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# AMELIORATIVE TENDENCY INHERENT IN SUB-CHRONIC CAFFEINE INGESTION ON PAIN HYPERSENSITIVITY AND BRAIN'S OXIDATIVE STRESS MARKERS' PROFILE IN WISTAR RATS.

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## ABSTRACT
Sensitivity to mechanical pain in animal could be heightened due to certain factors (chemical or physical) and this could induce myriads of chemical and molecular assaults in the system such as disruption in oxidative stress markers profile and moieties. The present study was therefore, designed to investigate the effects of sub-chronic caffeine ingestion on pain hypersensitivity and oxidative stress markers in wistar rats. To achieve this aim, twenty-five wistar rats with an average weight of 120g were sorted into five groups via; control (group 1) treated with distilled water only, group 2, 3 and 4 treated with caffeine at doses of 50, 100 and 150 mg/kg body weight for 3 weeks respectively. In addition, epinephrine drug was used as a positive administration as compared to the negative control. Effects of caffeine on episodic memory and pain hypersensitivity were measured using the Passive Avoidance Box test and the Analgesy-meter. Advanced Oxidative Protein Products (AOPP) and other stress markers such as Malondialdehyde (MDA), Superoxide dismutase Superoxide Dismutase (SOD), Catalase, Glutathione Reductase (GHS) Levels were quantified and assayed. Data were obtained and analyzed using standard statistical tools. Results shows that caffeine at sub-chronic administration significantly increased ($p \geq 0.05$) pain hypersensitivity in the passive avoidance test, at 50 and 100 mg/kg, and 150mg/kg dose concentrations when compared to the control groups thereby declining pain threshold and promoting exaggeration to sensation response. Step-through latency was seen to be decreased ($p \leq 0.05$) in similar fashion from low to high dose concentration leading to more time spent without in intact memory recall phase. Administration of sub-chronic caffeine significantly ($p \leq 0.05$) increased AOPP at 100 and 150mg/kg dose concentrations, while Catalase and Glutathione were decreased considerably leading to a significant protein breakdown and mild peroxidation by Caffeine at the end of the study, the levels of SOD was increased alongside the pro-oxidant Malondialdeyde (MDA) further suggesting a significant stressful state. In conclusion, there was significant increase in AOPP levels and other pro-oxidative stress markers compared to the control corroborating the scenario of stress. The data obtained from this study indicated that Caffeine a common psychoactive drug increases pain hypersensitivity upon chronic administration, however it can be used as an adjuvant pain analgestic agent in its therapeutic low dose. It also indicates that upon chronic administration of caffeine, homeostasis is altered in the face of cellular assault and challenge to cellular integrity.

**KEYWORDS:** Caffeine, wistar rats, pain, catalase, Superoxide Dismutase, Malondialdehyde.

## INTRODUCTION
Caffeine as the most widely consumed psychoactive substance, it is a potential stimulant of the central nervous system.[1] Unlike much other psychoactive substance, it is legal and unregulated. Uptake of caffeine occurs in a variety of forms, such as drinking coffee, tea, soft drinks, chewing cola nuts, consuming Cocoa products, such as energy drinks, or taking over-the-counter pain or slimming medications.[2] Caffeine is a bitter, white crystalline purine, a methylxanthine alkaloid, which is closely related to *adenine* and *guanine* contained in deoxyribonucleic acid (DNA).[3]

Prevalence of Caffeine Consumption. Caffeine is considered a "cradle to grave drug" because it is used nonmedically by young children and adults alike, which is true of no other psychoactive substance.[4] Good estimates of caffeine consumption around the world are

difficult to obtain because it is contained in so many different products, and how the product is prepared can make a difference in the caffeine content. However, from estimates derived, the U.S. average is triple the world average, and well over half is from coffee consumption.[5] Caffeinism is the term for caffeine intoxication. It has been reported following consumption of as little as 250mg of caffeine a day, the average for adults in the U.S. Generally, ingesting 600mg of caffeine a day greatly increases the chances of developing it.[6] Consuming over 1,000mg a day increases the risk of experiencing symptoms such as muscle twitching, rambling flow of thought and speech, cardiac arrhythmia, periods of inexhaustibility, and psychomotor agitation. Other symptoms that have been reported include ringing in the ears and seeing flashes of light. The lethal dose of caffeine when taken orally is 10 grams for adults and 100mgs for children. The adult lethal dose is equivalent to 75 cups of coffee, 125 cups of tea, 200 colas, or 100 NoDoz tablets.[7]

Hyperalgesia is an enhanced pain response. It can result from either injury to part of the body or from use of opioid painkillers. When a person becomes more sensitive to pain as a result of taking opioid medication, it's called opioid-induced hyperalgesia (OIH). Oxidative stress is defined as the imbalance in the redox characteristics of some cellular environment which can be the result of either biochemical processes leading to the production of reactive species, exposure to damaging agents (i.e., environmental pollutants and radiations), or limited capabilities of endogenous antioxidant systems.[8]There is disruption of all cellular biomolecules (lipids, sugars, proteins, and polynucleotides) which causes several defense systems to act to prevent uncontrolled ROS increase. These systems include non-enzymatic molecules (glutathione, vitamins A, C, and E, and several antioxidants present in foods) as well as enzymatic scavengers of ROS, with superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) being the best-known defense systems.[9,10].

As a result of the wide spread in discriminate use of caffeine for various purposes, its psychoactive activities could have a significant interference on cognitive activities, and by extension can affect pain pathway and neuronal constituents. Due to scanty literature on these observations, this study was designed to investigate possible caffeine's influence of pain hypersensitivity and Advanced Oxidative Protein Products (AOPP) and other oxidative scenarios.

## MATERIALS AND METHODS

### Procurement and Acclimatization of Experimental Animals

Healthy white wister rats, 120 – 150g, obtained from the Animal farm, University of Port Harcourt were used in the research. The rats were housed in a wooden cage and were fed with rat's diet and Distilled water under an environmental controlled room throughout the period of the experiment. The rats were acclimatized to the laboratory environment 20 days prior to experimentation. All experiments were subjected to Animal Ethical Procedures.

### Experimental Design

The animals were divided into groups based on various administration of drug according to body weight, four males and one female in each group.

Group 1 (Control): this group was administered rat's feed and distilled water without administration of drug throughout the experimental study.

Group 2 (Low dose): this group was administered with 50mg/kg of the diluted exogenous caffeine.

Group 3 (Moderate dose): this group was administered with 100mg/kg of the diluted exogenous caffeine.

Group 4 (High dose): this group was administered with 150mg/kg of the diluted exogenous caffeine.

Group 5: this group was administered with epinephrine at 10ml/kg.

### Determination of pain hypersensitivity with the Passive avoidance Machine.

The passive avoidance test, the laboratory animal (rat or mouse) learns to avoid an unpleasant stimulus by inhibiting locomotion and exploration, which is why it is also called the inhibitory avoidance test. At the start of the test, a rat, for example, is placed in a brightly lit compartment of the test box. As is the habit with rats, the animal very quickly moves into an adjacent, dark compartment where it receives an unavoidable shock. In the test phase, one notes whether the animal reenters the dark compartment. This is done to check for the pain threshold of the rats.

### Determination of pain hypersensitivity with the Analgesy-Meter.

Based on the method first described by Randall and Selitto in 1957. The main concept is to apply increasing force to an inflamed rat paw and to measure the force that causes the rat to withdraw its paw. This was used as a paw pressure machine which follows the method of the Randall-Stelitto. A force was applied on the animal's paw which was placed on the plinth. The force applied with a cone shaped pusher and with the aid of a pedal manipulated by the operator. At maximum amount of force, the rat removes the paw and then recordings were taken. The machine exerts a force that increases at a constant rat. The force exerted is measured on the scale calibrated in 10 gram.

### Determination of Oxidative Stress Markers

### Procurement and Preparation of tissue sample.

After sacrificing of the animals, collection of tissue and blood samples were done. The tissue of concern being the brain tissue at negative °C. The tissues were separately weighed and homogenized in 10 volumes of cold phosphate buffer (pH 7.4), using an automatic homogenizer. The homogenates were then centrifuged

with a cold centrifuge at 4966rpm for 10 min at 4 °C. Clear supernatants were used for the MDA, Catalase, SOD, GHS, and AOPP assays. Tissue protein levels were also measured at this step according to the method used by Lowry et al.

**Test for MDA/ Lipid peroxidation**

Materials used in the test included Trichloroacetic acid 15% (TCA), Tiobabuturic acid 0.65% (TBA) and Hydrochloric acid 0.25N (HCL). Preparation of reagents. 15g of Trichloroacetic acid was weighed alongside 0.65g of TBA into a beaker, 2.03 ml of HCL was measured also to give a molar mass of 100ml. 300 ml of sample (homogenized tissue and saline buffer) was mixed with 3ml of working reagent and boiled in the water bath for 15 minutes. The absolvent was measured at a wavelength of 532nm with a spectrometer.

**Test for SOD**

SOD an enzyme that catalyzes, converts superoxide anion to cingulate $O^2$ and $H^2O^2$. The materials used in the test included carbonate buffer with the pH of 10.2, 10mg of Adrenaline. Preparation for carbonate buffer ($Na^2CO^3$ and $Na^2HCO^3$) and solution. 100mm of solution (homogenized tissue and saline buffer) and 4ML of carbonate buffer was incubated at 37°C in 5 minutes. The reaction was then supplied with 100mm of Adrenaline and read with a spectrometer at 30 seconds and at 1 minute 30 seconds respectively at 480nm.

**Test for Catalase**

Catalase is an oxidative enzyme that converts $H_2O_2$ to $H_2O$ and $O_2$ The materials used in the test included for the Buffer solution of 0.05M and pH of 7.2 and $H_2O_2$ 0.036M. Preparation of Catalase and $H_2O_2$. Two solutions are prepared for Catalase, Solution; A 0.8g of $Na_2HPO_4$ was weighed with 50ml of $H_2O$ Solution B; 0.7g of $K_2HPO_3$ was weighed with the same 50ml of water. Solution B was further added to A to get a pH of 7.3. The above buffer was then added to 0.3ml of 6% of $H_2O_2$ to get 50ml together with the supernatant. The solution was read at 480nm or 240nm at 30 seconds and 3 minutes respectively.

**Test for GHS**

Materials used in this test includes sulfosalicylic acid, phosphate buffer, DTNB and the supernatant Preparation; 750Nl tissue supernatant and 750Nl 4% sulfosalicylic acid was incubated for 20 minutes and centrifuged at 5000pm for 10 minutes at 4°C, 500ml of DTNB and 500ml of phosphate buffer of 0.1M and pH of 7.4 was added. The resultant was read at 412nm.

**Test for AOPP**

Materials used in this test includes PBS, Potassium iodide, Acetic Acid and Chloramine T. Preparation; 400Nl of sample (supernatant) was diluted with 1600Nl of PBS, 200Nl of 1.16 potassium iodide was added and left to react, at 5 minutes the reaction was stopped with acetic acid of 100Nl. Standards where prepared with Chloramine T. The samples were read at 340nm.

**Data Analysis**

The data from the study and the variation and the statistical significance of the differences between the groups were determined by Analysis of Variance (ANOVA) and Turkey post Hoc test. The Analysis was performed using Statistical package for Social sciences (SPSS) software version 22.

## RESULTS

**Table 1: Pattern of memory recall and spatial response in the test and control rats in the three weeks of study using Passive Avoidance Box test.**

| Passive Avoidance Test | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | WEEK 1 Time(s) | | | WEEK 2 Time(s) | | | WEEK 3 Time(s) | | |
| **Groups** | **Treatment** | **Trial 1** | **Trial 2** | **Trial 3** | **Trial 1** | **Trial 2** | **Trial 3** | **Trial 1** | **Trial 2** | **Trial 3** |
| 1 | Control | 300.00 ±0.00 | 130.00 ±69.57 | 135.00 ±67.75[b] | 180.00 ±73.49 | 179.40 ±64.88 | 180.00 ±73.49 | 180.00 ±73.49 | 180.00 ±73.49 | 180.00 ±73.49 |
| 2 | 50mg/kg Caffeine low dose | 99.20 ±52.87[a] | 164.80 ±57.36 | 300.00 ±0.00[a] | 94.00 ±53.69 | 146.00 ±64.93 | 153.80 ±61.99 | 73.20 ±37.68 | 153.00 ±40.23 | 300.00 ±0.00[b] |
| 3 | 100mg/kg Caffeine low dose | 91.60 ±52.72[a] | 150.00 ±43.27 | 96.80 ±51.69[a] | 227.60 ±44.39 | 299.40 ±0.60[b] | 300.00 ±0.00 | 213.40 ±53.10 | 234.20 ±41.22[b] | 223.00 ±47.29[b] |
| 4 | 150mg/kg Caffeine low dose | 181.00 ±49.41 | 208.40 ±50.71 | 299.40 ±0.60 | 93.80 ±27.28 | 210.00 ±44.48 | 209.40 ±50.33 | 112.00 ±14.28 | 115.60 ±39.45 | 226.20 ±58.11[b] |
| 5 | Epinephrine | 120.00 ±73.49 | 60.00 ±60.00 | 60.00 ±60.00[a] | 120.00 ±73.49 | 60.00 ±60.00 | 60.00 ±60.00 | 120.00 ±73.49 | 60.00 ±60.00 | 60.00 ±60.00 |

Values are presented in mean ± sem. N = 5.**a** means values are statistically significant when compared to the negative control values and **b** means values are statistically significant when compared to the positive control values.

**Table 2: Pattern of noxious sensitivity and latency threshold in the test and control rats in the three weeks of study.**

| Analgesy-meter Test | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | WEEK 1 Load(g) | | | WEEK 2 Load(g) | | | WEEK 3 Load(g) | | |
| **Groups** | **Treatment** | **Trial 1** | **Trial 2** | **Trial 3** | **Trial 1** | **Trial 2** | **Trial 3** | **Trial 1** | **Trial 2** | **Trial 3** |
| 1 | Control | 18.02 ±4.38 | 21.36 ±3.64 | 25.00 ±0.00 | 22.62 ±2.28 | 18.32 ±4.55 | 13.64 ±3.01$^{b}$ | 20.80 ±2.80 | 21.80 ±1.45 | 21.72 ±2.08 |
| 2 | 50mg/kg Caffeine low dose | 19.52 ±3.36 | 18.18 ±2.81 | 25.00 ±0.00 | 15.88 ±3.87 | 13.10 ±1.65$^{b}$ | 12.86 ±4.14$^{b}$ | 6.50 ±2.74$^{ab}$ | 5.98 ±2.08$^{ab}$ | 16.10 ±5.45 |
| 3 | 100mg/kg Caffeine low dose | 21.00 ±2.62 | 18.80 ±4.00 | 25.00 ±0.00 | 14.90 ±3.59$^{b}$ | 9.60 ±3.15$^{b}$ | 8.28 ±1.85$^{b}$ | 8.72 ±2.24$^{ab}$ | 9.06 ±1.94$^{ab}$ | 3.90 ±0.61$^{ab}$ |
| 4 | 150mg/kg Caffeine low dose | 17.04 ±4.07 | 18.38 ±8.89 | 12.56 ±5.09$^{ab}$ | 9.96 ±4.09$^{ab}$ | 9.18 ±4.00$^{b}$ | 5.50 ±1.26$^{ab}$ | 13.70 ±4.75$^{b}$ | 3.12 ±0.52$^{ab}$ | 16.80 ±3.74 |
| 5 | Epinephrine | 25.00 ±0.00 | 24.00 ±2.24 | 25.00 ±0.00 | 25.00 ±0.00 | 24.00 ±1.00 | 25.00 ±0.00$^{a}$ | 25.00 ±0.00 | 24.00 ±0.52 | 25.00 ±0.00 |

Values are presented in mean ± sem. N=5. **a** means values are statistically significant when compared to the negative control value. **b** means values are statistically significant when compared to the positive control value.

**Table 3: Pattern of response of oxidative stress markers and OAPPs after 3 weeks-treatment in the test and control groups.**

| Groups | Malondialdeyde (mmol/ml ± sem) | Catalase (U/ml ± sem) | Superoxide dismutase (U/ml ± sem) | Glutathione reductase (U/ml ± sem) | AOPP (µmol/ml ±sem) |
|---|---|---|---|---|---|
| Control | 1.99 ± 0.16$^{b}$ | 36.60 ± 7.38 | 49.33 ± 4.49$^{b}$ | 5.41 ± 0.05 | 94.98 ±6.70 |
| 50mg/kg Caffeine low dose | 2.13 ± 0.10$^{b}$ | 20.38 ± 12.48 | 76.67 ± 2.04$^{a}$ | 4.34 ± 0.13 | 92.58 ±1.96 |
| 100mg/kg Caffeine low dose | 2.31 ± 0.04$^{ab}$ | 15.75 ± 1.13 | 52.67± 9.80$^{b}$ | 4.76 ±0. 03 | 122.33 ±1.80 |
| 150mg/kg Caffeine low dose | 1.74 ± 0.03 | 31.50 ± 11.91 | 63.33 ± 8.16$^{b}$ | 4.58 ±0.46 | 106.72 ±1.63 |
| Epinephrine | 1.65 ± 0.00$^{a}$ | 20.39 ± 1.70 | 92.67± 2.45$^{a}$ | 5.87 ±0.05 | 104.72 ±4.08 |

Values are presented in mean ± sem. N=5. **a** means values are statistically significant when compared to the negative control value. **b** means values are statistically significant when compared to the positive control value.

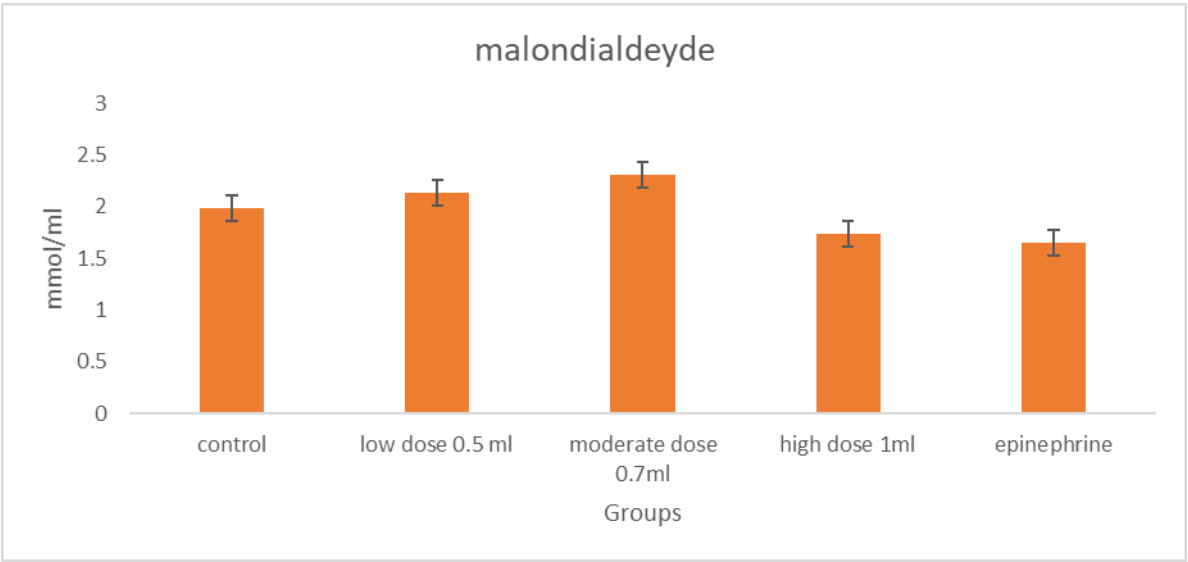


**Figure 1: Pattern of response of Malondialdeyde after 3 weeks treatment of sub-chronic caffeine treatment in the test and control groups.**

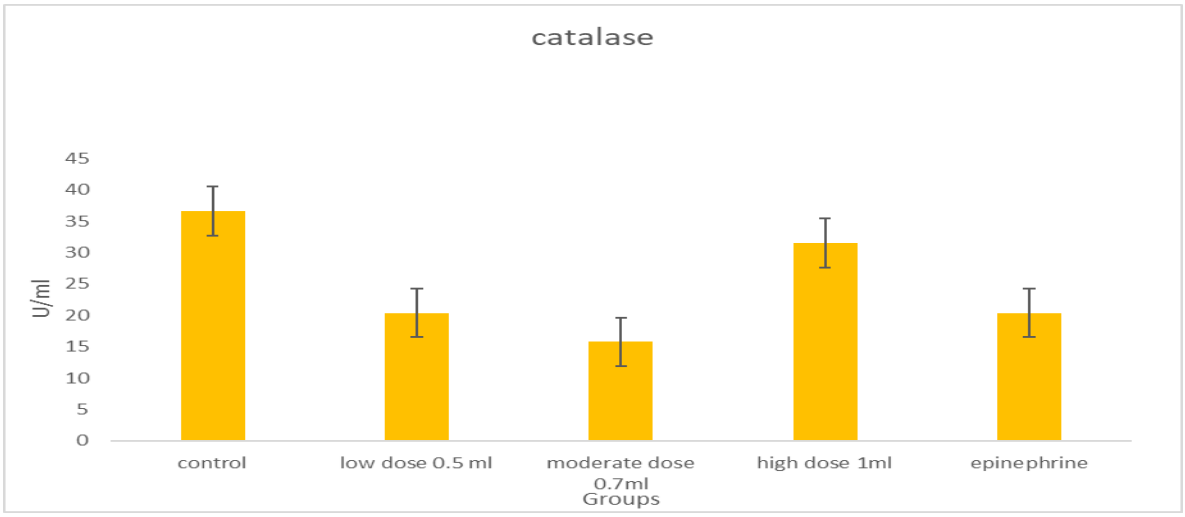


**Figure 2: Pattern of response of Catalase after 3 weeks of treatment of sub-chronic caffeine treatment in the test and control groups.**

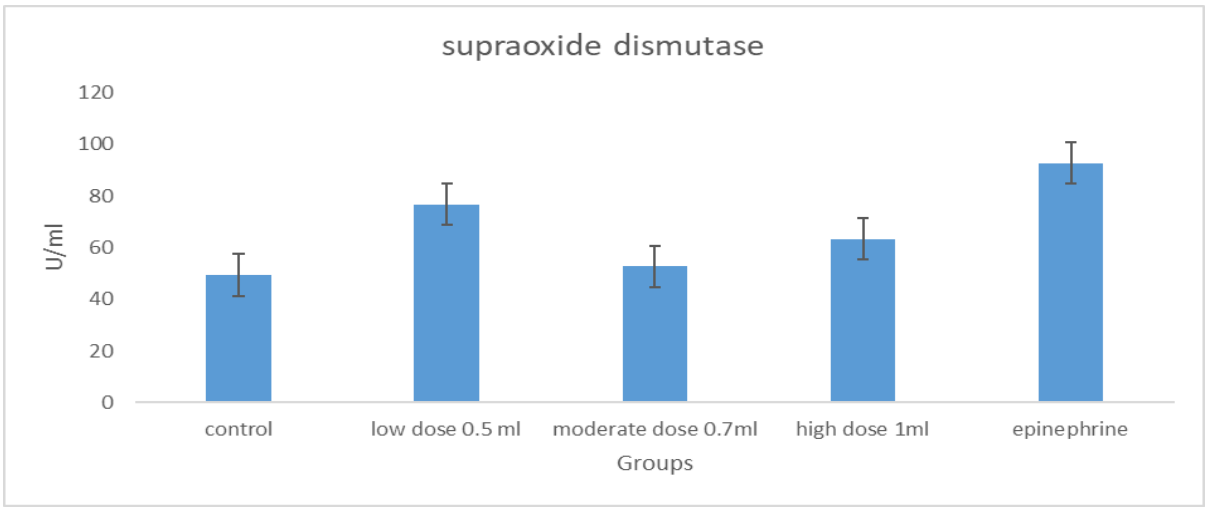


**Figure 3: Pattern of response of Superoxide dismutase after 3 weeks of treatment of sub-chronic caffeine treatment in the test and control groups.**

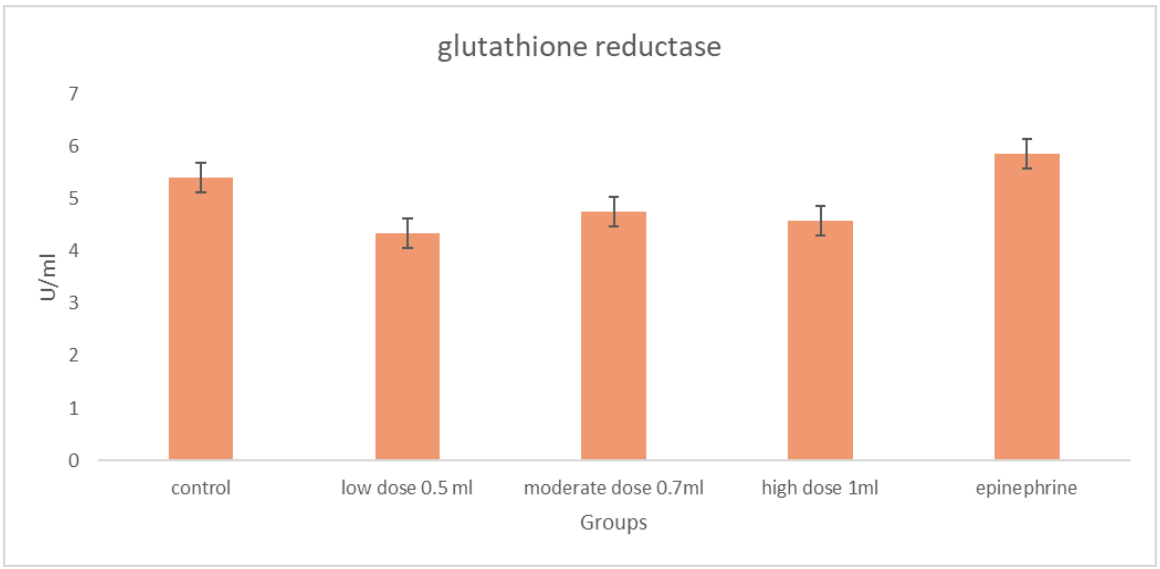


**Figure 4: Pattern of response of Glutathione reductase after 3 weeks of treatment of sub-chronic caffeine treatment in the test and control groups.**

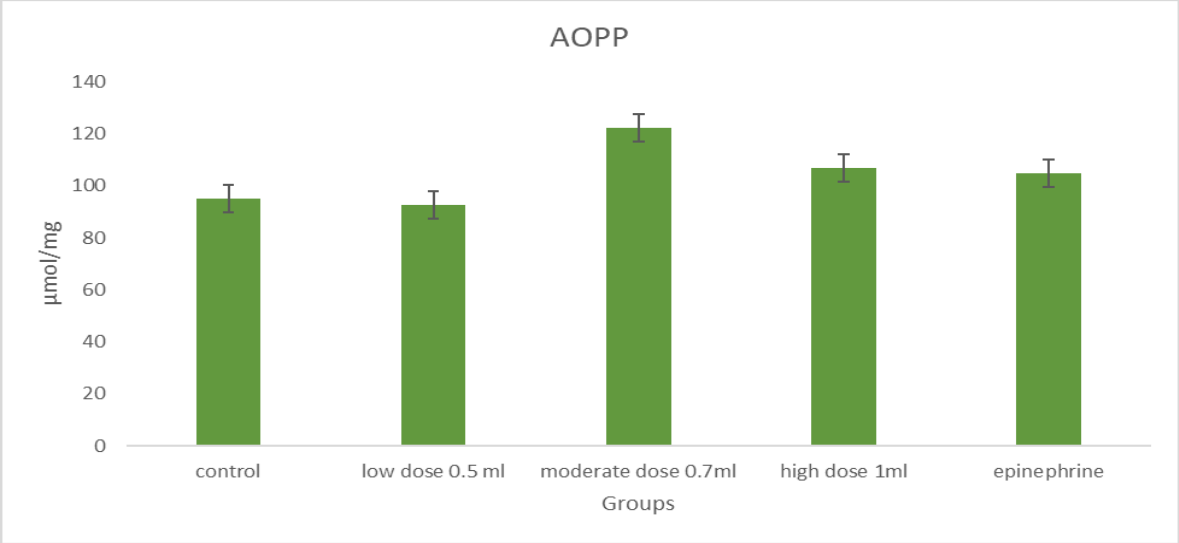


**Figure 5: Pattern of response of AOPP after 3 weeks of treatment of sub-chronic caffeine treatment in the test and control groups.**

## DISCUSSION

This research was designed to investigate the effect of sub-chronic administration of exogenous caffeine on advance oxidative protein products and pain hypersensitivity. Caffeine, which acts as an antagonist of adenosine receptors via G-protein pathway, has been found to increase pain hypersensitivity alongside advanced oxidative protein products. In other words, the implications of caffeine consumption from the data obtained in the 3 weeks of research showed that increase in hypersensitivity and oxidative protein products became more feasible in the consumption of moderate to high amounts of caffeine.

The effect of caffeine on pain hypersensitivity was investigated using the analgesy-meter. The pain threshold upon application of pressure on the paw of the rat was investigated. Table 2 data shows that rats in group 2, which were treated with 50mg/kg of caffeine, are relatively able to withstand the pain to the paw as in trial 1 and 2 week 1, giving a result almost similar to that of the rats in control group 1 that were given normal water. This was in agreement with the work of James Sawynok "*caffeine and pain"*[6] that lower dose of caffeine blocks antinociception and causes analgesic actions. This is in contrast from administration of caffeine in higher doses.[11]

Whereas in Table 2, group 3 and 4 which were treated with 100mg/kg and 150mg/kg respectively, exhibited hypersensitivity on their paws (group 4 with a pronounced increase) when tested with the machine. Rats in group 3 compared to that in group 1 removed their paws from the gripping hold of the machine earlier. This result was also noted in group 4, more so, in week 2 and 3 most of the rats couldn't complete the task properly and removed their paws almost immediately. This supports Alireza. *et.al,*[12]*the role of caffeine in pain management* that chronic ingestion of caffeine could lead to hypersensitivity and low threshold to pain. The effect of caffeine on pain-induced memory retrieval was investigated using the Passive Avoidance test. Following exposure to an inescapable shock at the dark compartment of the test apparatus, the rat's fear induced memory to the shock is used to evaluate the pain threshold level and hypersensitivity. As observed in table 2, the pain sensitivity which allows avoidance to the dark compartment is greatly increased in group 4.

The studies revealed that although hypersensitive, group 3 and 4, the initial latency that takes rats going away from light to dark compartment of shuttle box (before exposing to electrical shock) was decreased significantly, Step-through latency (moving from light to dark compartment) was increased significantly in group 2 versus the control rats (group 1). The group 1 rats couldn't complete the test, since there is no drug administered to them. There was no significant difference between Groups 3 and 4 as the both are hypersensitive to the shock but tended to go back to the dark compartment in lesser period of time than group 2 as seen in table 2.

MDA levels in high dose caffeine and epinephrine treated groups decreased significantly. Increase in MDA level is tantamount to a significant increase lipid peroxidation in the tissue. (Table 3, figure 1) The low level of this marker was an intrigue at this stage of the study considering the possibility of caffeine in causing imbalance to the homeostasis in the tissue due to Oxidative stress.[13,14] Activities of Catalase seen in the tissue after the three weeks of caffeine administration showed that there was a significant decrease ($p < 0.05$) from low dose to moderate dose of administration, however, there was a peak in the level of Catalase. (Table 3, figure 2) 50mg/kg and 100mg/kg showed lower catalase enzyme activities. This signifies that high

administration of caffeine could up regulate level of catalase activities.[16,17].

This increase in levels would be seen in both chronic administration and longer period of caffeine intake. Activities of caffeine contribution as an antioxidant to the reduction of Catalase as seen in the results this points to Anika *et al*, in "Role of Catalase in Oxidative Stress-free and Age- Associated Degenerative Diseases", which says Catalase is one of the crucial antioxidant enzymes which plays an important role by breaking down hydrogen peroxide and maintaining the cellular redox homeostasis. SOD activities of caffeine treated groups decreased significantly from the epinephrine treated group but was higher than the control set. Results showed a strong negative correlation between SOD activities of the control group and the low dose 0.5 ml administration.

Whereas moderate or mild dose of caffeine intake and high dose of caffeine intake had the same levels and a unit greater activity of SOD. (Table 3, figure 3). With the increase in dose the activities reduced a little giving an idea that chronic and prolonged caffeine intake could reduce SOD activity to an extent. Epinephrine treated group gave high activities of SOD as epinephrine alters equilibrium in the tissue giving rise to the increase. The results showed that consumption of all caffeine treated group decreased GHS activities to an extent compared to the control and epinephrine treated groups. This result is based on the fact that caffeine declined antioxidant's status which affected the stress marker,[18,19] GHS activities in the tissue upon administration evidently seen in the low and high dose of administration (Table 3, figure 4).

Unlike the control group, group three, moderate dose of caffeine (100mg/kg) caused a peak in advanced oxidative protein products which showed a statistically significant increase with caffeine intake. In group 4, high dose of caffeine administration (150mg/kg) however, showed increased activities of AOPP levels as seen from Table 3, Fig 5. Low dose of caffeine did not cause a visible effect on AOPP levels as compared to group two and four. The increase in advanced oxidative protein products levels shows that oxidative stress increased with increased caffeine intake. The relationship between increased in AOPP levels and increased caffeine intake. Finally the levels of both the control and group two of 50mg/kg administration were similar, showing that low doses of caffeine does not give a visible rise in AOPP levels.[20]

## CONCLUSION

From the data gotten from this research, it can be concluded that caffeine at lower doses acts as an antinociception agent and causes analgestic actions which supports the idea that caffeine can be used as an adjuvant analgestic agent, although this is different when sub-chronic ingestion of caffeine is given as it causes a hypersensitive relay of nociceptive pain to the test subject.

According to the experiment carried out, it can be confirmed that caffeine directly interferes with the oxidative stress markers. It can be affirmed that the moderate consumption of coffee does not represent a risk to health, presenting a protective effect in several pathologies, as seen in the presence of Catalase. The proven benefits of coffee justify its inclusion in the functional foods group, not only for caffeine but also for other compounds present in coffee. Current evidence has shown that moderate consumption of caffeine is a therapeutic resource as it promotes physical and mental performance. However, with further studies, and emphasis on neurobiology, it will be needed to elucidate the potential abusive effect on caffeine use, its risks and benefits.

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