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Caffeine pre- and postnatal exposure and its effects on brain histoarchitecture at puberty in mice

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ABSTRACT

This study investigated the effects of pre- and postnatal caffeine ingestion on the frontal cortex of experimental mice. Thirty-two (n=32) pregnant mice (*Mus musculus*) were divided into four groups: group A control, received distilled water; group B received caffeine- 10 mg/kg body weight; group C received caffeine- 50 mg/kg body weight; and group D received caffeine- 120 mg/kg body weight. The study was categorised into two phases; Phase I investigated prenatal caffeine exposure with half number of offspring sacrificed at birth. In Phase II, caffeine exposure continued till postnatal Day 35, marking puberty. Following sacrifice, brain specimens were processed using the H&E, Feulgen DNA and GFAP histological and histochemical techniques. Examination of cortical histoarchitecture showed that the caffeine- 120 mg/kg treated group had the most adverse effects with mortality soon after birth. Neuronal morphological heterogeneity was observed at doses higher than 10 mg/kg and these effects persisted till puberty. Astrocyte morphologies and patterns of dendrites were altered relative to the controls at puberty. Caffeine exposure altered the pattern of brain cell development by altering morphologies, patterns of elaboration and spatial distribution. Effects were dose-dependent.

Keywords: Brain, Frontal Cortex, Caffeine, Development, Neurons

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INTRODUCTION

Caffeine produces effects on mental functions including cognition[1] as well as brain structures and development.[2,3] Caffeine effects have genetic links[4]. However, it is difficult to state explicitly whether these effects are totally beneficial or deleterious. Cognitive enhancement or delayed decline in the elderly[5] has been mentioned as one of the main benefits of caffeine use.[6] It is however also important to note that caffeine influences natural neuronal communication, particularly by altering the activities of adenosine via adenosine receptors AR_1 and AR_2A primarily at physiological and moderate doses.[7] These receptors are widely distributed in the brain. There is evidence that suggests that continual use of caffeine might alter the brain's neurochemistry.[8]

An important perspective on the effects of caffeine on the brain is its effects on the developing brain. Caffeine has been reported to affect the process of neuronal development at certain stages[9], including memory mechanisms.[10] However, there remains a need to establish whether these effects on mental functions are persistently positive or otherwise. Previous report had stated that caffeine exposure during intrauterine life altered brain development and might produce negative effects that could impair the normal structural organisation of the brain, and consequently its functions.[11] Thus, elucidating the specific effects of caffeine on the brain during development requires conscientious investigation. If there are positive effects, the role of dosage variations is also important. If there are negative effects, it will be crucial to establish what they are. More importantly, the vulnerability of the brain to caffeine at the various stages of both intrauterine and postnatal development requires proper understanding. Such specific effects are yet to be properly documented whether in humans or in model or experimental animals, thus creating a knowledge lacuna. The period and patterns of brain development, as well as the dose of use, should be carefully and adequately modelled in experimental animals in order to obtain more reliable results.

The frontal cortex is the cortex of the frontal lobe, the anteriormost aspect of the cerebral cortex. It is the primary higher centre for executive functions which largely determine personality attributes as well as behaviour. These executive functions of the frontal cortex are however vulnerable to the effects of caffeine ingestion. Caffeine is an alkaloid with the chemical formula- $C_8H_{10}O_2N_4 \cdot H_2O$); it is naturally found in coffee, tea, cacao and some other plants. Caffeine is a central nervous system stimulant consumed globally either as an artificial additive to foods and drugs or in its natural sources including coffee and tea among others. It is the most popularly consumed stimulant.[12,13]

Currently, it is difficult to harmonise the results in existing literature because of irreconcilable variations in terms of sources and purity of caffeine ingested, the routes of administration, age and methods as well as the primary objectives of such research designs. The results thus appear discrete in terms of idea, methodology and objectives. What these facts present is that caffeine is yet to be adequately studied to appreciate its specific effects on mental health and brain development. This fact should be of major concern to the scientific world. The current

experiment used three doses that were carefully determined based on human caffeine consumption patterns. It should be noted that these values are lower than the recommended dose limit of 200-300mg/kg body weight[14], especially for pregnant women. The aim of this investigation was to assess the effects of pre and postnatal caffeine ingestion on the development of the frontal cortex of mice.

MATERIALS AND METHODS

Caffeine Preparation and Administration: Pure anhydrous caffeine was used for the investigation. It was obtained from Powder City, York USA; labelled as dietary supplement caffeine anhydrous with Batch Number 151219 and Lot Number CAFAH20151012. It was dissolved in distilled water at room temperature to produce the desired concentrations that were suitably administered to the mice.

Experimental Animals- Housing, Handling and Treatment: Mice (*Mus musculus*) were used for the investigation; a total of 32 (N=32) pregnant mice of approximately the same age (60 days old) and 21g average body weight were primarily recruited for the study. Mice were housed in a suitable, clean and dry animal holding facility. Drinking water was made available and accessible. Bedding in the cages was ensured to be non-allergenic, dust-free, inedible, absorbent, non-toxic and free of pathogenic organisms. Mice were properly fed to ensure adequate growth and development and prevent malnutrition. General requirements of standard housing measures were observed as recommended by Fawcett.[15]

Ethical approval: The research was approved by the Department of Anatomy Ethics Committee, Olabisi Onabanjo University, Nigeria- OOUANAT-16.

Research Design: The 32 female pregnant mice were divided into four groups labelled A, B, C and D which included the Control (administered distilled water), Low-Dose, Medium-Dose and High-Dose groups respectively. Groups B, C and D were given 10 mg/kg, 50 mg/kg and 120 mg/kg body weight of caffeine doses respectively. The mice as grouped were treated in two phases (Phase I and II). In Phase I, pregnant mice in experimental groups were administered caffeine from day 0 (E0) of pregnancy until parturition. The offspring from the Phase I experimental animals were used in Phase II for postnatal caffeine administration. Prenatal administration (Phase I) started on day 1 of pregnancy and ended at parturition while postnatal administration (Phase II) started at parturition and continued until day 35 of postnatal life when the offspring expectedly attained puberty. Phase I investigated the effect of maternal caffeine ingestion on intrauterine development of the brain while Phase II investigated the postnatal development of the brain of offspring (first pups, and thenceforth juvenile, then young adults) under the influence of caffeine.

Phase I: Prenatal Phase

The adult female mice (parents) were allowed to mate before the 00:00 hour of day 0 of pregnancy; ratio of male:female being 1:1. Mating, hence prospects of pregnancy was confirmed by the observation of the vaginal plug.[16] Female mice that observably mated were selected and appropriately grouped and the day was

recorded as the day 0 of pregnancy. Caffeine was administered through the enteral (oral) route from day 0 of pregnancy to parturition (approximately Day 21). At parturition, randomly selected offspring (mean = 12/group) were sacrificed. Their brains were excised, observed morphologically, fixed and processed for histological and histochemical analyses. The remaining first-generation offspring (mean = 12/group) of the treated female animals were sacrificed at parturition- end of Phase I; the rest were passed on to Phase II of the investigation so as to study the postnatal effects of caffeine on them.

Phase II: Postnatal Phase

In Phase II, half of the offspring continued with the postnatal treatment as Groups A (control), B^{C+}, C^{C+} and D^{C+}. These treated groups continuously received caffeine till puberty i.e. day 35 of postnatal life- first through their mothers' milk [up to day 25] and thereafter through orogastric administration of the same doses after weaning till puberty [day 35]. The second set of animals from caffeine-treated mothers of the same groups were left to develop without caffeine administration till D35 of postnatal life in order to observe the possibility and extent of the persistence of caffeine effects on the frontal cortex. They only received distilled water after weaning. These were grouped according to the pattern of grouping but designated with a different subscript as A, B^{C-}, C^{C-} and D^{C-}. The animals were sacrificed at D35 (Week 5) of the postnatal life of the animals recruited into Phase II. This marked the end of the animals' treatment in the course of the experiment.

Tissue Processing for Histological and Immunohistochemical Procedures: The haematoxylin and eosin (H and E) procedure was used for histological demonstration.[17, 18] Tissues were generally formalin-fixed using formal saline, and paraffin embedded. A routine processing procedure was followed to produce sections of 5µ that were mounted on histological glass slides subjected to the staining procedure. Procedures for demonstrating selected histochemical properties across the research groups included the cresyl fast violet (CFV) for the Nissl bodies[19], and the Feulgen DNA technique- for DNA.[20, 21] For the immunohistochemical procedure, the glial acidic fibrillary protein (GFAP) technique for astrocyte marker was used.[22]

Photomicrography: Photomicrographic images of the tissue sections were captured using the Photomicrographic set- Leica LCD. Analysis of the photomicrographs was done considering the general histoarchitectural features, and integrity of the tissue layers cells of the cortical layers. Cellular integrity was interpreted following the qualitative methods described by Garman[23]

RESULTS

The offspring in the group that was exposed to high caffeine dosage during pregnancy (D) all died by day 12 after birth, hence, the results available for this group included only those that were taken at birth. Offspring in the group in which caffeine exposure was limited to pregnancy periods died about the same time that offspring that were continuously exposed to caffeine during the pre- and postnatal periods died. Histological and histochemical observations of the frontal cortex samples were presented as

photomicrographs. Each set of photomicrographs compare the cortical structures and attributes of interest between the various groups. Emphasis was laid on observable and significant structural aberrations.

Caffeine Effects on Cortical Organisation and Cell Morphology: Figures 1-2: There are observations of caffeine's effects on the nervous tissues during development as variations in the morphology of individual cells and the spatial distributions of the cells within the frontal cortex. Caffeine at the lowest dosage produced relatively mild morphological cellular variations. Cortical histoarchitecture when medium caffeine dosage was administered showed morphological heterogeneities in cells even within the same cortical areas, and with observable distortions in spatial neuronal distribution and density. Certain cells have relatively less defined morphology relative to group (B) where the lowest dosage of caffeine treatment was administered. There is an observable disruption to the neuropil. Observations suggest that caffeine at this dosage still influenced the development and differentiation of cortical neurons- especially the pyramidal cells. The highest caffeine dose has the most deleterious effects on the cells and neuropil.

Caffeine's lowest dosage as administered continuously through pregnancy (to the mothers) and till puberty (to the offspring) (B^{C+}) influenced cell morphologies- especially the pyramidal cells. Most cells have normal morphologies and the granular cells are closely related in morphology to the counterparts in the control Group A. However, certain cells- observably pyramidal, exhibit relatively elaborate differentiation. The group administered the lowest caffeine dosage only during pregnancy (B^{C-}) helped to assess the possibility of the sustenance of observable caffeine effects on the frontal cortex tissue up to puberty even when the animals were only exposed to it in pregnancy. The cortical general histoarchitecture is largely preserved. There are only signs of mild morphological disruptions. The group administered the medium dosage of caffeine throughout pregnancy (to the mothers) and then continuously through the postnatal life till puberty (to the offspring) (C^{+}) had the brain cortical histoarchitecture preserved to a large extent; neurons and glia were demonstrated across the layers. Also, certain cells show a heterogeneous appearance relative to others and there is differential staining intensity among some others. A few cells have perinuclear spaces and glia are closely associated with many of them. The relative heterogeneity of cortical cells, even within the same cortical region or layer, points to the negative effects of caffeine administration on the brain tissue.

Cortical expression of Nissl bodies: Figures 3-4: Cortical expression of Nissl bodies at birth showed evidence of aberrations. This would further imply that rough endoplasmic reticulum-ribosome conjugates which are associated with cellular functions that are concerned with protein synthesis were altered at birth due to caffeine exposure. Heterogeneities in Nissl bodies were observable at birth. At puberty, however, there were nuclear aberrations that are indicative of pyknosis and karyorrhexis with these being most prominently observable in Groups that were administered the medium caffeine dose (C+ and C-).

Patterns on DNA distribution within cortical tissue: Figures 5-6: The Feulgen DNA Histochemical Technique (FDNA) demonstrates the DNA composition of the tissue which in primarily contained in the nucleus. The DNA materials primarily stain pink while the surrounding material takes on shades of green. Deoxyribonucleic Acid (DNA) is richly demonstrated in the cerebral frontal cortex of Group A animals. The general histoarchitecture shows an expanse of tissue cross-sectional area characterised by the adequately abundant distribution of the NDA materials throughout the cortex. The neuropil constitutes the environment of the cell-contained DNA materials. These observations are consistent with a normal cortical demonstration.

The cell-contained DNA material is well demonstrated in the cortex of the Group B animals whose regimen included the administration of low-dosage caffeine (to the mothers) throughout pregnancy. Slight aberrations relative to the control (group A) however include heterogeneities in the distribution of the DNA within cortical tissues. There are relatively large areas of DNA deposits indicating either larger cellular masses or clustering of cells. Also, the neuropil appears relatively quite abundant. Relative prominence or abundance of neuropil demonstration suggests more inter-soma fibre-occupying spaces. Observations, especially at the lower magnifications suggest that this occurrence might not be throughout the cortex. Thus, in line with morphological and myelination demonstrations; it is most likely that the areas of rapidly differentiate large cells that have less cell density per unit tissue volume consequently have abundant neuropil-areas surrounding the cells.

The pattern of DNA distribution as demonstrated in the group administered the medium dosage of caffeine showed no major aberrations. However, the mildly reduced staining intensities of DNA material and neuropil might indicate reduced cortical integrity relative to the control. When the highest dose of caffeine was administered (D), DNA-neuropil relative distribution also showed evidence of mild aberrations. Collectively, in the exposed groups, the observed mild aberrations persisted till puberty.

Caffeine Exposure and Effects on Astrocytes: Figures 7-8: At birth, astrocyte morphologies were relatively less defined in the groups administered caffeine relatively to the controls. These aberrations largely persisted till puberty. Photomicrographs of the Frontal Cortex of the group that was only exposed to the low caffeine dose (B^{C-}) in the pre-natal stage show that several astrocytes are relatively normal in morphologies relative to the control. However, certain astrocytes still exhibit deformed morphologies. Also, certain neurons are surrounded by dense astrocyte processes. While these observations largely show that the astrocytes in this group appear less affected morphologically than their counterparts that have pre- and postnatal caffeine exposure (B^{C+}). Also, photomicrographs of the frontal cortex of the group that had pre- and postnatal caffeine exposure (C^{+}) presented aberrations in branching patterns of astrocyte processes. The photomicrograph GFAP-demonstrated samples of the frontal cortex of the group that had prenatal caffeine exposure (C^{C-}), at puberty, show that the astrocytes are relatively heterogeneous in morphology.

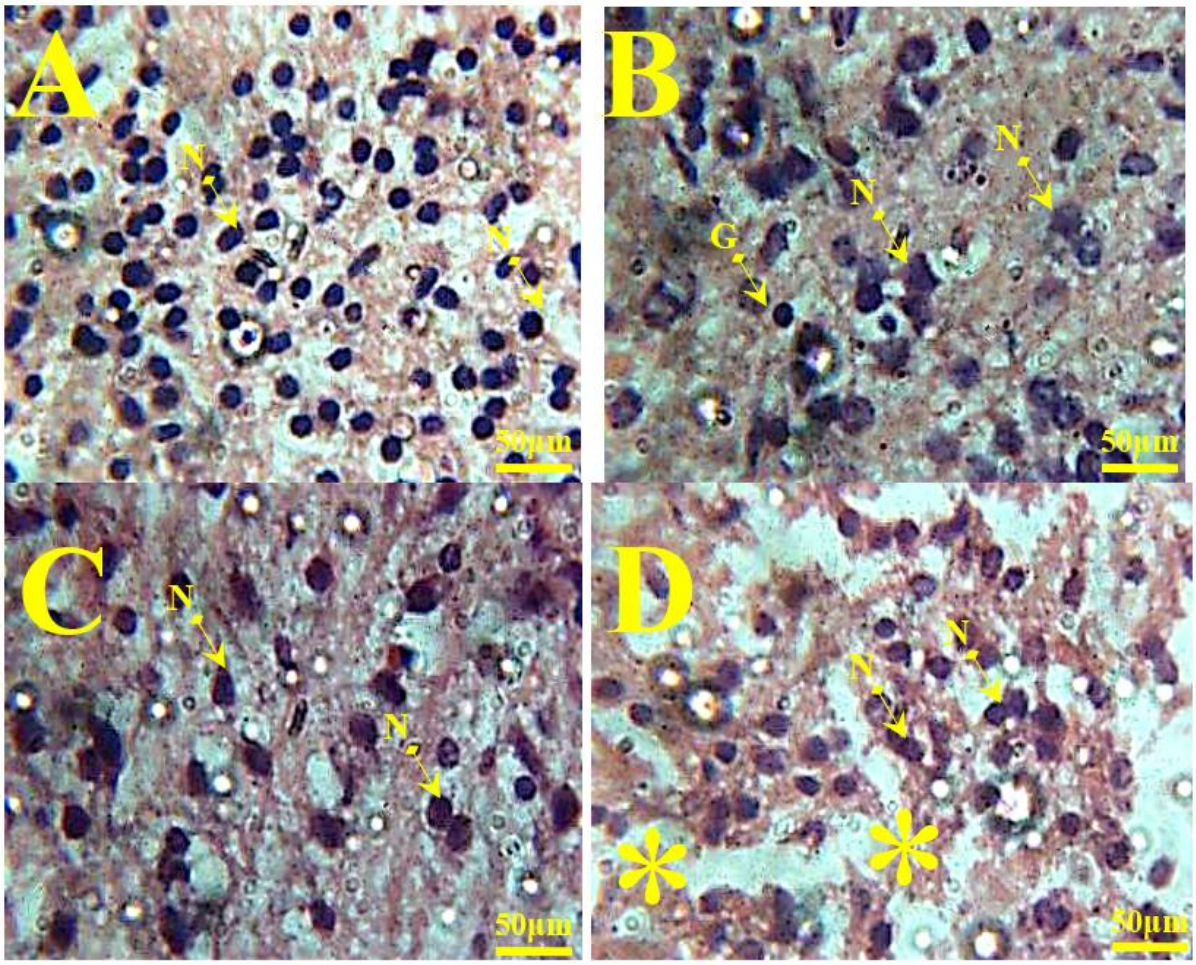

Figure 1: Photomicrographs of the frontal cortex of the mice litters sacrificed at birth (D0) (H and E; A= Group A; B= Group B; C= Group C; D= Group D). At birth, effects attributable to caffeine exposure during the intrauterine stage included alterations in the morphologies of cells in terms of size and shape. Cell populations per tissue cross-sectional areas were also reduced in a dose-dependent manner. Caffeine prenatal exposure altered cortical cell morphologies and their spatial distribution in the treated groups relative to the control. Effects became more prominent with increases in dosage. The brain cells of mice that were administered the lowest (A) and medium (C) doses of caffeine are relatively larger. When animals were exposed to the highest dose of caffeine, the cortical organisation of cells was disrupted relative to the control (Group A)**.**

N: Neuron; **G**= Glia.

* indicates an area of disrupted histoarchitecture

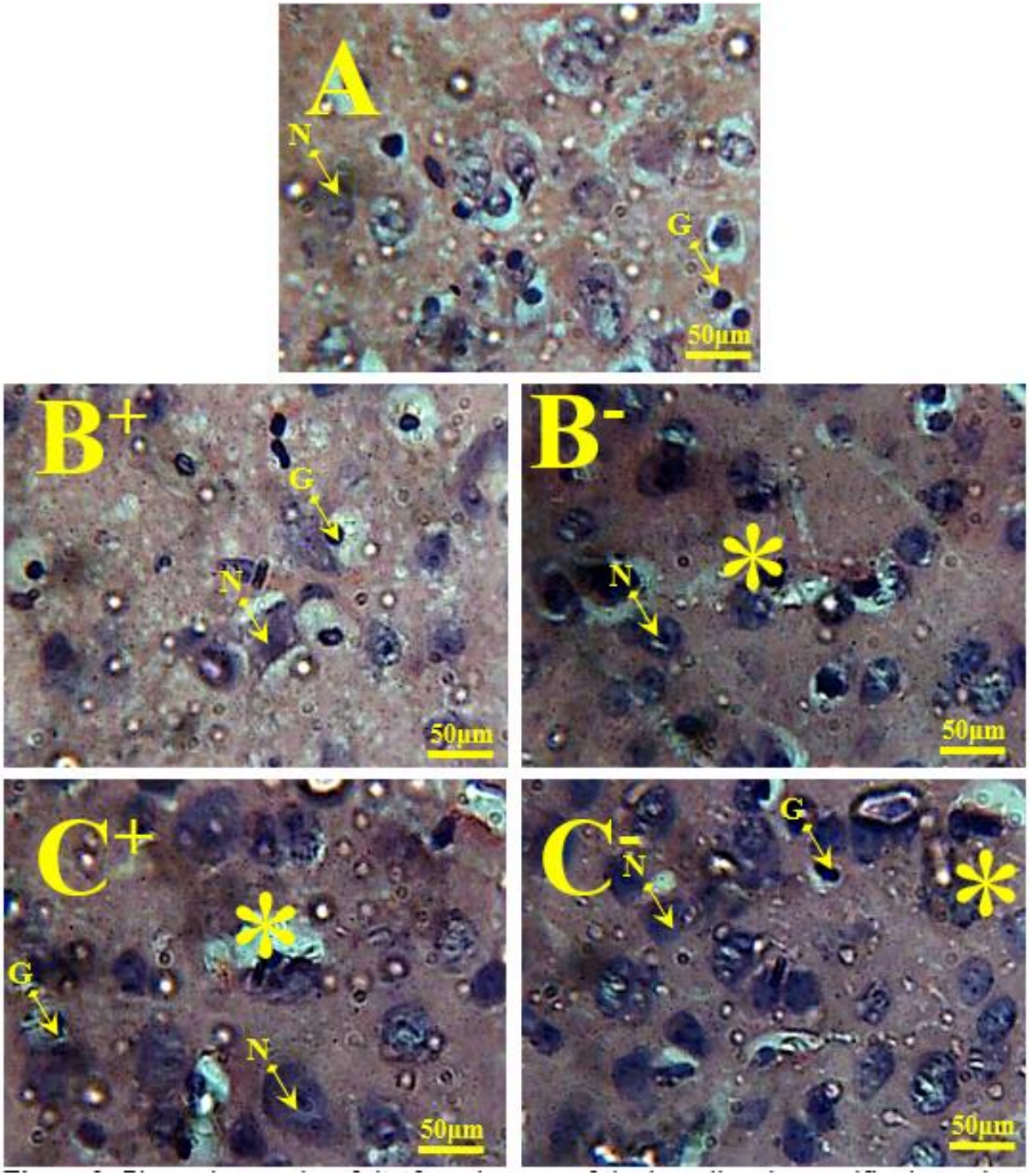

Figure 2: Photomicrographs of the frontal cortex of the juvenile mice sacrificed at puberty (D35) (H and E; A= Group A, control; B^+= Group B^+, administered low-dose caffeine during pre-and postnatal phases; B^- = Group B^-, administered low-dose caffeine during prenatal phase only; C^+= Group C^+, administered medium-dose caffeine during pre-and postnatal phases; C^- = Group C^-, administered medium-dose caffeine during prenatal phase only). The effects of caffeine exposure on neurons are persistent till puberty. Certain effects of caffeine exposure persisted till puberty and effects included persistent morphological alterations Certain cortical cells in the groups exposed to caffeine exhibited aberrations that are indicative of pyknosis and karyorrhexis (see asterisks*) [Scale bar = 50 µm**]**.

H and E: Haematoxylin and Eosin Staining Technique **N:** Neuron; **G**= Glia
* indicates the morphologically altered neuron

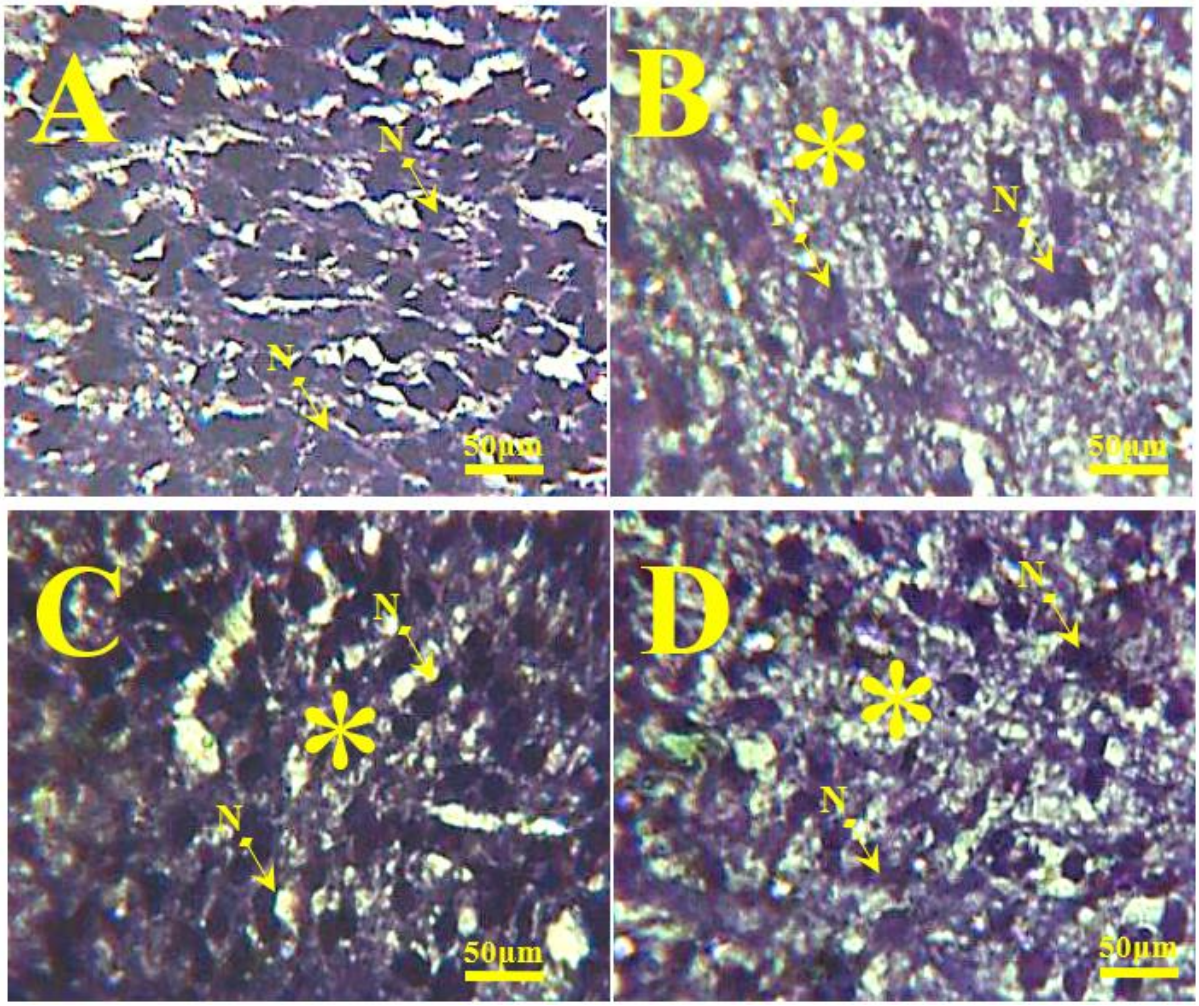

Figure 3: Photomicrographs of the frontal cortex of the mice litters sacrificed at birth (D0) (CFV; A= Group A; B= Group B; C= Group C; D= Group D). Cell morphologies vary and the Nissl bodies staining patterns in the treated groups suggest alterations in the activities of Nissl bodies. This indicated alterations in the activities of the rough endoplasmic reticulum and ribosomes involved protein synthesis. The pattern of cortical cell arrangement was altered. Cell morphologies appear more elaborate in B with consequential reduced Nissl density. Certain cortical areas of the treated group brains are relatively sparsely populated* [Scale bar = 50 µm**].**

N: Neuron

* indicates an area of altered histoarchitecture

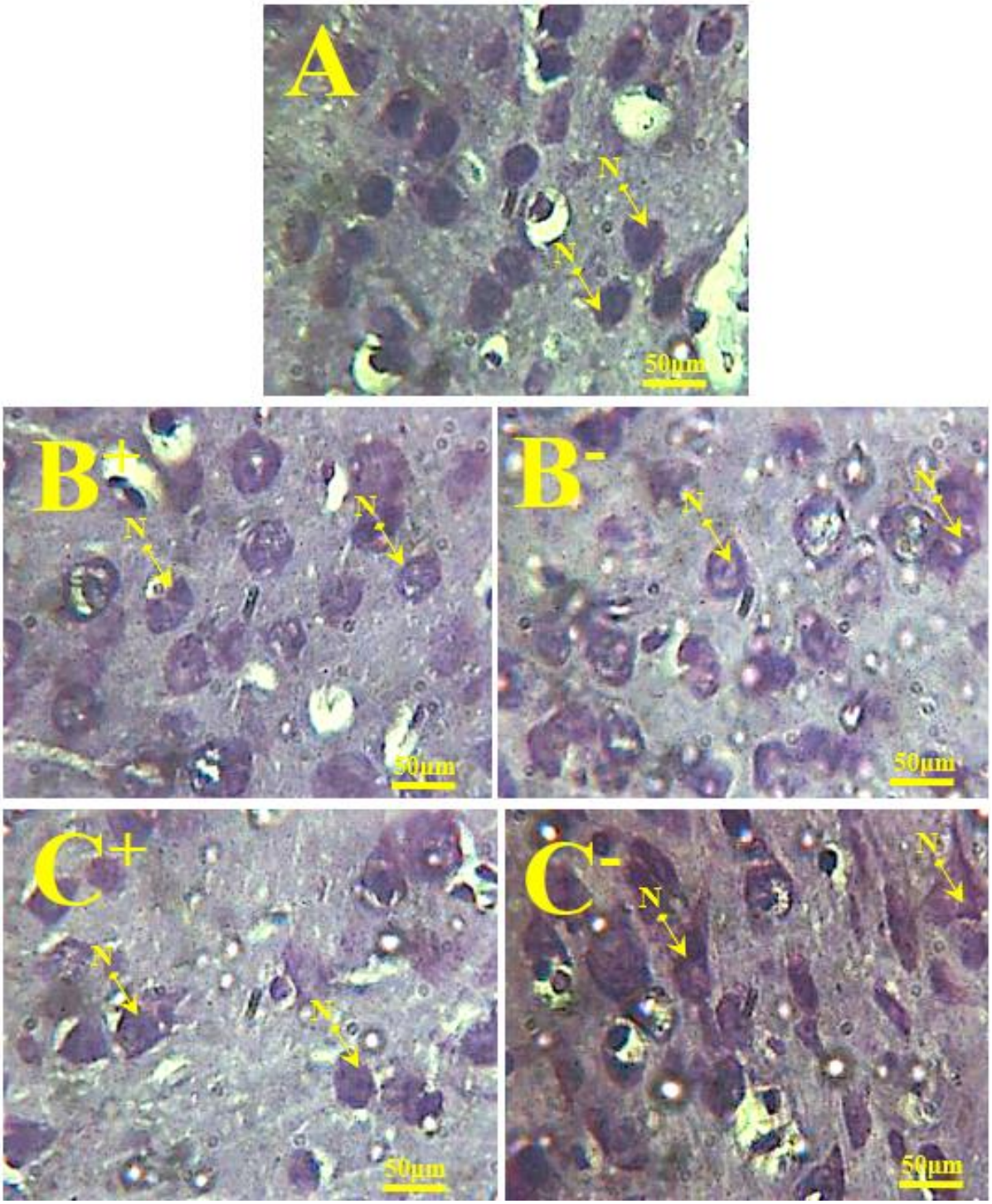

Figure 4: Photomicrographs of the frontal cortex of the mice sacrificed at puberty (D35) (CFV; A= Group A, control; B^+= Group B^+, administered low-dose caffeine during pre-and postnatal phases; B^- = Group B^-, administered low-dose caffeine during prenatal phase only; C^+= Group C^+, administered medium-dose caffeine during pre-and postnatal phases; C^- = Group C^-, administered medium-dose caffeine during prenatal phase only). Cortical neurons in groups that were administered the higher caffeine dose show persistent morphological heterogeneity There are nuclear aberrations that are suggestive of nuclear fading, pyknosis and karyorrhexis with these being most prominently observable in Groups C^+ and C^- [Scale bar = 50 µm**].**

CFV: Cresyl Fast Violet Staining Technique **N:** Neuron; G= Glia

* indicates morphologically altered neuron and histoarchitecture

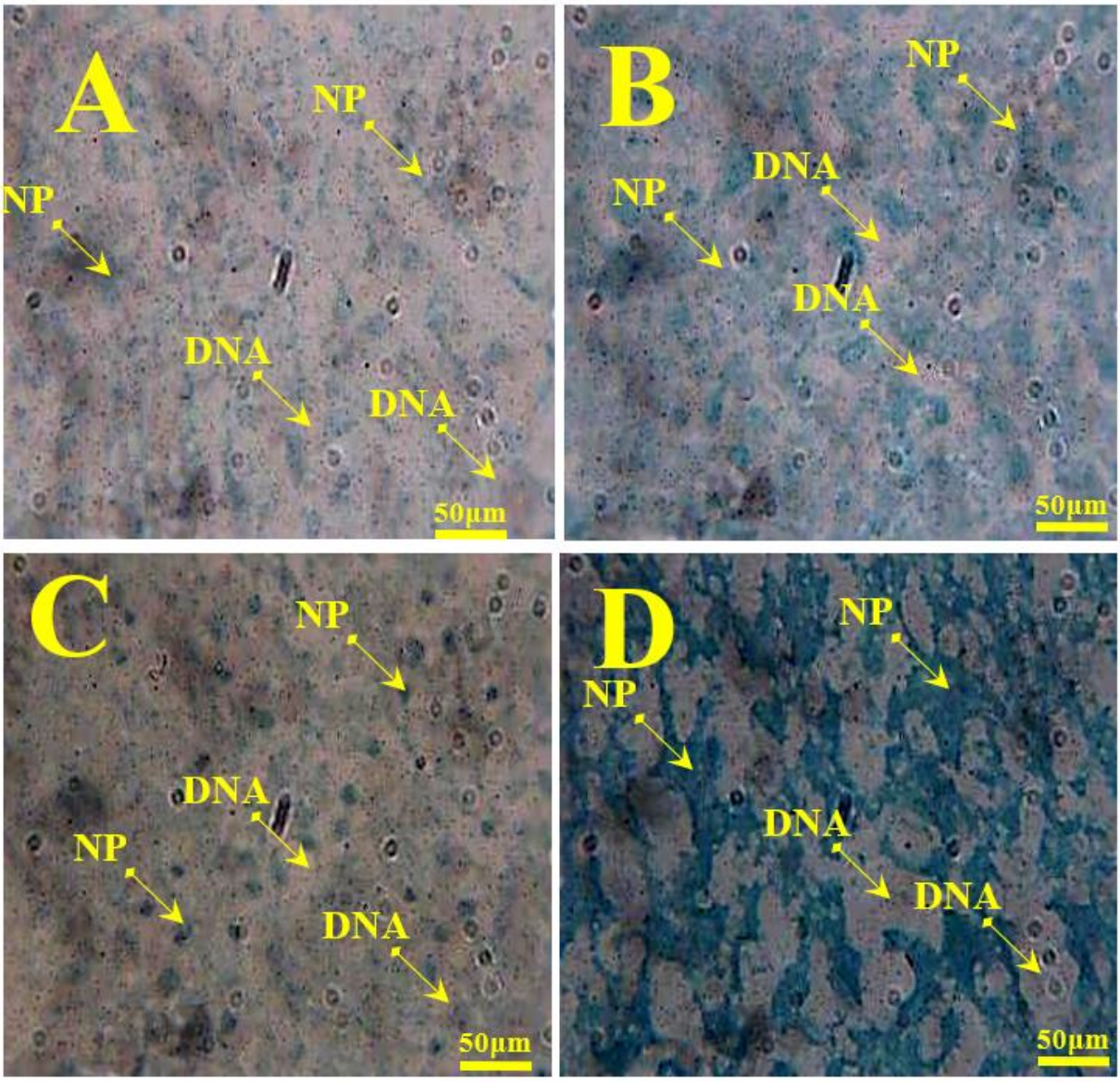

Figure 5: Photomicrographs of the frontal cortex of the mice litters sacrificed at birth (D0) (FDNA; A= Group A; B= Group B; C= Group C; D= Group D). DNA abundance in the cerebral cortex of Group D mice is relatively less abundant. Generally, the pattern of DNA distribution in the treated groups differed relative to the control Group A. Caffeine exposure affected the distribution of DNA in the treated groups (B-D) relative to the control (A). At the highest dosage- there is relatively less abundance of DNA indicating an aberration in DNA-tissue volume proportion and distribution [Scale bar = 50 µm**]**.

Key and Legend: FDNA: Feulgen DNA Histochemical Technique **DNA:** Deoxyribonucleic Acid; NP: Neuropil

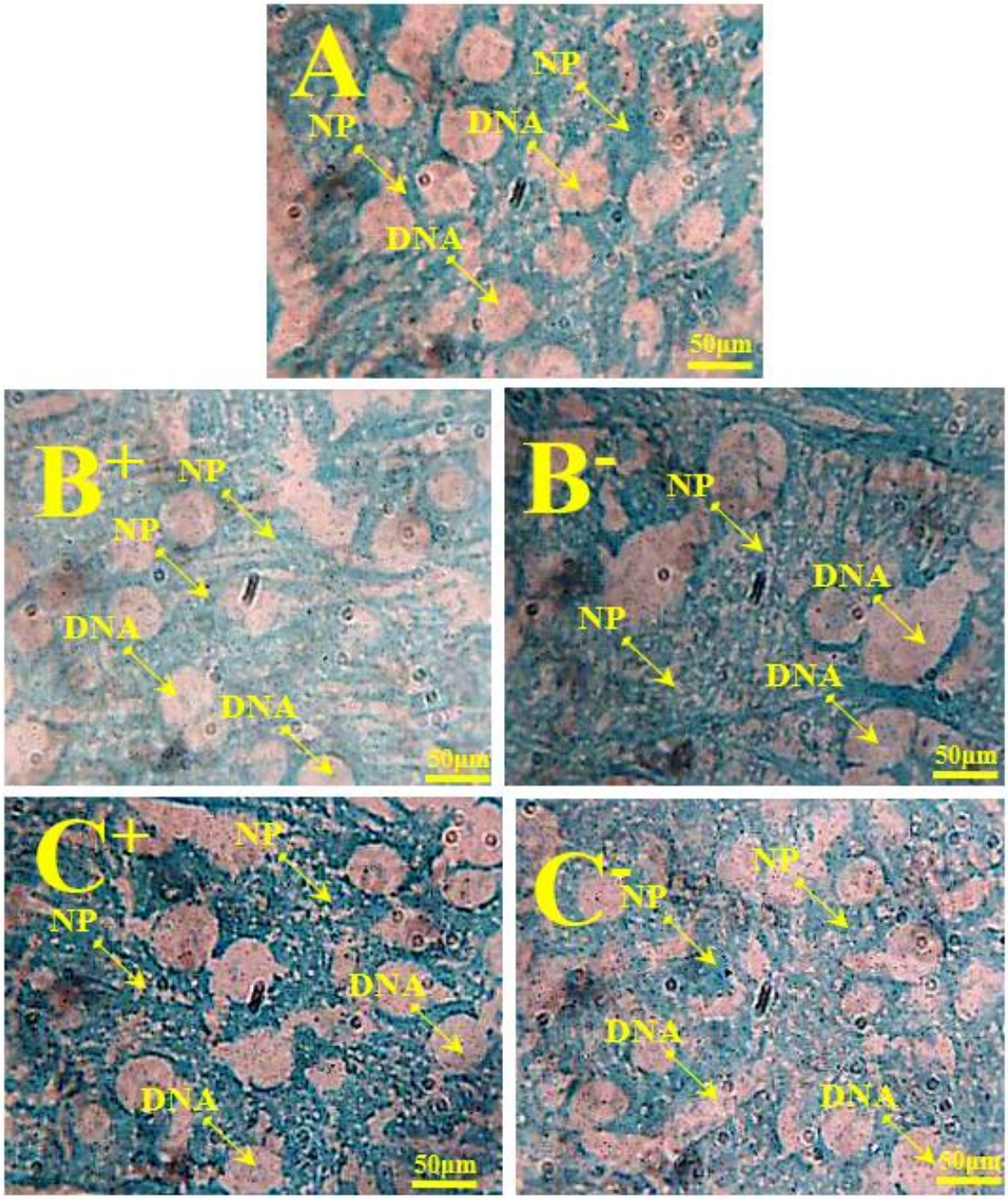

Figure 6: Photomicrographs of the frontal cortex of the juvenile mice sacrificed at puberty (D35) (FDNA; A= Group A, control; B^+= Group B^+, administered low-dose caffeine during pre-and postnatal phases; B^- = Group B^-, administered low-dose caffeine during prenatal phase only; C^+= Group C^+, administered medium-dose caffeine during pre-and postnatal phases; C^- = Group C^-, administered medium-dose caffeine during prenatal phase only). In Group B, there is a relatively wider area of DNA localisation per cell but sparse in terms of number per tissue cross-section area. Large deposit areas in Group B^- (exposed to caffeine during pregnancy only) coupled with relatively few areas of such deposits indicate larger nuclei and fewer cells per cross-section area of the tissue. Also, the less defined pattern of DNA distribution in Group C^+/C^- would also indicate the relative heterogeneity of DNA deposits [Scale bar = 50 µm**].**
FDNA: Feulgen DNA Histochemical Technique **DNA:** Deoxyribonucleic Acid; NP: Neuropil

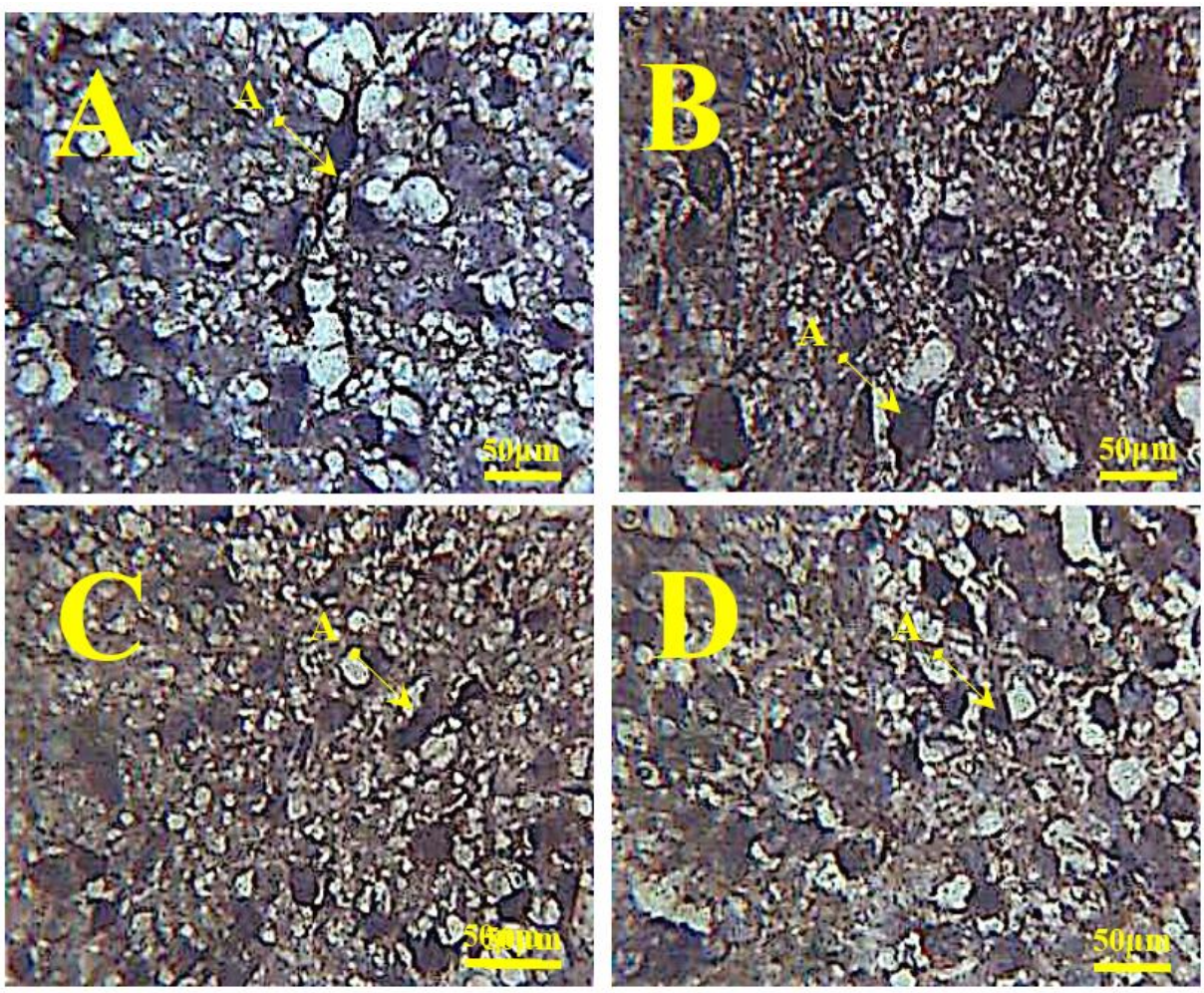

Figure 7: Photomicrographs of the frontal cortex of the mice litters sacrificed at birth (D0) (GFAP; A= Group A; B= Group B; C= Group C; D= Group D). Astrocytes in all the treated groups are less differentiated relative to the control. In the treated groups (B-D), cell bodies are demonstrated but the processes are poorly elaborated. Caffeine exposure in the treated groups affected astrocyte morphological elaboration at birth. In the treated groups (B-D), astrocytes were demonstrated but without typical patterns of processes. Caffeine exposure also affected cell sizes; astrocytes are relatively large in Group B and relatively smaller and less prominent in Group C and Group D [Scale bar = 50 µm**]**.

Key and Legend:

GFAP: Glial Fibrillary Acidic Protein Immunohistochemical Technique. **A:** Astrocyte

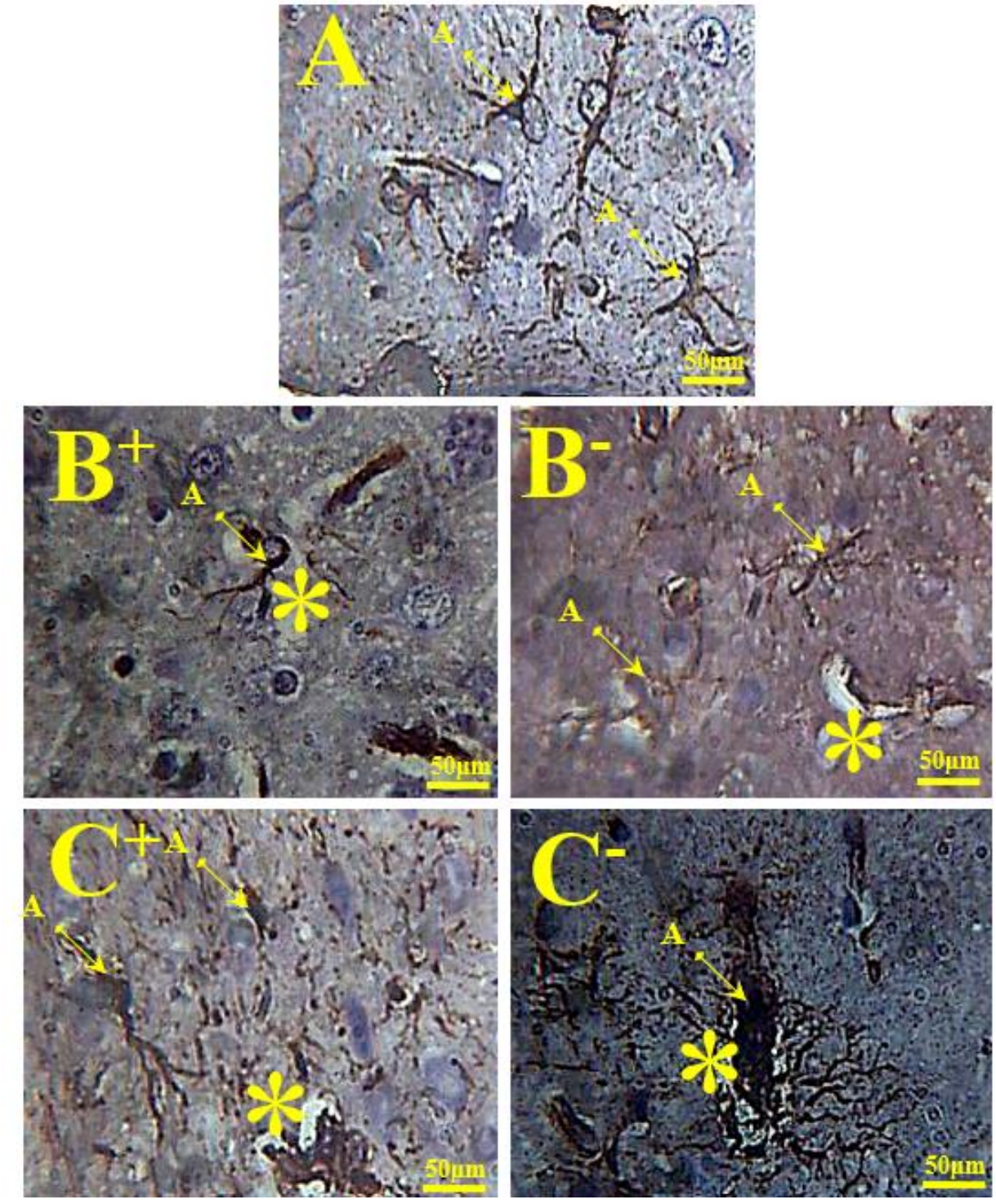

Figure 8: Photomicrographs of the frontal cortex of the juvenile mice sacrificed at puberty (D35) (GFAP). Astrocytes are observed in all groups. Groups that were exposed to caffeine had altered astrocyte morphologies. This effect remained persistent till puberty. Caffeine exposure affected astrocyte morphologies in all treated groups. Caffeine's exposure effects on the astrocytes remained persistent till puberty, even when discontinued at birth [Scale bar = 50 µm**].**

GFAP: Glial Fibrillary Acidic Protein Immunohistochemical Technique

A: Astrocyte. * indicates morphologically altered astrocyte

DISCUSSION

It is important to emphasise that the current investigation presents a multi-dimensional perspective into the effects of caffeine consumption on brain development, histological structure and functions using mice (*Mus musculus*) as a suitable model. There have been arguments in favour and against caffeine consumption in pregnancy as some reports have reported benefits[11,24] and others, deleterious influences.[25,26] The regimen, timing and perspective limitations have however played major roles in interpreting observations in most existing reports. This investigation has an edge by providing a multi-dimensional perspective of study and employment of dose and stage-based regimen design in addition to multi-perspective observation of parameters and interpretations of such. It is also crucial to state that the offspring from the group administered the highest caffeine dose did not survive till the end of Phase II of the experiment period of 35 postnatal days; the offspring died at approximately the Day 12 of postnatal life. This incidence strongly suggests that this dosage was grossly deleterious to the animals and even the consequences of prenatal exposure resulted in death about the same time that the animals of the same group that continued with the postnatal treatment died. In other words, the effects of the very high dosage of caffeine exposure in pregnancy resulted in death in the early days of postnatal life.

Caffeine Effects on Cortical Organisation and Cell Morphologies: Observed abbreviations in cells morphological are in line with previous investigations that suggested that the pyramidal cells were supposedly prone to positive postnatal caffeine effects by virtue of extensive dendritic differentiation and length.[11] It is important to note that the dosage of caffeine administered was relatively high and not a typical habitual caffeine consumer whether in natural coffee sources or in drinks and foods that have caffeine as an additive. Notably, while caffeine would primarily interact with the adenosine receptors AR1 and AR2A at conventional doses[7, 27]; it involves calcium intra-extra cellular movements at high doses and recruits AR2B and AR3 as well.[7,28] This would influence caffeine's extensive effects on the brain tissues at such high dosages.[7,29] However, this provides information on extreme cases of caffeine consumption which could reasonably be achieved by individuals that either consume caffeine pills or provided caffeine powder. The use of this dosage would expectedly also help in tracing the possible trends in the dose-effect caffeine interactions with the brain and link up the conventional caffeine dosages with the extreme dosages feasible. Interestingly, the mother mice tolerated this dosage throughout pregnancy and the offspring though reportedly fewer showed no sign of low birth weight.

The pattern of the observed effects of caffeine on the developmental and histological attributes of the frontal cortex of the experimental animals' brains shows that caffeine evidently and substantially influenced the morphological development of vital nervous elements. Results also show that variations in dosages produced variations in the nature of the effects rather than just the trend of effects. It is obvious that caffeine at the highest dosage caused extensive neuronal disruption in the frontal cortex at birth. Relatively scanty cells and disrupted neuropil suggest either a

disruption in cell differentiation or negative effects on primordial cells before reaching maturity and cortical destinations. The latter is suspected to be true in this instance; in addition, other evidence suggests a disrupted cortical process of migration. There have been reports of myelination disruption and astrocytogenesis inhibition caused by caffeine exposure.[24, 25] There was a downregulation of adenosine receptors when caffeine was administered to rats.[30,31] On the other hand, another report stated that caffeine neonatal administration caused a permanent increase in the dendritic length of rats prefrontal cortical neurons- a sign not reported as a negative effect.[11] Notably, it is therefore important to consider context and dose. For instance, protective effects of caffeine have been reported against chronic hypoxia-induced perinatal white matter injury.[32] On the other hand, neuronal death in neonatal rat brain and cortical cell cultures has been reportedly associated with caffeine effects.[33]

Cell morphology- including dendritic morphology by virtue of an increase in length persisting till puberty [11] - could be altered by agents and events- there have been reports of renovascular hypertension causing dendritic morphology alterations of pyramidal neurons. This shows that these neurons might be particularly susceptible to the influence of agents and events. Caffeine is an agent that could influence neurons as such.[30,34,35,36] Furthermore, caffeine has the potential to reorganise cortical network functions via synaptic mobilizations.[37] Change in pyramidal cell morphologies has been reportedly associated with caffeine's influence on pyramidal cells especially.[11] In the current study, cellular morphology is fairly heterogeneous. Aside from certain cells that exhibit relatively elaborate cellular differentiation; most other features of the cortex in this animal group are similar to the control. Cortical general histoarchitecture is largely preserved. Neurons and glia are observable in the superficial and deeper cortical layers. In previous similar research, caffeine postnatal treatment (50 mg/kg; postnatal day 1-12) reportedly enhanced pyramidal cell dendritic pattern by increasing the dendritic length up until puberty and much longer.[11]

When caffeine's highest dose was administered, brain cortical at birth (D0) had general histoarchitecture poorly preserved in the frontal cortex. There was evidence of disrupted neuropil and spatial distribution of cells. These provided evidence to support the fact that high-dose caffeine prenatal effect on the frontal cortex was largely negative and deleterious. The neuropil had an unusual feature- there were areas of extensive disruptions and other areas presenting dense materials accumulations not characteristic of the typical neuropil. The demonstrated cells were quite few relatively; with poorly defined morphology. Fibres were barely demonstrated in their characteristic pattern within the neuropil. These observations are similar to a number of previous reports whereby sustained disruptions in cortical fibre myelination have been reported for caffeine dosages above 20mg/kg body weight.[23] Also, permanent alterations in prefrontal cortical neurons due to neonatal caffeine administration have been reported in rats.[11] In addition, Park[38] reported that early-life caffeine exposure could have persistent effects on normative neuronal function.

Cortical expression of Nissl bodies: The Cresyl Fast Violet staining technique provides further insight into the cytological conditions of the demonstrated cells by demonstrating the Nissl bodies or materials in the cell. These materials are basically the rough endoplasmic reticulum[39] being demonstrated because of the rRNA materials associated with them.[40,41,42] The charged nuclear materials (DNA and RNA) are also differentially demonstrated as well. This provides reliable information not just on the cellular morphology and size that is typically observable, but the staining integrity also provides information on the functional status of the cell. This is because the level of activities at the level of the rough endoplasmic reticulum is a vital indication of the synthesising potentials of these cells. The rough endoplasmic reticulum state would be a marker cell's functional performance.[40,41,42]

Morphological observations in this study showed that cells were relatively larger. Also, there was evidence of altered cellular differentiation relative to the control. More so, these affected cells are of the deeper cortical layers showing a resemblance to pyramidal neurons more than any other cortical neuron type. Again, these observations suggest that caffeine altered certain cell morphological development and differentiation. Pyramidal cells are largely implicated. These observations are in line with the report of Mioranzza *et al.*[3] stating that even moderate caffeine consumption could alter foetal brain development.

The medium caffeine dosage employed in this investigation had its characteristic effects on the cell morphology and spatial distributions primarily. Unlike the lowest dosage that produced relatively altered and rapid cellular differentiation and development; caffeine medium dosage administration to this group of animals produced mild disruption of cell spatial distribution in certain cortical layers in the localised pattern. In addition, few cells show signs of morphological distortions. This might be a sign of impending degeneration or morphological alterations that might affect a number of cells and possibly their functions as a consequence. The nature of caffeine effects on the developing cortex could significantly vary just on the basis of dosage. This is reinforced by the effect of the medium dose relative to the low dose. These are possible indications that this caffeine dosage might have deleterious effects, however mild, on the developing brain. Mildly disrupted spatial arrangement, distribution and relationships between the neurons especially, and few instances of morphologically deformed cells might not be without accompanying functional consequences. Having stated the possible consequences of the structural aberrations in this group; it should be stated that the cells individually are largely normal in form, supposedly healthy in nature and adequate in the assessed intracellular activities or functions.

An excessively high dosage of caffeine was administered and tolerated by the mothers; their offspring at birth still had their general cortical histoarchitecture largely preserved. Most cells had normal morphology relative to the other animal groups including the Control Group A animals. However, there are observable aberrations resulting from the caffeine effects on the developing brains. There were localised regions of relative spatial disruptions. Certain cells in these localised regions of disruptions also exhibit morphological distortions. A keen

observation suggested that the primarily affected cells were somewhat more pyramidal than granular. Therefore, the supposedly deleterious effects affected the primordial pyramidal cells morphologically more than they probably affected the granular cells. Some of the cells affected as described also show slight deviations from the conventional staining pattern. Basically, this high caffeine dosage affected cells and compromised their morphologies in certain areas of the brain. The effects in the Nissl materials demonstration obviously point to functional integrity compromises. It is clear that cell function is largely limited when Nissl materials (rough endoplasmic reticulum, rRNA, ribosome etc) are compromised in such cells. These findings align with previous studies [43,44] about the dosage-and-time-dependent nature of caffeine effects on the brain.

The effects of caffeine on the brain when a medium dose was administered during the pre and postnatal stages of life until puberty was tested. The cortex generally presents normal histoarchitecture; cortical cells were demonstrated, and they were largely morphologically properly defined. The staining intensities of most cells appeared relatively normal and adequate. This observation suggests normal cytological structural and functional conditions to a large extent. Certain cells exhibit pericellular spaces and the neuropil in certain parts present acellular lacunae, also devoid of neuropil. Though these instances are quite a few relatively, they present aberrations relative to the control Group A. These observations are indicative of pyknosis and karyorrhexis. Although the frontal cortex of the counterpart group that was administered caffeine only during pregnancy had their prefrontal cortex largely preserved in terms of the histoarchitecture, there is evidence of sustained aberrations to suggest sustained consequence of caffeine prenatal exposure despite the stoppage at birth. This is in line with a number of previous investigations.[45,46]

Medium caffeine prenatal exposure also affected a few cells that appear to be morphologically deformed. Since this group only had caffeine exposure during pregnancy; the only logical deduction would be that these cells might have been affected by the medium dose of caffeine consumed during pregnancy. These effects were persistent. In the same vein, the cell did not die off but was persistently deformed. The fact that generally, the cortex in this group of animals did not show any sign of reduced cortical cell density buttresses the fact that the dosage did not necessarily induce cell death during pregnancy and its early developmental stage. Therefore, in addition to dosage regimens, the timing of administration influenced the pattern of cortical development and cellular distribution- a phenomenon earlier reported by Berger-Sweeney and Hohmann.[47]

Patterns on DNA distribution within cortical tissue: Though DNA and the neuropil were richly demonstrated in the cortical layer; there are aberrations that could be adduced to the effects of the high dosage of caffeine administered during pregnancy. The pattern of DNA staining suggests an amorphous rather than the characteristic patterned distribution of neuron-DNA materials in the brain cortex. There are relatively larger expanses of neuropil separating areas of DNA material deposits. Generally, the distribution of these materials also presents a less compact and

intact cortex in terms of cell and neuropil compaction. This could also mean fewer cell numbers, organised as clusters with abundant neuropil in their surroundings. This explanation logically conforms to other morphological interpretations of the results. The demonstrated cells though supposedly disrupted in the arrangement were supposedly functional to a large extent and this would explain their abundant and richly demonstrated neuropil. Caffeine at an excessively high dosage affected the cortical distribution of DNA and other materials.

Observation of the brain tissue that was exposed to low-dose caffeine during pre- and postnatal stages would not suggest inadequate DNA deposit (as an indication of relative neuronal de-population). Also, DNA was also abundantly demonstrated in the cortex when a low caffeine dose was only administered during the prenatal stage. However, the pattern of the DNA material relative to size and morphology suggest that the cells are relatively larger in this group in certain cortical regions or layers. This is in line with the morphological observations already stated. This does not in any way indicate relatively more abundant DNA since a cell irrespective of its size would have constant DNA functional mass. It however shows that the cells are morphologically larger, hence they occupy larger spaces within the substance of cortical tissue.

Additionally, the pattern of DNA-in-brain tissue demonstration when medium-dose caffeine was administered showed evidence of certain instances of cortical disruptions in DNA distribution patterns. Collectively, observations show that there are no observable neuronal losses or induced neurodegeneration as a result of caffeine administration during pregnancy. Hence, caffeine administration at this dosage does not produce cortical neuronal degeneration. Evidently, caffeine might not cause neuronal loss, however, it altered histoarchitecture and cell morphologies. This is similar to the report of Silva *et al.* [46] that caffeine altered foetal brain development in mice; the mechanism was postulated to be via its primary action as an adenosine antagonist. [46]

Caffeine Exposure and Effects on Astrocytes: Caffeine administration altered the pattern of glial differentiation, especially the astrocytes. The bodies of most astrocytes though demonstrated by the GFAP technique appear to have less well-defined processes. On the other hand, their relative abundance did not suggest any glial density anomaly such as gliosis in response to chemo-molecular assaults. Desfrere *et al.* [25] reported transient dose-dependent alteration of astrocytogenesis mediated by A(2a)R blockade. This might partially explain the current observation. The medium caffeine dose exposure affected glial morphology and general distribution within the cortex. Though glial cells are still demonstrated, and the astrocytes are observable; they have less defined morphology compared with control. This could be a pointer to various biological status and activity levels within the cells demonstrated. Caffeine administration at this dosage, therefore, affected glia differentiation and morphology. Rather than induce glial reaction or gliosis that characterises the response of astrocytes to chemical assaults within the brain; caffeine administration affected their morphologies.

High caffeine dosage exposure during pregnancy produced general cytological and

histoarchitectural disruptions and distortions that affected glia as well as other cell types. Cell staining patterns are largely heterogeneous as much as they are in morphological appearances. Cortex histoarchitecture, though fairly preserved in cross-sectional view has aberrations in forms of cell distributions and staining patterns heterogeneity. Cells were demonstrated but relatively sparse. A number of cells stained positive for GFAP, indicating that they are largely astrocytes. However, they had not morphologically developed the typical astrocyte morphology. Processes were quite sparse and hardly readily observable. These observations indicate possible limitations or retardations in astrocyte differentiation as a result of caffeine effects. In line with previous observations, this could be interpreted as another effect of general caffeine influence on the developing brain cells. The processes leading to this retarded astrocyte differentiation or development are most likely from a cascade of events that began from caffeine interactions with primordial brain cells. It is understood that caffeine's effects on neurons and glia have a genetic basis primarily; for instance, caffeine has been reported to affect cortical development[47] and to have induced the expression of the sonic hedgehog in astrocytes and neurons culture derived from the neocortex.[48]

Cortical tissues, after pre- and postnatal caffeine exposure, showed that certain astrocytes had distorted morphologies. These observations indicate that there are observable effects of continual caffeine administration on the glial morphology as well as differential densities across the cortical regions. While the differential densities might be mild; morphological distortions are being prominently demonstrated. Caffeine's influence on these cells alters their morphologies. Noting that these cells are vital for the nutritional support of the neurons and could contribute to neuronal functions, their altered morphology could be as a direct consequence of caffeine effects on them or a secondary consequence of their roles in supporting the primary cells (neurons) in their responses and interactions with caffeine. Caffeine had reportedly limited the proliferation of glia- astrocytes and oligodendrocytes in cultures. [49] Though it is logical to deduce that caffeine effects on astrocytes started during pregnancy, astrocytes appear less affected than when there was continual caffeine consumption. In the same vein, the extent of alteration could be directly proportional to the level of biological demand on these supportive cortical cells in response to caffeine interactions with neurons primarily. GFAP results provide insight into the nature and pattern of astrocyte alteration attributable to caffeine exposure; showing that alterations occurred and persisted until puberty whether caffeine exposure was withdrawn after birth puberty or not.

This study has contributed meaningfully to research on caffeine's effects on brain development and functions. It provides evidence that certain prenatal caffeine effects on neurons and astrocytes can persist till puberty. It is interesting to note that most investigations have reported caffeine or other stimulants' effects in the context of autogenesis[24]; the current investigation provides information on morphological alterations of the astrocytes. The persistence of caffeine effects as discussed supports a previous report that caffeine influences genetic expression, particularly the sonic

hedgehog.[48] The implications of these persistent effects should warrant critical investigation, should they have implications on brain health and mental health[50]. Zappettini *et al*[51] stated in their report that intrauterine exposure to caffeine could be a risk factor for the early onset of Alzheimer's disease-like pathology. It is also important to conscientiously work to use empirical evince from research to further understand specific and reported effects of caffeine ingestion on the brain, development, functional integrity of brain circuitry and behaviour[52, 53].

CONCLUSION

From the current investigation, caffeine exposure in pregnancy had persistent effects on brain cells and frontal cortex histo-architecture. Specifically, caffeine influenced brain development by altering the pattern of cell differentiation and established morphologies of astrocytes and pyramidal cells in a largely dose-dependent manner. Also, caffeine exposure caused alterations in the patterns of DNA-neuropil distribution that correlate with the observed morphological changes. Effects also included alterations in the patterns of astrocyte differentiation and caused morphological alterations in astrocytes that persisted till puberty. At relatively high doses, caffeine pre-natal exposure caused early postnatal life death. Altogether, evidence showed that caffeine prenatal exposure had observable effects that persisted till puberty.

RECOMMENDATIONS

Caffeine ingestion during pregnancy as well as the pre-pubertal stage should be further investigated in human conditions. If the results are consistent with the current experimental findings, adequate regulations should be put in place. Users should be adequately educated on the nature of caffeine's effects on the developing brain.

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