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Histomorphological Observations of Caffeine and Honey Effects on Choroid Plexus

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***Abstract**: Caffeine is ingested in form of its natural sources that chiefly include coffee and tea; it is also a major additive to soda or soft drinks as well as a number of medical drugs. Its wide range of availability and legal status in many countries make it available for consumption with little or no restrictions. Caffeine is a psychoactive agent with possibilities of affecting brain structures and functions including behaviours, especially when deliberately consumed at very high doses to alter mood and states of mind or levels of performances. Though the effects of caffeine on the choroid plexus has been fairly investigated, its effect in conjunction with honey has not been adequately researched. Sixty rats were used and there were six groups labelled: control group, honey only (0.5ml) group, low dose caffeine (0.025g/kg) group, high dose caffeine (0.050g/kg) group, low dose caffeine with honey and high dose caffeine with honey group. The analyses carried out were on physiological and morphological and histological features. Results from this study showed that long-term high dose consumption of caffeine altered the structural organisation of the choroid plexus. This could affect the production of Cerebrospinal Fluid. Histologically, caffeine ingestion mildly altered choroid plexus integrity and cellular organization while honey use alongside caffeine administration also mildly ameliorated the caffeine effects.*

***Key Words**: Caffeine, Choroid Plexus,Cerebrospinal Fluid, Honey, Antioxidants*

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I. Introduction

The choroid plexus is a multi-lobed leaf-like highly vascular structure of the pia mater that elongates into the ventricles of the brain (Redzic and Segal, 2004). There are two complexes and they can be found in the ventricular system (third, fourth and lateral ventricles), a communicating cavity within the brain. It is inserted into the sides of the lateral ventricles through a cleft called the choroid fissure. In terms of function, the choroid plexus are specifically recognized as producing a large amount of the Cerebrospinal Fluid (CSF), and acting as a site of blood- CSF barrier, a protective mechanism that ensures the stability of the CSF mileu (Davson *et al.,* 1963; Davson and Segal, 1996; Zheng and Chodoboski, 2005; Saunders *et al.,* 2008). Just like other brain barriers, the choroid plexus blood-CSF barrier is formed by presence of specialized junctions between adjacent epithelial cells. The function of these junctions is mainly to join the cells together to create a physical barrier to paracellular diffusion, allowing cells polarize with distinct luminal and abluminal components (Liddelow, 2015). Another function of these junctions is to allow cellular transporters to be effective in controlling the distribution of solutes on either side, thus setting up concentration gradients. These gradients are not only important for mature brain function, but are also significant for essential features of early brain development (cell division, differentiation, migration and synaptogenesis). The fact that over 65% of nearly 400 solute carriers (SLC) transporters are also expressed by cells of the choroid plexus is no news anymore (Saunders *et al.,* 2013). The choroid plexus is unique in the Central Nervous System (CNS) in the sense that once the cells are born and fully matured, they do not undergo replacement or degeneration under normal conditions. (Altman and Das, 1965).

Researchers have established that short- and long-term treatment with caffeine has different effects. Short-term treatment with caffeine decreases the threshold for convulsions (Albertson *et al.,* 1983; Ault, *et al.,* 1987) whereas long-term treatment with caffeine increases the threshold for convulsions by contrast (Georgiev *et al.,* 1993; Johansson *et al.,* 1996). Moreover, short-term treatment with caffeine worsens ischemia-induced damage (von Lubitz *et al.,* 1988), whereas, long-term treatment with caffeine reduces such damage (Rudolphi *et al.,* 1989; Sutherland *et al.,* 1991). Caffeine has been reported to have its own complimentary and positive health benefits (Yacoubi, 2003; Back *et al.,* 2006)

Honey, a natural food derivative, is a sweet, viscous substance that is formed from the nectar of flowers by honeybees (Apis mellifera; Family: Apidae). It has been reported that honey constitutes moisture and

carbohydrates including simple sugars such as fructose and glucose. It also contains enzymes like catalase and glutathione reductase, minerals such as iron and zinc, vitamins such as vitamins A and E as well as phenolic compounds and organic acids (Mahaneem *et al.,* 2011

In accordance with the previous studies, both short- and long-term supplementations with honey at a dose of 250 mg/kg body weight significantly decreased the lipid peroxidation in brain tissue with a concomitant augmentation of superoxide dismutase (SOD) and glutathione reductase activity. Thus, honey consumption ameliorates the defense mechanism against oxidative stress and attenuated free radical-mediated molecular damage (Oyefuga *et al*., 2008). Furthermore, honey reduced the number of degenerated neuronal cells in the hippocampal CA1 region, a region that is known to be highly susceptible to oxidative insult (Cai *et al*., 2011). Several lines of experimental evidence support the hypothesis that the neuropharmacological effects of honey are mediated via dopaminergic and nonopioid central mechanisms, such as the voltage-gated sodium channel blocking hypothesis, the activation of the noradrenergic inhibitory system and serotonergic systems, and the GABAergic system (Oyekunle *et al*., 2010 and Young & Gauthier, 1981).

In addition to the neural effects, glial cells may also respond to honey therapy because honey shows a neuroprotective effect in the cerebral focal-induced ischemia model in rats (Z´arraga-Galindo *et al*., 2011) Moreover, honey reduced ischemia-induced neuroinflammation by activating microglia, and neuroinflammatory processes in the brain are believed to play a crucial role in the development of neurodegenerative diseases as well as in neuronal injury associated with stroke (Frank-Cannon *et al*., 2009 and Carson *et al*., 2006). As interesting as it could be, ischemia-induced cognitive impairments that result from microglia- and/or astrocyte-mediated neuroinflammation were also significantly attenuated by honey therapy (Frank-Cannon *et al*., 2009 and Akanmu *et al*., 2009).

Table 1: Table illustrating of the major phytochemicals in honey and their functions.

PHYTOCHEMICALS	FUNCTIONS
Apigenin	Protects neurons against oxygen-glucose deprivation/reperfusion-induced injury in cultured primary hippocampal neurons by improving sodium/potassium-ATPase (Na^+/K^+-ATPase) activities (Shu-Qin *et al.,* 2010) & apigenin stimulates the adult neurogenesis that underlies learning and memory (Mishra, 2011).
Catechin	Contributes to the antioxidant activities of honey (Mohammad *et al.,* 2014), also, catechin possesses potent iron-chelating, radical-scavenging, and anti-inflammatory activities (Mandel *et al.,* 2003; Mandel *et al.,* 2004; Mandel and Youdim, 2004).
Ellagic acid	In addition to its antioxidant activity, ellagic acid exerts chemopreventive effects, as indicated by its antiproliferative activity (Seeram *et al.,* 2005)
Quercetin **Others:** Caffeic acid, Chlorogenic acid, Chrysin (5,7-dihydroxyflavone), p-Coumaric acid, Gallic Acid, Luteolin etc	Can inhibit oxidative insults as well as oxidative stress-dependent and independent apoptosis in a neural cell model (Chen *et al.,* 2006 & Mercer *et al.,* 2005). Another neuroprotective role confirmed for quercetin is the alleviation of neuroinflammation (Sharma *et al.,* 2007)

This research is basically aimed at studying and comparing the effects of caffeine, the most popular psycho-active stimulant on the structure and function of the choroid plexus and the lateral ventricle coupled with the possible and potential influence of honey on the choroid plexus of male juvenile Wistar rat.

II. Materials And Methods

Sixty male juvenile wistar strain rats were used for this research. The rats were aged 5 weeks old at the time of purchase, the average weight of the rats was 100-150g. The rats were purchased from Babcock University animal facility, Ogun state, Nigeria. The sixty rats were housed in plastic cages and were kept in a highly ventilated room in Babcock University animal facility, under standard conditions (12- hour light/dark cycles at a constant temperature of 25-29^{0}C) with free access to food and water. The rats were left to acclimatize in the environment for seven days before the research commenced.

There were six groups labelled: A- control group; B- rats administered honey only (0.5ml); C- rats administered the low dose caffeine (0.025g/kg); D- rats administered high dose caffeine (0.050g/kg); E- rats administered low dose caffeine and honey combined; and F- rats administered high dose caffeine with honey combined. **T**he pure/ anhydrous caffeine powder was purchased and was dissolved in distilled water in order to obtain the aqueous caffeine solution.

The honey used in this research was gotten from a tropical part of Nigeria, Okene, Kogi State. The Apis mellifica (honey bee) are the primary manufacturers of honey. The two main processes involved in honey making are harvest and extraction. To harvest the honey, the following are needed: beekeepers suite (mesh helmet and folding veil), smoker with fuel and a lighter, frame super (where frame with honey combs will be put), sting resistant gloves, and hive tool (to scrape wax). To extract the honey, the following tools are needed: heated knife, uncapping fork, tub for wax/honey, extractor, food grade bucket, double sieve, and containers. The process of honey harvest and extraction is presented in steps by (Sammataro and Avitabile, 2011); 0.5ml of honey was given to the rats across the groups.

After the thirtieth day of caffeine and honey administration, the animals were sacrificed by cervical dislocation. The brain was extracted and weighed on a sensitive weighing scale. Tissue homogenates were prepared using some of the brain specimen for biochemical assay.

MORPHOLOGICAL STUDIES

The rats were weighed using a weighing scale before the commencement and at the end of treatment. Also, the weight of the brain of each rat was weighed utilizing the sensitive weighing scale. The relative- brain weight was also calculated.

HISTOLOGICAL SAMPLE PREPARATION

Following the brain excision of the animals, the brain tissues were carefully grossed and sectioned in the planes that allowed the observation of all ventricles and the general procedure followed standard steps (Cardiff *et al.,* 2014) that included: Fixation, Dehydration, Clearing, Infiltration/Embedding, Sectioning, Mounting, Staining and Counter Staining.

HEMATOXYLIN AND EOSIN STAINING TECHNIQUE (Dhurba, 2015)

Harris Haematoxyline stain was counterstained by the Eosin stain. The tissue sections were deparaffinized; then, hydrated by passing them through decreasing concentration of alcohol baths and water (100%, 90%, 80% and 70%).The slides were immersed in hematoxylin for 3-5minutes.They were washed in running tap water until sections "blue" for 5 minutes or less and differentiated in 1% acid alcohol (i.e. 1% HCl in 70% alcohol) for 5 minutes. Again, the slides were washed in running tap water until sections were again blue by dipping in an alkaline solution (e.g. ammonia water) followed by tap water wash; followed by staining the slides in 1% Eosin-Y for 10 minutes. Then the slides were again washed in tap water for 1-5 minutes. The slides were dehydrated in increasing concentration of alcohols and clear in xylene. They were further mounted in a mounting media. The already prepared slides were then observed under the light microscope. Photomicrographs of the slides were taken.

CRESYL FAST VIOLET STAINING TECHNIQUE (Kádár *et al.***, 2009)**

This is used to demonstrate Nissl substance (Rough Endoplasmic Reticulum) in the cell. The Cresyl Violet Acetate solution was used in this technique. The histologically processed sections were de- waxed in two to three changes for three minutes each in xylene. The sections were then rehydrated in 100% alcohol (two changes for three minutes each).Then the sections were stained in 0.1% Cresyl Violet for four to fifteen minutes. The sections were quick rinsed in tap water in order to remove excess stain. After that, the sections were washed in 70% ethanol. The sections were dehydrated through two changes of absolute ethanol for three minutes each. Finally, the sections were cleared in xylene in two changes, mounted and then cover slipped.

GFAP- Glial Fibrillary Acidic Protein (IHC Protocols, 2017).

The free floating brain sections were washed four times in 0.01M phosphate buffer saline (PBS). In order to quench non- specific binding, the sections were incubated in 10% v/v normal donkey serum for one hour at room temperature. The sections were incubated in primary antibody overnight at room temperature. Then the tissue sections were washed 3-4 times in 0.01M PBS. The sections were incubated in secondary antibody for two hours at room temperature. The brain sections were then washed 3-4 times in phosphate buffer solution. Finally, the sections were mounted onto gelatinized or superfrost plus slide (BDH) with a fluorescence mounting medium.

RESULTS: HEMATOXYLIN AND EOSIN STAIN RESULT

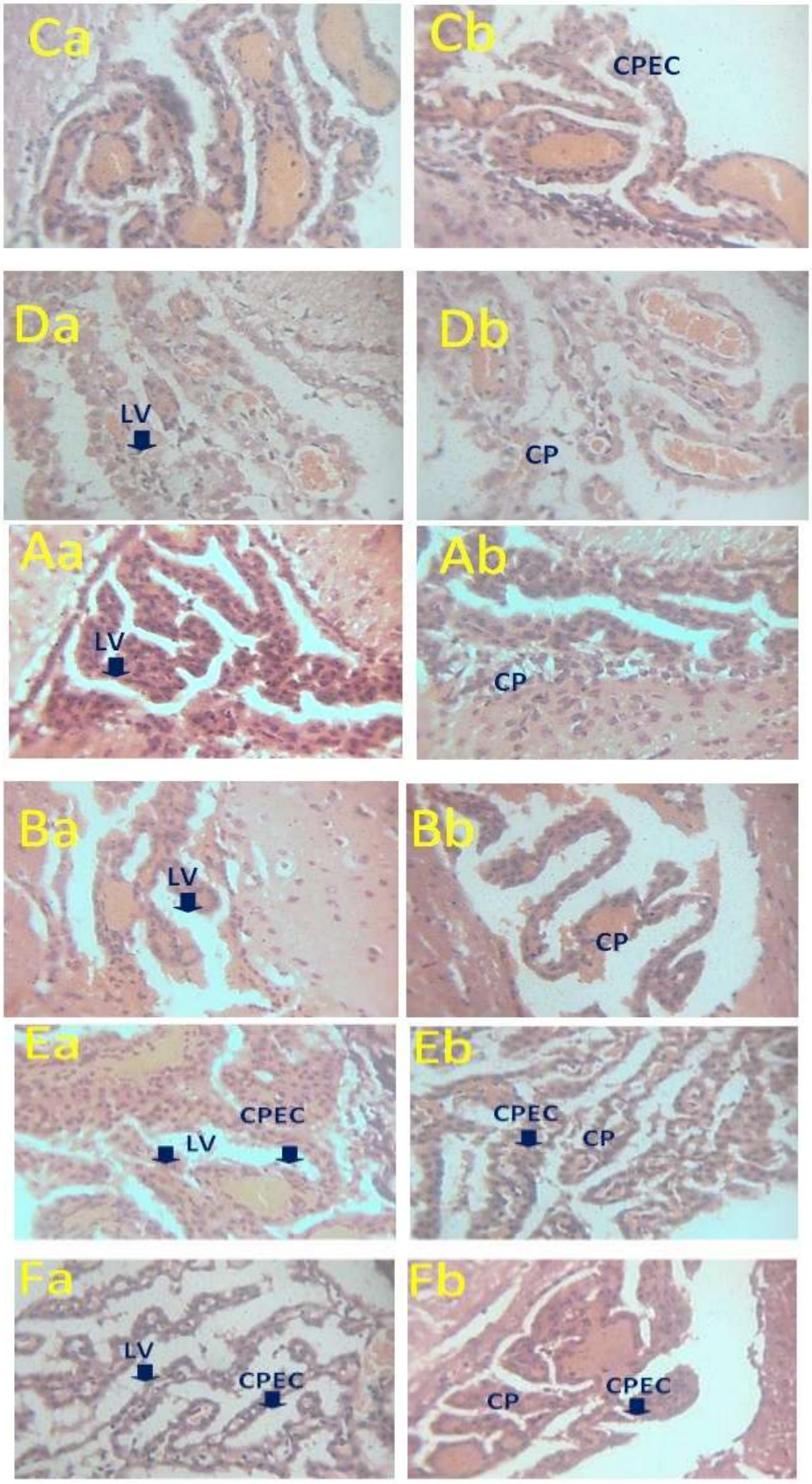

Figure 1: Photomicrographs of the choroid plexus of the Group A-F experimental animals [H&E; X640]. In Group A choroid plexus is normally distributed as well as its Ependymal cells that constitute its epithelium. Thus this Group A can suitably serve as a reference for the other Groups B-F. **In** the Group B experimental animals, ventricle appears normal and the choroid plexus is largely preserved. In the Group C experimental animals; Ventricle is relatively normal. The choroid plexus is slightly disrupted. In the Group D experimental animals; Ventricle is relatively normal but choroid plexus is relatively disrupted and the Ependymal cells are relatively sparse. In the Group E experimental animals; Ventricle is relatively preserved but choroid plexus is relatively disrupted and the Ependymal cells are relatively sparse in manner that appear to be more extensive with dose increase. In the Group F experimental animals, Ventricle is relatively preserved; but choroid plexus is relatively shrunken and its epithelial cells are disrupted. Also, the Ependymal cells are relatively sparse.

Legend: LV- Lateral Ventricle, CP- Choroid Plexus, CPEC- Choroid Plexus Ependymal Cells

4.3.2CRESYL FAST VIOLET (SPECIAL STAIN)

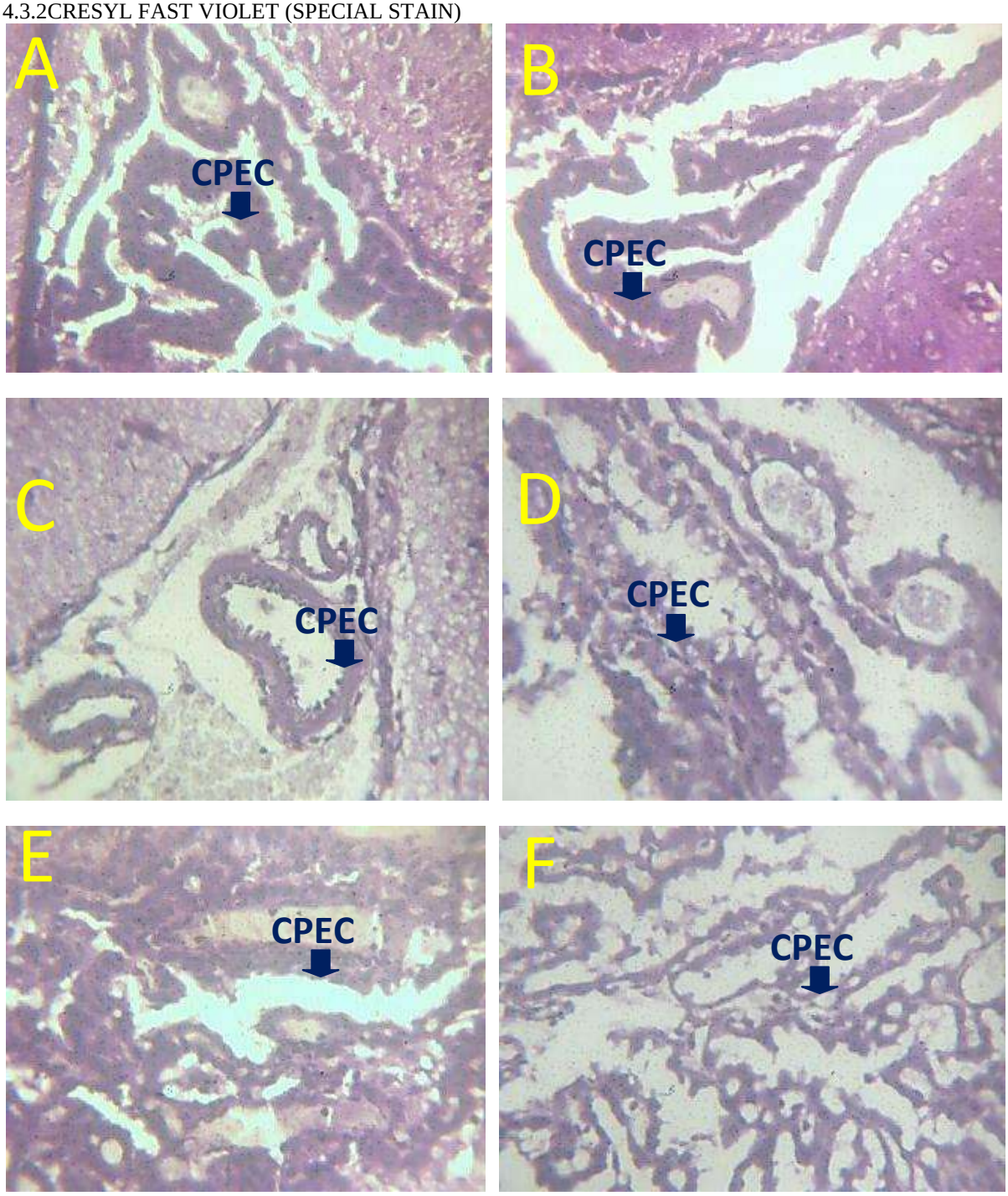

Figure 2: Photomicrographs of the choroid plexus of the Groups A-E experimental animals [CFV, X1600]. Ventricle is largely preserved across the groups but the treated Groups C, D, E and F choroid plexus and the surrounding brain tissues stain less intensely. Also, the choroid plexus is relatively disrupted and the ependymal cells are relatively sparse.

Legend: CPEC- Choroid Plexus Ependymal CellS, CFV- Cresyl Fast Violet.

IMMUHISTOCHEMISTRY: GFAP (GLIAL FIBRILLIARY ACIDIC PROTEIN)

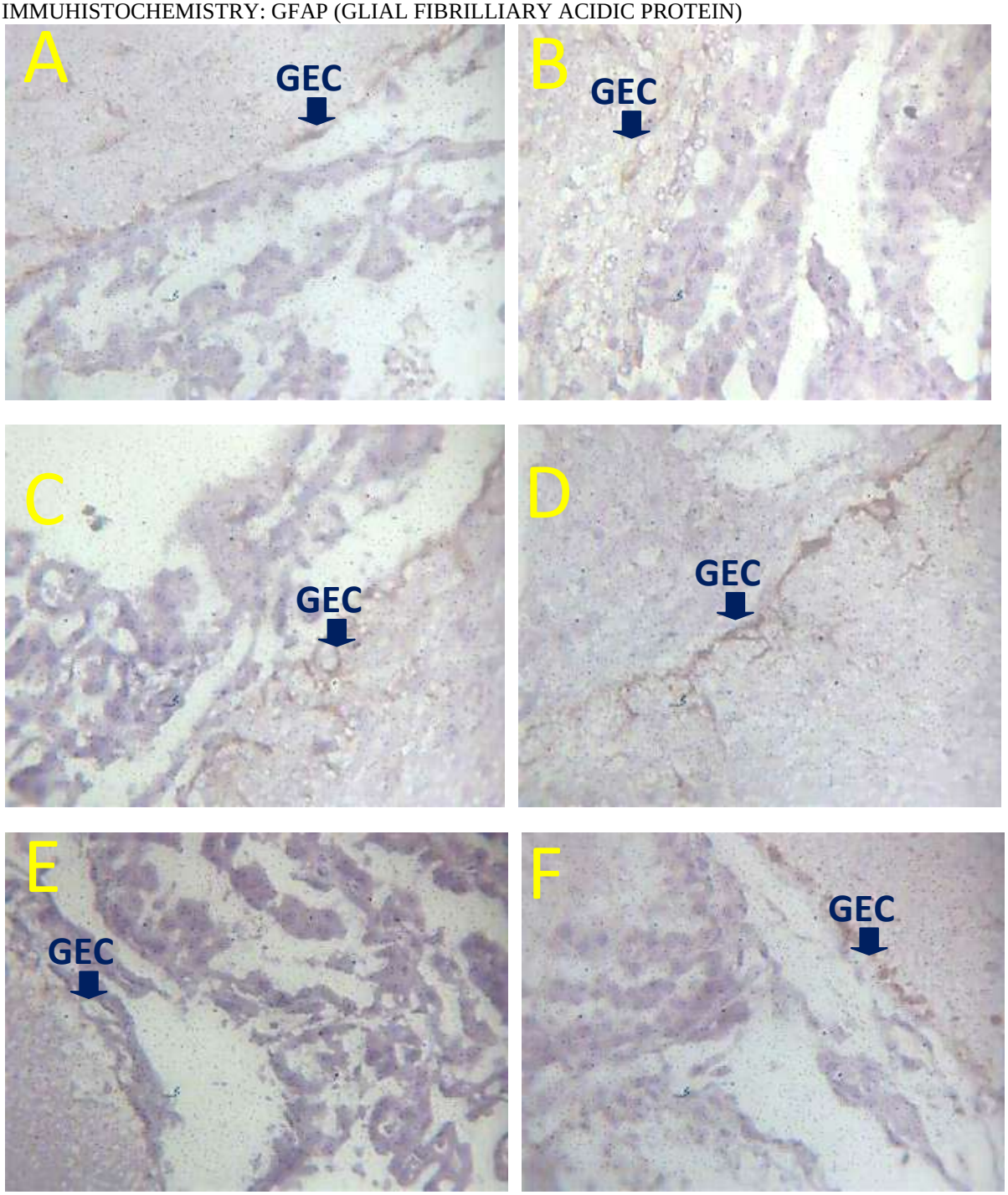

Figure 3: Photomicrographs of the choroid plexus of the Groups A-F experimental animals [GFAP, X1600] to demonstrate the expression the glial fibrillary acidic protein in the supporting and the surrounding tissues of the choroid plexus. GFAP is expressed in all groups and the treated groups, relative to the control do not show sign of Astrocytic reactions.

Legend: GEC- GFAP Expressing Cells, GFAP- Glial Fibrillary Acidic Protein

III. Discussion

Choroid plexus integrity is important in determining the normal production and maintenance of Cerebrospinal Fluid [CSF] in an around the brain. Histological demonstration of the structural integrity was done using the haematoxylin and eosin staining technique, then functional integrity of the nervous tissue was studied using the cresyl fast violet staining technique while the GFAP immunohistochemistry demonstrated possible reactions of the astrocyte of similar cells in the treated tissues.

General Structure of Tissues

Photomicrographs of the choroid plexus of the Group A experimental animals showed that the Choroid plexus is normally distributed as well as its ependymal cells that constitute its epithelium. The control, thus, suitably serves as a reference for the other Groups B-F. The photomicrographs of the choroid plexus of the Group B experimental animals show ventricles that appear relatively normal in terms of size and choroid plexus integrity. Hence, the choroid plexus and its constituent cells were largely preserved. The agent at the dose employed therefore did not produce extensive or observable structural alteration to the tissue. Photomicrographs of the choroid plexus of the Group C experimental animals also showed relatively normal ventricle. The choroid plexus is however slightly disrupted. Photomicrographs of the choroid plexus of the Group D experimental animals also showed that ventricle is relatively normal but choroid plexus is relatively disrupted and the ependymal cells are relatively sparse. Photomicrographs of the choroid plexus of the Group E experimental animals show that ventricles is relatively preserved but choroid plexus is relatively disrupted and the ependymal cells are relatively sparse.

In Group F, photomicrographs of the choroid plexus of the experimental animals showed that the ventricle is relatively preserved; but as observed in the previous groups, choroid plexus is relatively shrunken and its epithelial cells are disrupted. Also, the Ependymal cells are relatively sparse. Generally, histological results showed that caffeine use at the various doses did not cause extensive disruptions of the ventricle. The organisation of the ependymal epithelial cells however varied structurally. These effects are attributable to caffeine use. When the groups that received honey were compared with those that received caffeine only, structural improvements could be observed in the groups that received honey alongside caffeine, showing that honey may have played mild ameliorative role on the tissue of interest (Amy and Carlos, 1996; Schmitt-Schillig, 2005).

Demonstration of Nissl Bodies

The cresyl fast violet technique demonstrated Nissl bodies in the tissues; especially the adjacent cortical regions. Photomicrographs of the choroid plexus of the Groups A-E experimental animals show that ventricle is largely preserved across the groups but the treated Groups C, D, E and F choroid plexus and the surrounding brain tissues stain less intensely. This in an indication of relatively less cytoplasmic protein synthesis. Also, the choroid plexus is relatively disrupted and the ependymal cells are relatively sparse. However, other groups that received honey showed structural improvements in their choroid plexus ependymal cells and their organisation over the caffeine treated groups only (Sato and Miyata, 2000).

Immunohistochemical Demonstration of Astrocytes

Photomicrographs of the choroid plexus of the Groups A-F experimental animals showed that GFAP was used to demonstrate the expression the glial fibrillary acidic protein in the supporting and the surrounding tissues of the choroid plexus. GFAP is expressed in all groups and the treated groups. Relative to the control, the treated groups did not show signs of astrocytic reactions. Caffeine use in the treated group did not result in histological assault that would have demanded astrocytic reactions. Generally, caffeine ingestion mildly altered choroid plexus integrity and cellular organisation. Honey use alongside caffeine administration also mildly ameliorated the caffeine effects. Caffeine, however, did not produce effects that could be extensively disruptive or deleterious. Honey contained compounds appear to have contributed to the protection of the choroid plexus (Esposito, 2002; Carlos, 2011). Phenols, for instance have been appraised (Lau, 2005; Mandal and Jaganathan, 2009; Khalil, 2011, 2012).

Generally, there are evidences of honey befits when used alongside caffeine and this is in line with a number of previous reports (Mato *et al.*, 2003; Manyi-Loh *et al.*, 2011).

IV. Conclusion

Structurally, honey preserved the lateral ventricles and the choroid epithelial cells. Caffeine was not deleterious to the lateral ventricles choroid plexus but there was disruption in the choroid epithelial cells- that is the epithelium became thinner as the concentration of caffeine increased across the groups. In terms of astrocytic reaction, caffeine and honey didn't cause extensive changes in the histomorphology and histoarchitecture of the astrocytes. This appears to be one of the few researches to be conducted on the effects of

caffeine and honey on the structure and function on the choroid plexus, the results have shown positive- benefits and negative effects relative to doses and methods of use. Results show promise to contribute richly to the already existing knowledge of the choroid plexus.

V. Recommendation

Consumption of honey may be implemented in routine diets as it improves the activity of antioxidants and memory. Also, coffee and tea may be taken with honey as a sweetener because this combination may have better effects on the choroid plexus and enzymes compared to when caffeine is taken in its pure form. The results and discussions from this research could be serve as a basis for other researches to be done on this part of the brain; hence, advance research could be done on the choroid plexus (structure and function) and honey.

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