Research Square

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Histological Alteration in the CA4 and Dentate Gyrus Region of Hippocampus: Neurological and Oxidative Stress Change in Rats During Different Gestation Periods

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Abstract

Hippocampus consider on of most active region in nervous system, so it so sensitive to any alteration in metabolism or hormonal change during pregnancy. Present study demonstrated the oxidative stress as factor for alteration in normal histological architecture of hippocampus the Dente Gyrase and CA4 region during normal pregnancy in adult rats. The four-group allocated 40 adult female albino rat, G0 control group, G7 gestation stage at seven days, G14 the gestation day 14 and G19 the gestation day is 19.H&E staining dependent for histological evaluation, on another hand MDA use for detection oxidative stress by immunohistochemistry &biochemical lipid peroxidation assays. The progression in histological disruption of DG was detected in all gestational period, in which neuronal disorganization, widening of sub-granular cavity, and cellular cohesion level with highest deterioration in at 3rd ,4th group. The cell nerve in CA4 demonstrated size alteration (reduce in size), the density alteration in 3rd group, after that some degree of recovery occur in 4th period. The oxidative stress reach to highest level in G14 in which reflected by MDA expression tissue lipid peroxidation level and declined in 4th stage but remain higher than control group. The oxidative stress and normal histological deuteriation reported in present study. The fact of transient of oxidative stress and its effect on remodeling hippocampus dynamically during normal pregnancy, specially during mid-period of gestation, and in which contributed to maternal neurological adaptation through gestational period.

Introduction

The main region of hippocampus is Cornu Ammonis (CA), in addition to Fascia Dentata (FD). This region of Hippocampus up to cyto-Histological variation as build up unit of this neuroglial structure [(1)]. The Ammon's Horn is also common name for this neurological structure, there are four building Sub unit for CA which includes CA1,2,3 and CA4, The U Shape within DG is a form of CA. Histologically from inner side to outside started with stratum moleculare, stratum lacunosum (or lacunosum-moleculare), stratum radiatum, stratum lucidum (Restricted to CA3 region), stratum pyramidale, stratum oriens, and the last one is stratum alveus. The primary two layer contain cell body of pyramidal and granule cells respectively which know as stratum granulosum & pyramidale of Cornu Ammonis ,The radiatum layer of the CA region contain various of interneuron as mossy cells, pyramidal basket cell includes a variety of interneurons, such as mossy cells, pyramidal basket cells, axo-axonic cells (chandelier cells), fusiform cells, O-LM cells, IS neurons, the MOPP cell, and bistratified cells, In addition to the subiculum, presubiculum, parasubiculum, and entorhinal cortex[(2)].The Specicity of CA4 Region has variation in layers differentiation, the stratum pyramidale layer is notably thicker than the other, and stratum radiatum and stratum oriens being thinner,The 1st layer, the alveus, is absent, and the 6 th layer, stratum moleculare and lacunosum, remains without specific differentiation, There are variation in cell size in CA4 area vary with the stratum pyramidale containing larger cells than the stratum radiatum and Stratum oriens [(3)]. The oxidation change occurs in hippocampus due to nNOS cause alteration in neurons number within Hippocampus, the level of nNOS occur in both sex but the decrease neuron number in female is higher than male in addition to increase Ca + + contain with oxidant generation, the consequent of

oxidation is neuronal damage and neuronal loss [4]. Both long term and short term of hormonal fluctuation (estradiol (E_2) and Progesterone (P)) has it effect on Hippocampal dendritic spines concentration [5]. Present study will detection the fact of change in Hormones fluctuation in different gestation period by detection the Oxidation in CA4 region.

Material and Methods

Animal selection and Allocation

The Rattus Norvegicus Albinus Rat Use in present study total 40 Rat with wight average from 240 to 400 g, the animal age limited from 10 to 12 week. The animal source is Animal Resources Centre (Canning Vale, WA, Australia). The lab animal incubated in standard laboratory condition 25 centigrade with 2 above and below as average,light-dark cycle 12 hours, in addition food water supply .

The animal allocated in four group, below table illustrated the animal grouping.

Table 1
illustration the animal lab allocation according to gestation period .

Groups	Animal sex	Pregnant, Non-pregnant	Pregnant stage
Group 1 Control group	Female	Non-Pregnant	0 (G0)
Group 2	Female	Pregnant	7 Day(G7)
Group 3	Female	Pregnant	14 Day (G14)
Group 4	Female	Pregnant	19 Day (G19)

Note. The pregnant animal confirmed by present vaginal plague following animal matching after G0, every three female one male.

Anatomy and Histology.

Tissue collection and processing.

During normal pregnancy time we euthanized animal at G7, G14 and G19 Day, the authorization done according to institution animal ethic gridline. 10% natural formalin use for preservation whole animal brain tissue (NBF; Sigma-Aldrich, Cat. No. HT501128) for at least 48 hours.After fixation ,use gradating strategies for tissue dehydration by ethanol series (70%,90%100%), the xylene is agent for cleaning tissue (ThermoFisher Scientific, Cat. No. X5-1), then embedded in paraffin wax (Leica Biosystems, Cat. No. 39601095). The sections of preserved tissue by cut at 5 µm thickness using a Leica RM2235 rotary microtome and mounted onto SuperFrost™ Plus glass slides (Thermo Fisher Scientific, Cat. No. J1800AMNZ) for histological and immunohistochemical analyses.

Tissue staining protocol

The Hematoxylin and eosin (H&E) use staining procedure after sections of target tissue, the hippocampus evaluated by cytoarchitecture form and immunohistochemistry (IHC) to evolution Malondialdehyde (MDA), which is marker for detection Lipid peroxidation and oxidation stress.

The Immunohistochemistry staining, using following procedure.

- The xylene and graded ethanol use for deparaffinization and rehydration.
- The target Epitope restoration in 10 mM citrate buffer (pH 6.0) within 95°C for 20 minutes.
- The 3% Hydrogen peroxide use for quenching the endogenous peroxide activity, (Sigma-Aldrich, Cat, no.H1009).
- The 5% Normal goat serum use for blocking (Abcam, Cat.no.ab7481) 60 minutes at room temperature.
- The primary AB incubation :24 hors at 4°c with anti-MDA AB (Abcam, Cat, No. ab6463, dilution 1.200).
- The secondary immunodetection; Incubation with biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratory, Cat.No.BA-1000), followed by avidin-biotin-peroxidase complex (ABC Kit,vector Laboratories ,Cat.No.Pk-4000)and DAB substrate (Vector Laboratories ,Cat.No.SK-4100)
- Contrast staining; Light haematoxylin staining, pursued by dehydration and specimen mounting by DPX mounting medium (Sigma-Aldrich, Cat.No.06522).

Semi-quantitative image evaluation

Histological evolution, 5 separated high power fields (HPFs) used for both DG and CA4 area for each animal used ImageJ software (NIH, USA).

- Normal architecture abnormalities detected through irregular uniformed of granular layer, in which build up sub-granular cavities in addition to neuronal disturbance.
- The abnormalities in area evaluated by percentage of whole DG region.

The evaluation of Malondialdehyde through immunohistochemistry, by staining concentration was counted as **integrated optical density** (IOD). The average of IOD measurement in which compared Between groups to evaluated the oxidative stress degree.

Assessment lipid peroxidation

The oxidative stress degree with in hippocampus detected through lipid peroxidation, The terminal production of lipid peroxidation is MDA, in which evaluated by colorimetric lipid peroxidation (MDA) assay kit (Abcam, Cat.No. ab118970).

According to guideline of manufacture we use following protocol, in this test the MDA interact with Thiobarbituric acid (TBA) in high temperature environment and low pH condition (acidic) to build up MDA-TBA adducts, in which detected through colorimetric at 540 nm by using Bio-Rad iMark™ Microplate Reader. The result evaluated in form of µmol MDA per mg protein (µmol/mg protein) [6].

Biostatical analysis

The GraphPad Prism (Version X; GraphPad Software, San Diego, USA) used for analysis data collectively. Shapiro-Wilk test and Levenes tes use, the first for normality assessed and the second for homogeneity of variance verification. One way ANOVA use for parametric data analysis, followed by Tukey's post hoc test. On-another hand un parametric data done by Kruskal–Walli's test with Dunn's post hoc test and Bonferroni correction. All data presented form of mean ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

Results

The structural alteration of the DG during gestational period, histologically alteration in Hippocampus noted after use H&E in histologically satin in both CA4 and DG in the ulteration according to gestational stage. The G0 group (Control Group) the DG appeared normally in stricture in three-layer in compacted form molecular (M), granular (G), and polymorphic (P) layers. Granular (G) layer revealed densely with population of small, spherical, basophilic neuronal nucleus organized in four to five rows of cell, demonstrating robust cellular cohesion (Fig. 1). The DG architecture in G0 group (control group) no noted any alteration during all period of present study, this agrees with previous research about hippocampal normality in short term descriptive windows in mature female rodents.

In the second groups (G7 groups) illustrated primary alteration as gentle expanded in granular layer thickness and early signs of neuronal imbalance (Fig. 1B &E), in third groups G14 the disturbance increased and in significant form increase the cell rows six to seven layer, in addition to sub granular cavity (S) among granular and polymorphic layers (Fig. 1C and 1F).The granular nerve cell demonstrated decline in packing compactness & reduce in alignment ,in addition intracellular spaces increase in dorsomedial direction of dentate gyrus .figure 2 illustared the data from DG alteration throughout pregnancy stage, Fig. 3 outlined MDA expression degree and statistically density.

The fourth group (G19), sub-granular cavity expanded in addition neuronal alteration increased markedly, which give "lacy" characterised presented across higher in granular layer (Fig. 1D and 1G). The semi-quantitative analysis revealed increase around 30% alteration in architecture of DG region from G19 to G0, this disturbance in architecture is a indicate Pregnancy-stage-depending structural alteration in DG of hippocampus.

The CA4 region and its alteration in parallel with stage specification of pregnancy.

The CA4 region and its histological architecture analysis, the CA3 region extended into the polymorphic layer covered by the DG-illustrated pregnancy stage -relegated alteration in nerve cell morphology and arrangement. A huge neuron implanted within the neuroepithelium of the polymorphic layer demonstrated prominent perikaryal and nucleoli with shape variation and clustering throughout progressive stage.

The second group (G7) the nerve cell (neuron) showed massive and compactly arrangement with lowest immune-interaction with anti-MDA (Fig. 4AB).Third group (G14),nerve cell -neurone-demonstrated size reduction, a morphological alteration deviated to atypical form of pyramidal shape and atrophy in addition to reduction in integration with surrounded neuroepithelium-region associated with dendritic extended in different direction and synaptic connection .The target neurons positively stained with anti-MDA and demonstrated reduce in compact density (Fig. 4C,D).

The fourth group (G19), the size of neuron and compact density demonstrated some degree of recovery return to condition similar to G0 period (Fig. 4E, F). Measurable morphological evaluation of neuronal soma region in the CA4 was performed on neuron subset of ($n = 6–10$ for each group). A significantly reduced in mean soma region detected in third group (G14) in compare with first group and second group ($P < 0.05$), a headed by some degree of recovery in fourth group. This fact detected by evolution histological observation (Fig. 5).

The MDA as indicator for oxidative stress level in mid stage of gestation.

The MDA is detected by immunohistochemical, When MDA expressed demonstrated improvement change corresponding to histological observation. Within G0 group, the expression of MDA minimized and restricted to primary to scattered nerve cell inside granular layer (Fig. 6A). The Second group (G7), modest cytoplasmic staining detected, with elevation localization in the granular layer (Fig. 6B). Significant increase in MDA immunoreactivity observed in third group (G14) (Fig. 6C), the higher positive staining extended to all granular layer and into the buildup sub granular cavity. This a highest expression contributed to stage of pregnancy stage, in which the histological alteration initiated to be emerged. The fourth group (G19), MDA is continuous expressed in whole DG layers, in compare with G14 appear lower, and higher than G1group (Fig. 6D).

Table 2
the table illustrated the MDA level expression immunohistochemically in different gestation period, which indicated to oxidative stress in DG region. the intensity, localization and histological correlation of MDA immunoreactive throughout whole experimental groups.

Group	Pregnancy stage	MAD Expression	Extended of Staining	Histological alteration
G0	First group	Lowest level expression	Partially stained the neuron within granular layer	No-change appeared on architecture of normal tissue.
G7	Second group	Mild cytoplasmic staining	Extended the neuron staining within granular layer	Mild histological alteration occurs.
G14	Third group	Significant MDA expressed (Markedly increased)	More powerful staining the nerve cell in which included whole the granular layer and extended to sub granular cavity.	The alteration of normal architecture of normal tissue progressed.
G19	Fourth group	Continuous MDA express, Lower than third group and higher than first group)	The staining extended throughout whole DG layer.	The alteration of normal tissue continuous more severely but lowered than G14 and higher than G7 .

Lipid oxidative stress evaluation quantitatively

On another hand, evaluated lipid oxidative stress level by using the colorimetric Malondialdehyde assay (Fig. 7). Combination with histological and immunohistochemical data, the elevation of MDA occurs from G0 to G14, which indicated to oxidative stress restricted to mild-period of pregnancy. The 3rd (G14) demonstrated peak of MDA level, then with in 4th reduced level of expression, and remain higher than 1st group. This agrees with histological staining concentration pattern i.e. The oxidative restricted to mid stage of pregnancy.

The Aperio ImageScope software use for evaluation oxidative stress level quantitively. The (Fig. 3) illustrated the finding, in which highest in the G14 group (9,356,644.83 ± 918,505.50), followed by G7 (5,770,986.17 ± 1,180,373.26), control (4,531,670.17 ± 395,015.00), and G19 (5,133,079.83 ± 535,769.83). The MDA expression statistically between G0 and G19 ($p = 0.0077$), and G14 with G19 ($p = 0.0047$). The G0 and G14 compares is not significantly statistically ($p = 0.0771$). The moderate positive correlation between histological alteration and MDA expression in DG in which ($r = 0.62$, $p = 0.03$), which structural change and oxidative stress during pregnancy is indicated in present research, more details in discussion section.

Note

The MDA is indication of lipid peroxidation activity.

Table 3
this table illustrated quantitative evaluation MDA expression, demonstrated high oxidative stress in middle stage in which reach to peak in third stage G14 Group .

Group	Stage of pregnancy	Average of MDA expression ± Standard deviation (SD)	Level of oxidation stress	Statistically compare	P-Value
G0	Non	4,531.670.17 ± 395,015.00	baseline	G0 Vs G14	0.0771 non-S
G7	Early	5,770,986.17 ± 1,180,373.26	Moderate increase	-	-
G14	Mid	9,356,644.83 ± 918,505.50	High expression	G14 Vs G19	0.0047
G19	Late	5,133,079.83 ± 535,769.83	Decline by compare with G14 and remain higher than G0	G0 Vs G19	0.0077

Discussion

The present study demonstrated histological alteration in hippocampus (DG, CA4) according to gestational period, evaluation the alteration histologically and oxidative stress degree. The alteration includes sub-granular cavity formation, in addition disorganization the granular neuron layer, and alignment specially in 3rd and 4th period. In CA4 region morphologically in nerve cell on size level, alignment density in 3rd and reduced in 4th period. The MDA elevated in highest degree in G14 period as reflection for oxidative stress. This fact indicated for oxidative stress, normal nerve cell morphology relationship.

The oxidative stress and hippocampus normal architecture during pregnancy, The ROS elevation is a reflection in antioxidant defense mechanism [7, 8, 9]. The nature of hippocampus tissue is buildup by rich polyunsaturated FA with active metabolic characterized -the ROS induce lipid peroxidation, protein oxidation in addition to genetic material negative effect (DNA) [10, 11]. MDA, is by product of lipid peroxidation is an indicative marker for oxidative damage [12, 13]. The high metabolic activity during pregnancy and high level of O2 requirement the oxidation occur in maternal tissue generally including hippocampus, with advancing gestation time [14, 15]. The MDA expression in high level reported in normal and pathological gestation as pregnancy diabetes and preeclampsia [16, 17]. present study illustrated histological &biochemical disturbance of oxidative stress in different gestational stage in hippocampus. The normal tissue within DG and CA4 region influenced by MDA elevation which causes disturbance in normal tissue architecture in mentioned region specially in 3rd period. This fact confirmed by assessment assay test as in (Fig. 7), which demonstrated the level of MDA increase with progression in gestation time from 1st groups (0.299 ± 0.018 µmol/mg protein) to onset of gestation (G7: 0.397 ± 0.025

μmol/mg protein), with highest concentration at mid-gestation (G14: 0.617 ± 0.041 μmol/mg protein).in which 106% increase in MDA in compare with baseline, the oxidative activity and peroxidation of lipid throughout this period. The level of MDA restricted and declined in the last stage of gestation G19 significantly (0.371 ± 0.027 μmol/mg protein), but still higher than G0 groups when compared, which indicated partially recover occur before parturition. The G14 consider critical period on hippocampus due to highest concentration of ROS. The endogenous antioxidant system activation, enhance mitochondrial efficacy, or hormonal fluctuation oestrogen, prolactin and oxytocin which act as neuroprotective hormone during pregnancy thus factor contributed in ROS regulation &neuronal survival this illustrated reduce of MDA in 4th stage of pregnancy [18, 19].

Hormone & neuroplasticity, the high level of glucocorticoid, oestrogen, progesterone, prolactin, and oxytocin receptors influence on hippocampus and its plasticity [18, 20]. The level of corticosteroid in G14 increase significantly, which play role in decrease of dendritic complexity, neurogenesis and increase susceptibility to neurotoxic stressors [21, 22]. The reduced in size of nerve cell and synaptic disruption reported in CA4 of this stage which agree with this fact. The disturbance in dendritic nature and granular cell alignment in this stage has its effect on hippocampus normal function as memory encoding efficacy, pattern separation and emotional response. Increase of prolactin and oxytocin level in G19 period improve neurogenesis & structural recovery under stress [23, 24]. This agrees with partial recovery of neuronal morphology and restricted the MDA level at G19 period. On another hand the oestradiol improves neuronal survival and enhance neuroplasticity in hippocampus [25].

The stricture architecture modification which reported in pregnancy period is factor of adaptation plasticity that assist the cognitive &emotional requirement of motherhood [26]. The sub-granular cavity and the “lacy” which appeared in DG is like early indication synaptic compromise, which influenced on hippocampal operation in this transited time. The CA4 region is susceptible to oxidative and hypoxic stress inherently [27, 28]. The CA4 is recovery partially at G19 period and improve morphologically as result of hormonal modulation, this agrees with article which mentioned postnatal plasticity & recovery in CA4 [29].

The normal architecture of tissue and Oxidative stress, in present research the fact about moderate relationship between these two factors are detected ($r = 0.62$, $p = 0.03$) the first is MDA expression and second factor disturbance in normal histology of tissue architecture in DG. This agrees with hypothesis of ROS-induce Lipid peroxidation may contributed to histological disturbance in hippocampus tissue during different stage of gestation, DG have very high neurological activity is very high susceptible to oxidative stress and serve as important site of redox imbalance during gestational time with different stage [30, 31].

The mental health and future research, during pregnancy the cognitive change occur which called “pregnancy brain” in which explained by hippocampus structural alteration [32, 33]. The alteration in hippocampus normal alignment has role in memory impairment, learning in addition to mood regulation,

this generally expressed in perinatal period on another hand the pathophysiology of postpartum depression with anxiety [32, 33, 34, 35].

The present study detected fact the oxidative stress as biomarker for maternal mental health, any antioxidant agent uses for reduce oxidative stress as some supplement, hormones modulation agent may act as therapeutic agent. Future study concentrate on hippocampal change will persist in postpartum or crossed it and it effect on behaviors. More research on oxidative marker and antioxidant enzyme for deep understanding its mechanism. By different for detected the structural change are transit or indicative of long-term neuronal remodeling. understanding all previous fact will give chance in early intervention for improvement maternal mental health. The postpartum trajectory hippocampal disturbance and its effect on behaviors are underscored in present research which need more research in future in another hand this alteration in hippocampus is reversible or no, the antioxidant agent will service as significant factor for protective maternal mental health.

Conclusion

The present research demonstrated the hippocampus modulation in different normal gestational period in different level histologically, oxidation change and neuron morphology. The DG and CA4 region illustrate progressive neuronal deterioration in addition sub-granular cavity formation, nerve cell morphology alteration, in 3rd group (G14) highest MDA expressed and max change in cellular stricture occur with oxidative stress. In late stage(G19) some degree of recovery occurs, but persist disturbance is remained attributed to oxidative stress as main factor in hippocampus tissue. The fact of double natural of hippocampus plasticity throughout gestation: exposed to both oxidative and hormonal stress and capable of restriction structural restoration. This balance between exposing and adaptation has important complication for understanding maternal cognitive and emotional alteration throughout gestation in addition to postpartum period.

Declarations

Declaration of Interest

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

Ethical Approval

All procedures in studies involving human participants were performed in accordance with the ethical standards of the institutional Review Board at Al-Nahrain College of Medicine, Iraq (approval no. 36/IRB, dated 25 March 2025).

Consent for Publication

All authors consent to the publication of this manuscript and approve its submission to the journal.

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Author Contribution

Huda R. Kamoona: Conceptualization, methodology, histological investigation, data collection, writing of the original draft.Zahraa A. Mohssen: Laboratory work, immunohistochemistry, biochemical analysis, data interpretation.Saif Y. Hasan: Study design, supervision, statistical analysis, manuscript revision, corresponding author responsibilities.Hayder A. Al-Aubaidy: Scientific consultation, data interpretation, critical review, and final manuscript editing.All authors have read and approved the final manuscript.

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Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Figures

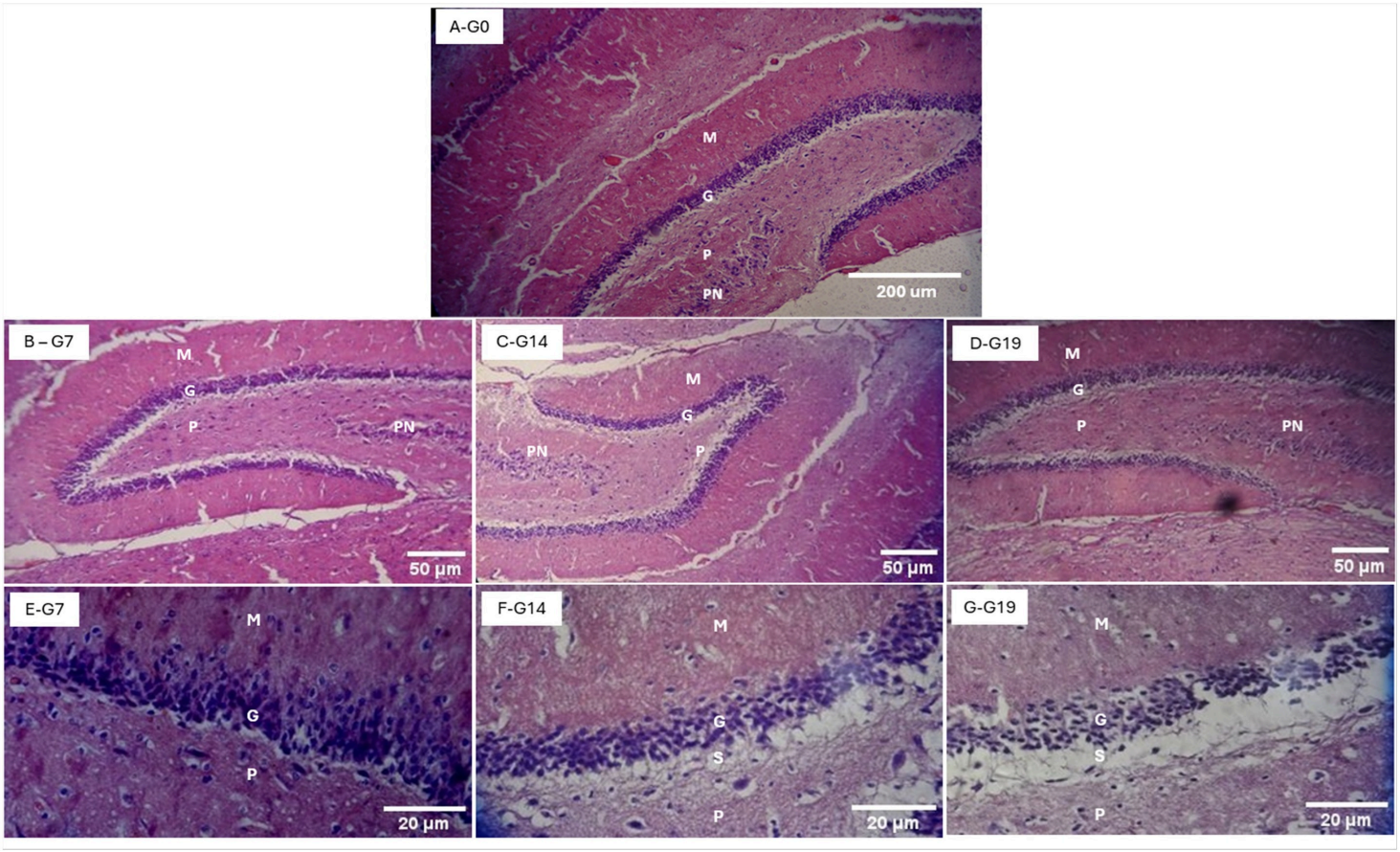


Figure 1

illustarate dentate gyrus after staining and secting in all four stage of pregnanacy ,G0,G7,G14,G19, staining by H&E. A: G0 Illustrate Molecular (M), Granular (G), and Polymorphic (P) layer (4 Power of magnification). B&D: G7, G14 &G19 respectively (10 power of magnification) illustrate granular disturbance of granular layer and emerging sub-granular cavity (Sub-Granular Space -S-). E&G: The power

of magnification is 40 for G7, G14 &G19 illustrated neuronal disturbance and loss in addition increase intracellular space (S).

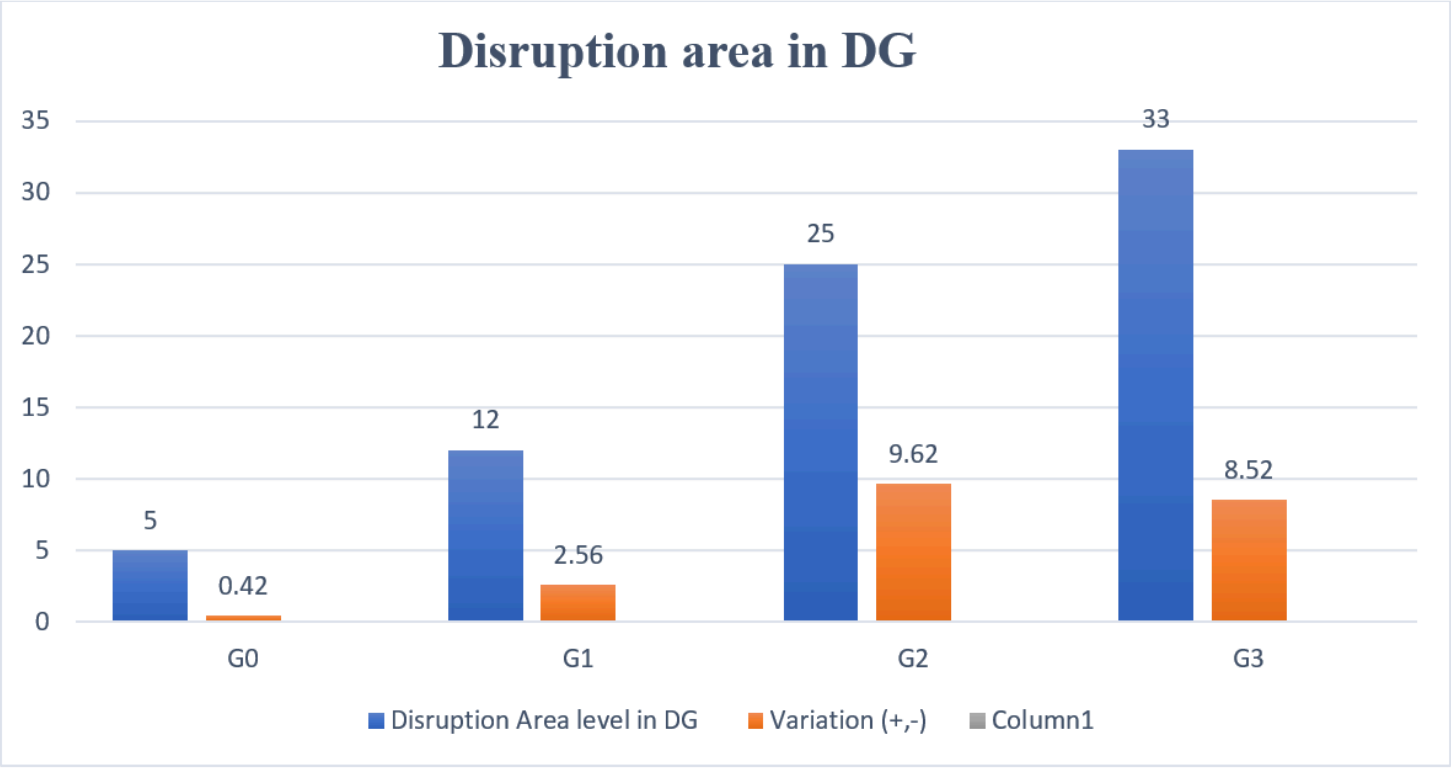


Figure 2

illustrated analysis quantitively of structural deterioration of dentate gyrus . The graph demonstrated mean percentage of deuteriation region (N=10 per group) ± SEM. A progression of deuteriation increase gradually reported from G0 to G19, reach to highest level in last gestation $p < 0.05$, $p < 0.01$, $p < 0.001$ demonstrate significant variation between groups

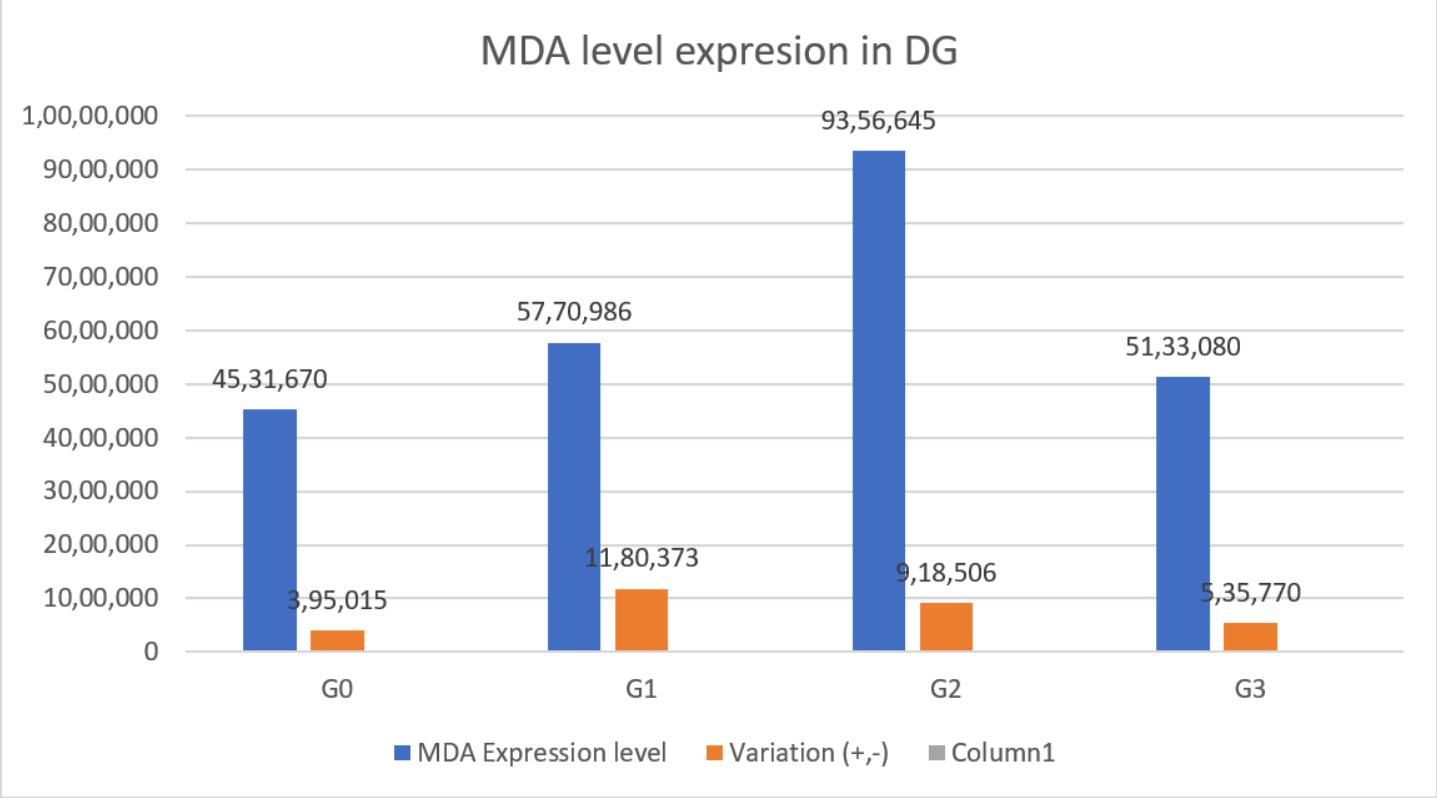


Figure 3

illustrated MDA expression which consider reflection for oxidative stress in dentate gyrus (DG), chart demonstrated mean of MDA expression (n=10 per group) ± SEM .highest level in G14,Lowest G0,The decline stared in G19 but still higher than G0.

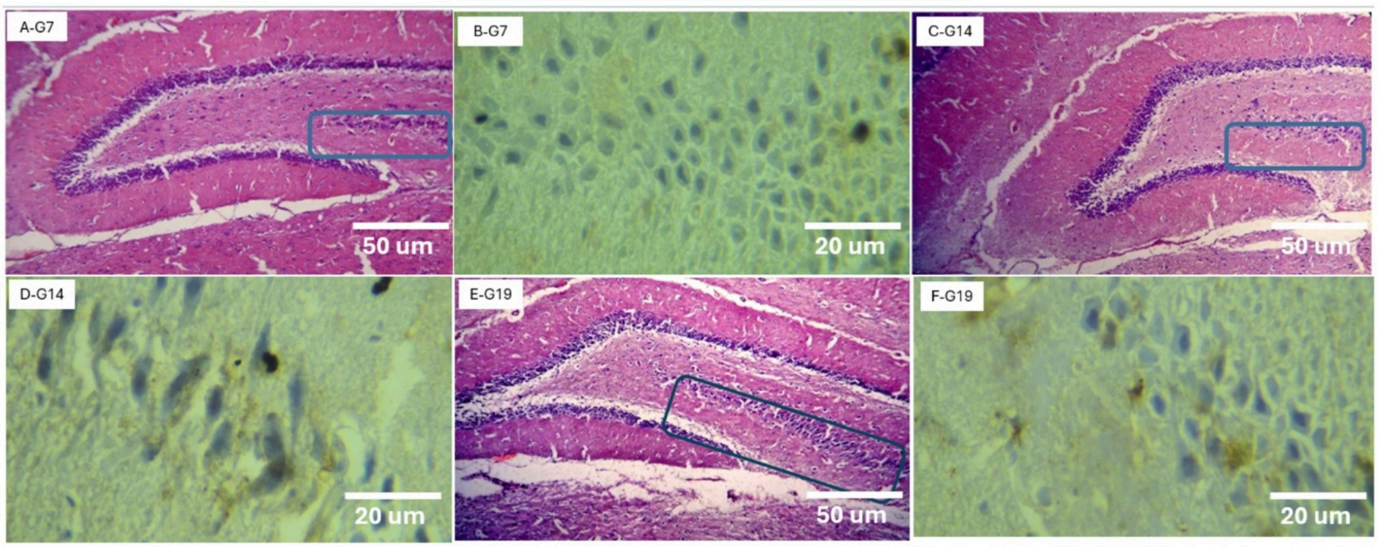


Figure 4

illustrated the CA4 region of hippocampus in all four group , immunohistochemically and histologically evaluation.The A, C&E H&E at power 10 , B,D&F immunohistochemical staining for MDA at power 40 .

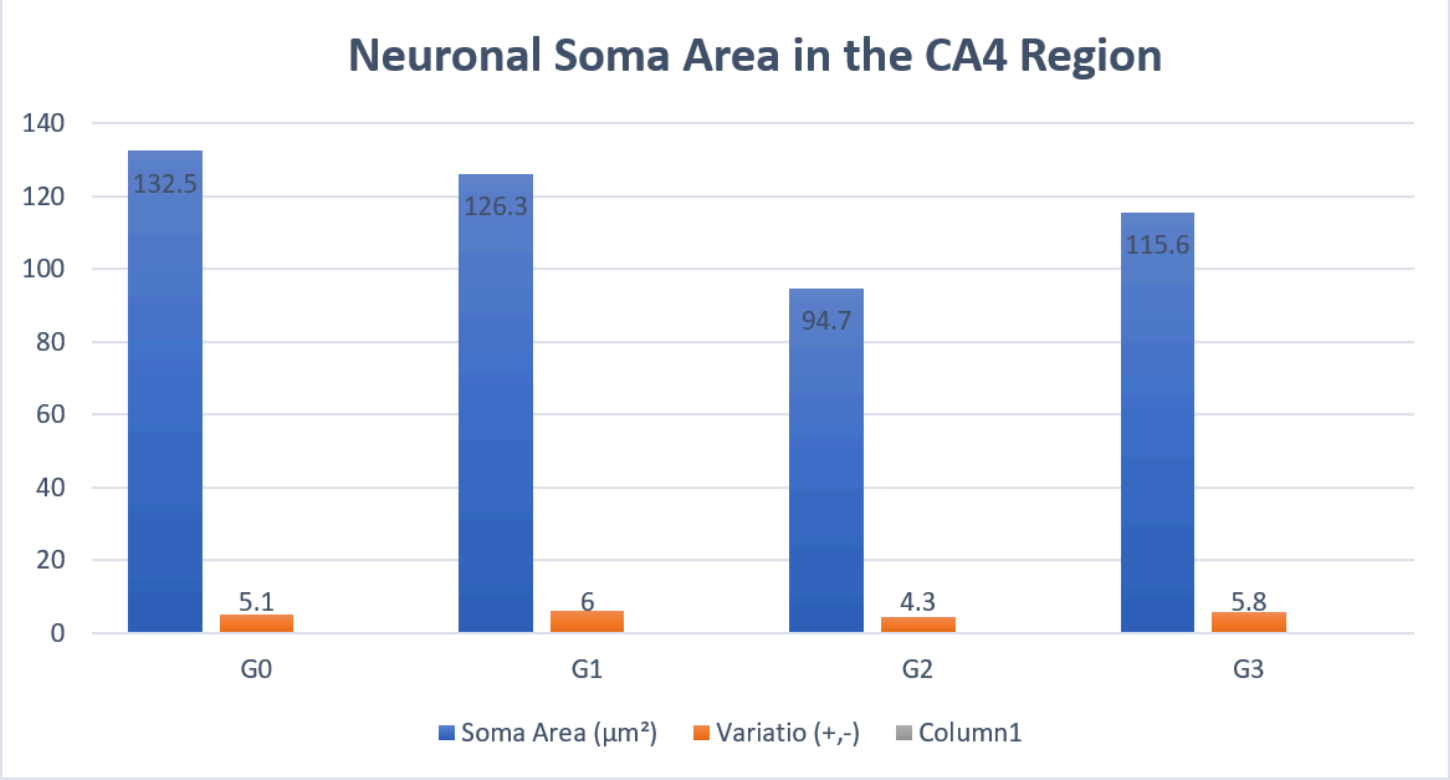


Figure 5

Neuronal soma area in the CA4 region of hippocampus , in which illustrated depicting mean soma area(µm²) for nerve cell in CA4 region (n=10 per group) ± SEM. The size variation reaches to highest level in G14, in compared with G0, partial recovery in 4th group.

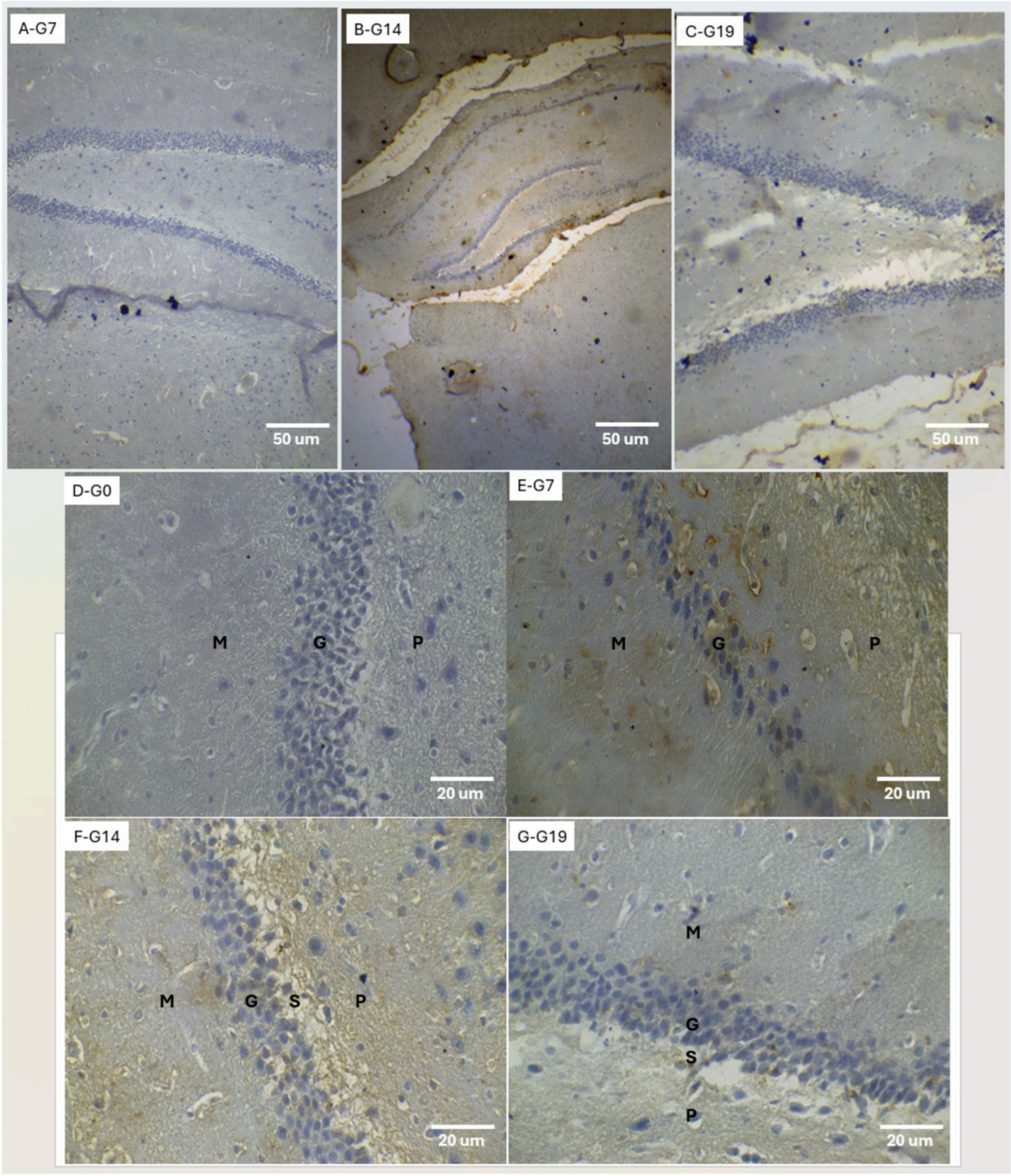


Figure 6

immunohistochemistry expression of Anti-MDA in the **DG**,

- A: G7, B: G14, C: G19(Power 10), D: G0, E: G7, F: G14, G: G19(power 40). The staining illustrated increase in MDA expression from G0 to G14 and reduced in G19.

-M. Molecular layer, G. Granular Layer, P. Polymorphic layer S. Sub-granular space.

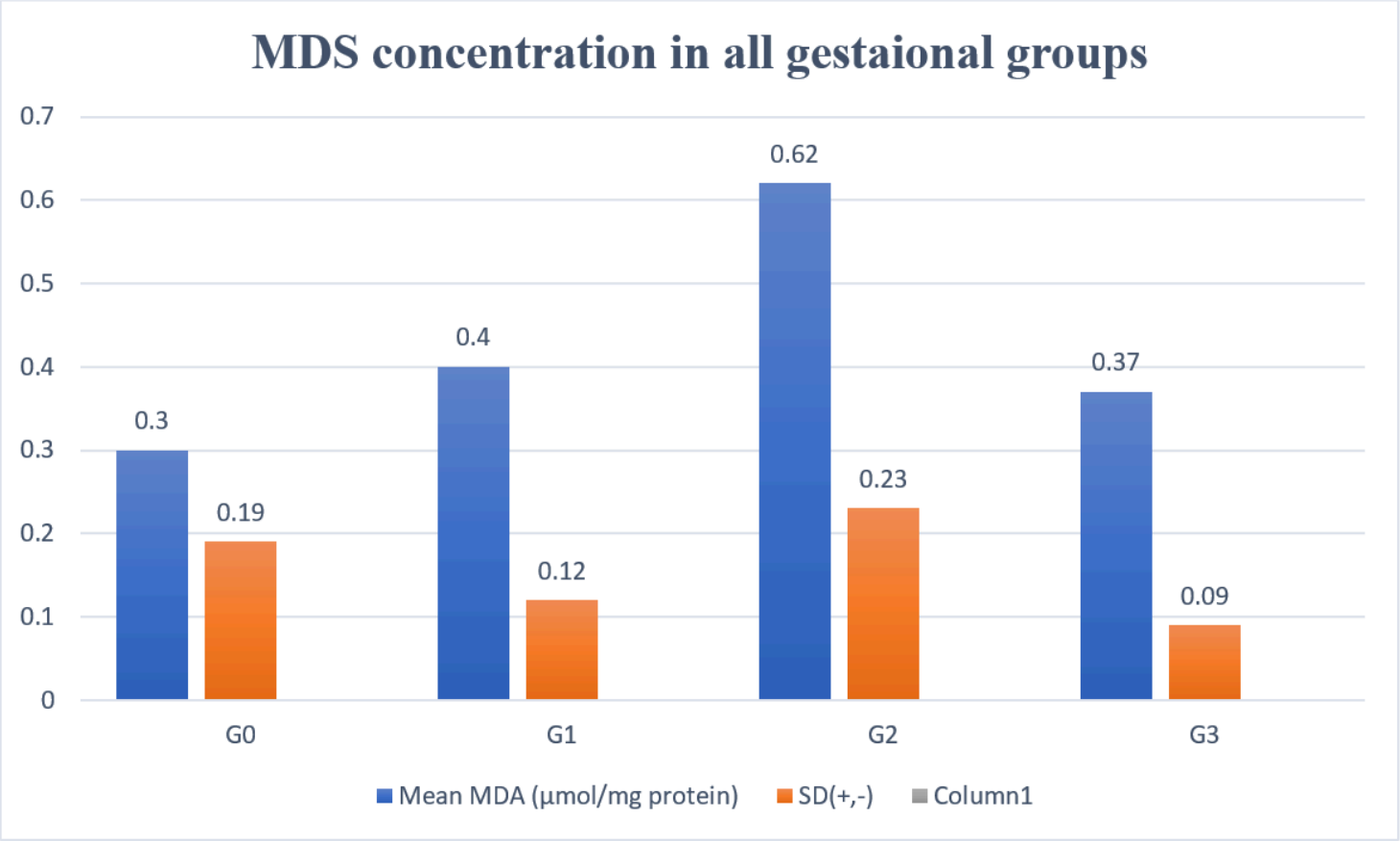


Figure 7

illustration the variation in MDA concentration in all gestational groups (hippocampus tissue)by (µmol MDA/mg protein) (n = 10 per group) ±SEM, determined using a colorimetric lipid peroxidation assay. Highest concentration occurs in (G14), followed by a significant decline at G19. $p < 0.05$, $p < 0.01$ indicate significant differences between groups.