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The effect of pembrolizumab on the healing of colonic anastomosis: a pre-clinical study in Wistar rats

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Abstract

Background Pembrolizumab is a monoclonal antibody that targets the programmed cell death-1 (PD-1) protein. Blocking this pathway alters T-cell activity, and has been approved for the treatment of several malignancies, including microsatellite instability-high (MSI-H) and mismatch repair-deficient (dMMR) colorectal cancers. The aim of this study was to evaluate the effect of pembrolizumab on colonic anastomotic healing in a rat model.

Methods Sixty male Wistar rats were randomly divided into 2 groups of 30: a control group, and an experimental group receiving pembrolizumab. Each group was further divided into 3 subgroups of 10 rats, sacrificed on postoperative day (POD) 3, 7 or 14. All animals underwent laparotomy, a 1-centimeter segmental colectomy, and an end-to-end colonic anastomosis. Postmortem evaluation included measuring anastomotic bursting pressure, tissue hydroxyproline levels, and histopathological assessment.

Results Statistically significant differences in bursting pressure (P=0.019) and rupture site (P=0.033) were observed between the groups on POD 7. Tissue hydroxyproline levels were significantly lower in the pembrolizumab-treated subgroups on POD 7 (P=0.003), and POD 14 (P=0.001). Histopathological analysis demonstrated significant differences on POD 3, in neovascularization (P=0.026), fibroblast ingrowth (P=0.005), and collagen deposition (P=0.030), suggesting impaired inflammatory-phase healing.

Conclusions This experimental study suggests that a high single dose of pembrolizumab may negatively affect colonic anastomotic healing in rats. Further studies are necessary to determine the safety of intestinal anastomosis in both emergency and elective clinical settings.

Keywords Pembrolizumab, immune checkpoint inhibitors, healing, anastomosis, colorectal cancer

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Conflict of Interest: None

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Introduction

Immune checkpoint inhibitors (ICIs) include monoclonal antibodies targeting various molecules, including programmed cell death protein 1 (anti-PD-1: pembrolizumab and nivolumab), programmed cell death ligand 1 (anti-PD-L1: atezolizumab), and cytotoxic T-lymphocyte antigen-4 (durvalumab) [1]. PD-L1 is expressed on antigen-presenting cells, tumor cells and some normal tissues [2]. The interaction of PD-L1 with PD-1, which is expressed on T-cells, inhibits T-cell responses, preventing autoimmune reactions and hindering cancer detection [2]. Conversely, blocking the

PD-1/PD-L1 pathway leads to overactivation of T-cells, enhancing the immune response and aiding in the detection of cancer cells [2].

Pembrolizumab, a humanized IgG4 monoclonal antibody against PD-1, has been approved for the treatment of various malignancies, including melanoma, squamous cell cancers, urothelial cancers, hepatocellular carcinoma, lymphomas, gastric and esophageal cancer, lung cancer, cervical cancer, triple negative breast cancer, and colorectal cancer (CRC)—which may be microsatellite instability (MSI)-high or mismatch repair-deficient (dMMR), or have a high tumor mutational burden [2]. A growing number of patients on immunotherapy may require surgery during their cancer journey. According to a 2023 review by Sindt *et al* [1], no specific wound healing complications were associated with ICI administration; therefore, no recommendations were made to delay ICI administration perioperatively. However, the administration of other monoclonal antibodies, such as bevacizumab, an antibody against vascular endothelial growth factor, has been related to impaired wound healing [3] and an increased risk of anastomotic leaks [4].

MSI-high and dMMR account for 10-15% of CRCs [5]. Currently, pembrolizumab is the first-line treatment for metastatic or unresectable dMMR/MSI-high CRC [6], while its use as neoadjuvant agent for localized or resectable dMMR/MSI-high CRC is under investigation. The good response rates reported in the literature [7], which convert inoperable to operable disease, suggest that an increasing number of patients treated with pembrolizumab will require surgery, including the performance of an anastomosis. The effect of pembrolizumab on the healing of colonic anastomoses has not yet been studied.

Wound healing is a complicated process, influenced by many factors, including malnutrition, age, steroids, diabetes, vascular disease and radiation [8]. It consists of 4 phases: hemostasis, inflammation, proliferation and remodeling [8]. The specific ways immunotherapy impacts wound healing are not yet fully understood. There is evidence that PD-1/PL-L1 inhibitors may prolong the inflammatory phase [8].

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The aim of our study was to investigate the effect of pembrolizumab on the healing of colonic anastomosis in a rat model. Anastomotic healing was evaluated using mechanical, biochemical and histopathologic parameters. Given the immunomodulatory mechanism of PD-1 inhibition, we hypothesized that administration of a single high dose of pembrolizumab in a rat model would impair colonic anastomotic healing and increase the risk of anastomotic leak.

Material and methods

Animals

A total of 60 male Wistar rats, 8-12 weeks old, weighing 250-450 g, were used. The animals were housed in Makrolon cages, 3 per cage, in a temperature-controlled environment (20-24°C), with 40-50% humidity, and an alternating 12-h light/dark cycle. They had free access to tap water and standard laboratory rat pellets. The animal study protocol was approved by the General Directorate of Veterinary Services of the Prefecture of Thessaloniki (protocol code 493560/2225, approved September 11, 2023), in accordance with the Greek legislation on ethical and experimental procedures and aligned with the provisions of European Union Directive 2010/63/EU. The procedures were performed in the Laboratory of Experimental Surgery, School of Veterinary Medicine (EL-54-BIOexp-10). The study protocol was also approved by the Bioethics and Ethics Committee of the School of Medicine, Aristotle University of Thessaloniki (approved November 7, 2023).

Experimental groups

The animals were divided into 2 main groups of 30 each. Normal saline was administered to the control group (Group C) and pembrolizumab at 10 mg/kg was administered intraperitoneally to the intervention group (Group P), 21 days before the procedure. For patients receiving pembrolizumab every 3 weeks, elective procedures typically occur 2-3 weeks after the last dose [8]. Each main group was further divided into 3 subgroups of 10 animals, based on the different stages of the healing process.

- Groups CA (n=10) and PA (n=10) were sacrificed on the 3rd postoperative day (POD).
- Groups CB (n=10) and PB (n=10) were sacrificed on the 7th POD.
- Groups CC (n=10) and PC (n=10) were sacrificed on the 14th POD.

Anesthesia and operative technique

The anesthesia was administered intraperitoneally using a combination of xylazine (5 mg/kg) and ketamine (50 mg/kg)

solutions. The abdominal area was shaved and sterilized with 10% povidone iodine solution. Under general anesthesia and aseptic conditions, the abdominal cavity was accessed via a midline incision. The ileocecal valve was identified. One segment of the transverse colon, 1 cm in length, was resected and an end-to-end approximating colo-colonic anastomosis was performed, in 1 layer, with interrupted absorbable 5-0 polyglactin (Vicryl) sutures. The abdominal wall was closed in 2 layers. A running 3/0 Vicryl suture was used for the abdominal muscle wall, and interrupted vertical mattress 3/0 silk sutures were used for the skin closure. Hypothermia was prevented, both intraoperatively with a warming blanket and during resuscitation. No antibiotics were administered and the animals had free access to food and water postoperatively.

Autopsy and macroscopic evaluation

The animals were sacrificed on the 3rd, 7th and 14th POD within the dependent subgroup. Euthanasia of the animals was performed via an intracardiac puncture. The abdominal cavity was reopened and assessed for intraperitoneal adhesions, anastomotic leaks, collections and abscesses. The Van der Ham scale [9] was used for the evaluation of adhesions as follows: 0=no adhesions, 1=minimal adhesions between the omentum and the anastomosis, 2=moderate adhesions between the omentum and the anastomotic site, and between the anastomosis and a loop of the small bowel; and 3=severe and extensive adhesions, including abscess formation.

Bursting pressure (BP) measurement

BP reflects the resistance of the intestinal anastomosis to increased intraluminal pressure. A 5-cm segment of colon containing the anastomosis in the middle was resected and connected to a digital manometer using a 3-way connector, while the other end of the colon was tightly ligated. The colon was immersed in a beaker filled with normal saline and povidone iodine was pumped into the 3-way inlet at a slow rate of 1 mL/min via a syringe. BP was recorded in bars and was determined as the pressure at which any leakage or rupture occurred. The specific location of the leakage, proximal or distal to the anastomosis, was also recorded.

Hydroxyproline examination

Collagen is the most abundant connective tissue protein, while overproduction and deposition of collagen occur during the healing process [10]. Tissue hydroxyproline levels reflect collagen concentration, because hydroxyproline is found almost exclusively in collagen and constitutes a substantial proportion of its amino-acid composition [11]. For the determination of hydroxyproline levels in the anastomotic area, a tissue sample was surgically excised and immediately stored at -80°C. The quantification of hydroxyproline was performed as previously described by Geropoulos *et al* [12]. Briefly, the tissue was mechanically homogenized by grinding, using mortar and pestle, and then subjected to several cycles of freezing/thawing of the tissues in liquid nitrogen. For the standard curve, serial dilutions of a collagen solution (Achilles tendon, SIGMA-ALDRICH) were used. Soluble collagen was initially extracted from the homogenized tissue sample by incubation with 0.5 N acetic acid. Aliquots of 70 μL from standards and tissue samples were hydrolyzed in 10.125 N NaOH by autoclaving for 20 min at 120°C. Following hydrolysis, all samples were cooled to room temperature, and the pH was adjusted to 6-7 using 8N HCl. Hydroxyproline oxidation was induced by incubation for 25 min at room temperature in a Chloramine-T solution (0.056 M in 10% isopropanol and 90% acetate-citrate buffer pH 6.5). Chromophore development was achieved by adding 1 M of Ehrlich's reagent for 20 min at 65°C. The absorbance at 550 nm was measured using a spectrophotometer. A standard curve was constructed by plotting absorbance values against hydroxyproline concentrations from the standards. Hydroxyproline levels in the tissue samples were then determined by interpolation from the standard curve and expressed as μg of hydroxyproline per gram of tissue (μg/g tissue).

Histopathological examination

After the BP measurement, a colonic segment, 1 cm in length, containing the anastomosis in the middle, was resected and rinsed with saline. The anastomotic site was incised longitudinally and was divided into 2 equal parts. One part was placed in 4% formalin solution for histopathologic examination and stained with hematoxylin–eosin. The specimens were evaluated for neovascularization, inflammatory cell infiltration, fibroblast ingrowth, and collagen deposition, according to the Ehrlich and Hunt scale [13], as modified by Philips *et al* [14], as follows: 0=no evidence, 1+=occasional evidence, 2+=light scattering, 3+=abundant evidence, 4+=confluent cells or fibers. For the assessment of epithelialization, a scale from 0 to 3 was used, as described by Verhofstad *et al* [15], where 3 indicates absent epithelialization, 2 incomplete cubic, 1 normal cubic, and 0 normal glandular. The acute and chronic infiltration by inflammatory cells was also estimated, and a scale from Ersöz *et al* [16] was used as an accessory tool for the quantitative interpretation of the results, as follows: no inflammatory cells 0, mild infiltration (1) if there were 5-10 cells per HPF (high power field), moderate (2) for 11-50 cells per HPF, and severe (3) for >50 in 1 HPF area. Neovascularization was scaled as absent (0), mild (1) if there were 1-5 capillary vessels in 1 HPF area, moderate (2) for 6-10, and severe (3) for >10 capillaries per HPF [16].

Statistical analysis

The G-Power software was used to calculate the sample size, based on a previously published animal study by Uzunkoy

et al [17]. An effect size of 1.25, a power of 80%, 2 groups of animals, and a significance level of P<0.05 indicated 9 animals per group. Taking into consideration possible dropouts, 10 animals per subgroup were used. SPSS Statistics Version 25 was used for the rest of the statistical analysis. A paired t-test for used for the evaluation of body weight, an independent *t*-test was applied for the comparison of continuous variables, while the Mann-Whitney test was used for non-parametric variables, after a normality test. Fisher's exact test was used for the categorical data. A value of P<0.05 was considered statistically significant.

Results

Evaluation of adhesions

The evaluation of adhesions was performed according to the van der Ham scale [9], and Fisher's exact test was used for the statistical analysis. No statistically significant difference in adhesion formation was noted between subgroups CA and PA (P=0.179), CB and PB (P=0.175), or between CC and PC (P=0.474), as shown in Fig. 1. No evidence of obvious anastomotic rupture, anastomotic leaks, collections, abscesses, or any signs of peritonitis were noticed during the relaparotomies.

BP

The BP was measured in bars using a digital manometer. It was recorded whether the rupture occurred proximally or distally to the anastomotic site. An independent *t*-test was applied for the BP, after a normality test, and the Mann-Whitney *t*-test was used for the non-parametric variables. Fisher's exact test was conducted for the determination of significance related to the site of bowel rupture.

Starting with subgroups CA and PA, in which the animals were sacrificed on the 3rd POD, no statistically significant difference was found regarding the BP (P=0.174), although in all cases the rupture occurred at the anastomotic site. In contrast, for subgroups CB and PB, where the animals were sacrificed on the 7th POD, there was a statistically significant difference in both BP, with mean values of 0.3053±0.1470 bar for CB and 0.1685±0.1042 bar for PB (P=0.019), and the site of the bowel rupture (P=0.033). Specifically, in all of the bowel segments of the PB subgroup the rupture occurred at the anastomotic site, whereas in the CB subgroup only half the segments had rupture at the anastomotic site. Lastly, for subgroups CC and PC, no statistically significant difference was noticed in either BP (P=0.221) or rupture site (P>0.99). The BP and the site of the rupture in all the subgroups are illustrated in Fig. 2,3.

Hydroxyproline concentration

The mean concentrations of hydroxyproline (± standard deviation) in the tissue samples, measured in μg per gram of tissue, were estimated as follows in the different subgroups: CA 264.5±44.86, PA 344.2±131.61, CB 419±150.67, PB 247±89.03, CC 379.1±140.45, PC 191.25±86.01. Statistical analysis revealed significant differences in tissue hydroxyproline levels between subgroups CB and PB (P=0.003) and between subgroups CC and PC (P=0.001), while no statistically significant difference was identified between subgroups CA and PA (P=0.086). Since hydroxyproline levels reflect collagen concentration, it appears that significantly less collagen was present in subgroups PB and PC. These results are illustrated in Fig. 4.

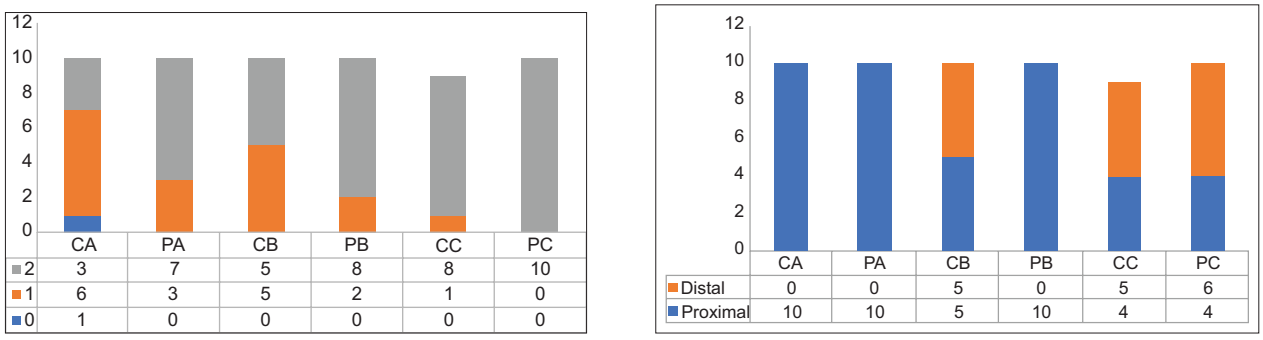

Figure 1 The evaluation of adhesions among the 6 subgroups according to the van der Ham scale

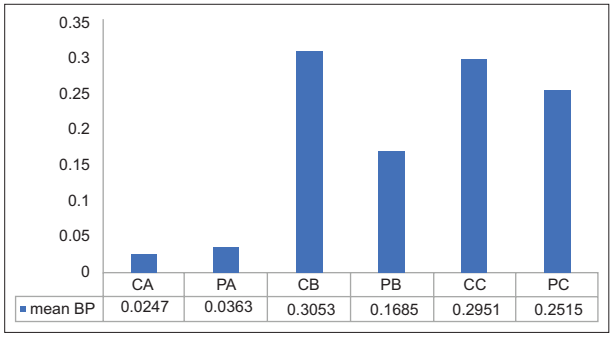

Figure 2 Mean BP in bars among the subgroups
BP, bursting pressure

Figure 3 Demonstration of the proximity of the bowel rupture to the anastomosis among subgroups

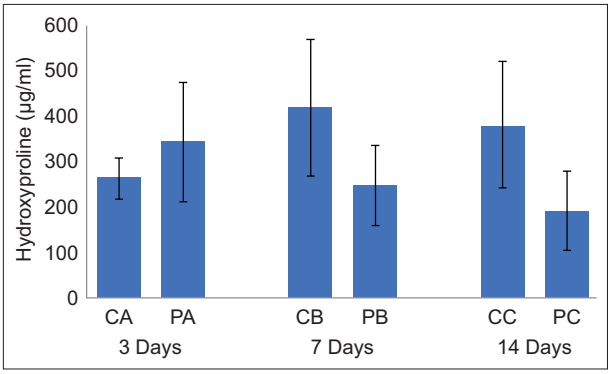

Figure 4 Concentration of tissue hydroxyproline among different subgroups

Histopathological examination

Between subgroups CA and PA, statistically significant differences were noticed in terms of neovascularization (P=0.026), fibroblast ingrowth (P=0.005), and collagen deposition (P=0.030), indicating a negative effect of pembrolizumab on the inflammatory phase of the healing process (Fig. 5). No statistically significant difference was noticed between subgroups CB and PB regarding any of the aforementioned parameters; neovascularization (P=0.707), fibroblast ingrowth (P=0.568), and collagen deposition (P=0.541) (Fig. 6). When comparing subgroups CC and PC, there was a statistically significant difference in neovascularization (P=0.000), but no difference in fibroblast ingrowth (P=0.474) or collagen deposition (P=0.1169) (Fig. 6). No differences between the subgroups were observed in acute or chronic inflammatory cell infiltration or in epithelization.

Discussion

There is no evidence in the literature from other experimental studies so far regarding any possible effect of pembrolizumab on the healing of colonic anastomosis. This is the first experimental study in a rat model exploring the effect of a single high dose of pembrolizumab on the healing of intestinal anastomosis during each healing phase. Although there was no obvious anastomotic rupture identified during autopsy, BP was significantly lower in the PB group, while the rupture occurred more frequently proximal to the anastomotic site. Hydroxyproline concentration, which reflects collagen deposition, was significantly lower in groups PB and PC. Histopathological evaluation revealed significantly less neovascularization, fibroblast ingrowth and collagen deposition during the inflammatory phase, while neovascularization also remained significantly lower during the remodeling phase.

Wound healing is a complex process that involves several phases, including hemostasis, inflammation, proliferation and remodeling [8]. The PD-1/PL-L1 pathway

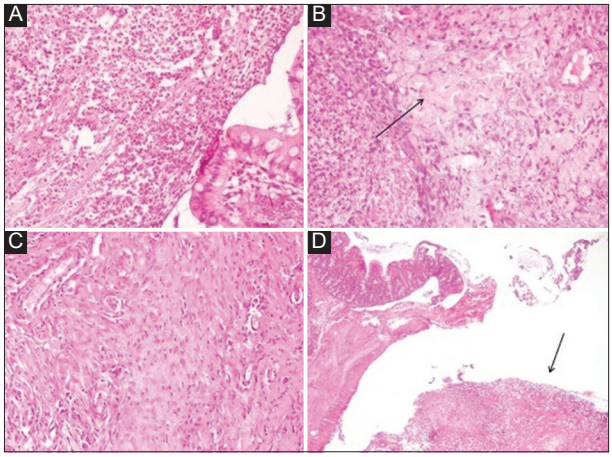

Figure 5 Histopathology review. H&E: (A) Inflammatory cells with predominance of neutrophils (case CA04) (H&E×200). (B) Fibroblast ingrowth (arrow) (case CA05) (H&E×200). (C) Collagen deposition (case PA03) (H&E×200). (D) Area of anastomosis with collagen deposition (arrow) (case PA09) (H&E×40)
H&E, hematoxylin-eosin

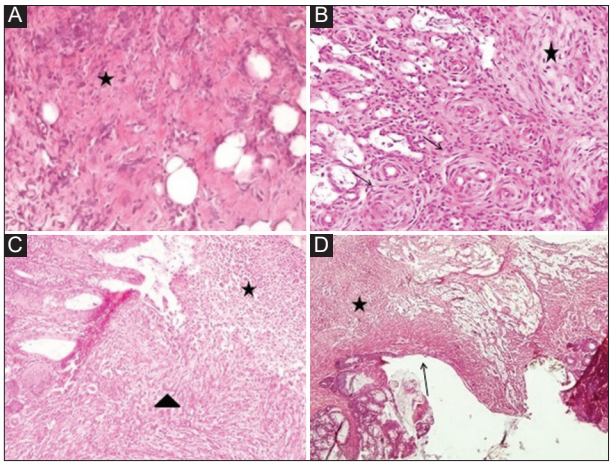

Figure 6 Histopathology review, H&E: (A) More collagen deposition (asterisk) (case CB02) (H&E×200). (B) Prominent vascular ingrowth (arrows) and fibroblast ingrowth (asterisk) (case CC08) (H&E×200). (C) Higher magnification of the inflammatory cells (triangle) in the anastomosis area, with visible fibroblast ingrowth (asterisk) (case PB02) (H&E×100). (D) Area of anastomosis with visible epithelium (arrow) and collagen (asterisk) (triangle in area of artifact) (case PC08) (H&E×40)
H&E, hematoxylin-eosin

normally helps resolve inflammation [8]. Increased T-cell activity caused by PD-1/PL-L1 antibodies may prolong the inflammation phase, resulting in delayed wound healing [8]. Overactivated T-cells may also attack normal cells [8]. PD-L1 is expressed on fibroblasts, which promotes the shift from proinflammatory M1 macrophages to pro-healing M2 macrophages [8]. PD-1/PL-L1 antibodies block the activation of M2 macrophages, negatively affecting granulation and remodeling [8]. The study presented here showed a potential adverse effect of pembrolizumab on the inflammatory healing phase, impairing neovascularization, fibroblast

ingrowth and collagen deposition, leading subsequently to decreased BP.

Pembrolizumab is given as neoadjuvant treatment for various unresectable or locally advanced tumors, achieving good responses and making tumors operable. A question of interest is whether pembrolizumab affects the wound healing process when administered preoperatively, involving either the skin or a possible anastomosis. Knowing about a negative impact of pembrolizumab administration prior to surgery could alter clinical practice concerning the dose administered, or the time interval between the last dose and the operation. Evidence suggests that neoadjuvant immunotherapy may increase complication rates after complex surgeries [8]. Elective procedures are commonly planned just before the next cycle of immunotherapy [8]. Initiation of adjuvant ICI treatment usually starts after proper wound healing, usually 2-4 weeks postoperatively [8]. In contrast, other retrospective studies indicate that ICI administration does not need to be discontinued in the perioperative setting [18]. However, careful patient selection is recommended [8].

A single-center retrospective study by Wang *et al* [19], which included 22 patients with locally advanced head and neck squamous cell carcinomas (HNSCC) who received neoadjuvant pembrolizumab in combination with chemotherapy, reported an higher incidence of delayed wound healing of up to 22.7%, making it the primary postoperative complication. These complications included infection around the tracheal stoma, pharyngeal fistula and lymphatic fistula, which were managed conservatively, but delayed the initiation of adjuvant radiotherapy. Another multi-center study by Mays *et al* [3], involving 132 patients, reported higher wound healing complication rates at the recipient site in patients who received neoadjuvant immunotherapy and underwent flap reconstruction after surgical treatment of HNSCC, while adjuvant-only ICIs did not show a greater risk. Healing complications may appear, even without surgery, in the case of recurrent or metastatic cancer responsive to treatment with anti-PD-1 therapy [20].

Regarding triple negative breast cancer, Barjot *et al* [21], in a retrospective single-centre study, reported that delayed wound healing was significantly more common in patients treated with pembrolizumab and chemotherapy than in those treated with chemotherapy alone. The same study reported that the time interval to surgery was also significantly associated with complication rates, as patients operated early (7-14 days) or late (more than 30 days), had higher complication rates compared to those operated between 21 and 28 days after the last dose of systemic treatment.

The safety of performing an anastomosis to ensure the continuity of the gastrointestinal tract in patients treated with pembrolizumab remains unclear. In patients suffering from locally advanced esophageal squamous cell carcinoma, the addition of pembrolizumab to chemoradiation in the preoperative setting did not raise the incidence of anastomotic leaks [22-24]. Another single-center study, involving patients with resectable gastroesophageal junction cancer who received neoadjuvant chemoradiotherapy combined with pembrolizumab prior to surgery, reported a 10.3% incidence of anastomotic leak [25].

Regarding CRC, in the RESET-C study a single dose of 4 mg/kg of pembrolizumab was administered 3-5 weeks prior to surgery in patients with stage I-III dMMR CRC [26]. The anastomotic leak rate was 3.5%, and the majority of patients with clinical stage I-II disease achieved a complete pathologic response. Gögenur *et al* [27], in a similar clinical trial where a single dose of 4 mg/kg of pembrolizumab was administered in 42 patients with stage I-III dMMR cancer, reported only 1 anastomotic leak. Elias *et al* [18] reported 5 bowel resection procedures with 7 bowel anastomoses, observing no anastomotic leak but 1 superficial wound separation. Calini *et al* [28], in a retrospective single-center study of patients treated with ICIs, reported 1 case out of 17 with anastomotic leak, highlighting the potential risk of colonic perforation due to ICI-induced colitis, which was managed with stoma formation and was associated with higher mortality. Bowel perforation due to ICI-mediated colitis or rapid tumor regression can present as acute abdomen [29,30]. There are some case reports supporting the safety of primary anastomosis after small bowel perforation in patients treated with pembrolizumab [31-33]. However, the safety of performing an anastomosis or primary closure especially in the acute setting has not yet been established [30]. The present study showed statistically significant lower BP and hydroxyproline levels in the experimental group on the 7th POD, but no obvious anastomotic leak was identified.

There are several limitations related to this study. In the experimental part, only 1 dose of 10 mg/kg of pembrolizumab was administered 21 days prior to surgery. Different protocol regimens for administering pembrolizumab exist in the literature, including variations in the dose, the cycles, the frequency and the time of discontinuation prior to surgery. A fixed dose of 200 mg administered every 3 weeks is the most common, while weight-dependent doses are less frequently used today. Regarding the literature review, there are limited data concerning both prospective and retrospective studies, especially large-scale studies, and bias due to heterogeneity across studies is another significant factor, as they usually include various ICIs.

In conclusion, the improved pathologic response rates highlight the significant role of neoadjuvant immunotherapy with pembrolizumab. A growing number of patients treated with immunotherapy will require surgical management during their cancer journey. This current study suggests a potential negative effect of a single high dose of pembrolizumab. However, further research is necessary to clarify the safety of conducting an intestinal anastomosis in the emergency or elective settings.

Acknowledgment

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Summary Box

What is already known:

- Pembrolizumab is a monoclonal antibody targeting the PD-1 protein.
- It has been approved for the treatment of several malignancies, including microsatellite instability-high and mismatch repair-deficient colorectal cancers
- The strong response, which converts inoperable cases to operable ones, suggests that an increasing number of patients treated with pembrolizumab will need surgery, including anastomosis
- There is evidence that PD-1/PL-L1 inhibitors may extend the inflammatory phase, but the specific effects of pembrolizumab on intestinal anastomosis have not yet been studied

What the new findings are:

- Histopathological review demonstrated significant differences on postoperative day (POD) 3 in neovascularization, fibroblast ingrowth and collagen deposition, suggesting impaired wound healing during the early inflammatory phase
- Bursting pressure on the 7th POD was significantly lower in the experimental group
- Tissue hydroxyproline levels were significantly lower on the 7th and 14th POD in the experimental group
- These results suggest that a single high-dose of pembrolizumab may negatively affect the anastomotic healing process

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