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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 target of the present study is to estimate the *in vitro* genotoxic potential of the volatile-anaesthetic halothane (Narcotan®) as well as the 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 that were exposed *in vitro* to halothane at concentrations of 0.1 or 1.0 mM for 10 and 30 min, respectively. The obtained results elucidated that anaesthetic halothane induced a significant increase in DNA damage that shown by 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 were orally received vitamin A (8000 IU $kg^{-1}$ body weight) for 15 days prior to *in vitro* halothane exposure. Vitamin A administration induced a significant repairing effect in lymphocytes-damaged DNA due to halothane exposure, comparing with the non-administrated group. The genotoxic effect of halothane might be attributed to its property as an oxidative agent capable to accumulate oxygen free radicals inside the cell and producing DNA damage. Thereby, the antioxidant property of vitamin A might counteract the hazardous effect of anaesthetic halothane. It is concluded that there is a great need of vitamin A intake for patients undergoing surgical operations with halothane anaesthesia and as a prophylactic therapy for the operation room personnel to antagonize the possible genotoxic effect of halothane.

**Key words:** Rabbits, lymphocytes, DNA, comet assay, halothane, vitamin A

## INTRODUCTION

In the current clinical practice many volatile anaesthetics are in use, including halogenated aliphatic compounds such as isoflurane, sevoflurane, enflurane, desflurane and others. Halothane is an inhalational anaesthetic used for general anaesthesia in surgical operations (Orth *et al*., 2006).

The potential health effects of patients exposure to volatile anaesthetics during surgical operations and operation room personnel, still remain an open question. Health hazards connected with an occupational exposure to inhalation 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 female operating room personnel or affect human reproduction in other ways (Bovin, 1997; Rosenfeld and Loose-Mitchell, 1998). In addition, some metabolites of anaesthetic gases are associated with toxic effects in certain tissues, such as liver, kidney or brain (Walker, 1996).

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Known detrimental effects of volatile anaesthetics are genotoxicity and cytotoxicity. *In vivo* and *in vitro* exposure of mammalian cells to various anaesthetics induced an increased number of chromosomal aberrations, an 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 reported to have genotoxic and cytotoxic effects as it increase DNA single strand breaks and induce DNA fragmentation (Jaloszynski *et al*., 1999a, 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 *et al*., 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).

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 a 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 E, vitamin A and ascorbic acid on the acute hypoxic ventillatory response with and without halothane and revealed a protective effect of the antioxidants against the genotoxicity of halothane. On the same line, Decoudu *et al*. (1992) concluded that vitamin A status of animals can influence the genotoxic activity due to 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. On the same line, Denli *et al*. (2003) reported that vitamin A supplementation in the diet (15000 IU $kg^{-1}$) provided a protection against the toxic effect of aflatoxin $B_1$ ($AFB_1$) in liver and kidneys of Japanese quails.

The *in vitro* carcinogenetic study, on hamster tracheal epithelium, indicated that both vitamin A and beta carotene caused an enhancement of DNA-repairing activities (Wolterbeek, 1995). The effect of vitamin A on B [a]p appeared to depend on the dose of B[a]p versus the concentration of vitamin A. Hoehler and Marquardt (1996) proved the effectiveness of vitamin A in reducing DNA damage 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 the 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 two concentrations of anaesthetic halothane. Moreover, to explore the role of vitamin A as an antioxidant to repair the resultant damage.

## MATERIALS 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) weighing 2-3 kg each. They were purchased from the Farm of the Egyptian Organization for Vaccine and Biological Preparations at Helwan, Cairo, Egypt. They were kept under hygienic 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 were allotted at random into two main equal groups, each of 25 rabbits. Animals of the first group were used to determine the possible genotoxic effect of halothane on blood lymphocytes at two different concentrations and time of exposure. Blood samples (3 mL) were collected from ear veins, of all rabbits, in heparinized sterile vacutainers and then the obtained samples were subdivided into five equal groups (5 samples/each). Lymphocytes were isolated by the standard method, including centrifugation of heparinized blood over ficoll at 3200 rpm for 20 min, then lymphocytes were aspirated from the plasma-ficoll interphase and the cells were suspended 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) at concentrations of 0.1 mM for 10 and 30 min, respectively. The third and fourth groups of lymphocyte cultures were exposed to halothane at 1 mM for 10 and 30 min, respectively. Lymphocytes that 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) were orally received vitamin A (8000 IU $kg^{-1}$ body weight) by gastric incubation 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 concentrations as described in the first main group. After halothane exposure, the cells were settled down by 10 min spinning (700 rpm) at 4°C and washed twice with fresh culture medium. Lymphocytes were then resuspended in RPMI 1640 medium and subjected to single cell gel electrophoresis (comet assay).

In the present study, 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**

Lymphocytes were subjected to comet assay after halothane exposure to estimate DNA damage of halothane-treated cells, as well as the protective effect of vitamin A. The alkaline comet assay was conducted as described by Jaloszynski and Szyfter (1999). For each cell, the length of DNA migration (comet tail length) was measured in micrometers from the center of nucleus to the end of the tail. 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 correspond 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 then statistically analyzed using Student's t-test. Results were considered significant when $p<0.05$ (Snedcor and Cochran, 1989).

## RESULTS

As shown in Fig. 1, the obtained results displayed a significant elevation of DNA comet tail length due to halothane exposure. Incubation of lymphocytes with 0.1 mM halothane for 10 and 30 min induced significant ($p<0.05$) increase of comet tail length with mean values of 11.20±2.51 and 19.07±3.2 μm, respectively (Fig. 3A, C). The obtained values were significantly ($p<0.05$) higher than that of the non-treated control (2.08±0.57) (Fig. 1, 2A). Similarly, lymphocyte treatment with halothane at 1.0 mM concentration for 10 and 30 min resulted in a significant ($p<0.05$) increase of tail length with 20.50±1.40 and 27.8±1.1 μm, respectively (Fig. 3B and D). The levels of DNA degradation (comet tail length) were dose- and time-dependent. Vitamin A administration prior to 0.1 mM halothane exposure for 10 and 30 min, produced tail length of 7.30±1.80 and 11.3±1.3 μm, respectively which were significantly ($p<0.05$) less than the corresponding 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 and 30 min produced comet tail length of 9.57±3.40 and 12.57±2.1 μm, respectively (Fig. 3E, F). These values were significantly ($p<0.05$) lower than those recorded for the similar non-vitamin A-administered groups (20.08±0.57 and 27.8±1.1). Vitamin A lymphocyte cultured-group which did not exposed to halothane, recorded 1.30±0.57 μm of DNA tail length.

Figure 4 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 and 30 min 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) that was supplemented with vitamin A only. On the same line, lymphocyte treatments with 1 mM halothane for 10 and 30 min 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. Vitamin A administration prior to 0.1 mM halothane exposure for 10 and 30 min caused a reduction in comet tail DNA concentration with recorded values of 5.2±0.52 and 9.3±1.05%, respectively which were significantly ($p<0.05$) less than the corresponding non vitamin A-administered groups. Vitamin A administration prior to 1 mM halothane exposure for 10 and 30 min 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. The supplemented-vitamin A group without exposure to halothane recorded 1.23±0.54% comet DNA concentration.

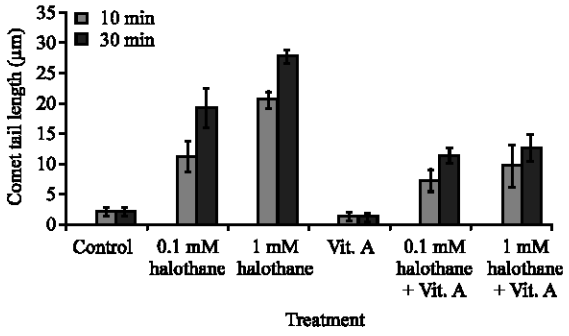


Fig. 1: Genotoxic potential of *in vitro* halothane exposure on rabbit lymphocytes and the effect of vitamin A supplementation. A measure of genotoxicity was an induction of DNA migration in comet assay (mean comet tail length±SE in μm)

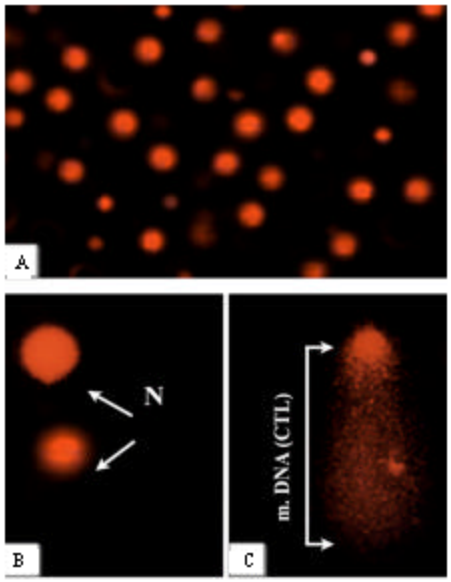


Fig. 2: (A) Lymphocyte nuclei from the non-treated control group, as established by single cell gel electrophoresis (comet assay) (B) Lymphocyte nuclei with normal (N) intact DNA and (C) Nucleus with migrated damaged-DNA (m.DNA) due to halothane incubation (1 mM for 30 min) with rabbit lymphocytes. The length of DNA migration (comet tail length, CTL)

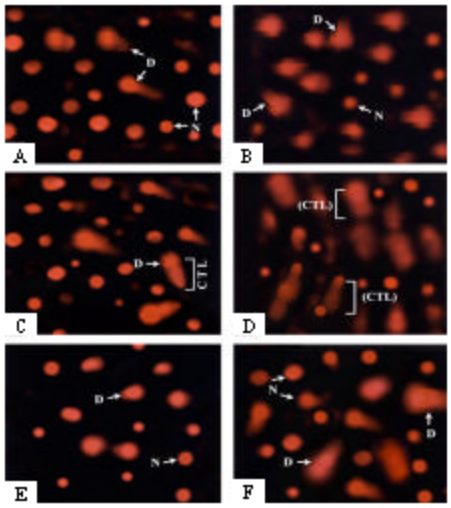


Fig. 3: Photomicrographs of rabbit lymphocyte nuclei exhibited DNA damage (comet tail) to different extents after to halothane exposure at various conditions, as established by comet assay: (A) After 10 min exposure to 0.1 mM halothane, (B) After 10 min exposure to 1.0 mM halothane, (C) After 30 min 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 and (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)

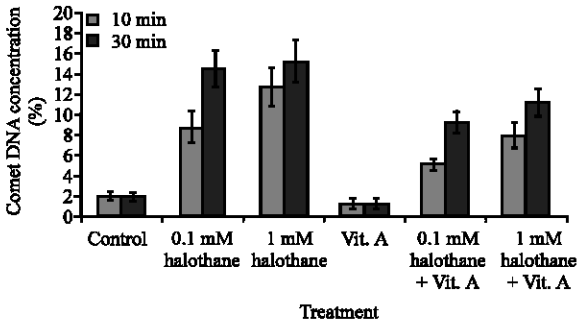


Fig. 4: Effect of *in vitro* halothane exposure and vitamin A supplementation on the percentage of comet DNA concentration (migrated damaged-DNA) in rabbit lymphocytes, as established by comet assay (mean comet DNA concentration±SE in %)

## DISCUSSION

From the obtained results, it is 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. The results displayed time- and concentration-dependent trend (Fig. 1, 4).

The obtained results may explain the findings of Bandoh and Fujita (1976)who reported that halothane caused a 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 by 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. The treated cells exhibited nuclear fragmentation and budding, early after treatment and 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 displaying 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 (Jaloszyñski *et al*., 1999a, 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 reductions in the DNA comet tail length and its concentration in all halothane treated lymphocytes (Fig. 1, 4).

Present results are in according to the findings of Jaruga *et al.* (2006). Who reported 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 later authors suggested that the formation of B[a]P-DNA adducts is considered to be an early step in respiratory tract carcinogenesis and 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 carotenoids supplementation is associated with decreased DNA damage, while the low carotenoids intake correlated with a high DNA damage, supporting a preventive role for carotenoids in bladder cancer (Matthew *et al.*, 2004).

For studying the mode of action of vitamin A, Huang *et al.* (2006) attributed the protective effect of vitamin A to its antioxidant effect. In their study, TUNEL assay and DNA electrophoresis indicated that vitamin A markedly blocked DNA fragmentation and apoptosis of microglia cells. Furthermore, the antioxidative enzymes as catalase (CAT) and Superoxide Dismutase (SOD) have been elevated after vitamin A treatment. The latter authors reported that vitamin A partially inhibited the activation of caspase-3, thereby both cleavage of poly-adenosine diphosphate ribose (ADP-ribose) polymerase (PARP) and degradation of inhibitor of caspase-activated DNase (ICAD) were effectively abolished. 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. In addition, 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 DNA of cultured lymphocytes. The recorded data are reinforced by the findings of Grandi *et al.*(2006). They clarified that comet test is an easily performed, rapid and highly sensitive genotoxicity assay; it requires small amounts of biological substrate and is applicable *in vivo* and *in vitro* to a wide variety of cells and tissues. However, modified versions of the comet test that able to detect oxidative DNA damage of individuals that are occupationally and environmentally exposed to genotoxic agents are available.

Giving the evidence, our findings reveal that genotoxicity could preclude the efficient use of halothane anaesthesia. Nevertheless, the current study advocates the need for vitamin A intake before anaethesia 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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