

# Ovarian Gene Transcriptional Responses to Anticonvulsant Drugs (Diazepam and Phenytoin) in Female Wistar Rats

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This study was designed to investigate ovarian gene transcriptional responses to selected anticonvulsant drugs (diazepam and phenytoin) in female rats. Fifteen female rats (120 – 140 g) were used for this study. Diazepam (0.14 mg/kg) and phenytoin (2.8 mg/kg) were given to the rodents orally for 50 days. The method of RT-PCR involving isolation of RNA, conversion of cDNA and electrophoresis was employed to investigate the expression of FSH-R, aromatase and GPX-1 genes. Graphics were generated as mean  $\pm$  SEM using Graph-pad Prism version 8.0. The expression of GPX-1 was significantly ( $p < 0.05$ ) up-regulated, while the FSH-R and aromatase expressions were significantly ( $p < 0.05$ ) and insignificantly ( $p > 0.05$ ) down-regulated respectively in the diazepam treated rats when compared with their respective controls. The FSH-R and aromatase expressions were significantly ( $p < 0.05$ ) up-regulated, while the GPX-1 expression was insignificantly ( $p > 0.05$ ) up-regulated in the phenytoin treated rats relative to their respective controls. Conclusively, it can be suggested that diazepam: inhibited follicular growth through the down-regulation of FSH-R expression, reduced estrogen level through the down-regulation of aromatase expression, inhibited the production of reactive oxygen species and oxidative stress through the up-regulation of GPX-1 expression. In addition, it can be suggested that phenytoin: induced follicular growth through the up-regulation of FSH-R expression, increased estrogen level through the up-regulation of aromatase expression, inhibited the production of reactive oxygen species and oxidative stress through the up-regulation of GPX-1 expression.

**Keywords:** Diazepam; FSHR; Phenytoin; RT-PCR; Rats.

Anticonvulsants are a group of drugs used to treat epilepsy<sup>1</sup>. They can be found to be useful in curing bipolar disorder<sup>2</sup> and personality disorder because many of them appear to behave like mood stabilizers and analgesics<sup>3</sup>. Anticonvulsants inhibit excess neuronal excitation during epilepsy as well as inhibiting epilepsy spread in the brain<sup>4</sup>. Anticonvulsant drugs may act as sodium channel blockers or potentiate GABA function with

many anticonvulsant drugs having numerous or unknown mechanisms of action; their targets include GABA receptors, calcium channels, SV2A e.t.c.<sup>5</sup> By obliterating the sodium or calcium channels, anticonvulsant drugs inhibit the release of glutamate which is responsible for excitation<sup>6</sup>.

The effects of anticonvulsant drugs on: pharmacokinetic activity in rats<sup>7</sup>, short-term cypermetrin poisoning in rodents<sup>8</sup>, behavioural

effect in rats<sup>9</sup>, prenatal and early post natal exposure<sup>10</sup>, rats' teratogenicity<sup>11</sup> as well as on pregnant rats renal corpuscle<sup>12</sup> have been reported.

But, as a result of limited information obtained from literature concerning the effects of anticonvulsant agents (diazepam and phenytoin) on ovarian gene expression in female rats, hence, this research intends to bridge this gap.

## MATERIALS AND METHODS

### Experimental Animals

Fifteen female rodents of weight range 120 – 140 g raised in the Animal Holding of ABUAD were used in the current study. These rodents were accommodated in a conducive laboratory atmosphere with unlimited supply of feed and water; the acclimatization period was for two weeks prior to starting the experiments. All animal experiments were done in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals.

### Drugs

Diazepam (Juhel Pharm, Nigeria Ltd.) and phenytoin (Merit Pharm, Ltd., India) were purchased from Danax Pharmacy, Ibadan, Nigeria. Among these, diazepam (5 mg) and Phenytoin (100 mg) were liquefied in 10 ml of distilled water to produce concentrations of 0.5 mg/ml and 10 mg/ml respectively. The dosages of the anticonvulsant agents considered in this study were in accordance with that reported by the manufacturing industries.

### Experimental Design

Fifteen matured female rats (five per group) used for this study received the following oral doses of the anticonvulsant drugs and distilled water (control) for 50 days as follows:

Group I rodents (control group) were given 0.5 ml/100 g of distilled water.

Group II rodents were given 0.14 mg/kg of diazepam.

Group III rodents were given 2.8 mg/kg of phenytoin.

On the next day after the last treatment (day 51), the rodents were euthanized by overdosing with diethyl ether (Standard Reagents Ltd.); ovaries were harvested with the fatty tissue removed and transferred quickly into TRIzol reagent (ThermoFisher Scientific) for isolation of RNA.

### Isolation of RNA

Total RNA was isolated from whole tissues following a method described by<sup>13</sup>. Briefly, tissues were homogenized in cold (4 °C) TRI reagent (Zymo Research, USA, Cat:R2050-1-50, Lot: ZRC186885). Total RNA was partitioned in chloroform (BDH Analytical Chemicals, Poole, England Cat: 10076-6B) following centrifugation at 15,000 rpm/15 min (Abbott Laboratories, Model: 3531, Lake Bluff, Illinois, United States). RNA from the clear supernatant was precipitated using equal volume of isopropanol (Burgoyne Urbidges & Co, India, Cat: 67-63-0). RNA pellet was rinsed twice in 70% ethanol (70 ml absolute ethanol (BDH Analytical Chemicals, Poole, England Cat: 10107-7Y) in 30 ml of nuclease-free water (Inqaba Biotec, West Africa, Lot no: 0596C320, code: E476-500ML). The pellets were air-dried for 5 min and dissolved in RNA buffer (1 mM sodium citrate, pH 6.4).

### Conversion of cDNA

Spectrophotometer (ThermoFisher Scientific Ltd.) was used to determine the purity and quantity of total RNA at an absorbance of  $A_{260}/A_{280}$  as described by<sup>13</sup>.

### Polymerase chain reaction (PCR)/ Electrophoresis

FSH-R, aromatase and GPX-1 genes were amplified by PCR targeting primers highlighted in the table below. A software called Primer3 was used to design the primers. The PCR amplification process was carried out as described by<sup>13</sup>.

Amplification products were electrophoresed in agarose gel (1.5%) using 0.5X TBE (Tris-borate EDTA, JHD chemicals, China) containing ethidium bromide at 100V for 60 minutes. The gel was visualized with UV light with the aid of a photo documentation system (ThermoFisher Scientific Ltd.) fitted with a camera (ThermoFisher Scientific Ltd.). Gel images were analyzed using keynote platform as previously described by<sup>14</sup> and Image J software (National Institute of Health) was used to quantify it. Graphpad prism version 8.0 was used to plot the graphs in the form of average +/- S.E.M.

## RESULTS

From figure 1, it was reported that FSH-R expression was down-regulated significantly

( $p < 0.05$ ) in the diazepam treated rats when compared with the control. Furthermore, from the results presented in figure 2, it was reported that aromatase was insignificantly ( $p > 0.05$ ) down-regulated in the diazepam treated rats as compared to the control. Similarly, figure 3 also suggested that GPX-1 expression was up-regulated significantly

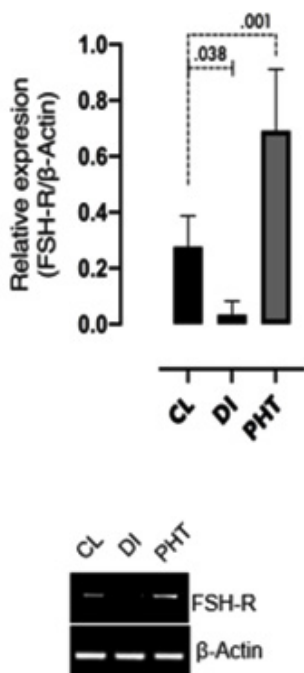
( $p < 0.05$ ) in the diazepam treated rats relative to the control.

In addition, results presented in figures 1 and 2 revealed that the expressions of FSH-R and aromatase were significantly ( $p < 0.05$ ) up-regulated in the phenytoin treated rats relative to their controls; while the GPX-1 expression was insignificantly ( $p > 0.05$ ) up-regulated in the phenytoin treated rats relative to the control.

## DISCUSSION

The FSH-R expression was significantly down-regulated in the diazepam treated rats which suggests that diazepam inhibited follicular growth. Similar result was reported by <sup>15</sup> in Pacific Oyster (*Crassostrea gigas*) treated rats, in which treatment with Pacific oyster (*Crassostrea gigas*) markedly decreased the expression level of FSH-R in the ovarian tissues of rats exposed to bisphenol. Contrarily, FSH-R expression was significantly up-regulated in the phenytoin treated rats which probably indicates that phenytoin induced follicular growth. Similar result was reported by <sup>16</sup> in Dandelion T-1 extract treated mice, in which FSH-R expression was significantly up-regulated in mice treated with Dandelion T-1 extract. These results were corroborated by the assertions of <sup>17,18</sup>.

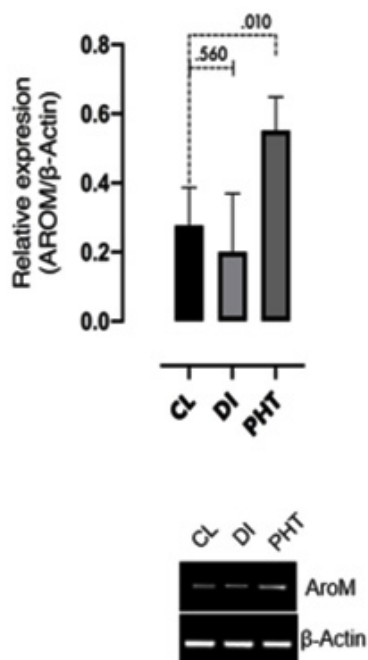
The aromatase expression was insignificantly down-regulated in the diazepam treated rats which suggests that diazepam reduced estrogen level. Similar result was reported by <sup>19</sup> in *Ginkgo biloba* extract flavonoids treated JEG-3 cells, in which three flavonoids (kaempferol, quercetin, and isorhamnetin) from *Ginkgo biloba* extract synergistically inhibit estrogen biosynthesis



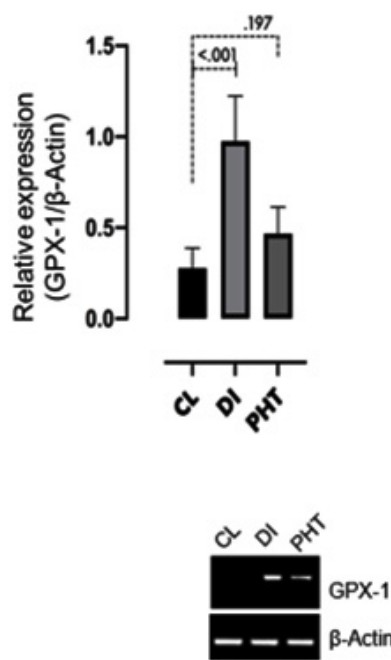
**Fig. 1.** Comparative expression of FSH-R in the ovary of rats treated with diazepam (DI) and phenytoin (PHT) as well as gel image expression patterns of FSH-R and  $\beta$ -actin for diazepam (DI) and phenytoin (PHT) treated rats ( $\beta$ -actin served as the internal control). The band density (image J) was plotted as a bar graph ( $n=5$   $p < 0.05$ )

**Table 1.** List of primers

Primer	Sequence	Product length	Annealing temperature
FSH-R	F:ATTCTTGGGCACGGGATCTG R:TGGTGAGCACAAACCTCAGTT	140	55.09 °C
Aromatase	F:GCTTCTCATCGCAGAGTATCCGG R:CAAGGGTAAATTCATTGGGCTTGG	192	60.00 °C
GPX-1	F:ATCAGTTCGGACATCAGGAGA R:TCACCATTACCTCGCACTT	124	53.93 °C



**Fig. 2.** Comparative expression of aromatase in the ovary of rats treated with diazepam (DI) and phenytoin (PHT) as well as gel image expression patterns of aromatase and  $\beta$ -actin for diazepam (DI) and phenytoin (PHT) treated rats ( $\beta$ -actin served as the internal control). The band density (image J) was plotted as a bar graph (n=5 p<0.05)



**Fig. 3.** Comparative expression of GPX-1 in the ovary of rats treated with diazepam (DI) and phenytoin (PHT) as well as gel image expression patterns of GPX-1 and  $\beta$ -actin for diazepam (DI) and phenytoin (PHT) treated rats ( $\beta$ -actin served as the internal control). The band density (image J) was plotted as a bar graph (n=5 p<0.05)

through aromatase inhibition using JEG-3 cells. In contrast, aromatase expression was significantly up-regulated in the phenytoin treated rats which could mean that phenytoin increased estrogen level. Similar result was reported by <sup>20</sup> in PELP1 treated mice, in which PELP1 (proline, glutamic acid, leucine rich Protein 1) which is a novel estrogen receptor co-regulator up-regulated aromatase expression via activation of aromatase promoter in transgenic mouse model. These results were validated by the assertions of <sup>21</sup>.

The GPX-1 expression was significantly and insignificantly respectively up-regulated in the diazepam and phenytoin treated rats which suggests that diazepam and phenytoin inhibited or decreased the production of reactive oxygen species and oxidative stress. Similar result was reported by <sup>22</sup> in young and aged bilaterally ovariectomized mice mitochondria oxidative stress investigation,

in which there was in increased GPX-1 expression in bilaterally ovariectomized mice tissues in conditioned ovarian media. These results were corroborated by the assertion of <sup>23</sup>.

### CONCLUSION

Conclusively, it can be suggested that diazepam: inhibited follicular growth through the down-regulation of FSH-R expression, reduced estrogen level through the down-regulation of aromatase expression, inhibited the production of reactive oxygen species and oxidative stress through the up-regulation of GPX-1 expression. In addition, it can be suggested that phenytoin: induced follicular growth through the up-regulation of FSH-R expression, increased estrogen level through the up-regulation of aromatase expression, inhibited the production of reactive oxygen species

and oxidative stress through the up-regulation of GPX-1 expression.

#### Limitation of the Study

Scantiness of prior research studies on the topic: There were limited information obtained from literature pertaining studies on ovarian gene transcriptional responses to anticonvulsant drugs (diazepam and phenytoin) in female rats prior to the commencement of this study.

#### Contribution to Future Studies

The mRNA (FSH-R, aromatase and GPX-1) expressed in this study should serve as precedence for future studies on their protein expressions.

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#### Conflict of Interest

There is absence of conflicting interests in this research work.

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