"Effects of Genistein on Pentylenetetrazole-induced behavioral and neurochemical deficits in Ovariectomized rats"

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(Pharmacology and Toxicology)

Presented **By:**

Amr Abdul-Hameed Ahmed

Bachelor of Pharmaceutical Sciences, 2011,
Faculty of Pharmacy, Ain Shams University

Under the supervision of:

Dr. Nagwa Ali Mohamed Sabri

Professor and Head of Clinical pharmacy department
Faculty of Pharmacy, Ain Shams University

Dr. Mariane George Tadros

Associate Professor of Pharmacology and Toxicology,
Faculty of Pharmacy, Ain Shams University

Ain Shams University
Faculty of Pharmacy
Department of Pharmacology and Toxicology
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Pre-requisite postgraduate courses:

Besides the work presented in this thesis, the candidate has attended the following courses:

General courses:

1. Instrumental analysis
2. Physical chemistry
3. Computer skills
4. Statistics

Special courses:

1. Basic pharmacology
2. Clinical pharmacology and therapeutics
3. Clinical toxicology
4. Selected topics in pharmacology and toxicology

He has successfully passed examination in these courses with general grade ***Very good***

Head of Pharmacology and Toxicology Department

Prof. Dr. Ebtehal El Demerdash Zaki

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List of Abbreviations

°C	Celsius
ɑ	Alpha
β	Beta
Δ	Gama
µl	Microliter
µM	Micromolar
µg	Micro gram
µl	Microliter
A	Absorbance
A%	Area percent
AED	antiepileptic drugs
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis Of Variance
AUC	Area under the curve
BSA	Bovine serum albumin
Ca^{+2}	Calcium
Cl^{-}	Chloride
Cl_{int}	Intrinsic clearance
Cm	Centimeter
CNS	Central nervous system
CSF	Cerebral spinal fluid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTNB	5,5' dithiobis (2-nitrobenzoic acid)
EC_{50}	Half maximal effective concentration
EEG	Electroencephalography
ELISA	Enzyme-Linked Immunosorbent Assay
EPSP	Excitatory postsynaptic potential
ER	Estrogen receptor
FDA	Food and Drug Administration
FSH	Follicle-stimulating hormone
G	Gram
GABA	Gamma-Amino Butyric Acid
GEFS+	Genetic Epilepsy with Febrile Seizures Plus
GFAP	Glial fibrillary acid protein
GGE	Genetic generalized epilepsies
GSH	Reduced Glutathione
GSSG	Glutathione disulfide
H	Hour
H & E	Hematoxylin and Eosin

H_2O_2	Hydrogen Peroxide
HDL	High-density lipoprotein
Hp	Hippocampus
HRP	Horseradish peroxidase
HVA	High voltage activated
i.p.	Intraperitoneal
i.v.	Intravenous
IC_{50}	Half maximal inhibitory concentration
IPSP	Inhibitory postsynaptic potential
KA	Kainic acid
kg	Kilogram
L	Liter
LDL	Low – density lipoprotein
LTP	Long-term potentiation
M	Molar
M.wt	Molecular weight
MDA	Malondialdehyde
MES	Maximal electroshock
mg	Milligram
Min	Minute
Ml	Milliter
mM	Millimolar
MRI	Magnetic Resonance Imaging
ng	Nanogram
nM	Nanomolar
nm	Nanometer
NMDA	N-Methyl-D-Aspartate
NMR	Nuclear magnetic resonance
NO	Nitric Oxide
OD	Optical Density
OVX	Ovariectomized
P.O	Per os (for oral administration)
PBS	Phosphate Buffered Saline
PE	Penicillin epilepsy
PRL	Prolactin
PSA	Prostate specific antigen
PTZ	Pentylenetetrazol
ROS	Reactive oxygen species
S	Second
SOD	Superoxide dismutase
SULT	Sulfotransferase
$T_{1/2}$	Terminal half-life

TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TBS	Tris Buffered Saline
T_{max}	Transport maximum
U	Unit
UGT	Uridine -glucuronosyltransferase
UV	Ultra Violet
V_{max}	Maximum velocity of metabolism
Zn^{2+}	Zink

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Abstract of the paper :

Estrogenic compounds have been documented in literature to exert neuroprotective effects. This study investigated the potential neuroprotective effect of genistein; a phytoestrogen in doses of 5, 10, 20 and 40 mg/kg p.o. in ovariectomized rats challenged with Pentylenetetrazole (PTZ) 90 mg/kg. i.p. Systemic acute administration of PTZ induced seizures, increased oxidative stress and caused apoptosis and histological abnormalities. Pretreatment with genistein delayed seizure onset, reduce the seizure duration, improved oxidative stress profile, decreased estrogen receptor expression, reduced apoptosis and improved the histopathological pattern. Overall, genistein doses (10 and 20 mg/kg) showed the most protective effects. In conclusion, the current study suggests a neuroprotective effect of genistein against PTZ-induced seizures. Such effects might be attributed to its estrogenic, antioxidant, and/or anti-apoptotic properties.

Key words:

Phytoestrogen, Genistein, Pentylenetetrazole, Neurodegeneration, Seizures, Ovariectomy.

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ORIGINAL ARTICLE

Effects of genistein on pentylenetetrazole-induced behavioral and neurochemical deficits in ovariectomized rats

Amr A. Elsayed[1] · **Esther T. Menze**[1] · **Mariane G. Tadros**[1] · **Bassant M. M. Ibrahim**[2] · **Nagwa A. Sabri**[3] · **Amani E. Khalifa**[1]

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Abstract Estrogenic compounds have been documented in literature to exert neuroprotective effects. This study investigated the potential neuroprotective effect of genistein; a phytoestrogen at doses of 5, 10, 20, and 40 mg/kg p.o. in ovariectomized rats challenged with pentylenetetrazole (PTZ) 90 mg/kg i.p. Systemic acute administration of PTZ induced seizures, increased oxidative stress, and caused apoptosis and histological abnormalities. Pretreatment with genistein delayed seizure onset, reduced the seizure duration, improved oxidative stress profile, decreased estrogen receptor expression, reduced apoptosis, and improved the histopathological pattern. Overall, the genistein doses (10 and 20 mg/kg) showed the strongest protective effects. In conclusion, the current study suggests that genistein exhibits neuroprotective effects against PTZ-induced seizures. Such effects might be attributed to its estrogenic, antioxidant, and/or anti-apoptotic properties.

Keywords Phytoestrogen · Genistein · Pentylenetetrazole · Neurodegeneration · Seizures · Ovariectomy

Abbreviations

OVX	ovariectomy
PTZ	pentylenetetrazole
GFAP	glial fibrillary acid protein
ER	estrogen receptors

✉ Esther T. Menze
esther.menze@gmail.com

[1] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt

[2] Department of Pharmacology, National Research Center, Cairo, Egypt

[3] Department of Clinical pharmacy, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt

Introduction

Epilepsy is one of the most well-known neurological maladies (Bell and Sander 2001). Epileptic seizures result from functional disorders of the brain, as a result of abnormal, excessive bioelectrical discharge in the nerve cells (Cavazos and Sanchez 2004). This issue can hypothetically happen in each populace of neurons; however, it is regularly seen in the unequivocal quality to natural mind harm, for example, a scar or a tumor. Epileptic seizures occur because of a sudden lopsidedness among excitatory and inhibitory procedures in the neural system (Engelborghs et al. 2000).

Pentylenetetrazole (PTZ), a tetrazol derivative with consistent convulsive actions in mice, rats, cats, and primates, is commonly used to study antiepileptic drug development. Many studies reported that the postulated mechanisms for PTZ-induced pathologic alterations involve imbalance of the inhibitory and excitatory neurotransmission systems, with loss of the inhibition mediated by GABA (Corda et al. 1992) and enhancement of the glutaminergic activity (Akdogan and Yonguc 2011).

Steroidal hormones, such as estrogen, impact the development, separation, and function of many target tissues other than the reproductive organs (Kuiper et al. 1998). Estrogens plays an important role in the central nervous system (CNS) (Turner et al. 1994). It was shown to have positive impact on rats' learning and memory (McEwen 2002). A positive relationship between estrogen levels and seizure exacerbation was described by Bäckström 1976. On the other hand, women with primary generalized epilepsy showed decreased seizure rate at their peak estrogen levels during ovarian cycle (Jacono

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and Robertson 1987). Similarly, during menopause in some women, improved seizure control was associated with estrogen replacement therapy (Peebles et al. 2000). Despite the fact that estrogen is advantageous, it has constrained clinical application in neurological diseases due to its proliferative and oncogenic effects on non-neuronal cells (Zeng et al. 2004).

Soy isoflavones, which are alluded to as phytoestrogens, are considered good alternatives as they can bind to and activate estrogen receptors (ERs) (Molteni et al. 1995). Pan et al. (2000) showed that oral administration of soy phytoestrogens enhanced the memory of ovariectomized rats. Likewise, huge changes in memory have been observed in human subjects eating a high-soy diet for 10 weeks (File et al. 2001). Genistein, one of the major components of soy, has been found to have a neuroprotective effect on cortical cell lines (Sonee et al. 2004). Moreover, high dietary consumption of genistein was found to result in memory improvement in male and female volunteers (File et al. 2001). Genistein binds differently to human ER-α and ER-β with priority to ER-β, to which it was found to have seven- to eight-folds binding affinity (Barnes et al. 2000). Genistein has a therapeutic potential against psychological decay and neurodegeneration related to menopause by reducing oxidative stress. Interestingly, genistein is powerful in both prophylaxis and treatment of hormone-dependent cancers. Therefore, genistein can eliminate the possible risk associated with the classically used estrogen replacement therapy (Herman et al. 1995).

This study was designed to investigate the protective effect of genistein, as an estrogen alternative, on PTZ-induced seizures in ovariectomized rats.

Materials and methods

Animals

Adult female Wistar rats weighing from 150 to 200 g were obtained from the Animal House Colony of the National Research Centre, Cairo, Egypt. All animals were maintained on a standard laboratory conditions. Food and water were provided ad libitum. After an acclimatization period of 1 week, the animals were housed in stainless steel cages in controlled temperature (23 ± 1 °C) and artificially illuminated (12-h dark/light, lights were turned on at 7:00 a.m.) room, free from any source of chemical contamination. On the day of the experiment, animals were left to habituate to the laboratory conditions for about 1 h. All the experiments were conducted between 8:00 a.m. and 3:00 p.m. The experimental protocol was approved by Ain Shams University Faculty of Pharmacy Ethical Committee for the use of animal subjects with a permit no. 149.

Surgery

Animals were anesthetized with a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg) i.p. (Shirke et al. 2008) and received injections of carprofen (5 mg/kg, s.c.) as an analgesic. Rats were bilaterally ovariectomized under aseptic conditions then left for 3 weeks to recover from surgery and for the cessation of the rats' estrous cycle (Túnez et al. 2006). During the recovery period, good care of the animals was taken, including daily cleaning of the cages and wound disinfection.

Materials

Genistein and PTZ were purchased from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). Genistein was dissolved in absolute alcohol:olive oil (2:3) and administrated orally (5 ml/kg). PTZ in saline was administrated i.p. in saline. Ketamine hydrochloride vials were used (Rotexmedica, Trittau, Germany). The caspase-3 kit was purchased from Glory science Co., Ltd., Del Rio, TX, USA. Glial cells fibrillary acid protein (GFAP) and estrogen receptors (ER) α and β antibodies (with product codes AHP1468, VMA00231, and MCA1974ST, respectively) were purchased from Bio-Rad Laboratories, UK. Glutathione, malondialdehyde, and nitric oxide kits were purchased from Biodiagnostics, Egypt. Other reagents were of the highest purity grade commercially available.

Experimental design

Rats were randomly divided into nine groups ($n = 8$). Group 1: sham-operated group. Group 2: control group of ovariectomized rats, received vehicles only. Group 3: PTZ-treated group of ovariectomized rats receiving a single dose of PTZ (90 mg/kg) (Hariry 2011; Hosseini et al. 2009) and genistein vehicle 30 min later. Groups 4 to 7: ovariectomized rats receiving genistein at doses of 5, 10, 20, and 40 mg/kg, respectively, 30 min before PTZ injection. Groups 8 and 9: ovariectomized rats receiving saline i.p. then 10 and 20 mg/kg of genistein alone, respectively (Hariry 2011). Twenty-four hours after the last PTZ injection, animals were sacrificed by decapitation (Abdallah 2010), then, skulls were split on ice cold phosphate-buffered saline. Whole brains were rapidly excised, washed with isotonic saline, dried, and weighed. Thereafter, brains were sagitally divided into two halves. From one half, the cortex was dissected then homogenized immediately in 0.1 M phosphate buffer saline at pH 7.4, to prepare 10% (*w*/*v*) homogenate. The homogenate was centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was used for biochemical analysis. The second half of each brain was fixed in 10% formol saline for histopathological and immunohistochemical examination.

Methodology

Seizure onset and duration

Immediately after PTZ injection, animals were individually placed in plastic cages and observed for 30 min. During this period, the onset and duration of seizures as well as mortality were recorded. The intensities of seizures were measured according to the scale introduced by Pohl and Mares (1987), as follows: 0, no changes in behavior; 1, isolated myoclonic jerks; 2, atypical minimal seizures; 3, full clonic seizure; 4, pattern of tonic-clonic seizures with a suppression of tonic phase; and 5, generalized tonic-clonic seizure. Mortality was evaluated during the observation period. Animals that showed no convulsions or convulsed for less than 30 min were observed for 24 h. Animals that showed convulsions for more than 30 min were euthanized by cervical dislocation (Hosseinzadeh and Khosravan 2002).

Histopathological examination

After fixation in formol saline for 24 h, brain samples were washed with tap water and then dehydrated in serial dilutions of alcohol. Specimens were cleared in xylene and embedded in paraffin at 56 °C in a hot air oven for another 24 h. Paraffin bees wax tissue blocks were prepared for coronal sectioning at 4-μm thickness. Sections were then collected on glass slides, deparaffinized and stained by hematoxylin and eosin (H & E) for examination under light microscope (Bancroft and Stevens 1996).

The doses of genistein that showed significant improvement in the two previous experiments were further assessed for the following.

Apoptosis (caspase-3 levels)

Caspase-3 detection depends on the color change, at certain wavelength, resulting from the formation of antibody-antigen-enzyme-antibody complex. Caspase-3 levels are expressed in nanogram per milliliter tissue.

Estimation of lipid peroxidation products

Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in acidic medium at 95 °C for 30 min. The absorbance of the resultant pink product was measured at 534 nm. MDA levels are expressed in nanomole per liter tissue.

Assay of reduced glutathione levels

It was carried out according to Ellman's method (Ellman 1959). The reduction of 5,5′ dithiobis (2-nitrobenzoic acid) (DTNB) with reduced glutathione (GSH) produces a yellow compound. This chromogen is directly proportional to GSH level, and its absorbance was measured at 405 nm. GSH levels are expressed in micromole per gram tissue.

Assay of nitric oxide levels

Nitric oxide measured as nitrite was determined using Griess reagent, according to the Moshage method (Moshage et al. 1995). In acidic medium, the Griess reagent reacts with nitrite forming nitrous acid diazotize sulphanilamide, which is coupled with *N*-(1-naphthyl) ethylenediamine. The resulting azo dye has a bright reddish-purple color, which can be measured at 540 nm. Nitric oxide (NO) levels are expressed as micromole per gram tissue.

Immunohistochemical estimation of GFAP, ER-α, and ER-β expressions (Buchwalow et al. 2002)

Sections (3-μm thickness) of paraffin-embedded tissues were rehydrated first in xylene and then in graded ethanol solutions. Sections were then blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 2 h, then immunostained with either mouse anti-human ER-β, mouse anti-human ER-α, or mouse anti-human GFAP antibodies. All the antibodies used have established cross reactivity with rat proteins. Slides were incubated in TBS (1:25) containing 5% BSA overnight at 4 °C. After washing the slides with TBS, the sections were incubated with goat anti-mouse secondary antibody, then washed with TBS, and incubated for about 10 min with 0.02% diaminobenzidine solution containing 0.01% H_2O_2. Counter staining was performed using hematoxylin. The quantitation of positively stained cells was performed using ImageJ software and represented by optical density (OD) and mean area % (A%).

Statistical analysis

Parametric data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey, post hoc test. Seizure scores, non-parametric data, were analyzed by Kruskal-Wallis followed by Dunn's test as a post hoc test. Percent of convulsed rats data and mortality rates are presented in contingency tables and analyzed by chi-square test. GraphPad Prism software (version 5.01, Inc., 2007, San Diego, CA, USA) was used for statistical analyses. *P* values of less than 0.05 were considered statistically significant.

Results

Effect of genistein on onset and duration of seizure and seizure score of PTZ-treated rats

Acute administration of PTZ (90 mg/kg i.p.) induced seizures in 100% of the rats. Genistein (5 mg/kg p.o.) did not significantly affect seizure duration. Genistein (10, 20, and 40 mg/kg p.o.) significantly decreased the duration of seizures in comparison to that of PTZ-treated group. However, genistein did not significantly decrease the number of convulsed rats, affect the onset of seizures, nor improved seizures scores compared to PTZ-treated rats (Tables 1 and 2).

Effect of genistein on mortality of PTZ-treated rats

Eighty-seven percent of rats treated with PTZ alone or PTZ and 5 mg/kg genistein died. While pretreatment with genistein (10, 20, 40 mg/kg) decreased the mortality rates to 25, 37.5, and 62.5%, respectively. Data analysis using chi-square test [df (40.5, 8), $P < 0.0001$] indicated that mortality is dependent on the treatments used (Table 1).

Histological examination of rat brains

Histological examination of the cortex sections from the sham-, ovariectomized-, and genistein-only-treated rats showed a normal histological structure. Administration of PTZ caused gliosis and congestion of blood vessels in the cortex. Pretreatment with genistein 5 mg/kg showed no enhancement in PTZ-induced brain injury while, pretreatment with genistein 10 mg/kg showed only mild focal gliosis. Pretreatment with genistein 20 mg/kg restored the cortex histological features. Congestion of blood capillaries was detected in the cortices of rats pretreated with genistein 40 mg/kg (Fig. 1).

Effect of genistein on GFAP expression of PTZ-treated rats

The effects of genistein and PTZ on glial cell recruitment were further investigated through the immunohistochemical staining of astrocytes-specific protein, GFAP. As shown in Fig. 2, PTZ significantly increased the expression of GFAP, compared to the sham and ovariectomized groups. While, genistein 10 and 20 mg/kg showed only faint immunoreactivity. These photomicrographs greatly correlate with the values of the mean OD and A%.

Effect of genistein on caspase-3 levels of PTZ-treated rats

One-way ANOVA showed significant differences among groups in cortical caspase-3 levels [F (6, 49) = 2.998, $P = 0.0142$]. Ovariectomy caused significant increase in cortical caspase-3 levels. Systemic administration of single dose of PTZ (90 mg/kg, i.p.) significantly increased cortical caspase-3 levels as compared to sham group, but showed non-significant increase compared to ovariectomized rats. Pretreatment with genistein (10 and 20 mg/kg, p.o.) did not significantly affect cortical caspase-3 levels as compared to PTZ-treated and ovariectomized rats (Fig. 3).

Effect of genistein on cortical MDA levels of PTZ-treated rats

One-way ANOVA showed significant difference in cortical MDA levels among the different groups [F (6, 49) = 13.25, $P < 0.001$]. Ovariectomy caused significant increase in MDA levels. Systemic administration of PTZ (90 mg/kg, i.p.)

Table 1 Contingency tables showing the effect of genistein on the onset of PTZ-induced seizures and mortality rates, respectively

Groups	No. of animals used	Seizures onset/occurrence			Mortality		
		Convulsed rats	Non-convulsed rats	% convulsed	Survived	Dead	% mortality
Sham	8	0	8	0	8	0	0
OVX + saline + vehicle	8	0	8	0	8	0	0
OVX + PTZ (90 mg/kg) + vehicle	8	8	0	100	1	7	87.5
OVX + PTZ (90 mg/kg) + Gen (5 mg/kg)	8	8	0	100	1	7	87.5
OVX + PTZ (90 mg/kg) + Gen (10 mg/kg)	8	8	0	100	6	2	25
OVX + PTZ (90 mg/kg) + Gen (20 mg/kg)	8	8	0	100	5	3	37.5
OVX + PTZ (90 mg/kg) + Gen (40 mg/kg)	8	8	0	10	3	5	62.5
OVX + saline + Gen (10 mg/kg)	8	0	8	0	8	0	0
OVX + saline + Gen (20 mg/kg)	8	0	8	0	8	0	0
Total	72	40	32	55	48	24	33