ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Morphometric Assessment of the Cerebellum of the Pre-implanted albino Rats Embryos after Maternal Exposure to AgNPs

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Abstract

Cerebellum (cb) is the most important and sensitive part of the central nervous system (CNS) after cerebrum. The exposure to any infection during embryogenesis produces abnormalities in the cerebellum function and morphology that effect on behavioral of offspring later. In the present study we used 30 mature female pregnant albino rats divided in to three groups, each group contain 10 females: G1 was considered the control group received D.W only, while G2 group treated orally with (2mg/kg /day) suspension of silver nanoparticles (AgNPs) and G3 group treated orally with (20mg/kg/day) AgNPs. The embryos retrieved in different embryonic days from ED12 to ED21. In this study morphometric analysis was measured in the developing albino rats cerebellum after maternal exposure to two concentrations of AgNPs from pre-implantation period until delivery . we measured the morphometric parameters (area, perimeter, anterior-posterior diameter APD and transverse diameter TD) of the selected embryonic days; ED12,ED15,ED18, and ED21 and retrieved the embryos from these days. The morphometric results in the control group(G1) showed that increased significantly (P≤ 0.05) in the area of the developing cerebellum from ED12 until reach the optimal level in the end of gestation at ED21 (14.0815mm2 to 18.969 mm2 respectively), perimeter (9.804 mm2 to 16.267 mm2) and TD (52.6308µm to 431.6 µm),while the APD value was increased significantly (P≤ 0.05) at ED12 and ED15 only (367.0041 µm, 454.011 µm)respectively but the orientation growth of the cerebellum decreased significantly (P≤ 0.05) at the later stage of the development at ED18 and ED21(364.48 µm, 225µm) respectively. In the present research both concentrations of the AgNPs (2mg and 20 mg/kg/ B.Wt) showed changes in the dimensions of the developing cerebellum cortex according to the studied morphometric parameters; in the 2 mg/kg/ B.Wt, the area of the developing cerebellum cortex was increased significantly (P≤0.05) from ED12 (47.437mm 2) and reached to maximum growth at ED18 (130.220 mm2), while it is growth inhibited at ED21(68.909 mm2) compared to control group, the values of the developing cerebellum cortex perimeter increased significantly (P≤0.05) from ED12 until ED15 (0.964 mm 2, 2.548 mm 2) respectively compared to control group; TD values of the developing cerebellum cortex increased significantly (P≤0.05) from ED12 until ED15 also (259.341 µm,436.203 µm) respectively compared to control group. While the APD of the developing cerebellum cortex was decreased significantly (P≤0.05) during late development of the cortex at ED18 and ED21(330.415 µm, 131.5 µm) respectively as in the control group. In G3 group which treated with 20mg/kg/ B.Wt concentration of AgNPs, the similar results of area were showed as in the lower concentration, area of the developing cortex increased significantly (P≤0.05) from ED12(80.11mm 2) and reached to maximum growth at ED18(140.56 mm2) and lower after that. the values of the developing cerebellum cortex perimeter increased significantly (P≤0.05) from ED12 until ED15 (0.256 mm2, 0.543 mm2) respectively compared to control group; while in the APD and TD values the effect of the higher concentration of the AgNPs was more effect on these dimensions; APD values decreased significantly (P≤0.05) at ED15 (360.48 µm) and not reach to the complete growth, also in TD values decreased at ED15 (400.5744 µm) and do not reach to maximum growth at end embryonic development compared to the embryonic development timing in the control group.

Keywords: *AgNPs, Pre-implantation, Cerebellum, ED, Albino rats.*

Introduction

Nanotechnology is the most important engineering technique development since the industrial period [1]. With the rapid development of nanotechnology and its applications, numerous nanotechnology based on consumer products have become available [2]. Silver nanoparticles (AgNPs) are emerging as one of the most usually used nanomaterials.

AgNPs exhibit strong antimicrobial and anti-inflammatory activity; thus, they have become widely employed in medical applications, personal care products, building materials, food packaging, and textiles [3]. Many studies on distribution of AgNPs demonstrated that these particles were transported mainly to the liver and the spleen but they were also found in brain, heart, lungs, kidneys and testes.

The blood-brain barrier (BBB) constitutes a distinctive and tightly regulated interface between the brain and the peripheral circulation [4]. AgNPs may disrupt the blood–brain barrier (BBB) integrity and reach the brain and inducing neuronal cell death. Continuous accumulations of AgNPs even at very low concentrations may result in neuronal degeneration and necrosis as AgNPs were proved to be highly neurotoxic [5].

To understanding how AgNPs induce the BBB dysfunction and the loss of brain protection could enable to develop nanomedical treatment and help to ensure that nanoparticles, which are not projected to reach the brain and do not cause adverse effects. Silver nanoparticles AgNPs are known to penetrate into the brain and cause neuronal death. Though, there is a small number studies examining the effect of AgNPs on the resident immune cells of the brain, microglia. Given microglia are implicated in neurodegenerative disorders such as Parkinson's disease (PD) as [6].

Material and Methods

Animal Housing (Mating the Animals and Timing of Pregnancy)

In this study, 30 mature female Sprague-Dawley albino rats were used. They were purchased from animal house of national center for drug control and research.

The averages of weights were between 100-300g.All animals housed in the animal house of the department of Biology, College of Science- University of Baghdad, in plastic cages with a metal network cover under climate conditions, with temperature 22±2°C. Cages were cleaned and sterilized in different time with 70% ethanol. Rats were provided with water and food ad libitum daily. In this study after isolation of the sexually mature females which at the estrous stage by examine the vaginal smears under light microscope.

The isolated females put in breeding cages (each 2-3 females with one mature male) and left overnight. In the next morning, copulation was confirmed by examining the vaginal smears. The gestational day zero was defined as the day when spermatozoa were observed in the smear of the vaginal smears, and then females were transferred to separate cages without males and stay until the appropriate days to isolate the embryos [7].

Silver Nanoparticles (AgNPs) Preparation

AgNPs were used in this study, it was purchased as grey black solid powder (purity 99.9 %, apparent density: 0.97g /ml, tap density: 2.16 g/ml and CAS NO.: 7440-22- 4) with an average diameter of (40-60) nm in diameter. AgNPs were prepared at a two concentrations, low concentration 2 mg/kg of body weight and high concentration 20mg/kg of body weight according to (3).

The AgNPs stock solution was prepared by suspending the calculated weight of AgNPs powder in a certain volume of deionized distilled water D.D.W in a sterile glass universal tube. The suspension was exposed to the ultrasonication technique by ultrasonic water bath for 2-3h in dark and under biological safety.

Characterization of AgNPs

Silver nanoparticles were characterized by scanning probe microscope (SPM) by using granularity cumulation distribution report, the average diameter in sample contain 260 grain was (40-60 nm) as the following report (Figure 1) and the spherical shape

nanoparticles under SPM were showed in) Figure 2).

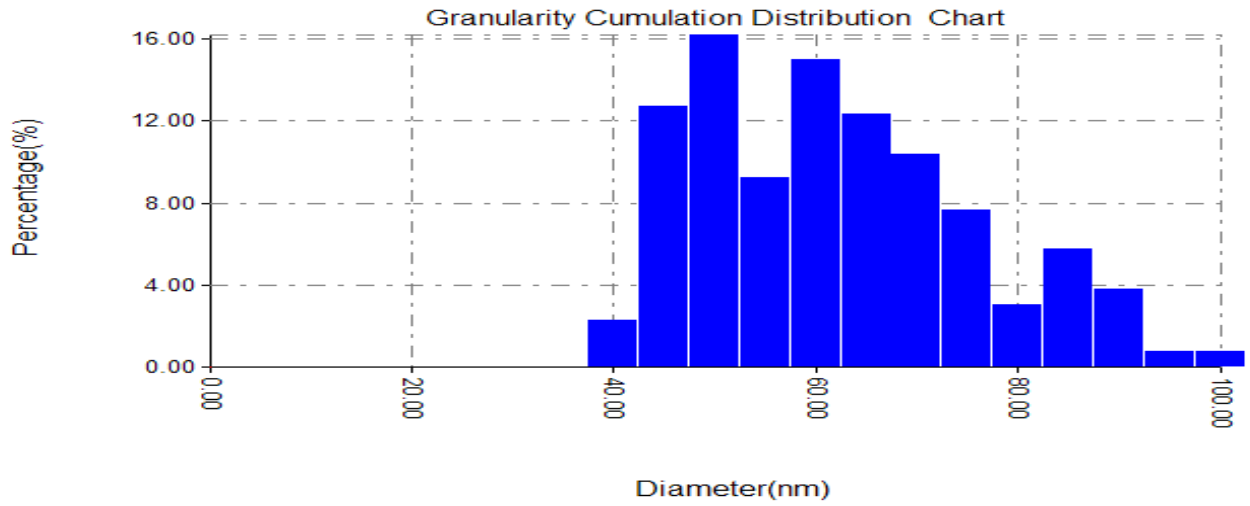

Figure 1: Granularity cumulation distribution

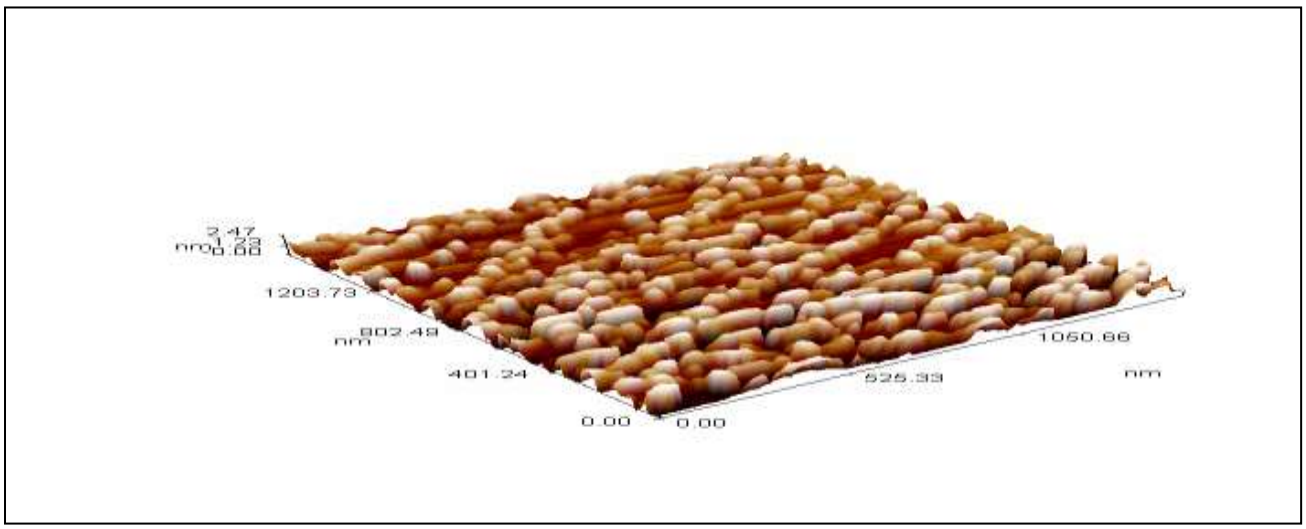

Figure 2: Spherical shape of AgNPs Chart

Administration of Ag NPs to Pregnant Female Rats

In this study, the AgNPs suspension was given to the treated groups (G2, G3) orally (gavage route) in a volume of 2 ml daily during pregnancy period by using polyethylene orogastric tubes (feeding tube) connected to a syringe in appropriate size . The dosage was in milligram per kilogram body weight (mg/Kg/B.wt). D.D.W was used as the vehicle for AgNPs preparation, while the control group G1 was given D.D.W only.

Embryos Retrieved

The pregnant female albino rats were fully anesthetized by diethyl ether for several minutes. The female were killed to remove the embryos in different gestation days (GD). In this study, we selected embryonic day from ED 12 to ED 21, in treated and control groups, abdominal midline incision was performed, the two uterine horns were exposed, the embryos were extracted from the placental sacs, and the extra-embryonic membranes were removed, rinsed in normal saline, then the embryo's brain were retrieved and stained in H&E for morphometrical examination.

ED12 transferred immediately to the Bouin's solution for fixation, embryos at ED15, 18 and 21 the skulls were removed and cerebellum was isolated from brain carefully by incision along the dorsal aspect, under the dissecting microscope, blotted dry with filter paper. All the samples were fixed in the Bouin's solution for 24-48h and were transferred to 70% ethanol until the time of the histological section [8]. Then everyone the sections prepared for morphometrical examination.

Morphometric Measurements

In this study the measurements of many parameters of the morphometry were done by

using the Motic Image Plus version 2.0 software programs 2004 on the personal computer, this version was downloaded to the computer and the recommended digital pictures of the many serial sections of the embryos were measured in some appropriate parameters of the morphometric parameters. The Motic Image plus 2.0 programs 2004, parameters were employed for the evaluation of the embryonic development of the cerebellar anlage during different embryonic stages by using only the serial midsagittal section of the cerebellum anlage.

The morphometric parameters were used to the midsagittal sections of the embryo's brain.

- Area mm2.
- Perimeter mm2.
- Antero-Posterior Diameter (APD µm) or called rostro-caudal diameter
- Transverse Diameter (TD µm)

Statistical Analysis

In this study the Statistical Analysis System-SAS was used to find the morphometric analysis of cerebellum during the embryo development, in this study the two treatment groups in the embryos were compared to their control group. The least significant difference-LSD test at the comparison between means was done on the level 0.05 to detect the significant effect of the different concentrations on the development of the cerebellum in embryos [9].

Results

Control Group

During normal development of the metencephalon and later the cerebellum in the embryos of the albino rats, these embryonic structures undergo several morphological changes due to the alterations in the dimension of their shape, this alteration we tried to detect them by using the morphometrical analysis through many selected parameters. In the present study, we attempted to evaluate the effect of AgNPs on the ontogenesis of the metencephalon quantitatively. Therefore (area, perimeter, anterior-posterior diameter and transverse diameter) were selected as morphometric parameters in this study.

The data of area, perimeter and transverse diameter (TD) of the normal development metencephalon and cerebellum cb was increased significantly ($P<0.05$) along the selected embryonic days until reach to the maximum growth at last stage of embryonic development at ED21, while the APD of the normal development of metencephalon and cb was also increased during embryonic development but decreased after ED18 (Figures 1, 2, 3 and 4) and also appearance in (Table 1).

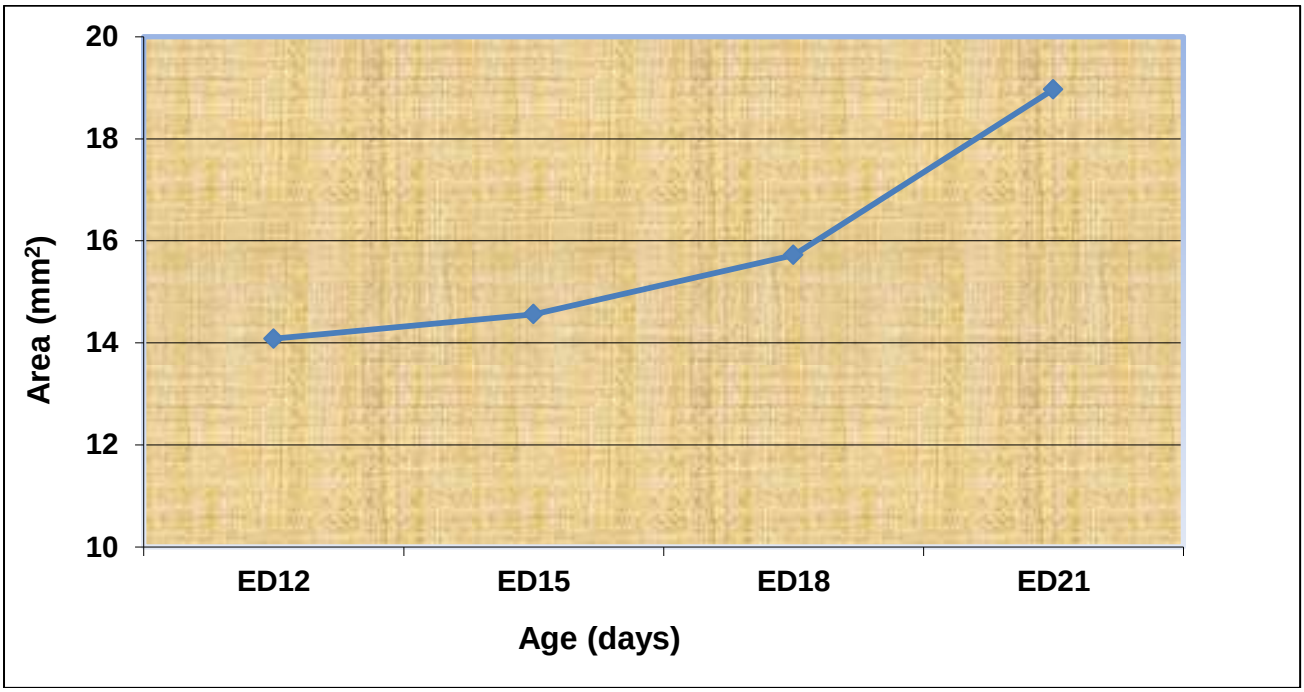

Figure 1: Effect of AgNPs on area development in control group

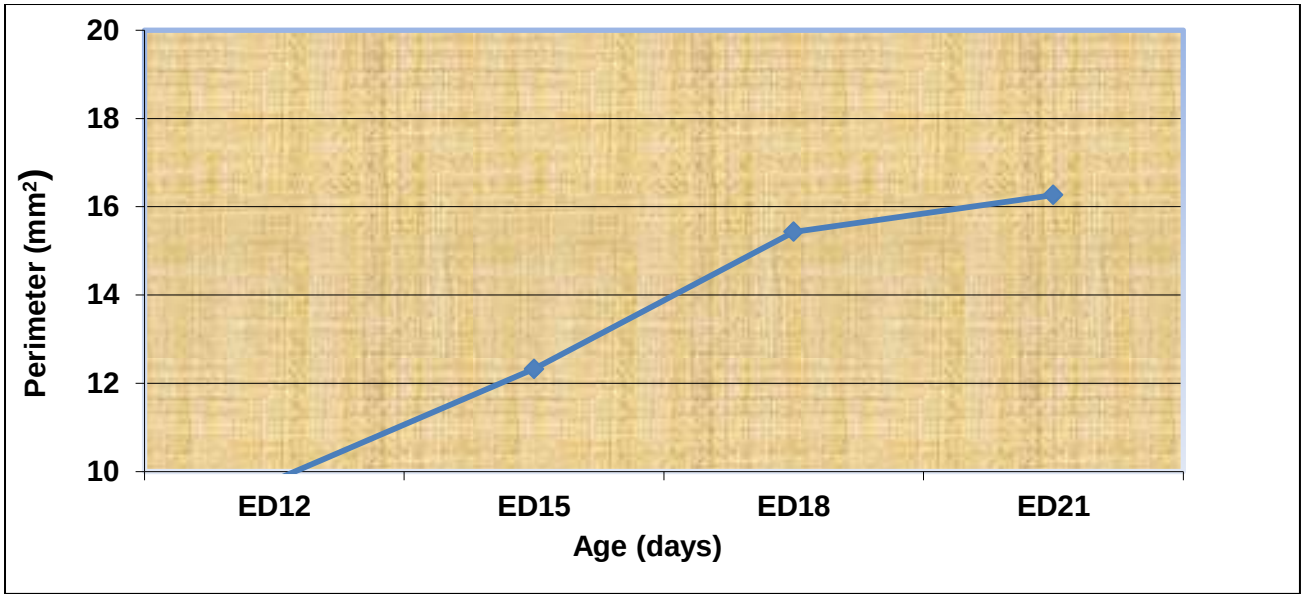

Figure 2: Effect of AgNPs on perimeter in control group

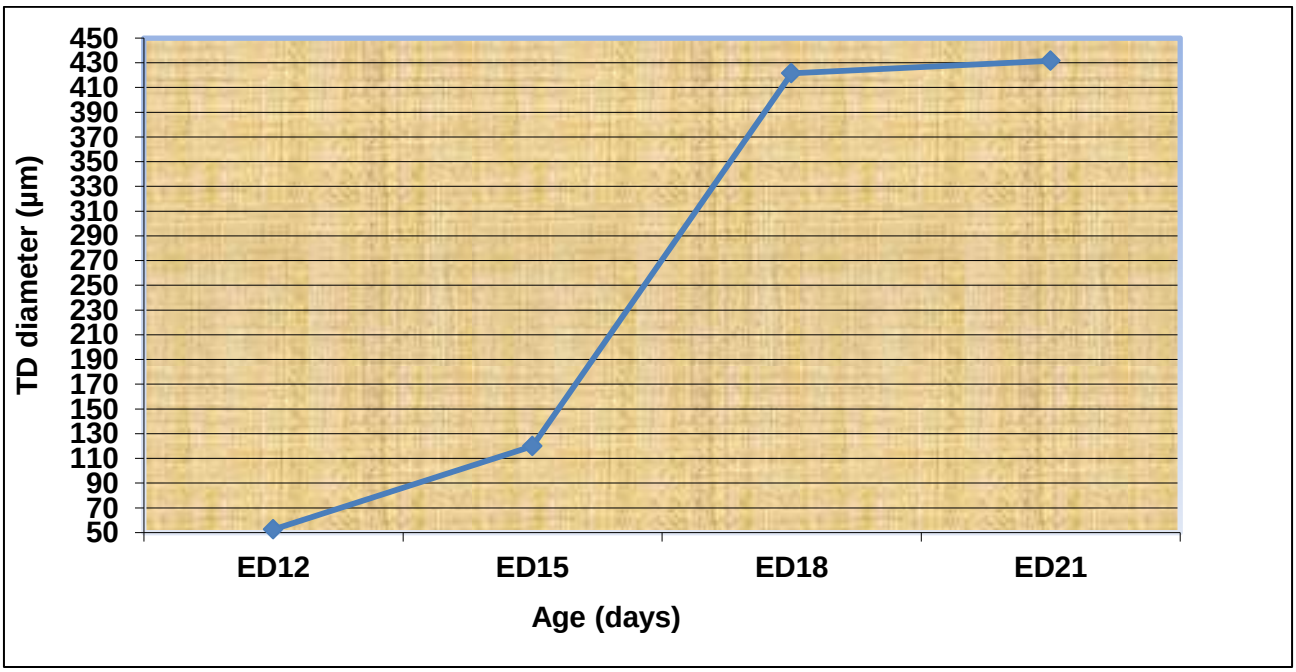

Figure 3: Effect of AgNPs on TD in control group

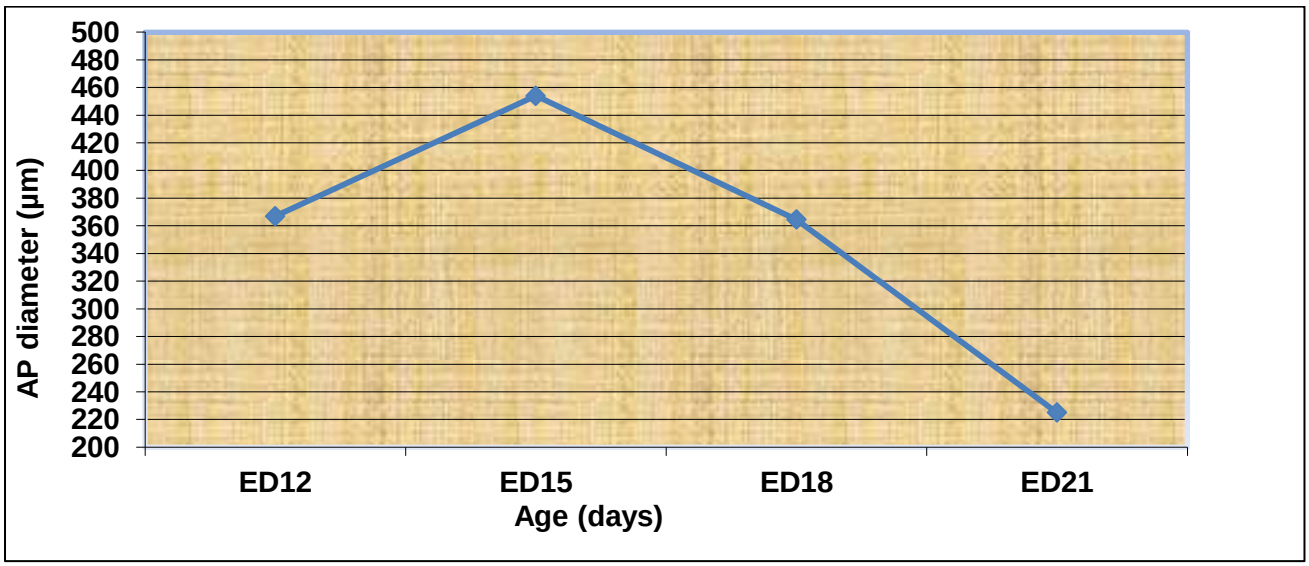

Figure 4: Effect of AgNPs on TD in control group

Table 1: morphometric analysis of cerebellum in control group in different selected embryonic stage

Age (days)	Area (mm^2)	Perimeter (mm^2)	AP diameter (µm)	TD diameter (µm)
ED12	14.0815	9.804	367.0041	52.6308
ED15	14.561	12.324	454.011	120
ED18	15.721	15.431	364.48	421.574
ED21	18.969	16.267	225	431.6
mean	15.83±1.10	13.45±1.48	352.62±47.3	256.45±59.2

Treated Groups

In the G2 and G3 treated groups, all the morphometric parameters of the development metencephalon except the area, the result showed significant increase (P≤0.05) until reach to the maximum growth at ED14. After this embryonic day the dimensions of growing cerebellum were decreased significantly (P≤0.05) compared with control group. In the area measurement, the results was found an increase in the total area of the cb as in the control group but not reach to the optimal growth at ED21, while reach to the maximum value at ED18 then the cb growth was inhibited (Figures 5, 6, 7 and 8) and in the G3group as showed in (Figures 9, 10,11and 12) The mean of this ED in both doses when compare with control group as showed in (Figures 13,14,15 and 16) and this parameters present in (Table 2 and 3). The statistical analysis between low dose (G2) and high dose (G3) of the AgNPs was shown that the higher dose has more deleterious effects on the area and the TD of the growing cb than the lower dose (Table 4).

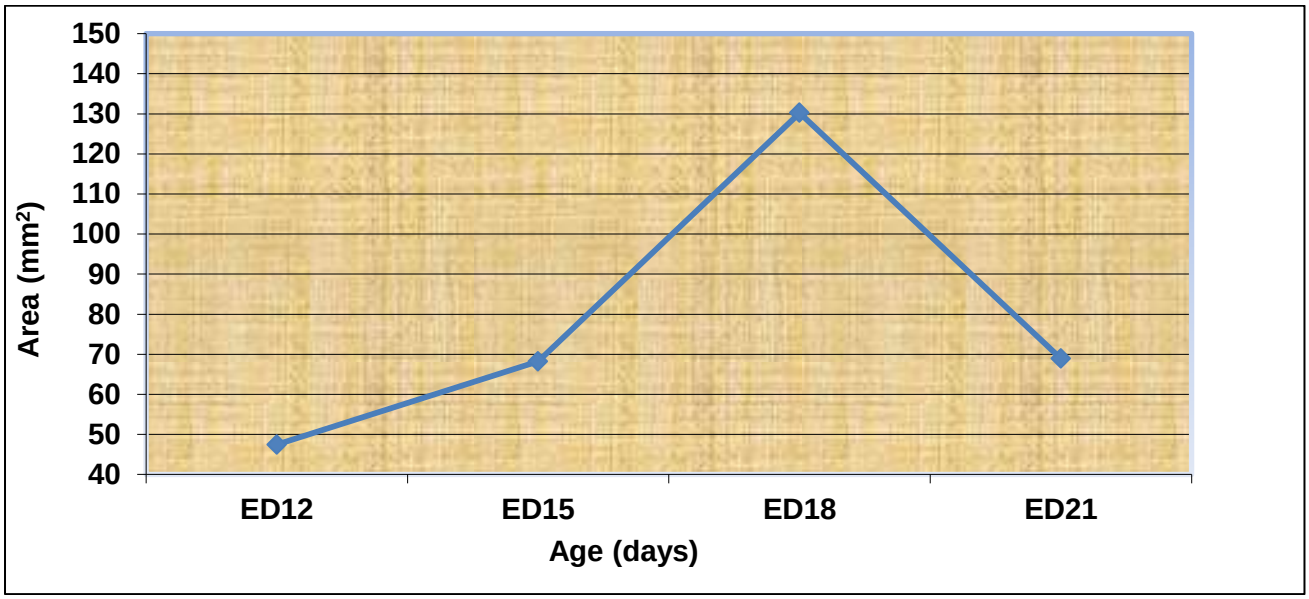

Figure 5: Effect of AgNPs in 2 mg concentration on area

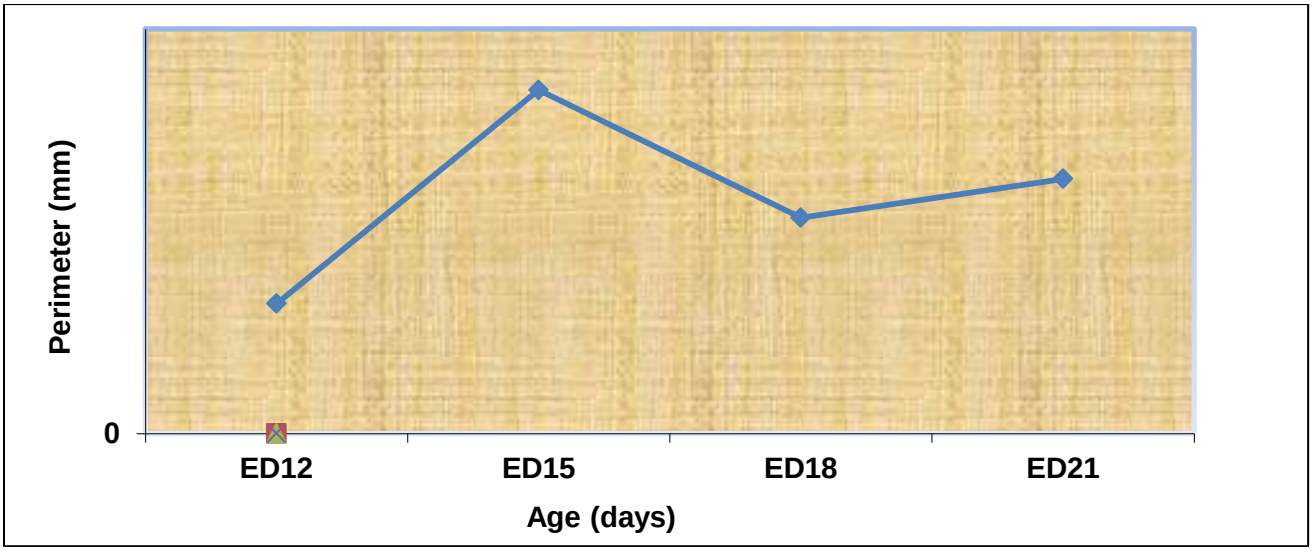

Figure 6: Effect of AgNPs in 2mg concentration on area

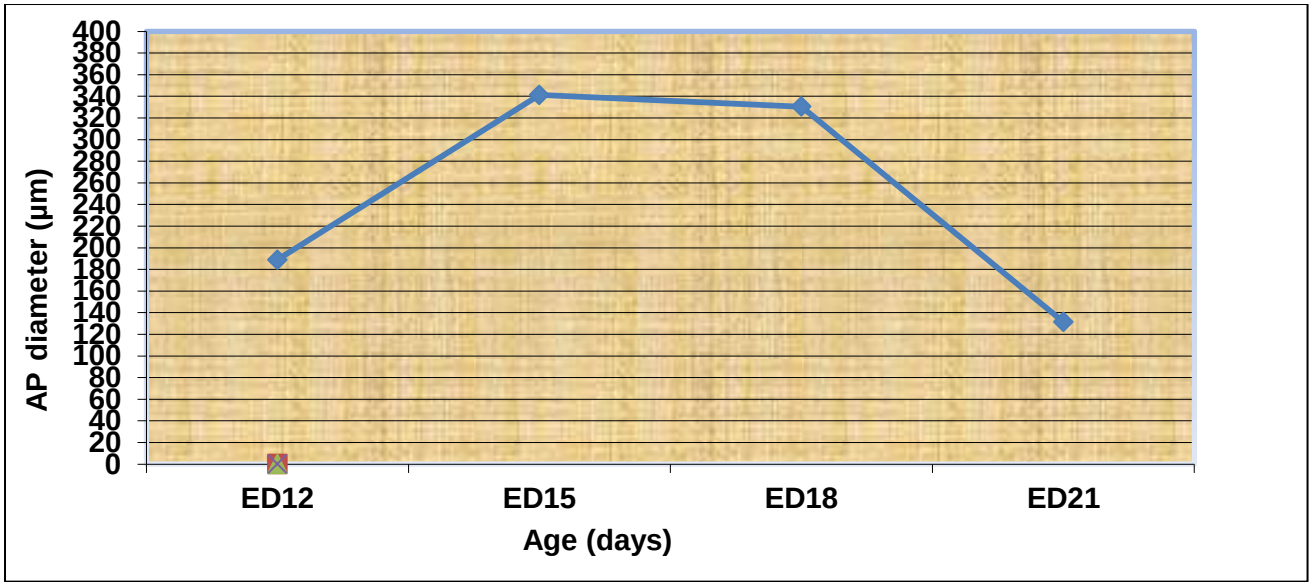

Figure 7: Effect of AgNPs in 2mg concentration on AP diameter

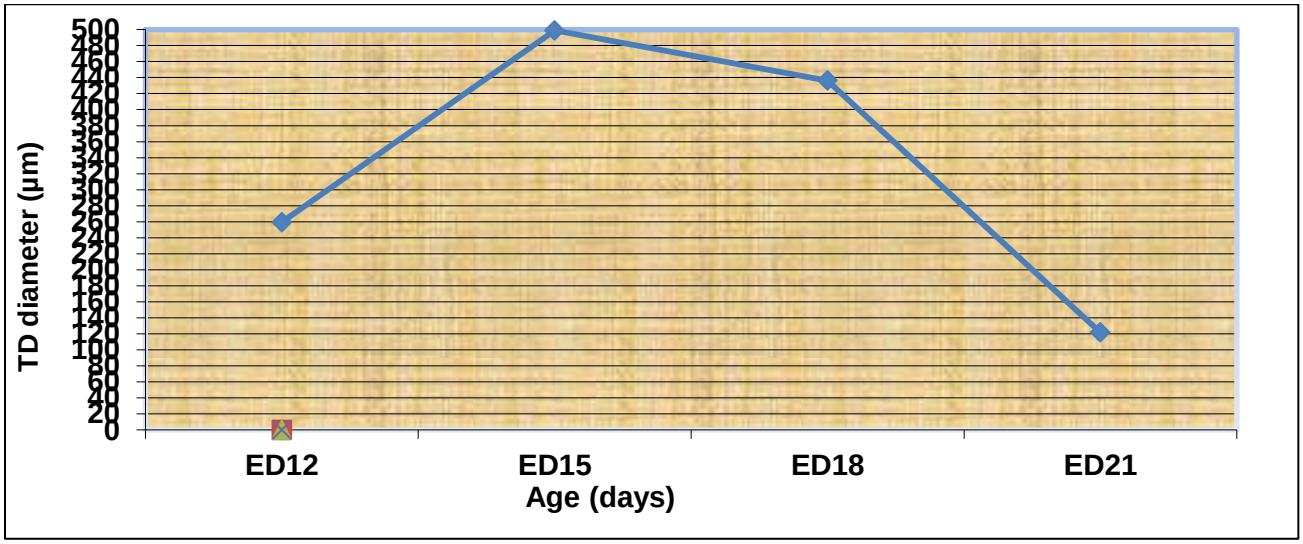

Figure 8: Effect of AgNPs in 2mg concentration on T diameter

And the results in higher dose showed as Figure 9, 10, 11 and 12.

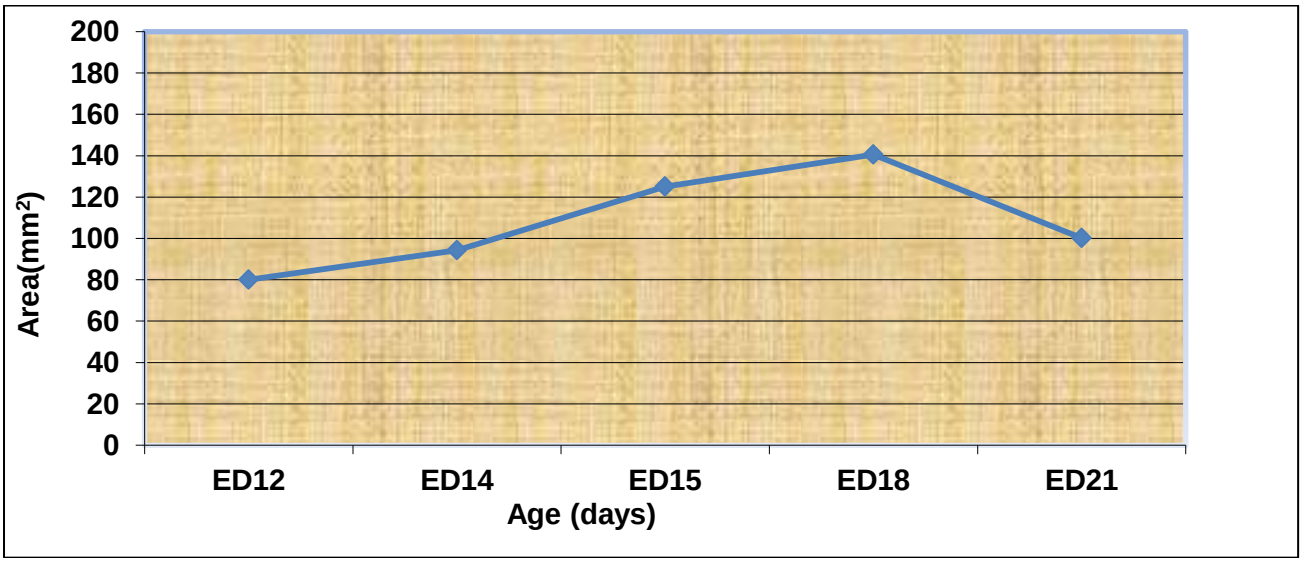

Figure 9: Effect of AgNPs in 20 mg concentration on the area

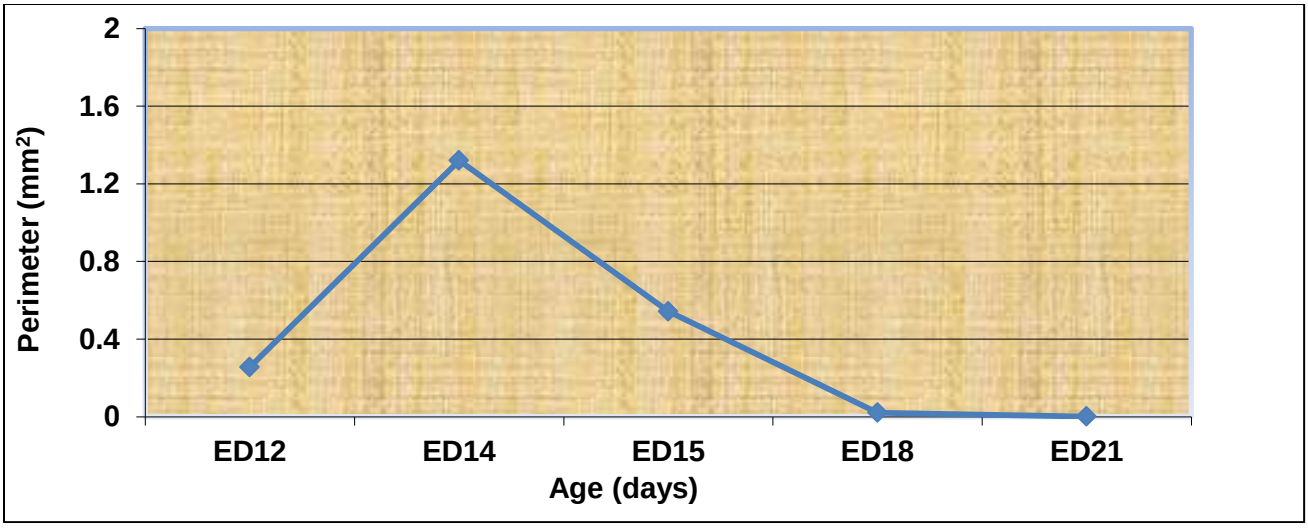

Figure 10: Effect of AgNPs in 20mg concentration on the perimeter

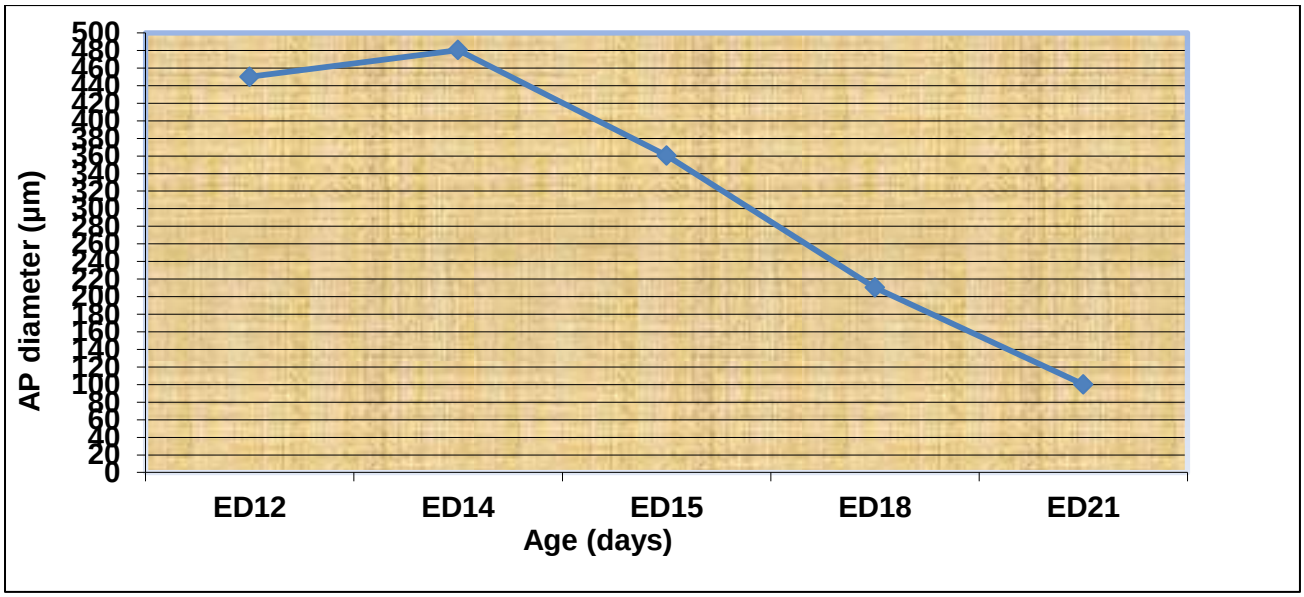

Figure 11: Effect of AgNPs in 20mg concentration on the AP diameter

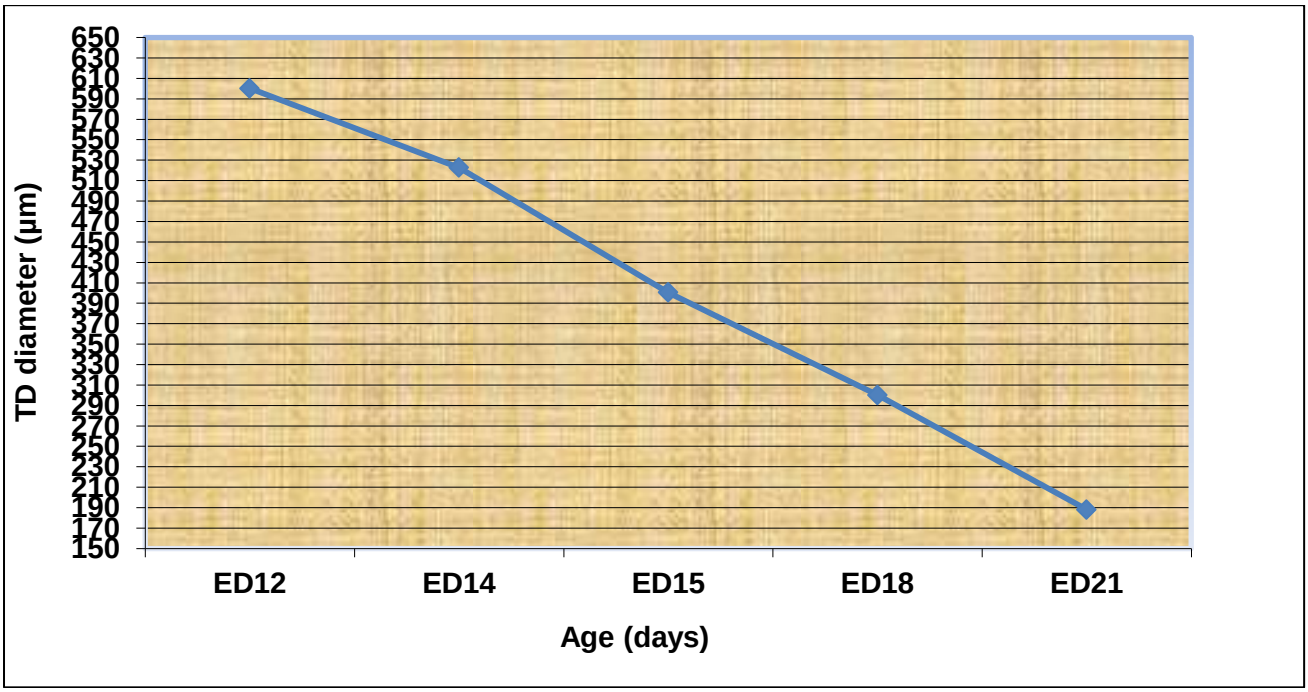

Figure 12: Effect of AgNPs in 20mg concentration on the T diameter

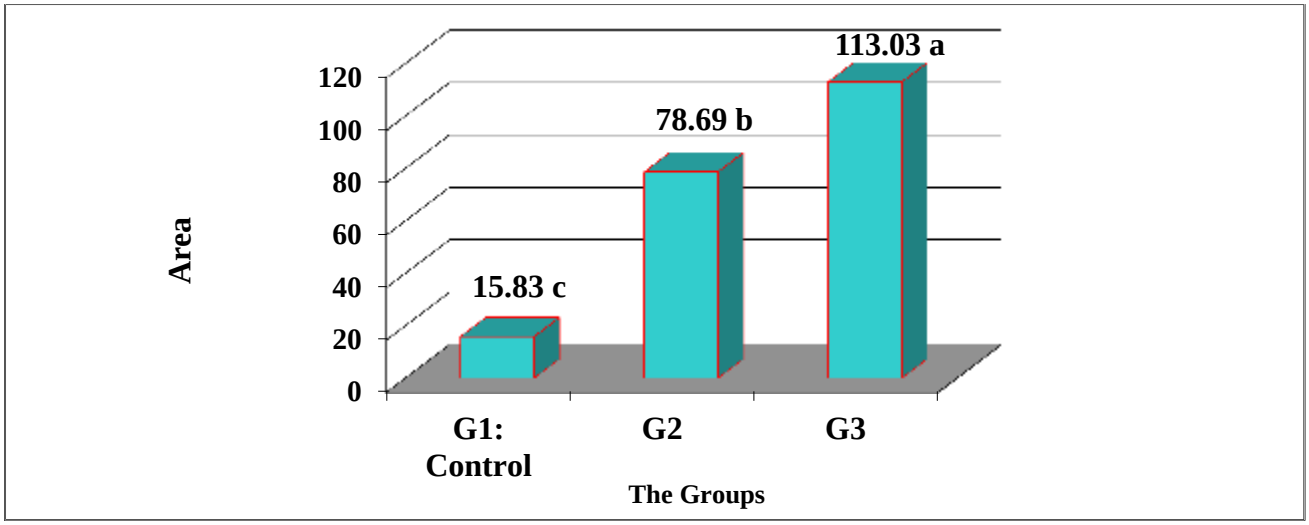

Figure 13: Effect of G2 and G3 of AgNPs on area of cb compare of control group

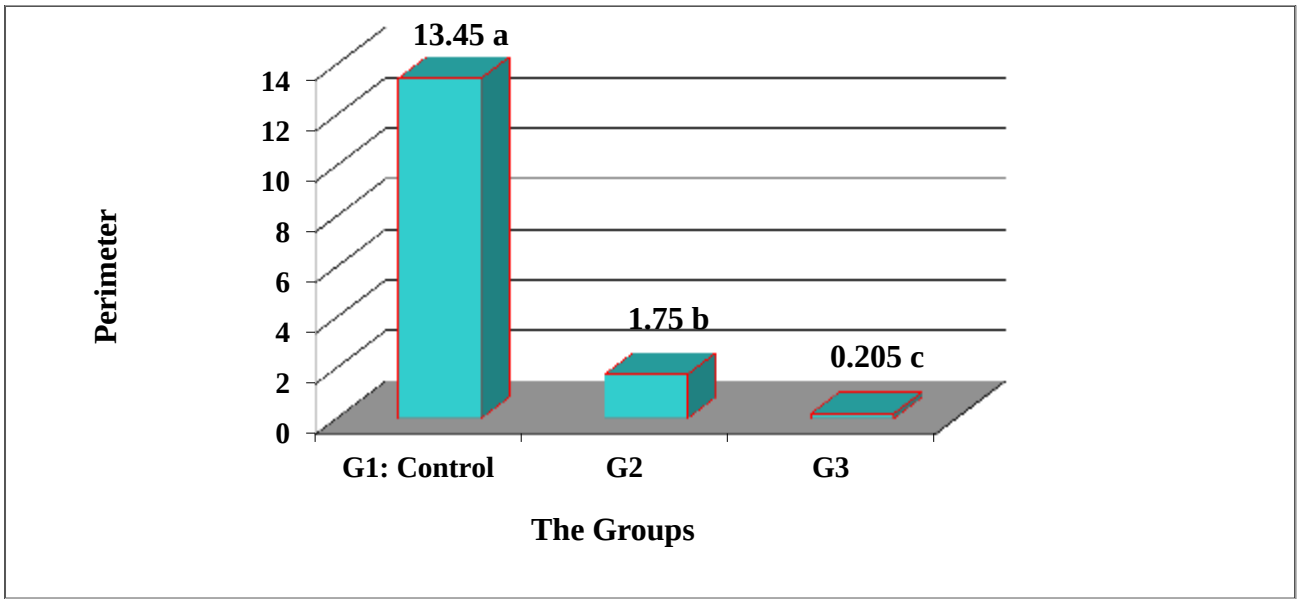

Figure 14: Effect of G2 and G3 of AgNPs on perimeter of cb compare of control group

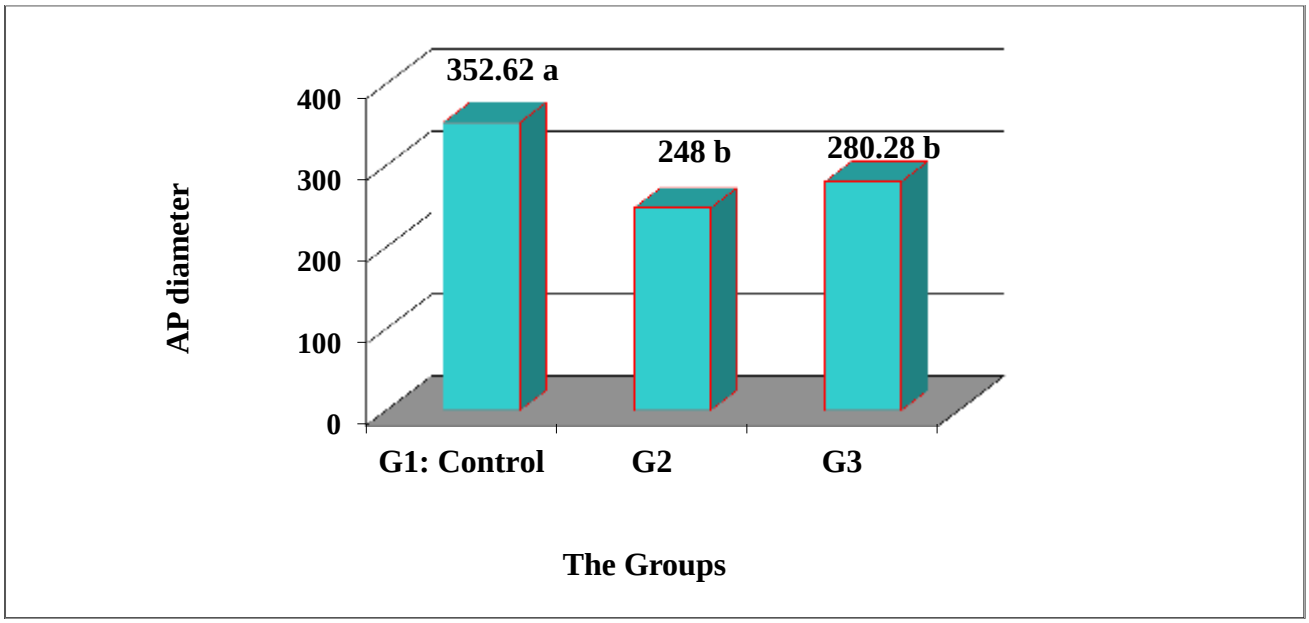

Figure 15: Effect of G2 and G3 of AgNPs on AP diameter of cb compare of control group

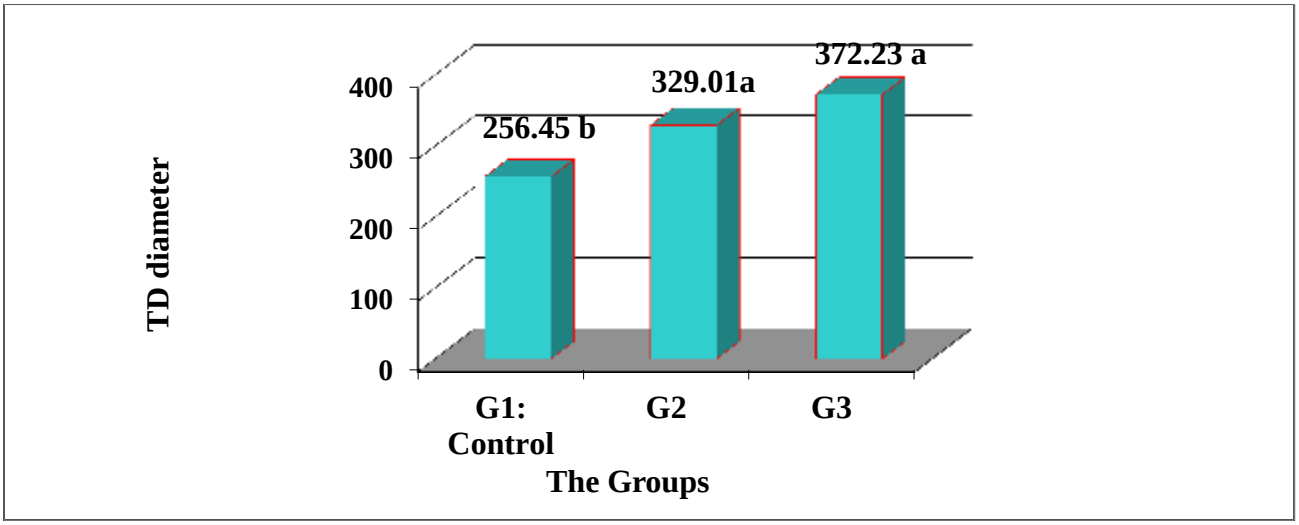

Figure 16: Effect of G2 and G3 of AgNPs on TD of cb compare of control group

Table 2: Effect of different parameters on development of cerebellum in treated group with 2mg/kg/B.wt pre implantation

Age (days)	Area (mm^2)	Perimeter (mm^2)	AP diameter (µm)	TD diameter (µm)
ED12	47.437	0.964	188.788	259.341
ED14	50.80	3.221	796.8767	606.2384
ED15	68.21	2.548	341.299	498.809
ED18	130.220	1.601	330.415	436.203
ED21	68.909	1.889	131.5	121.7
mean	78.69±17.88b	1.75±0.32 b	248.00±52.10 b	329.01±45.71a

Table 3: Effect of different parameter on development of cerebellum in treated group with 20mg/kg/B.wt pre implantation

Age (days)	Area(mm^2)	Perimeter (mm^2)	AP diameter (µm)	TD diameter (µm)
ED12	80.11	0.256	450.11	600.11
ED14	94.34	1.321	480.44	522.71
ED15	125.13	0.543	360.48	400.5744
ED18	140.56	0.021	210.33	300.11
ED21	100.21	0.001	100.21	188.12
mean	113.03±13.95	0.205±0.12	280.28±77.78	372.23±47.47

Table 4: Compare between G2 and G3 of AgNPs on development of cerebellum by using different parameters (pre-implantation)

The groups	Area (mm^2)	Perimeter (mm^2)	APD (µm)	TD (µm)
G2: 2mg / day	78.69±17.88 b	1.75±0.32 a	248.0±52.10 a	329.01±45.71a
G3: 20mg/ day	113.03±13.95 a	0.205±0.12 b	280.28±77.78 a	372.23±47.47 a
LSD	16.483 *	0.492 *	57.81 NS	64.07 NS
* (P<0.05), NS: Non-Significant				

Discussion

These normal morphometrical changes that occurred in the cerebellum may be due to the normal response of the early development of cb to changes in its shape. The vertical feature through early growth of the cerebellum anlage by projection from the metencephalon and undergo to several histological changes and formation of fissures and folia in the cortex of cb after ED15 when it's shape will convert to the vertical shape rather than horizontal shape [10]. From the previous results, observed many different alterations may be occurred on the dimension of the early developing cb when exposed indirectly to the action of the silver nanoparticles through the placenta and BBB during the neurogenesis process.

This process includes many dynamics movements of the stem cells from the neuroepithelium of the nerve tube and proliferate to many kinds of cells differ in morphology and physiology [11].The expansion in the cerebellum is linked with the proliferation of the EGL and migration of precursor cells to the IGL. The migration from the ventricular zone to the deep cerebellar nuclei is conclusion near gestational week 30 in human and after ED18 in rats [12, 13]. Increased the area of the development cerebellum may occurred result of nanoparticles effect on neuron and

glial cell in cerebellar cortex and formation depletion and dispersed and form intracellular space of cortex cells may be lead to increased the area in treated groups. On the other hand, found that AgNPs exposure could attenuate the viability of rat cerebellum granule cells through apoptosis and may be leaded to reduce neurons in the cortex then decreased the perimeter after ED15.

Reduce the formation and differentiation of the granule cells and Purkinje cells which are formed the developing cortex, as [14]. The cerebellar ratio of total brain volume found increased significantly from 2.4% to 3.7% more than during period of embryonic development in all mammals. Therefore; the cerebellum was increasing in size relatively faster than the cerebrum [15].

In the treated groups revealed significant decrease after ED15 compare of control group, this may be due to effect of silver nanoparticles on mitosis and proliferation of granular cells during development lead to less dense of EGL compare of control results and these unstable decreased results of unregulated migration of granule cells. Yin et al., (2015) suggested that when AgNPs treatment in (1 mg/kg/day) caused obvious distortions in both Purkinje layer and granular layer.

The granular layer was degenerated with loosen and separated structure, and the Purkinje layer was not distinct due to the deficiency of Purkinje cells, therefore observed unregulated development in both APD and TD.

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