Original article

Effect of prenatal exposure of green tea extract on the developing central nervous system of rat fetuses; histological, immune-histochemical and ultrastructural studies

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ABSTRACT

Although, several health benefits were associated with green tea, these effects may be beneficial up to a certain dose. Higher doses of green tea may cause several adverse effects. So, there is a need to test the potential negative effects of green tea during pregnancy. This study was designated to evaluate the effect of prenatal exposure of green tea extract on the development of the central nervous system of 20-day old rat fetuses. The pregnant rats were divided into 4 groups; the control group (received distal water) and the other 3 groups received green tea extract at different doses (200, 600 & 1000 mg/kg/day, respectively) from the 6th to 15th day of gestation i.e., during the organogenesis phase of development. Cerebral cortex, cerebellum and spinal cord specimens were subjected to histological, immunohistochemical and ultrastructure investigations. The body weight of both mothers and fetuses was significantly decreased in the groups that received 600 and 1000 mg green tea extract. Also, the neuronal tissues displayed various signs of degeneration which were evident with the 600 and 1000 mg doses. Green tea extract also increases the glial fibrillary acidic protein (GFAP) and decreases the proliferating cell nuclear antigen (PCNA) which were directly proportional with increasing the dose. Administration of green tea extract during rat organogenesis period induced various histological, immunohistochemical and ultrastructural degenerative changes in the cerebral cortex, cerebellum and spinal cord of 20-day old rat fetuses. These deleterious changes were directly proportional to increasing the green tea extract dose. Thus, it should be stressed that the effect of green tea is dose-dependent and therefore it can be either beneficial or adverse.
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1. Introduction

Tea is one of the most widely consumed beverages, which are commonly used all over the world and its medicinal properties have been widely explored (Yang and Landau, 2000). It is one of the most consumed herbs among pregnant women in the Middle East due to its relaxing features (John and Shantakumari, 2015). Generally, tea is consumed in the form of green, black and oolong tea, which are all derived from leaves of *Camellia sinensis* (*Theaceae* family), a small plant grown mainly in China, Japan and Southeast Asia. Green and black teas are differently manufactured. The freshly harvested leaves are steamed at high temperatures, rolled and then dried (Chopade et al., 2008).

Tea contains many polyphenolic components like epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG). These polyphenols may have antioxidant, anti-inflammatory, anti-carcinogenic and antimicrobial properties in numerous humans, animals either whole or *in vitro* studies (Alschuler, 1998; Pradhan and Dubey, 2019). Green tea contains other compounds such as flavanols, glycosides, chlorogenic acid, carotenoids, quinic acids, trigalloylglucose, protein, lignin, chlorophyll, minerals (aluminum or manganese, depending on

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the soil content), caffeine and methylxanthines (Wang et al., 2013; Baláži et al., 2016). Green tea can be used as dietary supplements in the form of a brewed drink or capsular extract (Sarma et al. 2008).

Green tea has several benefits, but there are hazardous side effects associated with its excessive use. These negative effects are mainly owing to its contents of caffeine, aluminum, and iron bioavailability due to polyphenols (Chacko et al., 2010). Green tea catechins have the potential to affect the absorption and metabolism of ions because flavonoids interact with a variety of metal ions (Mira et al., 2002). Costa et al. (2002) indicated that tea plants can accumulate high levels of aluminum which were responsible for neurological diseases.

Too much amount of caffeine (including caffeine from green tea), may cause nausea, vomiting, diarrhea, headache, and loss of appetite (Sarma et al., 2009; Emily Creasy, 2013; Nawab and Farooq, 2015). Central nervous system (CNS) stimulation like tremors, dizziness, confusion, restlessness and psychomotor agitation was associated with overconsumption of green tea with caffeine. Furthermore, caffeine can affect the CNS by acting as a potent adenosine receptor antagonist (Saito et al., 2011). In pregnancy, consumption of caffeine can cause adverse effects to the fetuses such as spontaneous abortion, low birth weight, increased risk of premature rupture of membranes and intrauterine growth retardation (Pacheco et al., 2008). Moreover, whole animal and *in vitro* studies have shown that green tea components were associated with many negative effects such as dermal and eye irritation (Isbrucker et al. 2006; Stratton et al. 2000). Also, consumption of four to nine cups/day of green tea have been associated with increased human lung cancer risk (Tewes et al. 1990), teratogenicity such as anencephaly and neural tube defects (Correa et al., 2000), the toxicity of the intestine and hepatotoxicity (Johnson et al. 1999; McCormick et al. 1999; Isbrucker et al. 2006; Teschke and Xuan, 2019). Large quantities of caffeine were consumed by mothers during pregnancy and lactation because it is desirable and has a psychostimulant effect.

Green tea extract (GTE) has not been tested in fetal CNS development, so the objective of this study was to evaluate the possible neurotoxicity of prenatal exposure to different doses of GTE during the organogenesis phase of embryonic development i.e., from the 6th to the 15th day of gestation. The planned aim included describing the histological, immunohistochemical and ultrastructural changes in the neuronal tissues i.e., cerebral cortex, cerebellum and spinal cord of 20-day old rat fetuses.

2. Materials and methods

2.1. GTE preparation:

Green tea was purchased from the local market at Shebeen El-Koom, Menoufia, Egypt. 250 g of green tea leaves were macerated using 1500 ml boiled deionized water for 15 min, then filtered to obtain the aqueous extract. The extract was then evaporated to dryness under reduced pressure conditions. Before administered to pregnant rats, the tea extract was dissolved in water at the dose of 200, 600 and 1000 mg/kg (Chengelis, et al., 2008; Morita, et al., 2009).

2.2. Animals and grouping

Principles of animal care and use were carefully followed during conducting the present study according to the guide for the care and use of laboratory animals approved by the faculty of science, Menoufia University, Egypt (Approval No. MNSE2199) and according to the National Institutes of Health guide for the care and use of laboratory animals (NIH publications No. 8023, received 1978).

Eighty healthy mature virgin females (60) and fertile males (20) of Westar albino rats (Rattus norvegicus), weighing 200 ± 15 g, were obtained from Vacsera (Helwan Animal Breeding Farm), Ministry of Health, Cairo, Egypt. Rats were kept in the laboratory for at least one week before initiation of the experiments for acclimatization. They were housed in suitable plastic rodent cages and maintained at 25 ± 2 °C in 12 h light and dark cycle. Free access to water and a standard diet was supplied. Mating was performed overnight by housing the females with the males at a ratio of one male with two females. The day at which vaginal smear was positive has been considered day zero of pregnancy. Injection started from the 6th to 15th day of gestation and day 20 was determined as the endpoint for experimentation (El-Borm et al., 2021). The weight (g) of both mothers and their fetuses of the control and experimental groups was recorded.

The pregnant rats were divided equally (15 in each group) into four groups as follows:

1) Control, orally administrated 1 ml distilled water.
2) GTE (A) group, given oral administration of 200 mg/kg.
3) GTE (B) group, given oral administration of 600 mg/kg.
4) GTE (C) group, given oral administration of 1000 mg/kg.

A total of 110 fetuses were produced and subjected to investigation.

2.3. HPLC analysis:

HPLC analysis was carried out using an Agilent 1260 series. The separation was carried out using the Kromasil C18 column (4.6 mm × 250 mm i.d., 5 μm). The mobile phase consisted of water (A) and 0.05% trifluoroacetic acid in acetonitrile (B) at a flow rate of 1 ml/min. It 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 injection volume was 10 μl for each of the sample solutions. The column temperature was maintained at 35 °C. The phenolic acids most consistently found in GTE are listed in Table 1.

2.4. Investigated parameters

2.4.1. Histological investigation

For light microscopic examination, specimens of the fetal brain cortex, cerebellum and spinal cord from both control and experimental groups were separated and immediately fixed in 10% buf-

Table 1
HPLC quantitative identification of phenolic acids in GTE.

No.	Retention time (RT)	Compound name	Molecular formula (M.F)
	Phenolic acids of GT aqueous extract Contents (μg/g Extract)		
1	3.310	Gallic acid	$C_7H_6O_5$
2	4.020	Chlorogenic acid	$C_{16}H_{18}O_9$
3	4.462	Catechin	$C_{15}H_{14}O_6$
4	5.659	Methyl gallate	$C_8H_8O_5$
5	6.005	Coffeic acid	$C_9H_8O_4$
6	6.299	Syringic acid	$C_9H_{10}O_5$
7	7.237	Pyro catechol	$C_8H_{12}ClNO_2$
8	7.489	Rutin	$C_{27}H_{30}O_{16}$
9	7.919	Ellagic acid	$C_{14}H_6O_8$
10	8.981	Coumaric acid	$C_{15}H_{18}O_8$
11	9.910	Vanillin	$C_8H_8O_3$
12	10.148	Ferulic acid	$C_{10}H_{10}O_4$
13	10.281	Naringenin	$C_{15}H_{12}O_5$
14	12.427	Taxifolin	$C_{15}H_{12}O_7$
15	14.474	Cinnamic acid	$C_9H_8O_2$
16	14.705	Kaempferol	$C_{15}H_{10}O_6$

fered formalin, washed in tap water, transferred to 70% ethanol, dehydrated in ascending series of ethanol, cleared in xylol, and embedded in molten paraffin. 5 μm sections were obtained using a rotary microtome (Leica, Model Rm 2125, Germany) then stained with Ehrlish's hematoxylin and aqueous eosin (Suvarna et al., 2018). Microscopical examination and photographing were performed using an Olympus microscope (BX41).

2.4.2. Immuno-histochemical investigation

Immunohistochemical staining involving proliferating cell nuclear antigen (PCNA) and glial fibrillary acidic protein (GFAP).

Paraffin sections were deparaffinized, rehydrated and the avidin–biotin-peroxidase rena complex method (Cattoretti et al., 1993) was used to determine immunoreactivity as follows: rinsing the sections in 0.01 M citrate buffer (PH 6.0) for 10 min to unmask the antigen; blocking the endogen peroxidase by incubation of sections in 0.3% hydrogen peroxide in methanol; incubation with 20% normal goat serum to block the non-specified associating of secondary antibodies; incubation with a solution of primary antibodies (1:100 monoclonal mouse anti GFAP ICN Pharmaceuticals; monoclonal anti-PCNA Boehringer Manheim 1:100) in PBS with 1% BSA (bovine serum albumin), 1 h; incubation with appropriate secondary antibody conjugated by biotin for 1 h; incubation with avidin–biotin-peroxidase complex for 30 min and stained reaction diaminobenzidine (DAB)–H_2O_2 for 5 min. In between all incubations, the rinsing in PBS was performed. Negative controls were processed according to the same protocol, except for the use of the primary antibody. Digital images were analyzed by a semi-quantitative scoring system (Fiji-Image J software, Java-based application for analyzing images). The brown stained immune-histochemical expressions of PCNA and GFAP were analyzed and the percentage-colored stained area (area fraction) per field area was determined by measuring six randomly photographed high-power fields (X400 magnifications) (Schindelin et al., 2012).

2.4.3. Transmission electron microscopy (TEM) investigation

Fetal brain cortex, cerebellum and spinal cord tissues were immediately fixed in 2.5% glutaraldehyde and 4.0% paraformaldehyde diluted in 0.1 M sodium cacodylate buffer for 24 h at 4°C and processed as described in El-Borm et al. (2021).

2.4.4. Statistical analysis

All data were expressed as means ± SEM. Differences between groups were evaluated by a one-way ANOVA test using a statistical package of social science (IBM SPSS) statistics software for windows, Version 22 (IBM Corp., Armonk, NY USA) followed by an LSD test for multiple comparisons. The significances were expressed as P < 0.05 and highly significant at P < 0.001.

3. Results

3.1. Mothers body weight gain

Figure (1a). Illustrated the effect of different concentrations of GTE on the average body weight gains per day (g) of the pregnant rats. The control group exhibited the highest weight gain per day (g) when compared to the GTE groups. The body weight gain reduced in the GTE groups compared with the control. Although these differences were statistically insignificant in GTE (A) but were significant in GTE (B) & (C) groups.

3.2. Body weight of fetuses

The body weight of 20-day old fetuses from the GTE (A) group exhibited an insignificant decrease (4.6) compared to the control

group (5.2). The other GTE (B) & (C) groups showed a highly significant decrease in body weight (3.6 & 2.8, respectively) compared with the control (5.2) (Fig. 1b).

3.3. Histological observation

3.3.1. Cerebral cortex

The control fetal cerebral cortex showed normal histological structure, as it consisted of five basic zones i.e., a cellular marginal zone (MZ), cell dense cortical plate (CP), intermediate zone (IZ) with linear arrays of cells, subventricular zone (SVZ) with densely packed neuronal cells and ventricular zone (VZ) with proliferated pseudostratified columnar epithelium which located close to the ventricular cavity of the brain. (Fig. 2a). The cerebral cortex contained different types of cells which are granular, astrocytes, microglial and pyramidal. The neuropil appeared homogenous without any vacuolation (Fig. 2b). The cells of the cerebral cortex of fetuses injected with a low dose of GTE (A) appeared with little signs of degeneration. Few granular cells appeared vacuolated with degenerated nuclei while other cells like astrocytes and microglial were shrunken with pyknotic nuclei. The surrounded neuropil showed some vacuolation (Fig. 2c&d). On the other hand, the fetal cerebral cortex from the GTE (B) and (C) groups displayed disruption in the layer architecture and evident neuronal cell degeneration compared with the control group. Cell necrosis was detected in different parts of the fetal brain (Fig. 2e&f). Vacuolation and pyknosis were prominent in the neuronal cells especially that of VZ and SVZ. The neuropil exhibited extensive vacuolation. Group C showed shrunken neuronal cells with small darkly stained nuclei, and there was an evident decrease in the cell number of VZ and SVZ. Moreover, collagen-like substances appeared in wide areas of the cerebral tissue (Fig. 2g&h).

3.3.2. Cerebellum

The cerebellum from the control group consisted of four layers which were the outer granular layer covering the surface of the cerebellum, the immature molecular layer with few numbers of basket cells, the Purkinje cell layer (ill-defined) with nearly mature cells and the thick inner granular layer with granule cells (Fig. 3a&b). H&E-stained areas of green tea injected groups showed different degrees of multifocal neuronal affection. GTE (A) group displayed a moderate degeneration of the cerebellar cells. A few Purkinje cells appeared shrunken. The inner granular cells showed many vacuoles and pyknotic nuclei. The molecular layer was less affected (Fig. 3c&d). GTE (B) & (C) groups showed severe disorganization of the cerebellar layers. In group B, the molecular layer became shrunken and ill-defined, while the Purkinje cells revealed marked degenerative changes, where the monolayer arrangement of them was disrupted. Irregular deposition of these cells was observed and was mostly pyknotic. The granular cells were shrunken with pyknotic nuclei and surrounded by many vacuoles (Fig. 3e&f). In GTE (C) group, most of the cerebellar cells appeared both shrunken and pyknotic. Moreover, the molecular layer appeared ill-defined, and most Purkinje cells were shrunken with an abnormal outline, while some Purkinje cells showed complete karyolysis. The monolayer arrangement of the Purkinje cells was sparse and disrupted in many areas and migrated downwards in the granular layer. Some degenerated and necrotic areas were seen in the granular layer which lost the normal organization. The surrounding neuropil showed severe vacuolation (Fig. 3g&h).

3.3.3. Spinal cord

The spinal cord of the control group showed a normal structure of white matter and gray matter. The glial cells and neurons were found in the gray matter and the axons of neurons and some neuroglial cells were in the white matter (Fig. 4a). Green tea-injected
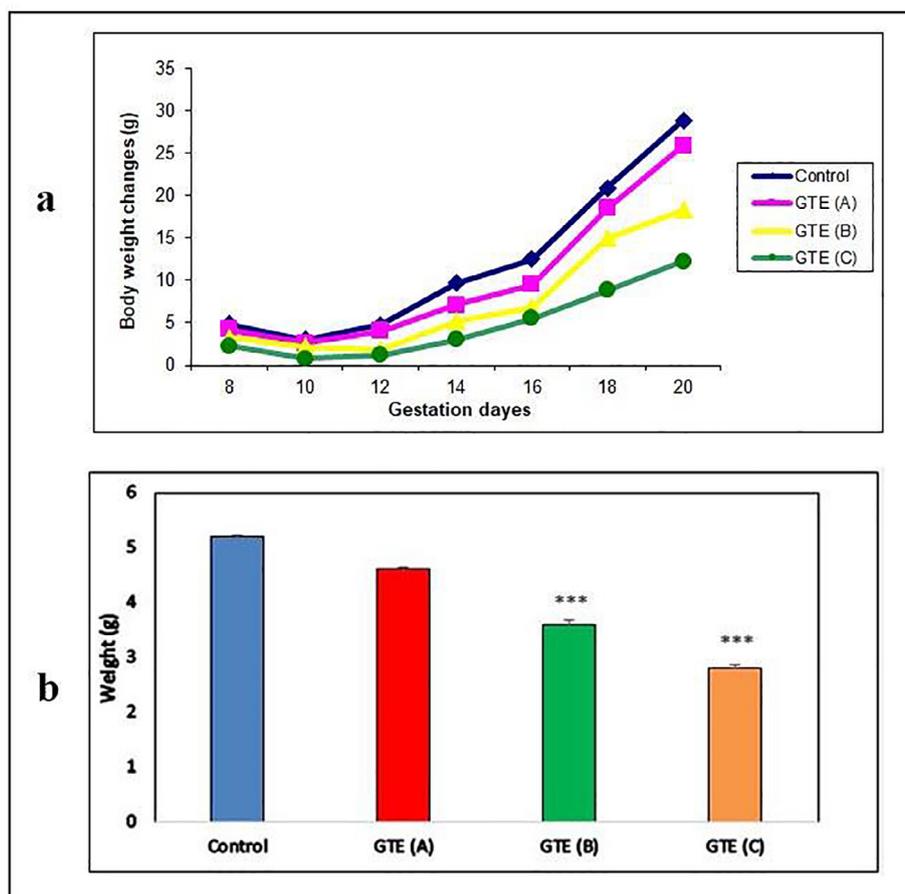
Fig. 1. Graphs showing: **(a)** changes in the average body weight gains per day (g) of mothers. **(b)** Body weight of 20-day old rat fetuses of different groups. Data are represented as mean ± SEM. Asterisks (***P > 0.001) highly significant compared with the control group.

groups showed various degrees of neurodegeneration. In GTE (A) and (B) groups, most neurons appeared vacuolated. Also, some pyknotic cells were noticed. Many cells lose the nuclear details and were surrounded by vacuolated neuropil especially in GTE (B) group. Other cells showed central chromatolysis (Fig. 4b&c). GTE (C) group showed intensive degeneration of the outer white matter and inner gray matter. There were many cells with degenerated nuclei and others with pyknotic nuclei. Also, more degenerated areas with huge sizes and number of vacuoles were noticed (Fig. 4d).

3.4. Immuno-histochemical observations

3.4.1. PCNA
3.4.1.1. Cerebral cortex. Immunohistochemically, PCNA-positive cells were detected mainly in the CP, SVZ, and VZ zones with a few PCNA-positive cells in IZ zone of the cerebral cortex of the control group. PCNA-positive cells decreased in the green tea treated groups and restricted mainly in VZ of the cerebral cortex, whereas the intensity of brown color significantly decreased gradually with the increase of green tea doses. The expression was highly decreased in both GTE (B & C) groups compared with the control group (Fig. 5a&b).

3.4.1.2. Cerebellum. The number of PCNA reactive cells in green tea-maternally treated fetuses (B group) was low significantly different from numbers in the control group which showed high expression in the four zones of the cerebellum. Both GTE (B & C) groups exhib-

ited a highly significant decrease in the PCNA-positive compared with control (Fig. 5a&b).

3.4.1.3. Spinal cord. Immunohistochemical expression of PCNA was increased in the neuroglial cells of the spinal cord of the control group. There was a low significant difference in the spinal cord of group B compared with the control. On the other hand, the PCNA positive cells in both GTE (B&C) groups were highly decreased compared with the control group (Fig. 5a&b).

3.4.2. GFAP
3.4.2.1. Cerebral cortex. Immunocytochemistry revealed the low intensity of brown immune-reactive areas, indicative of GFAP expression in the cerebral cortex of the control group. GTE (B&C) groups exhibited a highly significant increase in GFAP positive cells, whereas little change was observed in the GTE (A) group (Fig. 6a&b).

3.4.2.2. Cerebellum. The cerebellum of the control group showed few scattered positive fibrous astrocytes with thin processes in the molecular and granular layers, while green tea injected groups showed different degrees of glial fibrillary acidic protein expression. GTE (A) group showed little scattered positive immunostaining in the molecular and granular layers. While GTE (B) group revealed scattered positive immunostaining in the molecular and granular layers with the processes of many astrocytes. The cerebellar cortex of the GTE (C) group exhibited a more positive immunoreaction and seemed to be substantially larger within
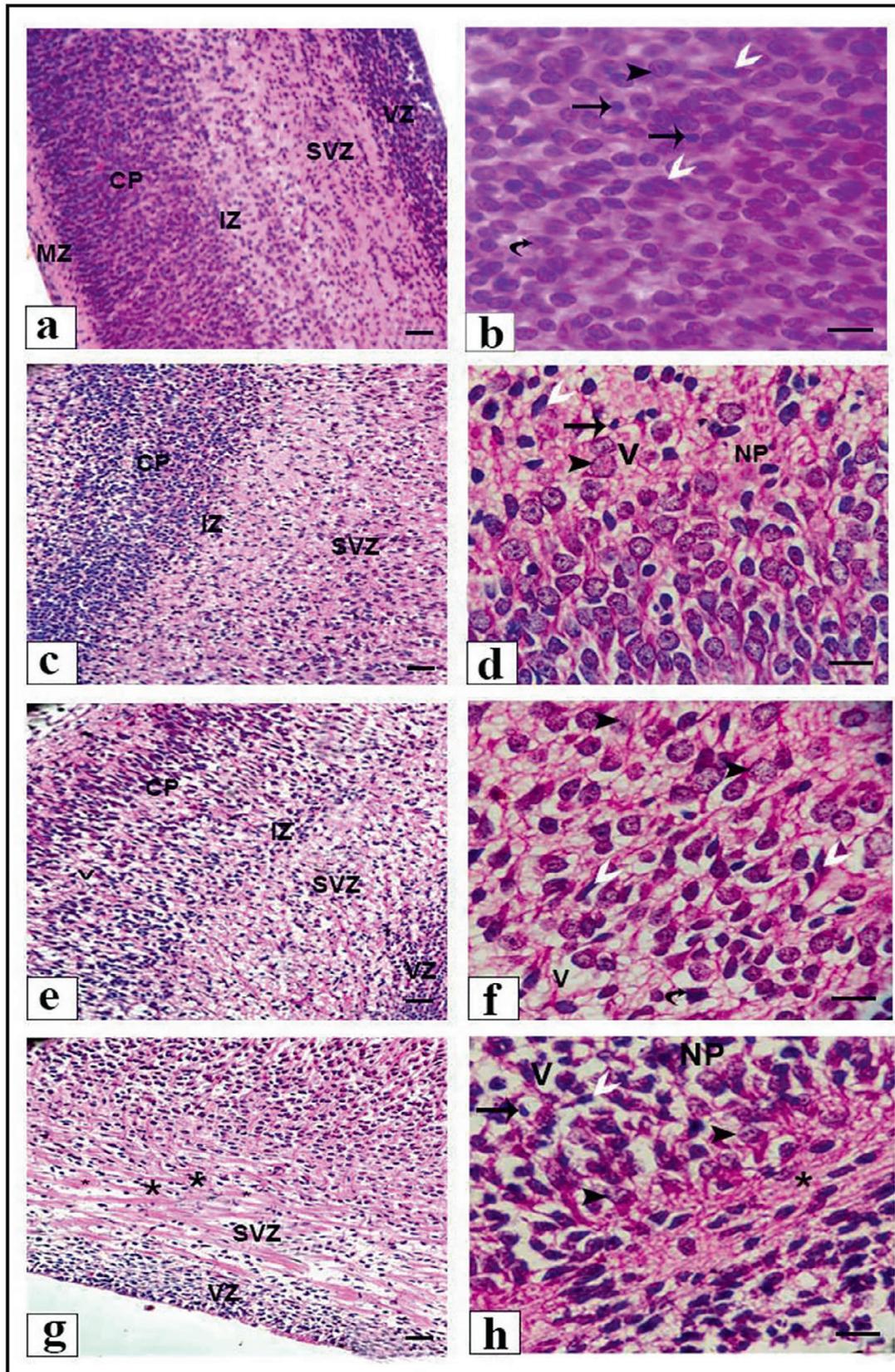
Fig. 2. Photomicrographs of cross-sections of the cerebral cortex of 20-day old rat fetuses. **(a-b)** control, **(c-d)** GTE (A), **(e-f)** GTE (B) and **(g-h)** GTE (C) groups showing marginal zone (MZ), cortical plate (CP), intermediate zone (IZ), subventricular zone (SVZ), ventricular zone (VZ), granular cells (Black Arrowhead), astrocytes (Arrow), microglial cells (White Arrowhead), pyramidal cells (Curved Arrow), degenerated granular cells (Arrowhead), pyknotic astrocyte (Arrow), pyknotic microglial cells (White Arrowhead), pyknotic pyramidal cells (Curved Arrow), vacuoles (V), neuropil (NP) and deposition of collagen-like substance (*). Scale bar = 0.059 mm (a, c, e, g) & 0.015 mm (b, d, f, h).
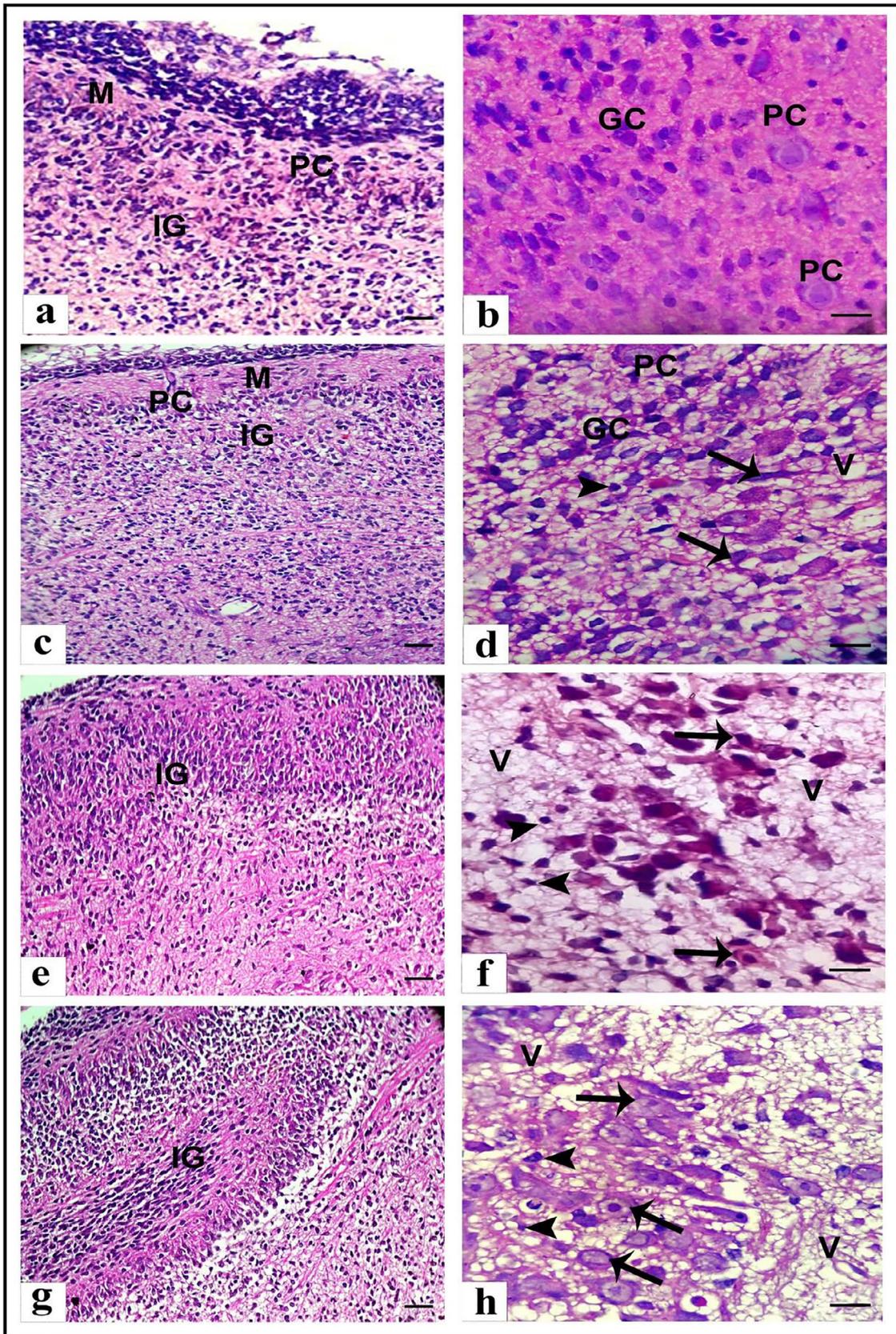
Fig. 3. Photomicrographs of sagittal sections in the cerebellum of 20-day old rat fetuses. **(a-b)** Control, **(c-d)** GTE (A), **(e-f)** GTE (B) and **(g-h)** GTE (C) groups showing molecular layer (M), Purkinje cell layer (PC), inner granular layer (IG) granular cells (GC), degenerated Purkinje cells (Arrow), pyknotic granular cells (Arrowhead) and vacuoles (V). Scale bar = 0.059 mm (a, c, e, g) & 0.015 mm (b, d, f, h).
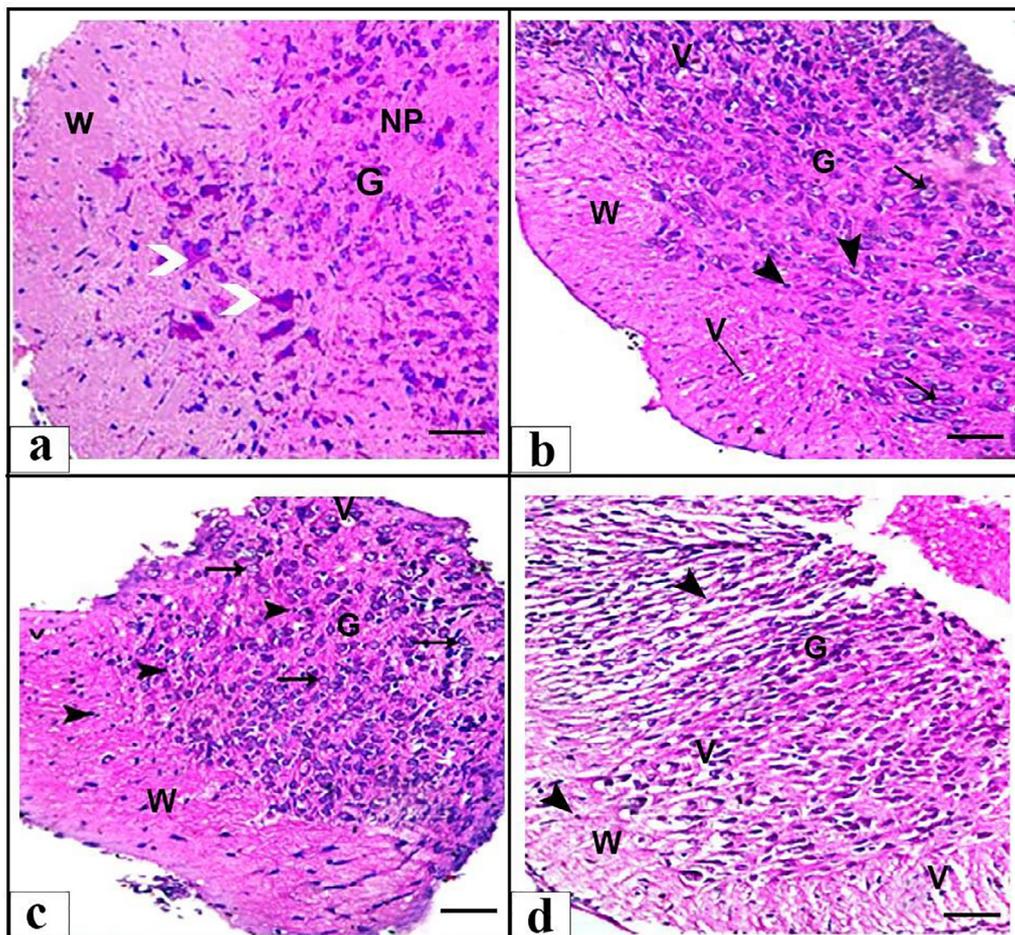
Fig. 4. Photomicrographs of transverse sections in the spinal cord of 20-day old rat fetuses. **(a)** Control, **(b)** GTE (A), **(c)** GTE (B) and **(d)** GTE (C) groups showing white matter (W), grey matter (G), neurons (White Arrowhead), neuropil (NP), vacuoles (V), vacuolated neurons (Arrow) and pyknotic neurons (Arrowhead). Scale bar = 0.059 mm.
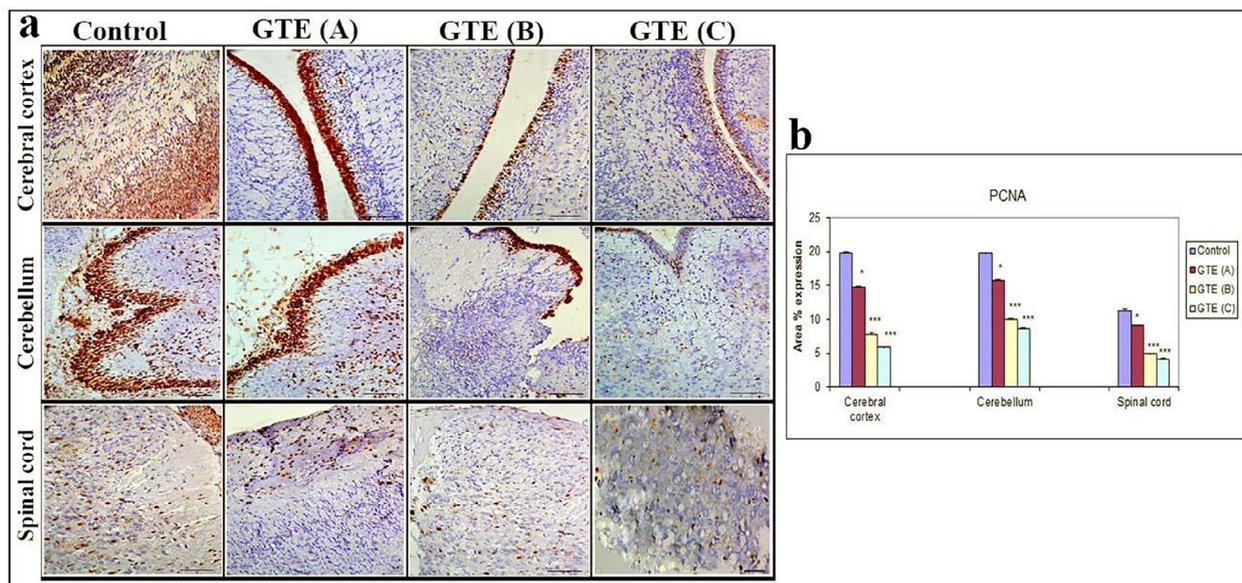
Fig. 5. Photomicrographs showing **(a)** immuno-histochemical localization of proliferating cell nuclear antigen (PCNA) in the cerebral cortex, cerebellum and spinal cord of 20-day old rat fetuses. GTE (A) group showed a moderate decrease in PCNA expression, GTE (B) and GTE (C) groups exhibited highly decrease in PCNA expression. **(b)** graph of the mean area % of PCNA expression in the cerebral cortex, cerebellum and spinal cord of different groups. Data are represented as mean ± SEM, (n = 6). Asterisks (***P > 0.001, *P > 0.05) refer to the P-value compared with the control group. Scale bar = 0.059 mm.
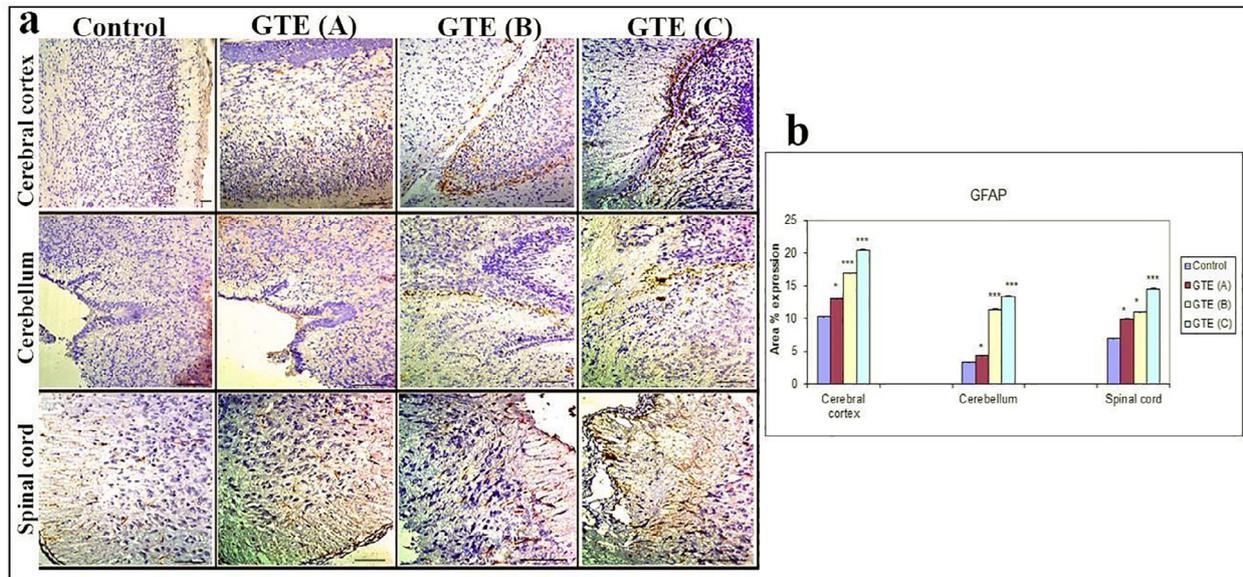
Fig. 6. Photomicrographs showing **(a)** immunohistochemical staining of glial fibrillary acidic protein (GFAP) for astrocytes and dendrites in the cerebral cortex, cerebellum and spinal cord of 20-day old rat fetuses. GTE (A) group showed a moderate increase of positive reaction of GFAP. GTE (B) and GTE (C) groups exhibited a strong positive reaction to GFAP. **(b)** graph of the mean area % of GFAP reaction in the cerebral cortex, cerebellum and spinal cord of different groups. Data are represented as mean ± SEM, (n = 6). Asterisks (***P > 0.001, *P > 0.05) refer to the P-value compared with the control group. Scale bar = 0.059 mm.

the 3 cortical layers, as well as apparent protoplasmic processes of many astrocytes (Fig. 6a&b).

3.4.2.3. Spinal cord:. In GFAP-stained sections, the control group showed few small astrocytes with short processes and a faintly brown color with GFAP immunostaining. The GTE (A&B) groups showed a significant increase in the number and size of astrocytes that had longer processes. On the other hand, the GTE (C) group displayed a highly significant increase in the number and size of astrocytes. These astrocytes have longer processes compared with the control group (Fig. 6a&b).

3.5. Ultrastructure observations

3.5.1. Cerebral cortex

Examination of the cerebral cortical cells of control fetuses showed a normal ultrastructural picture. The nerve cells contained large round euchromatic nuclei with prominent nucleoli and normal nuclear envelope, many rough endoplasmic reticula (rER), polyribosomes, spherical and elongated mitochondria and dense cytoplasm (Fig. 7a&b). The cortical neurons of green tea-treated groups exhibited different degenerative changes. The neurons of the GTE (A) group had irregular nuclei and nuclear envelopes folding and indentations. Other nuclei appeared shrunken with chromatin margination under the nuclear envelope. Areas of cytoplasmic loss are also seen (Fig. 7c&d). Most of the nerve cells of the GTE (B) group showed small pyknotic nuclei with very dense chromatin, fragmented nuclei and degenerated cytoplasm. The cytoplasm had degenerated mitochondria, swollen rER and the cell membrane appeared ruptured and discontinuous. The synaptic structures were disrupted, and the neuropil was swollen and degenerated (Fig. 7e&f). The cerebral cortical cells of the GTE (C) group exhibited severe degenerative changes. It had highly degenerated nerve cells with complete loss of architecture. The nerve cells exhibited evident nuclear degenerative changes which appeared in the form of small shrunken pyknotic, fragmented, or swollen nuclei. Most nerve cells appeared ruptured with dispersed organelles, swollen rER and mitochondria with destroyed cristae (Fig. 7g&i).

3.5.2. Cerebellum

The control group exhibited normal ultrastructure of the cerebellar cells. The granular cell layer contained many closed granular cells with large oval euchromatic nuclei. The surrounding cytoplasm was very little and contained organelles like mitochondria, rER and free ribosomes (Fig. 8a). Purkinje cells showed a nearly mature structure and contained large euchromatic nuclei with nucleoli. Their cytoplasm containing mitochondria, rER and free ribosomes (Fig. 8b). In GTE (A) group, the granular cells were similar to the control group. However, some granular cells had small pyknotic nuclei, dilated rER and degenerated mitochondria in their cytoplasm. The blood capillaries appeared congested with RBCs and the endothelial cytoplasm showed different size vacuoles (Fig. 8c&d). The Purkinje cells had abnormal irregular nuclei with deep indentation. Their cytoplasm showed some rarefied areas and contained small, packed mitochondria and a few fragmented rER. Also, the surrounding cell membrane appeared ruptured in some areas (Fig. 8e). GTE (B) group displayed more degeneration in both Purkinje and granular cells. The granular cells lost their closely packed organization. The majority of their cells appeared ruptured with irregular, pyknotic or fragmented nuclei, degenerated mitochondria and swollen rER (Fig. 8f&g). The Purkinje cells appeared shrunken with a fragmented nucleus. Their cytoplasm contained many degenerated mitochondria, and the other organelles could not be differentiated easily (Fig. 8h). The cerebellum of the GTE (C) group showed that most granular cells had degenerated nuclei which appeared either fragmented or pyknotic. The blood capillary appeared congested, and the endothelium had a shrunken nucleus and small vacuoles. Also, the neuropil around the capillaries was swollen. Other granular cells were ruptured with ill-defined organelles (Fig. 8i-k). The Purkinje cells had fragmented nuclei, rarefied cytoplasm and degenerated organelles (Fig. 8l).

3.5.3. Spinal cord

The spinal cord of the control group displayed a normal ultrastructure appearance. The spinal cord cells had regular euchromatic nuclei with finely dispersed chromatin, many rER, mitochondria, Golgi apparatus and many free ribosomes scattered
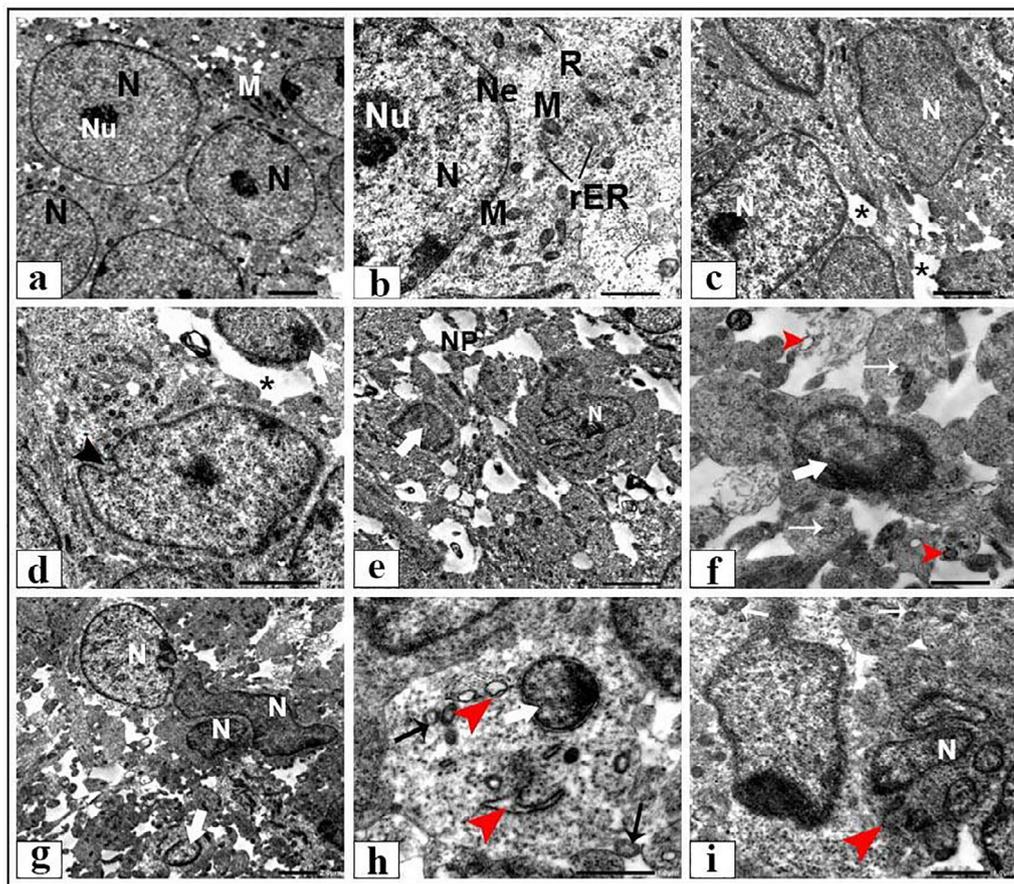
Fig. 7. Transmission electron photomicrographs of cerebral cortical cells of 20-day old rat fetuses. **(a-b)** control, **(c-d)** GTE (A), **(e-f)** GTE (B) and **(g-i)** GTE (C) groups showing nucleus (N) of nerve cells, nucleolus (Nu), nuclear envelope (Ne), mitochondria (M), rough endoplasmic reticulum (rER), ribosomes (R), cytoplasmic loose (*), nuclear envelopes indentation (Black arrowhead), swollen neuropil (NP), shrunken pyknotic nuclei (Thick white Arrow), fragmented nuclei (N), degenerated mitochondria (Arrow) and swollen rER (Red Arrowhead). Scale bar = 2.0 μm (a, c, d, e, g) = 0.1 μm (b, f, h, i).

between the organelles (Fig. 9a). The nerve cells of the GTE (A) group appeared with scanty cytoplasm containing a few ribosomes and several vacuoles and markedly irregular nucleus with condensed chromatin and degenerated mitochondria (Fig. 9b&c). Many of the nerve cells of the GTE (B) group were apoptotic and degenerative with condensed heterochromatic, shrunken nuclei and the cytoplasm contained some areas of vacuolation, lysosomes and degenerated mitochondria. Other Nuclei showed a marked indentation and chromatin condensation in the nucleus. In some nerve cells, less myelin sheath damage was seen (Fig. 9d-f). The ultrastructure of spinal cord cells in the GTE (C) group showed more degeneration. The cells had irregular shrunken and fragmented nuclei with condensed heterochromatin, vacuolated neuropil, many lysosomes, and empty spaces that could be detected in the cytoplasm. Their cytoplasm contained many degenerated mitochondria with crista loose, swollen rER, and a high degree of folding and delamination of myelin sheath laminae was detected (Fig. 9g-i).

4. Discussion

Studies on the effect of GTE on the development of the CNS are very limited. So, the present study was designated to explore the impact of perinatal exposure to three doses of GTE on the body weight of both mothers and fetuses, and the structure of the different parts of the rat fetal CNS. In the present study, the body weight gain of the pregnant rats was decreased gradually as doses increase. Also, the body weight of 20-day old fetuses was signifi-

cantly decreased in the GTE (B) and GTE (C) groups. This agrees with many other studies which stated that supplementation of polyphenols of green tea in water or GTE administration to rats caused body weight loss (Lu et al., 2012; Abo El-Khair et al., 2016). Similarly, Baláži et al. (2016) reported that green tea powder in the rabbit diet decreases the weight gains per week and the total average weight gains. Also, Morita et al. (2009) found that administration of two doses (600 mg/kg & 2000 mg/kg) of green tea catechins reduced the body weight gain of the pregnant rats. Moreover, Ajarem et al. (2017) demonstrated that prenatal administration of 20 and 50 g/L doses of green tea, significantly reduced the mice offspring's body weight. The explanation for body weight loss may be attributed to the presence of EGCG in green tea which reduces food intake, lipid absorption and stimulates the apoptosis of fat cells (Farooqui, 2012).

Tea has been reported to have beneficial effects, such as anti-cancer, antioxidant effects, anti-inflammatory and promoting weight loss but there are potential side effects which are shown on green tea high consumption (Tokunaga et al., 2002; Zhao, 2006). The nutritional benefits of tea owing to the presence of flavonoids. However, these flavonoids have been associated with some detrimental effects on human health when their consumption exceeds certain limits. The flavonoids toxicity can be attributed to the reactive oxygen species formation which causes damage to the DNA, lipid membranes and other biological molecules (Jain et al., 2013).

There is a widely held positive belief that causes women toa-reelieve that consumption of tea during pregnancy is a healthy
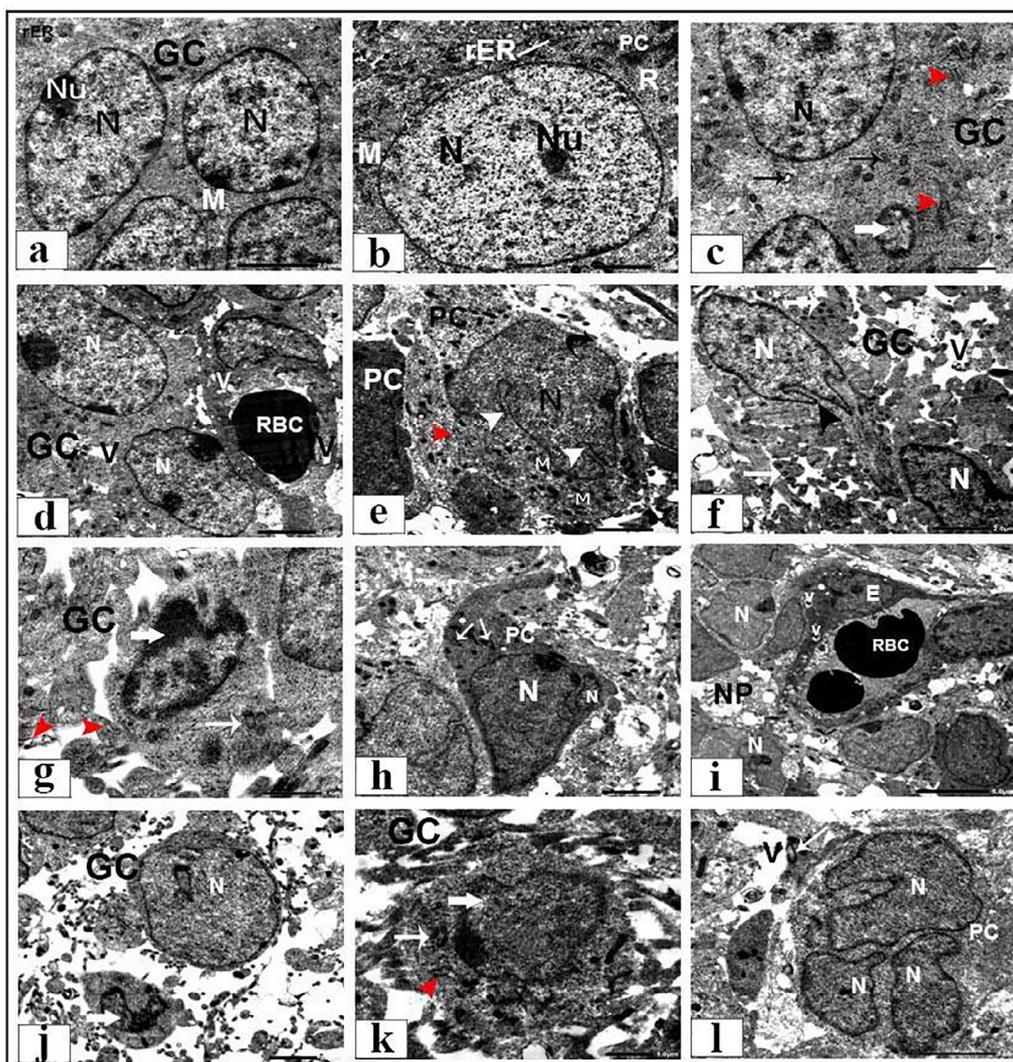
Fig. 8. Transmission electron photomicrographs of cerebellar cells of 20-day old rat fetuses. **(a-b)** control, **(c-e)** GTE (A), **(f-h)** GTE (B) and **(i-l)** GTE (C) groups showing granular cells (GC), nuclei (N), nucleolus (Nu), mitochondria (M), rough endoplasmic reticulum (rER), Purkinje cell (PC), ribosomes (R), shrunken nucleus (White thick Arrow), degenerated mitochondria (Arrow), swollen rER (Red Arrowhead), vacuoles (V), blood capillary conjected with red blood cells (RBC) nuclear indentation (White Arrowhead), small packed mitochondria (M), ruptured cell membrane (Curved Arrow), degenerated endothelial cell (E) with vacuoles (V) and degenerated neuropil (NP). Scale bar = 0.2 μm (a, b, d, e, f, h, j, l) = 0.1 μm (c, g, k) = 0.5 μm (i).

choice. Moreover, health care protors' recommendations on tea utilization during pregnancy is conflicting, although teas have been generally acknowledged to contain caffeine that related with negative pregnancy outcomes (Bakker et al., 2010). The present study demonstrated that prenatal exposure to GTE during rats' organogenesis caused adverse effects on the histological, immunohistochemical and ultrastructure of the fetal cerebral cortex, cerebellum and spinal cord if given in a high dose other than the reported optimum one. These effects were evident in the GTE high doses i.e., 600 & 1000 mg/Kg. It has been known that toxicity from GT occurs at high doses, but other harmful side effects were observed at lower doses also. Su-Yin (2009) concluded that caffeine is not the only factor responsible for GT toxicity. Other negative side-effects such as confusion, nausea, headache, and muscle pain were observed in people consuming decaffeinated tea. Dose-limiting side effects of GTEs have been observed in CNS, stomach and intestine (Chow et al., 2003; Inoue et al., 2011). It has been reported that the presence of xanthines, like caffeine, in tea was associated with various toxic effects such as convulsions and nervous irritability when given to a 7-week-old infant (Jain et al., 2013). In the same line, Correa et al. (2000) found that caffeinated

tea utilization by mothers during the *peri*-conceptional period was related to an increase in spina bifida rate, which could be a kind of NTDs, even though other caffeinated beverages were not related to that risk. It has been shown that caffeine easily crosses the placenta (Okubo et al., 2015) and accumulated in the brain of the fetus (Wierzejska et al., 2014). Additionally, the fetuses missing the enzymes responsible for caffeine metabolism, so that the half-life of caffeine in fetal tissue is more than fourfold that in adults and this may harm fetuses more than adults (Grosso and Bracken, 2005). Other detrimental and teratogenic effects of prenatal caffeine exposure on the newborn include learning and motility disabilities, anxiety, sleep disorders, craniofacial malformation, intrauterine growth retardation, neural tube defects (in high doses), and even abortion (Maslova et al., 2010; Greenwood et al., 2014; Li et al., 2016). Moreover, Shiraishi et al. (2010) demonstrated that besides caffeine, other tea substances, such as catechin, may influence the pathogenesis of NTDs.

It has been found that oolong tea or green tea consumption, with their EGCG, was related to the reduction of serum folate level, while black tea with little EGCG, did not affect the levels of serum folate (Nawab and Farooq 2015). Also, Augustin et al. (2009)
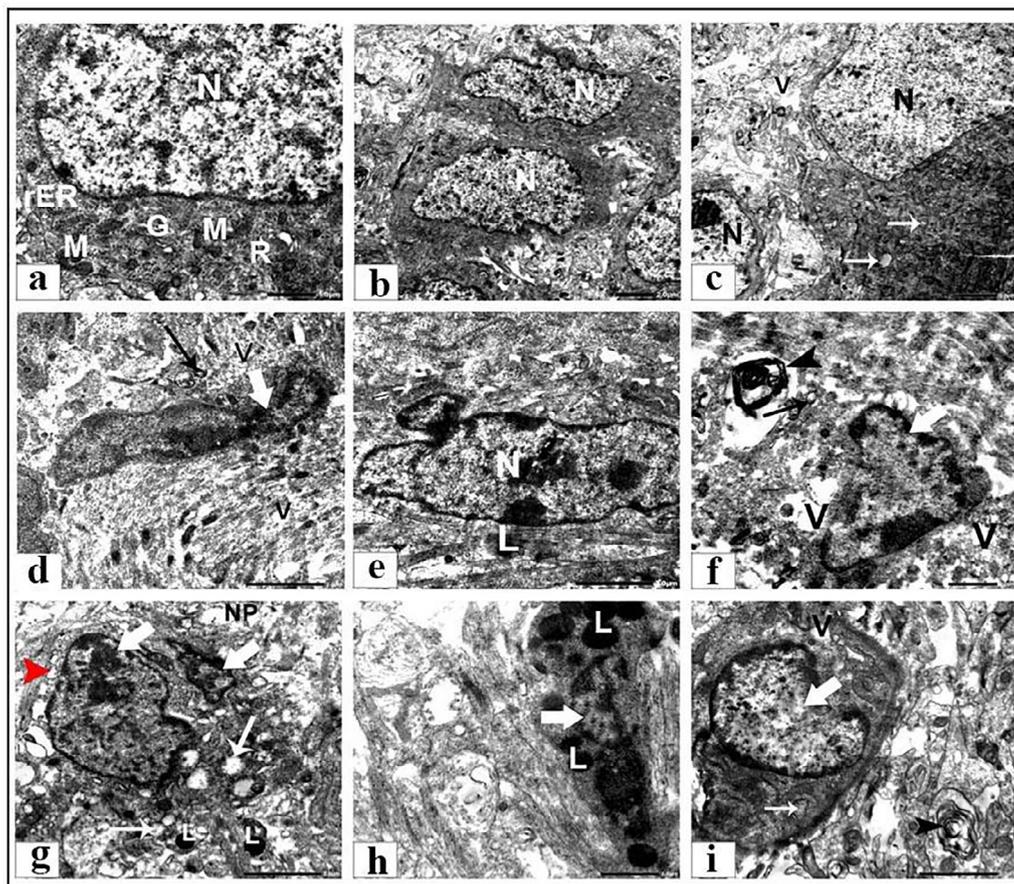
Fig. 9. Transmission electron photomicrographs of spinal cord cells of 20-day old rat fetuses. **(a)** control, **(b–c)** GTE (A), **(d-f)** GTE (B) and **(g-i)** GTE (C) spinal cells showing nucleus (N), mitochondria (M), rough endoplasmic reticulum (rER), Golgi apparatus (G), ribosomes (R), degenerated mitochondria (Arrow), degenerated nuclei which appeared either pyknotic with chromatin condensation (Thick Arrow) or nuclear indentation (N), vacuolation (V), lysosomes (L), demyelination area (Arrowhead), swollen rER (Red Arrowhead), vacuolated neuropil (NP), and myelin sheath wrinkle and demyelination (Arrowhead). Scale bar = 0.1 μm (a, f, h) = 0.2 μm (b, c, d, e, g, i) = 0.5 μm (i).

reported that consumption of green tea every day may influence folate levels in healthy peoples. Moreover, Lovblad et al. (1997) reported that folate and vitamin B12 deficiency can interfere in early brain development and function, by restricting the myelination and synaptic connectivity that occur early in life. Folate has many advantages in the body as it is needed for DNA making, protects against negative changes in the DNA from cancers, protects against many diseases and prevents anemia (Younes et al., 2018).

It was known that brain development mechanisms such as cell migration and synaptic formations could be affected by neurotoxins or environmental factors (Crandall et al., 2004; Salisbury et al., 2009). A study by Souza et al. (2015) indicated that caffeine consumption during pregnancy affects the development of neuromotor during brain development by interfering with cholinergic neurotransmission. Caffeine can pass through the placenta and concentrate in the fetal brain and could alter the CNS development and caused fetal growth retardation and even miscarriage (Mioranzza et al., 2014). A study by Correa et al. (2000) found that high maternal exposure to tea during the preconceptual period has been associated with developmental neural tube defects. Also, it has been reported that prenatal caffeine administration of 50 mg & 90 mg/kg/b.wt may induce fetal brain injury in 20-day old rat fetuses as it increased the number of hypertrophied astrocytes which is termed reactive astrocytes (Archibong et al., 2017). Besides, another study by Pintican et al. (2019) indicated that caffeine was associated with oxidative stress at the cerebral level. Archibong et al. (2017) reported that maternal administration of caffeine at a dose of 50 mg and 90 mg/kg caused pathological changes in the fetal and young cerebral cortex such as pyknotic nuclei, cell hypertrophy and vacuolations. Also, Tanaka et al. (1985) found that 0.04% maternal caffeine in drinking water during pregnancy caused a reduction in fetal cerebral weight and protein content. Furthermore, Nawab and Farooq (2015) reported that consumption of excessive green tea was associated with CNS stimulation such as insomnia, distraction, vertigo, tremors, impatience and psychomotor agitation.

Tannins like catechin and epicatechin in GT have been found to bind with non-heme iron in the body. This led to iron deficiency anemia because it interferes with the absorption of iron (Hamdaoui et al., 2003). A study by Dror and Allen (2008) revealed that newborn children of mothers with pernicious anemia shown neurodevelopment delay. Moreover, EGCG was found to has antifolate activity (Nawab and Farooq, 2015). Bhate et al. (2012) demonstrated that folate deficiency during pregnancy was positively correlated with offspring's brain development. Furthermore, it has been shown that folate deficiency during late gestation reduces progenitor cell mitosis and increases apoptosis in the fetal mice brain (Craciunescu et al., 2004). Similarly, Zhang et al. (2009) found that folate deficiency affects the neural stem cells in rat fetuses as it significantly decreases their proliferation and increases the apoptosis rate. Moreover, Costa et al. (2002) found that tea leaves can accumulate high levels of aluminum. Aluminum has been found to induce neurotoxicity in both human and animal studies (Shaw and Tomljenovic, 2013).

In this study, the fetal CNS of rats administrated 600 mg/kg and 1000 mg/kg of GTE during organogenesis showed a highly

significant increase in GFAP positive astrocytes. Astrocytes are neuroprotective cells and GFAP is a major component of its neurofilaments. Astrocytes and GFAP expression are upregulated in response to neurodegenerative diseases or exposure to neurotoxins. So, it was involved in neuron recovery and neuroinflammatory response in CNS pathology (Wang et al., 2006; Abo El-Khair et al., 2016). The main functions of astrocytes were providing nourishment, gases, metabolic support to neurons and remove the waste product between neurons in the CNS (Archibong et al., 2017). Moreover, Nakase et al. (2003) mentioned that astrocytes play a role in the regulation of ionic concentration for tight junctions and blood–brain barrier and this might reduce neuron apoptosis. The reactivity of astrocytes was associated with some morphological changes including hypertrophy, processes remolding and the overexpression of GFAP (Haim et al., 2015) so, this may explain, at least in part, the significant increase in GFAP expression in the neuronal tissues of the GTE (B&C) groups.

PCNA antigen determination is essential to clarify the biochemical pathways for the progression of the cell cycle. The PCNA is a member of the cyclin family which is a nuclear protein required for DNA replication and repair as it attaches to DNA delta polymerase. So, PCNA is abundantly expressed in proliferating cells and its presence was associated with the late G and S phase of the cell cycle (Ino and Chiba, 2000). In the present study, we used Anti-PCNA antibodies to determine the proliferative potential of fetal neuronal tissues. As mentioned before the PCNA expression was significantly decreased in the groups taken 600 mg & 1000 mg of GTE. This antigen localization proved that the high doses of GTE could decrease the proliferative capacity of the fetal neuronal tissue when administrated during rat organogenesis and this may serve as an early indication of abnormality.

It is concluded that prenatal exposure to GTE at doses of 200, 600 & 1000 mg/kg induced various deleterious changes in the cerebral cortex, cerebellum and spinal cord when administrated during the organogenesis phase of rats. Also, GTE was associated with a decrease in body weight of both mothers and fetuses. These deleterious changes were directly proportional to increasing the green tea extract dose. Thus, it should be stressed that the effect of green tea is dose-dependent and therefore it can be either beneficial or adverse.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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