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Impact of cyclophosphamide on the morphological and histological changes in polyglycolic acid spacers

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ABSTRACT

In radiotherapy for pediatric abdominal tumors, determining the effect of concurrent chemotherapy on polyglycolic acid (PGA) spacers is crucial; yet this effect has not been validated. Therefore, we aimed to evaluate the impact of cyclophosphamide (CPA) chemotherapy on the PGA spacer using a rat model. Twenty-four rats were implanted with the spacer, and morphological changes in the spacer were assessed on CT for both the CPA-dosed group (40 mg/kg) and the control group. The size and volume of the spacer were quantified using CT, while the degree of adhesion and microscopic examination of the tissue were determined using pathology specimens. Morphologically, the size of the spacer decreased over time in both the CPA-dosed and control groups, with no significant differences observed between groups. No significant differences in adhesion were observed between the two groups. Macrophages were observed around the PGA fibers, suggesting their involvement in the degradation of the PGA spacer. These results suggest that CPA does not cause significant clinically problematic degradation or adverse tissue reactions to the PGA spacer. This study reinforced the benefits of PGA spacers; however, future research focusing on *in vivo* longitudinal monitoring of individual rats, as well as on humans, is required.

Keywords: spacer; polyglycolic acid; PGA; cyclophosphamide; chemotherapy

INTRODUCTION

In radiation therapy, increasing the radiation dose is desirable for enhancing local treatment efficacy. However, when treating cancers near tissues at risk, the dose is often limited to protect surrounding healthy tissues. Even modalities like particle therapy cannot entirely eliminate adverse events. Developments in radiation therapy include techniques for artificially creating a 'space' between tumors and healthy organs [1–4]. Hydrogel spacers, known for their biocompatibility and ability to create space temporarily, are extensively utilized to distance the rectum from the radiation field, particularly in prostate cancer treatment. However, the injection area is limited [5, 6]. GORE-TEX® spacers (W. L. Gore & Associates, Inc., Newark, DE, USA) have been used to protect radiosensitive organs, such as the small and large intestines, when in close proximity to malignant tumors [7–9]. While

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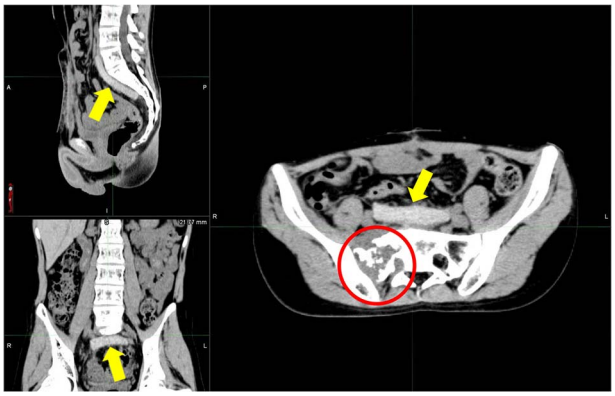

Fig. 1. Clinical application of PGA spacers. CT image of a 14-year-old boy with a primary Ewing-like tumor in the sacrum, a case of recurrence (circle) after previous proton therapy in the primary sacrum. A PGA spacer (arrow) was implanted to reduce the dose irradiated to the gastrointestinal tract as much as possible during re-proton therapy with irinotecan and CPA chemotherapy combination. PGA, polyglycolic acid; CT, computed tomography; CPA, cyclophosphamide.

these methods are effective in certain contexts, they often present limitations in terms of biodegradability, long-term safety and patient comfort. Polyglycolic acid (PGA) spacers have been developed and clinically adopted to overcome these limitations. In animal studies, PGA spacers have demonstrated biological safety, shielding efficacy and durability [10–14]. They represent a major advance in terms of reducing the long-term complications associated with conventional spacers and providing a biodegradable, patient-friendly alternative.

Chemotherapy prior to or in combination with radiation therapy is commonly used to treat pediatric malignancies, particularly Ewing sarcoma and rhabdomyosarcoma. There is concern that radiotherapy or particle therapy may cause significant disruption in later life, including growth retardation and infertility, if organs such as the spine, gastrointestinal tract and reproductive organs, are in close proximity to the tumor [14, 15]. Previous studies on PGA spacers have been validated primarily in specimens without chemotherapy. Although our institution has some experience with spacer implantation in combination with chemotherapy, it remains unknown how the PGA spacer is adversely affected when combined with chemotherapy in children (Fig. 1). Therefore, understanding how chemotherapy affects PGA spacers was the main focus of this study.

PGA spacers are rarely removed, and abdominal computed tomography (CT) is rarely performed to avoid radiation exposure. This leaves little opportunity for clinical evaluation of implanted PGA spacers and necessitates animal studies. In the present study, rats were selected for two reasons: they possess sufficient space in the abdominal cavity to implant the PGA spacer and for ease of experimentation.

Cyclophosphamide (CPA) is an alkylating agent that inhibits the synthesis of nucleic acids in cells and is used in many cancer types, including Ewing's sarcoma, rhabdomyosarcoma, multiple myeloma, malignant lymphoma and leukemia. It can be taken orally. CPA was chosen for chemotherapy because of its ease of administration in experimental animals and its common clinical use in pediatric malignancies. The main side effects are myelosuppression and hemorrhagic cystitis, with the former being used to determine the optimal dosage in this study [16, 17]. Therefore, this study aimed to investigate the effects of CPA on the PGA spacer in a rat model.

MATERIALS AND METHODS

Rats

This experiment was conducted after deliberation and approval by the Nagoya City University Animal Experiment Committee (Approval No. 20–039) and was conducted in compliance with the Code of Ethics. The experimental design necessitated considerations for the ease of blood collection, drug administration and the capacity of the abdominal cavity to accommodate the spacer. We utilized 24 male rats (Sprague–Dawley; closed colony, aged nine weeks). Three rats only with CPA and three controls were prepared in addition to the 24 rats with implanted spacers to determine the effect of CPA administration on the rats. A week-long acclimatization period to the laboratory environment was conducted prior to the study to minimize stress-related variables. The rats were housed under controlled 12-hour light/dark cycle conditions, with unrestricted access to food and water. Continuous monitoring of their health status and behavior was performed to ensure their welfare and to identify any signs of distress or illness. To maintain research standards as well as ethical considerations, humane euthanasia was performed on six rats every four weeks using carbon dioxide asphyxiation. This method was chosen to mitigate suffering and was performed within each housing cage to reduce stress among the cohort.

PGA spacer

The PGA spacer (NESKEEP®, Alfresa Pharma Corporation, Osaka, Japan) was designed to create a gap between normal organs and tumors, thereby facilitating increased dosage delivery to the cancer during radiotherapy and particle therapy. It is a 5-mm-thick non-woven fabric featuring numerous cavities. To remove internal air, the PGA spacer was previously injected with saline solution into its interior with a syringe. Subsequently, it was cut into 3-cm squares using a scalpel or surgical scissors. The prepared PGA spacer was meticulously wrapped on all sides with Seplafilm® (Kaken Pharmaceutical Co., Ltd., Japan) [18], a bioresorbable membrane comprising sodium hyaluronate and carboxymethyl cellulose, to prevent adhesion between the spacer and surrounding organs. This procedure was performed on all spacers.

The volume of the PGA spacer per body weight used in the rats was 7.5–9.0 cm^3/kg, which is comparable to implanting a 500–600 cm^3 spacer in a 70 kg adult and is considered to be clinically relevant volume.

Cyclophosphamide administration

In this experiment, 24 rats were randomly and equally divided into two groups: a CPA-dosed group and a control group. CPA was administered orally to the CPA-dosed group using a catheter. This method was chosen for its dosage accuracy and minimal stress imposition on the animals. Doses (40 mg/kg) were administered once every 4 weeks for 16 weeks. This dose was chosen to ensure the safety of the rats and

to prevent acute side effects. This CPA dose is the same dose given to pediatric patients with malignant tumors [19]. Furthermore, this dose was also validated in this experiment.

Blood sample

Blood samples were drawn from the tail artery of sedated rats. They were collected before each dose and at 0, 3-, 7-, 14-, 21- and 28-days post-dose in each cycle. The primary aim of these blood analyses was to monitor signs of pancytopenia, focusing on white blood cell counts (neutrophils), hemoglobin levels and platelet counts.

Surgery

The PGA spacer is typically handled in a sterile environment, such as an operating room; however, in this study, the constraints of the animal experimental setting necessitated the creation of a clean area, and the operation was performed within a quasi-sterile environment. For sedation, we administered inhalation anesthesia using isoflurane, which is known for its efficacy and safety in small rodents [20, 21]. A 6–7 cm incision was made in the midline of the rat's abdomen, and the PGA spacer was placed between the intestinal tract and the abdominal wall, with several sutures (blue nylon) securing it to the abdominal wall.

CT

CT was conducted using the Optima CT 580 (GE Healthcare, Japan). Imaging parameters were standardized with an X-ray tube voltage of 120 kV, gantry rotation time of 0.5 seconds, field of view of 10 × 10 cm, slice thickness of 0.625 mm, pixel size of 0.02 cm, and a matrix size of 512 × 512 pixels. These settings optimized image quality and consistency for precise assessments of the PGA spacers. Owing to our facility's limitations, continuous weekly CT and temporal monitoring of the PGA spacers in living rats were not feasible. To ensure hygienic conditions during the CT procedure, euthanized rats were meticulously prepared to prevent contamination of the CT scanner and the environment. CT was performed within 2 hours post-euthanasia to assess *in vivo* changes effectively. The rats underwent CT at 4, 8, 12 and 16 weeks to evaluate the dimensional changes of the PGA spacer over time.

Autopsy

All rats were dissected within 3 hours after CT. The abdominal cavity was opened via an incision, exposing the PGA spacer for initial observation. Observations were focused on assessing the extent of adhesions on the spacer's external surface; thereafter, its internal structure was examined through pathological analysis. A modified version of the adhesion scoring system, typically employed during surgical evaluations (Table 1), was utilized to assess the extent of dissection and adhesion of the extracted spacer [22]. The evaluation was performed by two people: the person who performed the dissection and an assistant dissector. In addition, photographs and other digital data were taken and acquired from two directions (back and front) to allow for reconfirmation and review of the evaluation. PGA spacers that were too small for accurate recognition and evaluation through CT or visual inspection were assigned a negative (−) rating. For spacers rated negatively, the presence of the blue nylon suture, used to secure the spacer to the peritoneum, served as an important marker for locating the reduced PGA spacer.

Pathology

Pathology specimens were fixed in formalin with the PGA spacer and adherent surrounding tissues (intestinal tract, peritoneum, liver, etc.), and hematoxylin–eosin staining was used to evaluate cell and tissue responses to the spacer. These specimens were prepared by the Pathology Department staff as well-preserved tissue samples to reproduce *in vivo* conditions accurately. Pathology experts performed histological evaluations and shared information on all pathology specimens. Particular attention was paid to identifying macrophages, inflammatory responses and histological changes.

Image-based measurement

The assessment focused on the spacer's length, width, thickness and volume. Following imaging, the CT data were imported into RayStation® (Raysearch Laboratories, Sweden), a radiation therapy planning system, to confirm the spacer's position and interaction with adjacent tissues, perform contouring, and measure the spacer's size and volume. To ensure uniformity, one of the study members, a radiation oncologist, performed all spacer contouring and size measurements.

Statistical analysis

Blood cell counts before and after CPA administration for each rat, as well as spacer size every 4 weeks were recorded and analyzed separately for the CPA-dosed and control groups. To eliminate individual differences, at least three sets of data at each time point were used. Given the data followed a non-parametric distribution, the Kruskal–Wallis test was chosen to compare variables between the defined groups. Statistical significance was set at $P < 0.05$. Data were analyzed using SPSS version 28 (IMB corp., Armonk, NY).

RESULTS

Assessment of cyclophosphamide dosage

Neutrophil counts in the CPA-dosed group were lower than those in the control group on day three after dosing. The decrease in neutrophils from day 0 to day 3 with CPA was 19% and 53%, respectively (Fig. 2A and B), while without CPA, the increase was 45% (Fig. 2C) ($P = 0.01$).

Comparative analysis of PGA spacer reduction on CT

Figure 3A illustrates the representative CT images of the control group. After 4 weeks of PGA spacer implantation, its morphology remained unchanged and appeared as a highly absorbent object on CT. After 8 weeks, it became thicker and slightly rounded, with reduced absorbency; by 12 weeks, it had become spherical with visible shrinkage; and by 16 weeks, the shrinkage was so pronounced that the spacer was no longer recognizable and had nearly vanished.

Figure 3B displays the CT images of the CPA-dosed group. The PGA spacer's shrinkage pattern was similar to that observed in the control group, with air pockets visible within the spacer at week 8.

Table 1. Adhesion score system for PGA spacer

Score	Description of score: separability, adhesion surface area (%)
0	No adhesion
1	Partially filmy adhesion: easy to separate with gentle traction, 1%–25%
2	Partially thick adhesion: separated with moderate traction, 26%–50%
3	Dense adhesion: not separate, 51%–75%
4	Very dense adhesion: not separate, 76%–100%
—	Obvious reduction, fibrous adhesions

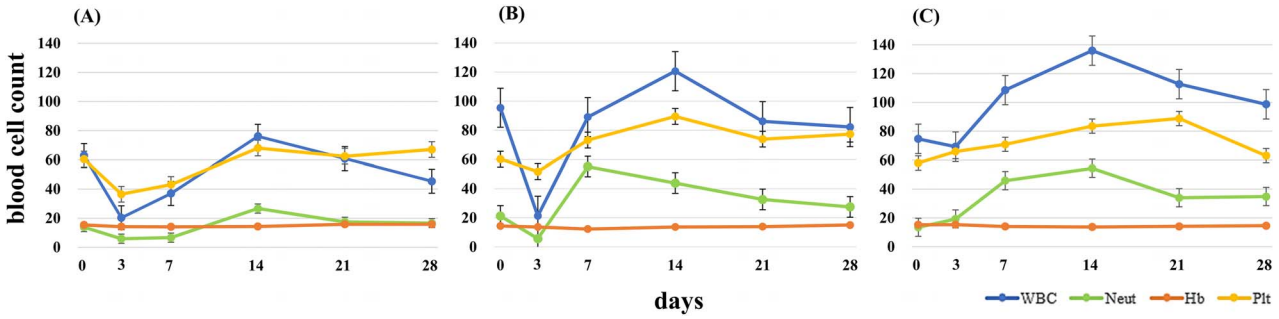

Fig. 2. Results of blood sampling in rats and controls following CPA administration. (A) CPA-dosed group without spacer surgery; (B) CPA-dosed group with PGA spacer surgery; (C) control group with PGA spacer surgery. Blood tests were performed immediately before and on day 3 and weeks 1, 2, 3 and 4 after the experiment began. The measurements include white blood cells (blue; ×100/mL), neutrophils (green; ×100/mL), hemoglobin (orange; g/dL), and platelets (yellow; $\times 10^3$/mL). The CPA-dosed group shows a decrease in neutrophils on the third day compared with the control group. CPA, cyclophosphamide; PGA, polyglycolic acid.

Figure 4 presents a graph depicting the size changes of the PGA spacer in both groups. The difference in size parameters between the two groups appeared to be more pronounced from 12 to 16 weeks; however, there was no statistically significant difference in spacer thickness between the two groups ($P = 0.12$).

Evaluation of adhesions

Small bands of remnants were observed on some of the spacers examined between 12 and 16 weeks, showing a decrease. In these instances, adhesions received a negative (−) rating. A trend toward fewer adhesion scores was observed in the CPA-dosed group relative to the control group (Fig. 5), although this difference was not statistically significant ($P = 0.21$).

Pathological analysis of microscopic changes in PGA spacers

The pathological examination elucidated the microscopic alterations occurring within the PGA spacer at different time points. Histological analyses revealed that macrophages enveloped the PGA fibers within the spacer (Fig. 6). Notably, marked fibrotic changes were observed on the spacer's surface, alongside adhesions to adjacent organs, including the peritoneum, the liver and muscle tissues. While inflammatory cells, such as neutrophils, were identified, there was no evidence of acute or chronic inflammation.

DISCUSSION

The PGA spacer is intended for implantation in the human body; therefore, ecological compatibility, actual contraction processes and behavior should be evaluated during chemotherapy. Although, in some cases, PGA spacers have been used clinically in combination with chemotherapy, existing studies have shown that *in vivo* evaluation using chemotherapy has not been adequately performed [10, 11, 14]. In the present study, using a rat model, we compared the CPA-dosed group with a control group and analyzed morphological changes in the PGA spacer over a 16-week period using direct observations of abdominal CT and extracted spacers. This provided a unique perspective for evaluating changes in the PGA spacer in the combined chemotherapy setting.

Although CPA was selected for chemotherapy because of its versatility in the treatment of pediatric tumors, the optimal CPA dose for rats has not been determined in existing experiments. Considering myelosuppression was observed on day three of CPA administration to the rats in the current study at a dose of 40 mg/kg based on pediatric malignancy dosing guidelines [19], it was determined that an appropriate pharmacological effect could be achieved in rats.

Similar changes in spacer dimensions, such as length, width, thickness and volume, were observed with and without CPA administration, suggesting that CPA does not influence PGA spacer shrinkage. Given the long duration of radiation and particle therapy in pediatric malignancies, ensuring that spacers do not degrade prematurely is critical.

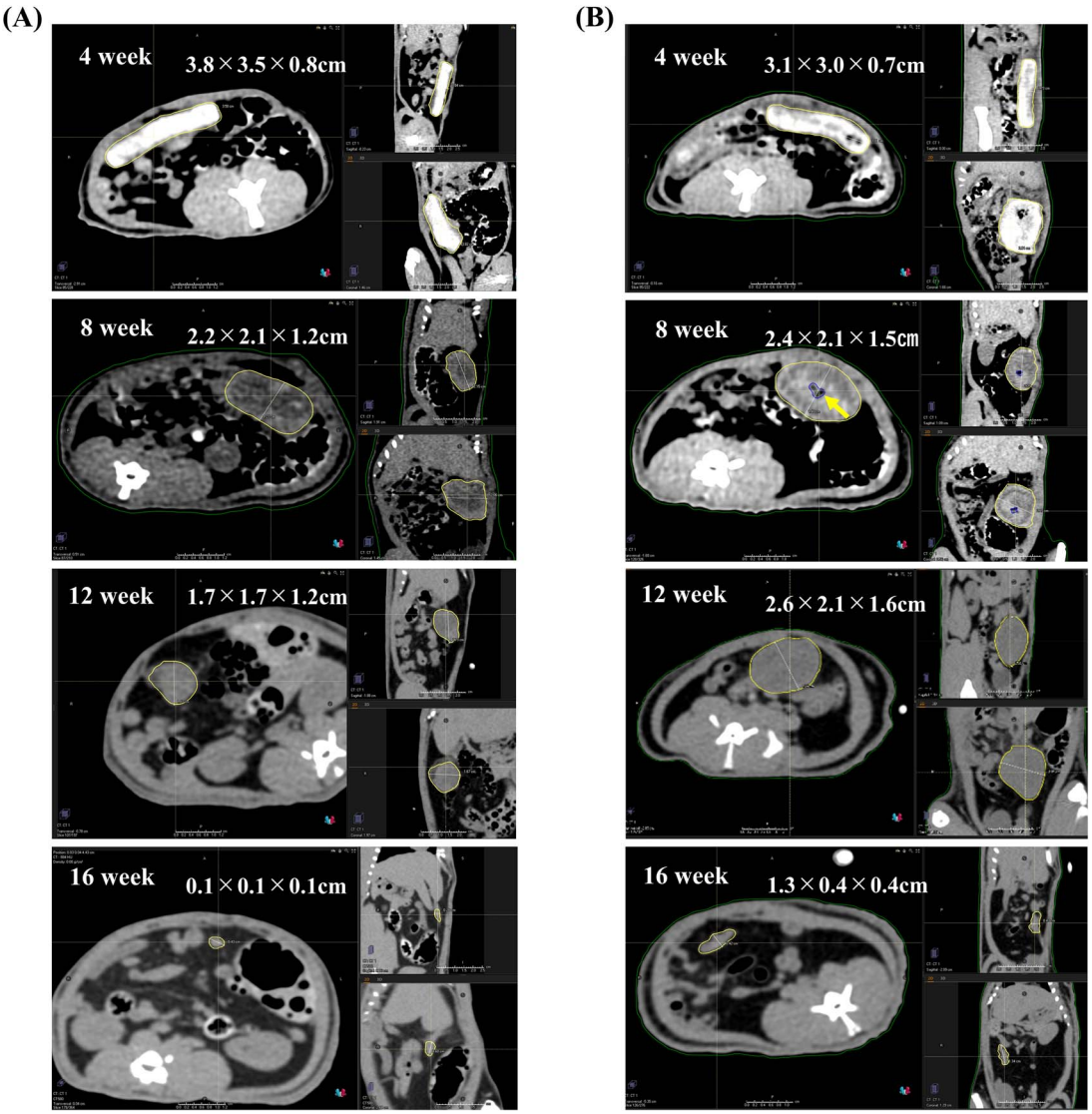

Fig. 3. Comparison of the two groups by CT. (A) CT of the control group, taken at four-week intervals, showed the PGA spacer within the rat's abdominal cavity. Over time, the spacer decreases in size, with noticeable morphological changes between weeks 4 and 8, including an increase in thickness and a more spherical shape. (B) CT of the CPA-dosed group, taken at four-week intervals, demonstrates a similar pattern of size reduction in the spacer. Notably, a 0.05-cm^3 pocket of air (arrow) is observed within the spacer at week 8. CT, computed tomography; PGA, polyglycolic acid; CPA, cyclophosphamide.

A characteristic change was observed both in the CPA-dosed group and the control group; specifically, the thickness of the PGA spacer transiently increased during degradation in the rat's body. This change may be due to the smaller size of the implanted spacer compared to that used in humans. The PGA spacer used in the current experiment was the same as that used in clinical practice, suggesting there may be periods of temporary increases or decreases in spacer thickness in spacers implanted in humans.

Contrary to our initial hypothesis that CPA administration may increase susceptibility to infection and adhesion formation, we observed a trend toward fewer adhesions in the CPA-dosed group compared to the control group. Although this difference was not statistically significant, it is possible that macrophages and other factors found in the surrounding area may be involved.

The results of the experiments suggest that hydrolysis plays a notable role in the degradation of the PGA spacer. However, the presence of macrophages around the PGA fiber also indicates that macrophages may contribute to the degradation process of the spacer, possibly as part of the body's response to the PGA fiber [23]. Based on pathological images, the absence of inflammatory reactions indicated no incompatibility reactions to the surroundings, including adherent organs, further emphasizing the biocompatibility

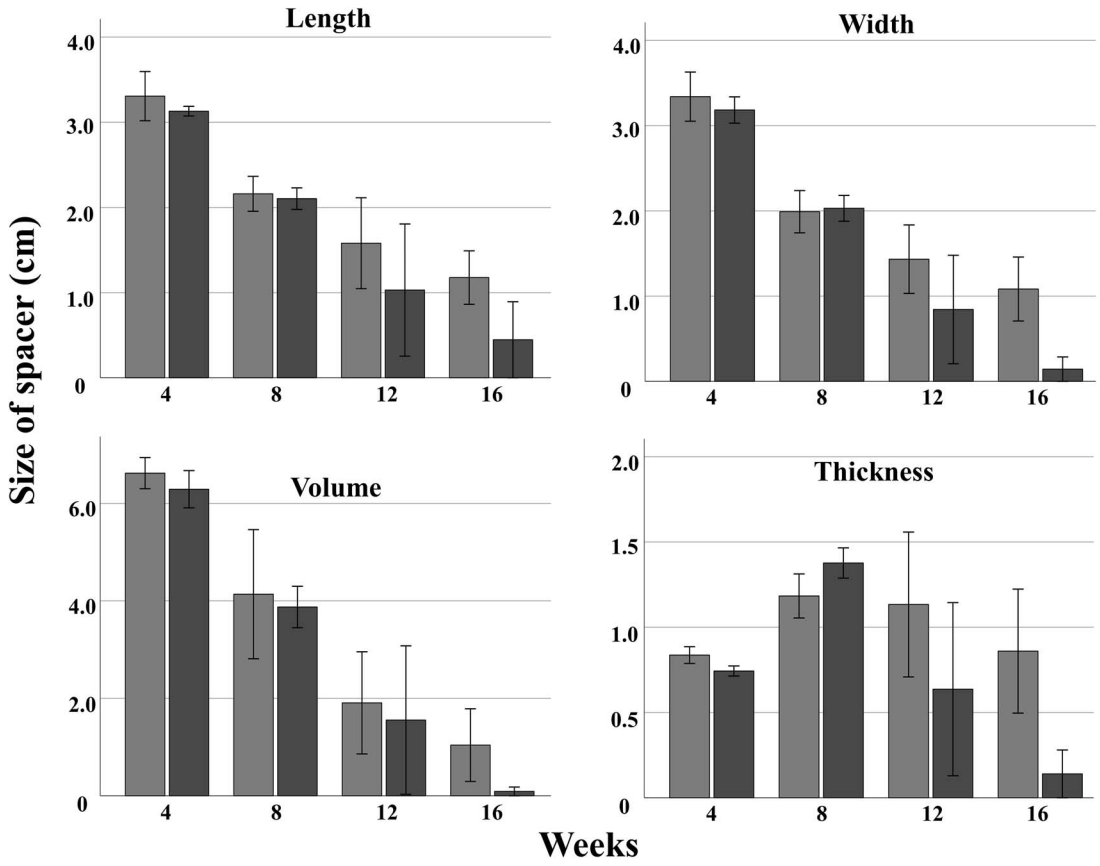

Fig. 4. Size change of PGA spacer in CPA-dosed and control groups. Gray: Control group with PGA spacer surgery; black: CPA-dosed group with PGA spacer surgery. Data are presented as mean measurements from three rats per group, showing no significant difference in size reduction between the groups ($P > 0.12$). PGA, polyglycolic acid; CPA, cyclophosphamide.

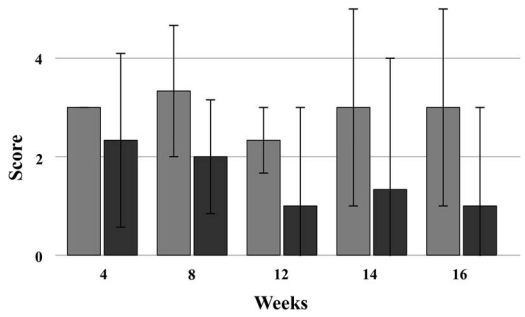

Fig. 5. Results of PGA spacer adhesion evaluation in CPA-dosed and control groups. Gray: Control group with PGA spacer surgery; black: CPA-dosed group with PGA spacer surgery. The figure shows the average adhesion score for spacers from both groups, obtained at four-week intervals, based on assessments from three rats in each group. PGA, polyglycolic acid; CPA, cyclophosphamide.

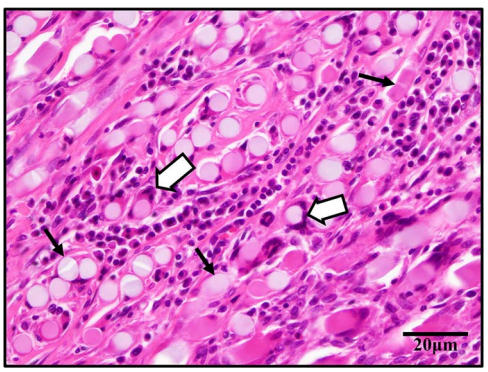

Fig. 6. Tissue specimen (hematoxylin–eosin stain) around the PGA spacer. The image displays PGA fibers (black arrows) contained within a circular or rectangular structure with a relatively homogeneous interior. These are surrounded by macrophages and multinucleated cells (white arrows) at 400× magnification. PGA, polyglycolic acid.

of PGA. Additionally, macrophages are assumed to be involved in the regulation of fibrosis [24]. This supports the safety profile of PGA spacers, although further detailed pathological studies and validation are necessary to consolidate these findings.

There were several limitations and constraints to the present study, the most significant being facility limitations, such as the inability to perform CT on live rats. Given it is desirable to evaluate the

same spacer using CT over time to eliminate individual differences, consideration should be given to coordinating other facilities for additional experiments. Another limitation is that chemotherapy was limited to CPA. Other anticancer drugs may affect the PGA spacer through different mechanisms. The current results may not apply to other chemotherapy regimens, and thus, further extensive evaluation is needed. Moreover, additional in vivo longitudinal studies in rats are needed to address the above limitations and confirm the clinical efficacy and safety of the PGA spacer. In addition, an ongoing Phase I study (UMIN 000039288) is expected to provide further insight and potentially expand the use of PGA spacers in cancer treatment protocols.

This study provides crucial insight into the impact of CPA chemotherapy on the morphological and histological characteristics of PGA spacers in a pediatric abdominal tumor model. Our results show that CPA administration does not significantly influence the degradation process of the PGA spacer nor cause adverse tissue reactions. These outcomes highlight the efficacy of the PGA spacer and confirm its compatibility with CPA chemotherapy and radiation therapy. Future research should expand on this investigation by further comparing outcomes between CPA-dosed and non-dosed groups in rats.

PRESENTATION AT A CONFERENCE

Presentation at the 35th annual meeting of the Japanese Society for Radiation Oncology, Hiroshima, 21–24 October 2022.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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