Possible Effect of Muscle-relaxant Anaesthetics on Invasion, Adhesion and Migration of Breast Cancer Cells

AIHUA JIANG[1,2], HUISHAN ZHAO[2,3,4], JUN CAI[2] and WEN G. JIANG[2*]

[1]*Department of Anesthesiology, Yuhuangding Hospital, Medical College, Qingdao University, Yantai, P.R. China;*
[2]*Cardiff–China Medical Research Collaborative, Cardiff University School of Medicine, Heath Park, Cardiff, U.K.;*
[3]*Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Beijing Key Laboratory for Cancer Invasion and Metastasis Research, Cancer Institute of Capital Medical University, Beijing, P.R. China;*
[4]*Capital Medical University–Cardiff University Joint Centre for Biomedical Research, Beijing International Cooperation Base for Science and Technology on China–UK Cancer Research, Capital Medical University, Beijing, P.R. China*

Abstract. *Background: Aggressive surgical removal of the primary tumour is the preferred treatment, but with tumour progression, some tumours cannot be completely removed surgically. Anaesthetics are administered to facilitate surgery. However, anaesthetics act as a potential factor in tumour recurrence or metastasis. Materials and Methods: Normal breast cells and cancer breast cells were treated with different doses of muscle-relaxant anaesthetics. The effects on breast cancer cell invasion, adhesion and migration of these anaesthetics were then investigated using in vitro models. Results: With increasing dose of rocuronium bromide and suxamethonium chloride CRS, the number of MCF-10A and MCF-7 cells, but not that of MDA-MB-231 cells, decreased. There was almost no difference in the number of cells when the three cell lines were treated with different doses of vecuronium bromide. The study also demonstrated that rocuronium bromide promoted the invasion, adhesion and growth of MDA-231 cells, while suxamethonium chloride CRS had no effect. Interestingly, vecuronium bromide did not affect the motility and invasion of breast cancer cells significantly. Conclusion: An understanding of the effect of anaesthetics and their impact on tumour metastasis is important, thus using an appropriate aesthetic strategy could improve long-term survival in some patients.*

Correspondence to: Professor Wen G. Jiang, Cardiff-China Medical Research Collaborative, Cardiff University School of Medicine, Heath Park, Cardiff CF14 4XN, U.K. E-mail: jiangw@cf.ac.uk

Key Words: Anaesthetics, metastasis, breast cancer, invasion, vecuronium bromide, vecuronium bromide, suxamethonium chloride CRS.

Breast cancer is the most common life-threatening disorder in females, with low survival rates due to metastatic lesions. The conventional treatment of breast tumour includes routine surgery, radiotherapy, hormone therapy and biotherapy. However, with tumour progression, some tumours cannot be completely surgically removed (1, 2). Factors including surgery, anaesthetics *per se* (3), stress response (4), acute postoperative pain, and opioid analgesics may be related to cancer recurrence or metastasis.

Tumour metastasis results from the unregulated and abnormal proliferation of primary tumour cells that can invade other tissues through the blood and lymphatic systems (5). The increasing rate of recurrence or metastasis puts an enormous responsibility on the anaesthesiologist, both during the perioperative period and during chronic cancer pain management. Anaesthetics may act as a potential factor in tumour metastasis (6, 7). Muscle relaxants, also called neuromuscular-blocking agents, block the nerve impulses to the muscles and are always used for general anaesthesia in surgery.

Recently, some anaesthetics have been suggested to have an impact on the metastasis of cancer. Some studies demonstrated that anaesthetics play an active role in tumour metastasis. Experimental evidence from a mouse model of breast cancer suggested that thiopental, ketamine and older volatile anaesthetics might promote tumour metastasis (8). Another study on breast cancer cell lines (MDA-231 and MCF-7) showed that morphine not only increased the migration of breast cancer cells but also induced higher expression of neuroepithelial cell transforming 1 (*NET1*) gene that is associated with cell migration (9). A review by Tavare *et al.* showed that hypoxia-inducible factor (HIF1α) was up-regulated in neoplastic cells when treated with volatile anaesthetics; HIF1α is associated with angiogenesis

and poor patient prognosis (10). However, some researchers showed that anaesthetics may act as inhibitors in the metastasis of the tumour. An experimental study suggested that isoflurane exposure (30 min at 1.2%) inhibited colon cancer cell growth by inducing apoptosis (11). Some volatile agents also modulated gene expression of two separate tumour cell lines *in vitro*: breast carcinoma and neuroblastoma (12). Furthermore, Muller-Edenborn *et al*. also found that pre-treating human neutrophils with desflurane and sevoflurane reduced the expression of matrix metallopeptidase 9 in colorectal cancer cells. MMP9 belongs to a family of multidomain, zinc-containing neutral endopeptidases which contribute to forming a microenvironment that promotes tumour metastasis during early stages of tumorigenesis (13, 14). In short, invasion and migration of colon cancer cells were inhibited (15).

There is scarce research on the effects of muscle relaxants on tumours, and whether they could affect tumour metastasis is not clear. We hypothesized that muscle relaxants may play a role in breast cancer cell metastasis. In this study, we studied the role it has in the development of breast tumour progression.

Materials and Methods

Cell culture. MCF-7 and MDA-231 cells are oestrogen receptor (ER)-positive and ER-negative breast cancer cells, respectively, and MCF-10A served as an example of normal breast epithelial cell. Cell lines were bought from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). MCF-7 and MDA-MB-231 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 HAM with L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) supplemented with penicillin, 10% foetal calf serum (FCS) (PAA Laboratories, Pasching, Austria) and streptomycin (Sigma-Aldrich, St. Louis, MO, USA), in an incubator at 37.0˚C, with 5% CO_2 and 95% humidity. MCF-10A cells were cultured in mammary epithelial cell medium (Lonza Biologics Inc. Portsmouth, NH, USA).

Muscle relaxant for anaesthesia. Three muscle-relaxant anaesthetics, namely rocuronium bromide (Rb), vecuronium bromide (Vb) and suxamethonium chloride CRS (SCC) were obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA). They were diluted in phosphate-buffer saline and stored at 4˚C. Their dose for research depended on the dose used by anaesthesiologists in clinical surgery. Immediately before the experiment, Rb, Vb and SCC were diluted with cell media to 8-80 μg/ml, 1.5-15 μg/ml and 20-200 μg/ml, respectively. These concentrations are similar to those obtained clinically during *i.v.* administration (16). The three cell lines were treated with different concentrations of muscle-relaxant anaesthetic.

3-(4,5-Dimethylthiazol-2-yl) 2,5diphenyltetrazolium bromide (MTT) assay. Cells (1×10^4 per well) were added to 96-well plates, and then anaesthetic was added after 8 h and cells were then cultured at 37˚C in 5% CO_2. MTT solution containing 5 mg/ml, 20 μl (Sigma-Aldrich, St. Louis, MO, USA) was added to each well after culturing for 48 h. Four hours later, the culture medium was removed. Precipitated formazan crystals were dissolved in 100 μl dimethyl sulfoxide (DMSO) added to each well and plates were incubated for 10 minutes. The absorbance of the dissolved dye was determined using an ELx800 spectrophotometer (BioTek UK, Swindon, UK) at a wavelength of 540 nm.

Electric cell-substrate impedance sensing (ECIS)-based analyses of cell adhesion and cell migration. An ECIS Ztheta instrument (Applied Biophysics Ltd, Troy, NJ, USA) was used to evaluated cell migration and adhesion as previously described by Jiang *et al*. (17). Special 96-well W96E1 microarrays were acquired for ECIS. DMEM (100 μl) with different concentrations of anaesthetic was prepared in 96-well W96E1 microarrays, then MDA-MB-231 breast cancer cells were seeded into the wells of the array immediately. Live tracking of cell adhesion over a range of frequencies was carried out from 1,000 Hz to 64,000 Hz using automated modules for 24 h. Confluent breast cancer cell monolayers in the arrays were electrically wounded (2,000 mA for 20 sec each), after which the migration of the cells was immediately tracked, again over a range of frequencies for 5 hours. All the experiments were conducted in triplicate.

In vitro cell-growth assay. Cell suspension was placed into 96-well plates (3,000 cells/200 μl/well). Cell growth was assessed after a period of incubation of up to 5 days in quadruplicate (overnight, day 3 and day 5). Cells were then fixed in 4% formalin and stained with 0.5% crystal violet. Crystal violet was extracted with 100 μl of 10% (v/v) acetic acid and the absorbance of the dissolved dye determined using an ELx800 spectrophotometer (BioTek UK, Swindon, UK) at a wavelength of 540 nm.

In vitro cell invasion assay. Transwell inserts with an 8 μm pore size were coated with 50 μg BD Matrigel™/100μl (BD Biosciences, Oxford, UK) and air-dried. Briefly, 10,000 cells/200 μl per well were added with 10% foetal calf serum and 1 ml with 10% foetal calf serum medium in the bottom well. After 72 h, the cells that had migrated through the matrix and pores were fixed with 4% formalin, stained in crystal violet. The plates were washed with tap water and allowed to be air dry. Acetic acid (10%) was added to each well for extraction dye. Absorbance of the staining was determined by an ELx800 spectrophotometer (BioTek UK, Swindon, UK) at a wavelength of 540 nm.

Statistical analysis. Statistical analysis was performed using the Minitab® 14 (Minitab Ltd. Coventry, UK) and Graphpad (Graphpad Software, Inc. La Jolla, CA, USA). Differences were considered be statistically significant at $p<0.05$. All experiments (triplicate per experiment) were repeated at least three times.

Results

The effect of different anaesthetics on different cell lines. When cells were treated with different doses of anaesthetics, the growth status of cells changed. With an increasing dose of Rb, the number of MDA-10A and MCF-7 cells gradually decreased, but Rb did not significantly affect the MDA-MB-231 breast cancer cell line (Figure 1A). SCC treatment had similar results but the influence was less than that of Rb

(Figure 1B). Intriguingly, SSC seemed to improve the growth of the breast tumour cells (Figure 1C).

Effect of Rb, Vb and SCC on the motility of MDA-MB-231 breast cancer cells in vitro. To study the role of different anaesthetics in breast cancer metastasis, we determined the migration rate of MDA-MB231 cells treated with Rb, Vb and SCC. Compared to the control group (no treatment), 8 μg/ml and 80 μg/ml Rb apparently promoted the migration of MDA-231 cells (Figure 2A), but this did not reach statistical significance. Vb at blood concentration had no detectable effect on MDA-MB-231 cell migration (Figure 2B). A similar lack of effect was also observed in the comparison between the control group (no treatment) and cells treated with SCC (Figure 2C). The data came from ECIS after wounding.

Differential inhibitory effects of Rb, Vb and SCC on MDA-MB-231 breast cancer cells in vitro. The growth assay showed that Rb and SCC at blood concentrations resulted in a dramatic increase at day 5 in the degree of proliferation compared to the group with no treatment (Figure 3A and C). However, Vb hardly influenced the proliferation of MDA-MB-231 cells (Figure 3B).

ECIS-based cell adhesion assay. We employed the ECIS method in tracking cell adhesion. The adherence of MDA-MB-231 cells treated with Rb increased compared to the control group (no treatment) (Figure 4A). In contrast, Vb and SCC had no detectable effect on MDA-MB-231 cell adhesion (Figure 4B and C).

Comparison of Rb, Vb and SCC with the control group (no treatment) for the invasion of MDA-MB-231 breast cancer cells in vitro. In searching for the effect of Rb, Vb and SCC on invasion of breast cancer cells, we carried out *in vitro* invasion assays. Rb and SCC significantly increased the invasion of MDA-MB231 cells as shown in Figure 5A and C but Vb did not (Figure 5B).

Discussion

Patients with breast cancer often exhibit heightened sensitivity to general anaesthetics; however, whether general anaesthetic agents influence breast tumour metastasis remains elucive. Rb, Vb and SCC are often used in surgery for patients with breast cancer. This study is the first to our knowledge to investigate the effect of these three anaesthetics directly on breast cancer cell function *in vitro*. We focused on finding the most appropriate anaesthetics with least effects on breast cancer progression.

Anaesthetic dose requirements vary for different patients and different diseases (18). Under different clinical conditions, for same patients, the anaesthetic dose for surgery also differs (19, 20). In our study, we adapted the dose based on the common clinical dosage use in surgery. We herein showed that a high dose of Rb and SCC can kill MCF-10A normal breast cells and MCF-7 benign breast cancer cells, but had no effect on the malignant breast cancer cell line MDA-MB-231. This result suggests anaesthetists should use the appropriate dose in surgery, and that a high dose may affect the condition of the normal cell. Furthermore, an inappropriate dose is harmful to the prognosis of patients.

In our study, we found Rb and SCC promoted the proliferation, migration and invasion of MDA-MB-231 cells. However, Vb had no effect on the metastasis of breast cancer cells *in vitro*. Our results provide an instructive dose in selecting anaesthetic in surgery of patients with breast cancer. Certain studies have reported the effect of some anaesthetics on breast cancer cells (21, 22). As a result, Vb is better for anaesthesia in surgery of patients with breast tumour. Recently, a study showed that morphine caused an overall increase in migration and proliferation of MCF-7 and MDA-MB-231 cell lines, and this result was more apparent in the oestrogen receptor-negative MDA-MB-231 cell line. This suggests that morphine directly induces growth and migration of breast cancer cells (9). Previous research using MCF-7 cells showed that morphine stimulates angiogenesis and indirectly increases tumour growth, although there is no evidence on the direct effects of morphine on cell migration and growth (23). However, another study suggested that the observed inhibitory effect of morphine on cell proliferation was dependent on stimulation of the tumour cells by 17β-oestradiol (24).

In conclusion, we found that high-dose Rb and SCC affect the growth of normal breast cells *in vitro*. Furthermore, Rb and SCC stimulate proliferation, migration and invasion of breast cancer cells *in vitro*. However, Vb is the mildest anaesthetic among these three muscle-relaxant anaesthetics. Thus, we suggest that it is better to use Vb in breast cancer surgery and to use Rb and SCC as little as possible. However, anaesthetists often use more than one kind of anaesthetic and different anaesthetics may interact with each other. In future studies, we would examine the effect on tumour metastasis when treated with several anaesthetics at the same time. Evidence accumulated over the years has given an insight into the importance of choosing the best anaesthetics for different diseases in attenuating tumour metastasis, thereby potentially facilitating a more favourable long-term outcome.

Acknowledgements

The Authors wish to thank Cancer Research Wales, Life Sciences Research Network Wales and the Albert Hung Foundation for supporting this study.

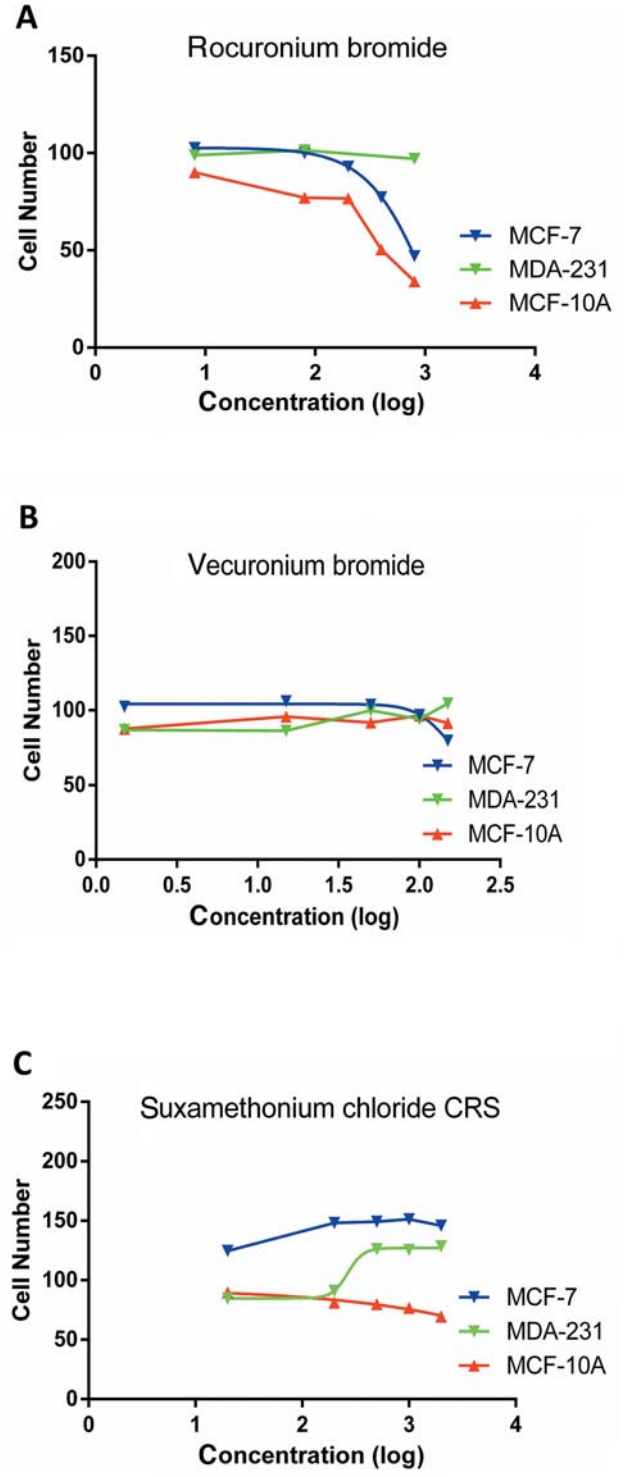

Figure 1. *Effect of rocuronium bromide (A), vecuronium bromide (B) and suxamethonium chloride CRS (C) in breast cells. A: High-dose rocuronium bromide inhibited the growth of MCF-10A and MCF-7 cells but not of MDA-MB-231 cells. B: Increasing doses of vecuronium bromide did not significantly affect breast cell growth. C: High-dose suxamethonium chloride CRS promoted the growth of MDA-MB-231 but had no effect on MCF-7 and MCF-10A.*

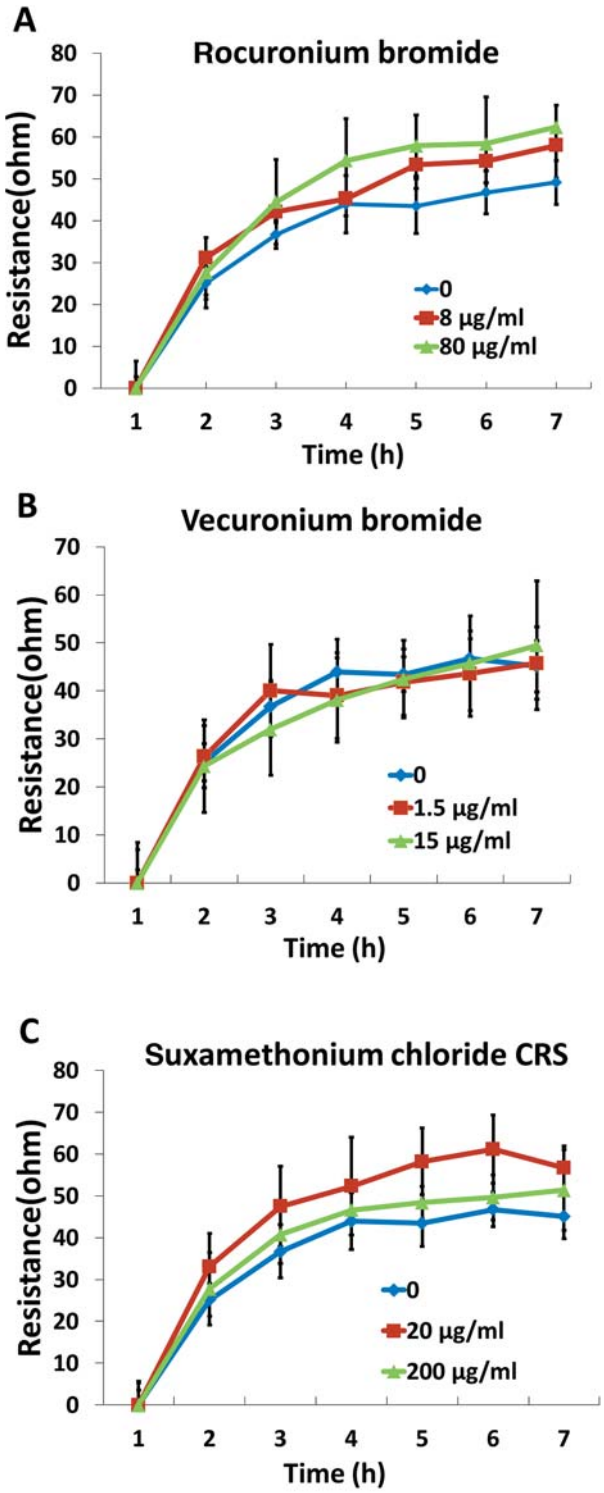

Figure 2. *The effect of rocuronium bromide, vecuronium bromide and suxamethonium chloride CRS on migration of breast cells in vitro. A: rocuronium bromide promoted the migration of MDA-MB-231 cells but did not reach significant difference in vitro. B: Vecuronium bromide had no effect on the migration of MDA-MB-231 cells in vitro. C: Suxamethonium chloride CRS did not significantly affect MDA-MB-231 migration in vitro.*

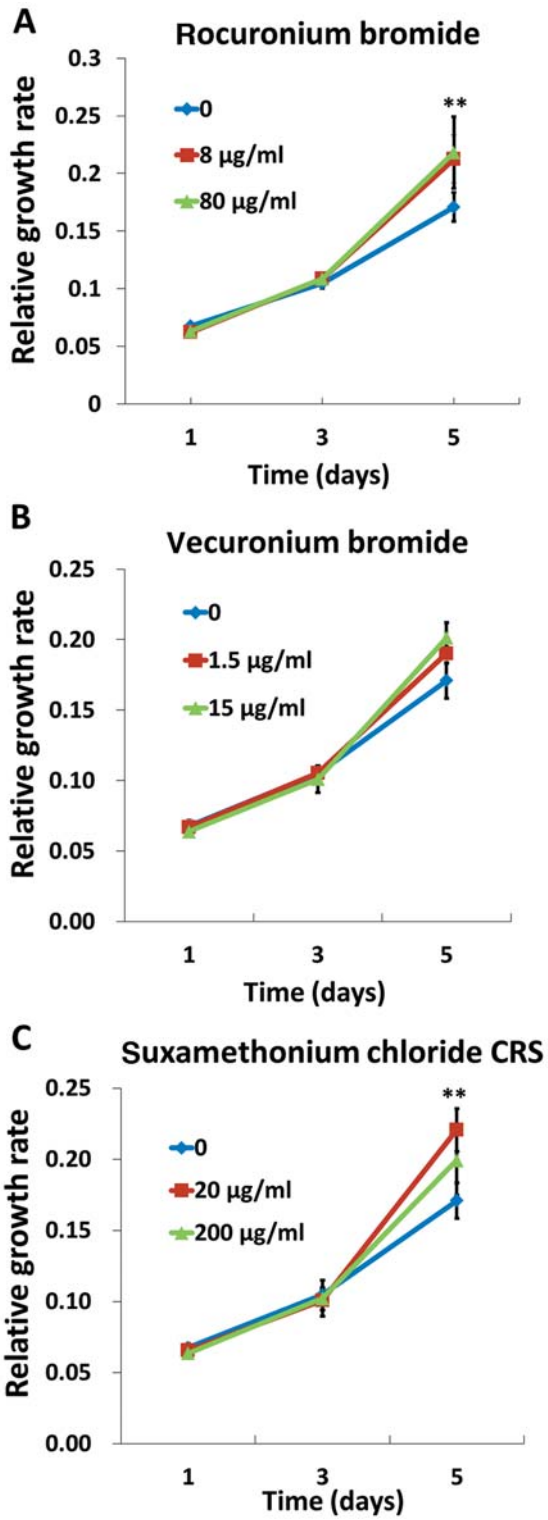

Figure 3. *Effects of rocuronium bromdide, vecuronium bromide and suaxmethonium CRS on proliferation of breast cancer cell in vitro. A: Rocuronium bromdide increased the proliferation of MDA-MB-231 cells in vitro. B: Vecuronium bromide had no significant effect on MDA-231 cells in vitro. C: Suaxmethonium CRS stimulate the growth of MDA-231 cells in vitro. **p<0.01 vs. control with no treatment.*

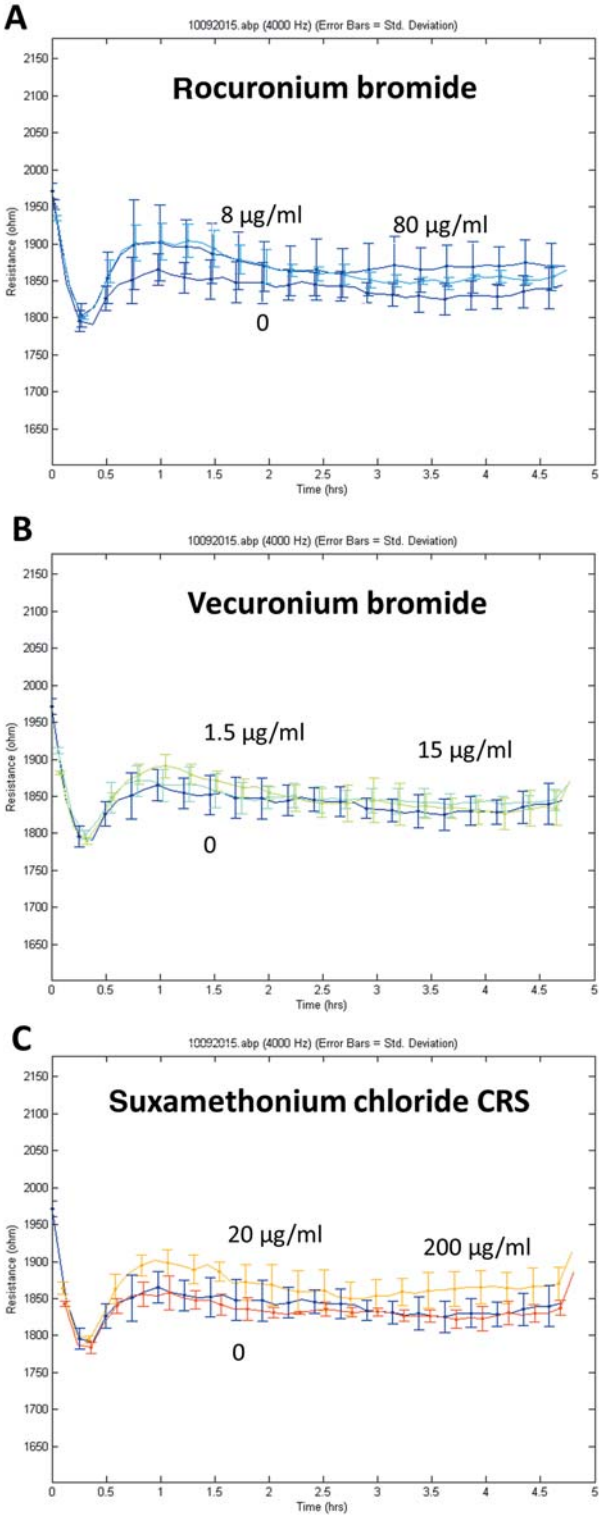

Figure 4. *Traces of MDA-231 cell response to rocuronium bromide, vecuronium bromide and suxamethonium CRS. A: Rocuronium bromide promoted the adhesion of MDA-MB-231 cells but not obvious. B: Vecuronium bromide had no effect on the adhesion of MDA-231 cells. C: Suaxmethonium CRS did not affect MDA-231 cell adhesion. Data are the mean±SD (n=3).*

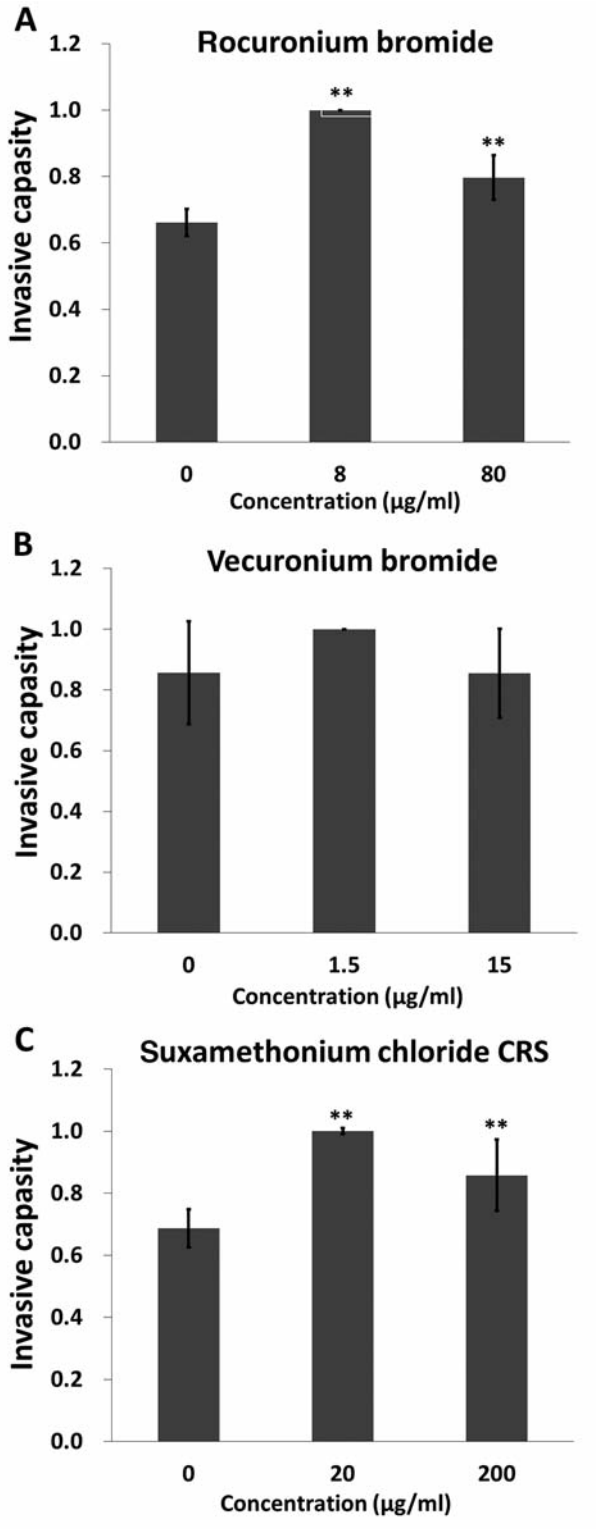

Figure 5. *Effects of rocuronium bromide, vecuronium bromide and suxamethonium CRS on invasion of MDA-231 cell in vitro. A: Rb significantly increased the invasion of MDA-MB-231 cells in vitro. B: Vb had no effect on the invasion of MDA-MB-231 cells. C: SCC promoted MDA-MB-231 cell invasion in vitro. *p<0.05, **p<0.01 vs. control with no treatment. Data are the mean±SD (n=3).*

References

1 Porter PL: Global trends in breast cancer incidence and mortality. Salud Publica Mex *51*: s141-146, 2009.

2 Gluck, S, Mamounas T: Improving outcomes in early-stage breast cancer. Oncology *24(11 Suppl 4)*: 1-15, 2012.

3 Brand JM, Kirchner H, Poppe C and Schmucker P: The effects of general anesthesia on human peripheral immune cell distribution and cytokine production. Clin Immunol Immunopathol *83(2)*: 190-194, 1997.

4 Ben-Eliyahu S, Page GG, Yirmiya R and Shakhar G: Evidence that stress and surgical interventions promote tumor development by suppressing natural killer cell activity. Int J Can *80(6)*: 880-888, 1999.

5 Fidler IJ: Timeline - The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. Nat Rev Cancer *3(6)*: 453-458, 2003.

6 Byrne K, Levins KJ and Buggy DJ: Can anesthetic-analgesic technique during primary cancer surgery affect recurrence or metastasis? Can J Anaesth 2015 Oct 23. [Epub ahead of print]

7 Le-Wendling L, Nin O and Capdevila X: Cancer Recurrence and Regional Anesthesia: The theories, the data, and the future in outcomes. Pain Med. 2015 Oct 6. doi: 10.1111/pme.12893. [Epub ahead of print]

8 Melamed R, Bar-Yosef S, Shakhar G, Shakhar K and Ben-Eliyahu S: Suppression of natural killer cell activity and promotion of tumor metastasis by ketamine, thiopental, and halothane, but not by propofol: mediating mechanisms and prophylactic measures. Anesth Analg *97(5)*: 1331-1339, 2003.

9 Ecimovic P, Murray D, Doran P, McDonald J, Lambert DG and Buggy DJ: Direct effect of morphine on breast cancer cell function *in vitro*: role of the NET1 gene. Br J Anaesth *107(6)*: 916-923, 2011.

10 Tavare AN, Perry NJ, Benzonana LL, Takata M, Ma D: Cancer recurrence after surgery: direct and indirect effects of anesthetic agents. Int J Cancer *130(6)*: 1237-1250, 2013.

11 Kawaraguchi Y, Horikawa YT, Murphy AN, Murray F, Miyanohara A, Ali SS, Head BP, Patel PM, Roth DM and Patel HH: Volatile anesthetics protect cancer cells against tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis via caveolins. Anesthesiology *115(3)*: 499-508, 2011.

12 Huitink JM, Heimerikxs M, Nieuwland M, Loer SA, Brugman W, Velds A, Sie D, Kerkhoven RM: Volatile anesthetics modulate gene expression in breast and brain tumor cells. Anesth Analg *111(6)*: 1411-1415, 2010.

13 Overall CM, López-Otín C: Strategies for MMP inhibition in cancer: innovations for the post-trial era. Nat Rev Cancer *2(9)*: 657-672, 2002.

14 Chakraborti S, Mandal M, Das S, Mandal A and Chakraborti T: Regulation of matrix metalloproteinases: an overview. Mol Cell Biochem *253(1-2)*: 269-285, 2003.

15 Müller-Edenborn B, Roth-Z'graggen B, Bartnicka K, Borgeat A, Hoos A, Borsig L and Beck-Schimmer B: Volatile anesthetics reduce invasion of colorectal cancer cells through down-regulation of matrix metalloproteinase-9. Anesthesiology *117(2)*: 293-301, 2012.

16 Wandel C, Kim R, Wood M and Wood A: Interaction of morphine, fentanyl, sufentanil, alfentanil, and loperamide with the efflux drug transporter P-glycoprotein. Anesthesiology *96(4)*: 913-920, 2012.

17 Jiang WG, Martin TA, Lewis-Russell JM, Douglas-Jones A, Ye L and Mansel RE: Eplin-alpha expression in human breast cancer, the impact on cellular migration and clinical outcome. Mol Cancer *7*: 71, 2008.
18 Eger EI: Age, minimum alveolar anesthetic concentration, and minimum alveolar anesthetic concentration-awake. Anesth Analg *93(4)*: 947-953, 2001.
19 Katoh T and Ikeda K: The effects of fentanyl on sevoflurane requirements for loss of consciousness and skin incision. Anesthesiology *88(1)*: 18-24, 1988.
20 McEwan AI, Smith C, Dyar O, Goodman D, Smith LR and Glass PS: Isoflurane minimum alveolar concentration reduction by fentanyl. Anesthesiology *78(5)*: 864-869, 1993.
21 Heaney A and Buggy DJ: Can anaesthetic and analgesic techniques affect cancer recurrence or metastasis? Br J Anaesthesia *109*: I17-I28, 2012.
22 Bajwa SJ, Anand S and Kaur G: Anesthesia and cancer recurrences: The current knowledge and evidence. J Cancer Res Ther *11(3)*: 528-534, 2015.
23 Gupta K, Kshirsagar S, Chang L, Schwartz R, Law PY, Yee D and Hebbel RP: Morphine stimulates angiogenesis by activating proangiogenic and survival-promoting signaling and promotes breast tumor growth. Cancer Res *62(15)*: 4491-4498, 2002.
24 Maneckjee R, Biswas R and Vonderhaar BK: Binding of opioids to human MCF-7 breast cancer cells and their effects on growth. Cancer Res *50(8)*: 2234-2238, 2009.

Received January 18, 2016
Revised February 22, 2016
Accepted February 23, 2016