

Brain-Choroid Plexus Chemistry Changes Linkable to Caffeine Ingestion and Accompanying Short-Term Memory Disturbances in Experimental Models

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Abstract: A number of literatures have reported that caffeine could have negative effects on mental health and brain structures and chemistry. There are also reported positive effects of caffeine, such as improvement in memory, especially for those who consume caffeine from its natural sources. Sixty male juvenile Wistar rats were grouped into six (6) groups of 10 rats in each. The groups were labeled A-F with group A being the control group and groups B, C, D, E, and F being the treated groups. This investigation complements our previous efforts to study the effects of caffeine on the choroid plexus microscopic structure, which is involved in the production of the cerebrospinal fluids. Caffeine alters the activities of key enzymes that are associated with the production of CSF. This showed that the structural changes have observable alterations of the brain chemistry. Also, memory, a major attribute of mental power was tested in the same models; results showed that caffeine affected the short term memories. The effects of caffeine was studied in models that ingested caffeine solely and in other modeled after human use by consuming it with honey, a natural antioxidant rich sweetener. In this study, it was observed that the long-term consumption and high amount of caffeine increased the expression of carbonic anhydrase enzyme in the choroid plexus, this enzyme plays a major role in the production of CSF. In the caffeine-treated rats, there was also increased expression of Na⁺/K⁺-ATPase which may be associated with changes in the choroid epithelial cells. Honey improved the glutathione peroxidase level. Results showed the use of caffeine with honey had positive effects against the observed effects caused by ingestion of caffeine only. This also showed that caffeine use effects would vary in users of pure caffeine as mere drugs; and habitual consumers as beverages. Generally, evidences lead us to conclude that alterations in enzymes activities are associated with choroid plexus changes that could affect CSF production. These effects are also associated with behavioural changes.

Keywords: Brain Chemistry, Caffeine, Choroid, Honey, Antioxidant, Cerebrospinal Fluid, Memory

1. Introduction

The pharmacology of caffeine explains how caffeine moves in the body to be absorbed, distributed, metabolized and excreted. All these actions that take place are known as the pharmacokinetics. For the metabolism of caffeine generally, the main route in humans is about 70-80% and this occurs from N-3 demethylation to paraxanthine (1, 7-dimethylxanthine, 17X) catalyzed by cytochrome (CYP) 1A2

in the liver. Other primary metabolites are theophylline and theobromine though smaller proportion is metabolized by CYP3A4, xanthine oxidase and N-acetyltransferase 2. In adult humans, the plasma half-life of caffeine is about 5 hours [1] (Han *et al.*, 2009), it is 0.7-1 hour in rats and mice, 1-1.6 hour in rabbits, 3-5 hour in monkeys, 4- 4.3 hour in dogs and 11-12 hour in baboons. Variability factors involved

in the metabolism of caffeine are divided into two: the primary which includes age, genetic factors such as men with higher CYP1A2 enzyme activity than women genetic polymorphism of CYP1A2, life-style factors e.g. caffeine intake (more than 2 cups of coffee daily), smoking (makes CYP1A2 activity increase) and (contraceptives which makes CYP1A2 activity decrease). Secondary factor is pregnancy because there's decrease in CYP1A2 activity (Ursula, 2015)

Antagonism of the adenosine receptors is known as the main mechanism of action of caffeine (Fredholm, 1999). Reported effects of caffeine on the choroid plexus and the ventricles include that ventriculomegaly caused by disturbances of CSF dynamics; the levels of Na⁺/K⁺-ATPase and Carbonic anhydrase were also increased (Han *et al.*, 2009). If the circulation pathway of the CSF is occluded, dilatation of the ventricles arises, since the production of fluid usually continues despite the obstruction, another effect is the overproduction of CSF (Han *et al.*, 2009). Hyperplasia of the choroid plexus can cause over production of CSF, also Na⁺, K⁺-ATPase establishes a sodium gradient across the choroid epithelial cells and so plays an important role in the production of CSF (Praetorius, 2007). Previous studies have shown that hypoxia-induced ventriculomegaly is mediated by the A1 adenosine receptor, which is a major target molecule of caffeine in the brain (Han *et al.*, 2009). Three findings from the previous study supports up regulation of the A1 adenosine receptor as associated with the observed effect inversion of caffeine. First, the expression of the A1 receptor was up-regulated in the caffeine treated rats. Second, A1 agonist treatment was associated with the development of ventriculomegaly. Third, the expression of Na⁺, K⁺-ATPase was increased in both the caffeine-treated and the A1 agonist-treated rats. Although this continues to be debated, many previous reports have demonstrated up-regulation of the A1 adenosine receptor in caffeine-treated rats (Han *et al.*, 2009).

Very few and current research projects investigate the nootropic and neuropharmacological effects of honey. Antioxidants such as polyphenols and flavonoids are two important bioactive molecules that are present in honey. Emerging evidence from recent studies has confirmed the presence of approximately 30 different polyphenols in honey (Carlos *et al.*, 2011 and Khalil *et al.*, 2011). The polyphenol content of honey has a totality ranging from 50 to 850 mg/kg, whereas the flavonoid content varies from 36 mg/kg to 150 mg/kg (Islam *et al.*, 2012, Alzahrani *et al.*, 2012 and Khalil *et al.*, 2012).

Choroid plexus produces a large proportion of the CSF (about 500 ml of cerebrospinal fluid per day), and acting as site of the blood-CSF barrier, a protective mechanism that ensures the stability of the CSF milieu (Davson and Segal, 1996; Zheng and Chodobski, 2005; Saunders *et al.*, 2008). The critical function of these junctions is to join the cells to create a physical barrier to paracellular diffusion, allowing cells to polarize with distinct luminal and abluminal components. Also, presence of these junctions between cells of the interface between the periphery and the CNS allows cellular transporters to be effective in controlling the

distribution of solutes on either side, and thus set up concentration gradients e.g. the most active transporter in the choroid plexus appears to be the Na⁺/K⁺ ATPase pump—integral for maintenance of the ion gradient that draws water into the ventricles via aquaporin-1(AQP1) channels in the plexus epithelium (Brightman and Reese, 1969). The presence of this functional barrier is an essential prerequisite for the establishment and maintenance of concentration gradient for ions and proteins between the blood (basal side of the cells) and the CSF on the apical side (Speake and Brown, 2004). Choroid plexus epithelial cells are also able to aid in removal of compounds toxic to the nervous system. The two components in the choroid plexus that have proved crucial for driving an influx of water and thus production of CSF are Na⁺/K⁺ ATPase and carbonic anhydrase (Davson and Segal, 1996; Catala, 1998; Zheng and Chodobski, 2005). Finally, the choroid plexus is responsible for regulation of the external environment of the brain by draining the excessive quantities of neurotransmitters present in the cerebrospinal fluid (Anirudh, 2014).

Results of the research conducted by Han *et al* in 2009 showed that long-term consumption of caffeine could cause ventriculomegaly (primary), which appears to be mediated, in part, by increased production of CSF. In addition to disturbances in CSF dynamics, periventricular leukomalacia (reduction in white matter) can cause secondary ventriculomegaly (Volpe, 2001). Periventricular leukomalacia can be caused by hypoxia, perinatal stress and sepsis. Previous studies have shown that hypoxia-induced ventriculomegaly is mediated by the A1 adenosine receptor, which is a major target molecule of caffeine in the brain (Turner *et al.*, 2003; Back *et al.*, 2006).

CSF is a clear, watery, cell free, nutritional fluid that fills the ventricles of the brain and the subarachnoid space around the brain and spinal cord, it contains sodium chloride, potassium, glucose and various proteins CSF is primarily produced by the epithelium of vascular bulges called choroid plexus ($\leq 70\%$ of the volume), (The Saliency, 2013); most of it is formed by the choroid plexus of the lateral ventricles. The rest of the CSF production is the result of transependymal flow from the brain to the ventricles (Elena, 2016). The fluid filled caverns and canals inside the brain constitute the ventricular system of the brain. It is formed by the process of secretion and/or filtration, which uses ATP, as an active transport mechanism is involved in the secretion of CSF (The Saliency, 2013). CSF flows from the lateral ventricles, through the interventricular foramens, and into the third ventricle, cerebral aqueduct, and the fourth ventricle. Only a very small amount enters the central canal of the spinal cord. CSF flow is the result of a combination of factors, which include the hydrostatic pressure generated during CSF production (known as bulk flow), arterial pulsations of the large arteries, and directional beating of the ependymal cilia. Hydrostatic pressure has a predominant role in the CSF flow within the larger ventricles, whereas the cilia favor the movement of the CSF in the narrow regions of the ventricular system, such as the cerebral aqueduct (Elena, 2016).

2. Method

2.1. Experimental Design

A sum total of 60 male juvenile Wistar rats were used for this experiment. The animals were grouped into six (6) groups of 10 rats in each. The groups were labelled A-F with group A being the control group and groups B, C, D, E, & F being the experimental groups.

Table 1. Table Illustrating the Grouping of Animals.

Groups	Animals No	Substance Given & Dosage
Control Group	10	1ml of Deionized water
Honey Only	10	0.5ml honey
Caffeine Low Dose	10	0.025g/kg caffeine+ 1ml deionized water to dissolve
Caffeine High Dose	10	0.050g/kg caffeine + 1ml deionized water to dissolve
Caffeine Low Dose + Honey	10	0.025g/kg caffeine dissolved in 1ml deionized water + 0.5ml honey
Caffeine High Dose + Honey	10	0.050g/kg caffeine dissolved in 1ml deionized water + 0.5ml honey

The administration of drugs was done for 30 days using the oral cannula (one per group) and the general behavior of the rats were monitored throughout experiment.

2.2. Neurobehavioural Study

The neurobehavioral study was conducted 24 hours after the day last of drug administration using the Barnes maze though the training started four (4) days prior to the test.

Barnes maze procedure (sunyer *et al.*, 2007)

The Barnes Maze, developed by Carol Barnes in 1979 and it is a dry land maze used for testing the spatial learning and memory in animals including rodents such as rats, mice, etc. There were three significant phases in the procedure and they are highlighted below:

The Adaptation period: in this phase, the rat was put in a cylindrical black start located in the middle of the maze. After 10 seconds, the chamber was lifted and the buzzer was switched on so as to guide the rat to the escape box gently. The buzzer, light and fan were switched off when the rat entered the escape box and the rat stayed in there for another two minutes.

The Spatial acquisition: in this phase, the rat was placed in the cylindrical black start chamber at the middle of the maze, after 10 seconds, the chamber was lifted and the buzzer was switched on the rat was allowed to explore the maze for 3 minutes in order to fine the box. While this was going on, the number of primary and secondary errors, primary and secondary latency were measured. Furthermore, immediately the rat entered the escape box, the buzzer was turned off and the rat was allowed to stay there for the next one minute. Any rat that didn't enter the box was guided to it and left for one minute there. At the end of this phase, the rat was placed in the home cage until the next trial.

Reference memory (Probe trial): on the fifth day which was the last training day, the probe trial was conducted. The animal was placed in the middle of the maze under the

cylindrical black start chamber and after 10 seconds, the chamber was lifted, the buzzer was switched on while the rat was allowed to explore the maze. The rat was removed after 3 minutes but within this time, the number of errors and latency were taken note of. This maze was used only to test for short term memory.

Animals were sacrificed by cervical dislocation at the end of the experiment. Brain tissues were excised and homogenates were prepared for enzymes assays.

2.3. Enzyme Assay

The blood samples taken were assayed for the plasma glutathione reductase level while the brain homogenates were assayed for Na⁺/K⁺-ATPase and carbonic anhydrase.

2.3.1. Glutathione (GSH) Redutase Assay

The total sulfhydryl groups, protein - bound sulfhydryl groups and free sulfhydryl groups like glutathione (GSH) in biological samples can be determined using Ellman's reagent, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) as describe by Sedlak and Lindsay (1968) and Jollow *et al*, 1974. In the procedure, 0.7ml of sample was pipetted in a clean test-tube and 1.5ml Ellman's reagent was added to the sample. The absorbance of the yellow color formed upon the addition of Ellman's reagent was read within 5minutes at 412nm using spectrophotometer. A plot of absorbance Versus Concentration of reduced GSH was then obtained from the serial dilution of the stock GSH which was prepared by the addition of 1.5ml of phosphate buffer and 1.5ml of Ellman's reagent. This assay was done using the Elisa kit.

2.3.2. Sodium/Potassium- ATPase Assay (Pradip, 2002)

The level of this enzyme was measured using the reaction mixtures: [1] 30 mM of imidazole- HCL, 130 mM of NaCl, 20mM of KCl, 4mM of MgCl₂ and [2] 1mM of ouabain at pH 7.4 each containing 20-50µg synaptosomal protein, 4mM of MgCl₂, 30 mM of imidazole- HCL.

In order to get complete ouabain binding, preincubation of the tubes in ice and dark for 60min was ensured. To commence the reaction, 4mM Tris- ATP (Tris hydroxymethyl-aminomethane salt of adenosine triphosphate, Sigma Co.) was added to brain homogenate and it was incubated at 37°C for 10min. the volume of the reaction mixture was 1ml. 100µl of 20% sodium dodecylsulfate was added to the reaction in order to halt the enzyme activity. The reaction mixture determined the Pi formation. Finally, the Na⁺/K⁺-ATPase level was calculated as difference in the Pi content in µgg⁻¹ between the first and second reaction media (formed from the reaction mixtures mentioned above).

2.3.3. Carbonic Anhydrase Assay (Worthington, 2017)

The method used in this assay was based on the electrometric method of Wilbur and Anderson (1948) at which the time (in seconds) required for a saturated Carbon (IV) Oxide solution to lower the pH of 0.012 M Tris HCL buffer from 8.3 to 6.3 at 0°C is determined. The unit of the time without enzyme is recorded at T₀; but with enzyme, the

unit of time is T.

Reagents included: [1] 0.02 M Tris HCL buffer, pH 8.0, stored in an ice bath at 0-4°C prior to and during use [2] Carbon dioxide saturated water, that is, bubbled CO₂ gas through 200ml ice cold water for 30 minutes before the assay. Water was stored at 0-4°C in an ice bath during saturation process.

To get the reaction mixture for the enzyme, lyophilized powder at a concentration of 0.1 mg/ml was dissolved in ice cold water and stored in ice bath before use. In the enzyme

determination, 6.0ml of chilled 0.02M Tris HCL buffer with pH was added to a 20ml beaker. The temperature was maintained at 0-4°C and the pH was recorded. 0.1ml of freshly diluted enzyme was also put in the reaction. 4ml of CO₂ saturated water was swiftly added and the time required for the pH to drop from 8.3 to 6.3 was recorded, likewise the time (T) as well.

To calculate the activity of the enzyme, the formula below is used:

$$\text{Units/ mg} = \frac{2 \times T_0 - T}{T \times \text{mg enzyme in reaction mixture}}$$

2.4. Statistical Analysis

The results that were collated were expressed as Mean \pm SEM for each group. All the grouped data were statistically evaluated utilizing the Graphpad prism 5 software using one way analysis of variance (ANOVA). Also, the Students-Dunnett test was used to compare all columns versus control columns. Confidence interval was placed at 95%, so that in all cases, a value of P < 0.05 was considered significant.

3. Results

The parameters in this research that are represented here are: Physiological and morphological results, enzyme and neurobehavioural test.

Morphological Results

In these results, the body weight and brain weight was used to determine the relative brain weights.

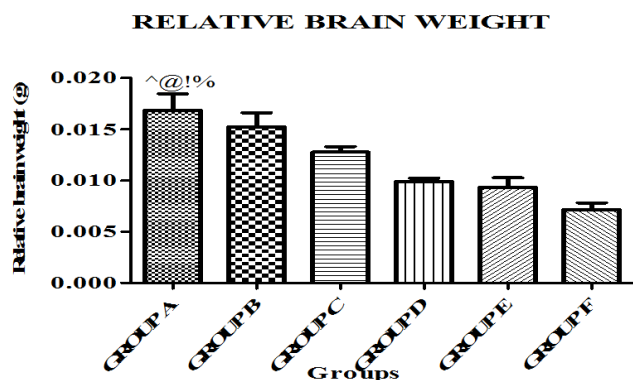


Figure 1. A Bar Chart Showing the Relative Brain Weight (R. B. W) of the Rats. The Relative Weights of the Brains Decreased in the Treated Groups.

Where ^: indicates that there is statistical significance when compared with Group C (p<0.05).

@: indicates that there is statistical significance when compared with Group D (p<0.05).

!: indicates that there is statistical significance when compared with Group E (p<0.05).

%: indicates that there is statistical significance when compared with Group F (p<0.05).

Biochemical Assay (Enzyme) Results

The results here entails the plasma glutathione reductase

level, Na⁺/K⁺-ATPase and Carbonic anhydrase.

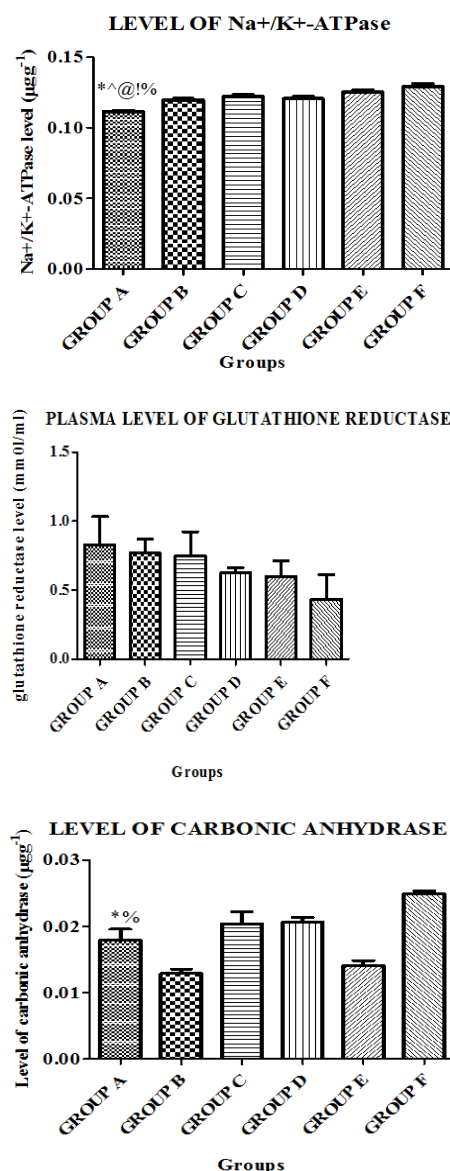


Figure 2. Bar charts showing the brain level of showing the level of Na⁺/K⁺-ATPase; plasma glutathione reductase and brain level of carbonic anhydrase of the rats. There was significant increase in the levels of sodium/potassium ATPase levels across the groups; reduced but insignificant levels of carbonic anhydrase and relative significant decrease in the levels of carbonic anhydrase in Groups B and F

Where *: indicates that there is statistical significance when compared with Group B ($p < 0.05$).

^: indicates that there is statistical significance when compared with Group C ($p < 0.05$).

@: indicates that there is statistical significance when compared with Group D ($p < 0.05$).

!: indicates that there is statistical significance when compared with Group E ($p < 0.05$).

%; indicates that there is statistical significance when compared with Group F ($p < 0.05$).

Neurobehavioural test

The results of the Primary/Secondary Latency and the Primary/Secondary Errors of the Barnes Maze Test.

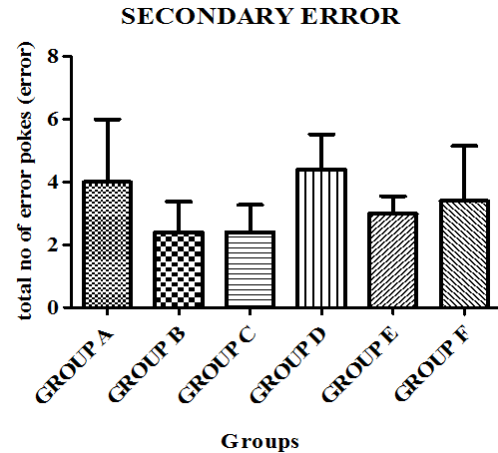
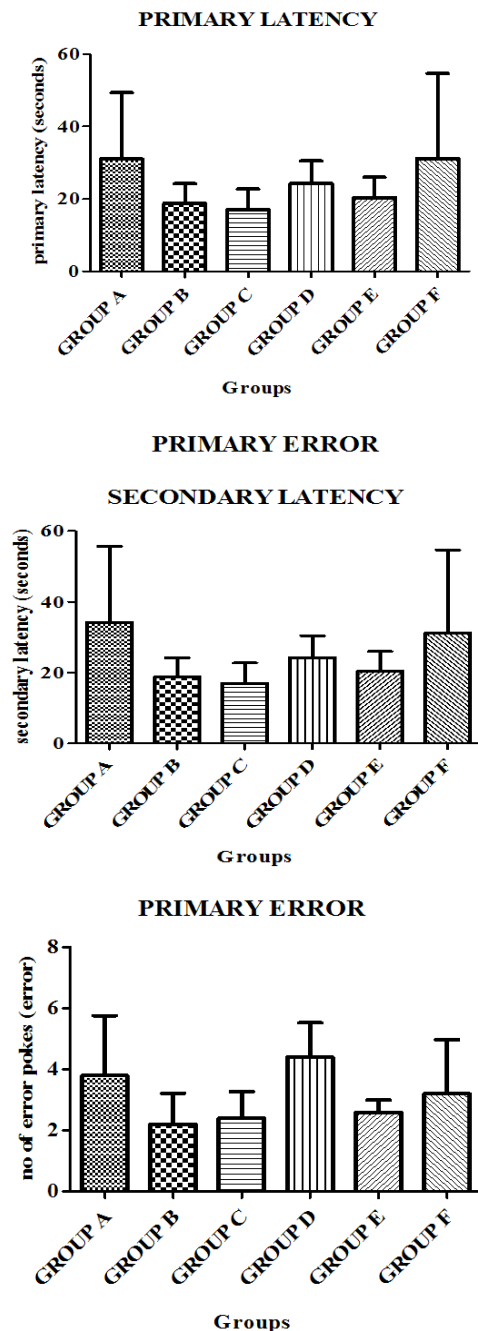


Figure 3. Bar charts showing the primary latency, secondary latency, primary error pokes and secondary error pokes as observed during the Barnes maze test in the rats. There was no significant difference observed across the groups.

4. Discussion

Enzymes Results

Glutathione reductase results showed no significant difference statistically when compared the experimental groups to the control group. Mere looking at the graph, there were slight reductions between the control group and the other groups most especially the high dose caffeine only group and the high dose caffeine and honey group. The honey only group when compared to the control group has no much difference at all, hence, the anti-oxidant (for glutathione reductase) level in the plasma of that group was maintained. The groups containing caffeine has slightly notable decrease in comparison with the control group meaning that caffeine could be responsible for causing reduction in the anti-oxidant (glutathione reductase) property of the plasma.

The Sodium/Potassium-ATPase level was measured and the result showed statistical significant difference of all the experimental groups when compared to the control group. Comparing the control group to the honey only group, they had almost the same values, but comparing the control group to the other groups with caffeine, there was progressive increase in the level of this enzyme most especially the group of animal treated with high dose caffeine with honey and the low dose caffeine. These results signifies that caffeine caused a relative increase in the level of Na^+/K^+ -ATPase, in a report given by Han *et al.*, (2009), it was documented that caffeine increased the activity of an increased level of these same enzymes was associated with an increase in the CSF production. Also, in Brightman and Reese, (1969), it was reported that the most active transporter in the choroid plexus appears to be the Na^+/K^+ ATPase pump and an increase in their levels could lead to over production of the Cerebrospinal Fluid.

Results obtained from the assay of the Carbonic anhydrase level showed in Figure 1 that there was a statistical significance in the graph. Comparing other groups to the

control group, the level of carbonic anhydrase reduced in the honey only group and the rest of the groups had a progressive increase in the level of the enzyme as caffeine concentration increased. The reason why the level of this enzyme reduced in the honey group is not well understood but for the low dose caffeine, high dose caffeine, low dose caffeine with honey and high dose with honey group the increase in the enzyme level signified that caffeine increases the activity of carbonic anhydrase and it is overly expressed in when caffeine is taken in its high dose, a supporting report stated that caffeine causes an increase in carbonic anhydrase level associated with an increase in the CSF production (Han *et al.*, 2009). Interestingly this result can be supported also by (Davson and Segal, 1996; Catala, 1998; Zheng and Chodobski, 2005), they reported that carbonic anhydrase is one of the two components in the choroid plexus that have proved crucial for driving an influx of water and thus production of CSF therefore an increase in the its levels could be associate with increased production of the Cerebrospinal Fluid.

Neurobehavioural Tests

Barnes maze test was conducted and there was no statistical significance when other groups were compared to the control group. Looking at Figure 3, a notable difference can be seen between the control group and the honey only group, the total latency (total time taken for the rat to find the escape box) and the total error pokes (the amount of times the rat poked its head into a box apart from the escape box) in the honey group was relatively low and this signified that the animals had an improved short term memory. This result is very much supported by a review article written by Mohammad *et al.*, 2014. In comparism with the control group, the caffeine treated groups as seen in Figure 3 was not statistically significant but mere looking at the graphs, the group that was administered high dose caffeine had a high total latency but not higher than the control group, this can be interpreted that the animals in this group had improved memory (short term) than the control group and this indicated that high dose of caffeine can enhance short term memory when taken for a long time, this result is supported by Angelucci *et al.*, 2002. The group treated with low dose caffeine was also not statistically significant but had a smaller total latency when compared to the control group; it can be said that low dose consumption of caffeine also improves short term memory. For the groups that were treated low and high dose caffeine with honey respectively, there were reductions in the primary & secondary latency and primary and secondary error when compared to the control group and this indicated that the memory of the animals in these groups improved, it can be said that the caffeine improved the short term memory of the rats and so did the honey that was administered alongside with it, the report that supports this result is given by Universidad de Barcelona, 2010.

Caffeine caused an increase in the size of the animal, decrease in the relative organ weight, this depicts the effects of caffeine on the feeding center of the rat's brain and its

metabolism. A decrease in the relative organ weight due to caffeine intake could be as a result of shrinkage of the rats brain (could be associated with ventriculomegaly), caffeine also caused increase in the activities of two the enzymes assayed for in this study (Na^+/K^+ -ATPase and Carbonic anhydrase) and a decrease in one of the enzymes (glutathione reductase). The inference derived from this is that the increase in the A1 receptor due to caffeine effects on the choroid plexus caused the rise in the level of Na^+/K^+ -ATPase which is associated with increased production of the Cerebrospinal Fluid. Also, the glutathione reductase level that was reduced could be as a result of the hyper metabolism that caffeine caused thereby creating much more free radicals, hence allowing depletion of the glutathione levels in the rat (depletion occurs when rate of free radicals produced at every metabolism is higher than the anti-oxidant level and the rate at which these antioxidants eradicate the free radicals). The effects of caffeine on the short term memory of the rats was positive, that is the memory improved more than those of the control group which shows that caffeine enhances memory. In the group administered honey only, there was no much weight gain, the relative organ weight was not reduced or increased, the glutathione level was increased being that honey contains polyphenols and flavonoids that have anti-oxidants property of which glutathione is one of them. The activities of Na^+/K^+ -ATPase were a little lesser than the control group hence no over production of CSF. Apparently, the honey only group was seen to have the lowest total latency in the short term memory test compared to the control group, therefore this signifies that the rats in this group had higher memory than those in the control group and it is impressive to know that honey has such tremendous memory improving ability.

5. Conclusion

Caffeine effects included tendencies to increase CSF production. There were mild alterations in behavioural attributes but not significant enough to be labeled aberrations or abnormalities. Caffeine, thus have potential to alter the chemistry of the choroid plexus vital enzymes that can influence the production of the cerebrospinal fluid to increase its production.

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