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IN VITRO GENOTOXIC EFFECT OF ANAESTHETIC HALOTHANE ON RABBIT LYMPHOCYTES AND THE PROTECTIVE ROLE OF VITAMIN A SUPPLEMENTATION

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ABSTRACT

The aim of the present study is to estimate the *in vitro* genotoxic potential of the volatile-anaesthetic halothane (Narcotan®) as well as the possible protective effect of vitamin A, as an antioxidant. To achieve this task, the alkaline single cell gel electrophoresis (comet assay) was applied as the extent of DNA fragmentation in rabbit lymphocytes (25 rabbits) that were exposed *in vitro* to halothane at concentrations of 0.1 or 1.0 mM for 10 and 30 minutes, respectively. The obtained results elucidated that the anaesthetic halothane induced significant increase in DNA damage that is demonstrable *via* comet DNA concentration and its tail length. The recorded elevation of DNA damage was clearly correlated with halothane concentration as well as the period of exposure. To mend the deleterious effect of halothane on DNA, 25 rabbits received vitamin A orally (8000 IU/kg body weight) for 15 days prior to *in vitro* halothane exposure. Vitamin A administration induced significant repairing effect in lymphocytes-damaged DNA resulted from halothane exposure.

The genotoxic effect of halothane may be attributed to its property as an oxidative agent capable of accumulating oxygen free radicals inside the cell, producing DNA damage. Thereby, the anti-oxidative property of vitamin A may counteract the hazardous effect of halothane.

The present investigation clarified the great need of vitamin A as a pre-anaesthetic medication for patients undergoing surgical operations and as a prophylactic therapy for the surgical operation room personnel to antagonize the possible genotoxic effect of the anaesthetic halothane

Keywords: Comet assay, Genotoxicity, Halothane, Inhaled anaesthetics, Vitamin A.

INTRODUCTION

In the current clinical practice many inhaled anaesthetics are in use, including halogenated aliphatic compounds such as isoflurane, sevoflurane, enflurane, desflurane and others. The oldest in this group is halothane. It was introduced as an anaesthetic in 1960 (Brown, 1960). Halothane (2-Bromo-2-chloro-1,1,1-trifluoroethane) is an inhalational vapour drug used for general anaesthesia in surgical operations (Orth *et al*., 2006). It is commercially known as Narcotan®.

The potential health effects of exposure to volatile anaesthetics by patients undergoing surgical operations and operation room personnel, still remain an open question. Health hazards connected with an occupational exposure to inhaled anaesthetics is not sufficiently documented, but some reports on nephrocytotoxicity, hepatotoxicity and carcinogenicity have been published (Gurguis *et al*., 1990; Lucchini *et al*, 1996). A chronic exposure to halogenated anaesthetics can also affect human reproduction. It was found that anesthetics can increase the frequency of spontaneous abortion among operating room females or affect human reproduction in other ways (Boivin, 1997; Rosenfeld and Loose-Mitchell, 1998). In addition, some metabolites of anaesthetic gases are associated with toxic effects in certain tissues, e.g. in the liver, kidney or brain (Walker, 1996).

Known detrimental effects of volatile anaesthetics are their genotoxicity and cytotoxicity. *In vivo and in vitro* exposure of mammalian cells to various anaesthetics, induced an increased number of chromosomal aberrations, increase in micronuclei formation and a high rate of sister chromatid exchanges (Karelova *et al*., 1992; Robbiano *et al*., 1998; Rozgaj *et al*., 1999 & 2001).

Halothane as one of the volatile anaesthetics was detected to have genotoxic and cytotoxic effects and it was

shown to increase DNA single strand breaks and induce DNA fragmentation (Jaloszyński *et al.*, 1999 a&b; Szyfter *et al.*, 2004; Karpiński *et al.*, 2005).

The genotoxic effect can be determined by the alkaline comet assay, which is also known as the single-cell gel electrophoresis (Singh *et al.,* 1988). It has been increasingly used during the last 10 years in diverse areas of genotoxic studies (Olive *et al.*, 1998). As a rapid, simple, sensitive and versatile technique, it has been found particularly useful for preliminary estimation of the genotoxic potential of drugs (Möller *et al.*, 2000). The technique is capable of detecting a wide variety of DNA damage and lesions such as DNA single strand breaks, double strand breaks, base damage as well as DNA repair (Tice, 2000). In this technique the migration of DNA in an electric field, supposed to be proportional to strand breakage, is a quantitative measure of genotoxicity (Duez *et al.*, 2003; Grandi *et al.*, 2006).

Vitamin A has long been recognized to be essential for healthy immune system and strong resistance to infection. This vitamin is necessary to the development of T lymphocytes, which are the front line troops fighting cancer, viruses and many other diseases including AIDS (Somner, 1995; Atkins, 1999). Many studies demonstrated that vitamin A provides protection against certain specific cancers, particularly those affecting lung, skin, prostate and other epithelial surfaces (Santillo and Lowe, 2006)

Teppema *et al.* (2006) examined the influence of vitamin A, vitamin E(alpha-tocopherol) and ascorbic acid on the acute hypoxic ventillatory response with and without halothane and revealed protective effect of the antioxidants for the genotoxicity of halothane. On the same line, Decoudu *et al.* (1992) indicated that vitamin A status of animals can influence the genotoxic activity induced by mycotoxins *in vitro* and *in vivo*. Gradelet *et al.* (1998) reported that carotenoids exert their protective effect throughout the deviation of metabolic pathway of cytotoxic drugs and mycotoxins toward detoxification. Moreover, Denli *et al.* (2003) indicated that vitamin A supplementation in the diet (15000 IU/kg) provided protection against the toxic effect of aflatoxin B_1 (AFB_1) in the livers and kidneys of Japanese quails.

The *in vitro* carcinogenetic study on hamster tracheal epithelium, indicated that both vitamin A and beta carotene caused a decrease in benzo (*a*) pyrene (B[*a*]P)- induced unscheduled DNA synthesis and DNA adduct levels by enhancing DNA-repair activities (Wolterbeek, 1995). The effect of vitamin A on B[*a*]P appeared to depend on the dose of B[*a*]P vis-à-vis the concentration of vitamin A. Hoehler and Marquardt (1996) indicated the effectiveness of vitamin A in reducing DNA damage that were induced by ochratoxin A and T-2 toxin in chicks. Moreover, Jaruga *et al.* (2006) illustrated that Vitamin A supplementation resulted in a significant decrease in the levels of all modified DNA bases in HIV infected patients (AIDS) when compared to the patients who received placebo.

The present investigation aims to cast light on the competence of comet assay to detect the potential genotoxicity as the extent of DNA damage in the cultured lymphocytes of rabbits that were subjected to different doses of the anaethetic halothane. Moreover, to explore the role of vitamin A as an antioxidant to recover the resultant damage.

MATERIAL AND METHODS

Chemicals

All chemicals were of analytical grade: normal melting point agarose and Triton X-100 (Sigma); dimethyl sulfoxide (DMSO) and Tris (Bio-Rad); low melting agarose (IITD, Poland); RPMI 1640 medium without L-glutamine (GIBCO); ficoll (Leciva Czech Republic); halothane [Narcotan® (2-Bromo-2-chloro-1,1,1-trifluoroethane) Medico-lab, Czech Republic]; vitamin A [retinyl-acetate; (Brolap R-M-H 3500, Agway, Syracuse, New York, USA)].

Animals

The experimental animals used in the present study were healthy adult male rabbits (New Zealand strain). They were purchased from the Farm of the Egyptian Organization for Vaccine and Biological Preparations at Helwan, Cairo. The used rabbits weighed 2-3 kg each. They were kept under hygienic/ aseptic conditions, housed in clean cages, bedded with wood shavings and were accommodated to the laboratory conditions for one week prior to the beginning of the experiment.

Experimental design:

Fifty adult male rabbits (2-3kg) were allotted at random into two main equal groups, each of 25 rabbits. Animals of the first main group were used to determine the possible genotoxic effect of halothane at different doses and time on blood lymphocytes. Blood samples (each 3 ml) from 25 rabbits were collected from ear veins, in heparinized sterile vacutainers ; the obtained samples were subdivided into five equal groups (5 samples/group). Lymphocytes were isolated by the standard method, including centrifugation of heparinized blood over ficoll at 3200 rpm for 20 min, then the lymphocytes were aspirated from the plasma-ficoll interphase and the cells were suspended and cultured in RPMI 1640 culture medium (Brulles and Wells, 1977). Afterwards, the first and second groups of lymphocyte cultures were exposed to halothane (dissolved in 1% DMSO on ice) at concentrations of 0.1 mM for 10 and 30 minutes, respectively. Meanwhile, the third and fourth groups of lymphocyte cultures were exposed to halothane at 1 mM for 10 and 30 minutes, correspondingly. However, the lymphocytes that were isolated from the fifth group of samples were treated only with 1% DMSO and kept as a negative control.

Animals of the second main group (25 rabbits) orally received vitamin A (8000 IU/kg body weight) by gastric

intubation for 15 days prior to blood sampling. Subsequently, blood samples were collected and subdivided into five equal groups (5 samples/group). Lymphocytes were isolated, cultured and halothane was added at the same conditions and concentraitons as described in the first main group.

After halothane exposure, cells were settled down by 10-min spinning (700 rpm) at 4°C and washed twice with fresh culture medium. The lymphocytes were then resuspended in RPMI 1640 medium and subjected to single cell gel electrophoresis (comet assay).

In the present work, the applied concentrations of halothane have been previously recommended by the published regimen of Szyfter *et al.* (2004) and Karpiński *et al.* (2005).

Alkaline comet assay

The lymphocytes were subjected to comet assay after halothane exposure to estimate DNA damage of halothane-treated cells, as well as the repair effect of vitamin A. The alkaline comet assay was conducted as described by Jaloszyñski *et al.* (1999). The lymphocyte suspension (30 µl) was mixed with 70 µl of 1% low melting point agarose solution in the RPMI 1640 medium at 37°C. The mixture was pipetted onto fully frosted microscope slides previously pre-coated with a layer of 1% normal agarose. The slides were immersed in lysing buffer solution (2.5 M NaCl, 0.1 M Na_2 EDTA, 10 mM Tris, freshly added 1% Triton X-100, pH 10 with NaOH) for 1 hour at 4°C to remove proteins. The slides were then placed in a horizontal electrophoretic tank filled with freshly prepared cold alkaline buffer (4°C, 3 M NaOH, 1 mM Na_2EDTA, pH 13.0) for 40 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was carried out in the former solution for 30 min (at 300 mA, 0.65 v/cm) using a compact power supply. After electrophoresis, slides were removed from the tank and immersed horizontally in neutralizing buffer (0.4 M Tris, pH 7.5) and stained with ethidium bromide (20 µg/ml distilled H_2O) for 1 hour. After staining, the slides were rinsed in distilled water and kept in a closed box at 4°C. The stained slides were observed under a Carl Zeiss fluorescence microscope at 40x magnification, using filter 15 (BP543/120 FT580, LP590). For each sample, 50 randomly selected cells, respectively, were examined and photographed using a 3-CCD camera and stored in separate files. The acquired images were pre-processed to remove artifacts. The cells with small heads and large fan-like tails were excluded under the principle that they represent apoptotic cells. The images were analyzed for measurement of DNA damage with Scion Image Software version 3b (National Institute of Health, USA). For each cell, the length of DNA migration (comet tail length) was measured in micrometers from the centre of nucleus to the end of the tail (Plate 1 C). The percentage of damaged-DNA concentration in the comet tail was determined by measuring the total intensity of ethidium bromide fluorescence in the cells, which was taken as 100 % and determining what percentage of this total intensity corresponds to the intensity measured only in the tail.

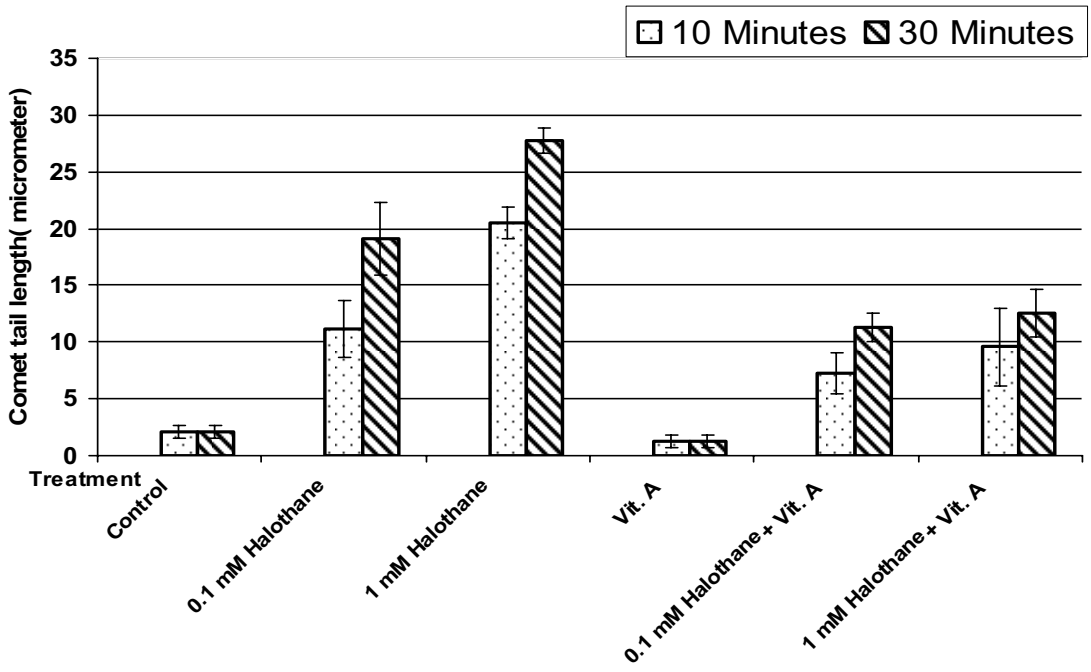
Statistical analysis:

The results of the different treated groups were recorded as mean ± standard error for each group and subsequently statistically analyzed using Student's *t* test. Results were considered significant when $p < 0.05$ (Snedcor and Cochran, 1989).

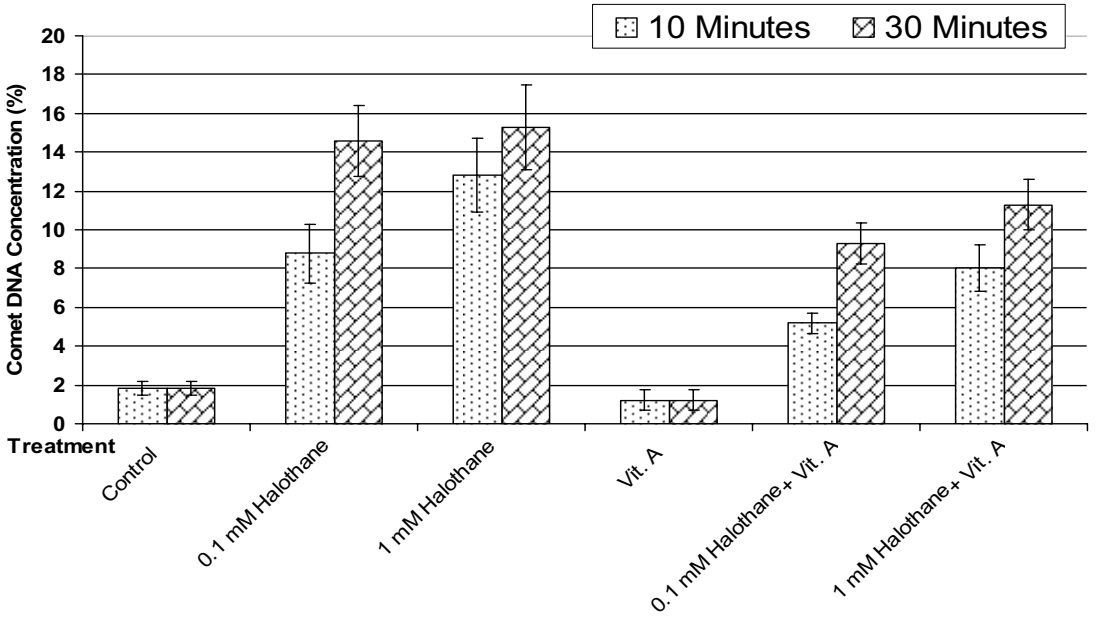
RESULTS

As shown in Graph 1, the results obtained from the present study displayed a significant damage of lympocytes DNA that was expressed by the elevation of DNA comet tail length (CTL) due to halothane exposure. Incubation of lymphocytes with 0.1mM halothane for 10 or 30 minutes induced significant ($P<0.05$) increase of comet tail length with mean values of 11.20±2.51 and 19.07±3.2 micrometers, respectively (Plate II A & C). The obtained values were significantly ($P <0.05$) higher than that of the non-treated control (2.08±0.57) (Graph 1 and Plate I A). On the similar ground, lymphocyte treatment with halothane at 1.0 mM concentration for 10 or 30 minutes resulted in a significant ($P<0.05$) increase of tail length with 20.50±1.40 and 27.8±1.1 micrometers, respectively (Plate II B&D). The levels of DNA degradation (comet tail length) were dose- and time-dependent. However, vitamin A administration prior to 0.1 mM halothane exposure for 10 or 30 minutes, produced tail length with 7.30±1.80 and 11.3±1.3 micrometers, respectively which were significantly ($P<0.05$) less than the correspondence non-vitamin A-administered groups (11.20±2.51 and 19.07±3.2). On the same line, vitamin A administration prior to 1 mM halothane treatment for 10 or 30 minutes produced comet tail length with 9.57±3.40 and 12.57±2.1 micrometers, respectively (Plate II E&F). These values were significantly ($P<0.05$) lower than that recorded for the similar non-vitamin A-administered groups (20.50±1.40 & 27.8±1.1). Meanwhile, cultured lymphocyte of vitamin A- treated group which were not exposed to halothane, recorded DNA tail length of 1.30±0.57 micrometers.

Graph 2 elucidated a significant ($P<0.05$) elevation in the percentage of comet DNA concentration due to halothane exposure in a dose- and time-dependent manner. Incubation of lymphocytes with 0.1 mM halothane for 10 or 30 minutes induced significant increase of comet DNA concentration, with values of 8.77±1.5 % and 14.58±1.8 %, respectively. These recorded values were significantly higher than that of the control group (1.84±0.54). On the same line, lymphocyte treatments with 1 mM halothane for 10 or 30 minutes exhibited a significant ($P<0.05$) increase in the percentage of comet DNA concentration with values of 12.84±1.9 and 15.3±2.2, respectively. However, vitamin A administration prior to 0.1 mM halothane exposure for 10 or 30 minutes created a reduction in comet tail DNA concentration with recorded values of 5.2±0.52 % and



Graph (1): Genotoxic potential of *in vitro* halothane exposure on rabbit lymphocytes and the effect of vitamin A supplementation. A measure of genotoxicity was an induction of DNA migration in comet assay (mean comet tail length ± SE in µm).



Graph (2): Effect of *in vitro* halothane exposure and vitamin A supplementation on the percentage of comet DNA concentration (migrated damaged-DNA) in rabbit lymphocytes, as established by comet assay (mean comet DNA concentration ± SE in %).

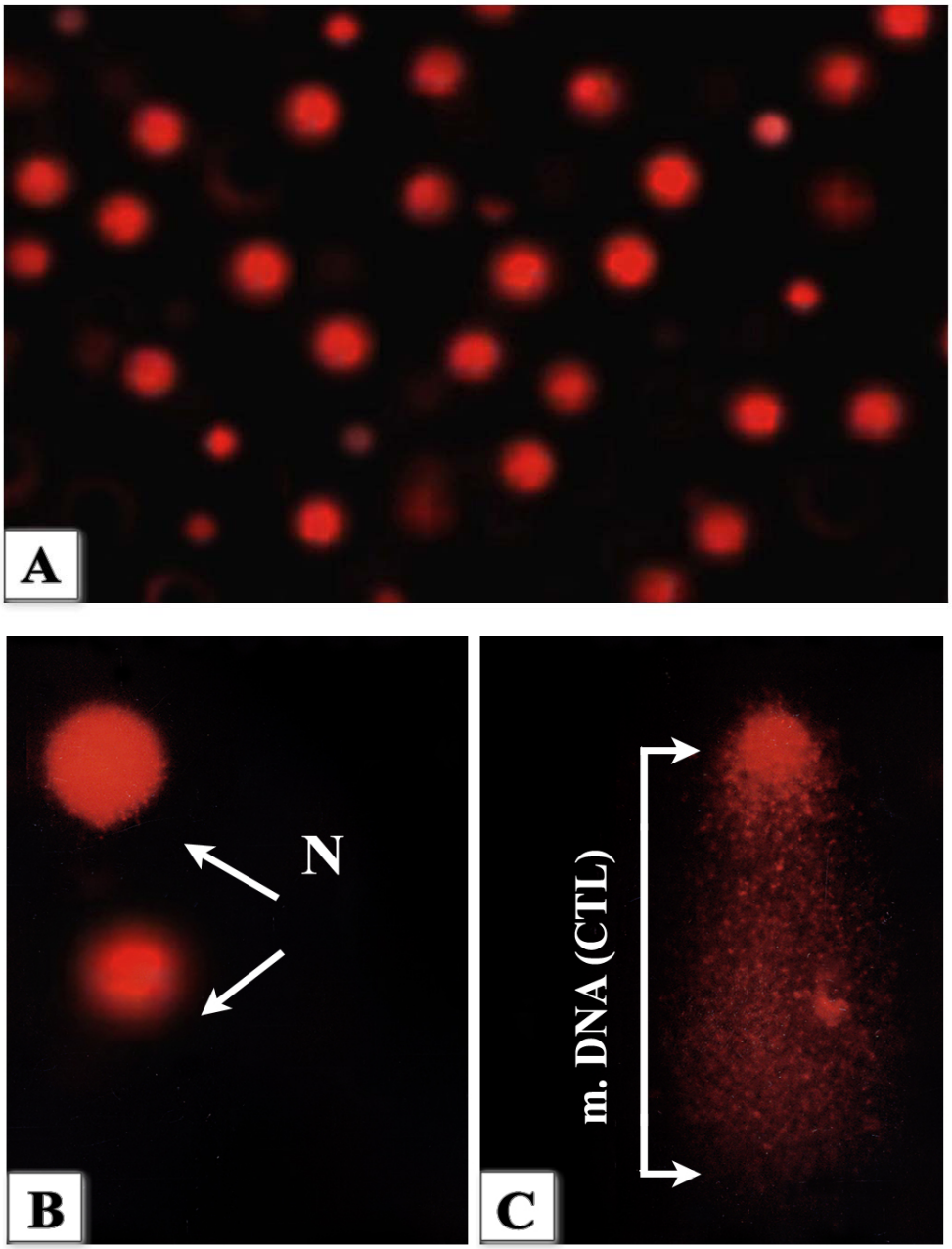


Plate I

(A) **Photomicrograph of rabbit lymphocyte nuclei of the non-treated control group, as established by single cell gel electrophoresis (comet assay).**

(B) **An image demonstrating lymphocyte nuclei with normal (N) intacted DNA.**

(C) **An image demonstrating a nucleus with migrated damaged-DNA (m.DNA) due to halothane incubation (1 mM for 30 min) with rabbit lymphocytes, as shown by comet assay. The length of DNA migration (comet tail length, CTL) was measured in μm from the center of nucleus to the end of the tail.**

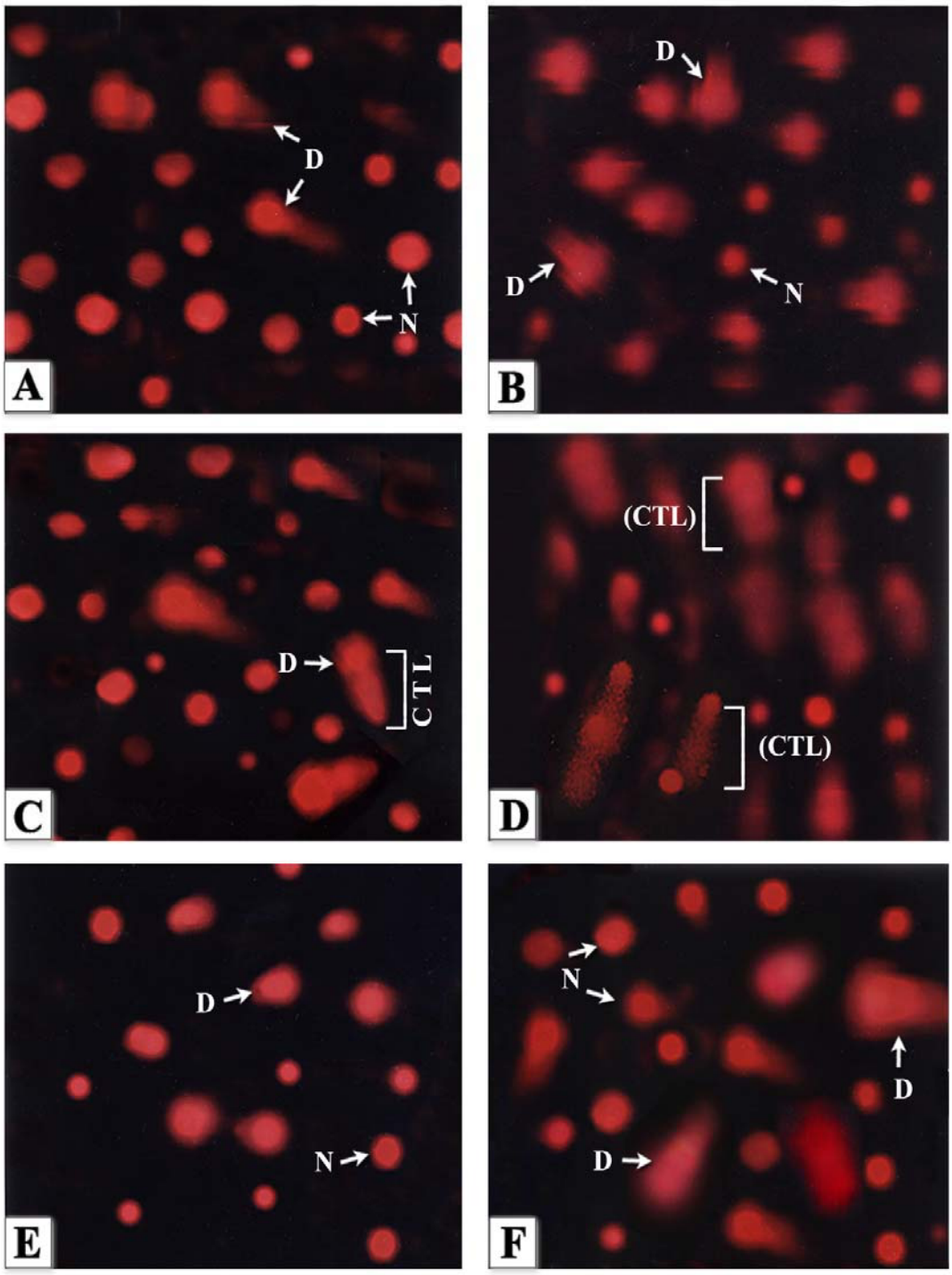


Plate II

Photomicrographs of rabbit lymphocyte nuclei exhibited DNA damage (comet tail) to different extents after to halothane exposure at various conditions, as established by comet assay: (A) After 10 min exposure to 0.1 mM halothane; (B) After 10 min exposure to 1.0 mM halothane; (C) After 30 min exposure to 0.1 mM halothane; (D) After 30 min exposure to 1.0 mM halothane; (E) After 30 min exposure to 0.1 mM halothane with vit. A administration; (F) After 30 min exposure to 1.0 mM halothane with vit. A administration.

(D = DNA-damaged nuclei; N = normal nuclei; CTL = comet tail length of migrated DNA).

9.3±1.05 %, respectively which were significantly (P<0.05) less than the correspondence non-vitamin A-administered groups. Moreover, vitamin A administration prior to 1 mM halothane exposure for 10 and 30 minutes exhibited tail DNA concentration with 8.05 ± 1.2% and 11.3 ± 1.3%, respectively. The reduction was significantly (P<0.05) lower than the similar non-vitamin A-administered group. Meanwhile, the supplemented-vitamin A group without exposure to halothane recorded 1.23± 0.54% comet DNA concentration.

DISCUSSION

From the obtained results, it was clear that halothane exposure induced a significant elevation in the DNA damage by increasing the comet tail length and the percentage of its DNA concentration compared to the control group. The results displayed time- and dose-dependent trend (Graphs 1 & 2).

The obtained results may explain the findings of Bandoh and Fujita (1976). They reported that halothane caused suppression of lymphocyte function. It is possible that halothane exposure induces changes in DNA construction, due to increased DNase I activity. The results of Reitz *et al.* (1993) suggested a mechanism by which chromosomal defects due to halothane treatment may be attributed to disturbances of DNA metabolism in the cells.

The cytotoxic and antiproliferative effects of halothane on human carcinoma cells (MIAPaCa-2) were investigated *via* radioactive precursors incorporation assay (Kvolik *et al.*, 2005). They found that the growth suppression in cells exposed to halothane was enhanced. Their results recorded reduction in DNA synthesis (52.4%), RNA synthesis (39.2%), and protein synthesis (19.2%). Moreover, the present results fit neatly with the data recorded by Topouzova-Hristova *et al.* (2006). They affirmed that the comet assay (single cell gel electrophoresis) clearly showed that, halothane applied *in vitro* at 3.0 mM concentration, caused DNA and cell damage. Treated cells exhibited nuclear fragmentation and budding, early after treatment; these events gradually increased during the next few days with apoptosis-like changes. They demonstrated that the majority of the affected cells did not recover and displayed signs of cell death. On the gene level, Pan *et al.* (2006) recorded that halothane produced significant changes in a few metabolic genes.

The elucidated DNA damage due to halothane exposure may be attributed to the accumulation of free oxygen radicals inside the cells after the oxidative phosphorylation process has been terminated. The accumulation of these radicals inside the cell may cause the cell to be apoptotic and increase the possibility of DNA damage (Jaloszyński *et al.*, 1999 a&b). Moreover, the genotoxic effect of halothane was shown to elevate DNA single strand breaks in the cells. It may open potassium channels in the plasma membrane and cause lipid peroxidation (Kharasch *et al.*, 2000), therefore halothane may cause an increase in the peroxides and other free oxygen species of radicals that can induce DNA damage and cell death (Karabiyik *et al.*, 2001).

In the present study, the recorded deleterious effects of halothane on the DNA of cultured lymphocytes were obtunded due to vitamin A administration. The obtained results provoked that vitamin A induced significant reduction in the DNA comet tail length and its concentration in all halothane-treated lymphocytes (Graphs 1&2).

Our results are in concordance with the findings of Jaruga *et al.* (2006). They found that vitamin A administration to HIV-infected patients induced a significant decrease in all modified DNA bases. Moreover, Wolterbeek *et al.* (1995) stated that vitamin A caused a decrease in B[*a*]P-DNA adduct levels by enhancing DNA-repair activities. The latter authors suggested that the formation of B[*a*]P-DNA adducts is considered to be an early step in respiratory tract carcinogenesis and that the enhancement of DNA-repair activities by vitamin A with the subsequent removal of DNA adducts, may be one of the mechanisms involved in vitamin A-mediated protection against cancer.

***In vitro* and *in vivo* studies have shown that high carotenoid supplementation is associated with decreased DNA damage, while the low carotenoid intake correlated with high DNA damage, supporting a preventive role of carotenoids in bladder cancer (Matthew *et al.*, 2004).**

In the field of studying the mode of action of vitamin A, Huang *et al.* (2006) referred the protective potential of vitamin A to its antioxidative effect. In their study, TUNEL assay and DNA electrophoresis indicated that vitamin A markedly blocked DNA fragmentation and apoptosis of microglia cells. This effect has been achieved via the elevation of antioxidative enzymes, catalase (CAT) and superoxide dimutase (SOD), as well as the partial inhibition of caspase-3 activation. The authors indicated that vitamin A effectively abolished both cleavage of poly-adenosine diphosphate ribose (ADP-ribose) polymerase (PARP) and degradation of inhibitor of caspase-activated DNase (ICAD). In addition, the expression of PARP for repair of impaired DNA was significantly increased by vitamin A treatment. Taken together, these data suggested that protective effects of vitamin A against oxidative DNA damage of microglia cells is exerted by the increased expression of DNA repair enzyme (PARP) and antioxidant enzyme activities. Moreover, vitamin A has been found as one of the antioxidants playing a role in scavenging free oxygen radicals and thus can recover the cell damage in case of apoptosis (Yan *et al.*, 2005). Very recent investigations revealed a protective strategy of vitamin A in minimizing the oxidative damage induced by anesthetics (Zhao *et al.*, 2006).

The present study elucidated that comet assay is a reliable method to estimate the genotoxic effect of halothane on the DNA of cultured lymphocytes. Giving the evidence,

the present recorded data highly light the notion that genotoxicity could preclude the efficient use of halothane in anaesthesia. Nevertheless, the current study advocates the need of preanaesthetic uses of vitamin A as a potent antioxidant for patients undergoing surgical operations as well as for the operation room personnel to antagonize the possible genotoxic effect of anaesthetic-halothane.

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