In utero exposure of green coffee extract alters rat fetal neurodevelopment in a dose dependent manner

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Abstract

Green coffee consumption has gained wide popularity, possibly due to its strong antioxidative activities and many beneficial effects in various human diseases. However, the effect of green coffee extract consumption on the development of the fetal central nervous system during pregnancy has not been elucidated. Consequently, the present study aimed to evaluate the effect of maternal administration of some doses of the green coffee extract on the development of the cerebral cortex, cerebellum, and spinal cord of rat fetuses in terms of histopathological, proliferation, astrogliosis, and ultrastructural investigations. Pregnant dams were divided into four groups; control group (administered distilled water) and three groups orally administered three different doses of green coffee extract, GC1 (200 mg/kg), GC2 (400 mg/kg), and GC3 (600 mg/kg) from the sixth day to the 15\textsuperscript{th} day of gestation. On the 20\textsuperscript{th} day, dams were sacrificed and fetal cerebral cortex, cerebellum, and spinal cord from different groups were fixed for subsequent investigations. The results showed that green coffee extract induced various histopathological changes in the three investigated organs including pyknosis, hemorrhage, and vacuolation. Immunohistochemical investigation revealed that green coffee extract decreased neuronal proliferation and increased reactive astrogliosis. Ultrastructurally, green coffee extract caused cytoplasmic rarefaction, neuronal degeneration, macrophage activation, and axon degeneration. Interestingly, the neurotoxic effects of green coffee on neuronal development were dose-dependent. Based on these results, the consumption of high doses of green coffee during pregnancy should be restricted. Moreover, further studies are needed to evaluate the long-term effects of green coffee ingestion on neuronal cognition and behavioral outcomes.

Keywords: Green coffee; Neurotoxicity; Astrogliosis; Neuredegeneration; Cell proliferation; Ultrastructure.
Introduction

Brain development occurs in early life and any disturbances or alterations in the development process, either anatomically or functionally, can have a detrimental effect on the developing fetus (Lenroot and Giedd, 2006; Zappettini et al., 2019). This disturbance can affect cell migration and proliferation, synapse formation, and cortical connectivity eventually resulting in improper neural development and leading to behavioral abnormalities later in life (Thompson et al., 2009; Guerrini and Dobyns, 2014). The neural development is not only controlled by gene expression but it is also affected by the external fetal environment (Silva et al., 2013), resulting in many pathologies either in early life including developmental delay, autism, epilepsy, or in adulthood leading to dementia (Borenstein et al., 2006; Barkovich et al., 2012; Stern, 2012; Seifan et al., 2015). Maternal lifestyle greatly affects fetal development, increases pregnancy complications, and may result in long-term diseases. Great caution and concern should be taken during pregnancy regarding the different chemicals that pass freely through the placenta to the fetus imposing great risk (van der Hoeven et al., 2017). In utero exposure to stimulants during pregnancy can alter the brain development of fetuses which may induce the development of many neural and even psychiatric disorders (Zappettini et al., 2019).

Coffee is one of the most popular consumed drinks worldwide due to its pleasant aroma and taste, as well as its stimulant and refreshing effect (Nebesny and Budryn, 2003). It constitutes one of the most cultivated plants in some countries such as Brazil, Colombia, Vietnam, Indonesia, and other countries whose economy relies mostly on coffee exports (Wanderley et al., 2017). The most common and preferred coffee species is Arabic coffee or *Coffea arabica*, which is thought to be originated in Ethiopia. The less preferred other coffee species is *Coffea canephora* or robusta which accounts for only 5% of the consumed coffee around the world (Babova et al., 2016; Tasew et al. 2020). Coffee could be consumed as black roasted beans or green raw unroasted beans (Al-Dujaili et al., 2016). In recent years, green coffee has gained great popularity as a healthy beverage with an increased consumption rate worldwide (Dziki et al., 2015). Green coffee is usually used as a dietary supplement in weight control and healthy diets (Abbass and El-Baz 2018). Due to its high consumption rate and effects on health, researchers have been interested to address its possible benefits or side effects on human health, especially on fetal development (Ma et al., 2012; Wanderley et al., 2017). Coffee has been reported to have antioxidant, anticancer, and antidiabetic properties (Sanlİer et al., 2018). The green coffee extract has been proved to have many beneficial effects on several health problems such as vascular abnormalities, blood pressure, metabolic syndrome, and type 2 diabetes (Butt and Sultan, 2011; Asbaghi et al., 2020). These effects may be because green coffee is rich in different bioactive compounds such as caffeine, chlorogenic acid, caffeic acid, quinic acid, and p-coumaric acid (Tanaka et al. 2009).

Although it is not recommended to consume coffee or any caffeine-containing beverages during pregnancy, it has been reported that about 70-95% of pregnant women consume 2 cups of coffee daily (Ma et al., 2012). Caffeine can cross the placental barrier and accumulate in the fetus (Paula et al., 2017). Consequently, caffeine consumption during pregnancy has been reported to induce adverse effects on the developing fetus and may increase abortion rates, induce limb and craniofacial malformations, as well as delayed ossification (Nehlig and Debry, 1994; Weng et al., 2008; Nikoui et al., 2013; Souza et al., 2016). Nehlig and Debry (1994) reported that maternal exposure to caffeine results in long-term consequences on learning abilities, sleep, anxiety, and locomotion in
rat offspring. Moreover, Ma et al. (2012) reported that caffeine administration during chick embryo development resulted in defective neural tube formation and altered neurodevelopment. In addition, it was found that prenatal and antenatal exposure to caffeine delayed migration and integration of GABA neurons, altered brain rhythms, and affected memory functions as well as enhanced seizures susceptibility in mice (Silva et al., 2013; Fazeli et al., 2017). Moreover, a human-based study found a relation between caffeine consumption during pregnancy and impaired cognitive functions in children (Galéra et al., 2015). It has been reported that caffeine metabolism is reduced during pregnancy with a half-life of 15 h. Fetuses are not able to metabolize caffeine as they lack the needed enzymes, leading eventually to its accumulation in the fetal brain which may affect their early development and childhood (van Dam et al., 2020).

Despite the high number of reports addressing the beneficial effects of coffee, either green or black, the consequences of prenatal exposure to coffee on neural development have not been fully fulfilled and are controversial (Zhang et al., 2021). Moreover, there has been no study investigating the effect of the green coffee extract on fetal neural development. Consequently, the purpose of the present study was to investigate the influence of maternal administration of three different doses of the green coffee extract on the development of different parts of the central nervous system, i.e., cerebral cortex, cerebellum, and spinal cord, in rat fetuses and to evaluate its possible neurotoxicity in terms of histological, immunohistochemical and ultrastructural effects.

Materials and Methods

Preparation of Green coffee (GC) extract

Green coffee beans, Coffea arabica, were purchased from a local market at Shebeen Elkoom, Menoufia, Egypt. Aqueous extract of green coffee was prepared according to El-Nabi et al. (2018) as follows: 250 gm of the beans were ground, macerated in 1000 ml of 55°C distilled water for 6 h. The supernatant was filtered 3 times, then it was air-dried at 55°C. The powder was stored at -20°C until use. The green coffee extract was dissolved in distilled water at doses of 200, 400, and 600 mg/kg body weight.

HPLC analysis of a green coffee extract

HPLC analysis was used to determine the different compounds in green coffee extract according to Croci et al. (2009). It was carried out using an Agilent 1260 series operated at 25 °C. Ten microliters of the extract were injected into the HPLC. Kromasil C18 column (4.6 mm x 250 mm i.d., 5 μm) was used for separation. The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) with a flow rate of 1 ml/min. The mobile phase was programmed consecutively in a linear gradient as follows: 0 min (82% A); 0–5 min (80% A); 5-8 min (60% A); 8-12 min (60% A); 12-15 min (85% A) and 15-16 min (82% A). The multi-wavelength detector was monitored at 280 nm. The phenolic compounds found in the green coffee extract are listed in the table (1).
Table (1): HPLC quantitative identification of phenolic acids in Green coffee (GC)

<table>
<thead>
<tr>
<th>No.</th>
<th>Retention time (RT)</th>
<th>Compound name</th>
<th>Molecular formula (M.F)</th>
<th>Area</th>
<th>Conc. (µg/ml = µg/15mg)</th>
<th>Conc. (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.313</td>
<td>Gallic acid</td>
<td>C₇H₆O₅</td>
<td>873.14</td>
<td>56.20</td>
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<td>2</td>
<td>4.043</td>
<td>Chlorogenic acid</td>
<td>C₁₆H₁₈O₉</td>
<td>9279.97</td>
<td>651.00</td>
<td>43400.26</td>
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<tr>
<td>3</td>
<td>4.546</td>
<td>Catechin</td>
<td>C₁₅H₁₄O₆</td>
<td>1033.01</td>
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<td>11441.27</td>
</tr>
<tr>
<td>4</td>
<td>5.575</td>
<td>Methyl gallate</td>
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<td>1066.00</td>
<td>17.39</td>
<td>3746.58</td>
</tr>
<tr>
<td>5</td>
<td>5.960</td>
<td>Caffeic acid</td>
<td>C₉H₄O₄</td>
<td>833.04</td>
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<tr>
<td>6</td>
<td>6.491</td>
<td>Syringic acid</td>
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<td>4691.15</td>
<td>152.93</td>
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<tr>
<td>7</td>
<td>7.136</td>
<td>Pyro catechol</td>
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<tr>
<td>8</td>
<td>7.601</td>
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<td>9</td>
<td>8.303</td>
<td>Ellagic acid</td>
<td>C₁₄H₆O₈</td>
<td>399.46</td>
<td>21.49</td>
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<tr>
<td>10</td>
<td>9.277</td>
<td>Coumaric acid</td>
<td>C₁₅H₁₈O₈</td>
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<tr>
<td>11</td>
<td>9.773</td>
<td>Vanillin</td>
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<td>1459.91</td>
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<tr>
<td>12</td>
<td>10.156</td>
<td>Naringenin</td>
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<td>9532.52</td>
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<tr>
<td>13</td>
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<td>Taxifolin</td>
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<tr>
<td>14</td>
<td>14.363</td>
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<td>515.18</td>
<td>5.25</td>
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<td>15</td>
<td>14.822</td>
<td>Kaempferol</td>
<td>C₁₅H₁₀O₆</td>
<td>148.59</td>
<td>8.87</td>
<td>591.44</td>
</tr>
</tbody>
</table>

**Phenolic acids of GC aqueous extract**

**Animals and husbandry**

All animal experimental procedures were conducted according to the guidelines of care and use of laboratory animals approved by the Faculty of Science, Menoufia University, Egypt (Approval No. MNSE2203) and according to the National Institutes of Health guide for the care and use of laboratory animals (NIH publications No. 8023, received 1978). Mature male and female Wistar Albino rats (200±15 g, 8±1 weeks old) were purchased from Vacsara (Hellwan Animal Breeding Farm), Ministry of Health, Cairo, Egypt. Rats were housed at 24°C on a 12:12 h light-dark cycle and had access to water and a standard diet composed of 50% ground barely, 20% ground yellow maize, 20% milk, and 10% vegetables ad libitum. Mating was achieved by housing two females with one male overnight and gestation day (GD) 0 was determined as the day on which sperm plug was detected. To confirm pregnancy, rat weight was monitored daily and those with increased body weight were selected. Day 20 was determined as the endpoint for experimentation.

**Experimental groups and treatment**

Four groups of pregnant mice were studied (each n=8), as follows:

1. The Control group received 1 ml of distilled water.
2. GC1 group received 1 ml of green coffee extract (200 mg/kg).
3. GC2 group received 1 ml of green coffee extract (400 mg/kg).
4. GC3 group received 1 ml of green coffee extract (600 mg/kg).

All injections were orally administered from GD 6 to GD 15. This study was based on 48 Adults (32 females and 16 males) and 124 fetuses.

**Histopathological and Immuno-histochemical investigation**

On GD20, dams were deeply anesthetized by halothane and underwent cesarean section where the whole uterus with fetuses was removed. Fetuses were anesthetized by halothane and the brain along with the cerebellum and spinal cord were removed, fixed by immersion in buffered 10% formalin for 24 hours, postfixed via washing under tap water for 12 h and finally were stored in 70% ethanol. The specimens were then dehydrated in ascending series of alcohol, cleared via xylene, infiltrated, and embedded in molten paraffin wax. Rotary microtome (Leica, Model Rm 2125, Germany) was used to produce five μm thick sections, mounted on albumen-coated slides and stored until staining. H&E staining was applied to study the histopathological features of the cerebral cortex, cerebellum, and spinal cord from different groups (Humason, 1979).

For immuno-histochemistry, the Avidin-biotin peroxidase immunohistochemical technique was performed according to Atallah et al. (2021). Briefly, sections were subjected to endogenous peroxidase blocking by immersing in methanol and H2O2. After a wash in PBS, sections were blocked in 5% bovine albumin serum with 0.3 % Tx100 for 1h. Sections were then incubated with the following primary antibodies overnight at 4°C: goat anti-GFAP (1:200; ab53554, Abcam, Cambridge, UK) and rabbit anti-PCNA (1:200; ab18197, Abcam, Cambridge, UK). Sections were washed with PBS, then incubated with biotinylated secondary antibody (1:500, Vector laboratories) for 2h. at RT, incubated with ABC Elite complex (Vector Laboratories) and visualized using DAB substrate in PBS. Sections were analyzed and photographed using an Olympus microscope (BX41).

**Ultrastructural investigation**

For ultrastructural investigation, which has been done using a transmission electron microscope, specimens of different groups were separated and immediately fixed for 4 h at room temperature in 2.5% Glutaraldehyde and 2% paraformaldehyde in 0.1 cacodylate buffer (PH. 7.4) and processed as described in Badawy et al. (2019).

**Quantitative analysis**

For the semiquantitative evaluation of immunohistochemical staining, six random areas from stained sections of each group were chosen and captured at 400X magnification using a light microscope (BX41; Olympus, Tokyo, Japan). The percentage of the stained area (area fraction) per field area was determined (Figi-Image J software, Java-based application for analyzing images; Schindelin et al. 2012).

**Data analysis**

All data were presented as mean±SEM. Data were analyzed statistically for normal distribution (student’s T-test) and homogeneity of variances (Levene test) independent-samples T-test using statistical package for the social sciences (IBM SPSS) statistics software for Windows, Version 22 (IBM Corp., Armonk, NY, USA). Differences were considered insignificant whenever P>0.05. The significances of the obtained data were classified into two categories, i.e. P<0.01 and P<0.05 according to the obtained P values.

**Results**

**The green coffee extract induced histopathological changes in the central nervous system**

**Cerebral cortex**

The cerebral cortex of rat fetuses from the control group showed normal histological structure and consisted of five distinct layers, namely, from outwards
inwards, small marginal zone (MZ) followed by large cortical plate (CP), then the intermediate zone (MZ), the subventricular zone (SVZ) and the last dense ventricular zone (VZ). The layers were clearly distinguished from each other, the neuropil appeared homogenous without any signs of vacuolations or hemorrhage. Different cell types were identified within the cerebral cortex including astrocytes (As), pyramidal cells (P), satellite microglial cells (S), and granular cells (G). Administration of the low dose of green coffee extract (GC1) didn’t alter the histological structure and the cerebral cortex showed similar five-layered architecture, though some cells were pyknotic and there were vacuoles in the neuropil. Contrarily, administration of 400 mg/kg of green coffee extract (GC2) induced various histopathological changes including vacuolation, pyknosis, necrotic foci, and ectopia within the pia matter, in addition to disruption in the layer architecture, where the IZ and SVZ zones could not be identified from each other. Administration of the high dose of green coffee extract (GC3) resulted in severe layer disruption, many necrotic foci, and evident micro-vacuolation and hemorrhage in the neuropil (Fig. 1A).

Cerebellum

The cerebellar cortex of 20-day-old rat fetus of the control group exhibited a three layered-structure, namely, the internal granular layer (IGL), the dense innermost layer lining the sulci formed of densely packed rounded cells, the Purkinje cell layer (PCL) formed of large pear-shaped Purkinje cells with extending dendrites arranged in a single row, molecular layer (ML) with scattered smaller cells. The fetal cerebellar cortex of the GC1 group displayed a similar structure to that of the control with minimal alterations, though vacuolation in the PCL layer was evident. On the other hand, prenatal administration of 400 mg/kg of green coffee extract (GC2) induced massive vacuolation in the ML with a spongiform appearance, IGL was packed with pyknotic ill-defined cells, the Purkinje cells were vacuolated and hardly identified and almost distorted. The high dose of green coffee extract (GC3) resulted in complete disruption of the layer arrangement, there was a complete separation between the IGL and ML with extensive hemorrhage between the two layers. The PCL migrated downwards towards the ML. The majority of Purkinje cells were shrunken, vacuolated, and disintegrated, even absent in some areas. Most of the granular and Purkinje cells were pyknotic. The neuropil showed evident vacuolation and fibrosis (Fig. 1B).

Spinal cord

The spinal cord of the control group was surrounded by the Pia matter (PM) and differentiated into the centrally located grey matter (GM) and the peripherally located white matter (WM). The gray matter consisted of neuroglial cells (NG) and neurons. Two types of neurons could be identified, namely, motor neurons (MN) which were large with a central vesicular nucleus and had many processes, and sensory neurons (SN) with a smaller centrally located nucleus. The white matter contained the cell axons and appeared homogenous with no signs of vacuolation. A similar histological structure was found in the fetal spinal cord of the GC1 group receiving the low dose of green coffee extract. On the other hand, the fetal spinal cord from the GC2 group showed pyknosis and vacuolation in the neuronal cells, hemorrhage was evident in the white matter, in addition to the spongiform appearance of the neuropil. Most of the neurons appeared shrunken and deeply stained. Fetuses maternally administered the high dose of green coffee (GC3) showed massive degenerative features including vacuolation, pyknosis, hemorrhage, and many necrotic foci within both the gray and white matter of the spinal cord. Most neurons lost their characteristic details and were ill-defined (Fig. 1C).
Fig. 1: Photomicrographs of H&E-stained coronal sections of the fetal cerebral cortex (A), cerebellum (B), and spinal cord (C) from different groups. A- Control group showing normal structure with distinct cortical layers. GC1 group showed nearly normal cerebral cortex structure, though the neuropil appeared vacuolated (V) with scattered pyknotic cells (arrow). GC2 group showing various histopathological alterations including loss of cortical layer arrangement, indiscrimination between IZ and SVZ layers, massive vacuolation of neuropil (V) along with necrotic areas (*), and ectopia in pia matter (arrowhead). GC3 group showing severe layer disruption, many necrotic foci (*), and evident micro-vacuolation and hemorrhage in the neuropil (h) as well as vacuolation (V) and pyknotic cells (arrow). B- Control cerebellar cortex showing a three layered-structure. GC1 showed a similar structure with minimal vacuolation (V) in the PCL layer. GC2 group showing vacuolation in the ML and Purkinje cells (PC). GC3 shows complete separation between the IGL and ML with extensive hemorrhage (h), neuropil vacuolation, and fibrosis. C- Control spinal cord differentiated into white matter (WM) and grey matter (GM) with neuroglial cells (NG), motor neurons (MN), and sensory neurons (SN). GC1 shows a similar structure. GC2 and GC3 groups show pyknosis (arrow) and vacuolation (V) in the neuronal cells, hemorrhage (h), and many necrotic foci (*). CP: cortical plate, Fi; fibrosis, GM; gray matter, h; hemorrhage, IGL; internal granular layer, IZ; intermediate zone, MLI; molecular layer, MN; motor-neurons, MZ; molecular zone, NG; neuroglial cells; Np; neuropil, PC; Purkinje cell, PCL; Purkinje cell layer, PK; pyknosis, PM; pia matter, SN; sensory neurons, SVZ; subventricular zone, V; vacuoles, VZ; ventricular zone, WM; white matter. Scale bar= 0.06mm, GC2A=0.12mm.
**Immunohistochemical results**

**Green coffee induced astrogliosis in a dose-dependent manner**

The expression of the astrocyte marker GFAP was investigated in the cerebral cortex, cerebellum, and spinal cord of rat fetuses maternally administered different doses of green coffee extract. The results showed that there was an increase in the expression of GFAP in the three organs in a dose-dependent manner. The expression of GFAP protein was in the form of brown staining of the cytoplasm and cell membrane of the astrocytes with lengthy and thin processes and was mainly located around blood vessels. In the cerebral cortex, few scattered cells were GFAP-immunopositive, mainly located in the subventricular zone. There was no significant difference in the immunoreactivity of GFAP between the GC1 and control group. On the other hand, GC2 and GC3 groups exhibited a significant increase (19.62±0.89 and 35.43±0.63, respectively) in the GFAP immunoreaction in the cerebral cortex compared with a control group (5.76±0.79) with the whole cerebral cortex was GFAP-immunoreactive. Similar results were found in the cerebellum, though the overall percentage area of expression was lower than that of the cerebral cortex in the four groups. Nevertheless, there was a significant increase in the immunoreactivity of GFAP in both GC2 (5.65±0.45) and GC3 (9.52±0.47) groups compared with the control group (1.12±0.53). Meanwhile, there was an insignificant difference between the control and GC1 groups (2.93±0.61). In terms of the spinal cord, there was a low distribution of GFAP-positive astrocytes in the control group (2.99±0.67). No substantial difference was observed between the GC1 (6.26±0.57) and control groups and the pattern of expression was quite similar. GFAP positive cells were increased significantly in the GC2 (18.91±0.42) and GC3 (27.01±0.76) groups as there was a marked increase in the thickness and branching of astrocytic processes with increased intensity of immunostaining due to astrocytic proliferation (Fig. 2, Table 2).

<table>
<thead>
<tr>
<th></th>
<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Spinal cord</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.76±0.79</td>
<td>1.12±0.53</td>
<td>2.99±0.67</td>
</tr>
<tr>
<td>GC1</td>
<td>11.13±1.08</td>
<td>2.93±0.61</td>
<td>6.26±0.57</td>
</tr>
<tr>
<td>GC2</td>
<td>19.62±0.89</td>
<td>5.65±0.45</td>
<td>18.91±0.42</td>
</tr>
<tr>
<td>GC3</td>
<td>35.43±0.63</td>
<td>9.52±0.47</td>
<td>27.01±0.76</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SEM.

* P < 0.05    ** P < 0.01 compared with the SV group. n=6.
Fig. 2. Photomicrographs showing immune-expression of GFAP in the cerebral cortex (A), cerebellum (B), and spinal cord (C) of 20-day-old rat fetuses from different GC extract groups. The Control group showed minimal immunoeexpression. GC1 group showed a slight increase in GFAP immunostaining in the three organs. GC2 and GC3 groups showed strong positive immunoreaction against the GFAP antigen. D- Graph showing the mean area % of GFAP expression in the three organs from different groups. Asterisks (*) - (**) refer to the p-value compared with the control group. (*) P < 0.05, (**) P < 0.001. n = 6 (number of sections). Scale bar = 0.06 mm.
Green coffee decreased neuronal proliferation in a dose-dependent manner

The immunoexpression of PCNA was investigated in the cerebral cortex, cerebellum, and spinal cord of rat fetuses maternally administered different doses of green coffee extract. The cerebral cortex of the control group showed the most increased expression of PCNA and the majority of cells were immunopositive. The immunoexpression was in the form of brown nuclear staining of the neuronal cells and was mainly found in the SVZ and VZ layers and around the lateral ventricle. There was an insignificant difference in PCNA immunoexpression between GC1 (23.04±0.79) and control (29.25±0.47) groups. Maternal administration of 400 mg/kg green coffee extract significantly decreased PCNA expression in the fetal cerebral cortex (7.49±0.48) compared with the control group and the immunostained cells were restricted to the VZ layer with fewer scattered cells in the IZ. Moreover, the intensity of brown staining was decreased compared with the control group. Concomitantly, the administration of the highest dose; 600 mg/kg, dramatically reduced PCNA immunoexpression and showed a highly significant decrease (3.27±0.56) compared with the control group with few cells scattered within the fetal cerebral cortex (Fig. 3A, Table 3).

In the cerebellum, the PCNA-immunopositive cells were mainly located in the granular layer and partially in the molecular layer of the cerebellar cortex and appeared with densely stained nuclei with a mean area percentage expression of 26.37±1.14 in the control group. Similarly, low dose administration showed insignificant change between the control and GC1 groups (16.29±1.01), and the PCNA-immunostained cells were mainly found in the granular layer. On the other hand, there was a significant decrease in PCNA immunoexpression in the fetal cerebellar cortex from the GC2 group compared with the control group with only 10.62±0.70% of cells expressing PCNA antigen. In addition, the brown color intensity was markedly decreased. In the same context, high dose administration showed a highly significant decrease in PCNA immunoexpression, and few scattered immunopositive cells with a light stain were identified in the fetal cerebellar cortex (Fig. 3B, Table 3).

The spinal cord showed the least PCNA immunoreactivity among the three organs. Immunostaining was mainly restricted to the grey matter where the neuroglial cells showed dense immunostaining in the control group (8.06±0.28). No significant difference was found between the control and GC1 (6.91±0.41) groups. The immunoreactivity of PCNA was significantly decreased in both GC2 (1.91±0.58) and GC3 (0.34±0.13) groups with few scattered cells were immunopositive (Fig. 3C, Table 3).

Table 3: Immuno-expression of PCNA in the fetal cerebral cortex, cerebellum, and spinal cord sections of different groups using immunohistochemistry staining.

<table>
<thead>
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<th>Cerebral cortex</th>
<th>Cerebellum</th>
<th>Spinal cord</th>
</tr>
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<tr>
<td>Control</td>
<td>29.25±0.47</td>
<td>26.37±1.14</td>
<td>8.06±0.28</td>
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<tr>
<td>GC1</td>
<td>23.04±0.79</td>
<td>16.29±1.01</td>
<td>6.91±0.41</td>
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<td>GC2</td>
<td>7.49±0.48*</td>
<td>10.62±0.70*</td>
<td>1.91±0.58*</td>
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<td>GC3</td>
<td>3.27±0.56**</td>
<td>2.67±0.74**</td>
<td>0.34±0.13**</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SEM.
* P < 0.05    ** P < 0.01 compared with the SV group. n=6.
Fig. 3. Photomicrographs showing immunohistochemical staining of PCNA in the rat fetal cerebral cortex (A), cerebellum (B), and spinal cord (C). The Control group showed strong positive immunoeexpression of PCNA in the three organs. The GC1 group showed no significant difference in PCNA immunostaining compared with the control group. GC2 and GC# groups showed a highly significant decrease in PCNA immunoexpression. D- Graph showing the mean area % of GFAP expression in the three organs from different groups. Asterisks (*) refer to the p-value compared with the control group. (*) P < 0.05, (**) P < 0.001. n = 6 (number of sections). Scale bar = 0.06 mm.
The green coffee extract induced ultrastructural changes in the rat fetal cerebral cortex, cerebellum, and spinal cord.

Cerebral cortex

The cerebral cortex of control fetuses showed normal ultrastructure. The neuronal cells had rounded euchromatic nuclei with prominent nucleolus and were surrounded by a smooth double-layered nuclear membrane. Intact spherical and elongated mitochondrial as well as rough endoplasmic reticulum (rER) surrounded the nucleus and were distributed within the homogenous electron-dense cytoplasm (Fig. 4A). Cortical neurons of rat fetuses maternally administered different doses of green coffee extract exhibited different degrees of degeneration. Low dose administration of green coffee extract resulted in cytoplasm rarefaction and vacuolation, an indented nuclear membrane with prominent infoldings, the organelles were somewhat intact with well-preserved Golgi apparatus, though some of the mitochondria were electron-dense and polymorphic (Fig. 4B&C). The fetal cortical neurons from the GC2 group exhibited severe nuclear changes. The nuclei were pyknotic, shrunken, and degenerated with unevenly distributed heterochromatin which was clumped beneath the irregular indented nuclear membrane. Additionally, the organelles were poorly defined within the thin rim of the cytoplasm (Fig. 4D&E). Macrophages occasionally appeared between the neurons within the degenerated neuropil (Fig. 4F). The most dramatic ultrastructure changes were seen in the cortical neurons from GC3. The nuclei were severely degenerated and appeared irregular with blebbing nuclear membrane, fragmented, and shrunken. In addition, the nuclear membrane disappeared in some parts. The cytoplasm appeared rarefied with many vacuoles and poorly distributed organelles (Fig. 4G-I).

Cerebellum

The ultrastructure of the cerebellar cortex of the control group showed that the granular cells had large rounded euchromatic nuclei with prominent nucleoli surrounded by a well-defined double-layered nuclear membrane. The electron-dense cytoplasm contained free ribosomes, well-preserved mitochondria, and rER (Fig 5A&B). The Purkinje cells of the fetal cerebellar cortex from the control group were identified by their large size and oval-shaped nucleus and a thin rim of cytoplasm. The nucleus appeared euchromatic surrounded by the continuous nuclear envelope. Mitochondria, rER, and polysomes were evident within the cytoplasm (Fig. 5C). The blood-brain barrier showed a complete and continuous structure. The capillaries consisted of a single layer of endothelial cells surrounded by a layer of basement membrane (Fig. 5D). The granular cells in the cerebellar cortex of rat fetuses maternal administered 200 mg/kg green coffee extract exhibited different degrees of neuronal degeneration including shrunken nuclei with the indented irregular nuclear membrane. In addition, the cytoplasm contained vacuoles fragmented rER (Fig. 5E&F). The Purkinje cells were darker, somewhat shrunken in size and the nuclear membrane showed convolutions. Within the cytoplasm, vacuoles and atrophied mitochondria were evident (Fig. 5G). The endothelial cells of the blood capillaries were hypertrophied (Fig. 5H). Severe ultrastructural changes were evident in both the granular and Purkinje cells in the cerebellar cortex of rat fetuses from the GC2 group. The granular cells have degenerated with enfolded nuclear envelope, rarefied cytoplasm with degenerated organelles. The nuclei were shrunken and exhibited clumps of heterochromatin on the margins of the irregular nuclear membrane. The neuropil showed signs of degeneration and vacuolation (Fig. 5I&J). The Purkinje cells were pyknotic, shrunken, and electron-dense with ill-identified organelles (Fig. 5K). Similarly, the endothelial cells of the blood capillaries
were swollen, dark, and vacuolated (Fig. 5L). Maternal administration of the large dose of the green coffee extract led to prominent ultrastructure alterations of the fetal cerebellar cortex. The granular cells were completely degenerated and lost most of their organelles, the nuclei were heterochromatic and showed blebbing. The neuropil lost its compartments and showed signs of edema and swelling with severe vacuolation (Fig. 5M&N). The Purkinje cells were destructed with the interrupted cell membrane and markedly increased electron density of the cytoplasm and nucleus which are characteristic features of so-called “dark neuron”. They contained dilated and fragmented rER. The nuclei were ill-identified, shrunken with folded nuclear membrane, and obscured (Fig. 5O). The blood capillaries were lined with degenerated endothelial cells, and, in some instances, the lumen contained macrophages (Fig. 5P).

**Spinal cord**

The fetal spinal cord of the control group showed normal ultrastructure. The neuroglial cells had either rounded or oval-shaped euchromatic nuclei surrounded by a regular nuclear membrane. The cytoplasm appeared homogenous with abundant mitochondria and rER. Glycogen granules were evident in the cytoplasm (Fig. 6A&B). The neurons from the GC1 extract exhibited different ultrastructural changes. The nuclei were surrounded by an irregular nuclear membrane, sometimes were shrunken and pyknotic. Vacuoles were evident in the cytoplasm and the mitochondria appeared atrophied (Fig. 6C-F). The axons were scattered between the nerve cells and appeared somewhat intact (Fig. 6F). Nerve cells from GC2 have degenerated and the nuclei exhibited large clumps of heterochromatin with the indented nuclear membrane. The predominant feature is that the axons were swollen, vacuolated, and degenerated (Fig. 6G-I). Macrophages were seen between the degenerated axons (Fig. 6G). Administration of the high dose of green coffee extract induced the most dramatic ultrastructural changes. Fibrosis and collagen deposition was evident (Fig. 6J). The neurons were pyknotic, shrunken, and severely degenerated (Fig. 6J-L). The axons were completely damaged, swollen, and degenerated (Fig. 6K). Hemorrhage was evident and RBCs were found between the degenerated nerve cells (Fig. 6L).
Fig. 4. Representative transmission electron photomicrographs of fetal cerebral cortex ultrathin sections. (A) Control, (B&C) GC1, (D-F) GC2 group, (G-I) GC3 groups. A- normal neuronal cells with a rounded euchromatic nucleus (N) and prominent nucleolus (n) surrounded by an intact nuclear membrane (Ne), rER, mitochondria (M), and continuous cell membrane (CM). B&C- neurons with indented nuclear membrane (thick arrow), cytoplasm vacuolation (*), well-preserved Golgi (G), and mitochondria (M). D&E- degenerated and shrunken nuclei (DN) and pyknotic cells (PK) with poorly defined organelles. F- macrophage (Ma) within the degenerated neuropil. G&H- fragmented (FN) and degenerated (DN) nuclei showing irregular and blebbing nuclear membrane and rarefied cytoplasm with large vacuoles (*). I- detached nuclear membrane and degenerated mitochondria (black arrowhead). Scale bar A-H= 2µm; I= 1µm.
Fig. 5. Representative transmission electron photomicrographs of fetal cerebellum ultrathin sections. (A-D) Control, (E-H) GC1, (I-L) GC2 group, (M-P) GC3 groups. A&B- granular cells (Gr) showing normal ultrastructure with large rounded euchromatic nuclei (N) and prominent nucleolus surrounded by a regular nuclear envelope. Well-preserved mitochondria (M) and rER appeared within the electron-dense cytoplasm. C- pear-shaped Purkinje cell (Pc) with a large oval nucleus and a thin rim of cytoplasm. D- blood capillary with normal endothelial cells resting on a basement membrane. E&F- granular cells exhibiting degenerated nuclei (DN) with indented irregular nuclear membrane (thick arrow), in addition to vacuolated cytoplasm (V). RBCs were found between the neuronal cells within the vacuolated neuropil. G- Purkinje cells (Pc) appeared darker and shrunk with convoluted nuclear membrane showed. H- the endothelial cells of the blood capillaries were hypertrophied. I&J- granular cells with severely degenerated nuclei (DN) with the enfolded nuclear envelope, rarefied cytoplasm with degenerated organelles (*) lying within the degenerated neuropil (Np). K- Purkinje cell (Pc) appeared pyknotic, shrunken, and electron-dense with ill-identified organelles. L- blood capillary endothelial cells (E) were swollen, dark, and vacuolated among the degenerated granular cells (DGr). M&N- nuclei of granular cells were heterochromatic, completely degenerated (DN), fragmented, and lost their organelles. The neuropil (Np) was edematous and vacuolated. O- Purkinje cell (Pc) was completely degenerated with a shrunk heterochromatic nucleus and interrupted cell membrane forming “dark neuron” with dark electron-dense cytoplasm, dilated and fragmented rER. P- blood capillary lined with degenerated endothelial cells (E) and macrophages (Ma) in the lumen. Scale bar A, C-L, P= 2µm; B, M-O= 1 µm.
Fig. 6. Representative transmission electron photomicrographs of fetal spinal cord ultrathin sections. (A-c) Control, (D-F) GC1, (G-I) GC2 group, (J-L) GC3 groups. A&B- neuroglial cells with rounded or oval nuclei (N) with homogenously dispersed euchromatin enveloped by a regular nuclear membrane. Glycogen (Gly) granules were evident in the cytoplasm along with mitochondria and rER. C-F- nuclei were surrounded by an indented nuclear membrane (arrow), and some of them were shrunken and pyknotic (DN). Vacuoles appeared in the cytoplasm (*) along with some atrophied mitochondria (M). The axons (Ax) appeared intact between the nerve cells. G-I- nerve cells with degenerated nuclei showing large clumps of heterochromatin with the indented nuclear membrane. Degenerated axons were swollen and vacuolated (DAx) with macrophage (Ma) in-between. J- collagen deposition (Cl) in the neuropil was evident along with pyknotic cells with degenerated nuclei (DN). K- the axons were completely damaged, swollen, and degenerated (DAx). L- hemorrhage was evident and RBCs were found between the degenerated nerve cells (DN). Scale bar A-H, J&K= 2µm; I&L= 5µm.
Discussion

Coffee is one of the most consumed psychoactive beverages around the world. It is rich in different phenolic and alkaloid compounds with strong antioxidative nature. Coffee had been reported to have various beneficial effects in the treatment of various human diseases such as cancer, diabetes, cardiovascular diseases, and atherosclerosis (Nkondjock, 2009; Jeszka-Skowron et al. 2016; Khojah 2016). Moreover, coffee has been claimed to possess a lot of different medicinal activities against infectious and non-infectious diseases (Tasew et al. 2020). However, various natural products gained their popularity owing to their significant therapeutic effects without any sufficient data regarding their toxic side effects (Alafiatayo et al., 2019). These plant products, used daily, are rich in different phytochemicals which may have a possible teratogenic effect (Seukep et al., 2014; Abebe et al., 2021). These chemicals can cross the placenta and may alter the placental functions and thus imposing great risk on the developing fetus (Dugershaw et al., 2020). To date, there has been no study addressing the possible teratogenic effect of green coffee on the developing rat fetuses, especially on their neural development. The present study evaluated the potential teratogenic effect of water extract of green coffee on the development of CNS of rat fetuses exposed to three different doses of green coffee; i.e. 200 mg/kg, 400 mg/kg, and 600 mg/kg, during the organogenesis period from gestation day 6 to 15 in terms of histopathological, proliferation, astrogliosis and ultrastructural investigations.

The current study showed that prenatal exposure to green coffee extract induced a plethora of histopathological alteration in the cerebral cortex, cerebellum, and spinal cord of rat fetuses in a dose-dependent manner. Fetuses exposed to the highest dose; 600 mg/kg, showed the most dramatic changes including severe layer disruption, necrosis, micro-vacuolation, hemorrhage, degeneration of cerebellar Purkinje cells, and fibrosis. Similarly, Abd El-Wahab and El-Dakdoky (2013) reported that in utero administration of caffeine resulted in dose-dependent alterations in the histological structure of fetal brains in the form of a decreased cell population, cell death, and vascular congestion. Archibong et al. (2017) reported similar results after prenatal administration of caffeine with pyknosis, vacuolation, and layer disruption in the cerebral cortex of rat fetuses exposed to 50 or 90 mg/kg caffeine. A recent study by El-Born and Abd El-Gaber (2021) reported similar results after in utero exposure of rat fetuses to different doses of green tea. The authors reported that the histopathological changes increased in a dose-dependent manner.

HPLC analysis of the present study illustrated that green coffee extract is rich in major phenolic and alkaloid compounds such as caffeic acid, cinnamic acid, gallic acid, and chlorogenic acid. The current study used the crude extraction of green coffee including all of its components; therefore, it is difficult to attribute the altered neural development to one specific group of compounds. Green coffee beans were reported to contain a two times higher level of 5-O-caffeoylquinic acid than in roasted coffee (Perrone et al., 2008). The concentration of caffeine in green coffee varies from one species to another. Jeszka-Skowron et al. (2016) showed that the levels of caffeine in green coffee brews range varies from 34.1 g kg⁻¹ d.m in Arabic coffees to 81.6 g kg⁻¹ d.m in Robusta coffees. Caffeine readily crosses the placenta and accumulates in the fetal brain. Abd El-Wahab and El-Dakdoky (2013) showed that the same concentration of caffeine was found in both the serum of mothers and cerebrum of fetuses. Wisborg et al. (2003) stated that high daily doses of caffeine during pregnancy increases stillbirth and late fetal death probably via the release of catechol from the kidney and thus leading to hypoxia. Hypoxia is known to have severe effects on fetal neural
development causing long-term neurologic deficits including mental retardation, learning disabilities, autism, seizure susceptibility, and cerebral palsy (Gonzalez-Rodriguez et al., 2014).

The present study showed that green coffee decreased the nuclear proliferation marker PCNA in a dose-dependent manner. Caffeine was suggested to inhibit the cell cycle and induce apoptosis via perturbing key regulatory proteins, including p53 (He et al., 2003). In the same context, Black et al. (2008) showed that cell death was increased in the cerebrum of rat pups shortly after exposure to caffeine. It has been reported that prenatal exposure to caffeine in high doses could disturb mitosis and decrease neuronal proliferation, as a result adversely affecting the brain development and functions leading to postnatal neurobehavioral disorders (Abd El-Wahab and El-Dakdoky, 2013). Moreover, the authors explained the neurotoxic effect of caffeine in rat fetuses by inhibition of protein and nucleic acid synthesis and thus inhibition of cell cycle, evidenced by reduced DNA, RNA, and protein contents. Similarly, Kang et al. (2012) reported that caffeine blocked the replication of DNA in E. coli.

Caffeine was found to affect the expression of TrkB, GAP-43, and Shh genes in the developing rat fetus brain (Mioranzza et al., 2014). It has been reported that the lack of expression of GAP-43 inhibits the proliferation of neuronal cells and hinders neuronal differentiation and maturation (Maier et al., 1999). Moreover, it was found that chronic administration of caffeine inhibited the neurogenesis and proliferation of the subgranular zone cells in the rat hippocampus (Han et al., 2007).

The current study revealed increased expression of the astrocytic marker, GFAP, which is clear evidence of reactive astrogliosis in the three parts of the developing central nervous system of rat fetuses subjected to in utero exposure to green coffee. Similarly, Archibong et al. (2017) reported that prenatal exposure to different doses of caffeine (50 mg/kg and 90 mg/kg) increased the number of reactive astrocytes in the fetal brain and suggested that maternal administration of caffeine could induce fatal brain injury. Reactive astrogliosis is characterized by a change in the morphology and number of astrocytes, in addition to increased expression of GFAP. Astrocytes serve many functions in the CNS; they are neuroprotective cells involved in neuroinflammatory responses (Haim et al., 2015). They provide metabolic support for neurons and synaptic communication in the brain (Peter et al., 1998). Additionally, they remove waste products between the neuronal cells (George et al., 1998). Previous reports have related CNS injury and reactive astrogliosis. These reports explained that, due to the neuroprotective role of astrocytes in the CNS, their number tends to increase to heal and recover the injured neurons and fill the injured zone (Ekanem et al., 2009). Kumar et al. (2005) related reactive astrocytes and cell loss, consequently, the effect of caffeine administration on astrocytes suggests a potential neurodegenerative effect and explains the long-term neurological deficits of caffeine on neurodevelopment (Archibong et al., 2017).

Ultrastructural investigation of the present study demonstrated that in utero exposure of green coffee altered the mitochondrial structure which was either vacuolated, degenerated, or polymorphic. Kasala et al. (2020) related the caffeine-induced neuronal apoptosis in rat pups to disruption in the expression of mitochondrial-associated genes. In addition, the ultrastructural investigation revealed that the highest dose of green coffee induced demyelination and degeneration of the axons in the fetal spinal cord. Interestingly, it has been shown that high doses of caffeine transiently reduce myelin synthesis in postnatal pups (Nakamoto and Joseph, 1991). Additionally, Huang et al. (2012) observed that maternal administration of caffeine-induced different
ultrastructural changes in the trophoblast cells in a dose-dependent manner. These changes included edematous endoplasmic reticulum, swollen cisternae of rER, degranulation of ribosomes, and heterochromatin clumping.

It has been postulated that psychoactive substances consumed during pregnancy may have deleterious effects on fetal neurodevelopment (Salisbury et al., 2009). Previous studies have ascertained that prenatal administration of caffeine slowed down the migration of the GABAergic neurons, increased neuronal loss and delayed postnatal development in rat pups (Silva et al., 2013), increasing susceptibility to epilepsy and hyperactivity, in addition to cognitive deficits in the offspring (Vesoulis et al., 2016; Fazeli et al., 2017; Zappettini et al., 2019). Caffeine was found to induce failure in neural tube closure in the cultured chick embryos (Marret et al., 1997). The same results were obtained in another study on rat embryos where 91% of embryos showed a failure of neural tube closure, specifically at the caudal region (Wilkinson and Pollard, 1994). In the same context, Ma et al. (2012) reported that in ovo injection of high doses of caffeine led to neural tube defects in chick embryos. The authors speculated this defect to an impaired elevation of the neural folds during the formation of the neural tube, in addition to defective migration and delamination of the neural crest cells. Moreover, caffeine treatment reduced the number of the proliferating neurofilament positive cells and decreased the length of their projections, thus inhibiting neuronal proliferation and differentiation. The severity of caffeine effects on neural tube defects was found to be dose-dependent (Li et al. 2012; Chian et al., 2021).

Previous studies have reported the deleterious effect of caffeine during pregnancy, though its consequence and mechanism of action on fetal neurodevelopment were obscure. Abd El-Wahab and El-Dakdoky (2013) assumed that caffeine may interfere with, and interrupt neurotransmitters responsible for maturation signals needed for brain development evidenced by increased dopamine levels in the fetal brain which leads to disorders in the developing fetus. Monoamine neurotransmitters regulate neurodevelopment and play important role in the architecture of a nervous system, possibly via maternal-fetal interactions, and whose interruption may have long-term adverse effects on mentality and behavior (Li et al., 2012). Monoamine neurotransmitters are expressed in the early embryogenesis process and increase gradually as embryonic development proceeds (Herlenius and Lagercrantz, 2004). These are responsible for transmitting maturation signals to different organs during development, any interruption in this process can result in fetal deficits (Lauder, 1993). Li et al. (2012) investigated the effect of caffeine administration on monoamine neurotransmitters and neural development using chick embryos. The authors found that caffeine administration led to the failure of neural tube closure, disrupted the development of a serotonergic system, and increased the risk of teratogenicity. The authors also showed that caffeine was accumulated in the brains of chick embryos without metabolization and concluded that caffeine can cross the blood-brain barrier and accumulate without being metabolized. Fetuses can’t metabolize caffeine, possibly due to the reduced activity of cytochrome P450 1A2 enzyme during pregnancy, which is one of the important enzymes needed to control caffeine metabolism (Singh et al., 2009). In addition, Zeidán-Chuliá, et al. (2013) attributed the neurotoxicity of caffeine to disruption of redox homeostasis. Other reports ascertained the neurotoxic effects of high doses of caffeine, either in vitro or in vivo, through neuronal cell death induction, possibly via activating the caspase-3 pathway, ROS production, and inhibition of
the Nrf2 pathway (Gepdiremen et al., 1998; Kang et al., 2002; Alasmari, 2020; Chian et al., 2021).

Among the phytochemicals of green coffee is gallic acid. The present study showed that prenatal administration of the high dose of green coffee induced hemorrhage in the cerebral cortex, cerebellum, and spinal cord of the developing rat fetuses. Gallic acid has been reported to be teratogenic at early embryonic development and induced severe cerebral hemorrhage in E9.5 embryos in a dose-dependent manner (Hsieh et al. 2015). This cerebral hemorrhage was attributed, partly, to the strong prooxidant nature of gallic acid (Lee et al., 2005; Eslami et al., 2010). Additionally, Severino et al. (2009) admitted that gallic acid increases the radical intensity and transforms the redox state to an oxidative environment, increasing the formation of Oxygen radicals, generating large amounts of ROS. It has been reported that a small concentration of gallic acid, about 1.02 mg/kg or more, can induce teratogenicity and result in cerebral hemorrhage. This concentration of gallic acid could be easily consumed in tea or coffee, imposing risk to the developing fetus (Hsieh et al. 2015). In addition to gallic acid, other polyphenols, present in the coffee extract, are considered to be ROS releasing compounds. For example, pyrogallol containing (–)EG) and (–)-EGCG can produce superoxide radicals from molecular oxygen (Kondo et al., 1999). Moreover, Boots et al. (2007) reported that orthoquinones from pyrogallol and catechol can produce ROS. All of these redox interactions can alter many biological functions during development, such as the cell cycle and apoptosis (Hsieh et al. 2015).

Another proposed mechanism of action of green coffee on fetal development is that caffeine decreases the zinc content and absorption in the developing fetal brain. Moreover, zinc content was restored after zinc supplementation in the maternal diet. It has been reported that zinc deficiency during pregnancy is associated with increased risks of fetal malformation, including neural tube defects (Moghimi et al., 2017; Brion et al., 2021). Previous animal studies reported that prenatal caffeine consumption could induce altered sleep and locomotion, anxiety, and learning abilities, in addition, it may interfere with brain zinc absorption (Cortés-Albornoz et al., 2021).

It is noteworthy that the induced neurotoxic effect of green coffee in the present study was dose-dependent. A recent systematic review admitted that caffeine could increase or decrease seizure susceptibility depending on many factors, such as dose, duration, and developmental stage at which caffeine is administered. Preterm infants exposed to high doses of caffeine showed a high incidence of seizures, moreover, caffeine administration decreased the efficacy of antiepileptic drugs (Vesoulis et al., 2016; van Koert et al., 2018).

**Conclusion**

The findings of the current study showed that in utero exposure of green coffee induced teratogenic effects in the developing rat fetuses and altered the neurodevelopment of different parts of the CNS in a dose-dependent manner. However, despite the current findings, further studies are needed to investigate the possible mechanisms of green coffee-induced neurotoxicity during pregnancy and assess its long-term effects on cognition and behavior, and develop new potential neuroprotective strategies to minimize the induced neurotoxicity. Nonetheless, it is important to increase public consciousness regarding the deleterious effects of green coffee and other psychoactive beverage consumption during pregnancy. It is recommended to avoid ingestion of large quantities of coffee during pregnancy, in addition, more attention
is needed to carefully monitor its impact during the critical periods of immature brain development.

Ethical Approval

All the experiments were conducted in compliance with the guide for the care and use of laboratory animals approved by the Faculty of Science, Menoufia University, Egypt (Approval No. MNSE2203).

Consent to Participate
Not applicable

Consent to Publish
Not applicable

Authors Contributions

AM conceived, designed, and carried out the experiments, analyzed data, interpreted the results, and wrote the manuscript. EA performed an HPLC experiment and analysis. All authors discussed the results and commented on the manuscript.

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The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material

All data supporting this work is included the manuscript. The corresponding author is responsible for supplying any additional data.

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