LigaSure® versus Clamp Tie Technique for Thyroid Surgery; Decreased Operative Time versus Increased Inflammatory Effect: a prospective randomized study

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Abstract. – OBJECTIVE: The aim of this study was to compare the LigaSure® Small Jaw Instrument (LSJI) with the conventional clamp-and-tie (CT) technique in thyroid surgery regarding complication rates (hematoma, hypocalcemia and recurrent nerve palsy), the duration of the operative procedure, and systemic and local inflammatory effects.

PATIENTS AND METHODS: Fifty-four consecutive patients were randomized prospectively into two groups, a Conventional Clamp-Tie (CT) group and a LigaSure® Vessel Sealing System (LVSS) Group. Pre- and postoperative blood plasma samples were taken to measure the tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), calcium, parathormon, CRP levels and WBC, as well as the lymphocyte subset (CD3, CD4, CD8, CD16/56, CD19) counts. The drain fluid samples were collected after the removal of the drains to measure the levels of IL-6 and TNF-α.

RESULTS: Both groups showed significant changes regarding peripheral blood CD3+, CD4+, and CD8+ T cell levels (p < 0.05). In the LVSS group, the level of CD16+/56+ NK cells showed a significant decrease compared with the CT group (p < 0.05). The IL-6 and TNF-α levels in the drainage fluid were significantly higher in the LVSS group.

CONCLUSIONS: We demonstrated that LSJI can decrease operative time. Although the systemic inflammatory effect of LSJI remains inconclusive, the local inflammatory effect was significant, which could cause early and late postoperative problems.

Keywords:
LigaSure®, Vessel sealing systems, Total thyroidectomy, TNF-α, IL-6, Lymphocyte subsets.

Introduction

Thyroid surgery is a common procedure in general surgery practice. However, there are serious complications associated with this surgery such as laryngeal recurrent nerve injury, hematoma and hypocalcaemia. The procedure involves meticulous devascularization of the thyroid gland, which has one of the richest blood supplies among the organs with numerous blood vessels entering its parenchyma.

The LigaSure® vessel sealing system (LVSS) (Covidien, Boulder, CO, USA) is an automatic vessel-sealing system for advanced vessel sealing through the application of a bipolar electro-coagulator. The efficiency and safety of the LVSS have been reported in many procedures such as gastrointestinal or laparoscopic operations in general surgery. LVSS has also been reported to be a safe and efficient vessel-sealing system.

A new electro-surgical device developed for open surgery, the LigaSure® Small Jaw Instrument (LSJI) (Covidien) was released in 2011 (Figure 1). This device has an angled jaw for the dissection (but not advanced dissection) of tissues. The device also has the ability to cut sealed tissue via its small blade, which can be used after sealing the tissue (Figure 2).

Over the last decade, a number of experimental and clinical studies have provided evidence that surgical and trauma injury markedly affects the immune system, including both the specific and non-specific immune responses. The protective immunity of the hosts may seriously de-
pend on an appropriate cytokine balance, the proper activation and recruitment of polymorphonuclear (PMNs) neutrophils and monocytes/macrophages and an intact macrophage-T cell interaction. This mechanism has also depended on CD4+ T helper and CD8+ T cytotoxic cell activation\(^\text{10,12}\). The injuries due to surgery and trauma potentially disintegrate these complex regulatory systems and induce the deterioration of immune function\(^\text{11}\). We used the cytokines IL-6 and TNF-\(\alpha\) to investigate the local or systemic humoral inflammatory responses. The proinflammatory IL-6 and TNF-\(\alpha\) cytokines play roles in many biological processes such as inflammation, sepsis and wound healing. IL-6 and TNF-\(\alpha\) play key roles in the cross talk between cytokines and are the most easily measured\(^\text{13,14}\).

Ergonomic designs of the LVSS led to a worldwide increase in the usage of the LVSS, and increasing attention has been paid to both the side effects and benefits of these devices in the literature. Despite the well-accepted clinical use of the LVSS, to date, the local/systemic inflammatory and immunologic effects of these devices have not been thoroughly investigated. In this study, we compared LSJI with the conventional clamp-and-tie (CT) technique in thyroid surgery regarding the complication rate (hematoma, hypocalcemia and recurrent nerve palsy) and the duration of the operative procedure in the light of systemic and local inflammatory and immunologic effects. In this respect, we would like to emphasize that, as opposed to the previous literature investigating the clinical benefits or side effects of these devices, we focused on the local and systematic inflammatory and immunologic effects of these devices by observing laboratory results in addition to their clinical effects.

**Patients and Methods**

This randomized prospective study was conducted between January 2\(^{\text{nd}}\) and April 30\(^{\text{th}}\) 2013 at the General Surgery Department of the Istanbul Research and Training Hospital of Baskent University, Turkey. A total of 54 consecutive patients underwent total thyroidectomy due to multinodular goiter either via LVSS or CT techniques. All subjects were fully informed about the study and were given an informed consent form. Patient confidentiality was ensured; the patient study number and hospital chart number were the only patient identifiers. A bilateral vocal cord exam was made preoperatively and postoperatively via videolaryngoscope. All thyroidectomies were performed by two surgeons (A.D and H.Y.B). Exclusion criteria were recurrent diseases, neck dissection requirement, malignity, and known systemic inflammatory diseases, such as scleroderma or rheumatoid arthritis.

The surgeons were blinded to the technique to be used until one was randomly assigned in the operating room. The technique to be used in the surgery was decided by rolling a die (1, 3, and 5 assigned the patient to Group 1 and 2, 4 and 6 assigned the patient to Group 2). Group 1 was the CT group; Group 2 was the LVSS group.

The duration of the procedure and the weight of the removed gland were recorded in the operating room. In the LVSS group, the LigaSure\(^\text{SM}\) Small Jaw Instrument (LSJI) (Covidien) was the primary device used. In the CT group, the thyroidectomy was performed with the standard technique for small vessels \(\leq 1\) mm, and conventional bipolar electro-cautery was used in both groups. Prednisolone 0.5 mg/kg was administered intravenously prior to the incision for protecting the recurrent
laryngeal nerves against neuritis. We performed all thyroidectomies with a mini incision without a flap dissection. In all of the cases, thyroidectomy was initiated by ligating or sealing the middle thyroid vein followed by ligating or sealing the upper pole. After identifying a recurrent laryngeal nerve (NLR) and at least one parathyroid gland on each side, the lower pole was ligated or sealed. The gland was removed by freeing it from its posterior vascular attachments. Thyroidectomy bed drainage was routinely performed with a suction drain with two branches for each side.

Directly after surgery, a vocal cord exam was made with a direct laryngoscopy by an experienced anesthesiologist in the operating room. If vocal cord palsy was detected, a repeat vocal cord exam was made at the 3rd postoperative month via a videolaryngoscope. No additional vocal cord exam was needed because of the absence of permanent palsy.

Preoperatively, just prior to the incision and on the 1st postoperative morning, venous blood samples were collected to measure the levels or counts of calcium, parathormone, CRP, IL-6, TNFα and WBC, CD3+, CD4+, CD8+ T cells, CD16+/56+ NK cells and CD19+B cells. The drain samples were collected after removing the drains to measure the levels of IL-6 and TNFα in the drain fluid. The IL-6 and TNFα levels were measured in either the serum or drainage fluids.

We measured the serum calcium and parathormone levels at postoperative day one, which is our routine procedure to detect hypocalcemia after total thyroidectomies. Serum calcium levels <8.5 mg/mL were defined as the presence of hypocalcemia, and <15 pg/mL parathormone levels were defined as hyperparathyroidism according to the standards of our hospital’s biochemistry laboratory. We prescribe 0.5-1 µg calcitriol per day in scenarios of <5 pg/mL parathormone levels and 0.25-0.5 µg in scenarios of <10 pg/mL parathormone levels. We also prescribe effervescent calcium carbonate tablets for any patients with < 8.5 mg/mL serum calcium levels at patient discharge.

The scientific and ethical committees of the Medical Faculty University of Baskent approved this study with the approval number KA12/206.

**Immunologic Assays**

**Lymphocyte subsets immunophenotyping**

Peripheral venous blood samples were collected into tubes with EDTA from all subjects and were subjected to flow cytometry within two hours. A total of 20 µl of fluorochrome-conjugated monoclonal antibody were added to 100 µl of whole blood in a tube; the mixture was vortexed gently and incubated for 20 minutes in darkness at room temperature. Then, 2 ml of 1 x Becton Dickinson (BD) FACS lysis solution was added and vortexed gently after incubating for 10 minutes in darkness at room temperature. The materials were centrifuged at 300 x g for 5 minutes, washed with 2 ml of BD cell-wash, centrifuged again at 200 x g for 5 minutes and the supernatant was removed. After 0.5 ml of BD cell-fix solution was added, the remaining cells were analyzed using BD FACS Calibur in the cell quest program. Immunophenotyping was performed in peripheral whole blood T cells and T cell subtypes (CD3+, CD3+CD4+, CD3+CD8+), Natural Killer (NK) cells (CD16+CD56+) and B-cells (CD19) using the following anti-mouse monoclonal antibodies: (BD Pharmingen /UK); CD3 FITC (lot: 2129648), CD4 APC (lot: 42305), CD8 PE (lot: 76700), CD45 PerCp (lot: 2129648), CD19 APC (lot: 2190760) and CD16 56 APC (lot: 80105). In the cytofluorometric data analysis (FACS Calibur, Becton Dickinson), the cell Quest program was used to optimize the gating of lymphocytes to provide an objective means of excluding both debris and erythrocytes. All assays were repeated pre- and postoperatively.

**Cytokine Assays**

Peripheral blood plasma samples were collected pre- and postoperatively. Drain fluid samples were collected after the removal of the drains at the 24th hour. All samples were stored at –80°C until assayed for IL-6. The peripheral blood plasma and drain fluid samples, as well as the IL-6 and TNFα levels were determined using enzyme-linked immunosorbent assays (ELISA). Their concentrations were assayed using AssayMax human anti TNFα and anti-IL-6 ELISA (lot: 02411327 and 03651312) kits (Assaypro LLC, St. Charles, USA). Standard curves were used to calculate the concentrations of each cytokine as expressed by ng/mL.

**Statistical Analysis**

A power analysis for the study was performed according to the wishes of the Ethical Committee of our institution, and a minimal number of patients for the study was calculated at 52.

All patient data were collected in a prospective manner with a dedicated electronic Microsoft office Excel Database (Microsoft Corporation, San Francisco, CA).
All numerical data are expressed as the means ± SEM. Outcomes in the groups were compared using the chi-square test for categorical variables and the independent samples t-test or dependent samples t-test for continuous variables. The normal distribution for the measured data was controlled using the Kolmogorov-Smirnov test. The statistical analysis was computed with the SPSS software program (version 11.0; SPSS Inc.; Chicago, IL, USA). A p < 0.05 was considered to be statistically significant.

## Results

A total of 54 patients underwent a total thyroidectomy between January and April 2013 and were randomized into either CT or LVSS groups. The groups had 22 and 32 patients, respectively. There were no significant differences between the two groups regarding age, gender distribution or the weight of the removed gland (Table I). The mean operation time was significantly shorter in the LVSS group (83.32±29.86 min. vs 58.94±23.56 min, respectively, p = 0.002) (Figure 3). The postoperative complications are detailed in Table II.

### Table I. General characteristics of the two groups.

<table>
<thead>
<tr>
<th></th>
<th>CT group</th>
<th>LVSS group</th>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Male (n)</td>
<td>8 (36.4%)</td>
<td>5 (15.6%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Female (n)</td>
<td>14 (63.6%)</td>
<td>27 (84.4%)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>56.23±17.34</td>
<td>52.50±14.03</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Weight of removed gland (grams)</td>
<td>52.45±45.17</td>
<td>52.56±42.73</td>
<td>&gt;0.05</td>
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</table>

The postoperative complication rates were similar among both groups. The estimated blood loss (EBL) did not differ significantly between the groups. There were a total of 5 vocal cord palsies among both groups (4 in the LVSS group and 1 in the CT group). All of the laryngeal recurrent nerve injuries were one-sided and temporary. The mean postoperative calcium level was 8.49±0.51 mg/dl in CT group and 8.26±0.59 mg/dl in the LVSS group. However, there was no statistically significant difference between groups (p > 0.05). The patients who developed postoperative hypoparathyroidism or hypocalcaemia healed within the follow-up period. Hematoma occurred only in one CT group patient. There was no between-group significance regarding hematoma rate (p = 0.41). All patients in CT group were hospitalized the night following

### Table II. Postoperative complications

<table>
<thead>
<tr>
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<th>CT group</th>
<th>LVSS group</th>
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<tbody>
<tr>
<td>Temporary Vocal cord palsy (n)</td>
<td>1 (4.54%)</td>
<td>4 (12.5%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Hematoma (n)</td>
<td>1 (3.12%)</td>
<td>0 (0%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Postop hypocalcemia (n) (&lt;8.5 mg/dL)</td>
<td>12 (54.5%)</td>
<td>10 (31.3%)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Postop parathormone (pg/mL, after 24 hours)</td>
<td>28.68±21.11</td>
<td>24.19±17.66</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Length of hospital stay (mean day)</td>
<td>1±0.00</td>
<td>1.13±0.34</td>
<td>&gt;0.05</td>
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</table>
surgery. In the LVSS group, 4 patients were hospitalized for 2 nights. The difference between the two groups was not significant ($p = 0.09$). The 24-hour collected mean drainages were similar among groups (34.86±13.04 ml and 31.56±19.77 ml, respectively, $p > 0.05$) (Figure 4).

To evaluate the systemic inflammatory effect of LigaSure®, the preoperative and postoperative plasma cytokine (TNF-α and IL-6) levels and plasma lymphocyte subset (CD3, CD4, CD8, CD19 and CD16/56) counts, as well as the CD4/CD8 ratio (as acute phase reactants), plasma WBC count and CRP levels were measured (Table III).

To determine the local inflammatory effect, the TNF-α and IL-6 levels in the drainage material were measured after the drains were removed. The mean TNF-α level in the drain fluid was significantly higher in the LVSS group (0.81±0.11 vs 1.37±0.16, $p < 0.001$). The mean IL-6 level in the drain fluid was significantly higher in the LVSS group (0.52±0.09 vs 0.68±0.06, $p < 0.001$) (Table IV).

The differences in real values (increases or decreases) of IL-6, TNF-α cytokine, CD3+, CD4+, CD8+ T cell, CD19+B cell, CD16+56+NK cell level and CD4+/CD8+ T cell ratio were also compared between the CT and LVSS groups.

Only the IL-6 plasma levels were found to be higher in the LVSS group compared with the CT group; this difference was statistically significant ($p = 0.003$). However, the difference in the CD4/8 ratio was significant according to the statistics ($p = 0.049$); however, when including the confidence interval value (1), this was not significant (Table V).

**Discussion**

The LVSS technique enables surgeons to apply high current and low voltage (<200 V) to achieve simultaneous vessel sealing and division.

![Figure 4. The 24 hour collected mean drainages.](image)

| Table III. Pre-postoperatively plasma cytokine, WBC, CRP and lymphocyte subset levels. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | **CT group**    |                 | **LVSS group**  |                 |
|                 | Preoperative    | Postoperative   | $p$             | Preoperative    | Postoperative   | $p$             |
| TNF-α ng/ml     | 0.150±0.02      | 0.290±0.01      | <0.05           | 0.140±0.02      | 0.270±0.03      | <0.05           |
| IL-6 ng/ml      | 0.030±0.02      | 0.140±0.01      | <0.05           | 0.028±0.002     | 0.168±0.09      | <0.05           |
| CRP             | 9.18±4.39       | 15.45±10.35     | <0.05           | 7.56±5.21       | 12.09±12.07     | <0.05           |
| WBC             | 6713.18±1948.48 | 12573.64±5379.69 | <0.05         | 6719.18±1779.20 | 11083.75±3688.95 | <0.05         |
| CD3+ %          | 67.72±3.74      | 66.89±2.08      | >0.05           | 72.22±3.64      | 70.43±4.86      | >0.05           |
| Total lymph.    | 38.53±2.67      | 34.81±3.45      | >0.05           | 44.87±4.36      | 42.82±3.6       | >0.05           |
| CD4+ %          | 38.02±3.61      | 29.97±2.62      | >0.05           | 26.04±4.83      | 25.52±3.95      | >0.05           |
| Total lymph.    | 12.34±3.86      | 17.09±3.37      | >0.05           | 12.79±3.62      | 15.66±4.57      | <0.05           |
| CD19+ %         | 17.27±2.18      | 13.89±3.12      | >0.05           | 12.98±2.71      | 12.15±3.26      | >0.05           |
| Total Lym.      | 1.33±0.04       | 1.19±0.06       | >0.05           | 1.72±0.04       | 1.68±0.06       | >0.05           |
| CD4+/CD8+ T cell ratio | 1.19±0.06       | 1.68±0.06       | >0.05           | 1.19±0.06       | 1.68±0.06       | >0.05           |
The device acts through the denaturation of the collagen and elastin in the vessel wall. The pressure applied by the jaws allows the proteins to form a seal.

The postoperative benefits of LVSS have been controversial in thyroid surgery. In a retrospective case-control study, Petrakis et al.\(^{15}\) reported fewer complications and shorter operative and hospitalization times for the LigaSure group. In a systematic review and meta-analysis study, Hou Shan Yao et al.\(^{16}\) reached the same results.

In our study, the LVSS group had a shorter operative time, which is preferable.

In their systematic review and meta-analysis study, Hou Shan Yao et al.\(^{16}\) reported no differences between LigaSure vs conventional thyroidectomy regarding hospitalization period. Although our study had a limited number of patients, we found that hospitalization length did not differ between the groups.

In a prospective randomized study, Saint Marc et al.\(^{17}\) found no significant difference between LigaSure and conventional surgery regarding surgical complications such as hypocalcemia, RLN injury or hematoma development.

Some studies\(^{18-20}\) have reported that transient hypoparathyroidism after thyroidectomies with or without the use of sealing systems may be noted in as many as 15\% of patients according to clinical symptoms but in as many as 80\% of patients when laboratory criteria are considered. In our study, the hypocalcemia rate was slightly higher in the CT group but was not statistically significant. Significance could potentially be found in a larger series.

In our study, all of the hypocalcemia cases and low parathyroid levels were transient. The 22 patients (12 (54.5\%) in the CT group and 10 (31.3\%) in the LVSS group) who developed hypocalcemia were discharged with a prescription for a therapeutic regimen of oral calcium and cholecalciferol. Similar postoperative calcium levels and parathormone levels were found among the groups.

RLN injury is an important morbidity of thyroid surgery. In our study, all RLN injuries were transient and were primarily in the LVSS group. There was 1 (4.54\%) case of RLN palsy in the CT group and 4 (12.5\%) cases of RLN palsy in the LVSS group. This transient injury may be related to thermal energy spreading to the surrounding tissue. In some studies, transient recurrent nerve paralysis was observed in 8.7\% to 39.0\% of patients\(^{20-23}\) and was not completely avoidable even with systematic laryngeal nerve identification\(^{14,20}\).

<table>
<thead>
<tr>
<th>CT Group (mean±SD)</th>
<th>LVSS Group (mean±sd)</th>
<th>p</th>
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<tbody>
<tr>
<td>Pre and postoperatively Difference of TNF-α</td>
<td>0.136±0.14</td>
<td>0.131±0.39</td>
</tr>
<tr>
<td>Pre and postoperatively Difference of IL-6</td>
<td>0.0196±0.012</td>
<td>0.027±0.013</td>
</tr>
<tr>
<td>Pre and postoperatively Difference of CD3+</td>
<td>-0.83±0.26</td>
<td>1.79±5.12</td>
</tr>
<tr>
<td>Pre and postoperatively Difference of CD4+</td>
<td>1.72±6.8</td>
<td>2.05±8.51</td>
</tr>
<tr>
<td>Pre and postoperatively Difference of CD8+</td>
<td>1.95±5.54</td>
<td>-0.52±3.46</td>
</tr>
<tr>
<td>Pre and postoperatively Difference of CD16/56</td>
<td>-3.38±5.57</td>
<td>-0.83±4.97</td>
</tr>
<tr>
<td>Pre and postoperatively Difference of CD4/8</td>
<td>-0.084±0.34</td>
<td>-0.036±0.48</td>
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</table>
The local inflammatory effects of the vessel-sealing systems such as LigaSure and harmonic focus devices remain unclear. Inflammatory cytokine levels in the drain fluid samples of breast cancer surgery have been shown to be significantly higher when using electrocautery compared with a scalpel and ultrasonic dissector; however, there were no differences regarding the complications of mastectomy with the exception of seroma.

Cytokines in the wound healing process play an important role in initiating, controlling, and terminating cellular events such as angiogenesis and extracellular matrix formation. TNF-α in surgical wounds is secreted into the surgical field by monocytes and macrophages. Their rise in the blood is limited and slow. The minimum detectable level of TNF-α in the blood is 2 pg/mL. IL-6 is secreted from activated monocytes and macrophages, and the secretion period and half-life of IL-6 is longer than that of TNF-α. The minimum detectable IL-6 level in the blood is 1 pg/mL, and its detection in the blood is relatively easier than TNF-α. Although TNF-α levels begin to increase earlier than those of IL-6, both cytokines reach their maximum levels within 24 hours. The amounts of cytokines secreted are also associated with the duration of surgery. It has been postulated that longer operations are associated with higher cytokine levels. It Reith et al. and Kristiansson et al. demonstrated that laparoscopic cholecystectomies with less tissue trauma and shorter surgery durations induce less cytokine release than open surgery. Despite the shorter surgery duration, the LVSS group demonstrated higher cytokine levels in our study; this may demonstrate the local inflammatory effect of LVSS.

An increase in the white blood cell and polymorphonuclear cell (PMNc) counts, together with a decrease in the number of T-lymphocytes and natural killer (NK) cells, is characteristic of acute-phase response after surgery. A decrease in circulating CD4+ T-helper cells appears shortly after the operation, thus, disturbing the balance with CD8+ cytotoxic/suppressor T cells. A drop in the circulating CD4/CD8 T cell ratio has been suggested to reflect the degree of surgical trauma. Data on lymphocyte subsets after laparoscopic versus open surgery are incoherent. Most randomized studies have found no differences with respect to CD4+ and CD8+ T cells, as well as B cells and NK cells. However, other studies demonstrated an increase in or the earlier normalization of the lymphocyte count and CD4/CD8 ratio after laparoscopy compared with open surgery. The largest series of Tang et al. with a total of 161 colonic resections did not demonstrate any differences in lymphocyte subsets between the two operative procedures. Nevertheless, the measurements were performed only once, on postoperative day 3. Any differences between the groups may have been missed; Walker et al. demonstrated that significant changes occur on postoperative day one. The influence of surgery on NK cell cytotoxicity has not been extensively studied. A profound suppression of NK cell function occurs 24 hours after abdominal surgery.

In our study, both groups showed significant changes within their groups of peripheral blood CD3+, CD4+ and CD8+ T cell levels. However, if the groups are compared with each other, there is no difference between them: the surgeries affected the groups in a similar manner. This could be interpreted to mean that the suppression of T lymphocyte subset levels in systemic inflammation did not differ between the two groups. However, the relative decrease in the level of CD16+56+ NK cells in the LVSS group showed that NK cell functioning was more significantly suppressed in this group. Only the increase rate for the IL-6 plasma levels was found to be higher in the LVSS group than the CT group; this difference was statistically significant. We observed that in the drain fluids (which we consider local inflammation) in both groups, the postoperative IL-6 and TNF-alpha levels reached higher values than the plasma postoperative cytokine values. The cytokine levels of the patients in the LVSS group led us to think that the local trauma in this procedure may have further exacerbated inflammation. We also think that the cell analysis, which will be performed using the drain fluid, will be important for investigating local inflammation.

**Conclusions**

LSJI has a world-wide accepted usage in surgical era. However the safety and efficiency of the device has reported in many papers in the literature, the immunologic and inflammatory effects have not been well investigated. In this study we found that the local inflammatory response is increasing with the usage of the device; but the systemic immunologic and inflammatory response was normal.
Competing of interest
The authors declare that they have no competing interests.

Acknowledgements
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References


