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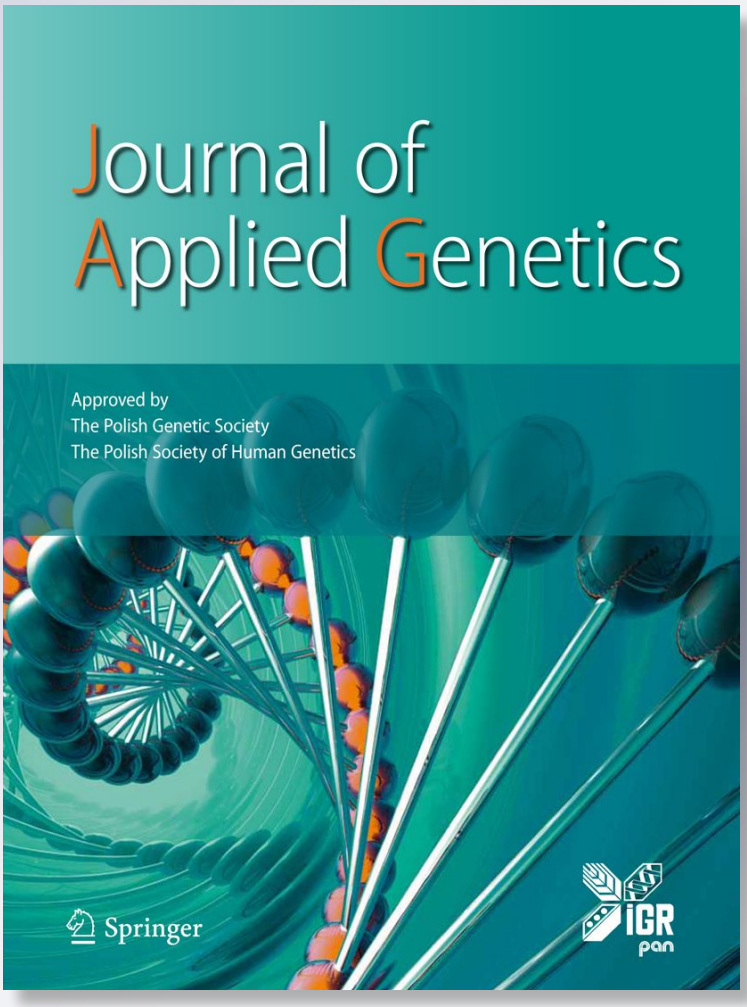

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The in vivo genotoxicity of cisplatin, isoflurane and halothane evaluated by alkaline comet assay in Swiss albino mice

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Abstract The aim of this study was to evaluate the genotoxicity of repeated exposure to isoflurane or halothane and compare it with the genotoxicity of repeated exposure to cisplatin. We also determined the genotoxicity of combined treatment with inhalation anaesthetics and cisplatin on peripheral blood leucocytes (PBL), brain, liver and kidney cells of mice. The mice were divided into six groups as follows: control, cisplatin, isoflurane, cisplatin–isoflurane, halothane and cisplatin–halothane, and were exposed respectively for three consecutive days. The mice were treated with cisplatin or exposed to inhalation anaesthetic; the combined groups were exposed to inhalation anaesthetic after treatment with cisplatin. The alkaline comet assay was performed. All drugs had a strong genotoxicity ($P<0.05$ vs. control group) in all of the observed cells. Isoflurane caused stronger DNA damage on the PBL and kidney cells, in contrast to halothane, which had stronger genotoxicity on brain and liver cells. The combination of cisplatin and isoflurane induced lower genotoxicity on PBL than isoflurane alone ($P<0.05$). Halothane had the strongest effect on brain cells, but in the combined treatment with cisplatin, the effect decreased to the level of cisplatin alone. Halothane also induced the strongest DNA damage of the liver cells, while the combination with cisplatin increased its genotoxicity even more. The genotoxicity of cisplatin and isoflurane on kidney cells were nearly at the same level, but halothane caused a significantly lower effect. The combinations of inhalation anaesthetics with cisplatin had stronger effects on kidney cells than inhalation anaesthetics alone. The observed drugs and their combinations induced strong genotoxicity on all of the mentioned cells.

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Introduction

The effects and toxicity of potential interactions of drugs used in anaesthesia and cancer therapy have been poorly investigated but, due to the fact that a growing number of cancer patients undergo cytoreductive surgeries under general anaesthesia soon after receiving chemotherapy, this issue needs to be addressed. Furthermore, chemotherapy treatments are occasionally applied during a surgery. Therefore, the pharmacological interactions between anti-cancer and anaesthetic drugs are important to consider (Zaniboni et al. 2005). The main disadvantages of chemotherapeutic agents are their relatively non-selective damage of normal tissues as well as tumour cells and synergistic toxicity with other drugs (Hegarty and Chisholm 2002).

Cisplatin is one of the most useful anti-neoplastic drugs with DNA as its critical target. Jirsova et al. have shown that, using a comet assay, cisplatin caused a dose- and time-dependent increase of the number of cells with DNA damage (Jirsova et al. 2006). These genotoxic effects may lead to the initiation of unrelated tumours years after the chemotherapy cessation. Besides that, it has been demonstrated that cisplatin has a potential for genetic events in the non-tumour cells of both humans and animals. DNA damage is a commonly accepted reason for platinum agents' cytotoxic properties (Aly et al. 2003). Chemotherapy drugs can cause a wide variety of effects on the liver, renal impairment, neurotoxicity and myelosuppression.

Among the exogenous compounds, inhalation anaesthetics, commonly used in general anaesthesia procedures, have recently attracted a lot of attention due to their potential mutagenic/genotoxic effects. Generally, the induction of halogenated inhalation anaesthetics in clinical practice was associated with the incidence of DNA damage (Rozgaj et al. 2009; Karpiński et al. 2005; Topouzova-Hristova et al. 2006). Because of its cost, halothane was the leading anaesthetic for a very long time, mostly in less developed countries, although it was known to induce a fulminant hepatitis in susceptible individuals (Jaloszyński et al. 1999). Today, isoflurane as well as sevoflurane are widely used in current clinical practice. Experimental and epidemiological studies suggest that genotoxic effects could arise from inhalation anaesthetics. In fact, genetic damage has been observed in operating room staff who have been exposed to traces of anaesthetic gas concentrations (Rozgaj et al. 2009). The investigation by Kvolik et al. (2005) has shown *in vitro* that halothane and isoflurane used in anaesthetic doses have cytogenetic and anti-proliferative effects on both tumour and normal human cells. In addition, cytogenetic changes have been observed in the kidneys of albino rats treated with different anaesthetics (Robbiano et al. 2004).

There are very few reported drug interactions between chemotherapy and anaesthetic drugs. We suggest that the concomitant use of inhalation anaesthetic with cisplatin may decrease the efficacy of cisplatin by reducing its damaging capacity, by the mechanism of competitive binding at the same site of drugs binding. On the other hand, there is a possibility that some inhalation anaesthetics could, perhaps, interfere with the effect of cisplatin and augment its toxicity. The fact that some anaesthetics could have a significant influence on cytostatic or cytotoxic drug metabolism gives great importance to the selection of an appropriate anaesthetic during the treatment of cancer patients (Zaniboni et al. 2005; Brozovic et al. 2008, 2009). The anaesthesiologist must be familiar with the drug interactions of the cytostatic drugs used in the course of the treatment and their consequences. Most of the observations due to such reactions are based on *in vitro* and animal studies because controlled clinical trials of anaesthetics and cytostatic interactions are rare (Kvolik et al. 2003).

With respect to the fact that cancer patients are frequently exposed to repeated surgical procedures under general anaesthesia and, increasingly, receiving chemotherapy in the perioperative period or even intraoperatively, we evaluated the DNA damage of healthy cells and tumour cells of Swiss albino mice induced by sevoflurane and cisplatin using the alkaline comet test in mice in our previous papers (Brozovic et al. 2008, 2009). The metabolism of the inhalation anaesthetics takes place mainly in the liver, and, to a lesser extent, in the kidney and lungs. All agents are metabolised in the liver by cytochrome P450, especially by the isoenzyme 2E1, with a dominating oxidative route. The toxic reactions caused by the inhalation anaesthetics are very much connected to their metabolism. The metabolism of isoflurane is significantly lower than the metabolism of other inhalation anaesthetics, such as halothane and sevoflurane. The inhalation anaesthetics have been determined in various tissues, including lung, brain and spinal fluid, but the blood is the most useful material for the analysis (Pihlainen and Ojanperä 1998).

Evidence-based significantly different influence of inhalation anaesthetics on the genotoxic, as well as on the cytotoxic, effects of cisplatin on healthy cells *in vivo* suggested multiple interaction mechanisms with either augmenting or decreasing effects. Intrigued by the previous observations, we decided to evaluate the DNA damage after repeated exposure to isoflurane or halothane and compare their detrimental effects with the genotoxic effects of cisplatin as a known genotoxin. In addition, we also tried to determine the possible genotoxic properties of combined treatment with isoflurane and cisplatin and halothane and cisplatin on samples of peripheral blood leucocytes (PBL), brain, liver and kidney cells of Swiss albino mice.

Materials and methods

All experiments were performed in accordance with the relevant Croatian guidelines, including the Guidelines for the Care and Use of Laboratory Animals, approved by the Ethical Committee of the Faculty of Science and Medical School (University of Zagreb, Croatia). The inhalation anaesthetic isoflurane (Forane) was provided by Abbott Laboratories (Queenborough, UK) and halothane (Fluothane) by Zeneka (Macclesfield, Cheshire, UK). The chemotherapeutic anti-cancer drug cisplatin was supplied by Pliva (Zagreb, Croatia).

The male Swiss albino mice used in this study (2 months old, 20–25 g body weight) from our own conventional mouse colony, were housed in cages in the groups of five and maintained on a pellet diet and water *ad libitum*. The mice were divided into six experimental groups as follows: control group, cisplatin group, isoflurane group, cisplatin–isoflurane group, halothane group and cisplatin–halothane group. Each group comprised five healthy mice. In the cisplatin group, the mice were treated with cisplatin at a dose of 5 mg kg^{-1} via the i.p. route daily, in the isoflurane group, the mice were exposed to isoflurane at a dose of 1.7 vol% for 2 h daily and in the halothane group, the mice were exposed to halothane at a dose of 1.5 vol% for 2 h daily. In the combined cisplatin–isoflurane group, the mice were treated with a combination of cisplatin at a dose of 5 mg kg^{-1} via the i.p. route daily and, afterwards, exposed to isoflurane at a dose of 1.7 vol% for 2 h daily. Mice in the cisplatin–halothane group were treated with cisplatin at a dose of 5 mg kg^{-1} via the i.p. route daily and then exposed to halothane at a dose of 1.5 vol% for 2 h daily. The mice were treated for three consecutive days. The used doses of isoflurane and halothane were equivalent to clinical doses in humans. The depth of anaesthesia was considered to be satisfactory when the mice were sleeping calmly, breathing spontaneously and not moving their tails. The dose of cisplatin used in this study was slightly different from human doses due to the pharmacokinetic differences between humans and small laboratory animals (Máthé et al. 2006).

Anaesthesia was maintained with isoflurane (1.7 vol%) or halothane (1.5 vol%) in oxygen (3 l/min) in a specially designed incubator connected to an anaesthetic machine Sulla 800 (Dräger, Germany) using a isoflurane or halothane vaporiser. Fresh gas was flowing in one direction and exhaled gases were released through the exhaust pipe into the atmosphere. The depth of anaesthesia was considered to be satisfactory when the mice were sleeping calmly, breathing spontaneously and not moving their tails. The mice were sacrificed on the third day after the treatment. Samples of blood and tissues were collected for analysis. Blood samples of mice were collected from the tail vein. The brain, liver or kidney tissues were minced and passed through a stainless steel mesh and single-cell suspensions were made in the homogenisation buffer solution (at the ratio 1 g tissue/1 ml buffer) at pH 7.5 (0.075 M NaCl and 0.024 M Na_2EDTA) and then cooled to 4°C. The comet assay was carried out under alkaline conditions, as described by Singh et al. (1988). A freshly prepared cell suspension obtained as a mixture of cells with 100 μl of 0.5% LMP agarose was placed onto pre-cleaned microscope slides, previously pre-coated with 300 μl of 0.6% NMP agarose. After cooling on ice for 10 min, the slides were covered with 0.5% LMP agarose. After the agarose gel had solidified, the slides were immersed for 1 h in ice-cold lysis solution, consisting of 100 mM EDTA, 2.5 M NaCl, 10 mM Tris-HCl and 1% sodium sarcosinate, adjusted to pH 10 with 1% Triton X-100 and DMSO, added just prior to use. Prior to electrophoresis, the slides were removed from the lysing solution and placed for 20 min in a horizontal electrophoresis unit (near the anode) filled with an alkaline buffer, in order to allow the unwinding of the DNA and to express alkali-labile damage. The electrophoresis alkaline solution consisted of 1 mM EDTA and 300 mM NaOH, pH 13. After the unwinding of the DNA, electrophoresis was carried out in the same solution for 20 min at 25 V (300 mA). Alkaline unwinding and electrophoresis were performed at 4°C under dimmed light. After electrophoresis, the gels were neutralised by the rinsing of slides with Tris buffer (0–4 M Tris-HCl, pH 7.5). After neutralisation, the slides were stained with the fluorescent dye, ethidium bromide (20 μg ml^{-1}). A total of 500 PBL, brain, liver or kidney cells from each group were analysed under a fluorescent microscope (Opton, Germany) at 160× magnification.

The degree of DNA damage was determined visually by the categorisation of comets into different "classes" of migration in a value range from 0 to 4 arbitrary units, according to the degree of DNA damage. The comet tail lengths were evaluated in order to determine the DNA damage. Analysis was performed on coded slides by one reader to avoid variability due to the subjective scoring.

Results from the comet assay were analysed using descriptive statistical methods. Non-parametric Kruskal–Wallis analysis of variance (ANOVA) and median test were used for the statistical analysis of differences between particular groups and *post hoc* analysis was performed with the Mann–Whitney *U*-test. The software package Statistica 6.0 (StatSoft, Inc., Tulsa, OK, USA; for Windows) was used. Statistical significance was set at $P<0.05$.

Results

Cisplatin, isoflurane and halothane produced a strong genotoxic effect ($P<0.05$ vs. control group) in all of the

observed types of cells of Swiss albino mice (Table 1). Isoflurane generated stronger DNA damage of the PBL and kidney cells in contrast to halothane, which had a stronger genotoxic effect on brain and liver cells. Isoflurane had the strongest genotoxic effect on PBL, but a combined treatment with cisplatin and isoflurane caused a statistically significant lower genotoxic effect than isoflurane alone ($P<0.05$). Halothane also caused damage to PBL DNA versus the control group ($P<0.05$), but this effect was lower than the effect of isoflurane ($P<0.05$) and cisplatin ($P<0.05$) alone. A combination of cisplatin and halothane increased the effect of halothane ($P<0.05$) and cisplatin alone (not statistically significant). The enhanced DNA damage of kidney cells was observed in the cisplatin group versus the isoflurane group (not statistically significant) and halothane group ($P<0.05$). Both combinations of inhalation anaesthetics and cisplatin increased the genotoxic effect of cisplatin on the kidney cells of mice ($P<0.05$).

The strongest DNA damage of mice brain cells was expressed after repeated treatment with halothane ($P<0.05$ vs. all other groups). High genotoxicity of cisplatin on brain cells decreased in combination with isoflurane ($P<0.05$), but the combination with halothane showed similar values as cisplatin alone. In all groups of treated animals, strong DNA damage of liver cells versus the control group ($P<0.05$) was noted. In the halothane group, the liver cells of Swiss albino mice sustained the highest DNA damage. DNA damage in the cisplatin group was lower, but was not statistically significant so. Isoflurane produced a significantly lower genotoxic effect ($P<0.05$ vs. halothane and cisplatin group). Combined treatment with cisplatin and halothane had the strongest genotoxicity on the liver cells of Swiss albino mice; the effect was stronger than the effect of cisplatin or halothane alone ($P<0.05$). The combination of cisplatin and isoflurane had a lower genotoxic effect than cisplatin ($P<0.05$) and isoflurane alone.

Discussion

The present *in vivo* study was designed to determine the differences in possible genotoxicity of repeated exposure to isoflurane and halothane and to compare their detrimental activity with the genotoxicity of cisplatin on PBL, brain, liver and kidney cells of Swiss albino mice. Moreover, we also wanted to assess the genotoxic effect of the combined application of cisplatin and above-mentioned inhalation anaesthetics on the same type of cells.

Inhalation anaesthetics are very often used for inducing or maintaining anaesthesia in cancer patients, as the length and complexity of the surgical procedure cannot be predicted due to intraoperative surgical and pathohistological findings. Besides that, disease very often requires repeated operations for the removal or reduction of the primary tumour, regional metastases, recidivism, pathological fractures or surgery complications. A growing number of patients undergo surgical procedures with general anaesthesia soon after receiving chemotherapy, and, occasionally, such treatment has been applied during surgery already. Therefore, it is worthwhile and prudent to consider the pharmacological interactions between anti-cancer and anaesthetic drugs. Since the number of individuals receiving chemotherapy in the peri-operative period is on the increase, patients who might have potentially dangerous and unwanted effects are also due to increase in number (Zaniboni et al. 2005).

Inhalation anaesthetics are mostly eliminated from the body by respiration, but a small amount is metabolised in the liver, such as cytostatics via the cytochrome P450 oxidase family, and is excreted via the kidneys, so their toxicity has always attracted considerable interest from investigators. If isoflurane or halothane reacts directly with DNA, the most feasible alkali-labile modification may be alkylation at the N-7 position of purines. Another explanation is that inhalation anaesthetics undergo a residual metabolic oxidation or reduction, giving rise to reactive products. In fact, it is known that free radical species are capable of directly attacking DNA (Alleva et al. 2003). Therefore, the determination of DNA damage, especially of repeated anaesthesia, is particularly important, and studies by the comet assay are needed in order to clarify the grade of damage that might appear with different anaesthetics (Kaymak et al. 2004). The alkaline comet assay can detect strand breaks as well as alkali-labile sites, including abasis sites.

Isoflurane, in contrast to halothane, is metabolised to a minimal degree (0.2%). Halothane undergoes extensive biotransformation, with approximately 50% of an administered dose metabolised by reductive and oxidative pathways (Spracklin et al. 1997). Recent studies indicate that general anaesthetics are much more selective than is usually appreciated and may act by binding to proteins rather than lipids and only to specific targets in the central nervous system (Franks and Lieb 2004).

Taking into the account a difference of isoflurane and halothane in the magnitude of reactive metabolites, the aim was to find out whether it had a direct influence on the DNA damage intensity of the of healthy mice cells. The overall results obtained in this study confirmed the genotoxicity of both inhalation anaesthetics, but the direct influence on the DNA damage intensity could not have been confirmed because the extent of DNA damage depends on both the inhalation anaesthetic applied and the type of cells observed.

Cisplatin is a known anti-cancer drug, active for a variety of solid tumour types and it is generally considered

Table 1 Results of the alkaline comet assay for peripheral blood leucocytes (PBL), brain, liver and kidney cells of mice treated with cisplatin, isoflurane and halothane or their combinations (500 comets per group)

Treatment	Leucocytes						Brain cells						Liver cells						Kidney cells					
	Comet class*					TCS	Comet class*					TCS	Comet class*					TCS	Comet class*					TCS
	0	1	2	3	4		0	1	2	3	4		0	1	2	3	4		0	1	2	3	4	
Control	460	40	0	0	0	40	429	68	2	1	0	75	327	167	5	1	0	180	379	107	14	0	0	135
	0.08±0.013						0.15±0.018						0.36±0.025						0.27±0.025					
Cisplatin	7	385	90	17	1	620	2	31	237	204	27	1,225	68	107	109	209	7	980	8	152	272	68	0	900
	1.24±0.026[a]						2.45±0.035[a]						1.96±055[a]						1.8±0.69[a]					
Isoflurane	119	150	68	108	55	830	79	210	104	86	21	760	8	258	163	68	3	800	13	228	138	118	3	870
	1.66±0.067[ab]						1.52±0.054[ab]						1.6±0.038[ab]						1.74±0.043[ab]					
Cisplatin + isoflurane	97	201	79	86	37	765	26	202	107	156	9	920	2	318	145	33	2	715	7	163	175	133	22	1,000
	1.53±0.06[acd]						1.84±0.048[abcd]						1.43±0.032[abd]						2±0.045[abcd]					
Halothane	88	371	39	2	0	455	15	9	85	303	88	1,440	0	258	326	204	12	1,015	105	269	71	46	9	585
	0.91±0.02[abc]						2.88±0.041[abc]						2.03±0.041[ac]						1.17±0.046[abc]					
Cisplatin + halothane	0	348	144	8	0	660	12	29	198	219	42	1,250	4	105	149	241	1	1,130	6	142	176	173	3	1,025
	1.32±0.025[abd]						2.5±0.041[abce]						2.26±0.040[abcde]						2.05±0.042[abcd]					

*The results are expressed in arbitrary units (a.u.) ranging between 0 and 4, with the total comet score (TCS) calculation. TCS = 0 × N0 (number ofcomets in category 0) + 1 × N1 (number of comets in category 1) + 2 × N2 (number of comets in category 2) + 3 × N3 (number of comets incategory 3) + 4 × N4 (number of comets in category 4). The second lines show mean±SEM of the referred group. The small letters denote significant differences ($P<0.05$) from the control, cisplatin, isoflurane, halothane and cisplatin + isoflurane group

that it induces a cytotoxic effect by binding to DNA, resulting in mutation induction. The high mutagenic potency of cisplatin raises the concern that its use in cancer chemotherapy may be responsible for secondary malignancies, which have been observed in animals and some cured patients who were treated with cisplatin. Cisplatin combined with other drugs can lead to multiple pathways of different pharmacokinetic interactions, increased intracellular drug accumulation, enhanced uptake, reduced efflux or reduced inactivation, enhanced binding to DNA, decreased repair or bound DNA, or a difference in cellular response to DNA damage (Crul et al. 2002). The alkaline comet assay is a simple and inexpensive method that only requires a small number of cells for estimating DNA damage. This assay has already been described as being suitable for the detection of crosslinks induced by a variety of agents (Speit and Hartmann 2005).

The results of this study have shown that both cisplatin and the mentioned inhalation anaesthetics and their combinations induced strong *in vivo* genotoxic effect on the PBL, brain, liver and kidney cells of mice. In general, the brain cells were the most sensitive type of cells. These results concur with the results of previous investigations of sevoflurane and cisplatin (Brozovic et al. 2008). Halothane had the strongest effect on the brain cells of mice, even stronger than cisplatin ($P<0.05$), while isoflurane caused considerably less damage of the DNA in the brain cells ($P<0.05$ vs. halothane and cisplatin). The combination of cisplatin and halothane had a lower effect than halothane alone ($P<0.05$), but although the above-mentioned combination increased the genotoxic effect of cisplatin, it was not statistically significant. The combination of isoflurane and cisplatin decreased the genotoxicity of cisplatin ($P<0.05$).

According to their sensitivity to the mentioned drugs, the liver cells sustained the largest DNA damage from the repeated treatment with halothane and cisplatin (the difference between the two drugs was not statistically significant). The combination of halothane and cisplatin had caused even stronger genotoxicity than each of the halothane ($P<0.05$) and cisplatin ($P<0.05$) treatments alone. Isoflurane had a smaller influence on the DNA of liver cells ($P<0.05$ vs. halothane, $P<0.05$ vs. cisplatin). The combination of isoflurane and cisplatin decreased the genotoxicity of cisplatin ($P<0.05$) and isoflurane alone (not statistically significant). The DNA of kidney cells was very sensitive to cisplatin and isoflurane (the difference between the groups was not statistically significant). Repeated anaesthesia with halothane had a much lower influence on the mentioned cells ($P<0.05$ versus cisplatin and isoflurane). The magnitude of the DNA damage of isoflurane or halothane in combined application with cisplatin increased the DNA damage induced by cisplatin alone ($P<0.05$ versus cisplatin in both combinations). At the end, the PBL cells were the most resistant type of described cells. All of the investigated drugs caused DNA damage of PBL (statistically significant vs. control), but the genotoxicity was considerably lower when compared to the other types of cells. If we keep in mind that blood is the most applied material for genotoxic studies, we suspect that the genotoxicity of the drugs tested is much stronger on the other cell types of the tested organisms than the results on blood.

Conclusion

The *in vivo* results reported here indicate a necessity for further observations to find out the mechanism(s) by which inhalation anaesthetics separately and/or in combination induce genetic damage. Due to its sensitivity, rapidity, small cell sample and low cost, the alkaline comet assay is a good method for the detection of DNA damaged cells, but it cannot directly distinguish between the dead cells and the cells that have a heavily damaged DNA, but are still able to recover. Therefore, we feel that there is a lack of knowledge about the subsequent repair of the mentioned cells after repeated cell exposition to inhalation anaesthetics and their combinations with chemotherapy drugs.

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