

Peer Review File

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Responses to reviewer #1:

Comment 1: Only one question: Has this "carbon nanoparticles" been approved by the FDA or other government agencies in other countries?

I wonder if this "carbon nanoparticles" has been sufficiently evaluated for how it is metabolized in the human body, allergic reactions and side effects to the patient.

I think authors need to reinforce about the safety of "carbon nanoparticles"..

Reply 1: As we know, carbon nanoparticles have been only used as a novel lymph node tracer in malignant tumors since China Food and Drug Administration approval in 2007 (approval number: Zhunzi H20073246).

Carbon nanoparticles comprises nanosized carbon with an average diameter of 150 nm, which ensures that these CNs pass through the lymphatic vessels (diameter: 120–500 nm) rather than blood capillaries (diameter: 20–50 nm). CNs are captured rapidly by macrophages. They enter the lymphatic vessels and accumulate in the lymph nodes, and they are eventually excreted by the lung and intestines within a few months (1).

Previous studies have demonstrated the safety of CNs (2). the accumulation and toxicity of CNs were preliminarily evaluated after intravenous injection in mice, where no apparent toxicity was observed, Which confirms the biosafety of CNs that entered the blood circulation during the intratumoral injection. This was consistent with the clinical observations that only several cases in all the treated patients (over 500,000) showed very short hyperpyrexia after the regional injection of CNs (3).

Changes in the text: We have reinforced the safety of carbon nanoparticles in the introduction section (see Page 6, line 84-88 and ref. 14&15).

Responses to review #2:

Major concerns:

Comment 1: In this study, preoperative injection of CNs has less chance of spillage

than intraoperative injection. Could you discuss the possible reasons? Is it because the strap muscle still in contact with the thyroid capsule and compress the thyroid during the preoperative injection?

Reply 1: In our opinion, several reasons might account for less chance of spillage in the preoperative injection of CNs than intraoperative injection. Firstly, the trap muscle still in contact with the thyroid capsule could play an important role in compressing the injection site, which prevents CNs spillage from the thyroid. Secondly, we could ensure a proper injection depth in the preoperative injection of CNs under ultrasonic guidance. Thirdly, 0.2ml saline administered to clean the needle track might reduce the chance of spillage before the needle was withdrawn from the thyroid.

Changes in the text: We have added a more detailed interpretation regarding the possible reasons for less chance of spillage in the discussion section (see Page 18, line 298-303).

Comment 2: The drawback of preoperative injection is that you cannot see if leakage occur at the time of injection. On the contrary, the leakage can be easily seen if you do intraoperative injection under 3D magnified view of robotic system in real time and proper measure can be taken to prevent further leak. What's your opinion about this?

Reply 2: Based on our experience, when we perform the intraoperative injection of CNs, we find that CNs can easily spill from the injection point of the thyroid, especially in case of an improper depth. Moreover, the leak during surgery is difficult to handle. Sometimes, it is difficult to obstruct the extravasation of CNs from the spilled point even with electrotome cauterization or compression with gauze. On the other hand, the injection procedures are complicated during robotic surgery because the needle does not have enough length. In addition, we can not control the depth of injection and can not ensure that the CNs is not injected into a blood vessel without ultrasound guidance. Therefore, we prefer the preoperative injection of CNs in endoscopic surgery.

Comment 3: In this study, 0.2ml saline was administered in addition to the 0.15mL

of CNs to avoid staining the skin. Do you think that the increase of injection volume (total 0.35mL) might higher the risk of CNs leakage?

Reply 3: As discussed in the discussion (page 17, line 293), leakage of CNs occurred in five patients during the early stage of our study. By replacing a new syringe with 0.2ml saline to clean the needle, the risk of CNs leakage can be avoided and no further leakage occurred in the following 54 patients injected with CNs. Thus we do not think that the increase of injection volume (total 0.35mL) higher the risk of CNs leakage.

Changes in the text: To be more clear and in accordance with the concern, we have added a brief description in the result section (see Page 14, line 230).

Comment 4: In your results, 3 patients had CNs leakage out of the thyroid gland and stained the peri-thyroid area black which might impair the ability to identify the parathyroid gland and recurrent laryngeal nerve. Did incidental parathyroidectomy and/or recurrent laryngeal nerve injury occur in these 3 patients?

Reply 4:

These 3 patients did not have incidental parathyroidectomy and recurrent laryngeal nerve injury. The first two patients underwent lobectomy and central neck dissection. Both of them had 2 PGs preserved in situ and did not have postoperative hoarseness. The postoperative PTH levels were 24.77 and 21.59 pg/mL, respectively. The other patient underwent total thyroidectomy and ipsilateral central neck dissection. This patient had 3 PGs preserved in situ and 1 PGs autotransplantation, and did not have postoperative hoarseness. The postoperative PTH level was 20.20pg/mL.

Changes in the text: To be more clear and in accordance with the concern, we have added a brief description in the result section (see Page 14, line 234).

Comment 5: In your conclusion, one advantage of preoperative injection of CNs is a higher black-stained rate of the central lymph node. Is it because of the time duration? How long do you think that is enough for CNs to stain the lymph node adequately?

Would it be the same or worse if you do the injection in the operating room just prior to the surgery after the patient was anesthetized? Or you prefer to inject the CNs as early as possible? In your reference 18, the authors inject the CNs one month before the surgery.

Reply 5: Regarding a higher black-stained rate of the central lymph node in the preoperative injection of CNs, as discussed in the discussion (Page 19, line 320), we speculate that the time effect may play a part in CNs diffusion and staining lymph nodes. In addition, potential damage to the lymphatic network around the thyroid might happen when performing an intraoperative injection of CNs.

Consistent with other studies (4,5), Five minutes was needed for particle diffusion based on our experience in the intraoperative injection of CNs. The black-stained rate of total LNs was 91.71% (data not shown in this study) in patients with the intraoperative injection of CNs in our team, which is lower than the black-stained rate (96.21%) in this study.

We have not injected CNs at other different times (after anesthesia or other different times before surgery), and we plan to do further studies on the application of preoperative CNs at different times and under different surgical approaches.

Comment 6: In Table 2, the detection rate of Delphian node was higher in the CNs group. Is the detection rate also higher for lymph nodes in other locations? (e.g. paratracheal, pretracheal)

Reply 6: Thank you for your careful review. In our opinion, we could dissect more black-stained lymph nodes located in the potential occult position with CNs, such as the prelaryngeal, the right paraesophageal, RLN laryngeal entry point, and low central zone. However, we did not classify the central compartment by region. In our observation, the detection rate might be also higher for lymph nodes in the paratracheal (more nodes dissected in paraesophageal and RLN laryngeal entry point) and pretracheal region (more nodes dissected in the low central zone).

Minor issue

Comment 1: line 164: typographical error, “d” in the sentence, “Furthermore, we would d thoroughly dissect “

Reply 1: Thank you for underlining this deficiency. We have modified the sentence in the revised manuscript as the following: “Furthermore, we would thoroughly dissect”.

Changes in the text: We have modified our text as advised (see Page 11, line 171).

Comment 2:line 177: it seems not suitable to use “were” in this sentence. “All patients were empirically intravenous calcium gluconate (0-2.0 g/day) as well...”

Reply 2: We have modified the sentence in the revised manuscript as the following: “All patients are empirically intravenous calcium gluconate (0-2.0 g/day) as well”.

Changes in the text: We have modified our text as advised (see Page 11, line 185).

Comment 3: How do you count the number of black-stained lymph node in this study? Do you have a clear definition? Or it is just relied on pathologist’s discretion?

Reply 3: As shown in the picture below, we divided the specimen of nodes into two parts(black-stained and unstained) before they were sent to pathological examination.



Reference:

1. Hagiwara A, Takahashi T, Sawai K, et al. Lymph nodal vital staining with newer carbon particle suspensions compared with India ink: experimental and clinical observations. *Lymphology* 1992;25:84-9.
2. Xie P, Yang ST, He T, et al. Bioaccumulation and Toxicity of Carbon Nanoparticles Suspension Injection in Intravenously Exposed Mice. *Int J Mol Sci* 2017;18.
3. Chongqing Lummy Pharmaceutical Co. L, Chongqing, China. Internal data of carbon nanoparticles suspension injection. 2017.
4. Liu JS, Xu CW, Wang R, et al. Do carbon nanoparticles really improve thyroid cancer surgery? A retrospective analysis of real-world data. *World Journal of Surgical Oncology* 2020;18.
5. Yu WB, Cao XL, Xu GH, et al. Potential role for carbon nanoparticles to guide central neck dissection in patients with papillary thyroid cancer. *Surgery* 2016;160:755-61.