J Anesth (2012) 26:675–681
DOI 10.1007/s00540-012-1427-1

ORIGINAL ARTICLE

The effects of intravenous anesthetics on mouse embryonic fibroblast viability and proliferation

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Received: 18 October 2011 / Accepted: 25 April 2012 / Published online: 20 June 2012

Abstract
Purpose The aim of this study is to evaluate the cytotoxic and antiproliferating effects of intravenous anesthetics on an mouse fibroblast in vitro cell culture system.
Methods The cells were exposed to the usual clinical plasma concentration of intravenous anesthetics, i.e., midazolam (0.15 μg/ml), propofol (2 μg/ml), remifentanil (2 μg/ml), thiopental (10 μg/ml), for 4, 8, or 24 h. Cell proliferation ($n = 6$ for each) under intravenous anesthetics was analyzed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cytotoxicity ($n = 6$ for each) of intravenous anesthetics was investigated using a LIVE/DEAD viability assay kit.
Results Intravenous anesthetic exposure time did not affect the proliferation rate of mouse fibroblasts. The cytotoxicity of intravenous anesthetics did not differ in accordance with exposure time.
Conclusion Our results showed that intravenous anesthetics may not affect mouse fibroblast proliferation and viability.

Keywords Fibroblast · Midazolam · Propofol · Remifentanil · Sedation · Transplantation

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Introduction

A fibroblast is a type of connective tissue cell that synthesizes the extracellular matrix rich in collagen and other macromolecules which maintain the structural framework (stroma) for many tissues [1]. Fibroblasts have a morphologically heterogeneous appearance according to their diverse location and activity. For example, in the heart, composed of cardiac myocytes and nonmyocytes, particularly fibroblasts, cardiac fibroblasts lead to interstitial fibrosis with proliferation and increase of the deposition of extracellular matrix proteins [2–4] and play a consequential role in the enhancement of intrinsic myocardial stiffness, resulting in diastolic dysfunction [2, 3, 5, 6]. In the lung, fibroblast proliferation performs an important function in the late phase of acute lung injury [7]. In addition, fibroblasts play an important role in wound healing [1]. To consider these characteristics may have potential therapeutic benefits on clinical use. A previous report recommended the transplantation of genetically engineered cardiac fibroblasts to produce recombinant human erythropoietin for repairing the infarcted myocardium [8]. Further, double transplantation of gene-nucleofected fibroblasts can be used to promote collateral vessel growth and remodeling of circulation in ischemic limbs [9]. In addition, autologous cultured fibroblast injection as cellular therapy can be considered for facial contour deformities

[10, 11]. Therefore, induction and maintenance of general anesthesia as well as sedation may be needed during these managements. However, the effect of intravenous anesthetics on fibroblasts is controversial.

Intravenous anesthetics including midazolam, ketamine, thiopental, and propofol do not have an antiproliferative effect on lung fibroblasts [12]. However, one recent study suggests that propofol prevents cardiac fibroblast proliferation by interfering with the generation of reactive oxygen species [13]. The conflicting results may be, in part, caused by the differences of investigated cell types. Another reason may be suspected to be the difference in the propofol concentration and time for incubation. However, the direct effect of intravenous anesthetics on proliferation of fibroblasts during various times remains unclear.

The aim of this study is to examine the effect of thiopental, remifentanil, midazolam, and propofol on cytotoxicity and proliferation of mouse embryonic fibroblasts using an in vitro culture system. We selected a fixed plasma drug concentration that is commonly used in clinical practice and followed up cell cytotoxicity and proliferation for 4, 8, and 24 h.

Methods

Materials

Cell culture studies were conducted using a mouse embryonic fibroblast cell line (MEF) obtained from the Department of Biomedical Engineering in Korea University (from Yongdoo Park, PhD). Clinical doses of midazolam (Midazolam inj®; Bukwang Pharmaceutical, Seoul, Korea) [14], propofol (Fresofol®; Fresenius Kabi Korea, Seoul, Korea) [15], remifentanil (Ultiva®; GlaxoSmithKline Pharmaceuticals, Uxbridge, UK) [16], and thiopental sodium (Pentothal Na®; Jungwae Pharmaceuticals, Seoul, Korea) [17] were used in this study (Table 1).

Cell culture

Equal numbers of MEF cells (1×10^4 cells/well) were plated onto 96-well microplates. Eagle's minimum essential medium (Invitrogen, Carlsbad, CA, USA) provided with 10 % horse serum, streptomycin (100 μg/ml), and penicillin (100 U/ml) was used to culture fibroblasts. Cells with 80–85 % confluence were obtained from T-flask cultures by trypsinization and incubated onto the disk samples. Briefly, cells were washed with phosphate-buffered saline (PBS), incubated with 0.25 % trypsin/0.53 mM ethylenediaminetetraacetic acid (EDTA) for 5–7 min to detach the cells from the Petri dish, dispersed in trypsin/EDTA, transferred to a centrifuge tube, and centrifuged at 322 *g* for 5 min.

Table 1 The concentration of intravenous anesthetics

Intravenous anesthetic	Concentration (μg/ml)
Midazolam	0.15
Propofol	2
Remifentanil	2
Thiopental	10

Cell cytotoxicity and proliferation

The initial fibroblast attachment and viability on different wells was calculated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at 4, 8, and 24 h as previously described [18, 19]. The samples were washed twice with PBS and incubated with fresh culture medium containing MTT (0.5 mg/ml medium) at 37 °C for 4 h in the dark. The unreacted dye was then removed, and dimethyl sulfoxide (DMSO) was added to release the intracellular purple formazan product into solution. The absorbance of this solution was quantified by photospectrometry at 570 nm with a plate reader (Bio TEK Instrument EL307C). The cell proliferation rate (%) was calculated and compared with the control group. Moreover, cytotoxicity was investigated using a LIVE/DEAD viability assay kit (Molecular Probes) according to the manufacturer's instructions. Fibroblasts were incubated with two probes, calcein-AM (green color) and ethidium homodimer-1 (EtdD-1, bright red color), for intracellular esterase activity and plasma membrane integrity, respectively. Then, specimens were observed under a three-dimensional (3D) fluorescence microscope (Olympus SZX12 stereomicroscope; Olympus, Japan). All experiments were performed in triplicate.

Statistical analysis

Results are expressed as mean ± SD. Statistical analysis was performed with one-way analysis of variance (ANOVA), followed by the Kruskal–Wallis test, using SigmaStat 3.5 for Windows (Systat Software, Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

Result

In each well, all fibroblasts showed viability and proliferation. Proliferation rate under intravenous anesthetics during various times was showed as percent of control (Fig. 1). The proliferation rates (% of control) of 4-h

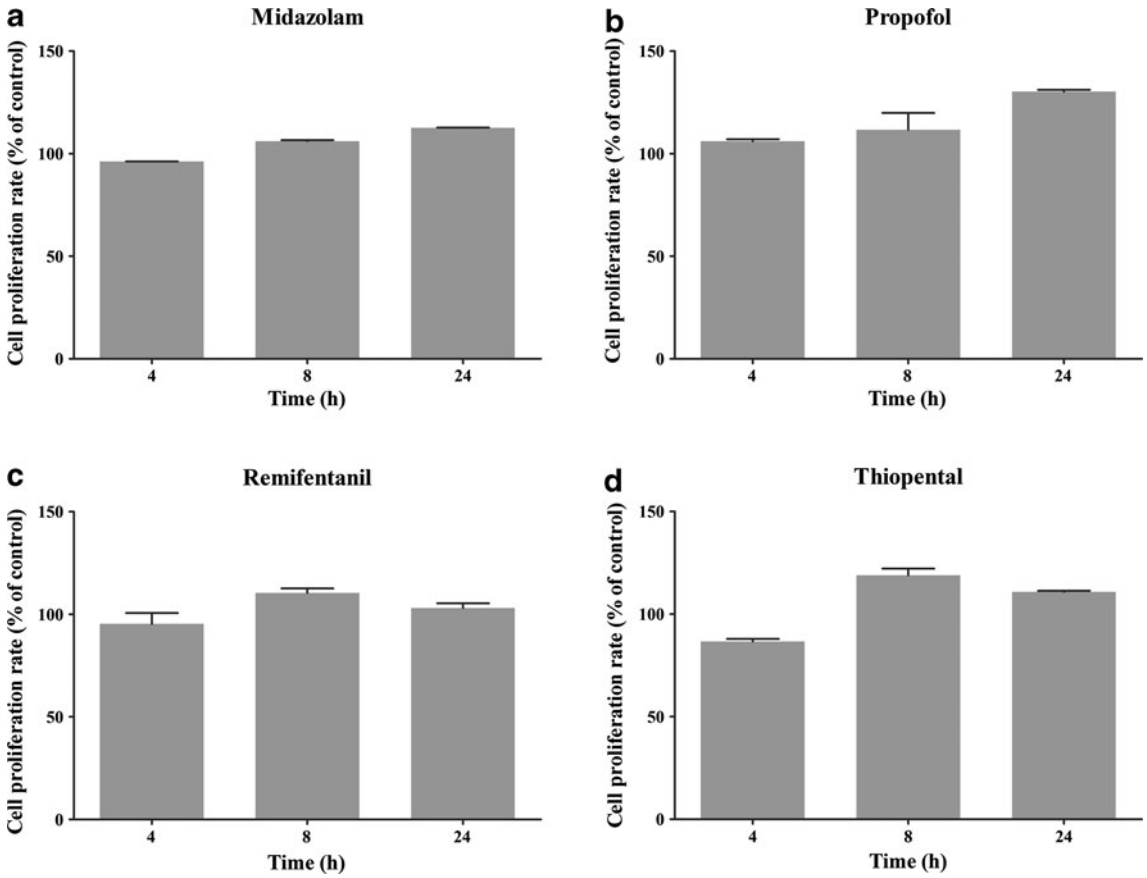

Fig. 1 Direct effects of midazolam (**a**), propofol (**b**), remifentanil (**c**), and thiopental (**d**) on proliferation rate during various times. Data (% of control) presented as mean ± SD ($n = 6$)

incubation groups ($P = 0.143$) are as follows ($n = 6$): control, 0.28 ± 0.17; midazolam, 0.27 ± 0.02 (96 %); propofol, 0.3 ± 0.02 (106 %); remifentanil, 0.27 ± 0.04 (96 %); and thiopental, 0.24 ± 0.02 (87 %), respectively. Proliferation rates (% of control) of 8-h incubation groups ($P = 0.586$) are as follows ($n = 6$): control, 0.33 ± 0.02; midazolam, 0.3 ± 0.02 (90 %); propofol, 0.32 ± 0.06 (96 %); remifentanil, 0.31 ± 0.03 (94 %); and thiopental, 0.34 ± 0.04 (101 %), respectively. Proliferation rates (% of control) of 24-h incubation groups ($P = 0.082$) are as follows ($n = 6$): control, 0.33 ± 0.02; midazolam, 0.32 ± 0.02 (96 %); propofol, 0.37 ± 0.03 (111 %); remifentanil, 0.29 ± 0.03 (88 %); and thiopental, 0.31 ± 0.02 (95 %), respectively. There was no statistical difference between control group and experimental groups at each time point.

Furthermore, fibroblasts that were exposed to variable anesthetics for 24 h are shown with calcein-AM (green color) in Fig. 2 and with ethidium homodimer-1 (EtdD-1, bright red color) in Fig. 3. Cytotoxicity under intravenous anesthetics during various times was shown as percent of control (Fig. 4). The cytotoxicity (live cell % of total cell count) of 4-h incubation groups is as follows ($n = 6$): control, 94.8 ± 2.87; midazolam, 95.0 ± 2.53; propofol, 94.6 ± 2.11; remifentanil, 95.0 ± 2.53; and thiopental, 95.9 ± 2.3, respectively. Cytotoxicity (live cell % of total cell count) of 8-h incubation groups is as follows ($n = 6$): control, 93.6 ± 3.06; midazolam, 93.1 ± 2.74; propofol, 94.4 ± 2.19; remifentanil, 95.0 ± 2.53; and thiopental, 93.8 ± 3.18, respectively. Cytotoxicity (live cell % of total cell count) of 24-h incubation groups is as follows ($n = 6$): control, 95.0 ± 3.44; midazolam, 95.7 ± 2.84; propofol, 96.2 ± 2.22; remifentanil, 95.2 ± 3.04; and thiopental, 94.9 ± 3.4, respectively. There was no statistical difference between control group and experimental groups at each time point.

Discussion

Our results showed that intravenous anesthetics in the clinical dose of plasma concentration might not affect the proliferation and viability of mouse fibroblast.

If intravenous anesthetics have cytotoxic effects on fibroblasts, the fibroblasts may go through necrosis, in which they undergo loss of membrane integrity and die as a

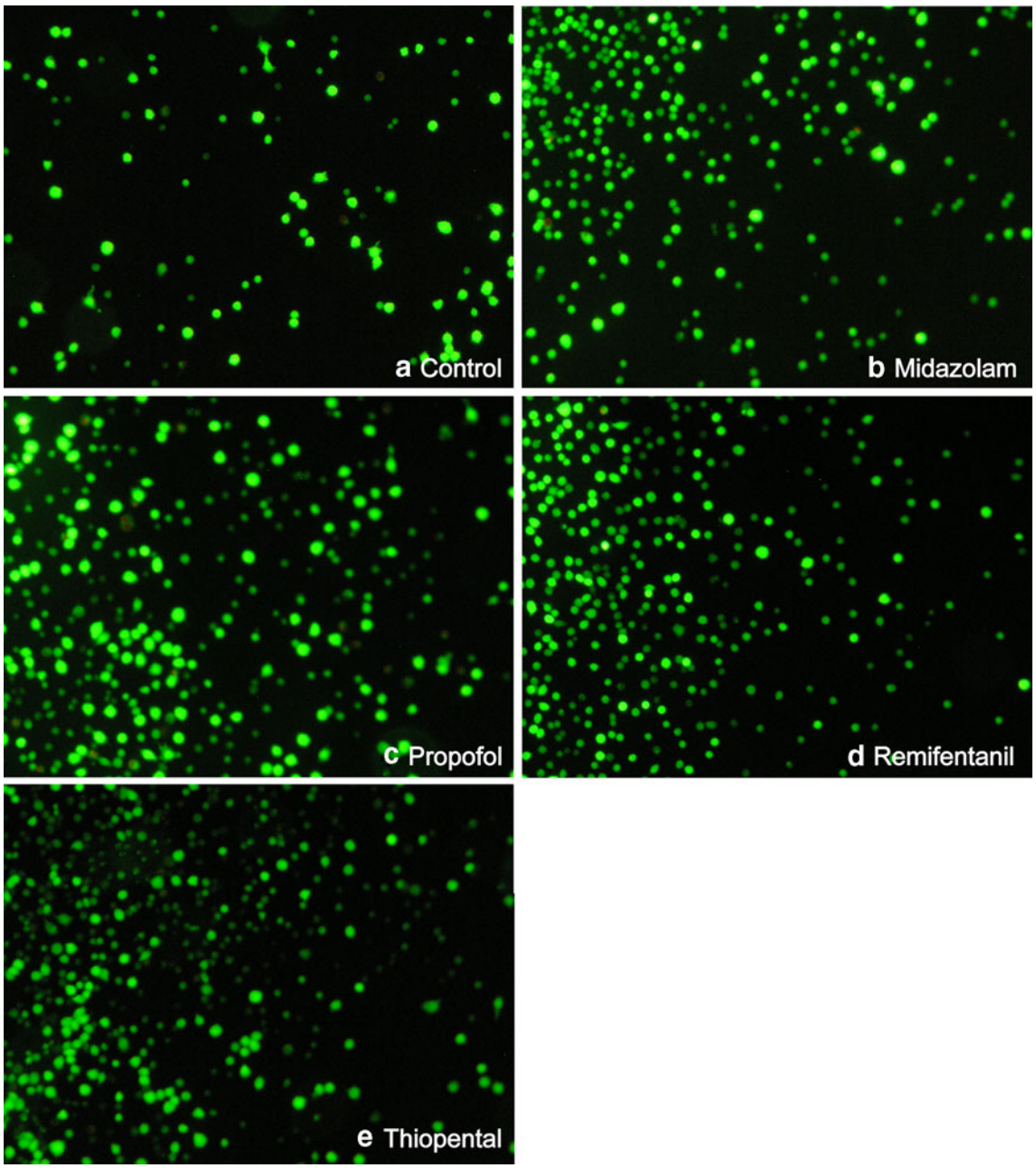

Fig. 2 Fibroblasts incubated with calcein-AM (*green*), exposed to control (**a**), midazolam (**b**), propofol (**c**), remifentanil (**d**), and thiopental (**e**) for 24 h

direct result of cell lysis; or, the fibroblasts can stop actively growing and dividing (a decrease of cell viability); or, the fibroblasts can activate a genetic process of controlled cell death (apoptosis). These fates of fibroblasts may have an influence on transplanted fibroblast survival and proliferation. Accordingly, the cytotoxic and antiproliferative effect of intravenous anesthetics on transplanted fibroblasts may be considered by the anesthesiologist and surgeon performing fibroblast transplantation.

Several studies have revealed that some intravenous anesthetics have an antiproliferative effect on diverse mature cells. Chanimov et al. [20] showed that thiopental sodium, etomidate, fentanyl, and lidocaine significantly hindered phytohemagglutinin P-induced 3H-thymidine incorporation of rat peripheral blood mononuclear cells in culture. They also reported that propofol, midazolam, and ketamine seemed to suppress lectin-induced cell proliferation, but there was no statistical difference. Similarly, it is

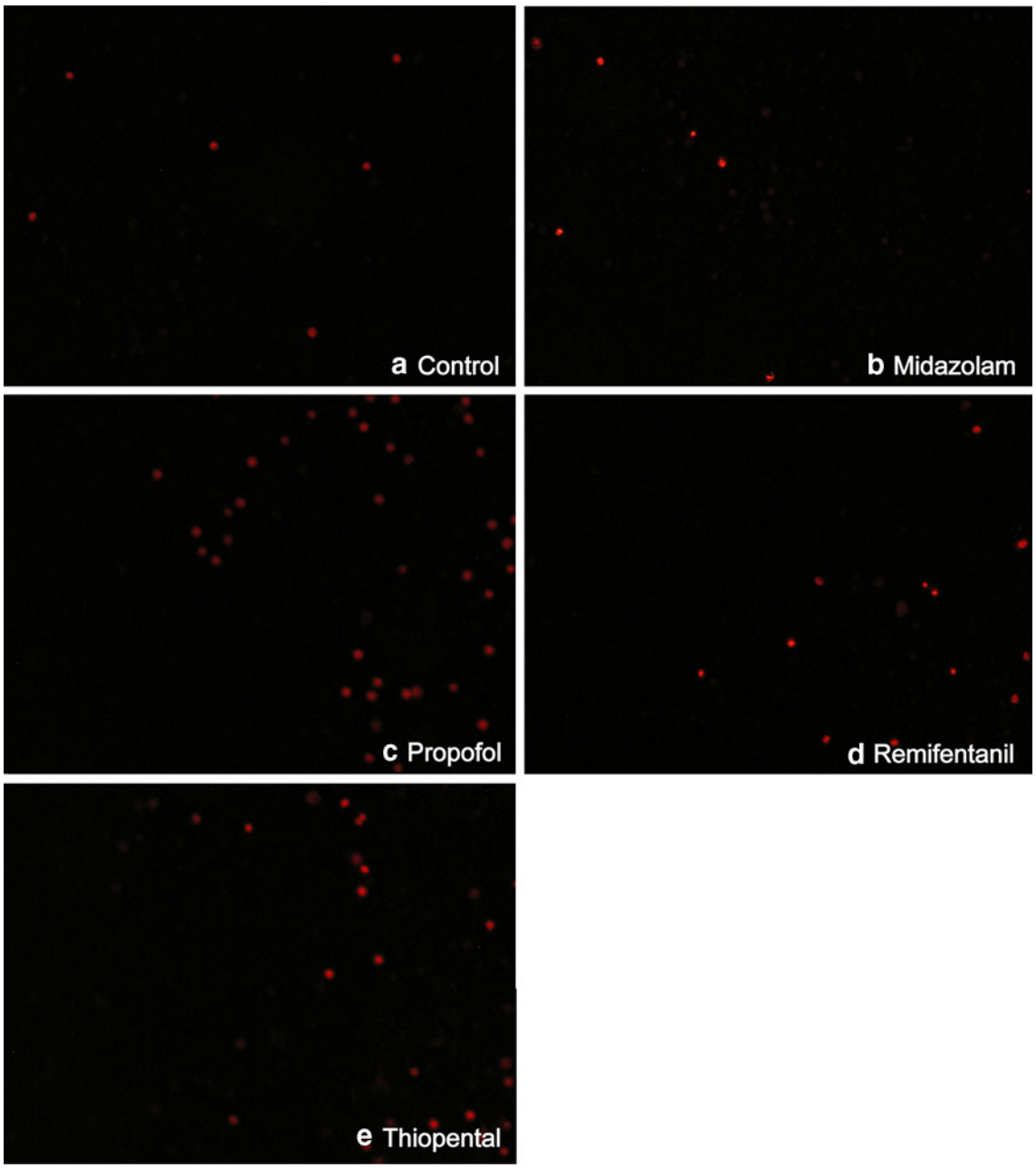

Fig. 3 Fibroblasts incubated with ethidium homodimer-1 (EtdD-1; *bright red*), exposed to control (**a**), midazolam (**b**), propofol (**c**), remifentanil (**d**), and thiopental (**e**) for 24 h

reported that ketamine inhibited rat mesangial cell proliferation but propofol did not [21], because ketamine has an inhibitory effect on IL-l, IL-6, and TNF-α, which leads to mesangial cell proliferation.

The effect of intravenous anesthetics on immature cells is controversial. Intravenous anesthetics such as midazolam, propofol, thiopental, and ketamine do not seem to have an antiproliferative effect on fibroblasts [12]. However, one recent study suggested that propofol prevents fibroblast proliferation by interfering with the generation of reactive oxygen species [13]. The conflicting results may be, in part, caused by the differences of investigated cell types. Another reason we think may be the difference in propofol concentration and time for incubation. In the previous fibroblast study, they used a propofol concentration of 5, 50 and 500 μg/ml and incubated the cells for 48 h. In another study, they used a propofol concentration of 1, 3, 10, and 30 μg/ml and a 24-h incubation. In the present study, we simulated actual clinical practice. The authors selected clinically relevant serum concentrations of

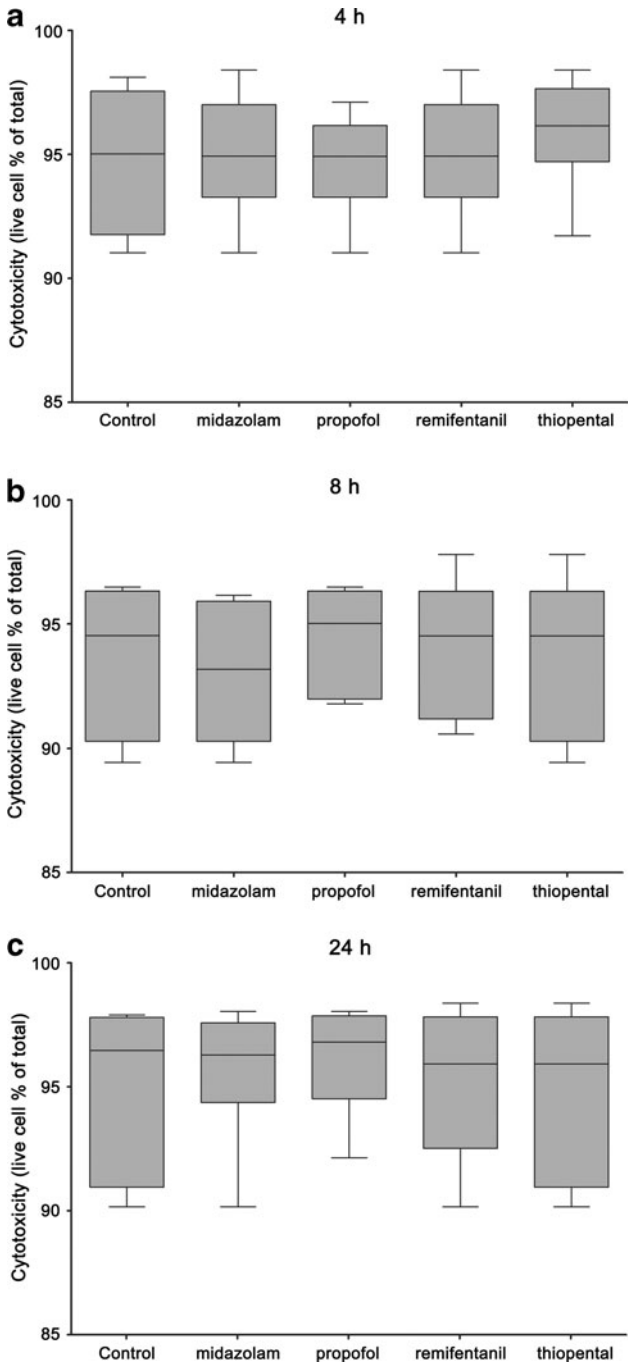

Fig. 4 Cytotoxicity (live cell % of total cell count) under intravenous anesthetic during various times: 4 h (**a**), 8 h (**b**), and 24 h (**c**). Data (% of control) presented as mean ± SD ($n = 6$). The *line within the box plots* indicates the mean value; the *lower and upper sides of the box* represent the 25th and 75th percentiles; the *error bars* indicate the 10th and 90th percentiles

intravenous anesthetics for sedation or general anesthesia and incubated the cells for variable times. In addition, we selected more immature fibroblasts, i.e., embryonic fibroblasts, to evaluate the effect of anesthetics on proliferation of the fibroblasts.

However, this study has limitations. Within a living body, the amount of drug needed to reach the target concentration may vary because of metabolism or distribution, especially protein binding. In addition, we used variable doses of anesthetics according to clinical conditions. As a result, the effects of anesthetics that act on fibroblasts in vitro can be different from those in vivo.

Our preliminary results showed that intravenous anesthetics may not affect mouse fibroblast proliferation and viability in an in vitro culture system. However, further study is needed to determine the effect of anesthetics in fibroblast proliferation and viability in humans.

Acknowledgments This study was supported by Korea University Research Grant.

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