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SURGERY | RESEARCH ARTICLE

A comparative study of the acute and long-term prognosis for mouse models undergoing laparoscopic surgery under continuous intra-abdominal perfusion with either CO_2 gas or saline

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Abstract: *Background*: We developed a water-filled laparoendoscopic surgery (WaFLES) method using an isotonic irrigant like physiological saline instead of carbon dioxide (CO_2) insufflation. Although surgical experimentation in porcine models has revealed some advantages, the effect of perfusate absorption remains uncertain. Here, we examined the acute and long-term prognosis of this method in mouse models. *Materials and methods*: CO_2 gas or physiological saline was continuously perfused into mice for 1 h. Body-weight fluctuation was observed for 100 days in the two groups and control anesthesia group of mice. In addition, the induction of stress proteins and cytokines was evaluated immediately after perfusion. *Results*: Mice perfused with saline showed a temporary 30% increase in body weight during perfusion; however, this increase was not significant when assessed one day later. There was no significant increase in either IL6 or TNFα levels in the peritoneal lavage fluid obtained from any of the three groups. There were no significant changes in the expression of HSP70 or BiP.

ABOUT THE AUTHOR

We are interested in responses to cellular stress and focus on the function of the endoplasmic reticulum (ER) and its clinical applications. Previously, we have attempted to elucidate the adaptive response of intracellular trafficking between the ER and other organelles, such as the Golgi complex and the mitochondria, in response to various pathological situations. Evaluating adaptive responses can be useful in assessing cellular injuries, while the regulation of adaptive responses can be useful in treating diseases caused by such cellular injuries. We have evaluated the functions of stress proteins, such as BiP, in relation to neuronal development and neurodegeneration. As stress proteins function as molecular chaperones, the modulation of stress responses may be useful against a variety of human diseases. In the present study, we evaluated the effects of intraperitoneal perfusion with CO_2 gas or physiological saline in a mouse model by analyzing the expression of specific stress proteins.

PUBLIC INTEREST STATEMENT

Minimally invasive surgical techniques are an essential component of enhanced recovery after surgery (ERAS). Endoscopic surgery is a potent tool that reduces postoperative complications and pain while providing the additional advantages of a small incision size, a minimally invasive approach, and reduced hospital stays. Conventional laparoscopic surgery and robot-assisted surgery routinely apply carbon dioxide (CO_2) gas to widen the body cavity and obtain an adequate surgical field. Although both CO_2 insufflation and liquid irrigation may have some adverse effects, liquid irrigation would provide us with the opportunity to perform lavage that would help to maintain an appropriate local/body temperature, avoid the desiccation of organs, and would allow us to use a favorable navigation system that permits simultaneous monitoring by both ultrasonography and laparoscopic imaging. Laparoscopic surgery under continuous intra-abdominal perfusion may be a possible alternative method for minimally invasive surgical techniques.

All mice survived over the long-term observation period of 100 days without any evidence of body-weight fluctuation ($P = 0.7408$, $N = 5$ for each group). *Conclusion*: WaFLES showed a good prognosis in a mouse model, thus indicating significant potential for clinical application.

Subjects: Medicine, Dentistry, Nursing & Allied Health; Medical Technology & Engineering; Anesthesiology; Surgery; Urology

Keywords: water-filled laparoendoscopic surgery (WaFLES); laparoscopy; saline; carbon dioxide; mice models; stress proteins

1. Introduction

Minimally invasive surgical techniques are essential components of enhanced recovery after surgery (ERAS) (Ljungqvist, Scott, & Fearon, 2017). Endoscopic surgery is a potent clinical tool that reduces postoperative complications and pain (Pedziwiatr et al., 2014, 2016) while providing the additional advantages of a small incision size, a minimally invasive approach, and reduced hospital stays. Conventional laparoscopic surgery and robot-assisted surgery routinely apply carbon dioxide (CO_2) gas to widen the body cavity and obtain an adequate surgical field. In contrast, for endoscopic surgery in small, closed cavities, such as the urinary tract, medullary cavity, and joint spaces, the surgical field of view is commonly secured by irrigation with physiological saline or other isotonic solutions instead of CO_2 gas, thus allowing endoscopic surgery to be performed safely (Aziz & Ather, 2015; Koga et al., 2012). Laparoscopic surgery under continuous intra-abdominal perfusion may represent a possible alternative method for minimally invasive surgical techniques.

In 2012, Igarashi et al. developed a water-filled laparoendoscopic surgery (WaFLES) method for use in the peritoneal and retroperitoneal cavities; this method was tested in a porcine model and allowed the continuous irrigation of physiological saline (Igarashi et al., 2012). A subsequent study showed that this method provided a good field of view via the use of perfusion when supported by a high-volume pump, extracorporeal cistern, bacterial filter, and a closed reperfusion circuit (Igarashi et al., 2016).

Both CO_2 insufflation and liquid irrigation may have some adverse effects. The insufflation of CO_2 is associated with risk of gas embolism (Hong, Kim, & Kil, 2010), desiccation of tissue, a reduction in body temperatures, and/or postoperative tissue adhesion (Molinas, Binda, Manavella, & Koninckx, 2010). The accumulation of dissolved CO_2 in body fluids may also cause acidosis (Gutt et al., 2004). The most common intra-operative complication is subcutaneous emphysema with hypercapnia (Lu et al., 2012). The irrigation of isotonic liquid may avoid the disadvantages of CO_2 gas and provide a lavage effect; however, the absorption of large volumes of an irrigant could also cause concern. For example, this process might provoke an overload upon the cardiopulmonary system and cause serious damage in a number of vital organs. Furthermore, both CO_2 gas and irrigant can increase the internal pressure of the body cavity and cause a reduction in cardiac output owing to reduced venous return, as well as a reduction in blood flow to the intestinal tract, liver, and kidneys (Hatipoglu, Akbulut, Hatipoglu, & Abdullayev, 2014).

Previously, we examined the short-term effects associated with the abdominal irrigation of physiological saline using a porcine model in comparison with CO_2 gas insufflation (Ishii, Igarashi, Naya, Aoe, & Isono, 2016); this study revealed that saline irrigation was not associated with any life-threatening reactions. In the present study, we used a mouse model to evaluate the effects of intraperitoneal perfusion with CO_2 gas or physiological saline and investigated the acute and long-term prognosis of this technique by allowing our experimental mice to breed after perfusion.

2. Materials and methods

2.1. Perfusion

This study was approved by the local ethics committee of Chiba University.

Perfusion experiments were conducted on C57BL/6 mice (mean age: 1 year, mean weight: 33 g). Mice were deeply anesthetized with an intraperitoneal injection of 0.08 mg/g pentobarbital (Dainippon Sumitomo Pharma, Tokyo, Japan) and were then fixed on their backs such that they were able to breathe spontaneously. Blood pressure and respiratory rate were measured at 20-min intervals. Mean arterial blood pressure and tail blood flow were measured non-invasively using a tail cuff (CODA Monitor; Kent Scientific Corporation, Torrington, CT, USA). An inlet tube (22-gauge cannula) and an outlet tube (18-gauge cannula) were inserted into the peritoneal cavity. CO_2 gas, or physiological saline, was continuously perfused for 1 h based on preliminary experiments (data not shown), such that adequate anesthesia could be maintained throughout perfusion. Mice in the control anesthesia group underwent the same procedure without perfusion. After completion of the experiment, body-weight fluctuations and the feeding behavior of experimental mice were observed for 100 days in the long-observation mice (five mice each for the CO_2 gas group, physiological saline group, and control anesthesia group).

Mice in the acute experiment group (two mice for each group) were perfused in a similar manner and sacrificed after the perfusion experiment for biochemical and histological examinations.

2.2. IL6 and TNFα measurements

Peritoneal washings were obtained on the first and seventh day after perfusion experiments in the long-observation mice using 5-ml of saline. IL6 and TNFα were then measured using DuoSetELISA Mouse IL-6 and DuoSetELISA Mouse TNFα kits (R&D Systems, Minneapolis, MN, USA) on a SYNERGY2 microplate reader (BioTek Instruments, Winooski, VT, USA), in accordance with the manufacturer's protocol.

2.3. Western blot analysis

One day after perfusion experiments, the acute-experiment mice were sacrificed by deep anesthesia with pentobarbital. Livers were then collected and homogenized by sonication (UR-20P, TOMY, Tokyo, Japan) in a buffer containing 0.4% (w/v) Nonidet P-40, 0.2% N-lauroylsarcosine, 10 mM Tris/HCl (pH 8.0), 30 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 30 μg/ml N-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN, Sigma-Aldrich Japan, Tokyo, Japan), and 1 mM sodium orthovanadate (Sigma-Aldrich). The lysates were then centrifuged, and the resultant supernatants were resuspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer. Proteins in the lysates were then separated by SDS-PAGE under reducing conditions. Separated proteins were then transferred from gels on to polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp., Billerica, MA, USA), and Western blotting was performed as described previously (Hamada et al., 2004). Membranes were exposed to the following antibodies: mouse mAb SPA-827 against immunoglobulin heavy chain-binding protein (BiP)/glucose regulated protein (GRP) 78 (KDEL sequence, Enzo Life Sciences, Farmingdale, USA), mouse mAb SPA-810 against heat shock protein (HSP) 70 (Enzo Life Sciences), and mouse mAb against γ-tubulin (Sigma-Aldrich). Images were acquired by LAS-1000 and Image Gauge software (Fuji Photo Film Co. Ltd., Tokyo, Japan).

2.4. Histological examination

For each mouse in the acute-experiment, a part of the liver was isolated and fixed in 4% paraformaldehyde for 24 h. After fixation, livers were dehydrated in increasing concentrations of ethanol and then embedded in paraffin wax. Sections measuring 5 μm in thickness were then prepared and stained with hematoxylin and eosin. Finally, sections were observed under a microscope using an N-Achroplan 40× NA 0.65 objective (Axio Imager A1, Carl Zeiss, Oberkochen, Germany). Brightness and contrast were optimized using AxioVision Rel.4.7 software (Carl Zeiss) and images were captured with a digital camera (AxioCam MRc, Carl Zeiss).

2.5. Peritonitis model

To induce peritonitis, two mice were intraperitoneally injected with 1 mg of zymosan A (Sigma-Aldrich) (Aoe, Okamoto, & Saito, 1995). After 6 h, peritoneal washings with saline were collected from each of the two mice to allow the measurement of IL6 and TNFα. Then, the two mice were sacrificed by deep anesthesia with pentobarbital, and livers obtained for Western blotting and histological examination.

2.6. Statistical analysis

All data are expressed as mean ± standard error of the mean. Statistical analysis was carried out using the paired *t*-test, one-way analysis of variance (ANOVA), and two-way repeated measures ANOVA followed by Bonferroni post-hoc tests. Analysis was carried out using Prism 4.0 (GraphPad Software, San Diego, CA, USA) and P values < 0.05 indicated statistically significant differences.

3. Results

CO_2 gas was continuously injected into the abdominal cavity of experimental mice at a flow rate of 70 ml/min; then the pressure in the abdominal cavity was equivalent to 2 cm water column (H_2O). The abdominal cavity was fully expanded, but care was taken to ensure that the mice were still able to breathe normally. Next, mice were perfused with physiological saline at a rate of 60 ml/h for 1 h; during this time, we monitored blood pressure and respiration rates. Figure 1 shows a side view of a mouse undergoing physiological saline perfusion. In this state, approximately 10 ml of saline, or CO_2, was contained in the abdominal cavity of each mouse.

Respiration rate was compared across the three groups of mice (CO_2 insufflation, saline perfusion, and control); controls were fitted with a cannula under anesthesia but without any perfusion (Figure 2). There were significant increases in the respiratory rate of both the CO_2 insufflation during perfusion ($P < 0.01$, $P < 0.001$) and saline perfusion groups at the end of perfusion ($P < 0.05$) compared to the control group. The observed increases in respiratory rate were more prominent in the group undergoing CO_2 insufflation

To evaluate the short-term effects of perfusion, samples were collected one day after perfusion experiments (the acute group; $n = 2$) for biochemical and histological investigations. Hematoxylin and eosin staining of liver tissue samples showed that neither CO_2 insufflation nor saline perfusion caused marked pathological findings, whereas vacuolar degeneration was observed in the zymosan-induced peritonitis model (Figure 3). We also investigated the expression of stress proteins in the livers of experimental mice by Western blot analysis to evaluate the effects of perfusion at the molecular level

Figure 1. Perfusion experiment. Mice were deeply anesthetized with an intraperitoneal injection of pentobarbital, fixed on their backs, and allowed to breathe spontaneously. Blood pressure and respiratory rate were measured at 20-min intervals. Mean arterial blood pressure and tail blood flow were measured non-invasively using a tail cuff. An inlet tube (22-gauge cannula), and an outlet tube (18-gauge cannula), were inserted into the peritoneal cavity. CO_2 gas or physiological saline was continuously perfused for 1 h.

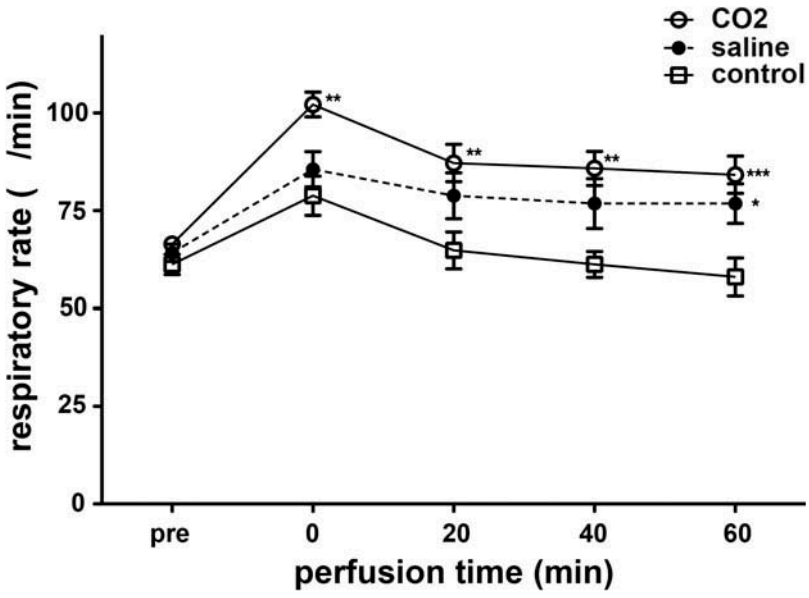

Figure 2. Respiratory rates during perfusion. Respiratory rates were measured intermittently every 20 min. We compared the respiration rate in three groups (*N* = 5 per group): CO_2 insufflation, saline perfusion, and controls (which were fitted with a cannula under anesthesia but without any perfusion). All data are expressed as mean ± standard error of the mean (SEM). Two-way repeated measures analysis of variance (ANOVA) was used for statistical analysis, followed by Bonferroni post-hoc tests. Values for the three groups were significantly different (*P* = 0.0084). Saline *vs* control; **P*-value <0.05. CO_2 *vs* control; ***P*-value <0.01, ****P*-value <0.001. Saline *vs* CO_2; not significantly different.

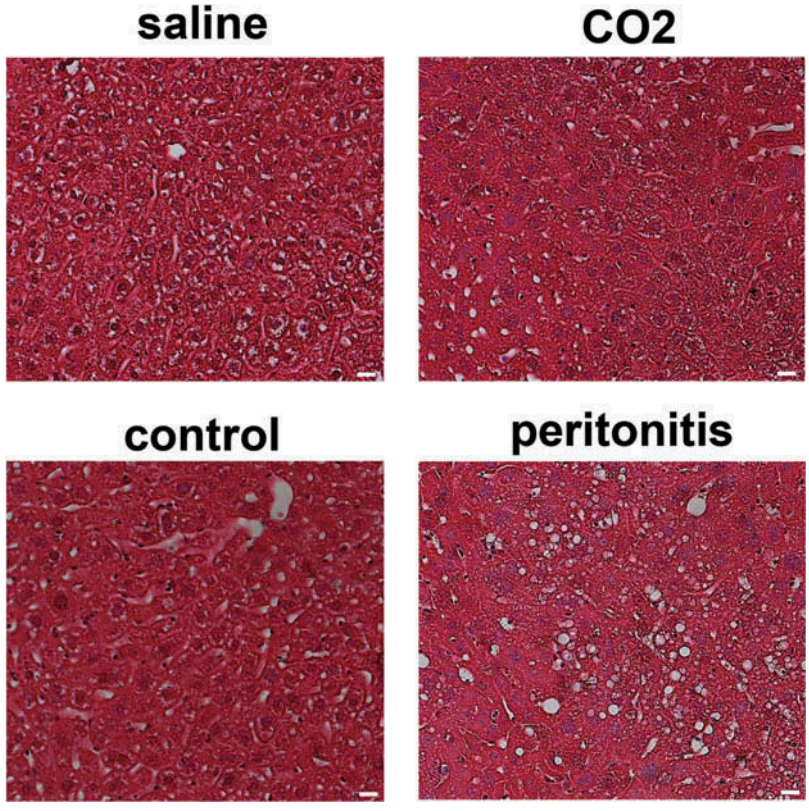

Figure 3. Samples of liver tissue stained with hematoxylin and eosin staining. One day after the perfusion experiments, mice were deeply anesthetized with pentobarbital and sacrificed. Livers were then isolated for analysis. For the peritonitis model, mice were inoculated intraperitoneally with 1 mg of zymosan. Six hours later, a sample of liver was acquired and stained in hematoxylin and eosin. Finally, sections were examined under a microscope. Scale bar represents 10 µm.

(Figure 4). BiP is an endoplasmic reticulum stress protein, and HSP70 is a cytoplasmic stress protein. Although zymosan treatment enhanced the expression of BiP and HSP70 in the liver, CO_2 and saline perfusions did not enhance their expression. Peritonitis caused by zymosan induced higher expression levels of both TNFα (Figure 5A) and IL6 (Figure 5B) in peritoneal lavage solutions, while cytokine

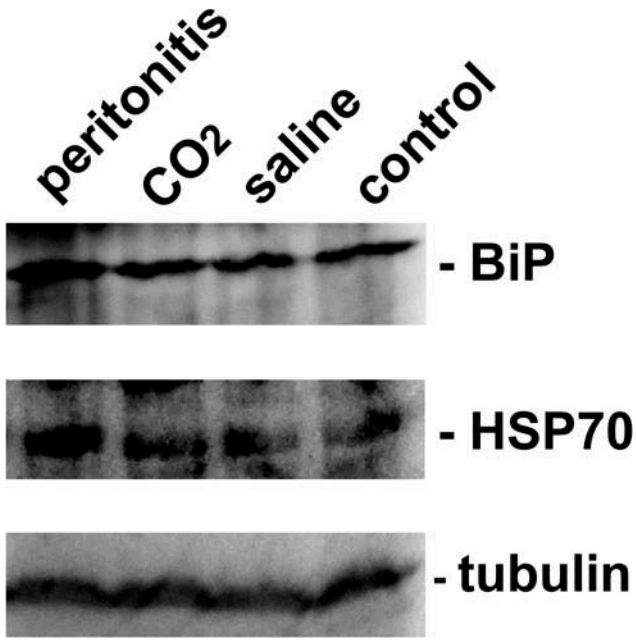

Figure 4. The expression of stress proteins in the liver was examined by Western blot analysis. Zymosan treatment enhanced the expression of BiP and HSP70 in the liver, while CO_2 insufflation and saline perfusion did not significantly alter the expression of BiP and HSP70 in the liver.

production in CO_2 insufflation and saline perfusion was similar when these two groups were compared to the control when measured on day 1 or day 7. The non-infectious zymosan-induced peritonitis model was used as a positive experimental control for inflammation (Aoe et al., 1995). In fact, peritonitis induced by zymosan caused obvious inflammation. In contrast to zymosan-induced peritonitis, both CO_2 insufflation and saline perfusion did not induce a significant acute inflammatory response.

Next, we investigated feeding behavior and changes in body weight over a period of 100 days after perfusion. Although mice perfused with saline experienced a significant, but temporary, 30% increase in body weight during perfusion compared to that of pre-perfusion (P = 0.0037), this excessive weight was lost immediately after perfusion finished (Figure 6A). Body weights of mice in the CO_2 insufflation and control anesthesia groups did not change significantly during perfusion (Figure 6B). Furthermore, there was no significant difference (P = 0.7408) in body weight during the observation period when compared across the CO_2 insufflation, saline perfusion, and control anesthesia groups. None of our mice showed abnormal feeding behavior over the 100-day period of observation.

4. Discussion

Our research aims to develop a means of performing endoscopic surgery with fluid irrigation in a larger volume of space, such as the abdominal and retroperitoneal cavities (Igarashi et al., 2016). This form of surgery would provides us with the opportunity to perform lavage that would help to maintain an appropriate local/body temperature, avoid the desiccation of organs, and allow us to use a favorable navigation system that permits simultaneous monitoring by both ultrasonography and laparoscopic imaging (Vapenstad et al., 2010). The present study therefore investigated the safety and physiological benefits of laparoscopic surgery with saline irrigation.

The retention of perfusate in the peritoneal cavity would be expected to represent a significant burden on respiration and circulatory dynamics due to suppression in the movement of the diaphragm and by the inhibition of venous return due to compression of the inferior vena cava and other abdominal vessels. Extensive absorption of perfusate into the circulation, and other extracellular spaces, may represent another major concern. In our previous research, we used a porcine model to perform endoscopic abdominal surgery under general anesthesia using a

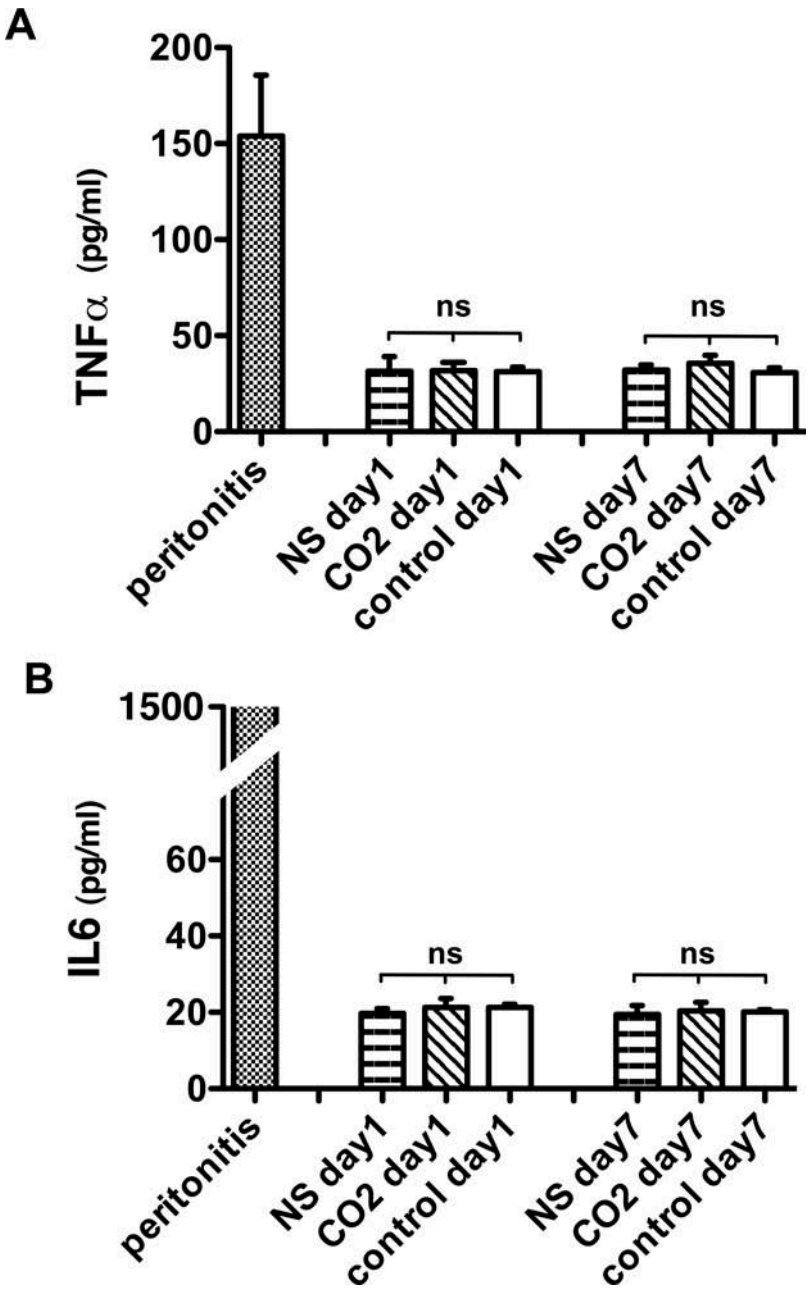

Figure 5. Saline perfusion did not induce a significantly enhanced production of cytokines. Peritonitis caused by zymosan induced a significant expression of TNFα and IL6 in peritoneal lavage solutions, while both CO_2 and saline perfusions did not lead to the increased production of TNFα and IL6 on either the first or seventh day after perfusion. Peritonitis group; *N* = 2, other groups; *N* = 5. Bar graphs represent mean ± standard error of the mean (SEM). One-way ANOVA was used for statistical analysis. (A) There were no significant differences of TNFα when compared across the saline perfusion, CO_2 insufflation, and control anesthesia groups on day 1 (*P* = 0.9962) or day 7 (*P* = 0.5687). (B) There were no significant differences of IL6 expression when compared across the saline perfusion, CO_2 insufflation or control anesthesia groups on day 1 (*P* = 0.7410) or day 7 (*P* = 0.9392).

perfusion system that had been developed for humans (Igarashi et al., 2016). The mean body weight of our pigs (28.5 kg) increased by 2.5% immediately after WaFLES compared to that before surgery; this gain in weight lasted for 3–5 h (Ishii et al., 2016). According to arterial blood gas analysis, we observed minor reductions in pH, but no significant change in hematocrit. We did not observe any specific problems during surgery with the WaFLES method compared to conventional endoscopic surgery using CO_2 insufflation (Ishii et al., 2016).

In the present study, we evaluated the effect of peritoneal perfusion with physiological saline in a mouse perfusion model. Due to the fact that a mouse has a body weight of only 30 g on average, we opted to maintain spontaneous breathing rather than ventilator management; the perfusion time was 1 h. Approximately 10 ml of CO_2 or physiological saline was retained in the peritoneal

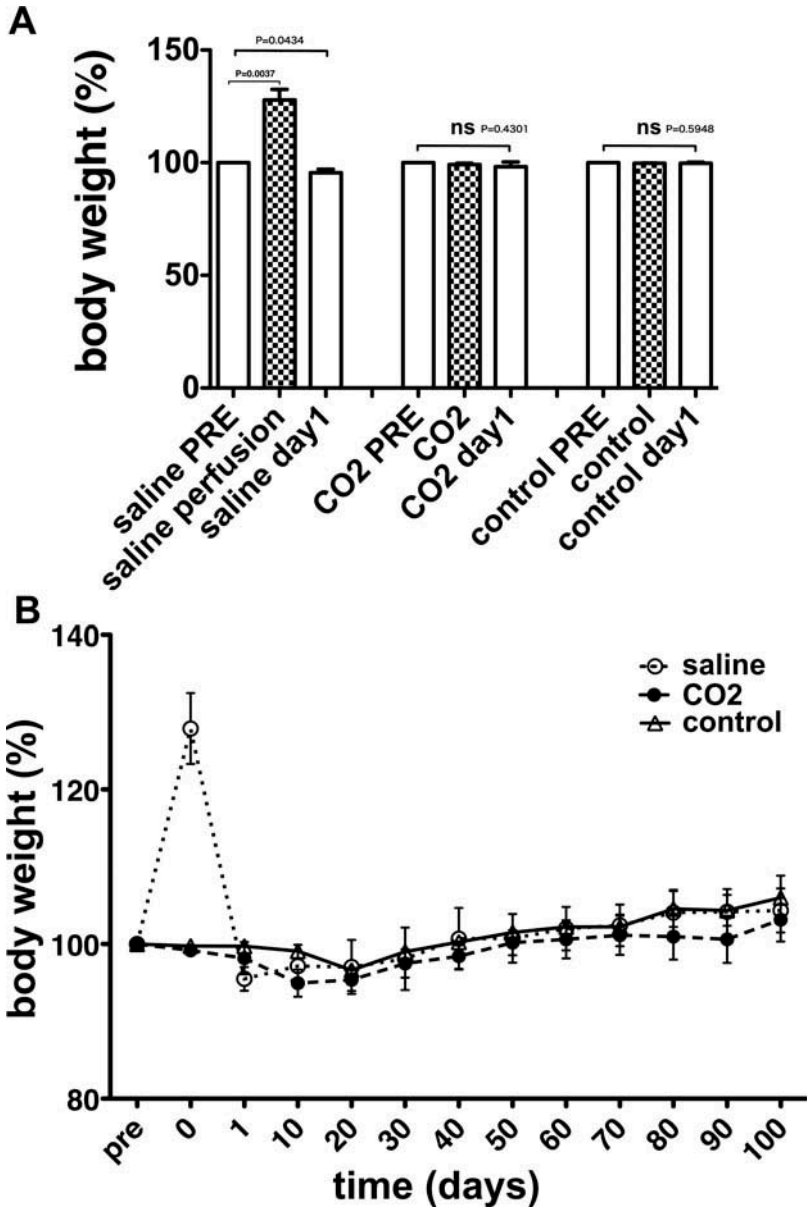

Figure 6. Changes in body weights during the 100-day observation period following perfusion. (A) Mice perfused with saline showed a temporary 30% increase in body weight during perfusion compared to that of pre-perfusion (*P* = 0.0037). The post-perfusion body weight in the saline group was significantly reduced compared to the pre-perfusion weight (*P* = 0.0434). However, there were no significant changes in the body weights in the CO_2 insufflation (*P* = 0.4301) and control anesthesia (*P* = 0.5948) groups when compared between pre- and post-perfusion (paired *t*-test). (B) The body weights of mice in the saline perfusion, CO_2 insufflation, and control anesthesia group were not significantly different after perfusion for 100 days (*P* = 0.7408). *N* = 5 for each group. All data are expressed as mean ± standard error of the mean (SEM). Two-way repeated measures analysis of variance (ANOVA) was used for all statistical analysis.

cavity during perfusion, which may have created an extra load on the dynamics of the respiratory and circulatory systems. Blood pressure remained stable in both of these groups. In both the CO_2 and saline perfusion groups, respiratory suppression might have occurred owing to an increase in intraperitoneal pressure. In the CO_2 group, we observed a larger increase in respiratory rate, which appeared to be influenced by CO_2 burden. Body weight increased by 27.9% in the saline group during perfusion, but on the following day, this excessive weight gain disappeared.

In terms of intraperitoneal pressure, the inhalation of CO_2 exerts a uniform pressure on the abdominal cavity, thus creating a wider field of view. In contrast, the pressure created by a liquid perfusate depends on gravity and is generally not uniform. In our current experiments, involving the mouse model, the intra-peritoneal pressure experienced by the CO_2 group was approximately 2 cm of H_2O, which allowed spontaneous respiration to be maintained. If this was translated to the human situation, the water pressure only at the bottom of the abdominal cavity may be

approximately 15 cm of H_2O (10.95 mmHg), which is believed to be equivalent to conventional CO_2 insufflation endoscopic surgery. Therefore, the mean pressure load of CO_2 insufflation would be greater than that produced by the liquid perfusate.

We assumed that the absorption of perfusion fluid was negligible in our present mouse model and the porcine model we used previously (Ishii et al., 2016). Even if absorption had occurred, it is likely to be only a small amount that will dissipate by the following day. We have gained a significant body of information relating to fluid retention in the peritoneal cavity through the management of peritoneal dialysis (Kim & Biesen, 2017; Li, Ng, & McIntyre, 2017). In human clinical peritoneal dialysis, 1–2 l of electrolyte solution is retained in the peritoneal cavity for several hours, or sometimes, an entire day. However, it is well established that the abrupt absorption of dialysis solutions into cells, blood circulation, or other extracellular tissue space does not occur. Moreover, the load exerted on respiration and circulation is only mild and does not affect a patient's quality of life. Because of their impaired renal function, patients undergoing peritoneal dialysis may have an increased risk of over-hydration due to the absorption of dialysate and the consumption of excessive drinks and food. However, if a patient has normal renal function, the absorption of a small amount of perfusion fluid during the WaFLES method may not cause a serious problem.

The perfusion of liquid or gas into the abdominal space may cause peritoneal inflammatory reactions. Patients undergoing peritoneal dialysis are exposed for long periods to a dialysis solution containing high levels of glucose, which stimulates the synthesis of pro-inflammatory factors such as IL-6 and TNFα (Li et al., 2017). CO_2 insufflation can cause tissue dryness, which may also cause inflammation. However, the retention of physiological saline in the peritoneal cavity lasts only for a few hours during the WaFLES method. Thus, the use of physiological saline may not exert significant influence upon the inflammatory response when compared to CO_2 insufflation, as tissue drying can be avoided. In our previous porcine experiments, there was no significant difference between the WaFLES group and the CO_2 group in terms of IL-6 concentration in the peritoneal lavage solution immediately after perfusion (Ishii et al., 2016).

In the present mouse experiment, peritoneal lavage fluid was collected on the first and seventh day after perfusion and the values of TNFα and IL-6 were determined. The non-infectious peritonitis model induced by zymosan showed a significant increase in the levels of TNFα and IL-6 in the peritoneal lavage fluid (Aoe et al., 1995), while no such increase was observed in the WaFLES or CO_2 insufflation groups. Histological examination of the liver on the first day after perfusion showed vacuolar degeneration in the peritonitis model but not in the WaFLES, CO_2 insufflation, or anesthesia control groups.

Upon cellular insults such as ischemia, hypoxia, inflammation, or exposure to toxic substances, a series of compensatory reactions occur within the cell. The enhanced expression of stress proteins such as BiP and HSP70, which serve as molecular chaperones, ensures protein folding and prevents protein denaturation. The expression of HSP70 is induced by a heat shock response in the cytoplasm (Aoe et al., 1997) while that of BiP is induced by an unfolded protein response in the endoplasmic reticulum (Mimura et al., 2007). Such a compensatory response at the cellular level was observed in the peritonitis model, whereas the expression of BiP and HSP70 was not enhanced in either the WaFLES or CO_2 insufflation groups.

Collectively, our results relating to the production of inflammatory cytokines, our histological findings, and the expressions of stress proteins provide evidence that adverse events such as ischemia, hypoxia, or inflammation did not occur in either the WaFLES group or the CO_2 insufflation group. In fact, during the observation period of 100 days following perfusion the survival of mice in both groups was similar to that of mice in the anesthesia control group.

There are several limitations to this research. For small animals, it was easier to maintain spontaneous breathing rather than ventilator management, and the perfusion time was limited to 1 h. In future, we may need to manage the anesthesia of mice during perfusion in a better way so that perfusion can be maintained for a longer time period. As this was a pilot study using mice over a long observation period, the sample size was within our ability to manage the experiment.

In conclusion, the acute and long-term observation of mice irrigated with physiological saline did not reveal specific problems. Perfusion could wash out pathogens and cancer cells, and therefore potentially improve the long-term prognosis of patients in a manner that is better than that of CO_2 insufflation or the open abdominal operation (Hesami et al., 2014; Misawa et al., 2014; Shimada, Kuramoto, Marutsuka, Yagi, & Baba, 2011). This study shows that laparoscopic surgery under continuous intra-abdominal perfusion is a possible alternative for minimally invasive surgical techniques.

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Competing Interests
The authors declare no competing interest.

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