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Experimental Research

Combined spinal and general anesthesia attenuate tumor promoting effects of surgery. An experimental animal study

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ABSTRACT

Background: Radical prostatectomy, a standard management approach for localized Prostate Cancer (PC), may cause a stress response associated with immune modulating effects. Regional anesthesia was hypothesized to reduce the immune effects of surgery by minimizing the neuroendocrine surgical stress response, thus mitigating tumor cells dissemination. Our primary objective was to investigate whether the use of spinal blocks attenuates PC tumor cells dissemination on an animal model. We also assessed the number of circulating NK cells and the amount of inflammatory and anti-inflammatory cytokines.
Materials and methods: A subcutaneous tumor model, with PC-3M cell line transfected with a luciferase-producing gene (PC-3M-luc-C6) was used. After proper tumor establishment and before tumors became metastatic, animals were submitted to tumor excision surgeries under general or combined (general and spinal) anesthesia. A control group was only anesthetized with general anesthesia.
Results: The subcutaneous tumor model with PC-3M-luc-C6 cells was effective in causing distant metastasis after 35 days. The number of circulating tumor cells increased in animals that underwent surgery under general anesthesia alone compared to the group submitted to combined anesthesia. Interleukin 6 levels were different in all groups, with increase in the general anesthesia group.
Conclusion: Our results suggest that combination of spinal and general anesthesia may attenuate the suppression of innate tumor immunity and it might be related to a reduction in the neuroendocrine response to surgery.
Institutional protocol number: Animal Ethics Committee 1332/2019.

1. Introduction

Mortality from Prostate Cancer (PC) is usually attributable to distant organ metastasis despite surgical resection with curative intent. Surgical resection is the primary definitive treatment for localized PC, but minimal residual disease in the form of microscopic foci not removed by radical surgery may be unavoidable in some patients [1]. Whether these residual cells result in clinical relapse is a function of host defense and tumor survival and growth [2].

At least two major factors shift the balance towards dissemination of minimal residual disease during the perioperative phase. First, local tissue damage can activate the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis with subsequent releases of catecholamines and glucocorticoids, both with immunomodulating effects that can last for several days [3]. Imbalance between pro and anti-inflammatory cytokines results in decreased number of circulating natural killer (NK) cells, cytotoxic T lymphocytes, dendritic cells and T helper cells after major surgeries [2,4,5]. Second, drugs used in anesthesia, such as opioids and volatile agents, may suppress cell mediated and humoral immunity on host defenses [1,2] and promote tumor growth in animal models [4–6].

Regional anesthesia, including epidural and spinal block, attenuates

Abbreviations: ANOVA, analysis of variance; ICTC, circulating tumor cells; IFN, interferon; IL, interleukin; IVIS, In Vivo Imaging System; FC, flow cytometry; NK, natural killer; NOD/SCID, non-obese diabetic/severe combined immunodeficiency; PC, prostate cancer; SD, standard deviation; TNF, tumor necrosis factor.

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or prevents the immune effects of surgery and anesthesia by minimizing the neuroendocrine surgical stress response, thereby reducing the need for both volatile anesthetic and opioids [7]. Despite the potential ability of neuraxial blockages to improve long-term cancer outcomes, to date evidence is scant and inconsistent. Data on this topic have been limited to animal studies and retrospective human comparisons with variable outcomes and results [4,5,8,9].

Thus, in the current study, we sought to investigate whether spinal block attenuates the PC tumor cells' surgical dissemination using an animal model. Because previous data suggest that innate immunity plays a significant role in antitumor responses, as secondary outcome we also assessed the number of circulating NK cells and the amount of inflammatory and anti-inflammatory cytokines after regional anesthesia.

2. Methods

This study was conducted in accordance with the guidelines of the Animal Ethics Committee of our institution and adhered to the applicable ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines [10].

2.1. Animals

Male, non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (15–20 g), aged 12 weeks, were maintained and fed under specific pathogen-free conditions according to a 12:12 h lighting regimen. Animals were acclimatized to the vivarium for one week. All experiments were conducted in our laboratory during the first half of the light phase. To calculate our experimental groups sample size, we used the resource equation approach [11,12].

2.2. Study design and procedure

Based on previous data [13,14], we conducted a preliminary study to evaluate the PC progression on a subcutaneous tumor model. Under sterile conditions, 1.5×10^6 tumor cells were injected under the lower right flank of NOD/SCID mice (n = 5). All animals were monitored weekly for five weeks (7, 14, 21, 28 and 35 days). Tumor growth was evaluated by in vivo bioluminescence imaging (IVIS® Spectrum, PerkinElmer, USA) and by caliper measures. After five weeks, all animals were euthanized and selected tissues were analyzed by ex vivo imaging and processed for subsequent histology. The most commonly affected tissues were chosen based on our cell lineage metastasis: jaw, ribs, femur, lungs, liver, heart, kidneys and brain.

The experimental groups included: a control group (anesthetized only with general anesthesia) (n = 7), an intervention group with surgery and general anesthesia only (n = 7) and an intervention group with surgery and combined anesthesia (general and spinal block) (n = 7). Mice were housed four or five per cage with free access to food and water.

Surgery was performed four weeks after subcutaneous injection, before tumors became metastatic, and blood was withdrawn 24 h after the procedure. Blood withdrawal or tumor injection was completed within 1 h in all animals. Mice were euthanized with isoflurane overdose. Each experiment was conducted separately, and mice were not used for more than one experiment.

Before each experiment, mice were randomly selected from their cages and placed back after they returned from anesthesia. To avoid potential order effects, mice from the different experimental groups were alternately treated throughout the study. The same investigator conducted all experiments, and blood analyses were made by a blinded researcher. There were no excluded animals during tests and data analyses.

2.3. General anesthesia, morphine and spinal block

General anesthesia was induced with isoflurane and maintained at 1.2% isoflurane in room air via a vaporizer. Mice breathed spontaneously throughout the anesthesia, and the isoflurane concentration was adjusted according to the animal's respiratory pattern.

The spinal injection technique was adapted from a previously described method [4,5,15,16]. After isoflurane induction, mice were gently held and a 30 Gauge needle was inserted between the L5 and L6 vertebrae. Penetration into the spinal canal was confirmed with tail or hind leg movement and 5 μl bupivacaine, 0.5%, containing 1.25 μg morphine sulfate was injected. This drug regimen was adapted from previous experimental studies [4,16].

Analgesia for animals without regional anesthesia was provided with morphine sulfate prepared in a concentration of 5 mg/ml in saline and injected intraperitoneally in a dose of 10 mg/kg. The injection was performed after isoflurane induction, 15 min before the abdominal incision.

Analgesia was evaluated by the tail-flick test. Pain threshold was assessed by measuring the latency to tail withdrawal after immersion in 50 °C water. To validate the analgesic effect of systemic morphine and spinal blockade, we performed measurements: 5 min before spinal or systemic analgesia, 10 min after spinal or systemic analgesia and 5 min after awakening from general anesthesia in all animals. The baseline tail-flick latency, 5 min before spinal injection or intraperitoneal morphine, was 2–4 s. A 20 s cutoff time was set to avoid tissue damage.

2.4. Tumor excision

An elliptical incision, centered over the subcutaneous tumors, was made. The incisions spanned one-third the length of the lesion, with lateral margins of 2 mm. Skin flaps were elevated to expose the adherent tumor. Once tumors were dissected clear of the adjacent fascia, the pedicles were tied with 5-0 vicryl ties. Wounds were closed using vicryl interrupted sutures. Mice awoke 3–5 min after suturing.

2.5. The PC-3M-Luc-C6 cell line

The PC-3M cell line was originally derived from bone metastasis of human prostatic adenocarcinoma. PC-3M-Luc-C6 (American Type Culture Collection, Manassas, VA, USA) is a luciferase-expressing cell line derived from PC-3M human adenocarcinoma cells by stable transfection of the North American Firefly Luciferase gene. PC-3M-Luc-C6 cells were maintained in minimum essential media (Life Technologies, Waltham, MA, USA) with 10% fetal bovine serum at 100% humidity and 5% CO_2 at 37 °C.

2.6. Blood collection

Blood was obtained by terminal blood collection in all mice from the Inferior Vena Cava. The abdominal cavity was opened with a 1 cm longitudinal incision, intestines were shifted over to the left and the liver was pushed forward. The Inferior Vena Cava was then easily located and blood was drawn. All animals were anesthetized before this procedure and were euthanized after blood collection.

2.7. Circulating tumor cells analysis

CTC were analyzed based on the bioluminescence produced by the PC-3M-Luc-C6 cell line after in vivo reaction with luciferin (MISSION® Light Switch Luciferase Assay Reagent, Merck, Darmstadt, Germany). Blood was collected and 100 μl of whole blood was exposed to the luciferase assay reagent. Cells were then treated with lysing solution (BD FACS™ Lysing Solution, New Jersey, NJ, USA) and analyzed by flow cytometry (FC). Wavelengths emitted by this reaction was around 570 nm, which was able to be detected by the flow cytometer (Muse™ Cell

Analyzer, Millipore, Hayward, CA, USA) fluorescence detectors (photodiodes yellow - 576/28).

2.8. NK cells number assay

One hundred μl of whole blood was drawn and NK cells were identified using fluorescein R-phycoerythrin conjugated with anti NK1.1 monoclonal antibody (BD Pharmingen, San Diego, CA, USA). The stained samples were treated with a lysing solution and washed. The absolute number of NK cells was then assessed by FC analysis.

2.9. Assays for cytokines production

Cytokines analyses were prepared in a 96-well plate utilizing a seven cytokines kit, Milliplex Multiple Analyte Profiling, Mouse Cytokine/Chemokine Magnetic Bead Panel (Millipore, Billerica, MA, USA), following specific protocols provided by Millipore. We measured the concentrations of interleukins (IL) 2, 4, 6, 10, 12, interferon (IFN) -γ and tumor necrosis factor (TNF) -α. All cytokines were measured in triplicates.

2.10. Counting of tumor metastasis

Based on preliminary tests evaluated by in vivo bioluminescence imaging, we selected tissues for subsequent histology analyses. After blood collection, mice were killed and we removed jaw, ribs, femur, lungs, liver, heart, kidneys and brain. These organs were fixed in buffered formalin and stained with Hematoxylin-Eosin for analysis.

2.11. Statistical analyses

Statistical analyses were performed using the GraphPad Prism 8.0.1 software. After performing Levene's test to check homogeneity of variances, one-way analysis of variance (ANOVA) and Kruskal Wallis test were used to compare CTC, NK cell counts and cytokines blood concentration between groups. We also used Student's t-test and Mann-Whitney as post-hoc tests to compare both intervention groups (general and combined anesthesia). Results are expressed as mean ± SD unless stated otherwise. For all statistics, we considered a significance level of 5% ($p \leq 0.05$).

3. Results

3.1. Subcutaneous tumors growth and distant metastasis model

Tumor growth was monitored weekly by in vivo imaging and caliper measurements. Signals at the injection site were visible on day 0. Tumor growth was noted by week 3 in all animals (Fig. 1).

In order to detect low signals from secondary metastasis, primary tumors were shielded and metastatic signals appeared after 5 weeks. Selected tissues were then analyzed by ex vivo imaging and processed for subsequent histology, which confirmed low levels of small neoplastic lesions within jaws, ribs and lungs parenchyma (Fig. 2).

3.2. Evaluation of analgesia

Systemic injection of morphine (5 mg/kg) resulted in analgesia beginning 10 min after injection and lasted for 120 min. Spinal injection of 25 μl bupivacaine with 1.25 μg morphine showed analgesia beginning 5–10 min after injection and lasting for 120–150 min. Both these analgesic techniques showed a tail withdrawal latency higher than our 20 s cutoff time. Flaccidity of lower limbs started less than 5 min after intrathecal injection and dissipated within 30 min.

3.3. Effect of surgery and anesthesia on number of natural killer cells

FC showed that surgery with general anesthesia alone did not change the number of circulating NK cells 24 h after the procedure. Also, the

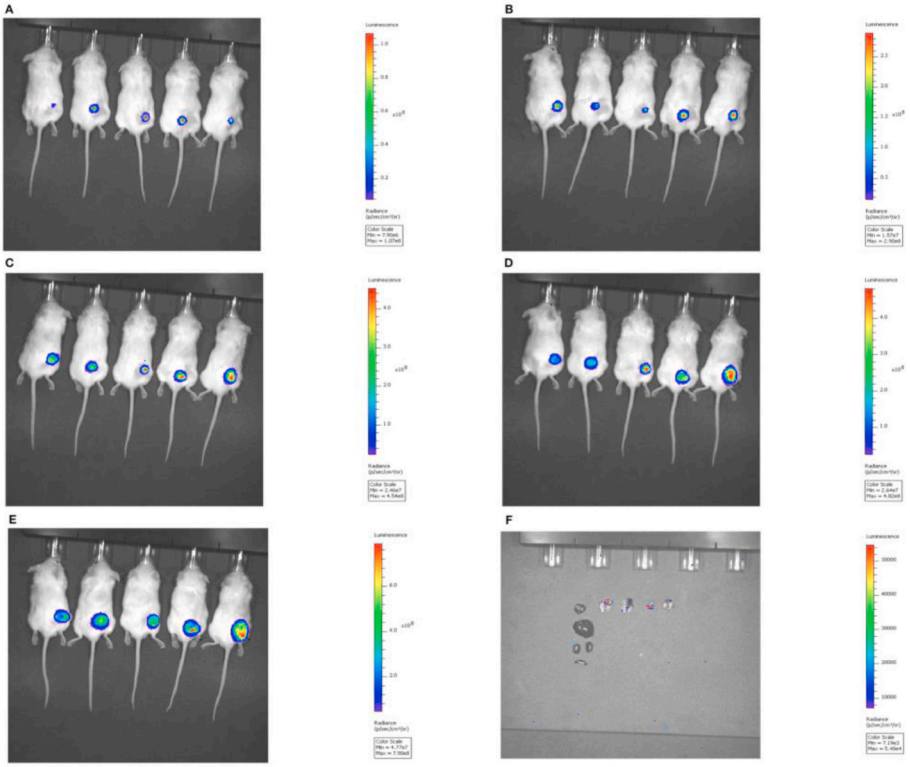

Fig. 1. Tumor bioluminescence evolution after PC-3M-lucC6 subcutaneous injection. A: 7 days, B: 14 days, C: 21 days, D: 28 days, E: 35 days, F: ex vivo imaging of selected tissues.

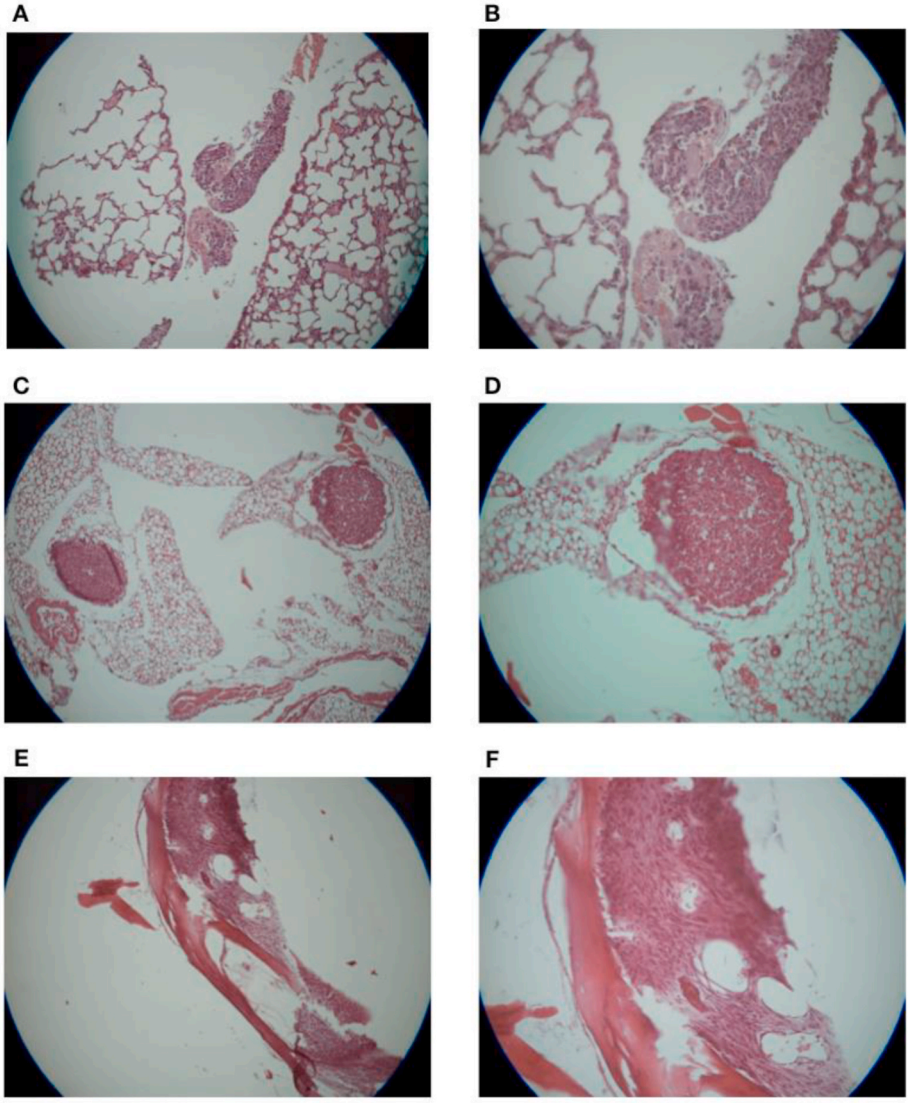

Fig. 2. Histopathology analysis of: A. lung lobes 20x; B. lung lobes 40x; C. ribs 20x; D. ribs 40x; E. jaw 20x; F. jaw 40x.

addition of spinal block to general anesthesia did not modify the number of these cells (Figs. 3 and 4; Table 1).

3.4. Effect of surgery and anesthesia on circulating tumor cells

The addition of spinal block to general anesthesia significantly reduced the number of CTC compared to animals submitted to general

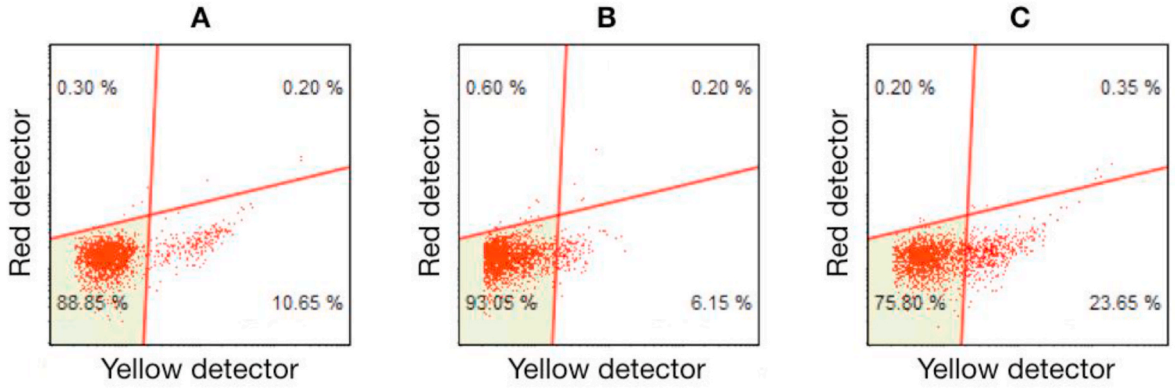

Fig. 3. Examples of experiments in the three groups. The graph of NK cells labeled with antibodies conjugated to fluorochromes detected in the FC as yellow (PE, wavelength 576/28 nm). A, control group; B, general anesthesia group; C, combined anesthesia group. FC, flow cytometry. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

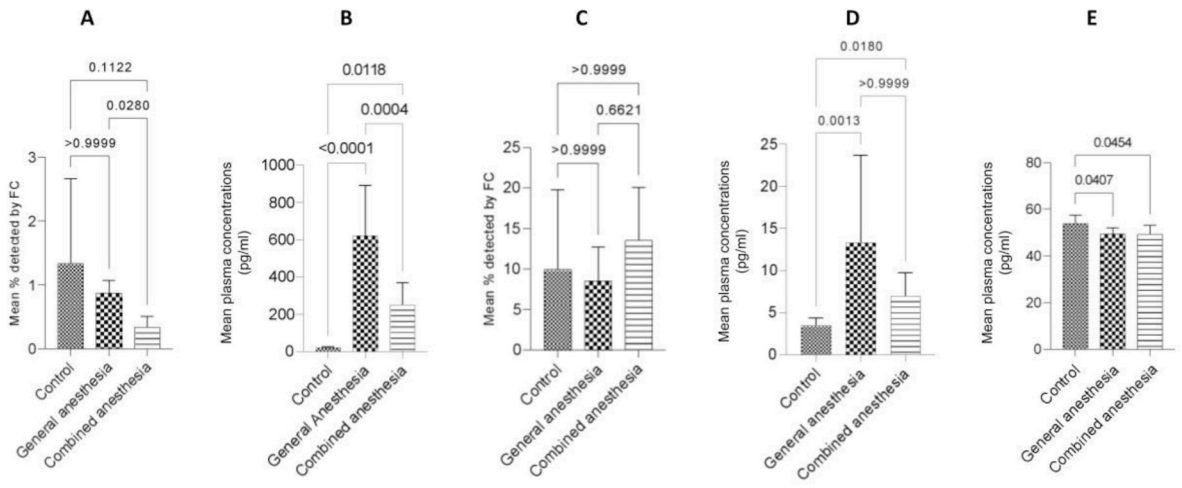

Fig. 4. Comparisons between groups. All values are means with standard deviation. A: Circulating tumor cells (CTC); B: Interleukin-6 (IL-6); C: Natural killer (NK) cells; D: Tumor necrosis factor-α (TNF- α); E: Interleukin-4 (IL-4).

Table 1
NK cells, CTC and cytokines in each group.

	Mean value per group ± SD (%)		Mean value of cytokines per group ± SD (pg/ml)						
	NK cells	CTC	TNF-α	IL-12	IL-2	IL-4	IL-6	IL-10	IFN-γ
Control	9.94 ± 4.8	3.64 ± 0.5	3.48 ± 0.89	42.82 ± 3.89	5.61 ± 0.49	53.86 ± 3.63	21.52 ± 3.76	9.80 ± 1.11	16.73 ± 2,.32
General anesthesia	8.53 ± 4.18	0.87 ± 0.2	13.30 ± 2.07	43.40 ± 4.71	5.40 ± 0.33	49.33 ± 2.77	621.75 ± 170.64	12.74 ± 3.89	18.46 ± 3.46
Combined anesthesia	13.54 ± 4.5	0.34 ± 0.17	6.96 ± 2.79	43.50 ± 1.49	5.27 ± 0.31	49.20 ± 3.95	251.59 ± 118.41	16.63 ± 5.56	25.17 ± 2.41
p	0.662	0.028	<0.001	0.0887	0.328	0.013	<0.001	0.070	0.569

Values are mean (% measured in flow cytometry or pg/ml). CTC: circulating tumor cells; IL: interleukin; IFN: interferon; NK: natural killer; SD standard deviation; TNF: tumor necrosis factor.

anesthesia alone (0.87 ± 0.2 in the general anesthesia group and 0.34 ± 0.17 in the general anesthesia plus spinal block group; p = 0.028; Fig. 4). However, no significant difference was found between the two groups that underwent surgery and the control group (p > 0.05 for both) (Figs. 4 and 5; Table 1).

3.5. Effect of surgery and anesthesia on cytokines production

Plasma cytokines mean values for each group are presented in Table 1.

IL-6 analyses showed that surgery increased this cytokine production in both groups submitted to surgery when compared to the control group (p = for p < 0.001 general anesthesia alone; 0.0118 for combined anesthesia; Fig. 4) and the addition of spinal block significantly attenuated this effect.

Production of TNF-α significantly increased in response to surgery, compared to control (p = 0.0013 for general anesthesia alone; p = 0.0180 for combined anesthesia; Fig. 4). Despite an apparent difference in the amount of this cytokine between both general and combined anesthesia groups, this variation was not shown to be significant (p > 0.99).

IL-4 level decreased significantly in response to surgery, compared to control group (p = 0.04 for general anesthesia alone; p = 0.045 for combined anesthesia; Fig. 4). However, this difference was not observed between both anesthesia groups.

No significant difference was observed between groups when

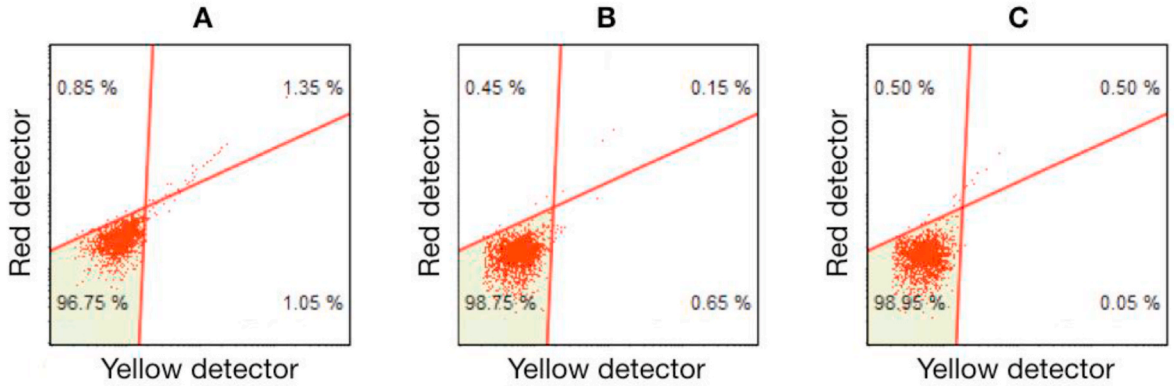

Fig. 5. Examples of experiments in the three groups. Graphs of CTC stimulated by the enzyme luciferase and detected in FC with yellow color (PE, wavelength 576/28 nm). A, control group; B, general anesthesia group; C, combined anesthesia group. FC, flow cytometry. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

analyses of other cytokines, Il-2, IL-10, IL-12 and IFN-γ, were made.

4. Discussion

Local tissue damage can reduce the production of cytokines involved in the activation of innate immunity cells. Regional anesthesia, including epidural and spinal block, may attenuate or prevent the deleterious immune effects of surgery by minimizing the neuroendocrine surgical stress response [3]. Despite the potential ability of neuraxial blockages to improve long-term cancer outcomes, to date animal and retrospective human data are scant and inconsistent [8,9]. Our study suggests that innate tumor immunity may be impaired by the neuroendocrine response triggered after surgical trauma and regional anesthesia can attenuate this effect, thereby inhibiting the release of PC-derived tumor cells into the bloodstream after surgery in an animal model.

Evidence shows that inflammatory response to surgery leads to a suppression in the immune system that begins in the wake of surgery and persists for weeks after [3]. Mechanisms for depressed immune function include suppression of NK cell activity, function and responsiveness [17,18]. This cell population spontaneously recognizes and destroys cancer cells and has been shown to be especially important in the control of CTC and micrometastasis [5,18]. We observed a slight reduction in the number of NK cells in the group submitted to general anesthesia after surgery, but this difference was not statistically significant. Previous data indicate that suppression of NK cell activity occurs within hours of surgery, lasts a few days, and is proportional to the procedure's invasiveness [17,19]. We hypothesize that the short interval of 24 h between blood collection and subcutaneous incision for tumor removal may have contributed to this absence of significance.

Another perioperative factor that may contribute to minimal residual disease is the release of CTC after surgery [20–22]. Previous clinical investigations showed that malignant cells are shed into the bloodstream during surgical manipulation of a primary tumor, leading to an increased incidence of distant metastases [21,23]. As outlined above, regional anesthesia might be able to reduce the stress response induced by surgery and also preserve NK cells activity as one of the most important factors for the elimination of CTC [24]. We noted a significant difference in CTC counts when combined and general anesthesia groups were compared. This finding contributes to the hypothesis that regional anesthesia has benefits not only for pain management and perioperative complications, but also for the control of tumor cells dissemination in the postoperative period. Although clinical results have been contradictory [7,9], other similar studies with metastatic tumor models in animals have also shown a reduction in the invasiveness of tumor cells after the use of neuraxial anesthesia [4,5].

The inflammatory response after a surgical insult promotes the healing of damaged tissue and can paradoxically result in an improved microenvironment for tumor growth [3,25]. Besides the suppression of NK cells activity, mechanisms for depressed immune function also include the production of anti-inflammatory cytokines and attenuation of inflammatory cytokines [26]. IL-6 plays an important role in determining the local and systemic inflammatory response and it has been used in many studies as an indicator of surgical stress [18]. Levels of IL-6 correlate with the magnitude of the injury and the risk of postoperative complications [27]. Scant information about regional anesthesia and postoperative IL-6 production reported that the anesthetic management may alter the postoperative IL-6 production [28,29]. In our study, compared to control and combined anesthesia groups, IL-6 concentration was significantly higher in animals that received only general anesthesia. When reviewing the literature [27,29], it is feasible that spinal anesthesia was important to attenuate the production of this cytokine. In addition, the fact that IL-6 levels were also elevated in the combined anesthesia group further suggests that spinal block reduces partly, but not totally, the neuroendocrine stress response [27].

Differences in two other cytokines, IL-4 and TNF-α, were seen when comparisons between intervention and control groups were made. IL-4 has a great capacity to suppress NK cells in vitro [30] and it reduces the production of inflammatory cytokines induced after treatment with IL-12 in human NK cells [31]. In an animal study, Salman-Ehr et al. [32] demonstrated that IL-4 is expressed during the early period of wound healing, but most of this cytokine was found in tissues located near the wounds. In fact, IL-4 has anti-inflammatory properties and exerts its regenerative effects directly on damaged cells [33,34]. Considering the systemic pro-inflammatory state immediately after surgery and the IL-4 localized effects, a blood reduction of this interleukin concentration could be expected in the postoperative period. A case-control study in humans who suffered traumatic brain injuries also showed a reduction in serum IL-4 post-injuries [35]. Our results are in agreement with these findings. On the other hand, there were no apparent differences between the quantities of this molecule in general anesthesia and combined anesthesia groups. Therefore, combined anesthesia seems to have no effect on this cytokine production in our animal model.

TNF-α is an important regulator of innate immunity in a variety of diseases, in both patients and animal models [31]. TNF-α can induce NK cell activation and enhance NK cell cytotoxicity [36]. We noted an increase in TNF-α levels in all animals after surgery, but we did not find a significant difference between general and combined anesthesia groups. Besides being an important component of the immune defense, TNF-α plays a role in collagen synthesis and wound healing [36]. It seems to be increased in pro-inflammatory states [31,37], which is in line with our postoperative results. However, the almost ubiquitous expression of TNF-α receptors in various cells, their production by different cell types under a variety of immune conditions, and the complex interaction with other cytokines make the definition of the physiological role of TNF-α a difficult task. Because of that, it is unclear how regional anesthesia would affect this interleukin. In a trial of 32 patients who were randomly assigned to general anesthesia alone or general anesthesia combined with femoral nerve block for hip surgery, a lower mean TNF-α level was noted with femoral nerve block [38]. The findings of this study suggest that regional anesthesia may attenuate the postoperative elevation of this interleukin.

Similar to TNF-α, IFN-γ has multiple functions related to the immune system and increases the cytotoxic activities of NK cells [36]. IFN-γ is also released by NK cells [37]. Angka et al. demonstrated that NK cell IFN-γ secretion is significantly suppressed for up to two months following colorectal cancer surgery [17]. We noticed a small increase in IFN-γ concentrations after surgery, higher in the combined anesthesia group. Although it was not statistically significant, increases in this molecule in the group submitted to neuraxial anesthesia could indirectly represent an improved NK cells activity in these animals [18].

IL-10 is an immunosuppressive cytokine, and its ability to reduce inflammation and promote repair of lesions is well documented [39]. No difference in anti-inflammatory IL-10 concentration was observed between groups. Tumor cells secrete IL-10 in PC and this cytokine is elevated in PC patients [39], which may explain the consistent IL-10 levels in our results. Additionally, other studies comparing the effects of general and regional spinal or epidural anesthesia in different types of surgeries revealed no difference in IL-10 levels before and after the procedures [28].

IL-2 and IL-12 did not show differences in their levels between the three groups. IL-2 and IL-12 are related to the increased activity of NK cells [18] and these cytokines may play an important role for NK cells after an inflammatory stimulus [40,41]. Literature data about effect of regional anesthesia on postoperative IL-2 and IL-12 are contradictory, with some reporting increased levels with regional anesthetic but others not [41–43]. Our results suggest that surgery and regional anesthesia exerted a minor influence on these serum cytokines. We hypothesized that a significant factor contributing to the similar levels of these two interleukins between groups is that they are largely produced by T-lymphocytes [44], absent in the NOD/SCID mice, and by antigen-presenting cells, which have limited functions in these animals.

These immunological dysfunctions are an important limitation to our study. Although the multiple defects in innate and adaptive immunity in these mice lead to the high engraftment levels of xenografts, they also may cause a reduced cytokine production capability. Since this mouse strain lacks functional T-lymphocytes, interleukins produced by these cells or their interaction with other cells are the most affected by this immunodeficiency.

Our results provide evidence that spinal and general anesthesia may attenuate the suppression of innate immunity. This association can reduce cancer cells dissemination in the postoperative period by attenuating the neuroendocrine response to surgical trauma. However, the clinical significance of attenuation of postoperative immunosuppression by regional anesthesia is still unclear and controlled clinical studies should be considered. As far as we know, the present study provides the first experimental evidence that regional anesthesia can reduce postoperative metastatic development in PC.

5. Conclusion

The subcutaneous PC model with bioluminescent cells is feasible and reproducible in NOD/SCID mice to assess types of anesthesia and their impact on this neoplasm.

In vivo, a reduction in the total number of circulating tumor cells was observed, as well as differences in the expression pattern of the cytokines IL-4, IL-6 and TNF-α, involved in the inflammatory process and activation of innate immunity cells.

Ethical approval

This study was conducted in accordance with the guidelines of the Animal Ethics Committee at the University of Sao Paulo (reference number: 1332/2019), Brazil and adheres to the applicable CONSORT guidelines.

Sources of funding

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Author contribution

Gustavo N. C. Inoue: This author helped in conceptualization, methodology, investigation, writing - original draft and funding acquisition; Ruan Pimenta: This author helped in formal analysis and software; Juliana A. Camargo: This author helped in data curation; Nayara I. Viana: This author helped in resources; Vanessa R. Guimarães: This author helped in validation; Miguel Srougi: This author helped in supervision; William C. Nahas: This author helped in supervision; Katia R. Leite: This author helped in visualization; Sabrina T. Reis: This author helped in the project administration and writing - review and editing.

Registration of research studies

1. Name of the registry:
2. Unique Identifying number or registration ID:
3. Hyperlink to your specific registration (must be publicly accessible and will be checked):

Guarantor

Gustavo N. C. Inoue.
Sabrina T. Reis.

Consent

Animal studies. Not human studies.

Data statement

The authors confirm that the data supporting the findings of this study are available within the article or its supplementary materials.

Declaration of competing interest

None.

Provenance and peer review

Not commissioned, externally reviewed.

Declaration of competing interest

None to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.amsu.2022.103398.

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