DNA Damage Induced in the Germ and Bone Marrow Cells of Mice by Caffeine

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Abstract: Caffeine is a major constituent of many drugs and also widely and immensely consumed in beverages and soft drinks. The public health implication of the habitual and indiscriminate consumption of caffeine containing products has prompted studies on its safety evaluation. In this study, we investigated the potentials of caffeine to induce abnormal sperm cells in the testes and Micronuclei (MN) in the bone marrow cells using the sperm morphology and MN assays in mice. For these assays, mice were administered Intraperitoneally (IP) caffeine at dose levels of 0.39, 0.78, 1.56 and 3.12 mg kg⁻¹ bwt (body weight) and normal saline and cyclophosphamide were used as negative and positive controls, respectively.

Key words: Caffeine, genotoxicity, mice, micronucleus, spermatozoa, health risk, saline

INTRODUCTION

The prevalence of self-medication practice in Nigeria is a phenomenon that calls for rapid intervention. The Over-the-Counter (OTC) healthcare market is the largest and most vibrant for medical products and has experienced strong steady growth in recent years (Euromonitor International, 2008). This growth was stimulated by self-medication which is the leading form of healthcare among many Nigerians given the problems and expense of visiting a medical doctor. It is common in Nigeria to see petty traders selling kola nuts, cigarettes and oranges, among other items in kiosks, motor parks and roadside establishments also hawking medical products ranging from OTC drugs to antibiotics (Euromonitor International, 2008). Onochowski and Olasehinde (2004) have shown that medications like pain-relievers, anti-malarials, blood tonics and antibiotics were common in homes of secondary school students in Ibadan, Nigeria. As a result of ignorance on the use of drugs, illiteracy, poverty and general lack of understanding of the consequences of the interaction of the drug components with the body systems, they are often used indiscriminately in wrong doses and/or in combination with other drugs. Some of the drugs utilised in this way include analgesics, diuretics, cough medicines and several energy drinks. A common constituent of some of these is caffeine, the most psychophysiological active drug (Pollard, 2004). Caffeine whose primary biological effect is the antagonism of the A1 and A2 subtypes of the adenosine receptors is used as an analgesic adjuvant in aspirin and acetaminophen where it serves to increase their effectiveness. It is also used in combination with ergotamine for treatment of migraine and cluster headaches as well as in >200 other preparations with other drugs (IARC, 1990). Caffeine is naturally found in the leaves, seeds and fruits of >60 different plants (Al-Shoshan, 2007) and as an additive in coffee, tea, kola nuts, chocolate and soda beverages; thus it is widely and immensely consumed. Caffeine has been shown to have various pharmacological and cellular responses in a wide spectrum of biological systems (Dews, 1984). These include stimulation of the central nervous system (Dash and Gummadi, 2008) and cardiac muscle, increased urinary output and relaxation of smooth muscles (Yukawa et al., 2004). It increases endurance in well trained athletes thereby increasing the athlete’s ability to increase the intensity or duration of exercise (Sinclair and Geiger, 2000).

It is also able to inhibit the mutagenic effects of numerous chemicals (Nehlig and Deby, 1994). The use of caffeine has some negative consequences as a result of its molecular interactions with biological macromolecules. Cardiac arrhythmias and other symptoms in newborn infants were associated with maternal caffeine use of >500 mg day⁻¹ in comparison to the offspring of women who used <250 mg day⁻¹ of caffeine (Hadeed and Siegel, 1993). Hinds et al. (1996) reported that mothers who consumed >300 mg day⁻¹ of caffeine had a relative risk of giving birth to a low-weight infant with a mean difference of 105 g compared with infants of non-consumers of caffeine. Rumsby et al. (1982) showed that caffeine has various effects on DNA metabolism including enhancement of the damage caused by various types of

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radiation and chemicals. Caffeine has been implicated as interfering with cellular repair mechanism, inhibiting some enzymes and also inhibiting RNA and protein synthesis (Kanemaru et al., 1992). Selby and Sancar (1990) demonstrated that caffeine inhibits photoreactivation of damaged DNA in Escherichia coli while Ostertag et al. (1965) reported chromsome breakage in cultured human cells following treatment with caffeine. Zhou et al. (2000) indicated that caffeine abolishes the mammalian G2/M DNA damage checkpoint by inhibiting Ataxia-Telangiectasia-mutated kinase activity. Caffeine has also been reported to reduce the cytogenotoxicity of Methotrexate (MTX) in vivo in mice (Palo and Choudhury, 2006) by binding to MTX-induced damaged site. This prevents DNA repair and causes the cell to switch to the apoptotic pathway before meiosis, leading to a reduction in the number of cells with abnormal DNA. Bakare et al. (2007) further reported that caffeine combined with paracetamol reduced the genotoxic effect of paracetamol as evident in reduction of DNA damage in the germ cells of mice. There are controversies concerning the genotoxic and mutagenic effects of caffeine in biological systems. Considering human consumption of this substance worldwide, there is therefore the need for further studies on the genotoxicity and mutagenicity in mammalian system. In this study, the potentials of caffeine to induce genetic damage in the germ and bone marrow cells of male mice have been investigated.

MATERIALS AND METHODS

Biological materials: Pathogen free male Swiss albino mice (Mus musculus) of ages 4 and 10 weeks were obtained from the animal breeding facility of the Cell Biology and Genetics Unit, University of Lagos, Nigeria. They were acclimatized for 2-4 weeks at the animal house of the Department of Zoology, University of Ibadan, Nigeria where they were maintained under alternating periods of 12 h light and darkness. Their bedding was usually cleaned and they were supplied with feed (Ladokun pelleted feeds® Ibadan) and potable water ad libitum. The mice were then divided into 2 groups (a group for the MN test and the other for the sperm morphology assay).

Sperm morphology assay: Induction of sperm abnormalities was done according to Wyrobek et al. (1983) and Bakare et al. (2005). Mice of 12-14 weeks of age were used for this test. About 4 concentrations of caffeine (CAS No.: 58-08-2, purchased from Sigma Aldrich Cheme GmbH, Germany): 0.39, 0.78, 1.56 and 3.12 mg kg⁻¹ bwt corresponding to ×0.0625, ×0.125, ×0.25 and ×0.33 of the IP LD₅₀ together with the positive (cyclophosphamide 20 mg kg⁻¹ body weight) and negative (normal saline) controls were considered in each of the two exposure periods of 35 days (5 weeks) and 70 days (10 weeks). There were 4 mice dose⁻¹ and for each of the control group. A single Intraperitoneal (IP) injection of 0.5 mL/dose/mouse was administered daily for 5 consecutive days.

The IP route was favored since, it is one of the fastest and most efficient means of delivering test-chemicals into test-animals in a short-term-assay (Wyrobek et al., 1983). Sperm was sampled from the caudal epididymes at 5 and 10 weeks from the first injection. The mice were sacrificed by cervical dislocation and their epididymes surgically removed; sperm suspensions were then prepared from the caudal of each testis by mincing the caudal in physiological saline. Smears were prepared on grease-free slides after staining with 1% Eosin Y for 45 min. The slides were air-dried and coded for subsequent microscopic examination at 1000. Each mouse, 1000× sperm cells were assessed for morphological abnormalities according to the criteria of Wyrobek and Bruce (1975).

Micronucleus assay (MN): Mice of 6-8 weeks of age were used for this assay (4 mice per treatment group and the treatment were as for the Sperm morphology test). About 6 h after the last injection, bone marrow preparation for MN assessment was done according to standard procedure (Schmid, 1975; Bakare et al., 2009). Briefly, the animals were sacrificed by cervical dislocation. The femurs were removed and bone marrow flushed from the bones with Foetal Bovine Serum (Sigma Aldrich Cheme GmbH, Germany). Cells were centrifuged at 2000 rpm for 10 min and slides stained with May-Grunwald and Giemsa stains. At least 2000 cells/mouse were scored microscopically at 1000× for MN in Polychromatic Erythrocytes (MNPCE).

Statistical analysis: The SPSS® 15.0 statistical package and SAS® Version 8 (2003) were used for data analysis. Data obtained were expressed as percentage frequency and mean±standard error. Significance at the different dose-level of each assay was tested by using the Students t-test and Dunett test. Differences between the negative control-group and individual dose-groups were analyzed at the 0.05 and 0.001 probability levels. Pearson product moment correlation coefficient (r) was calculated to show concentration-response relationship in the two assays.

RESULTS AND DISCUSSION

Sperm morphology assay: In the two exposure periods, caffeine at the different doses induced dose-dependent increase in abnormal sperm morphology in mice (Table 1).

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At the 5th weeks exposure period (r = -0.20), the frequency of abnormal sperm cells in the negative and positive controls was 2.05 and 14.75% (p<0.05), respectively. There were 2.55, 2.58, 4.75 and 5.53% (p<0.05) of abnormal sperm cells at the different doses of 0.0625, 0.125, 0.25 and 0.33 of caffeine, respectively. At same doses for the 10 weeks exposure period (r = -0.23), there were 3.58, 3.98, 8.65 and 10.90% of abnormal sperm cells and the negative and positive control values were 2.15 and 18.95% (p<0.05), respectively. These values were significant at the >.05 and >.33 of caffeine.

Figure 1 shows the frequency of the different types of abnormal sperm cells observed. Sperm cells with short hook had the highest frequency of abnormal cells at the 5th week exposure period while it was sperm with amorphous head at the 10th week exposure phase. Sperm with rubbed hook and double-tailed sperm cells were the least prominent for the two exposure periods, respectively.

**Micronucleus assay:** Figure 2 shows the MN induced in the bone marrow cells after exposure to mice of different doses of caffeine. Compared with the negative control, there was a significant (p<0.01, p<0.05) increase in MN at >0.25 and >0.33 doses. This was however not dose-dependent (r = -0.03). The positive control induced a significant (p<0.05) induction of MN in positive control groups.

Cells that multiply rapidly like sperm precursor cells and immature red blood cells are easy target for chemical mutagens which may be present in the circulatory system. In this study, caffeine induced abnormal sperm morphology and MN in treated mice.

The responses are believed to be positive for both assays; there was an increase in abnormal sperm morphology at least double the control level in two of the treated groups and this increase was significant (p<0.05) at the highest tested doses and significant (p<0.05) induction of MNPCs in mice bone marrow cells though not dose-dependent. This is believed to be due to the ability of caffeine to disrupt the DNA repair processes in the rapidly multiplying germ and bone marrow cells of mice.

Caffeine has been known to potentiate rather than to induce chromosomal aberrations and also to transform sub-lethal damage of mutagenic agents into lethal damage (Burgen and Mitchell, 1978). Herein, caffeine may have potentiated spontaneous damage in the DNA of sperm and bone marrow cells.

As caffeine is structurally similar to DNA, it interacts chemically with rapidly replicating DNA in growing cells, thus inducing mutation (Pollard, 2004). Thus, it inhibits DNA repair either by binding itself to the single stranded DNA regions or by interfering in the specific binding of repair enzymes and thereby inhibiting the DNA repair process.

This is supported by a report (Selby and Sancar, 1990) that caffeine intercalates into nucleic acids and hinders digestion of damage by DNAase I in vivo, producing sequence specific inhibition of repair. The abnormal sperms may have been due to induced mutation caused by caffeine in the DNA of the early spermatocytes and spermatogonia at the pre-meiotic stages of spermatogenesis. The induction may also have been during the last few weeks of spermatogenesis (post-meiotic) (Shelby, 1996; Marchetti and Wyrobek, 2005).

The high sensitivity of the post-meiotic period to mutagenic exposure has been associated with the reduced DNA repair capacity of late spermatids and sperm as compared to early spermatids and other spermatogenic cell types (Sotomayor and Segà, 2000; Baarends et al., 2001; Olsen et al., 2005). Some other reports (Loon et al., 1993; Hamer et al., 2003; Xu et al., 2005) have also shown that all major DNA repair pathways seem to be less functional in late spermatids and sperms. Caffeine has a metabolic halftime of 3-5 h in man (Adler, 1966) and so long-term exposure, therefore cannot be expected to lead to an accumulation of the substance in the body. In the mouse, it takes 34.5 days for spermatogenesis to

<table>
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<tr>
<th>Table 1: Summary of morphologically abnormal sperm cells induced in mice by caffeine</th>
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<tr>
<td>Dose levels (mg kg⁻¹ bw)</td>
<td>5 weeks</td>
<td>10 weeks</td>
<td>5 weeks</td>
</tr>
<tr>
<td>Normal saline</td>
<td>82</td>
<td>86</td>
<td>2.05</td>
</tr>
<tr>
<td>Cyclophosphamide (20 mg kg⁻¹ bw)</td>
<td>590</td>
<td>758</td>
<td>14.75*</td>
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<tr>
<td>0.39 (0.0625)</td>
<td>102</td>
<td>143</td>
<td>2.55</td>
</tr>
<tr>
<td>0.78 (0.125)</td>
<td>103</td>
<td>159</td>
<td>2.58</td>
</tr>
<tr>
<td>1.56 (0.25)</td>
<td>190</td>
<td>346</td>
<td>4.75</td>
</tr>
<tr>
<td>2.08 (0.33)</td>
<td>221</td>
<td>436</td>
<td>5.53*</td>
</tr>
<tr>
<td>Total</td>
<td>1288</td>
<td>1084</td>
<td>8.05*</td>
</tr>
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*The percentages and the means are for groups of four mice for each dose. §4000 sperms were assessed for morphological damage/dose and the controls.

*Significantly higher (p<0.05) than the negative control.
the action of caffeine especially as the distribution of caffeine into semen rapidly equilibrates with that in the blood (Beach et al., 1984; Ramlau-Hansen et al., 2008). The inability of sperm to repair DNA lesions as they occur may make them particularly susceptible to lasting exposure to a chemical. This suggests a possible mechanism of caffeine accumulation/retention in the body, causing genomic damage to stem cells, late spermatids and sperms which present as aberrant sperm cells. The observation is in concert with those of Ekaluo et al. (2005, 2009) who reported sperm abnormalities in rats treated with caffeine combined with some other analgesics and also with those of Bakare et al. (2007) wherein the presence of various types of abnormal spermatozoa in mice treated with paracetamol combined with caffeine was reported.

Wyrobek and Bruce (1975) indicated that elevated levels of sperm head abnormalities in both animal models and humans following exposure to a physical or chemical mutagen may not necessarily be as a result of mutations in the germ line, it may also be due to the production of some somatic changes in the animal which in turn results in the increased production of aberrant sperm. The findings also show increased genetic damage in the somatic cells (bone marrow cells) of treated animals and might have some correlation with observed sperm abnormalities.

The MN assay was derived primarily for evaluating the ability of test agents to induce structural and/or numerical chromosome damage. Both types of damage are associated with the appearance and/or progression of tumors and with adverse reproductive and developmental outcome (Krisha and Hayashi, 2000). In this study, caffeine induced the formation of MN in PCEs of caffeine-treated mice. This was believed to be as a result of the ability of caffeine to cause DNA strand to break and also affect spindle formation and function. It is consistent with earlier reports in some animal models (Dambrosio, 1994; Palo and Choudhury, 2006) and with those of Choudhury et al. (2001) wherein caffeine was found to induce dose-related cytogenotoxicity especially at high doses. MN form as a result of the aggregation of whole chromosome or chromosome/chromatid fragments as well as disturbance of the spindle fibres therefore, the increased frequency of MN in caffeine-treated mice suggests its possible clastogenic and aneugenic activity. Therefore, high concentrations of caffeine in living cells could be of genetic consequence. The public health implication of the habitual consumption of caffeine containing products has prompted many recent studies on its safety evaluation. The induction of abnormal sperm cells and MN with increasing doses of caffeine in mice in this study indicates that caffeine is a potential germ and somatic cell mutagen. Abnormal sperm morphology has
been associated with reduced fertility in both human and animal studies (NRC, 1989). Bone marrow MN induction is a clastogenic effect that has been associated with carcinogenesis in human and experimental animals (Oshiro et al., 2001). These could be of significant public health interests. Further studies are ongoing on the potential of caffeine to induce genetic instability in rat.

CONCLUSION

The study showed that the sperms of mice from the caudal epididymes examined at 5th and 10th week from the 1st day of treatment showed that caffeine induced dose dependent significant (p<0.05) formation of abnormal sperm cells. Induction of micronucleated polychromatic erythrocytes was not dose dependent but statistically significant (p<0.05) at the doses of 1.17 and 1.56 mg kg⁻¹ bwt. These suggest that caffeine may be a potential germ and somatic cell mutagen and thus, its indiscriminate use may be of potential human health risk.

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