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Research Article
Effect of Epidural Anesthesia on the Immune Function of Patients with Non-small Cell Lung Cancer

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Abstract

Background and Objective: Epidural anesthesia is reduced opioid and anesthetic consumption, facilitates pain control and ameliorates surgical stress. Propofol and sevoflurane have also made changes in immune responses. The objective of the study was to evaluate the effect of pre-operational epidural sevoflurane and propofol anesthesia on cellular immunity of patients following lung cancer surgery. **Materials and Methods:** Total 246 patients with lung cancer who had planned for thoracotomy subjected to randomize (1:1 ratio) and divided into two groups of 123 patients each. Anesthesia was maintained by sevoflurane at 2 μg mL^{-1} alveolar concentration (group I) or a continuous infusion of propofol at 2-4 μg mL^{-1} steady-state plasma concentrations (group II). The killing activity of natural killer T-cells, western blot, T-helper cells assay, phagocytosis assay and cell proliferation assay had been evaluated. Wilcoxon test/Tukey's *post hoc* tests were performed between pre-operational and post-operative data at 95% of confidence interval. **Results:** Sevoflurane ($p = 0.6217$) had no effect and propofol ($p<0.0001$, $q = 10.835$) had depressed CD 4^{+}/CD 8^{+} ratio. Sevoflurane ($p<0.0001$, $q = 40.473$) had decreased but propofol ($p = 0.058$) had no effect on interferon-γ; Interleukin-4 ratio. Sevoflurane had decreased and propofol had boosted the counts of T-cells, T-helper cells and killing activity natural killer T-cells. Unlike, sevoflurane, propofol had increased interleukin-5 counts. Propofol and sevoflurane both had a significant cell proliferation and phagocytosis effects. **Conclusion:** Type of anesthetic used modulates the immunity of patients undergoing lung cancer resection.

Key words: Cell proliferation, epidural anesthesia, natural killer T-Cells, phagocytosis, propofol, sevoflurane

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lung cancer is the most occurring cancer in Chinese men and women[1]. About one-third portion of lung cancers are the non-small cell lung cancers (NSCLC) type[2]. T-cells and T-lymphocytes can promote immunity of β-cells[3]. Human T-follicular helper cells can also contribute immunity against tumors[4]. T-helper cells and T-lymphocytes have an important role in immunity during NSCLC[5]. $CD4^+$, $CD8^+$ and $CD4^+$:$CD8^+$ ratios have a significant effect on immunity of patients suffering from lung cancer[6]. Natural killer T (NKT)-cells have cell-mediated controls on cancer formation[7]. Therefore, they are considered to be hall marks of cancer formation, sepsis and infections[5].

The pre-operative, intra-operative and post-operative periods of any major surgery have been decreased the immunity of the patients[8]. However, epidural anesthesia has an important effect on immune system because it has reduced opioid and anesthetic consumption, facilitates pain control and ameliorates surgical stress[9]. Several studies reported that epidural anesthesia may positively affect the number and function of NKT-cells, preserves $CD4^+$: $CD8^+$ ratio and restores the TH_1 and TH_2 balance following major surgeries[10]. Moreover, propofol[11] and sevoflurane[12] have made changes in immune responses of patients such as counts of T-cells, T-helper cells and T-lymphocytes following any major cancer surgery and ultimately affect the cancer progression.

The primary aim of the study was to anesthetize the patients before thoracotomy by pre-operational epidural anesthesia (propofol or sevoflurane). The secondary endpoint was to compare the effect of sevoflurane and propofol on T-cells, T-helper cells, T-lymphocytes and killing activity of NKT-cells of NSCLC patients following surgery.

MATERIALS AND METHODS

Materials: Propofol was purchased from Corden Pharma S.P.A, Caponago, Italy. Rocuronium bromide (Esmeron) and Noradrenaline injections were purchased from N.V. Organon, the Netherlands. Sevoflurane was purchased from Abbvie, Shanghai, China. Phosphate buffered saline (PBS), CO_2, propidium iodide (PI), carboxyfluorescein succinimidyl ester (CFSE), protease inhibitor (PRI) and NF-phosphatase inhibitor (NFPI) were purchased from Sigma Aldrich, US. Erythroleukemic cells (TF1a (ATCC® CRL2451™) were purchased from American Type Culture Collection, MN, USA.

Ethical consideration and consent to participate: The ethical approval (protocol: ZHA001) had obtained for the prospective anesthetic experimental human study from Zhejiang Cancer Hospital, PR China for the trial. The study had been registered in Research registry (http://www.researchregistry.com), UID No.: research registry 3391 dated, 14 November, 2014. The written informed consent form had been signed by all participants before study regarding interventions, surgeries and publications of materials (including images of particular patients if any) in all forms (hard and/or electronic) irrespective of time, place and language. The datasets created during and/or analyzed during the current study available from DICOM files of patients of Zhejiang Cancer Hospital, PR China. The study adhered the law of PR China, 2013 Declaration of Helsinki and CONSORT guidelines[13].

Inclusion criteria: Patients who had admitted to the Department of Oncology, Zhejiang Cancer Hospital and the Hospital of Zhejiang Province, Hangzhou, China during December, 2014-February, 2017 for thoracotomy and age greater than 18 years included in the randomized trial. Patients who had ASA (American Society of Anesthesiologists) physical status of 1-4 included in the study. Patients with all stage of cancer (as per biopsied of the pathological studies) were included in the study.

Demographic and clinical characteristics of enrolled patients for thoracotomy are shown in Table 1. The enrolled patients for thoracotomy had same demographic and clinical characteristics between the groups ($q<4.152$ for all parameters).

Exclusion criteria: Patients who had aged younger than 18 years and older than 75 years excluded from the study. Patients who had ASA physical status of 5 also excluded from the study. Patients who had a recent history of opioid, alcohol and/or steroid consumption excluded from the study. Patients who had chronic depression, moderate to severe dementia, severe bipolar disorder and acute stroke history excluded from the study. Patients who refused to sign, an informed consent form and do not follow study protocol excluded from the study.

Design: Total 246 patients who had planned for thoracotomy subjected to simple randomization (1:1 ratio) allocation. The prior sample size was calculated by Open Epi 3.01-English (Epidemiologic Statistics for Public Health, USA) and was

Table 1: Characteristics of enrolled patients for thoracotomy

	Groups			
	I	II		
	n = 123	n = 123	Comparison of groups	
Characteristics	Sevoflurane	Propofol	p-value	q-value
Age (years, Mean±SD)	43.95±2.01	46.39±1.85	0.0920	4.026
Gender				
Male	85 (69)	78 (63)	0.0076	1.306
Female	38 (31)	45 (37)		
ASA physical status				
1	73 (59)	69 (56)	0.0024	0.7699
2	12 (10)	13 (11)		
3	08 (07)	09 (07)		
4	30 (24)	32 (26)		
TNM cancer stage				
I	12 (10)	15 (12)	0.0089	2.746
II	25 (20)	29 (24)		
III	86 (70)	79 (64)		
Smoker	85 (69)	81 (66)	0.0056	0.9397
Tobacco consumer	45 (37)	55 (45)	0.0073	2.247
Origin				
Chinese	120 (98)	119 (97)	0.1515	N/A
Non-Chinese	03 (02)	04 (03)		
BMI (kg m^{-2}, Mean±SD)				
Obese	35 (28)	43 (35)	0.0078	1.508
Non-obese	88 (72)	80 (65)		
Interleukin-5 (ng L^{-1})	64.04±2.16	63.87±1.67	0.4780	N/A
Percentage phagocytosis	24.15±1.25	23.71±1.28	0.0068	3.258
Percentage cell proliferation	68.54±2.25	67.85±1.91	0.0101	N/A

BMI: Body mass index, N/A: Not applicable. ASA (American Society of Anesthesiologists) physical status, 1: Needed emergency surgery, 2: Contraindication to epidural analgesia, 3: Contraindication to any other drug used in the treatment, 4: Recent history ($\leq$8 weeks) of chemotherapy or radiotherapy, Constant data were represented as a number (percentage) and continuous data were represented as Mean±SD. One-way ANOVA following Tukey's *post hoc* test was used for statistical analysis. A $p<0.01$ and $q>4.152$ were considered as significant

found to be 123 for both groups. The other parameters, percentage frequency outcome was 95±5% and the confidence level was 95%. CONSORT flow diagram of the study is represented in Fig. 1[14].

***Epidural anesthesia* procedure:** Anesthesia was maintained in group I by sevoflurane at a minimum alveolar concentration of 2 µg mL^{-1} (n = 123). Group II had received a continuous infusion of propofol anesthesia via target-controlled infusion aimed at achieving steady-state plasma concentrations of 2-4 µg mL^{-1} (n = 123). Both groups were reported for hypnosis using processed EEG (BIS XP®, Aspect Medical Systems, Newton, MA). The BIS target value was 45-60. Neuromuscular relaxation was achieved with Esmeronat a perfusion rate of 1-2 µg kg^{-1} min^{-1}. Analgesia was performed with remifentanil (Ultiva, Mylan Institutional, USA) infusion of 0.1–0.2 µg kg^{-1} min^{-1}. On completion of the procedure, the patients were transferred to the intensive critical care unit (ICCU), where the group I patients had received sevoflurane via the Anesthetic Conserving Device (AnaConDa device®, Sedana Medical, Uppsala, Sweden) to obtain sedation with bispectral index values ranging from 60-70 and sevoflurane end-tidal concentrations were maintained 0.5-0.7% and group II patients had received propofol infusion to obtain sedation with bispectral index values ranging from 60-70 and plasma propofol concentrations ranging from 1-1.5 µg mL^{-1}. Patients were extubated when they were within normal ranges. Hemodynamic stability was achieved by continuous infusion of noradrenaline if the cardiac index was <2.21 L^{-1} min^{-1} m^{-2}. Pulmonary capillary wedge pressure was <15 mm Hg and vascular resistances were normal[15]. Anesthesia was performed by co-authors and operation theater assistance staff.

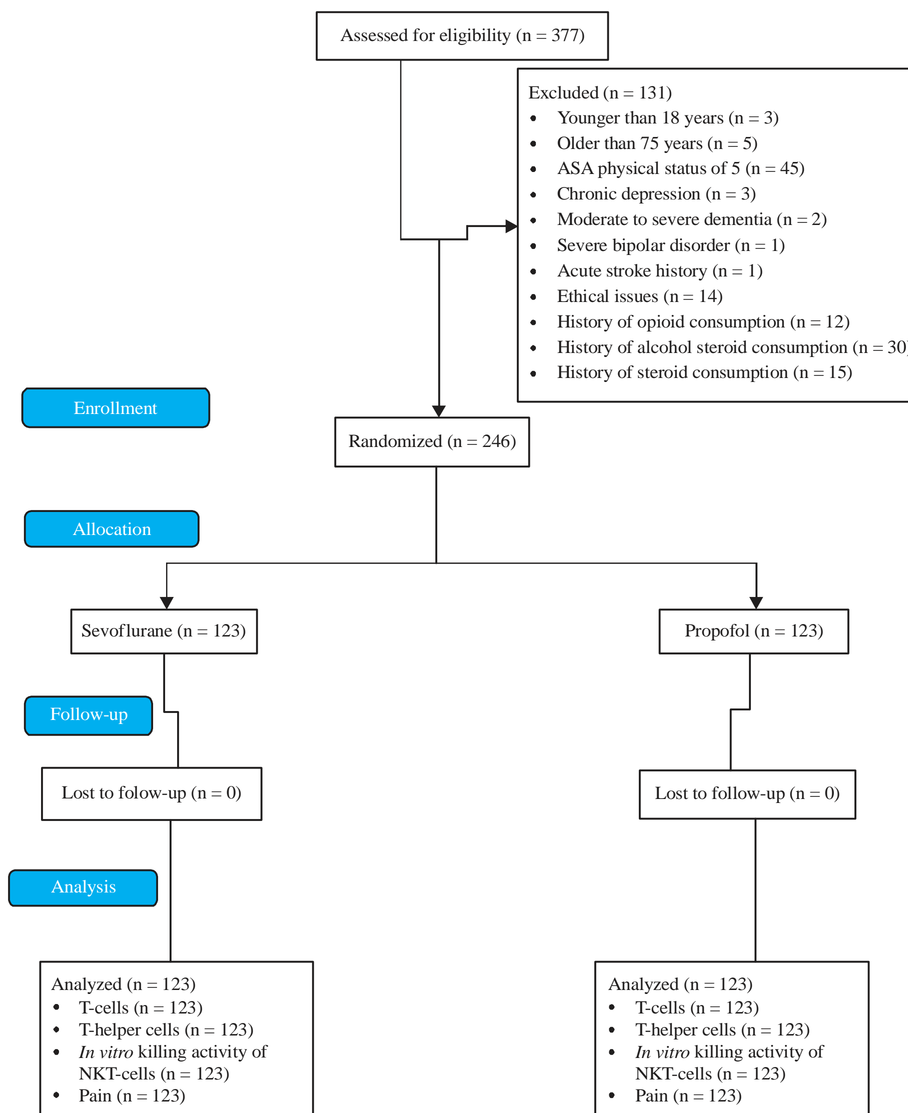

Fig. 1: Flow diagram of the study. NKT-cells: Natural Killer T-cells. Population size (N): 246, percentage frequency outcome: 95±5%, confidence level: 95%

Thoracotomy: All patients were subjected to thoracotomy or surgical resection by a cardiothoracic surgeon in the major operation theater. The lung isolation was carried out for tumor resection. Normothermia was controlled by forced-air[16]. The surgery was performed in the early morning on empty stomach.

Blood sample collection: For analysis of cytokine and lymphocyte, venous blood samples were collected at baseline and at one-day after surgery (in morning) in citrate cuvettes (Shenzhen Sinothinker Technology Co., Ltd., China). For mononuclear cell isolation, blood samples were collected in a cuvette with heparin sulfate. Density gradient centrifugation technique (Ficoll-Paque, GE Healthcare, Sweden)[5] was performed for separation. $CD4^+$, $CD8^+$ and regulatory T-cells were isolated from the blood sample by negative selection using amagnet[17].

Western blot assay: The collected blood sample was activated with anti-CD 4^+ and anti-CD 8^+ and anti-regulatory T-cells for different times, 0, 6, 20 and 40 min separately as per instructions of the manufacturer of kits. The cells were lysed in 0.21 mL of PBS (pH 7.4) containing PRI and NFPI individually. Protein concentrations in the PRI-soluble fractions were determined by Clean-Blot™ IP Detection Kit (Thermo Scientific, Waltham, USA). The cell protein samples of 22 μg were

separated by 10% SDS-PAGE and transferred to PVDF membranes (BioRad, Hercules, California, USA). The membranes were incubated with the primary antibodies overnight at 4.1°C after a 120 min-block in 4.95% skim milk at room temperature, followed by incubation with secondary antibodies for 125 min. The signals were visualized using the Western Blotting Substrate (Thermo Scientific, Waltham, USA)[17].

T-helper cells assay: T-helper cells were extracted from the collected blood sample (250 μL). Serum plasma levels of interferon-γ (INF-γ), interleukin-4 (IL-4), interleukin-2 (IL-2), interleukin-5 (IL-5) were assessed by HBsAg Elisa Diagnostic Kit (Daan Gene Co., Ltd. of Sun Yat-Sen University, Guangdong, China) using My-B026 Fully Automatic Lab Elisa Reader (Guangzhou Maya Medical Equipment Co., Ltd., Guangdong, China) according to manufacturer of kit instructions[18].

Evaluation of killing activity of NKT-cells: NKT-cells were separated from the blood sample by Magnetic Coolant Separator (Great Magtech Electric Co., Ltd., Xiamen, China). NKT-cells were treated with PBS (pH 7.4) for 1 day. Then incubated with TF1a and stained with CFSE for 5 h at 37°C and 5% CO_2. After incubation, 1 μG mL^{-1} of PI was added. The CFSE and PI double staining is allowing discrimination of living and dead NKT-cells[19].

Assay for phagocytosis: The phagocytosis assay was performed with 2 μm fluorescence-labeled latex beads (Polysciences, Inc, USA) at 30°C and 30 mm Hg pressure using phosphate saline buffer (pH 7.4). Tissue culture medium (1 mL) containing total 1×10^6 cells of blood plasma was supplemented with 10% PBS in a petri dish, mixed with latex beads at a multiplicity ratio of 1:5 and incubated for 2 h at 37°C. The scales used for differentiation purpose were cells with 1 bead, with 2-5 beads, with 6-10 beads and with more than 10 beads[20]. Percentage phagocytosis was calculated as per Eq. 1:

$$\text{Phagocytosis (\%)} = \frac{\text{Cells with at least one bead}}{\text{Total number of cells subjected to count}} \times 100 \quad (1)$$

Assay for cell proliferation: The cell proliferation assay was performed using a colorimetric crystal violet staining method (Molecular Devices, USA). Cells were seeded with culture plates (100,000 cells/well 6-well) for 24 h. One-third cells were serum starved for 1 day. These cells were put to normal growth medium at 37°C and 30 mm Hg pressure for 1 day, rinsed with PBS and fixed in absolute glacial acetic acid: Ethanol (1:3 v/v) for 590 sec at 37°C and left to air dry, stained with crystal violet for 10 min. The excess of crystal violet was removed by decantation and washed thrice with distilled water. The optical density was measured at 550 nm by Thermomax microplate reader (Molecular Devices, Ramsey, USA)[20].

Pain score: The pain score was measured when patients become conscious. Pain score had been recorded on a verbal numeric rating scale. The coding of pain was 0: Absent paint and 10: The worst possible pain. No any extra painkiller was given during surgery[21].

Statistical analysis: Data were represented as Mean±SD all of them. One-way analysis of variance (ANOVA)(considering $p<0.01$ as significant, Microsoft Excel® 2016, Microsoft, USA) following Tukey's *post hoc* test (considering critical value $q>4.152$ as significant)[22] was performed for demographic and clinical characteristics of the enrolled patients who had planned for thoracotomy[23]. Pre-operative and post-operative data were compared using Wilcoxon t-test[24] following Tukey's *post hoc* test (considering $q>3.329$ as significant)[22] for Western blot assay, T-helper cells assay and killing activity of NKT-cells. Two-tailed paired t-test[14] following Tukey's *post hoc* test (considering $q>3.329$ as significant)[22] were performed for phagocytosis assay, cell proliferation assay, pain score and predictive values of T-cells and T-helper cells after surgery between the groups. The intention-to-treat method of analysis was performed using InStat (GraphPad, USA). A $p<0.05$ was considered significant for effects of anesthetics on immunity cells.

RESULTS

Sevoflurane ($p = 0.6217$) had no effect and propofol ($p<0.0001$, $q = 10.835$) had depressed $CD4^+$:$CD8^+$ ratio. Sevoflurane ($p<0.0001$, $q = 40.473$) had decreased but propofol ($p = 0.058$) had no effect on INF-γ: IL-4 ratio. Sevoflurane had decreased counts of T-cells, T-helper cells and percentage killing activity NKT-cells, however, propofol had boosted the same counts of the immunity cells and percentage killing activity NKT-cells (Table 2). Unlike, sevoflurane, propofol had increased IL-5 counts as represented in Fig. 2 ($p<0.0001$, $q = 52.954$).

Table 2: Effects of interventions on T-cells, T-helper cells and killing activity natural killer T-cells

Cells	Group I (sevoflurane anesthesia)				Group II (propofol anesthesia)				Statistical analysis between groups I and II¶			
	BS	AS	*SA		BS	AS	*SA		at BSφ		at AS	
	n = 123	n = 123	p-value	q-value	n = 123	n = 123	p-value	q-value	p-value	q-value	p-value	q-value
T-cells (pg mL^{-1})												
CD 4$^+$	40.03±0.87	35.85±1.060	<0.0001	49.552	39.10±1.86	40.46±0.93	<0.0001	9.346	<0.0001	8.088	<0.0001	50.132
CD 8$^+$	26.39±1.78	23.33±1.364	<0.0001	20.549	23.89±1.97	26.99±1.87	<0.0001	17.768	<0.0001	15.002	<0.0001	27.425
CD 4$^+$:CD 8$^+$	1.52±0.11	1.51±0.130	0.6217	N/A	1.65±0.15	1.51±0.11	<0.0001	11.195	<0.0001	10.835	0.7968	N/A
Regulatory	1.81±0.73	1.15±0.360	<0.0001	11.394	1.25±0.44	1.64±0.70	<0.0001	8.018	<0.0001	9.458	<0.0001	26.241
T-helper cells (ng L^{-1})												
INF-γ	34.14±1.310	27.02±1.420	<0.0001	58.477	33.32±1.310	35.24±1.780	<0.0001	14.345	<0.0001	6.942	<0.0001	58.797
IL-4	48.51±1.120	46.55±1.120	<0.0001	19.428	50.29±1.040	52.51±1.120	<0.0001	23.098	<0.0001	18.073	<0.0001	59.512
INF-γ: IL-4	0.70±0.035	0.58±0.031	<0.0001	40.473	0.66±0.029	0.67±0.035	0.058	N/A	<0.0001	13.929	<0.0001	30.564
IL-2	24.58±1.140	21.98±1.580	<0.0001	22.064	25.48±1.140	27.51±1.120	<0.0001	19.889	<0.0001	8.778	<0.0001	42.419
IL-2: IL-5	0.39±0.021	0.37±0.0282	0.0015	5.713	0.40±0.021	0.42±0.016	<0.0001	10.099	<0.0001	7.532	<0.0001	19.657
Killing activity of NKT-cells (%)	30.62±1.010	25.50±1.560	<0.0001	53.175	29.81±1.350	34.46±1.710	<0.0001	34.963	<0.0001	7.779	<0.0001	72.787

BS: Before surgery, AS: After surgery, SA: Statistical analysis between BS and AS within the group. All data were represented as Mean±SD, n = 123. INF-γ: Interferon-γ, IL-4: Interleukin-4, IL-2: Interleukin-2, IL-5: Interleukin-5, NKT-cells: Natural killer T-cells. * Wilcoxon test following Tukey's *post hoc* test was used for statistical analysis, ¶Two-tailed paired t-test following Tukey's *post hoc* test was performed for statistical analysis. The p<0.05 and q>3.329 considered were as significant. φp<0.01 and q>4.152 considered were as significant. N/A: Not applicable

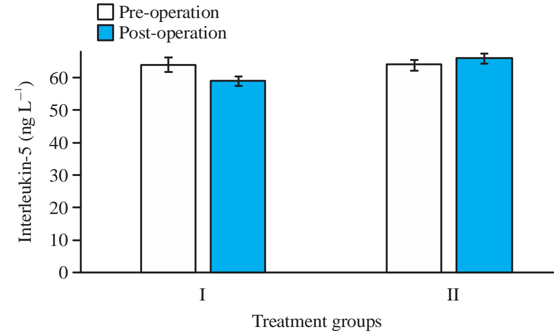

Fig. 2: Effects of anesthetics on interleukin-5. The p-values and q-values between pre-operative and post-operative conditions of patients for the group I (sevoflurane anesthesia) were <0.0001 and 28.903 and for group II (propofol anesthesia) <0.0001 and 14.475, respectively. A p-value and q-value between groups were <0.0001 and 52.954

Wilcoxon test (within the group) or two-tailed paired t-test (between groups) following Tukey's *post hoc* test was used for statistical analysis. The p<0.05 and q>3.329 were considered as significant

Propofol and sevoflurane both had a significant effect over cell proliferation and phagocytosis but propofol had higher phagocytosis effect than sevoflurane (Fig. 3a) and sevoflurane had higher cell proliferative effects than propofol (Fig. 3b).

Propofol and sevoflurane both had a reduction in post-operative pain but this was due to the epidural type of anesthetic technique.

DISCUSSION

The study demonstrated that sevoflurane had slightly depressed immunity after surgery. However, propofol had boosted the immunity. These results were some what similar to the results are reported by the available studies[11,12]. In respect to the selection of anesthetics and the method of anesthesia for thoracotomy, the study had provided a good choice of anesthesia to improve immune system following major cancer surgery.

In the study, sevoflurane had depressed and propofol had boosted the killing potential of NKT-cells. NKT-cells play a key role in cancer immune response[25]. However, till date, available studies do not evaluate the effect of sevoflurane[26] and propofol[11] on the killing potential of NKT-cells. With respect to the design of the study, the finding was added valuable literature in the available state-of-art.

In the study, it was reported that sevoflurane had depressed and propofol had boosted the counts of T-cells

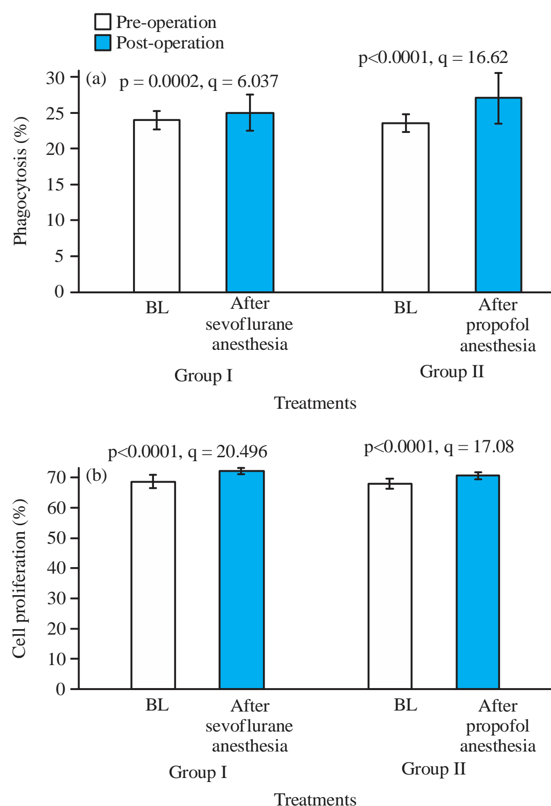

Fig. 3: Effect of anesthesia on cell activity, (a) Phagocytosis assay. A $p<0.0001$ and $q = 9.808$ between groups after surgery, (b) Cell proliferation assay. A $p<0.0001$ and $q = 16.537$ between groups after surgery. The p and q-values indicated in the figure were represented that off within the group between BL and after anesthesia. All data were represented as Mean$\pm$SD, n = 123
Wilcoxon test (within the group) or two-tailed paired t-test (between groups) following Tukey's *post-hoc* test was performed for statistical analysis. The $p<0.05$ and $q>3.329$ were considered as significant. BL: Baseline

and T-helper cells. The available studies are shown that anesthetics have no significant effect on T-cells and T-helper cells[5,27]. However, some literature reported that anesthetic has influenced the treatment of the cardiac surgeries[28] and liver surgeries[29]. According to the results of the study, the study provided the important data regarding the effect of anesthetics on T-cells and T-helper cells during major surgeries.

The study reported that sevoflurane had no any effect and propofol had decreased $CD4^+/CD8^+$ ratio. The previous studies are reported the same results regarding sevoflurane only[12]. However, the study first time ever provided depressed action of propofol on $CD4^+/CD8^+$ ratio. In respect to the results for T-cells of the study, the finding was unique in nature itself.

Propofol and sevoflurane both had the same effect on post-operative pain (p = 0.067). These results were in the line of presently available studies results[30]. With respect to the selection of anesthetics for thoracotomy, they were decreased post-operative pain.

Propofol and sevoflurane both had considerable phagocytosis and cell proliferative effects. Propofol and sevoflurane had immuno-protective effects[31]. In respect to the anesthetic agent used for thoracotomy, the study would contribute valuable state-of-art to improve immune system after the lung cancer surgery.

In limitations of the study, for example, the study had not evaluated the effect of anesthetics during follow-up periods. The study was not developed effects of anesthetics on post-surgical recovery. All selected patients were young. The age also affects immunity of patients. Age oscillation study was not performed. There were significant discriminations for T-cells, T-helper cells and percentage killing activity of NKT-cells between groups at baseline.

CONCLUSION

A randomized trial concluded that patients undergoing thoracotomy and who received anesthesia following sedation had shown depressed T-cells, T-helper cells and T-lymphocytes including killing activity of natural killer T-cells by sevoflurane and boosted by propofol. The type of anesthetic used modulates the immunity of patients undergoing lung cancer resection.

SIGNIFICANT STATEMENT

The anesthetic experimental study discovered the effect of pre-operational epidural sevoflurane and propofol on T-cells, T-helper cells and T-lymphocytes of patients following lung cancer surgery. The finding will help the surgeons to uncover the critical areas of effects of epidural anesthesia on immunities that many anesthesiologists were not able to explore.

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