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INHALATION ANESTHETICS PROVOKE REORGANIZATION OF NUCLEAR COMPONENTS IN HUMAN BRONCHIAL CELLS

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ABSTRACT. The inhalation anesthetics, alone or in combination with intravenous anesthetics, are applied in human and veterinary surgery. It is considered that they will influence different cells in the body. Unfortunately data showing changes in lung cells at cellular and molecular level are insufficient, despite the fact that they are the first to get in contact with the anesthetics. Depending on the influence, the possible outcomes for the cells are membrane damage or disruption of membrane receptors functions; activation of intracellular signaling pathways; adaptation and restoration of cell processes; or induction of programmed cell death.

The aim of our study was to follow the impact of the inhalation anesthetics halothane and penthrane on the nuclear structures in human bronchial cells during *in vitro* exposure. For assessment of the nuclear morphology a mixture of two DNA-binding fluorescent dyes Hoechst 33342, and Propidium iodide was applied. Alkaline comet assay was used to determine the DNA damage in individual cells.

We established decreased survival as well as DNA fragmentation and heterochromatin clumps in treated cells. Both anesthetics possessed cytotoxic effect on human bronchial cells. The halothane showed dose-dependent, rather than time-dependent effect. In contrast, the penthrane did not have dose dependence but we observed correlation with duration of the treatment. Both anesthetics provoked DNA fragmentation in bronchial epithelial cells. We demonstrated dramatic reorganization of the chromatin in large heterochromatin clumps as a result of treatment with both anesthetics.

KEY WORDS. inhalation anesthetics, human bronchial cells, DNA injury, chromatin condensation

INTRODUCTION

The inhalation anesthetics, alone or in combination with intravenous administrated anesthetics, are applied in human and veterinary surgery. The intravenous concentrations can be precisely controlled and their effect is aimed at definite target cells and membrane receptors. They metabolize relatively slowly, mainly trough the kidney or after degradation in the liver. In contrast, clearance of inhalation anesthetics during breathing is faster and mostly trough the lung, because they are poorly soluble in blood. It is difficult to control accurately their concentration and the mechanism of action is still not completely understood. According to the Molecular Theory of General Anesthesia, postulated in 1961 by Linus Pauling, anesthetics such as halothane change fluidity of the cell membranes, forming microcrystals with stricture similar to the known hydrate crystals [1]. This hypothesis is supported by a number of experimental data, demonstrating changed surface tension of pulmonary surfactant and model lipid films after exposure to inhalation anesthetics [1]. According to the theory of Frank и Lieb (1982), anesthetics exert their effect by binding to specific proteins, either membrane associated or not, and inhibiting their normal functions [2]. Several studies proved affected ion channels by volatile anesthetics and ion balance changes of the cell [3]. Genetic evidences also support this theory – point mutations in certain ion channels lead to decrease of sensitivity to halothane and isoflurane [4]. Whatever the molecular mechanism for general anesthesia is, the agents inevitably will influence different cells in the body. Depending on the degree of influence, the possible outcomes for the cells in contact with an inhalation anesthetic are membrane damage or disruption of functions of membrane receptors; activation of intracellular signaling pathways; adaptation and restoration of cell functions (after weak injury); or activation of programmed cell death (after accumulation of significant damages that can't be repaired effectively).

Most of the recent studies are focused on the effect of the inhalation anesthetics on the neurons (which are the final target cells) and changes of different physiological indexes at the level of the whole organism [5, 6]. Treatment of laboratory animals with 2.5 % halothane and up to 1 % penthrane) leads to disruptions of physico-chemical properties of lung surfactant [7]. Another study reports impairment of physiological parameters in humans and animals [8]. According to clinical studies, carried out in Scandinavian countries during 20 years, high risk of development of childhood brain tumors exist, when penthrane had been applied during the birth [9, 10]. A comparative study revealed the effect of a number of inhalation agents on the mitotic activity in sea urchin embryo used as a model system [11] and anti-proliferative effect of many inhalation agents [12]. Data *in vitro* and *in vivo* are available for their genotoxic effect on human peripheral lymphocytes [13, 14]. Unfortunately data showing changes in lung cells on cellular and molecular level are insufficient, despite the fact that they are the first to become affecting by the anesthetics.

One of the used inhalation anesthetic is penthrane (methoxyfluran, 2,2-dichloro-1,1-difluoroetilmetil ether). Because of the proved nephrotoxicity due to its

metabolism, penthrane is applied mainly in veterinary medicine. In human medicine this anesthetic is replaced by new, more stabile metabolic derivatives isoflurane, enflurane and sevoflurane. However, disadvantage of these anesthetics is formation of methoxyflurane during metabolic processing. Another nowadays used inhalation anesthetic is halothane (2-bromo-2-chloro-1,1,1-trifluoroethane), have been introduced in practice since 1956 as substitute of the chloroform and ether. In comparison with the new anesthetic (e.g. isoflurane and enflurane) it possesses relatively faster induction and recuperation of patients after anesthesia and lower inflammation effect of the lung [15].

On the basis of our previous results for the effect of both anesthetics halothane and penthrane on the alveolar cells, we presumed that their impact lead to induction of programmed cell death in bronchial epithelial cells and as a consequence to changes in chromatin compactization and DNA fragmentation. To check this hypothesis, we focus our attention on the impact of the inhalation anesthetics halothane and penthrane on the nuclear structures in human bronchial cells during *in vitro* exposure. We established a decreased level of cell survival as well as DNA fragmentation and heterochromatin clumps in treated cells. These allow us to suggest that the result of treatment is the induction of cell death.

MATERIAL AND METHODS

Cell cultures

The experiments were performed with bronchial epithelial cells 16HBE14o$^-$, normocytes kindly provided by Priscilla Kimberly, University of Vermont, Burlington, USA), grown in standard conditions until the cells reached 80 % confluence.

Cells were treated for 40 or 80 min with halothane (Narcotane, Leciva) at 1.5 and 3 mM concentration or 0.5 – 1 % penthrane solution in DMEM. After treatment the cells were washed with phosphate buffer saline (PBS), pH 7.4, the medium was changed and the cells were maintained up to 5 days without anesthetic. Cells treated with 100 μm H_2O_2 were used as a positive control. Every 24 h cells were counted using haemocytometer and survival coefficient was calculated as percent from untreated cells (the number of untreated cell is accepted to be 100 %).

Nuclear staining

For assessment of nuclear morphology, cells were grown on sterile coverslips, treated with both anesthetics for 40 or 80 min, and allowed to recover for several days in fresh medium. Samples were taken every 24 h, washed twice with PBS, pH 7.4 and stained *in vivo* for 5 min at 37°C with a mixture of two DNA-binding fluorescent dyes (2 μg/ml Hoechst 33342, and 5 μg/ml Propidium iodide, Sigma) in PBS, pH 7.4. The samples were analyzed with Leica fluorescent microscope, equipped with a CCD-camera.

Evaluation of DNA integrity

Alkaline comet assay was used to determine the DNA damage in individual cells. Cells were analyzed using Leica Q-Win software. Two parameters were estimated for each comet: (1) integral fluorescence, which is proportional to the DNA content in the tail or head, and (2) tail length [16]. The amount of damaged DNA in each cell was calculated on the basis of the fluorescence in the tail as a percent of integral fluorescence in the comet. All experiments were performed three times and for each experimental point at least 50 comets were measured and the data represent mean values ± SE. Statistical processing of data was made using GraphPad InStat 3.01 and results were compared by one-way ANOVA. A level of probability P = 0.05 was accepted for statistical significance testing.

RESULTS

Cell survival index

The cell survival and growth are the cell culture characteristics that change after negative impact. After exposure of human bronchial cells to both anesthetics (Fig. 1) we established high mortality and suppressed growth. While the halothane showed a clear dose-dependent effect (Fig. 1 a), we did not observe any statistically significant differences in response to both applied concentrations of penthrane. The survival coefficient (in comparison with untreated cells) was about 30 % at the end of observed period (Fig. 1 b). Exposure to lower concentration of halothane led to relatively long period of adaptation and suppressed growth (about tree days). In contrast, after treatment with 3 mM halothane we observed continuously decreased level of survival and permanently suppressed cell growth, which was a sign for strong cytotoxic effect.

Genome integrity

Using alkaline comet assay, we detected DNA breaks induced by 20 to 80 min treatment with 1.5 mM halothane or 0.5 % penthrane. Our results demonstrated that in non-treated cells high molecular weight DNA was localized only in the head of the comet, but after treatment with both anesthetics, significant amount of released DNA was apparent in the comet tails (Fig. 2 and 3). Damaged DNA was quantified by calculating the percentage of DNA in the tail versus total DNA in the comet for each individual cell.

The statistical data analyses confirmed the strong *in vitro* genotoxic effect of halothane and the damaged DNA (mean value 58 ± 3.79, $P<0.001$), which occurs even after 20-minutes of exposure. The most powerful effect on DNA (mean value 68.66 ± 3.44, $P<0.001$) was detected in bronchial cells after 80-minutes treatment (see Fig. 2).

The effect of 0.5% penthrane was strongly time-dependent. The DNA in these cells was relatively preserved after 40 min treatment (mean value 47.6 ± 3.44) but after 80 min cells demonstrated strongly damaged DNA (mean value 75.14 ± 3) (Fig. 3).The large number of cells with injured DNA caused by this anesthetic showed that

penthrane has more sever effect in comparison with halothane for the same duration of treatment.

Reorganization of the chromatin

Following the halothane treatment (1.5 mM, 40 min), reorganization of chromatin stricture in bronchial cells was observed. On the first day most of the cells possessed large heterochromatin regions in the nuclei (Fig 4 a). In addition, the later periods were characterized by nuclear fragmentation and budding (Fig 4 c). To assess the nuclear morphology and cell vitality, we used mixture of two fluorescent DNA-binding dyes - Hoechst 33342 and Propidium iodide. Hoechst 33342 penetrates through cell membranes and "stains" all nuclei producing light blue fluorescence, when the second dye can pass only trough impaired membranes of death cells and their nuclei display red fluorescence. All cells with nuclear fragmentation and budding were with intact membranes (negative for propidium iodide staining). Furthermore, no mitotic figures were detected during analyzed period hence mitotic activity was suppressed.

Conversely, after penthrane treatment (0.5 %, 40 min) most of the cells were propidium iodide positive, but without nuclear fragmentation, which indicated initiation of necrotic cell death (Fig.5). The cell number dramatically decreased soon after treatment and a small number of cells remained during post-treatment period. On the firs day after treatment we observed reorganization of chromatin compactization which was similar to that provoked by halothane (Fig.5 a), but two days later all cells had either normal or completely condensed (picnotic) nuclei (Fig.5 c).

DISCUSSION

The reorganization of nuclear components in human bronchial cells provoked by the inhalation anesthetics halothane and penthrane corresponded to the changes of alveolar cells, which we have found in our previous studies [17, 18, 19]. Our preceding results showed that inhalation anesthetics penthrane and halothane, applied at clinically relevant concentrations (up to 2 % and 4 mM respectively), impaired both morphology and physiological parameters of human alveolar type II cells. The suppressed cell growth in culture is a clear indication of cytotoxic action of analyzed agents. After such treatment affected cells might adapt and restore their normal functions or activate programmed cell death that allows replacement by new, healthy cells.

Chromatin condensation and DNA fragmentation occur very early during apoptosis and it is accepted as a marker for the programmed cell death [20]. Our results revealed DNA fragmentation even after short treatment with both anesthetics, which can be due to both direct injury and/or activation of intracellular endonucleases [20]. DNA cleavage by endonucleases is accompanied with dramatic changes in chromatin condensation and subsequent nuclear and cellular fragmentation. These two distinct morphological features were only manifested after exposure to halothane. Treatment with penthrane led mainly to chromatin condensation followed by

necrotic-like cell death, accompanied with prominent membrane damage. Our results indicated that induction of apoptosis-like cell death is the main effect on bronchial cells after treatment with 3 mM halothane.

CONCLUSIONS

In conclusion, both studied anesthetics possessed cytotoxic effect on human bronchial cells. Unlike penthrane, the halothane showed clear dose-dependent, but not time-dependent effect. Both anesthetics provoked DNA fragmentation in bronchial epithelial cells. We observed dramatic reorganization of the chromatin in large heterochromatin clumps as a result of the treatment.

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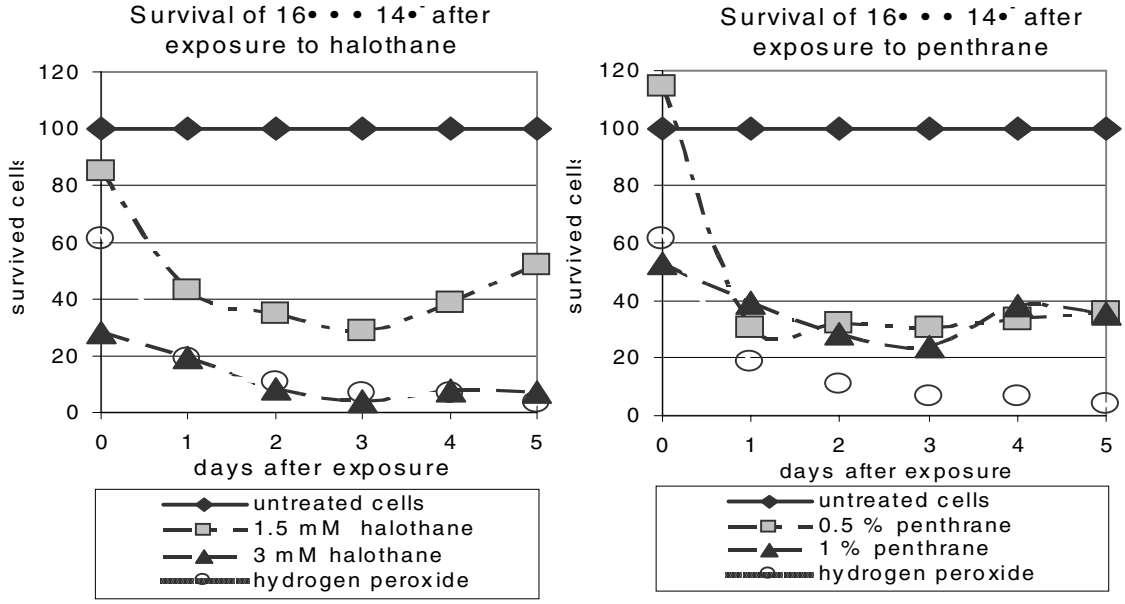
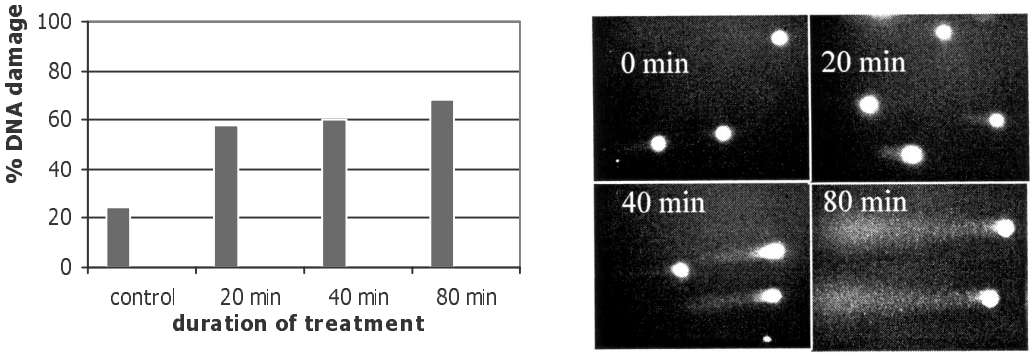


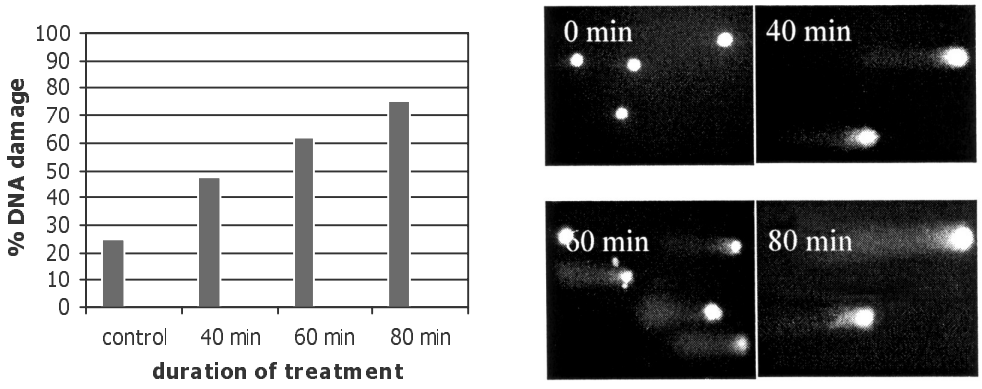
a b

Fig. 1 Survival coefficient of human bronchial cell line 16HBE14o- after treatment with the inhalation anesthetics halothane (a) and penthrane (b)



a b

Fig. 2 Effect of treatment with 1.5 mM halothane on the genome integrity of 16HBE14o- cells. Average levels of damaged DNA (a) and typical images of comets for the indicated time of treatments are shown



a b

Fig. 3 Effect of treatment with 0.5 % penthrane on the genome integrity of 16HBE14o- cells. Average levels of damaged DNA (a) and typical images of comets for the indicated time of treatments are shown.

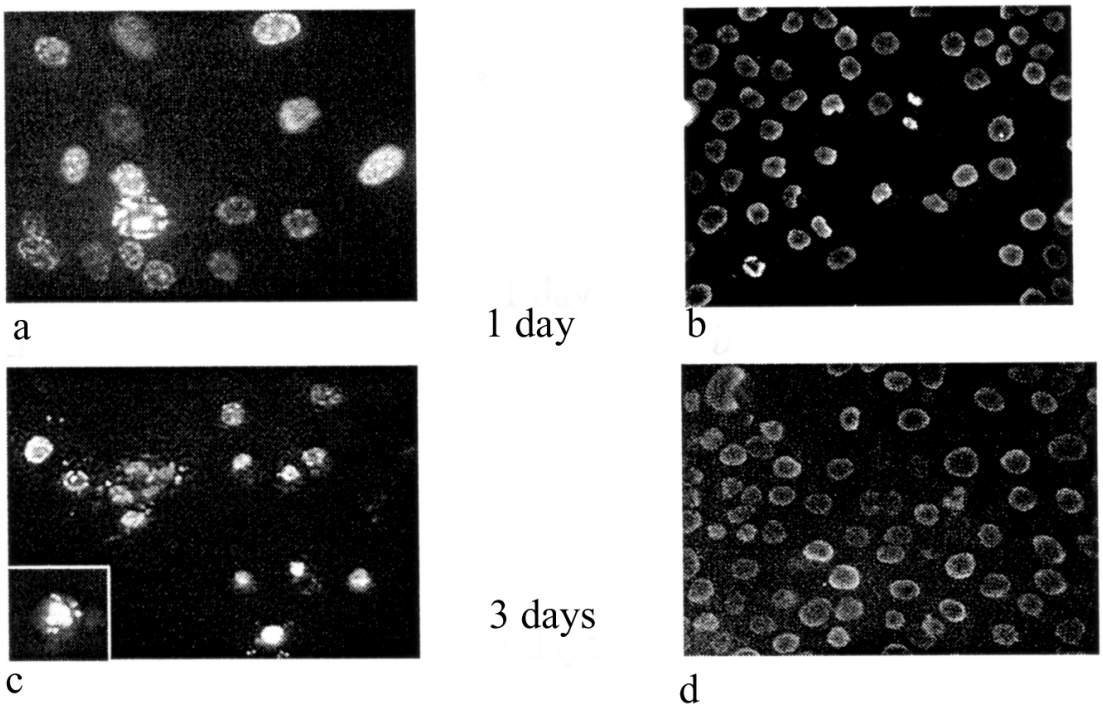


Fig. 4 Reorganizations of chromatin components after 40 min of treatment of 16HBE14o$^-$ cells with 1.5 mM halothane.
a and *c* – treated cells; *b* and *d* – control
(original magnification of *a* and the insert in *c* – 600 x; *b, c* and *d* – 250 x)

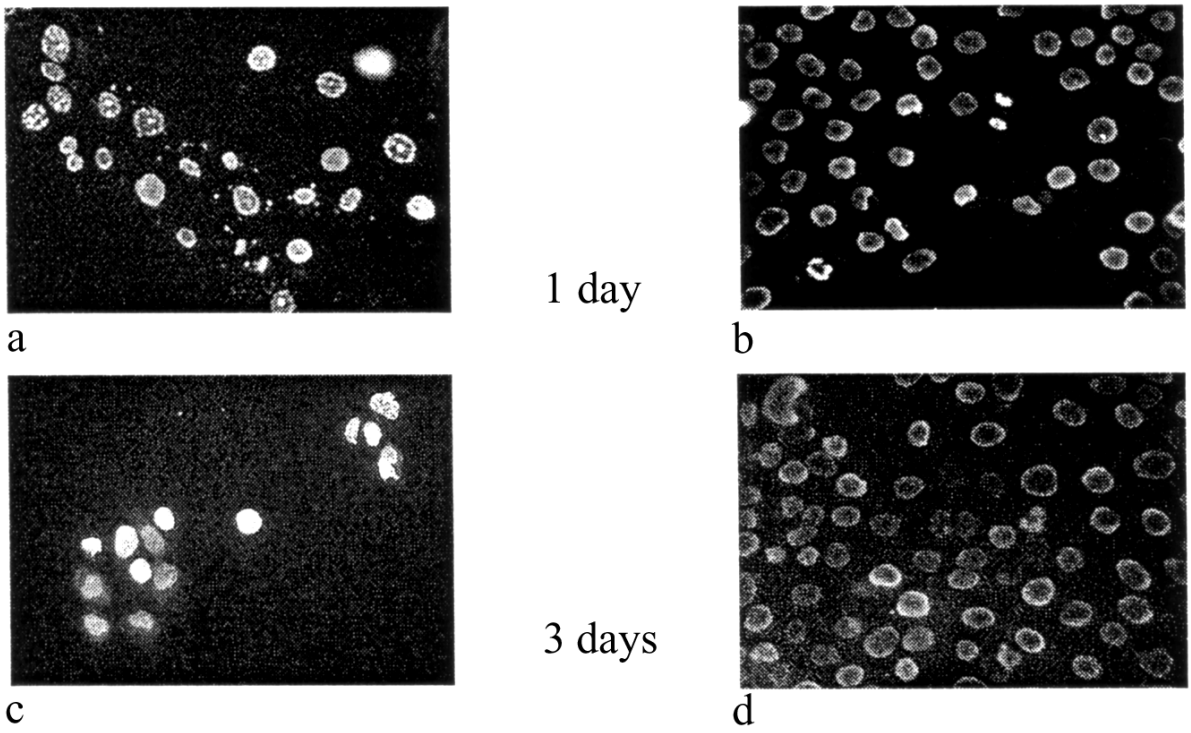


Fig. 5 Reorganizations of chromatin components after 40 min of treatment of 16HBE14o$^-$ cells with 0.5 % penthrane.
a and *c* – treated cells; *b* and *d* – control (original magnification is 250 x)