Cytotoxicity and Type of Cell Death Induced by Midazolam in Human Oral Normal and Tumor Cells

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Abstract. *Background: Intravenous anesthetics have been used during the treatment of various malignant tumors, however, their effects on oral tissues is not well-understood. In the present study, the cytotoxicity of five intravenous anesthetics towards oral tumor and normal cells was compared. Materials and Methods: Tumor specificity index was determined by the ratio of the mean 50% cytotoxic concentration for normal cells to that for tumor cells. Apoptosis induction was monitored by internucleosomal DNA fragmentation and caspase-3, -8, and -9 activation. Fine cell structure was observed under transmission electron microscopy. Results: Benzodiazepines (midazolam and diazepam) exhibited higher cytotoxicity than barbiturates (thiopental and thiamylal), whereas propofol had the intermediate range of cytotoxicity. Midazolam showed the highest cytotoxicity. HL-60 cells were the most sensitive to midazolam, followed by epidermal keratinocytes, oral squamous cell carcinoma (OSCC), glioblastoma and then oral normal cells. Midazolam did not induce the production of apoptosis markers such as internucleosomal DNA fragmentation and activation of caspase-3, -8 and -9, but did induce the appearance of many vacuoles, mitochondrial swelling and cell membrane rupture in OSCC cell lines (HSC-2 and HSC-4) cells. The cytotoxicity of midazolam was not reduced by pre-treatment with autophagy inhibitors (3-methyladenine and bafilomycin A1). Conclusion: These results suggest that midazolam may induce necrotic cell death, rather than apoptosis or autophagy, in OSCC cell lines.*

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Intravenous anesthetics for general anesthesia have been reported to exert anesthetic action by binding to subtype A γ-amino butyric acid (GABAA) receptor, inhibiting *N*-methyl-D-aspartic acid (NMDA) receptor activity (1), but can induce neuronal degeneration such as delirium and postoperative cognitive dysfunction (POCD) (2-5). However, it is difficult to study such neuropathy due to the lack of biomarkers for cerebropathy and the difficulty of histological analysis. Anesthetics have been reported to induce toxicity and damage to various tissues and cells (6-11).

Nowadays, intravenous anesthetics are frequently used in the inspection, treatment and palliative care of malignant tumors. Surgery for malignant tumor may stimulate the metastasis and recurrence of the tumor (12). It has been reported that the invasive nature of surgery may lower immunocompetence and cellular immunity (13). Furthermore, anesthetics have been reported to affect the progression of malignant tumor, metastasis and recurrence, depending on the administration method (14). Retrospective cohort studies of patients with breast and prostatic cancer have shown that simultaneous treatment with local anesthesia or analgesics significantly reduced the postoperative recurrence of cancer (15, 16). However, whether the anesthetics presently utilized have antitumor potential is not well-understood. Therefore, investigation of the effect of anesthetics on malignant cells is crucial.

Recently, propofol was reported to induce antitumor activity towards human promyelocytic leukemia (HL-60) (17) and human breast cancer (MDA-MB-231) cells (18) at clinical concentrations (below 50 μM), in addition to its antioxidant, anesthetic and sedative actions (19, 20). However, longer administration of anesthetics may reduce physiological function and cause complications both during and after surgery. Although intravenous anesthetics have been utilized for surgery of malignant head and neck tumors, how they affect malignant and normal cells is not clear. We therefore investigated the relative cytotoxicity of five intravenous anesthetics, namely benzodiazepine derivatives: midazolam and diazepam, barubiturates: thiopental and

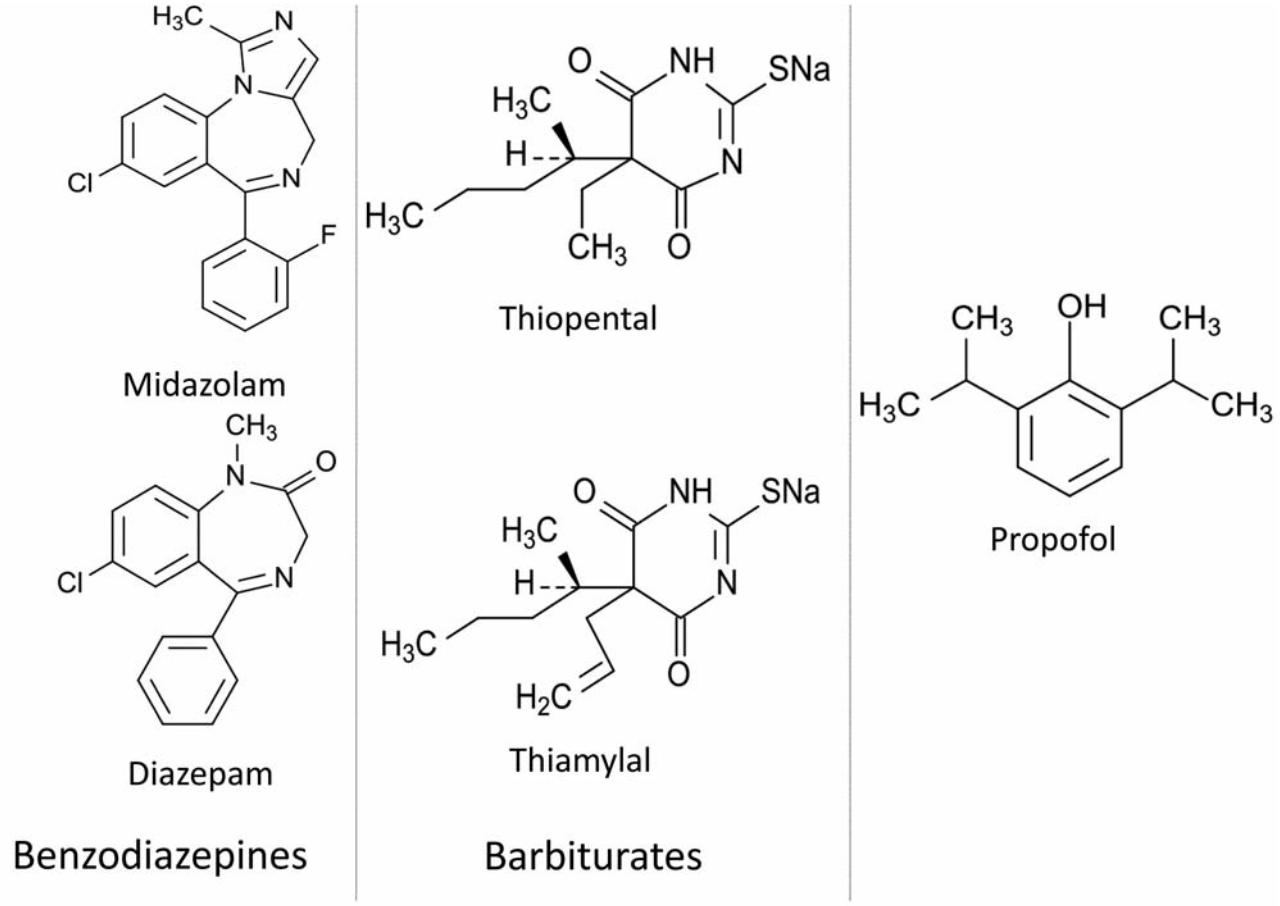

Figure 1. *Chemical structure of five intravenous anesthetics.*

thiamylal, and propofol (Figure 1) towards normal oral cells and malignant tumor cells. Since midazolam exhibited the highest cytotoxicity, the type of cell death induced by midazolam in oral squamous cell carcinoma (OSCC) cell lines was also investigated.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: RPMI-1640, Dulbecco's modified Eagle medium (DMEM) from Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS), 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT), thiamylal sodium (MW=276.3) and 3-methyladenine (3-MA) from Sigma Chem. Ind., St. Louis, MO, USA; dimethylsulfoxide (DMSO), NaI, propofol (MW=178.3), sodium thiopental (MW=264.3), midazolam (MW=325.8) and diazepam (MW=284.8) from Wako Pure Chemicals, Osaka, Japan; RNase A, Proteinase K, ethidium bromide, agarose S (NIPPON GENE Co., Ltd, Toyama Toyama); DNA molecular marker from Bayou Biolabs, Harahan, CA, USA; 100 mm-plastic dish, 24-well plate and 96-microwell plate from Becton Dickinson, Franklin Lakes, NJ, USA); substrates of caspase-3, -8 and -9 [DEVD- *p*-nitroanilide (pNA), IETD-*p*NA, LEHD-*p*NA, respectively] from MBL, Aichi Prefecture, Japan); HuMedia-KG2 from KURABO, Osaka, Japan; bafilomycin A_1 (BAF) from Cosmobio, Tokyo, Japan. Intravenous anesthetics were dissolved in DMSO at 100 mM before use, and diluted with medium.

Cell culture. HL-60 cells (Riken, Tsukuba, Japan) were cultured at 37˚C in RPM-I1640 supplemented with 10% heat-inactivated FBS. Human OSCC cell lines (HSC-2, HSC-3, HSC-4, NA and Ca9-22) were kindly provided by Professor Nagumo and human glioblastoma cell lines (T98G and U87MG) by Dr. Iida, Showa University, Japan. These adherent cells were cultured in DMEM supplemented with 10% heat-inactivated FBS. Normal human oral cells, gingival fibroblast (HGF), pulp cells (HPC) and periodontal ligament fibroblast (HPLF) were prepared from periodontal tissues, according to the guideline of the Intramural Ethic Committee (no. A0808) after obtaining informed consent from the respective 12-years-old patient at the Meikai University Hospital (21). Since normal oral cells have a limited lifespan of 43-47 population doubling-levels (PDL), they were used at 8-15 PDL. Human skin keratinocytes (HEKa, HEKn) (purchased from Kurabo, Osaka,

Table I. *Cytotoxic activity of intravenous anesthetics towards human normal cells, oral squamous cell carcinoma (OSCC) and glioblastoma cells. The 50% cytotoxic concentration (CC_{50}) values were calculated from the dose–response curve shown in Figure 2. Each value represents the mean±S.D. from four or five independent experiments. The log P value was cited from Chem Spider (http://www.chemspider.com/Chemical-Structure).*

	CC_{50} (μM)				
	Benzodiazepines		Barbiturates		
	Midazolam	Diazepam	Thiamylal	Thiopental	Propofol
Log P	3.93	2.80	3.14	2.38	3.83
OSCC					
HSC-2	119±70.8	148±29.1	412±45.9	527±86.4	269±68.8
HSC-3	232±159.0	248±87.2	403±109.1	621±193.1	400±76.8
HSC-4	148±59.8	190±40.4	333±68.4	491±78.9	264±49.6
NA	91.2±48.9	146±54.7	316±105.4	428±129.2	308±79.6
Ca9-22	86.0±46.5	100±35.6	223±97.3	280±93.7	195±24.9
Glioblastoma					
T98G	175±133.1	250±98.7	513±112.4	658±47.7	384±126.9
U87MG	137±67.7	223±173.9	606±89.8	734±102.4	364±59.7
HL-60	43.0±6.0				
Normal cells					
HGF	349±176.5	484±135.1	706±59.9	>854	376±62.5
HPC	281±177.7	362±65.7	662±65.7	>840	371±40.3
HPLF	313±95.8	367±64.2	686±32.5	777±96.6	362±4.5
HEKa	137±33.9				
HEKn	113±17.0				

Japan) were cultured in HuMedia-KG2 supplemented with insulin, human recombinant epidermal growth factor (EGF) (hEGF), hydrocortisone, gentamicin, amphotericin B and bovine pituitary gland extract (BPE).

Assay for cytotoxic activity. All cells were inoculated at 5×10^3 cells/well in 96-microwell plate (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 48 h, the medium was removed by suction with an aspirator, and replaced with 0.1 ml of fresh medium containing different concentrations of the test compounds. The same concentrations of vehicle (DMSO) were added to control. The cells were incubated for another 48 h, and the relative viable cell number was then determined by the MTT method (22). In brief, the cells were washed once with phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(–)], and replenished with fresh culture medium containing 0.2 mg/ml MTT. After incubation for 4 h, the cells were lysed with 0.1 ml of DMSO, and the absorbance at 540 nm of the cell lysate was determined using a microplate reader (Biochromatic Labsystem, Helsinki, Finland). From the dose–response curve, the 50% cytotoxic concentration (CC_{50}) was determined.

Assay for DNA fragmentation. Cells treated with midazolam were lysed by lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsalcosinate]. The lysate was incubated with 0.4 mg/ml RNase A and 0.8 mg/ml proteinase K for 1-2 h at 50˚C, and then mixed with 50 μl NaI solution (7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0), and 100 μl of ethanol. After centrifugation for 20 min at 20,000 ×*g*, the precipitate was washed with 1 ml of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 3-5). The sample (10-20 μl) was then applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0) (22). DNA molecular marker (Takara) and the DNA from HL-60 cells induced to apoptosis by ultraviolet (UV) irradiation were used for calibration. The DNA fragmentation pattern was examined in photographs taken under UV illumination.

Assay for caspase activation. Cells treated with midazolam were washed with PBS(–) and lysed in lysis solution (MBL, Nagoya, Japan). After standing cells for 10 min on ice and centrifugation for 5 min at 10,000 ×*g*, the supernatant was collected. The lysates (50 μl, equivalent to 200 μg protein) were mixed with 50 μl 2× reaction buffer (MBL) containing substrates for caspase-3 (DEVD-*p*NA), caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*NA). After incubation for 4 h at 37˚C, the absorbance at 405 nm of the liberated chromophore pNA was measured by a microplate reader (22).

Autophagy assay. The possibility of autophagy induction was investigated, using two autophagy inhibitors, and by observing the fine cellular structure with electron microscopy described below.

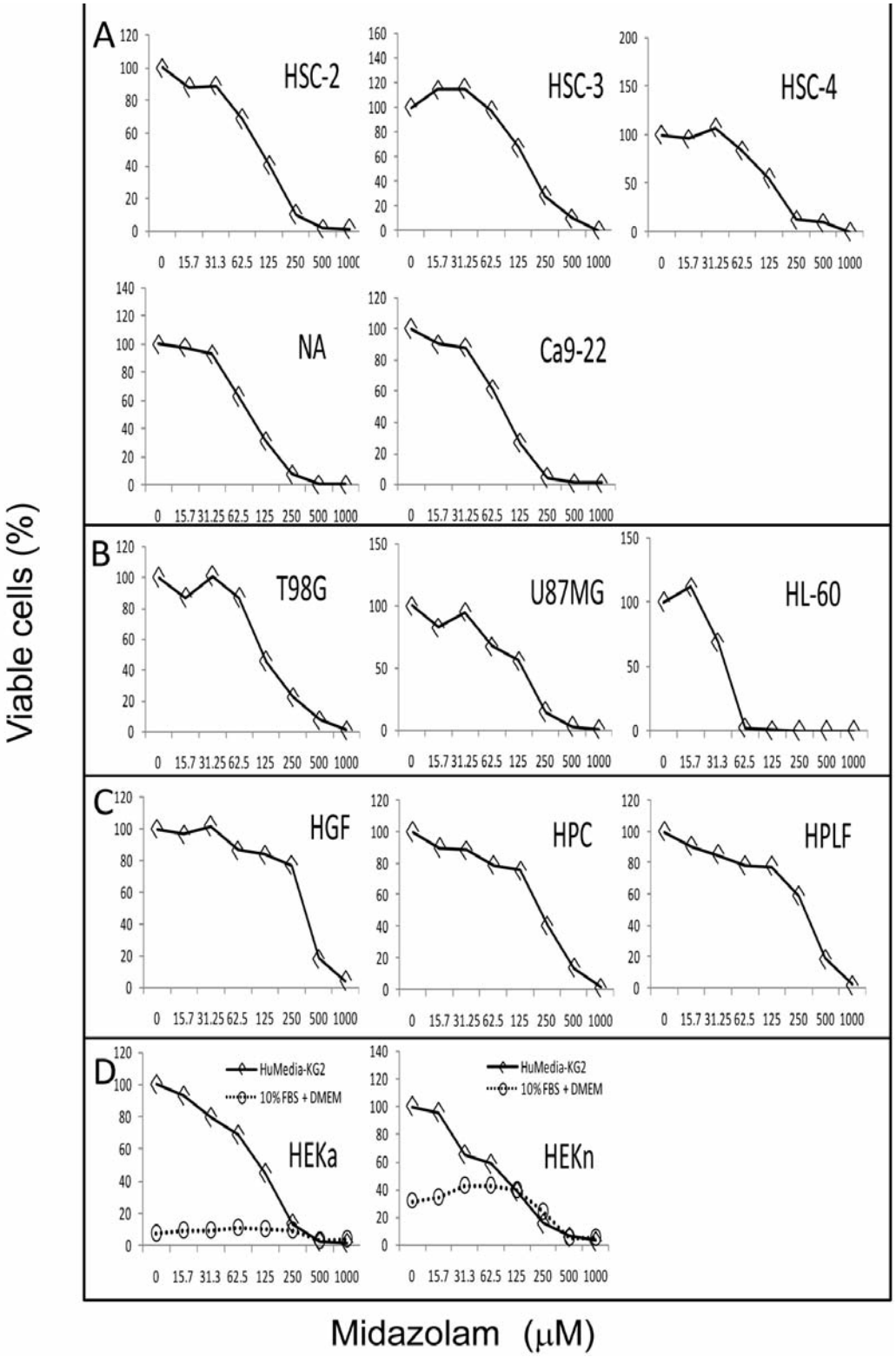

Figure 2. *Cytotoxic activity of midazolam towards human normal and tumor cells. Human oral squamous cell carcinoma (OSCC) (A), T98G, U87MG and HL-60 cells (B), human oral normal cells (C) and human normal epidermal keratinocytes (D) were treated for 48 h with the indicated concentrations of midazolam or without the agent (control). The viable cell number was then determined by 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide method, and expressed as a percentage of that of the control. Each value represents the mean±S.D. from four or five independent experiments.*

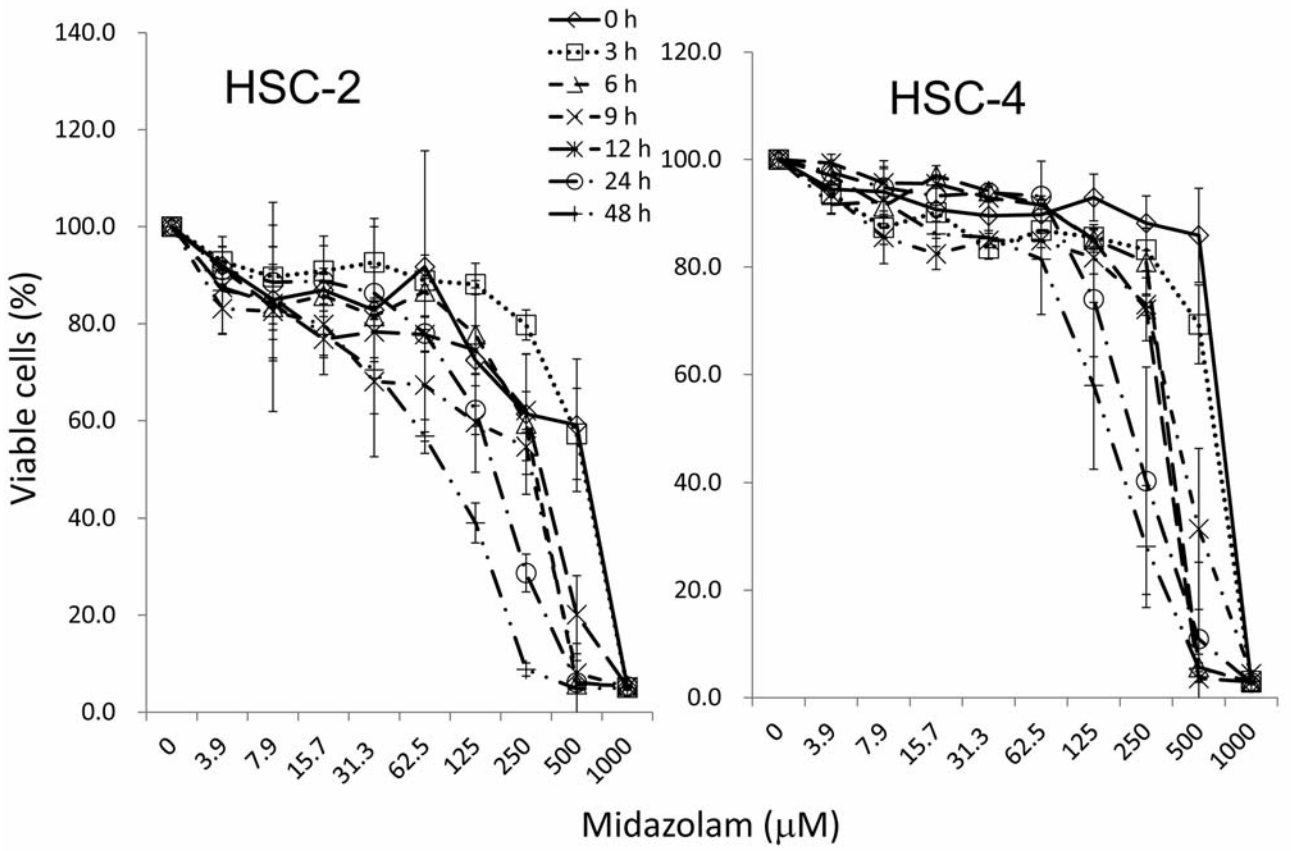

Figure 3. *Effect of treatment time on the induction of cytotoxicity in HSC-2 and HSC-4 cells. Cells were treated for the indicated times with the indicated concentrations of midazolam or without the agent (control), after inoculation of the cells. The viable cell number was then determined by 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide method, and expressed as a percentage to that of the control. Each value represents the mean±S.D. from three independent experiments.*

Electron microscopy. Midazolam-treated cells were harvested by trypsin-EDTA and were pelleted by centrifugation at 1000×*g* for 5 min. The cells were fixed for 1 h with 2% gultaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4˚C, dehydrated and then embedded in Araldite 502 (CIBAGEIGY Swiss; NISSHIN EN Co., Ltd., Tokyo Japan). Fine sections were stained with uranyl acetate and lead citrate, and were then observed under a JEM-1210 transmission electron microscope (JEOL) at an accelerating voltage of 80 kV (23).

Results

Cytotoxicity of local anesthetics. The cytotoxcity of five intravenous anesthetics towards tumor cells was in the order of midazolam>diazepam>propofol>thiamylal>thiopental, while that towards normal oral cells was midazolam> propofol>diazepam>thiamylal>thiopental (Table I). The cytotoxicity of benzodiazepine derivatives (midazolam and diazepam) was found to be higher than that of barubiturates (thiamylal and thiopental). Since midazolam exhibited the highest cytotoxicity, subsequent experiments were carried out with this drug.

Midazolam exhibited dose-dependent cytotoxicity towards human OSCC, globlastoma, human promyelocytic leukemia and normal oral cells (Figure 2). As compared with normal oral cells, OSCC cell lines exhibited relatively higher sensitivity to midazolam. HL-60 cells were the most sensitive to midazolam (Figure 2B). It is most appropriate to use oral keratinocytes for the comparison of midazolam sensitivity of OSCC cell lines. However, it is difficult to establish oral keratinocytes, and therefore we used human skin keratinocytes (HEKa, HEKn) for this comparison. We found that both HEKa and HEKn cells ceased to grow when cultured in regular culture medium (DMEM+10% FBS) (Figure 2D), and it was necessary to culture them in growth factor-enriched medium (HuMedia-KG2). Skin keratinocytes exhibited higher sensitivity to midazolam as compared with normal oral cells when cultured in HuMedia-KG2 (Figure 2D, Table I).

The cytotoxicity of midazolam towards HSC-2 and HSC-4 cells was increased with the treatment time (0-48 h) and concentration (0-1000 μM) (Figure 3). Midazolam had cytotoxic, but not cytostatic effects on the cells.

Type of cell death induced by midazolam in OSCC. Midazolam induced internucleosomal DNA fragmentation in HL-60 cells (used as positive control due to its high sensitivity to various apoptosis inducers), but not in OSCC cell lines (HSC-2, HSC-4), regardless of incubation time (6 or 24 h) (Figure 4). Similarly, midazolam did not activate caspase-3 in HSC-2 and HSC-4 cells, in contrast to significant ($p<0.01$) elevation of caspase-3 activity in UV-induced apoptotic HL-60 cells (Figure 5). These data suggest that midazolam does not induce apoptosis of OSCC cells.

Next, the possibility of autophagy induction was assessed using autophagy inhibitors. Pre-treatment of OSCC cells with either 3-MA or BAF failed to inhibit the cell death of OSCC induced by midazolam (Figure 6). These data suggest that autophagy is not involved in midazolam-induced cell death of OSCC cell lines.

To assess the possibility of induction of other types of cell death, the fine cell structure after midazolam treatment was observed under transmission electron microscopy (Figure 7). When HSC-2 cells were treated for 24 h with 60 μM midazolam, no apparent change in mitochondrial morphology was detected (Figure 7B and 7F). At 120 μM midazolam, mitochondria were damaged with the formation of vacuoles in some parts of the cytoplasm (Figure 7C and 7G). At 240 μM midazolam, swelling of mitochondria with many vacuoles throughout the cytoplasm were observed (Figure 7D and 7H). When HSC-4 cells were treated for 24 h with 75 μM midazolam, mitochondria began to swell (Figure 7J). At higher concentrations (150, 300 μM) of midazolam, mitochondrial damage became much more prominent, with disruption of the plasma membrane and leakage of cellular substrates, and rapid cell breakage (Figure 7K, 7L). Cellular damage of HSC-4 cells, induced by midazolam, was more rapid, than that of HSC-2 cells (Figure 7A-H).

Discussion

Total intravenous anesthesia (TIVA) is a fundamental element of general anesthesia. TIVA uses a combination of opioids (such as morphine, fentanyl and remifentanil) for analgesia, and intravenous anesthetics (such as the ones used here) for sedation to minimize the awakening delay and tissue injury. Although the balanced anesthesia (24) is realized again, the postoperational complications of general anesthesia are still reported (8-11). Opioids (*e.g.* codeine and morphine) have been investigated for their effects on malignant tumor cells (25-27), however, information regarding intravenous anesthesia is limited.

The present study demonstrated that five intravenous anesthetics were cytotoxic towards tumor cells as well as normal cells. To our knowledge, it was revealed here for the first time that benzodiazepine derivatives (midazolam and diazepam) exhibited higher cytotoxicity than barbiturates

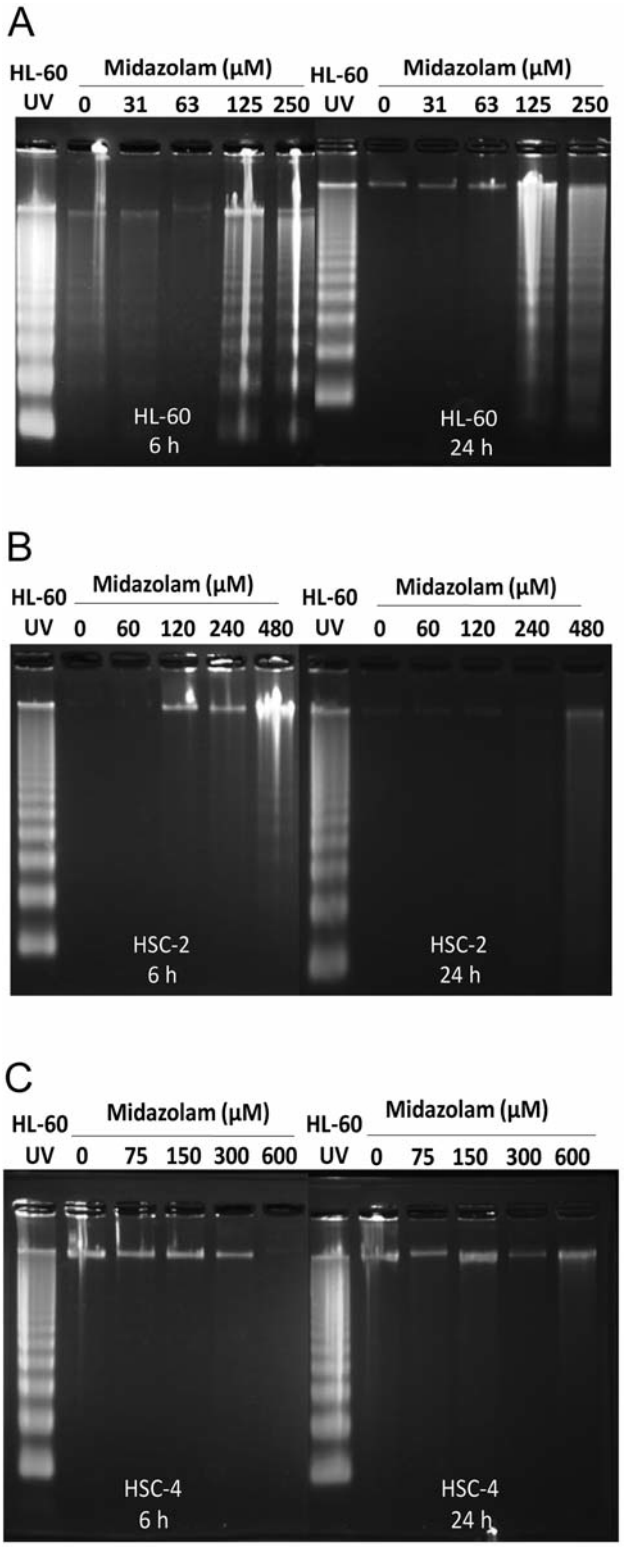

Figure 4. *Effect of midazolam on DNA fragmentation in HL-60 (A), HSC-2 (B) and HSC-4 cells (C). Cells were incubated for 6 or 24 h with the indicated concentrations of midazolam or without the agent (control). DNA was then extracted and subjected to agarose gel electrophoresis. UV: DNA from HL-60 cells induced to apoptosis by UV irradiation.*

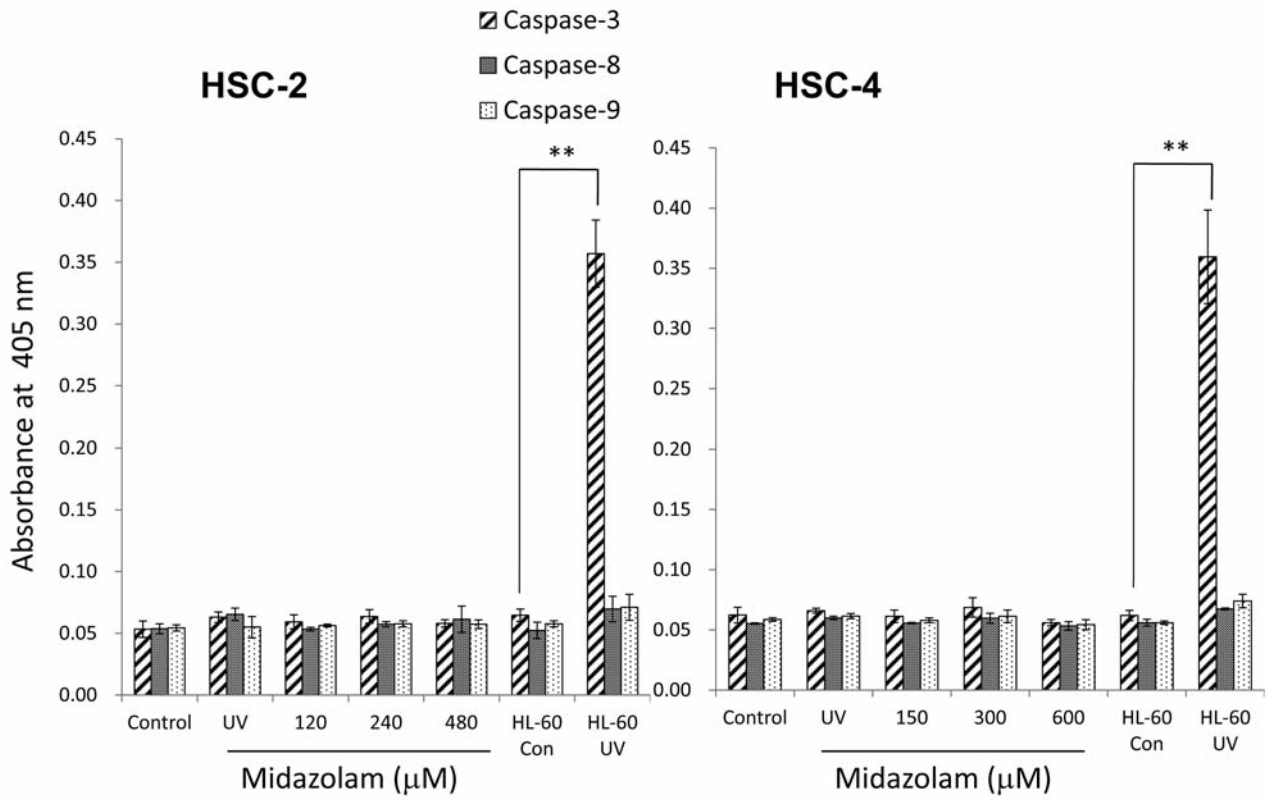

Figure 5. *Effect of midazolam on caspase activity in HSC-2 and HSC-4 cells. Cells were incubated for 24 h with the indicated concentrations of midazolam or without the agent (control) and then assayed for caspase-3, -8 and -9 activity (expressed as absorbance at 405 nm of cleaved product for each substrate). Data are expressed as the mean±S.D. **Significantly different from the HL-60 control (p<0.01). UV: HL-60 cells were exposed to 1 min UV irradiation, followed by 3 h incubation.*

(thiopental and thiamylal). We also found that there was no apparent correlation between the cytotoxicity (as evaluated by CC_{50}) and membrane permeability, as evaluated by octanol:water partition coefficient (log P) of five intravenous anesthetics (Table I). This indicates that membrane permeability may not be involved in cytotoxicity induction by intravenous anesthetics. We found that five intravenous anesthetics were most cytotoxic towards OSCC, followed by glioblastoma and then oral normal cells. Furthermore, midazolam exhibited cytotoxicity towards OSCC, glioblastoma and skin keratinocytes more potently than towards normal oral cells.

The present study demonstrated that (i) midazolam had the highest cytotoxicity among five intravenous anesthetics, but it did not induce apoptosis [internucleosomal DNA fragmentation, caspase-3 activation, hallmarks of apoptosis (28)]; (ii) the cytotoxicity of midazolam was not affected by pre-treatment with autophagy inhibitors (3-MA, BAF); and (iii) the electron microscopy study demonstrated that midazolam induced mitochondrial swelling, injury and disruption of plasma membrane without induction of autophagosome which is characteristic of autophagy. These data suggest that midazolam may induce necrotic cell death of HSC-2 and HSC-4 cells. On the other hand, it has been reported that inhalation anesthetics induced apoptosis of normal peripheral lymphocytes (10). Propofol (at less than 50 μM) induced apoptosis of human promyelocytic leukemia (HL-60) (17), human breast cancer cell (MDA-MB-231) (18) and lipopolysaccharide-treated mononuclear cells and lymphocytes (29). Thiopental induced apoptosis of Jurkat T-cell leukemia cells (30). Furthermore, even midazolam, at 150 μM, a concentration higher than the one clinically used, induced apoptosis of mouse Leydig cells (31). These data indicate that the type of cell death induced may differ depending on the type of both the anesthetics used and target cell.

Propofol (28 μM) has been reported to inhibit the infiltration of cultured tumor cell lines (HeLa, HOSm RPMI-7951, HT1080), suggesting its potential for inhibiting the infiltration and metastasis of tumor cells (32). However, at the concentration much higher than that used clinically, we found that propofol exhibited potent cytotoxicity towards both tumor and normal cells. This suggests the necessity of investigating its side-effects.

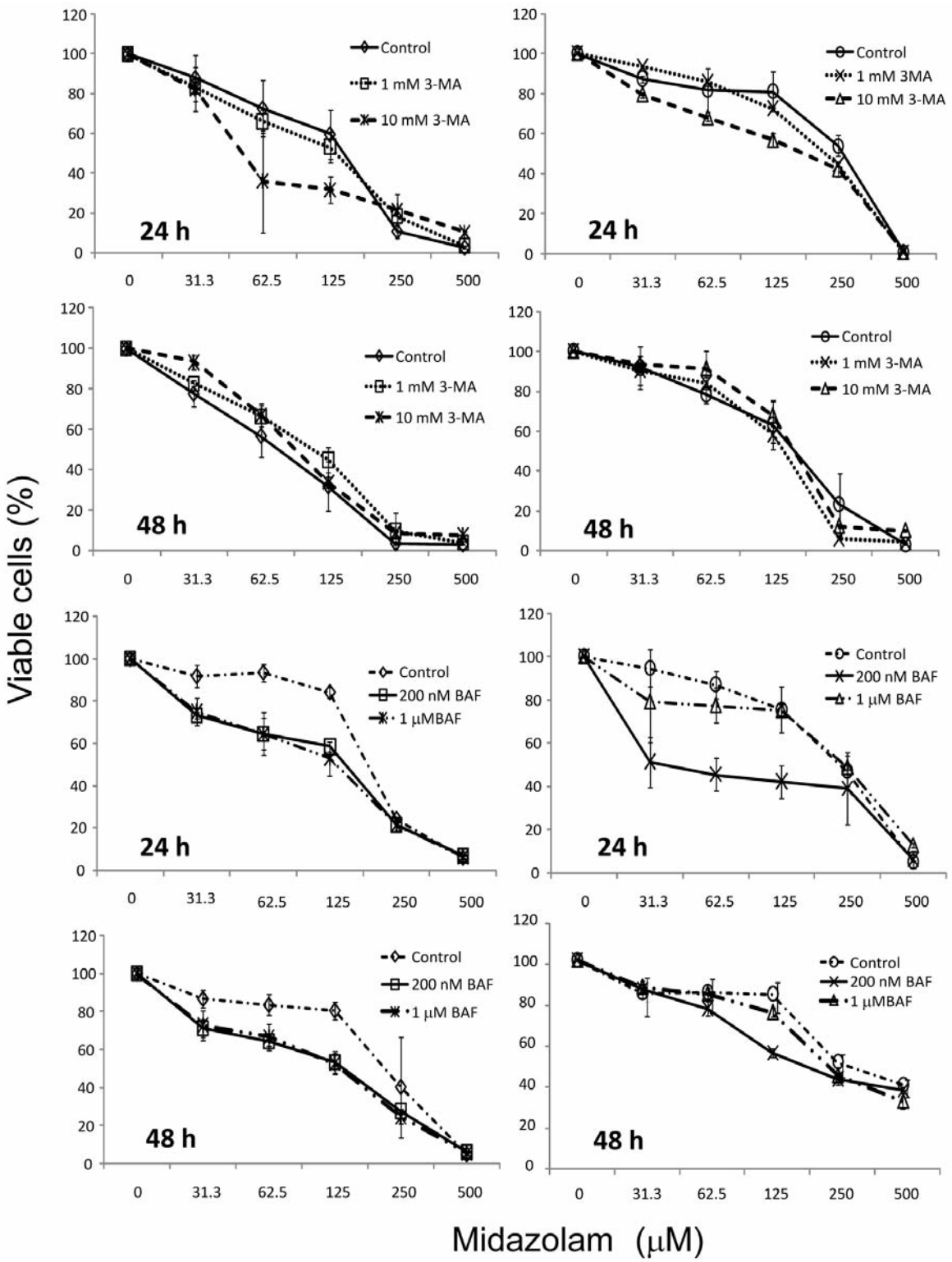

Figure 6. *Effect of autophagy inhibitors 3-methyladenine (3-MA) and bafilomycin A1 (BAF) on midazolam-induced cytotoxicity towards HSC-2 and HSC-4 cells. HSC-2 and HSC-4 cells were pre-treated for 1 h with the indicated concentrations of autophagy inhibitors, and then incubated for 24 or 48 h with the indicated concentrations of midazolam or without the agent (control). The viable cell number was then determined by 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide method, and expressed as a percentage to that of the control. Each value represents the mean±S.D. from triplicate determinations.*

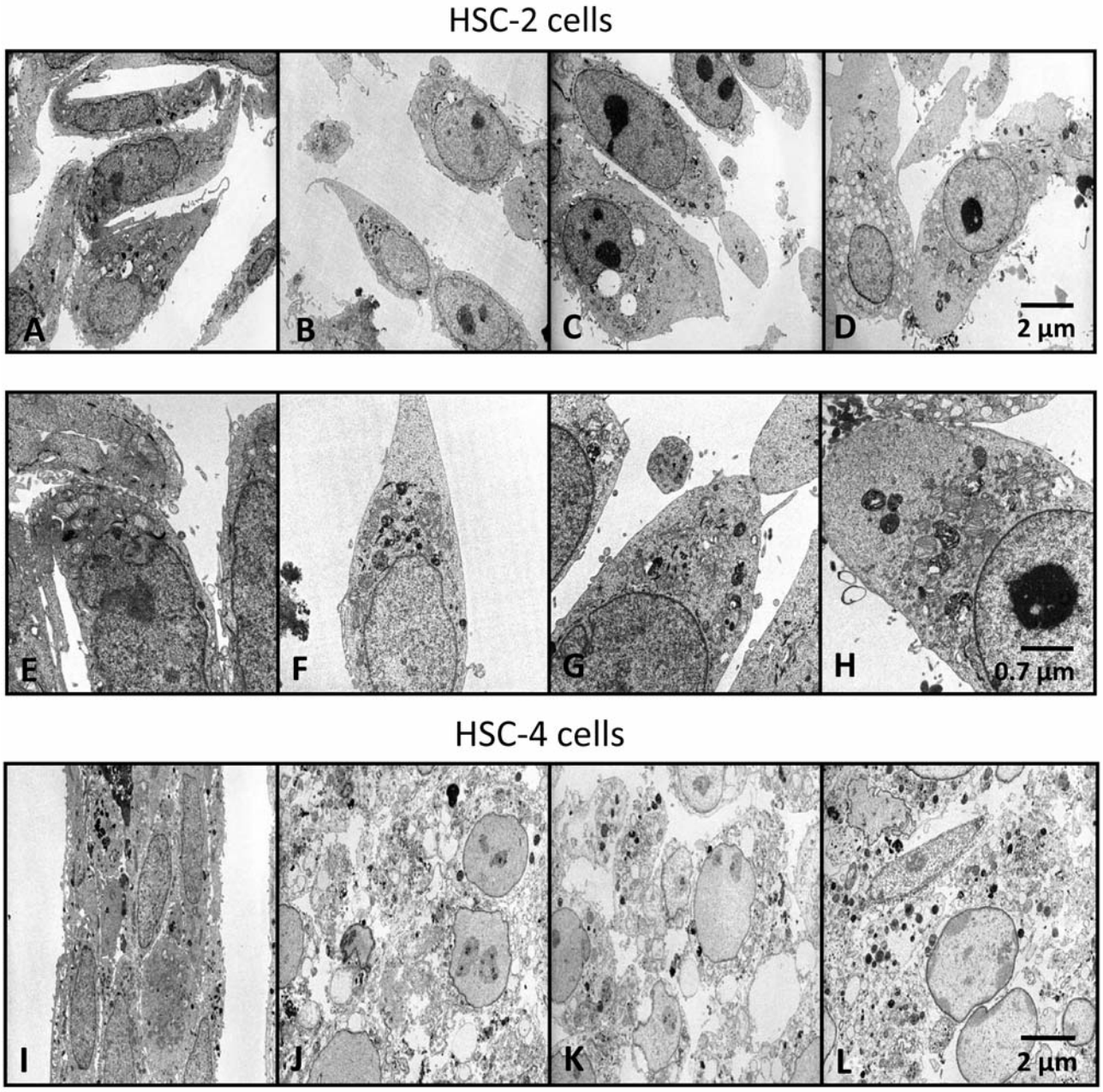

Figure 7. *Changes in the fine cell structure of oral squamous cell carcinoma cells induced by midazolam. HSC-2 (A-H) and HSC-4 (I-L) cells were treated for 24 h with the indicated concentrations of midazolam or without the agent (control) and then processed for electron microscopy. HSC-2: control (A, E); 60 μM (B, F); 120 μM (C, G); 240 μM (D, H); HSC-4: control (I); 75 μM (J); 150 μM (K); 300 μM (L).*

Diazepam has been reported to enhance the cytotoxic activity of lonidamin towards five human glioma cell lines (SNB-56, SNB-78, SNB-19, TG-8-OZ and U251) (33) and that of tegafur, a pro-drug of 5-fluorouracil against mouse sarcoma 180 (34). However, the interaction between anesthetics and anticancer drugs is not clear (35). General anesthesia has been used in most surgical treatments for malignant tumors. Surgery greatly affects the prognosis of patients, and may trigger metastasis and recurrence of the tumor (12), possibly due to the influx of tumor cells into the blood, reduced production of anti-vascularization factors and invasion due to stress-induced immunosuppression (36-44). We found that the cytotoxicity of five intravenous anesthetics was only apparent at much higher concentrations than those

used clinically. Surgery-induced direct invasion, bleeding, hypotension, transfusion, hypothermia and elevation of blood nitric oxide concentration may all reduce the threshold of intravenous anesthetics, so as to cause their cytotoxicity to be manifested at much lower concentrations. Recently, mortality has been reported to be increased with the depth of anesthesia (45). In order to prevent cytotoxicity due to anesthesia, it is essential to maintain an optimal depth of anesthesia by monitoring the bispectral index.

In conclusion, the present study suggests that midazolam induces necrotic cell death, rather than apoptosis or autophagy, in OSCC cell lines. The side-effects of midazolam due to injury towards normal cells should be monitored.

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