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Effect of Isolated Serum from Breast Cancer Patients with Pectoral Nerves Block on Breast Cancer Cell Line (MDA-MB-231) Apoptosis Index

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Abstract

Background: Breast cancer (BC) is the most frequent cause of cancer death in women. The thoracic pectoral nerve (PECS) block has been described as the gold standard analgesic modality for BC surgery. It has been previously reported that PECS is associated with decreased BC recurrence post-mastectomy. Although several anesthetic drugs and techniques are used in surgical oncology, their effects on the behavior of cancer cells are yet to be known and the key question of whether the anesthetic technique affects cancer outcome remains unresolved.

Objectives: Since anesthetic drugs and techniques and post-operative pain may affect BC recurrence, this study aimed to determine whether the anesthetic choice and technique, PECS II block, affects in vitro apoptosis of the MDA-MB-231 BC cell line.

Methods: Twenty-two female BC patients, 20 to 75-years-old, with the same pathologic grades were included in this study. The patients were randomly divided into two groups. The first group received propofol general anesthesia (PGA) associated with PECS and the second group received standard PGA. Blood was sampled pre and post-operation from all patients. The sera were isolated and then exposed to the MDA-MB-231 human BC cell line. The mean percentage of apoptosis indices was analyzed by flow cytometry using Annexin V-fluorescein isothiocyanate 24 hours after treatment with patients' sera.

Results: A significant decrease was seen in the mean viability percentage of BC cell line in the PECS group, besides a significant increase in the mean percentage of necrosis and late apoptosis indices compared to the control group after exposure to sera collected from patients post-operation. Intra-group analysis of the control group showed that the exposure of the tumoral cell to post-operation sera resulted in a significant increase in the mean percentage of necrosis and late apoptosis index compared to pre-operation sera exposure. In the PECS group, the exposure of the tumoral cell to post-operation sera resulted in a significant increase in the mean percentage of cell viability and late apoptosis index compared to pre-operation sera exposure.

Conclusions: In conclusion, anesthesia and BC surgery may induce apoptosis indices in the MDA-MB-231 human BC cell line. We also found that sera collected from PECS II block patients with BC could induce more apoptosis in the MDA-MB-231 cell line compared to collected sera from systemic analgesia alone after BC surgery.

Keywords: Breast Cancer, PECS, Apoptosis, Flow Cytometry

1. Background

Breast cancer (BC) is the most frequent cause of cancer death in women. This cancer is the second most common and the fifth cause of overall cancer death worldwide (1). Surgery is the first-line treatment for the management of BC (2). However, metastatic recurrence post-BC surgery remains common and is a major cause of morbidity and mortality (2). It has been demonstrated that various factors such as acute pain, anesthetic drugs, type of anesthesia technique, and opioids can complicate the metastatic process (3, 4). Apoptosis is an important step of malignant tumor metastasis such as in BC, but whether it is influenced by the anesthetic drug is unknown.

Post-surgery pain is one of the important patient concerns following any surgery such as BC. Although BC

surgery is minimally invasive, it is associated with an increased incidence of moderate to severe postoperative pain (5, 6). The PECS II block has been recently introduced to provide a longer duration of analgesia and great pain relief and safety in patients undergoing radical mastectomy (7-9).

2. Objectives

Although several anesthetic drugs and techniques are used in surgical oncology, their effects on the behavior of cancer cells are yet to be known and the key question of whether the anesthetic technique affects cancer outcome remains unresolved. Since anesthetic drugs and techniques, as well as post-operative pain, may be affected by BC recurrence, this study was designed to answer the key question of whether the anesthetic choice and technique, PECS II block, affects in vitro apoptosis of BC cells.

3. Methods

3.1. Patients

Twenty-two female BC patients, 20 to 75-years-old, with the same pathologic grades were included in this study. The patients were randomly divided into two groups. The first group received PGA associated with PECS (48.67 $\pm$ 12.63-years-old) and the second group received standard PGA (46.3 $\pm$ 11.02-years-old). In the operating room, all patients were routinely monitored for vital signs. The patients were premedicated with the intravenous administration of fentanyl 2 μg/kg and midazolam 0.02 mg/kg to create a desirable BIS index (40 - 60) (10). Before surgery and post-induction of anesthesia, 5 mL/kg of normal saline solution was injected for all patients. Atracurium 0.5 mg/kg and propofol 1 - 2 mg/kg were intravenously injected to induce anesthesia. The anesthesia was maintained by infusion of propofol 100 - 200 μg/kg/min. After establishing the general anesthesia, PECS-I and PECS-II, respectively, were performed using 20 and 10 mL of 0.25% ropivacain-molteni (5 mg/mL) with an S-Nerve ultrasound apparatus (SonoSiteInc, Bothell, USA) and a 10 to 15 MHz linear transducer (SonoSiteInc, Bothell, USA). In the control group, normal saline was injected in the same manner.

3.2. Sampling from Patients

Immediately after the induction of anesthesia, as well as one hour post-surgery, 20 mL of blood sample was collected from each patient, centrifuged at 5,000 rpm for 5 min, and the isolated serum was stored at -20°C until use.

3.3. Cell Culture

The MDA-MB-231 human breast cancer cell line was purchased from the Cell Bank of the Pasteur Institute, Tehran, Iran. The cells were cultured at 5×10^5 cells/well in six-well plates in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% Fetal Bovine Serum (FBS) and penicillin (100 U)-streptomycin (100 μg/mL) and incubated at 37°C in a humidified atmosphere of 5% CO_2. The culture medium was changed every 48 h until the tumor cells reached ~90% confluency. Before adding the sera to the tumor cell culture, the complement proteins of sera were inactivated at 56°C for 30 min. To examine the effect of the BC patients' sera on tumor cell line apoptosis, the sera collected from the patients were exposed to BC cell lines. The apoptosis parameters were analyzed 24-h post-exposure.

3.4. Apoptosis Assay

The mean percentage of apoptotic cells was measured using an Annexin V-FITC Apoptosis Detection kit (Sigma Aldrich, USA) according to the manufacturer's protocol. Briefly, the cells were suspended in four microtubes (5×10^5/mL each), washed two times with PBS, and re-suspended in 1X binding buffer (400 μL). The cells in each microtube were stained with l μL of FITC-conjugated Annexin V (10 mg/mL), 2 μL of Propidium Iodide (PI) (50 mg/mL), and 2 μL of PI along with 1 μL of FITC-conjugated Annexin V, while the last microtube was unstained. The cells were incubated in dark for 10 min at room temperature after mixing. All experiments were done triplicate and the mean values were used for statistical analysis. The cells were analyzed by using flow cytometry (FACScan, Becton-Dickinson, USA). The BC cells stained with both Annexin V and PI and Annexin V alone were considered as being in late and early apoptosis, respectively.

3.5. Ethical Statement

This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (ethical no.: SBMU.MSP.REC.1397.110). All patients signed informed consent forms.

3.6. Statistical Analysis

After testing for the normality of data with the Kolmogorov-Smirnov normality test, the effects of PECS-II and PECS-I on the mean percentage of apoptotic cells were compared between the groups using the independent *t*-test. The intra-group comparison was done using the paired *t*-test. Data analyses were done using the SPSS-22 software package (SPSS Inc., Chicago, Ill., USA). All data are expressed as mean $\pm$ standard deviation. The P-values of less than 0.05 were considered significant.

4. Results

As PECS was done under the ultrasonography guide, no complications such as pneumothorax, hematoma, and nausea were seen. The control group showed no significant difference in the mean percentage of viability, necrosis, and early and late apoptosis of breast cancer cell line after treatment with collected sera from patients at pre-operation compared to the PECS group at the same time ($P > 0.05$). While a significant decrease was seen in the mean viability percentage of breast cancer cell line in the PECS group compared to the control group after exposure to collected sera from patients post-operation ($P = 0.004$) (Table 1). A significant increase was seen in the mean percentage of necrosis and late apoptosis index of breast cancer cell line in the PECS group compared to the control group after exposure to patients' sera post-operation ($P = 0.050$, $P = 0.004$). There was no significant difference between the PECS and control groups in terms of early apoptosis index after exposure to collected sera from patients post-operation (Table 1).

Intra-group analysis showed no significant difference in terms of the mean percentage of tumor cell viability and early apoptosis index after exposure to patients' sera collected pre- and post-operation in the control group (Table 2). Whereas, a significant difference in the mean necrosis and late apoptosis index was seen in the control group after exposure to patients' sera collected pre and post-operation (Table 2).

Intra-analysis in the PECS group showed a significant difference in terms of the mean percentage of tumor cell viability and late apoptosis index after exposure to patients' sera collected pre- and post-operation in the control group (Table 2). In contrast, there was no significant difference in the mean necrosis and early apoptosis index in the PECS group after exposure to patients' sera collected pre- and post-operation (Table 2).

Table 1. Mean Percentage of Cell Viability, Necrosis, Early and Late Apoptosis Index in Groups of Cells Exposed to Patients' Sera Collected Pre- and Post-operation

	Mean ± SD	P-Value
Viability before operation		0.381
Control	89.20 ± 7.49	
Case	85.36 ± 10.62	
Viability after operation		0.004
Control	86.13 ± 8.94	
Case	62.83 ± 18.61	
Necrosis before operation		0.311
Control	3.88 ± 3.27	
Case	5.80 ± 3.70	
Necrosis after operation		0.050
Control	0.79 ± 0.48	
Case	6.12 ± 5.16	
Early apoptosis before operation		0.471
Control	2.02 ± 0.60	
Case	1.68 ± 1.27	
Early after operation		0.807
Control	2.62 ± 0.081	
Case	2.75 ± 144	
Late before operation		0.313
Control	4.03 ± 2.00	
Case	5.42 ± 2.95	
Late after operation		0.041
Control	9.25 ± 2.89	
Case	16.54 ± 7.81	

5. Discussion

This study was designed for answering the key question of whether sera collected pre- and post-operation from patients who have undergone the PECS II block affect the survival rate and apoptosis indices of BC cell line compared to controls or patients without the PECS block. In addition, the intra-group analysis of apoptosis indices and survival rate in each group when BC cells were exposed to sera collected pre- and post-operation was also investigated using flow cytometry. Heterogeneous cell populations that contain viable, necrotic, and apoptotic cells cannot be distinguished by standard bulk techniques such as Western Blot, DNA-electrophoresis, and colorimetric enzyme assays (11). Flow cytometry is an alternative technique for investigating these heterogeneous cell populations (12). Anesthetics may play an important role in the postoperative outcome of cancer surgery due to their immunomodulatory activity and through pro-apoptotic mechanisms (13). The obtained data from inter-group comparison showed no significant difference in the mean percentage of all apoptotic indices, as well as the mean percentage of viability rate, in BC cells when the cells were exposed to sera collected from the patients at pre-operation in both groups. These findings show the patients' sera collected from both groups had the same effects on BC cells before intervention. In contrast, we found not only a significant decrease in the mean viability percentage of BC cells but also a significant increase in the mean percentage of necrosis and late apoptosis index of BC cells in the PECS group compared to the control group after exposure

Table 2. Intra-group Analysis of the Mean Percentage of Cell Viability, Necrosis, Early and Late Apoptosis Index in Groups of Cells Exposed to Patients' Sera Collected Pre- and Post-operation

Time	Mean ± SD	P-Value
Control		
Live before	88.78 ± 7.90	0.528
Live after	85.50 ± 9.34	
Necrosis before	3.88 ± 3.27	0.049
Necrosis after	0.96 ± 0.45	
Early before	2.02 ± 0.60	0.066
Early after	2.47 ± 0.84	
Late before	4.91 ± 2.28	0.002
Late after	9.80 ± 3.10	
Case		
Live before	83.75 ± 11.37	0.002
Live after	62.83 ± 18.61	
Necrosis before	5.80 ± 3.70	0.892
Necrosis after	6.12 ± 5.86	
Early before	1.81 ± 1.28	0.061
Early after	2.75 ± 1.44	
Late before	6.17 ± 3.09	0.026
Late after	12.56 ± 3.02	

to collected sera from patients post-operation. The thoracic PECS block has been described as the gold standard analgesic modality for BC surgery (14). The analgesic effectiveness of the PECS II block versus systemic analgesics alone and paravertebral block for BC surgery has been recently demonstrated (15). Acute pain, anesthetic drugs, and type of anesthesia technique can contribute to complicate the metastatic process (3, 4). It has been previously reported that PECS is associated with decreased BC recurrence post-mastectomy (16, 17). Gong et al. (18) hypothesized that regional anesthetic reduces cancer recurrence, as it decreases opioid consumption and blunts the neuroendocrine stress response to surgery and resultant inflammation, both of which inhibiting the immune system to scavenge metastatic cells. Local anesthetics increase the concentration of intracellular calcium via either releasing from intracellular stores or external influx (19). Furthermore, they can inhibit energy production in the mitochondria by activating certain kinases. In this process, apoptosis is a mechanism of cytotoxicity of local anesthetics in vitro (20). Necrosis is mostly activated by extrinsic factors. Necrosis is characterized by the progressive loss of cytoplasmic membrane integrity and rapid influx of water, Na^+, and Ca^{2+}, leading to cytoplasmic swelling and nuclear pyknosis (13). It has been recently reported that sevoflurane induces apoptosis and autophagy in colorectal cancer cells via inactivating Ras/Raf/MEK/ERK signaling (21). Since the late apoptosis and necrosis index show the loss of plasma membrane and primary necrotic cells (12), our findings showed that the local anesthetic in the PECS group may induce the loss of plasma membrane and extensive membrane rapture of tumoral cells. There was no significant difference between the PECS and control groups in terms of early apoptosis index after exposure to sera collected from patients post-operation. Early-stage apoptosis is represented by changes to and ultimate loss of the mitochondrial membrane potential (12). Our findings showed that the local anesthetic in the PECS group could not lead to the loss of the mitochondrial membrane.

In the second step of this study, the intra-group analysis of apoptosis indices was performed. The loss and rupture of the plasma membrane are represented by late apoptosis, and necrosis can result from changes in sera during surgery consisting of systemic anesthesia drugs and local anesthetic drugs in these groups.

5.1. Conclusions

We found that anesthesia and BC surgery can induce apoptosis in the MDA-MB-231 human BC cell line. We also found that the PECS II block induces more apoptosis in the MDA-MB-231 cell line when compared to systemic analgesia alone after BC surgery. However, a study in a larger group is suggested.

Footnotes

Authors' Contribution: AM and DA designed the study. All the authors contributed to data collection and analysis. All authors read and approved this study.

Conflict of Interests: The authors have no conflict interest.

Ethical Approval: This study was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran (ethical no.: SBMU.MSP.REC.1397.110).

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