.30±1.458) had the lowest.

LH levels were more pronounced in uterine fibroid animals than in the control animals. Also there was a decrease in the level of LH in the ginger-administered groups.

FOLLICLE-STIMULATING HORMONE (FSH) LEVEL
Group A: Control
Group B: MSG only

Group C: Ginger only
Group D: Ginger → MSG
Group E: Ginger + MSG
Group F: MSG → Ginger (Low dose)
Group G: MSG → Ginger (High dose)

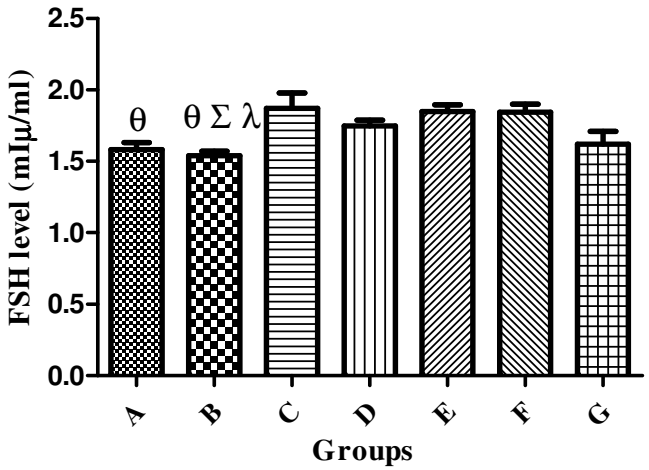

Fig 1.3. Bar graph showing FSH levels (mIμ/ml) of control and experimental groups
θ=P<0.05 when compared with Group C
λ=P<0.05 when compared with Group F
Σ=P<0.05 when compared with Group E
FSH levels were slightly decreased in uterine fibroid animals than in the control animals. Also there was an increase in the level of FSH in the ginger-administered groups.

4. DISCUSSION

Results from an attempt to confirm the presence of uterine fibroid in this study showed that the groups induced with uterine fibroid showed increased levels of estradiol - E2 (the form of estrogen found in non-pregnant conditions) and progesterone when tested. There was a remarkable rise in the estradiol level of groups E and F when compared to the control group. This can be attributed to the fact that estrogen promotes the growth of uterine fibroids, as these benign tumors have been described as estrogen-feeding tumors [38,39]. MSG which was used to induce the uterine fibroid has been reported to increase estrogen levels in experimental animals thus causing fibroid growth. The mechanism behind the MSG-induced fibroid formation and growth doesn't just involve the hormones estrogen and progesterone but also circulating enzymes and hormone receptors. Aromatase and 17beta-hydroxysteroid dehydrogenase enzymes are aberrantly expressed in fibroids. Some of the known factors that increase aromatase activity are age, insulin, obesity, gonadotropins, and alcohol [40]. Aromatase and 17beta-hydroxysteroid dehydrogenase convert circulating androstenedione (a pro-hormone released by the adrenal cortex & ovaries) into estradiol [68]. Estradiol action is then mediated by its

nuclear receptors ERα and ERβ to promote other uterine-fibroid-inducing conditions, amongst which is to induce the production of Progesterone Receptor (PR) which is then responsible for the response of uterine tissues to progesterone secreted by the ovaries. Upon ligand binding, these receptors act as transcriptional factors that up- or down-regulate gene expression by interacting with the regulatory regions of target genes. Estrogen also promotes fibroid growth by up-regulating Transforming Growth Factor-β3 which leads to increased cell proliferation, Insulin Growth Factor-1, Epidermal Growth Factor Receptor, and Platelet-derived Growth Factor (PDGF), and also promotes the abnormal survival of leiomyoma cells by reducing apoptosis through down-regulation of p53, increasing expression of the anti-apoptotic factor PCP4 and antagonizing PPAR-gamma signaling [41]. Progesterone also promotes growth of leiomyoma through up-regulating EGF, TGF-beta1 and TGF-beta3, and promotes survival through up-regulating Bcl-2 expression and down-regulating TNF-alpha [42,43]. Estrogen and Progesterone, through their up-regulation of TGF-β3 and down-regulation of p53, increases cell proliferation and survival and enhances extracellular matrix formation through induction of fibrosis which is characterized by resistance to apoptosis leading to the persistence of cells, and secretion of collagen and other ECM components by those cells leading to abundant disposition of highly cross-linked, disoriented, and often widely dispersed collagen fibrils. Also, the stiffness of ECM that surrounds the cells depends on the amount of cross-linking of the newly secreted altered collagen. ECM stiffness have been reported to contribute to fibroid growth. [44].

Histologically, in conjunction with hormonal assay of LH and FSH, the photomicrographs of the pituitary gland were captured a magnification of x100 for better appreciation of cellular components. Neurohypophysis is well demonstrated while adenohypophysis is not conspicuous at this cortical section, pars distalis, pars intermedia and pars nervosa are the various parts of the hypophysis associated with different secretions. Results showed that Plate 1A, which represents the control group at a magnification of x100, had no abnormality, unlike the negatively-induced group where there was degeneration of pituicytes. Across the groups, degenerative changes as well as presentation of red and inflammatory cells are seen present in treated group B & F (yellow arrows) as against the control group A & C with well outlined panoramic cytoarchitectural presentation (black arrows). Hence, it is safe to say Monosodium Glutamate treatment induces degenerative changes in the cytoarchitectural presentation of the pituitary gland as demonstrated by H&E stain. Treatment received by groups D, E & G showed a mild degenerative change – not very conspicuous. These findings can be attributed to the fact that in group B, MSG-induced uterine fibroid caused over-production of estrogen and LH which has been reported to be a trigger for estrogen hormone also. This finding is in conjunction with works done by Danuta Plewka et al. in 2014 [46]. As seen, compared to the negative induced group, LH levels were lower in all ginger-treated groups especially in the prophylactic and high-dosed curative groups. FSH levels in the present study were

not increased in the uterine fibroid group, but were seen to increase in the group administered ginger only.

In a research article titled, “Receptors of Hypothalamic-Pituitary-Ovarian-Axis Hormone in Uterine Myomas” by Danuta Plewka et al. in 2014, the expression of GnRH, FSH, LH, amongst other parameters were examined in uterine myomas of women in reproductive and perimenopausal ages, results from this study showed that in myomas of women in reproductive age, independently of their size, expression of GnRH, FSH, and LH receptors was more pronounced than in myometrium. In women of peri-menopausal age, independently of myoma size, expression of LH was higher while expression of GnRH receptors was lower than in myometrium. FSH receptor expression was not observed.

These findings can be compared to results from the study “The anti-oxidant effects of ginger and cinnamon on spermatogenesis dys-function of diabetes rats” by Khaki *et al.* in 2014 [47].

Also, published in the New York Times, an article titled, “Fibroids In-Depth Report” stated that in women, six hormones serve as chemical messangers that regulate the reproductive system: the hypothalamus first relaeases the GnRH which then stimulates the pituitary gland to produce FSH and LH [48]. Estrogen, progesterone and the male hormone testosterone are then secreted by the ovaries at the command of FSH and LH. As stated earlier, it is not clear what causes fibroids, but estrogen and progesterone have been reported to play a role in their growth. Also, an article titled, “Zoladex (goserelin)” in 2009 [49], reported that the amount of FSH and LH released from the pituitary gland is controlled by another hormone called gonaderelin (LHRH). This hormone acts on LHRH receptors in the pituitary gland, causing the release of LH and FSH, hence subsequent release of estrogen in women. GnRH is secreted into the portal pituitary circulation, reaching the anterior pituitary to affect FSH and LH release from the anterior pituitary.

Although there is very little that has been reported on the relationship between uterine fibroid and the master gland, With respect to the anterior pituitary gland in this study, there were degenerative changes in the negative-induced and low-dosed curative groups. These changes were seen to have been prevented, reversed or reduced in the prophylactic, protective and high-dosed curative groups.

5. CONCLUSION

Conclusions to be drawn from this study is that Uterine fibroid affects the brain at the level of the pituitary gland as a result of over-production of FSH and LH which in turn increases estrogen and progesterone production that causes further increase of the fibroid volume, as fibroids are estrogen-feeding tumors. While ginger prevented and reversed these effects.

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