

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). Onohwosafe and Olaseha (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 Debry, 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

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 chromosome breakage in cultured human cells following treatment with caffeine. Zhou *et al.* (2000) indicated that caffeine abolishes the mammalian G₂/M DNA damage check point 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 acclimatised 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 beddings were regularly changed 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 favoured 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. For 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 Dunnett 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).

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 $\times 0.0625$, $\times 0.125$, $\times 0.25$ and $\times 0.33$ 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% 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 $\times 0.25$ and $\times 0.33$ 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 nubbed 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 of mice to different doses of caffeine. Compared with the negative control, there was a significant ($p < 0.01$, $p < 0.05$) increase in MN at $\times 0.25$ and $\times 0.33$ 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 MNPCEs 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 Segal, 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 half-time 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 1: Summary of morphologically abnormal sperm cells induced in mice by caffeine^{a,b}

Dose levels (mg/kg bwt)	Total abnormal sperm		Abnormalities (%)		X±SE	
	5 weeks	10 weeks	5 weeks	10 weeks	5 weeks	10 weeks
Normal saline	82	86	2.05	2.15	7.45 ± 3.14	14.33±5.95
Cyclophosphamide (20 mg kg ⁻¹ bwt)	590	758	14.75*	18.95*	53.64± 41.91	126.33±57.33
0.39 (×0.0625)	102	143	2.55	3.58	9.27 ±3 .55	23.83±10.08
0.78 (×0.125)	103	159	2.58	3.98	9.36 ± 3.15	26.50±8.94
1.56 (×0.25)	190	346	4.75	8.65*	17.27± 5.09	57.67±27.96
2.08 (×0.33)	221	436	5.53*	10.90*	20.09± 5.317	72.67±35.02
Total	1288	1084	8.05*	6.78*	117.09± 62.16	180.67±80.84

^aThe percentages and the means are for groups of four mice for each dose. ^b4000 sperms were assessed for morphological damage/dose and the controls. *Significantly higher ($p < 0.05$) than the negative control

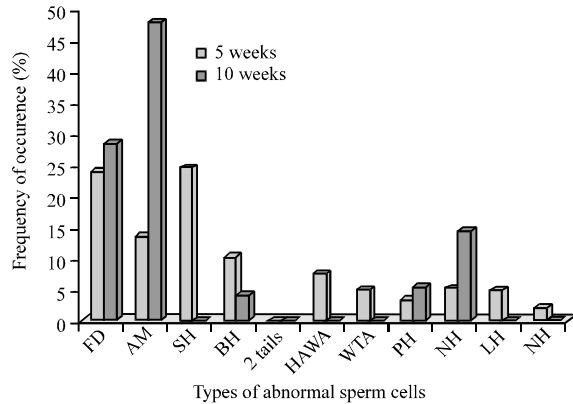


Fig. 1: Frequencies of the different types of sperm abnormalities observed in mice treated with different doses of caffeine after 5 and 10 weeks exposure period (FD: Folded; AM: Amorphous; SH: Short Hook; BH: Banana Head; HAWA: Hook At Wrong Angle; WTA: Wrong Tail Attachment; PH: Pin Head; NH: No Hook; LH: Long Hook; NH: Nubbed Hook; $\times 1000$)

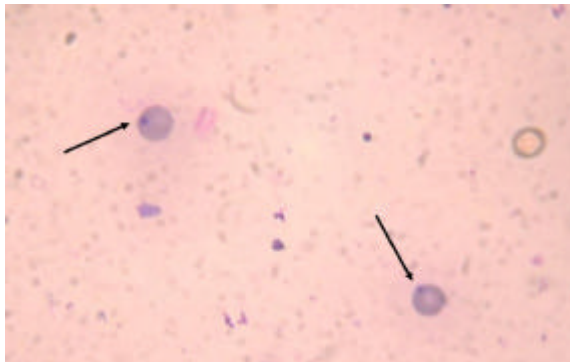


Fig. 2: The MN induced in the bone marrow cell after exposure of mice to different doses of caffeine

complete (Bartke *et al.*, 1974). This implies that cells from subsequent spermatogenic processes should not be affected by previous exposure to caffeine. If these cells must be affected, they would have to be treated repeatedly via re-exposure. Results from the 10 weeks exposure period, however suggest retained genetic damage to spermatogenic stem cells. The implication of this is that caffeine had an effect on sperm cells which had arisen from treated spermatogonial cells causing damage to pre and post meiotic stages of spermatogenesis. Monesi (1962) reported that during spermatogenesis, DNA synthesis occurs before the pre-meiotic phase and no further synthesis occurs throughout the duration of spermatogenesis in the cell cycle. Pollard (2004) reported that sperm precursor cells are particularly susceptible to

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 (Krishna 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.

ACKNOWLEDGEMENTS

This study was partly funded by the University of Ibadan Senate Research Grant SRG/FSC/2006/17A given to AAB. The researchers are grateful to Dr. Oluwatoyin Odeku of the Faculty of Pharmacy, University of Ibadan, Nigeria for her comments on the draft of this manuscript and Ikpe Ibanga for his assistance during data collection.

REFERENCES

Adler, I.D., 1966. Cytogenetic investigations of mutagenic action of caffeine in premeiotic spermiogenesis in mice. *Hum. Genet.*, 3: 82-83.

Al-Shoshan, A.A., 2007. Predictors of caffeine consumption among young women. *Pak. J. Nutr.*, 6: 597-602.

Baarends, W.M., R. van der Laan and J.A. Grootegoed, 2001. DNA repair mechanisms and gametogenesis. *Reproduction*, 121: 31-39.

Bakare, A.A., A.A. Mosuro and O. Osibanjo, 2005. An *in vivo* evaluation of induction of abnormal abnormal sperm morphology in mice by landfill leachates. *Mutat. Res.*, 582: 28-34.

Bakare, A.A., K.A. Oshodi and A.T. Adelodun, 2007. Paracetamol and caffeine induced sperm shape abnormalities in mice. *Afr. J. Anim. Biomed. Sci.*, 2: 11-16.

Bakare, A.A., A.A. Okunola, O.A. Adetunji and H.B. Jenmi, 2009. Genotoxicity assessment of a pharmaceutical effluent using four bioassays. *Genet. Mol. Biol.*, 32: 373-381.

Bartke, A.J., A. Weir, P. Mathison, C. Roberson and S. Dalterio, 1974. Testicular function in mouse strains with different age of sexual maturation. *J. Heredity*, 65: 204-208.

Beach, C.A., J.R. Bianchine and N. Gerber, 1984. The excretion of caffeine in the semen of men: Pharmacokinetics and comparison of the concentrations in blood and semen. *J. Clin. Pharmacol.*, 24: 120-126.

Burgen, A.S.V. and J.F. Mitchell, 1978. *Gaddum's Pharmacology*. 8th Edn., Oxford University Press, Oxford Medical Publications, London, New York, pp: 269.

Choudhury, R.C., S.K. Ghosh and A.K. Palo, 2001. Potential transmission of the cytogenetic toxic effects of methotrexate in the male germline cells of Swiss mice. *Environ. Toxicol. Pharmacol.*, 10: 81-88.

Dambrosio, M.S., 1994. Evaluation of the genotoxicity data on caffeine. *Reg. Tox. Pharm.*, 19: 243-281.

Dash, S.S. and S.N. Gummadi, 2008. Inhibitory effect of caffeine on growth of various bacterial strains. *Res. J. Microbiol.*, 3: 457-465.

Dews, P.B., 1984. *Caffeine: Perspectives from Recent Research*. Springer-Verlag, Berlin.

Ekaluo, U.B., A.E. Udokpoh, U.U. Udofia and R.O. Ajang, 2005. Comparative toxicity of five commonly used analgesics on sperm count and sperm head abnormalities. *Glob. J. Pure Applied Sci.*, 11: 81-84.

Ekaluo, U.B., E. Ikpeme and A.E. Udokpoh, 2009. Sperm head abnormality and mutagenic effect of aspirin, paracetamol and caffeine containing analgesics in rat. *Int. J. Toxicol.*, Vol. 7.

Euromonitor International, 2008. OTC health care in Nigeria. http://www.the-infoshop.com/report/eo_726_64-otc-healthc.html.

Hadeed, A. and S. Siegel, 1993. Newborn cardiac arrhythmias associated with maternal caffeine use during pregnancy. *Clin. Pediatr.*, 32: 45-47.

Hamer, G., H.L. Roepers-Gajadien, A. van Duyn-Goedhart, I.S. Gademan, H.B. Kal, P.P. van Buul and D.G. de Rooij, 2003. DNA double-strand breaks and gamma-H2AX signaling in the testis. *Biol. Reprod.*, 68: 628-634.

Hinds, S.T., L.W. William, M.K. Enid and F.H. Barbara, 1996. The effect of caffeine on pregnancy outcome variables. *Nutr. Rev.*, 54: 203-207.

IARC., 1990. *Monographs on the evaluation of carcinogenic risks of humans*. Pharm. Drugs IARC Lyon, 50: 307-322.

Kanemaru, Y., M.J. Rossowska, C.H. Narayanan and T. Nakamoto, 1992. Effect of caffeine and zinc on DNA and protein synthesis of neonatal rat cardiac muscle cell in culture. *Res. Exp. Med.*, 192: 115-122.

- Krishna, G. and M. Hayashi, 2000. *In vivo* rodent micronucleus assay: Protocol, conduct and data interpretation. *Mutat. Res.*, 455: 155-166.
- Loon, A.A., E. Sonneveld, J. Hoogerbrugge, G.P. van der Schans, J.A. Grootegoed and P.H. Lohman, 1993. Induction and repair of DNA single-strand breaks and DNA base damage at different cellular stages of spermatogenesis of the hamster upon *in vitro* exposure to ionizing radiation. *Mutat. Res.*, 294: 139-148.
- Marchetti, F. and A.J. Wyrobek, 2005. Mechanisms and consequences of paternally-transmitted chromosomal abnormalities. *Birth Defects Res. C. Embryo Today*, 75: 112-129.
- Monesi, V., 1962. Autoradiographic study of DNA synthesis and the cell cycle in *Spermatonia* and *Spermatocytes* of mouse testis using titrated thymidine. *J. Cell Biol.*, 14: 1-18.
- NRC., 1989. *Biologic Markers of Human Male Reproductive Health and Physiological Damage*. National Academy Press, Washington, DC., pp: 83-106.
- Nehlig, A. and G. Debry, 1994. Potential genotoxic, mutagenic and antimutagenic effects of coffee: A review. *Mutat. Res.*, 317: 145-162.
- Olsen, A.K., B. Lindeman, R. Wiger, N. Duale and G. Brunborg, 2005. How do male germ cells handle DNA damage. *Toxicol. Applied Pharmacol.*, 207: 521-531.
- Onohwosafe, P.S. and I.O. Olaseha, 2004. Factors influencing self-medication among students of Abadina College, Ibadan, Nigeria. *Int. J. Health Prom. Educ.*, 42: 27-32.
- Oshiro, Y., P.S. Balwierz and G. Witz, 2001. Micronucleus formation in mouse bone marrow cells *in vivo* in response to trans, trans-muconaldehyde. *Toxicol. Lett.*, 121: 159-166.
- Ostertag, W., E. Duisberg and M. Sturmman, 1965. The mutagenic activity of caffeine in man. *Mutat. Res.*, 2: 293-296.
- Palo, K.A. and C.R. Choudhury, 2006. Modulation of methotrexate-induced cytogenotoxicity in mouse *Spermatogonia* and its transmission in the male germline by caffeine. *Environ. Toxicol. Pharmacol.*, 21: 254-259.
- Pollard, I., 2004. New dangers of caffeine exposed. Macquarie University News. http://www.postgradandbeyond.mq.edu.au/enewsletter/05-health/issue_11_stories/story3.htm.
- Ramlau-Hansen, C.H., A.M. Thulstrup, J.P. Bonde, J. Olsen and B.H. Bech, 2008. Semen quality according to prenatal coffee and present caffeine exposure: Two decades of follow-up of a pregnancy report. *Human Reprod.*, 23: 2799-2805.
- Rumsby, P.C., H. Kato, C.A. Waldren and D. Patterson, 1982. Effects of caffeine on pyrimidine biosynthesis and 5-phosphoribosyl 1-1 pyrophosphate metabolism in Chinese hamster cells. *J. Biol. Chem.*, 257: 11364-11367.
- Schmid, W., 1975. The micronucleus test. *Mutat. Res.*, 31: 9-15.
- Selby, C.P. and A. Sancar, 1990. Molecular mechanisms of DNA repair inhibition by caffeine. *Proc. Nat. Acad. Sci. USA.*, 87: 3522-3525.
- Shelby, M.D., 1996. Selecting chemicals and assays for assessing mammalian germ cell mutagenicity. *Mutat. Res.*, 352: 159-167.
- Sinclair, C.J. and L.D. Geiger, 2000. Caffeine use in sports: A pharmacological review. *J. Sport Med. Phys. Fitness*, 40: 71-79.
- Sotomayor, R.E. and G.A. Sega, 2000. Unscheduled DNA synthesis assay in mammalian spermatogenic cells: An update. *Environ. Mol. Mut.*, 36: 255-265.
- Wyrobek, A.J. and W.R. Bruce, 1975. Chemical induction of sperm abnormalities in mice. *Proc. Natl. Acad. Sci. USA.*, 72: 4425-4429.
- Wyrobek, A.J., L.A. Gordon, J.G. Burkhardt, M.W. Francis and R.W. Jr. Kapp *et al.*, 1983. An evaluation of the mouse sperm morphology test and other sperm tests in non-human mammals. A report of the US Environmental Protection Agency Gene-Tox Program. *Mutat. Res.*, 115: 1-71.
- Xu, G., G. Spivak, D.L. Mitchell, T. Mori and J.R. McCarrey *et al.*, 2005. Nucleotide excision repair activity varies among murine spermatogenic cell types. *Biol. Reprod.*, 73: 123-130.
- Yukawa, G., S. Mune, M. Otani, H. Tone, X-M. Liang and H. Iwahashi, 2004. Effects of coffee consumption on oxidative susceptibility of low-density lipoproteins and serum lipid levels in humans. *Biochemical*, 69: 70-74.
- Zhou, B.B.S., P. Chaturvedi, K. Spring, S.P. Scott and R.A. Johanson *et al.*, 2000. Caffeine abolishes the mammalian G2/M DNA damage checkpoint by inhibiting ataxia-telangiectasia-mutated kinase activity. *J. Biol. Chem.*, 275: 10342-10348.