Indian Journal of Animal Sciences **82** (6): 602–604, June 2012

Evaluation of lymphocytic DNA fragmentation in rats exposed to fluoride along with buffalo pineal proteins and melatonin

VIJAY K BHARTI[1] and R S SRIVASTAVA[2]

Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

Received: 4 September 2010; Accepted: 9 November 2011

Key words: Apoptosis, Buffalo pineal proteins, DNA fragmentation, Fluoride, Lymphocytes, Melatonin, Rat

Oxidative DNA damage and production of apoptotic bodies in fluoride-induced oxidative stress might be the outcome of the altered and hostile internal milieu in the body (Chinoy and Patel 2000, He and Chen 2006). Recent experimental findings indicated the linkage between physiological processes of oxidative stress and extent of apoptosis in lymphocytes. Oxidative DNA damage and apoptotic cell death are possible mechanism by which peripheral blood lymphocytes may be depleted and immunity is affected in oxidative stress (Haddad and Mashaly 1992, Bharti 2008). Therefore, peripheral blood lymphocytes represent a physiologically and toxicologically relevant model for studying the mechanism of NaF toxicity and antioxidant action *in vivo*. The DNA fragmentation assay has been widely used to study oxidative DNA damage in isolated lymphocytes from animals exposed to high level of fluoride (Jing *et al.* 1999, Anuradha *et al.* 2001). Fluoride induces a change in the levels of expression of some apoptotic genes that leads to cellular DNA damage (Anuradha*et al.* 2001, Otsuki *et al.* 2005). Since, free radicals can start a chain reaction that lead to poor cell function or may cause cellular damage in animals exposed to high level of fluoride (Kanduc *et al.* 2002). As a result, some regulatory genes (such as *p53*, *Bcl-2*) may be damaged, causing DNA breakage and abnormal rate of apoptosis (Ha *et al*. 2004). To prevent free radical damage the body has a defense system as antioxidants. It was observed that various antioxidants can inhibit cell death and apoptosis (Serbecic and Beutelspacher 2005, Bharti 2008). Earlier few studies reported antioxidants role of buffalo pineal proteins and melatonin on fluoride-induced oxidative stress (Chawla *et al.* 2008, Bharti and Srivastava 2009a,b). In this study, we analyzed the lymphocytic DNA damage on fluoride administration alone or along with buffalo pineal proteins and melatonin in rats.

All the procedures, conducted on the experimental animals were duly approved by the Institutional Animal Ethics Committee (IAEC) of control and supervision of experiments on animals.

Experimental design: The details of experimental animals (48 adult female, Wistar albino rats) including various groups, doses, route of exposure and duration of treatment made are presented in Table 1. On arrival at the laboratory all rats were

Table 1. Distribution of experimental rats to different treatments

Groups	Body weight (g)	Treatments	Dose	Route of administration
1	139.16 [a]±3.51	Drinking water	*Ad lib*	Oral
2	141.66 [a]±3.57	Drinking water+normal saline	*Ad lib*	OralIntra peritoneal
3	125.16±3.11	Vehicle		Intra peritoneal
4	130.00±3.41	Pineal proteins	100 µg/kg BW	Intra peritoneal
5	135.00±5.92	Melatonin	10 mg/kg BW	Intra peritoneal
6	123.33±1.05	NaF +Normal saline	150 ppm F	Oral with drinking water, Intra peritoneal
7	142.17 [a]±5.66	NaF +Pineal proteins	150 ppm F100 µg/kg BW	Oral with drinking water, Intra peritoneal
8	137.50 [a]±4.79	NaF +Melatonin	150 ppm F10 mg/kg BW	Oral with drinking water, Intra peritoneal

Values (n,6; means±SE) in the same column bearing no superscript ([a]) common vary significantly (P<0.05).

Present address: [1]Scientist C (vijaykbharti@rediffmail.com), Nutrition and Toxicology Laboratory, DIHAR, DRDO, Ministry of Defence, C/o- 56 APO, Leh 194101, India.

[2]Ex-Principal Scientist (srivarsvet@yahoo.co.in), Neurophysiology Laboratory, Division of Physiology and Climatology.

examined for any abnormality or overt signs of ill health. Rats were housed in polypropylene cages in a light/dark (LD) cycle of 12 h in a pathogen-free, temperature and humidity-controlled environment (set at 21±2°C and relative humidity at 50±10%, respectively). After a week long acclimatization period, they were weighed and randomly assigned to 6 groups so as to give approximately equal initial group mean body weights (Bharti and Srivastava 2009a,b). The animals were checked daily for the health and husbandry conditions.

Dose of F was based on previous published reports and our experience to induce oxidative stress in experimental rats (Bharti and Srivastava 2009a,b). Fluoride level in drinking water was calculated and thereafter required concentration of F was made by addition of sodium fluoride daily for 28 days. Appropriate dosages of MEL and PP were optimized from experience in our previous work and thereafter-required amount was calculated based on body weight before administration at exactly 17:00–17:30 h daily for 28 days (Bharti and Srivastava 2009a,b).

Sample collection: The blood samples were collected from cardiac puncture under ether anesthesia at the end of experiment (28 day). Immediately, lymphocytes were separated from the blood using for genomic DNA isolation.

DNA fragmentation assay: It was performed in genomic DNA to evaluate apoptotic DNA by using agarose gel electrophoresis. Genomic DNA extraction was done from isolated lymphocytic cell suspension as per method described by Hermann *et al*. (1994) for isolation of apoptotic DNA. The concentration and purity of the isolated DNA were determined by UV/VIS Spectrophotometer. Thereafter DNA ladder pattern was assessed by agarose gel electrophoresis in a submarine horizontal electrophoresis unit. In brief, a 1.4% agarose gel was prepared for electrophoresis at 40 volts for 3 h and progress of the mobility was monitored by the migration of dye. The DNA migration and ladder pattern were examined by UV transillumination technique and the picture was documented by photography.

The oxidative DNA damage is emerging as a biomarker in studies assessing the health risks of occupational and environmental exposures to chemical that are capable of inducing oxidative stress (Toraason *et al.* 1999). Since, DNA is subject to continuous oxidative damage and leads to variety of DNA lesions due to free radicals exposure (Breen and Murphy 1995). We examined ladder pattern of DNA fragmentation by agarose gel electrophoresis to evaluate apoptotic DNA damage due to F-induced oxidative stress (Fig.1). Characteristic inter-nucleosomal double-stranded breaks, producing DNA fragments in multiples of 180bp-200bp can be visualized as characteristic DNA ladder by agarose gel electrophoresis following total DNA extraction. However, we did not observe DNA ladder pattern of apoptotic DNA in control (0 day and 28 day), vehicle, and PP, MEL, F + PP, and F + MEL groups. Surprisingly, agarose gel electrophoresis applied in present study did not resolve small characteristic DNA fragments and show ladder pattern in fluoride treated animals (Fig. 1).

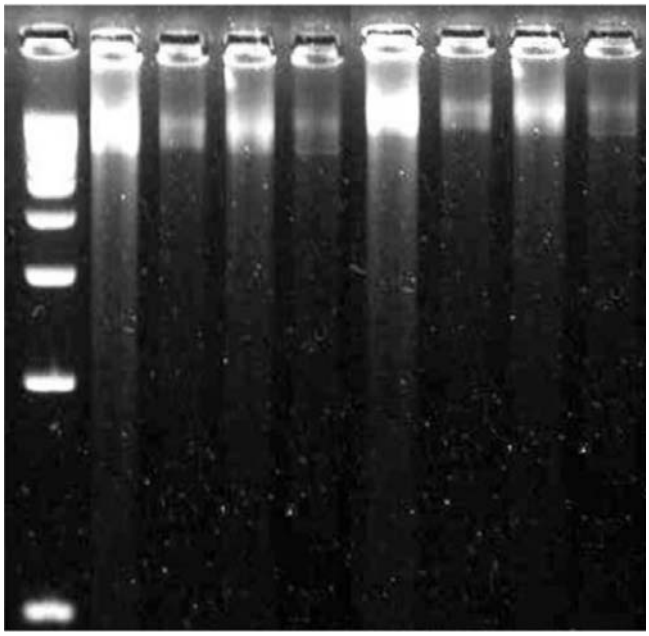

Fig. 1. DNA fragmentation assay (DNA ladder pattern) by agars gel electrophoresis. M, marker; C-F, Fluoride Control; F+PP, Fluoride +Pineal proteins; F+MEL, Fluoride + Melatonin; C-PP, pineal proteins control; C-MEL, melatonin control; C-Veh, vehicle control; C-28D, control 28 days; C–0D, control 0 day.

Our findings indicate that vehicle, pineal proteins, melatonin, F alone in general have no adverse effect on DNA fragmentation. Although, apoptosis is a normal phenomenon in the body, but in oxidative stress apoptotic cells number increases as compared to that in control. Oxidative stress DNA damage, apoptosis and modification of membrane lipids can be induced in cells by excess fluoride (Bharti 2008). Reactions between cellular components and free radicals lead to DNA damage, mitochondrial malfunction, cell membrane damage and eventually cell death (apoptosis) (Chlubek *et al.* 2003). Therefore, one of the main features of the apoptotic process is the characteristic breakdown of DNA. Apoptotic DNA degradation proceeds *via* characteristic inter-nucleosomal double-stranded breaks, producing DNA fragments in multiples of 180–200 bp (Satosh *et al.* 2005); whereas, in present study NaF did not cause ladder pattern of DNA fragment on agarose gel electrophoresis. This may be interpreted as insufficient insult to show nucleosomal breakage of DNA due to F-induced oxidative stress.

Agarose gel electrophoresis, applied in present study did not resolve small characteristic DNA fragments. Numerous studies now indicate that classical oligonucleosomal DNA fragmentation does not occur in apoptotic cell death. This might be due to absence of small DNA fragments without induction of internucleosomal DNA fragmentation, possibly due to weak activation of caspases-3, –8, and –9. However,

sodium fluoride may cause large, as well as small DNA fragments (internucleosomal DNA fragments) (Bharti 2008).

SUMMARY

Fluoride-induced characteristic inter-nucleosomal double-stranded DNA breaks in rat's lymphocytes was studied to find out protecting effect of melatonin (MEL) and buffalo pineal proteins (PP) on DNA fragmentation *in vivo*. For this, we tested MEL (@) 10 mg/kg BW, i.p.) and PP (@100 µg/ Kg BW, i.p.) alone or with F (@150 ppm) in adult female Wistar rats (123–143 g BW) for 28 days. Following anaesthesia, blood was collected for lymphocytes separation and genomic DNA isolation to examined ladder pattern of DNA fragmentation by agarose gel electrophoresis. Interestingly, we did not observe any DNA ladder pattern of apoptotic DNA in control (0 day and 28 day), vehicle, and PP, MEL, F, F+PP, and F+MEL groups. These findings indicate that buffalo pineal proteins and melatonin in general have no adverse effect on DNA fragmentation and fluoride did not cause ladder pattern of DNA fragment on agarose gel electrophoresis.

ACKNOWLEDGEMENTS

Research grant in the shape of Institute's Senior Research Fellowship to the first author and facilities provided by Indian Veterinary Research Institute for conducting this study is duly acknowledged. We also acknowledge the tireless efforts of our lab and animal shed assistants.

REFERENCES

Anuradha C D, Kanno S and Hirano S. 2001. Oxidative damage to mitochondria is a preliminary step to caspase-3 activation in fluoride-induced apoptosis in HL60 cells. *Free Radical Biology and Medicine* **31**: 367–73.

Bharti V K. 2008. Studies on buffalo (*Bubalus bubalis*) pineal proteins on fluoride- induced oxidative stress and apoptosis in rats'. Ph.D. thesis. Indian Veterinary Research Institute, Izatnagar, India,.

Bharti V K and Srivastava R S. 2009a. Fluoride-induced oxidative stress in rat's brain and its amelioration by buffalo (*Bubalus bubalis*) pineal proteins and melatonin. *Biological Trace Element Research* **130**: 131–40.

Bharti V K and Srivastava R S. 2009b. Pineal proteins up-regulate specific antioxidant defense systems in the brain. *Oxidative Medicine and Cellular Longevity* **2**: 88–82.

Breen A P and Murphy J A. 1995. Reactions of oxyl radicals with DNA. *Free Radical Biology and Medicine* **18**: 1033–77.

Chawla S L, Yadav R, Shah D and Rao M V. 2008. Protective action of melatonin against fluoride-induced hepatotoxicity in adult female mice. *Fluoride* **41**: 44–51.

Chinoy N J and Patel T N. 2000. The influence of fluoride and/or aluminium on free radical toxicity in the brain of female mice and beneficial effects of some anti-dotes. *Fluoride* **33**: S8

Chlubek D, Grucka H E, Polaniak R, Stawiarska P B and Duliban H. 2003. Activity of pancreatic antioxidant enzymes and malondialdehyde concentrations in rats with hyperglycemia caused by fluoride intoxication. *Journal of Trace Element Medical Biology* **17**: 57–60.

Ha J, Chu Q, Wang A, Xia T and Yang K. 2004. Effects on DNA damage and apoptosis and p53 protein expression induced by fluoride in human embryo hepatocytes. *Wei Sheng Yan Jiu* **33**: 22–26.

Haddad E E and Mashaly M M. 1992. Augmentation of natural cell-mediated cytotoxic activity by supernatant from in vitro mitogen-stimulated, *in vivo* hormone-treated lymphocytes in immature male (K strain) chickens. *Immunological Investigations* **21**: 365–75.

He L F and Chen J G. 2006. DNA damage, apoptosis and cell cycles changes induced by fluoride in rat oral mucosal cells and hepatocytes. *World Journal of Gastroenterology* **12**: 1144–48.

Hermann M, Lorenz M H, Voll R, Grunke M, Woith W and Kalden J R. 1994. A rapid and simple method for the isolation of apoptotic DNA fragments. *Nucleic Acid Research* **22**: 5506–07.

Jing L, Shao Z and Ren L. 1999. Hepatocyte apoptosis in fluorosis rats. *Chinese Journal of Epidemiology* **18**: 10–15.

Kanduc D, Mittelman A, Serpico R, Sinigaglia E, Sinha, A A, Natale C, Santacroce R, Di Corcia M G, Lucchese A and Dini L. 2002. Cell death: Apoptosis versus necrosis (review). *International Journal of Oncology* **21**: 165–70.

Serbecic N and Beutelspacher S C. 2005. Antioxidative vitamins prevent lipid- peroxidation and apoptosis in corneal endothelial cells. *Cell Tissue Research* **320**: 465–75.

Toraason M, Clark J, Dankovic D, Mathias P, Skaggs S, Walker C and Werren D. 1999. Oxidative stress and DNA damage in Fischer rats following acute exposure to trichloroethylene or perchloroethylene. *Toxicology* **138**: 43–53.