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Investigation of blood coagulation effect of non-thermal multi-gas plasma jet *in vitro* and *in vivo*

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Author contributions:
YN, TT, MY and TA designed and conducted the experiment.
HK, HM and AO developed and setup the plasma device.

ABSTRACT

Background: Non-thermal atmospheric pressure plasma (NTAPP) has recently received attention as a novel tool in medicine. It is thought that plasma components yield plasma effects such as sterilization, blood coagulation and wound healing. These effects are produced without thermal damage. We investigated the blood coagulation effect of NTAPP by using a multi-gas plasma jet.

Materials and Methods: Multi-gas plasma jets can generate NTAPP by several gas species. In this study, argon, oxygen, helium, nitrogen, mock air and carbon dioxide were used to generate NTAPP and blood coagulation times were compared with each plasma treated sample. The NTAPP blood coagulation effects on whole blood with four different anticoagulants were investigated. Additionally, in this study, the effects of plasma treatment on porcine tissues and organs were investigated as *in vivo* experiment.

Results: A tendency to coagulate later with argon gas plasma than others was shown. There were no significant differences between oxygen, helium, nitrogen, mock air and carbon dioxide. Whole blood with each anticoagulant demonstrated fast coagulation by NTAPP treatment. Fast control the bleeding lesions on porcine stomach and liver by plasma treatment was observed, and no tissue damage due to the plasma treatment was detected by optical microscope.

Conclusions: These experiments suggest the potential of various gas NTAPPs as a novel medical device to control bleeding lesions.

1. Introduction

Gastrointestinal bleeding is a situation that we frequently encounter clinically and can be lethal. Some endoscopic treatments are employed for bleeding i.e. endoscopic clipping, injection therapy (absolute ethanol or hypertonic saline), high frequency coagulation and argon plasma coagulation (APC) [1-3]. However, there are problems with each of these therapies. Endoscopic clipping needs to hold a bleeding vessel precisely. Furthermore, it can be difficult to observe the target lesion in detail and to perform additional therapy after endoscopic clipping. Absolute ethanol injection therapy has tissue toxicity and high frequency coagulation and APC lead to thermal damages to the treated tissue that can cause ulceration or perforation of the gastrointestinal wall. Non-thermal atmospheric pressure plasma (NTAPP) has recently received attention as a novel tool in medicine. Plasma is composed of several active species such as charged particles, electronically excited atoms and molecules, radicals and ultraviolet photons [4, 5]. It is thought that these generated components yield plasma effects such as sterilization [6], blood coagulation [7-10] and wound healing [11-13]. These effects are produced without thermal damage [14].

Two different approaches are used for plasma treatment. One approach is called direct plasma: plasma is generated between an electrode covered by a dielectric material and the sample tissue forms the second electrode. Thus in this approach, the sample tissue contacts the discharge directly, but it has a need to maintain a range for atmospheric discharge between the electrode and the sample. The NTAPP jet source offers another approach called indirect plasma: this can treat a distance of several millimeters by a gas flow delivering the plasma components [15-17]. After glow of NTAPP components can be seen during treatment, so it is easy for everyone to irradiate the target lesion without special training. Locations requiring hemostasis treatment during surgical or endoscopic operations have irregular surfaces as they are organic structures or lesions, and may also move due to breathing or pulsation in many cases. So it is considered that remote treatment by indirect plasma is more suitable for medical use.

In many previous studies, helium or argon was used for plasma generation. The effects and safety of NTAPP are still unclear. We used a multi-gas plasma jet that can generate NTAPP from several gas species. Our previous study showed that the kinds and amounts of active species vary by gas species. In addition, the effects of hydrophilization and sterilization changed significantly with gas species[18]. We determined to investigate the difference of the coagulation effect by gas species. Thus, in this study, the potential of NTAPP as a novel endoscopic device for blood coagulation without thermal and discharge damage was investigated *in vitro* and *in vivo*.

2. Materials and methods

2.1. Plasma source

The multi-gas plasma jet source [19-22] has a columnar body like a pen and is connected to an AC power supply (Plasma Concept Tokyo, Inc., Tokyo, Japan) of 16 kHz and 9kV and a gas cylinder. Plasma is

generated between a stick electrode at the center of the body and a cylindrical electrode around the stick electrode. The generated plasma components are delivered by gas flow through a hole (2 mm diameter). The multi-gas plasma jet source can generate atmospheric plasma of various gas species including argon, oxygen, helium, nitrogen, mock air (N_2:O_2=4:1) and carbon dioxide at low gas temperatures (<57 ºC) [23]. The generated plasma has a different character depending on the gas species.

2.2. Coagulation effect of NTAPP on human blood

Human blood was observed after treatment with NTAPP. Blood was drawn from healthy adult volunteers who had no history of hematologic disease and did not recently take medicine. Experiments using human blood were approved by the research ethics committee of Kobe University Hospital (No. 160110).

The experiments were performed as follows. Blood with anticoagulants, in a volume of 10 μL, was set on an adjustable platform and the multi-gas plasma jet was fixed at 7 mm from the jet hole to sample. The gas flow was set 5 L/min as shown in Fig. 1.

First, the blood coagulation time was compared for each plasma treated sample. Blood samples with 3.2% sodium citrate (TERUMO corporation, VENOJECT II VP-CA053K), volume 10 μL, were treated by argon, oxygen, helium, nitrogen, mock air and carbon dioxide plasma. We measured the time to clot the whole surface of each blood sample.

In this study, four different anticoagulants were used: sodium citrate, ethylenediaminetetraacetic acid (EDTA), heparin and EDTA with acetylsalicylic acid (ASA). The difference in blood coagulation by NTAPP treatment was investigated. This investigation was expected to help to elucidating the mechanism of blood coagulation by NTAPP, because each anticoagulant affects a different point of the coagulation cascade. Drawn blood was divided in four vacuum tubes (TERUMO, VENOJECT II) with 3.2% saline citrate (VP-CA053K), EDTA-2K (VJ-DK052E004), heparin (VP-H052K) and EDTA-2K including ASA (100mM), and 10 μL samples from each tube were treated by NTAPP. Nitrogen and carbon dioxide were used for treatment, and the surface of the blood sample was observed after 30s NTAPP treatment. A sample treated only with gas flow was also observed as a control.

2.3. Investigation of NTAPP hemostasis *in vivo*

We investigated the NTAPP coagulation effect and biological safety using porcine stomach and liver as an *in vivo* experiment. Artificial bleeding lesions (about 3 mm) were made by biopsy forceps (FUJIFILM, BF2316DF4) in the stomach and liver of an adult female LDW pig, and the lesions were treated manually using the NTAPP jet described above. NTAPP treatment was continued until the bleeding stopped visually. Nitrogen and carbon dioxide were used to generate NTAPP. These gas species were selected because both nitrogen and carbon dioxide are biologically safe. As nitrogen generates a NTAPP jet with a clear and long after glow, this allows the surgeon to irradiate the target precisely [23]. Carbon dioxide has been used widely in the medical

field because of its high bio-absorption.

All experiments were performed under deep anesthesia. After incision of the abdomen wall, the stomach was incised and the gastric wall reversed to treat the gastric mucosa directly by NTAPP jet. The surface of the liver was also treated by NTAPP jet. Treatment by only gas flow was performed as a control. To investigate the tissue damage by NTAPP jet treatment, liver tissue and gastric mucosa with no bleeding lesion were removed after the NTAPP treatment and observed for tissue changes with hematoxylin and eosin stain by optical microscope. Moreover, to investigate damage to tissue by plasma treatment, immunostaining was conducted using an In situ Apoptosis Detection Kit (MK500, Takara Bio Company, Japan). The kit can detect fragmented chromatin DNA with TUNEL assay as its measurement principle. The operation was performed according to the manufacturer's recommended protocol. In the observed pathologic sample, fragmented chromatin DNA was stained brown by diaminobenzidine, and normal cells were stained green by methyl green.

All the procedures and protocols were approved by the animal care and use committee of IVTeC Japan (IVT15-05). The pig received intramuscular injections of ketamine (10 mg/kg) (DAIICHI SANKYO PROPHARMA), xylazine (2 mg/kg) (Bayel HealthCare) and atropine (0.5 mg/head) (Mitsubishi Tanabe Pharma Corporation), and inhalation of 5% isoflurane (Merck) and oxygen (3 L/min) as induction agents for anesthesia. The pig was connected to an anesthesia apparatus with endotracheal intubation and general anesthesia was maintained by inhalation of 1~3% isoflurane (Merck). Lactated Ringer's solution (60 ml/h) was administered through auricular veins of the pig during operation. The pig's abdominal aorta was incised for euthanasia and we confirmed the pig's pulse had stopped by percutaneous arterial blood oxygen saturation degree apparatus. After euthanasia, the liver tissue and gastric mucosa were removed for preparation.

3. Results

3.1. Blood coagulation by various plasmas

Blood coagulation of the surface of blood samples commenced rapidly upon plasma irradiation by all gas species. The period for whole surface coagulation of blood sample was measured. Figure 2 shows a tendency for coagulation time with argon gas plasma to be slower than with other gas species plasmas. No significant differences between oxygen, helium, nitrogen, mock air and carbon dioxide were detected. The mean of coagulation times by argon, oxygen, helium, nitrogen, mock air and carbon dioxide were 14.7, 7.6, 6.8, 9.2, 6.8 and 7.9 s, respectively. While coagulation was not detected in all control samples after treatment by gas flow alone for 30 s.

3.2. Plasma treatment for blood with various anticoagulants

The blood coagulation process is affected by many factors and reactions. Some activated coagulation factors activate the next reaction and thrombin is generated. Thrombin is an important enzyme for platelet

activation and fibrin polymer cross-linking. Each anticoagulant has different effective points in the blood coagulation process. In this study, sodium citrate, EDTA, heparin and EDTA with ASA were used. The coagulation factors as they relate to each anticoagulant. Sodium citrate combines with calcium ion, which is a requisite factor for some reactions in the coagulation cascade. EDTA captures a divalent ion with a chelate bond, therefore a calcium ion is captured and blood coagulation is inhibited. Heparin combines with antithrombin III and this combined body inhibits some coagulation factors and thrombin, which is an important factor for blood coagulation as an activator of some coagulation factors and platelet [24]. ASA inhibits cyclooxygenase-1 of platelets and this reaction suppresses the agglutinability of platelets by reducing the generation of thromboxane A2. However ASA alone cannot impede blood coagulation completely. Therefore ASA was added to blood with EDTA, and we observed the difference in blood coagulation effect of NTAPP treatment compared with EDTA alone. Figure 3 shows that the clot layer was confirmed on the whole surface of blood samples with each of the four anticoagulants after 30 s of nitrogen NTAPP treatment (Fig. 3 CI, CII, CIII, CIV) and carbon dioxide (Fig. 4 DI, DII, DIII, DIV), while no clot was confirmed after treatment with only gas flow (Fig. 3 AI, AII, AIII, AIV, BI, BII, BIII, BIV). Coagulation on the surface of blood sample began soon after exposure to NTAPP. The samples treated by only gas flow showed some changes by drying, but there was no coagulated blood.

3.3. Investigation of the blood coagulation effect by NTAPP *in vivo*

NTAPP treatment was performed to bleeding lesions on porcine gastric mucosa and liver. Fast control of a bleeding lesion on the gastric mucosa by nitrogen NTAPP jet is shown in Figure 4 (AI, AII). Using a carbon dioxide gas NTAPP jet, a bleeding lesion was also controlled immediately (Fig. 4 (BI, BII)). The time required for hemostasis of bleeding lesions on porcine gastric mucosa by NTAPP treatment was about 10 seconds with both by nitrogen and carbon dioxide gas.

A bleeding lesion on a porcine liver was also controlled by NTAPP jet treatment as in Figure 4 (CI, CII, DI, DII). After NTAPP jet treatment, the treated areas of gastric mucosa and liver tissue were removed and compared with normal tissues by optical microscope.

Crypt structures were observed in normal gastric mucosa. No change was detected and the crypt structures were observed in the gastric mucosa after NTAPP jet treatment with nitrogen and carbon dioxide (30 s). After 1 s application of APC to a porcine gastric mucosal bleeding lesion, the thermal damage was confirmed by optical microscope. The crypt structures were destroyed by thermal damage from the surface of the tissue to a depth of 1.4 mm (Fig. 5). Hepatic lobule structures were observed in normal liver tissue. No change was detected in the liver tissue after NTAPP treatment by nitrogen and carbon dioxide gas, as was the case with the gastric mucosa. In the case of APC treatment to liver tissue, hepatic cells at the surface of the treated area of liver were intensely damaged and hepatic lobule structures from the surface to a depth of 1.6mm were destroyed (Fig. 6). To assess the DNA damage by NTAPP treatment, an in situ Apoptosis Detection Kit (TAKARA BIO INC.) was used. No change was detected after nitrogen or carbon dioxide NTAPP jet treatment. In addition, some staining indicating an area of DNA damage was detected after 1 s APC treatment of gastric mucosa (Fig. 7).

4. Discussion

In this study, blood coagulation effects by NTAPP jets generated from several gas species were measured and compared. The mechanism of blood coagulation by NTAPP is considered to be due mainly to ROS such as OH radical [24, 25]. In our results, the coagulation time by argon gas NTAPP jet was longer than the others, and the coagulation time by other gas NTAPP jets were virtually all the same as each other. This suggests that blood coagulation by NTAPP is affected by not only the OH radical but also some other ROS. Our previous study indicated that the quantities and kinds of reactive species generated by plasma differ between gas species [18], and the sterilization effect and hydrophilization effect of argon plasma were weakest among the gas plasmas we examined [23, 26, 27]. This suggests that argon plasma cannot generate sufficient reactive species for equivalent effects.

The blood coagulation effect by NTAPP jet was confirmed even in the presence of four different anticoagulants. This result suggests that plasma affects not only a single factor but several factors or affects a terminal factor in the blood coagulation process, for example fibrin polymer or activated platelet aggregation [10, 24].

Furthermore, fast hemostasis by NTAPP jet on porcine gastric mucosa and liver tissue was confirmed. The hemostasis effects by nitrogen and carbon dioxide NTAPP jets on porcine gastric mucosa are shown in supplementary movies 1 and 2. Both treatments stopped the bleeding within 15 s. In this evaluation, to quantify a hemostasis effect is difficult. The degree of difficulty to control the bleeding lesions depends on presence of vessel, especially artery, in addition there may be cases where an area had no bleeding after chipping by forceps at the porcine gastric mucosa. This diversity of bleeding lesion makes it difficult to quantify or to provide a technique for objectively evaluating the coagulation effect by NTAPP. Therefore, the quantification of hemostasis is forthcoming challenge.

We confirmed that NTAPP treatment is free from damage by pathological observation. In addition to hematoxylin and eosin stain, TUNEL stain was used and DNA damage by NTAPP treatment was evaluated. No change was detected after NTAPP treatment, while thermal damage and DNA damage were considered after APC treatment for 1 s. It is assumed that the mechanism of NTAPP in stopping bleeding is not thermal degeneration of capillary vessel but activation of blood coagulation reaction directly. Therefore, structural changes of tissue were not detected. Moreover, to compare with other methods, conventional dielectric barrier discharge (DBD) plasma by helium [28] and only gas flow (5 L/min of nitrogen gas) were observed. A longer treatment time by helium DBD plasma was needed for bleeding control. With only gas flow, a change of drying was detected but the bleeding was not controlled. The change of drying was only observed at the surface of blood.

NTAPP jet treatment can control a bleeding lesion with less tissue damage than APC treatment, and more rapidly than conventional helium DBD plasma treatment. It was reported that this plasma jet source can be miniaturized for endoscopic use by 3D printer with low cost (less than $100) [29]. Moreover, it has an advantage that the plasma source can generate NTAPP by carbon dioxide, which shows high bio-absorption.

And carbon dioxide costs lower than argon, so running costs for NTAPP jet treatment are not higher than APC. We have great expectation that it can be used routinely in many surgery situations not only in stomach or liver. The electrical safety of the machine has been confirmed by use in several industrial fields. These results suggest great potential of the NTAPP jet as a novel tool for safety and effective hemostasis in the endoscopic and surgical fields.

5. Conclusion

We confirmed the efficacy of the blood coagulation by NTAPP jet treatment not only with blood samples *in vitro* but also with porcine gastric mucosa and liver tissue *in vivo*. The differences in blood coagulation effect with several gas species were investigated for the first time. In addition, the safety of NTAPP jet treatment to living tissues and its hemostasis effect were confirmed. Our results indicate that nitrogen and carbon dioxide gas NTAPP jets have the potential to be used for hemostasis in various surgical fields.

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Figure legends

Fig. 1. Multi-gas plasma jet for blood coagulation setup

Fig. 2. Relationship of coagulation time (s) and 95% confidence intervals for each gas plasmas (n=3)

Fig. 3. The clotting layer of human blood with anticoagulants after NTAPP jet treatment

Human blood was combined with citrate (AI, BI, CI, DI), EDTA-K (AII, BII, CII, DII), heparin-Na (AIII, BIII, CIII, DIII) and EDTA-K + aspirin (AIV, BIV, CIV, DIV). Blood samples were observed after NTAPP jets of nitrogen (CI, CII, CIII, CIV) and carbon dioxide (DI, DII, DIII, DIV) and compared with the samples after only gas flow (nitrogen (AI, AII, AIII, AIV), and carbon dioxide (BI, BII, BIII, BIV)).

Fig. 4. NTAPP treatment for bleeding lesion on porcine tissues

Gastric mucosa was observed before treatment (AI) and after treatment (AII) by nitrogen gas NTAPP jet and before treatment (BI) and after treatment (BII) by carbon dioxide gas NTAPP jet.

Liver tissue was also observed before treatment (CI) and after treatment (CII) by nitrogen gas NTAPP jet and before treatment (DI) and after treatment (DII) by carbon dioxide gas NTAPP jet.

Fig. 5. Gastric mucosa damage by NTAPP jet was observed by optical microscope (AI, BI, CI, DI×1.25) (AII, BII, CII, DII ×20)

(AI) and (AII) are normal gastric mucosa for control, (BI) and (BII) are after treatment by nitrogen gas NTAPP jet for 30 s, (CI) and (CII) are after treatment by carbon dioxide gas NTAPP jet for 30 s, (DI) and (DII) are after treatment by APC for 1 s.

Fig. 6. Liver tissue damage by NTAPP jet was observed by optical microscope (AI, BI, CI, DI ×1.25) (AII, BII, CII, DII ×20)

(AI) and (AII) are normal liver tissues for control, (BI) and (BII) are after treatment by nitrogen gas NTAPP jet for 30 s, (CI) and (CII) are after treatment by carbon dioxide gas NTAPP jet for 30 s, (DI) and (DII) are after treatment by APC for 1 s.

Fig. 7. Imaging of pathologic sample of gastric mucosa was investigated by using *In situ* Apoptosis Detection Kit (AI, BI, CI, DI ×1.25) (AII, BII, CII, DII ×20)

(AI) and (AII) are normal gastric mucosa for control. (BI) and (BII) are after treatment by nitrogen gas NTAPP jet for 30 s. (CI) and (CII) are after treatment by carbon dioxide gas NTAPP jet for 30 s, (DI) and (DII) are after treatment by APC for 1 s.

Figure1
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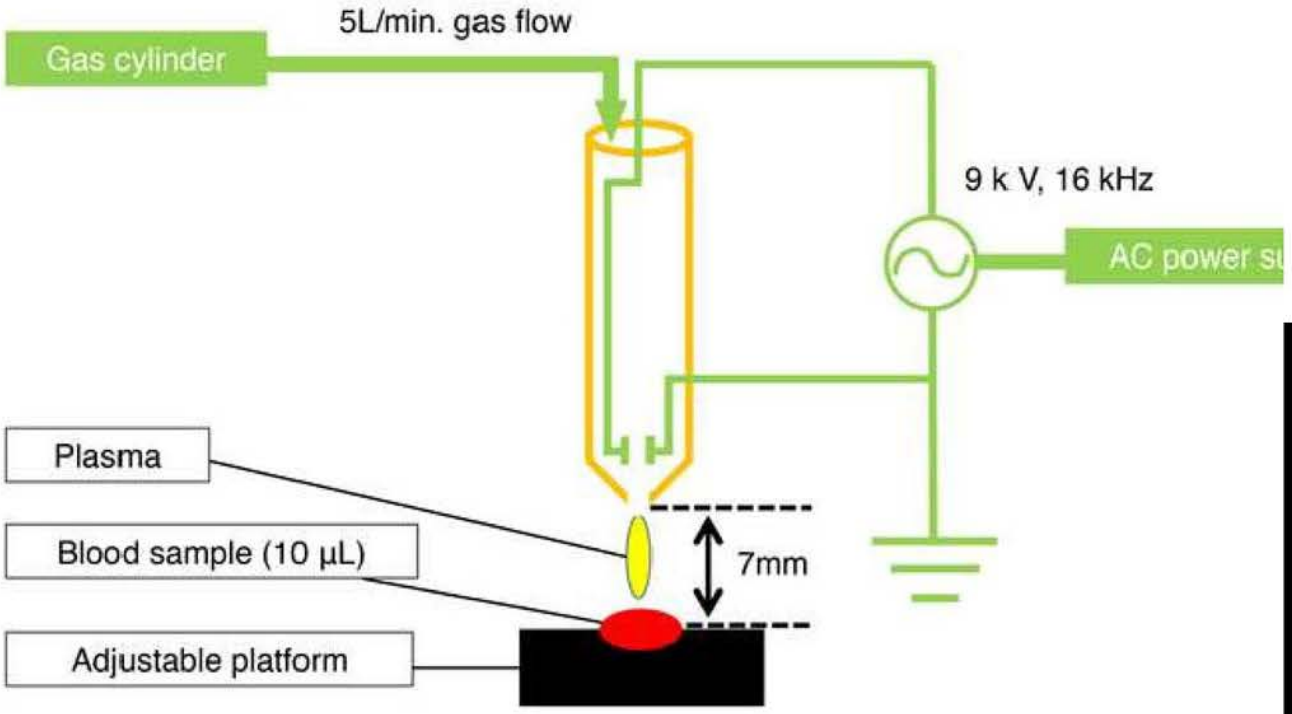

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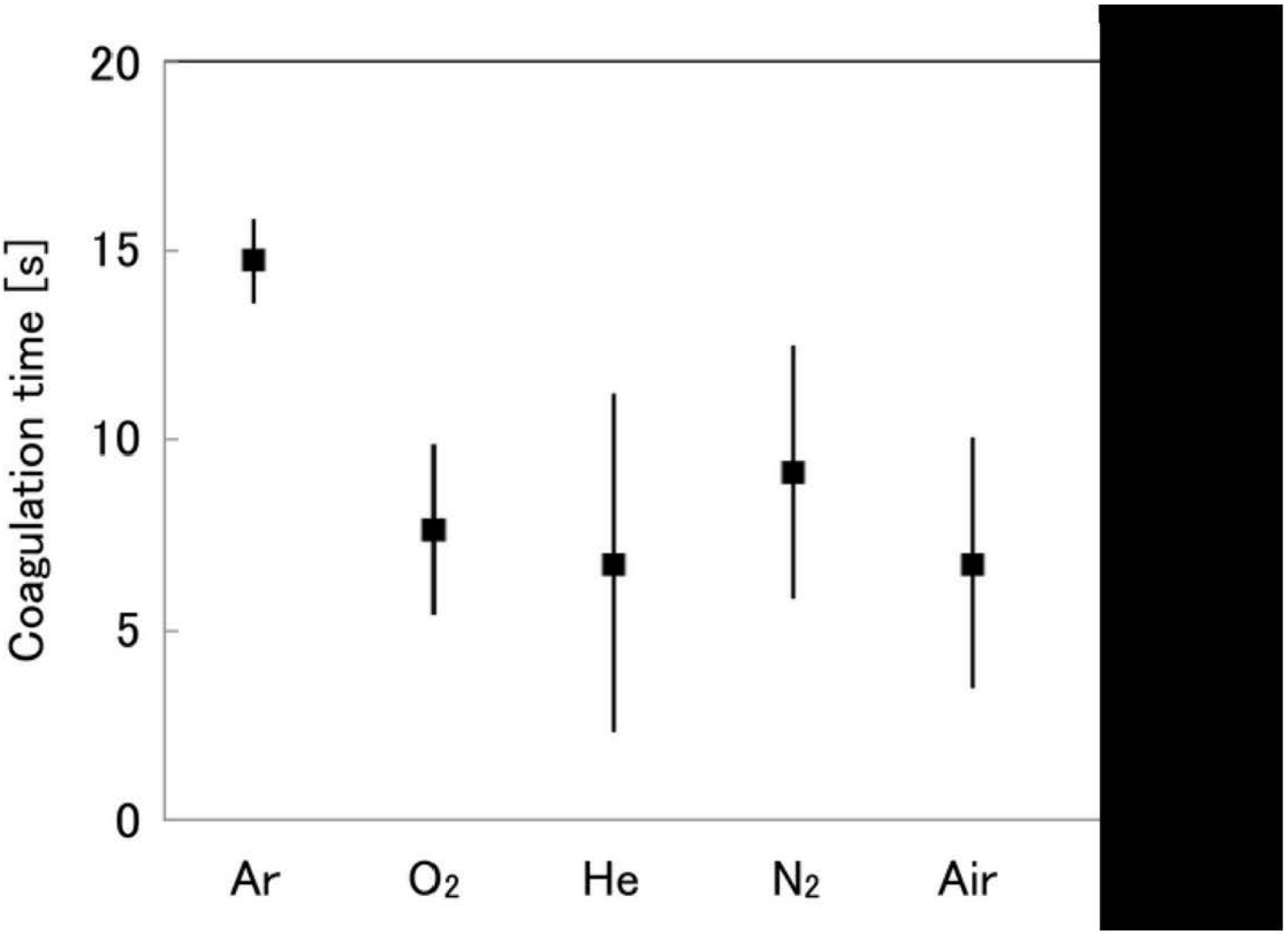

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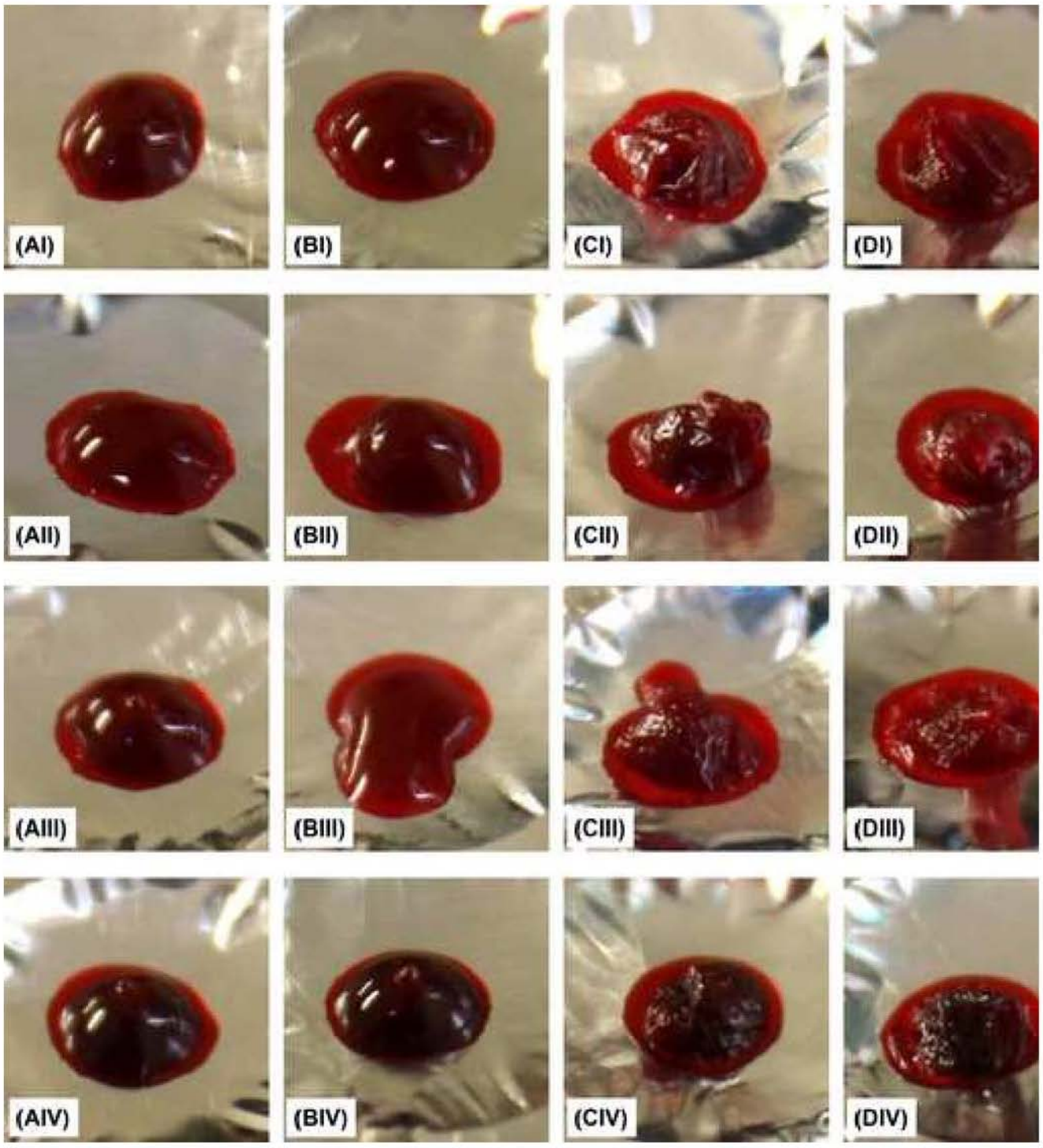

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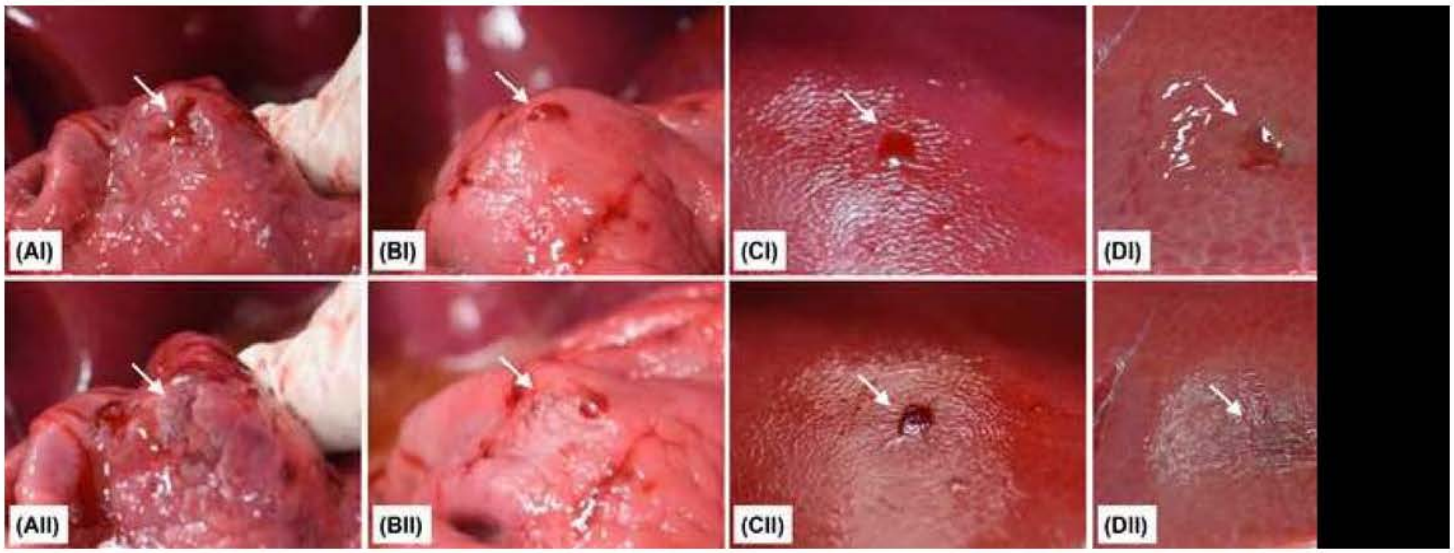

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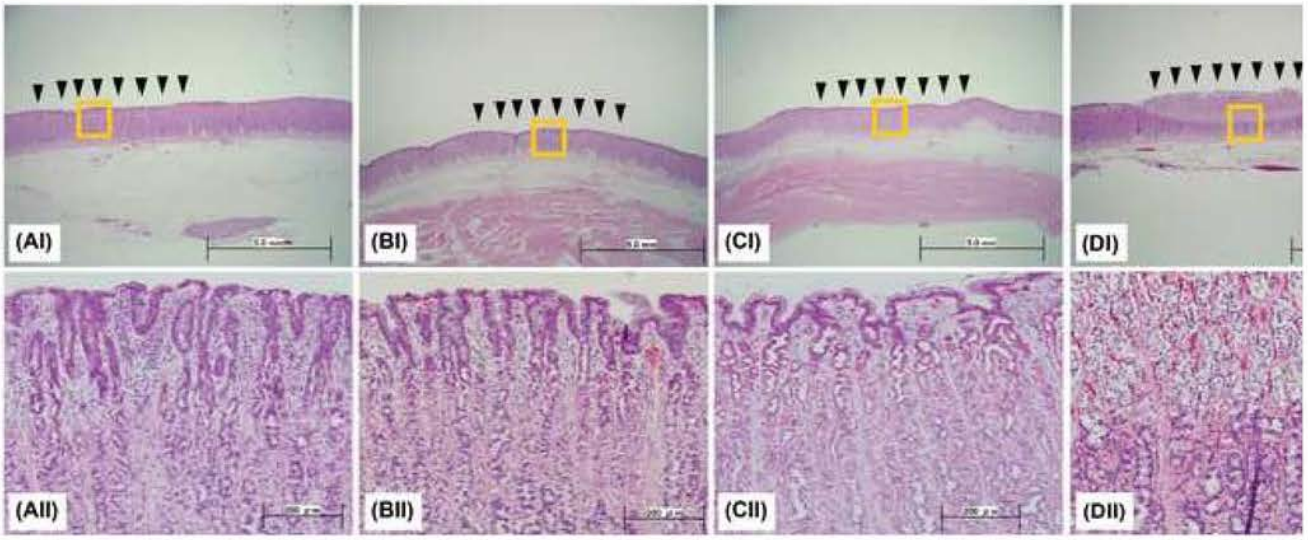

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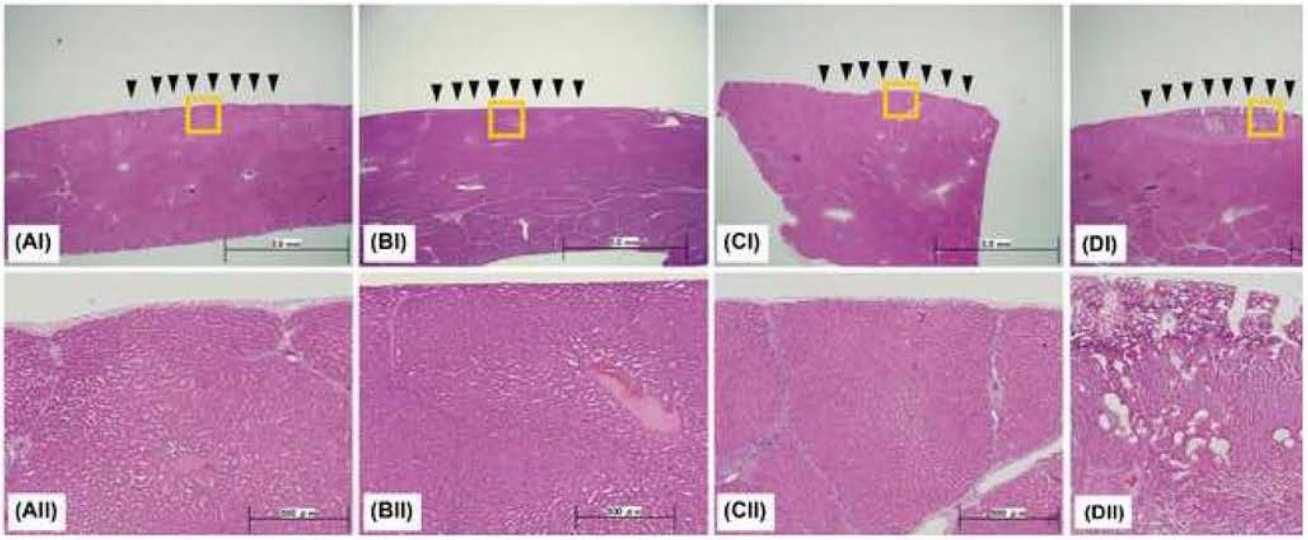

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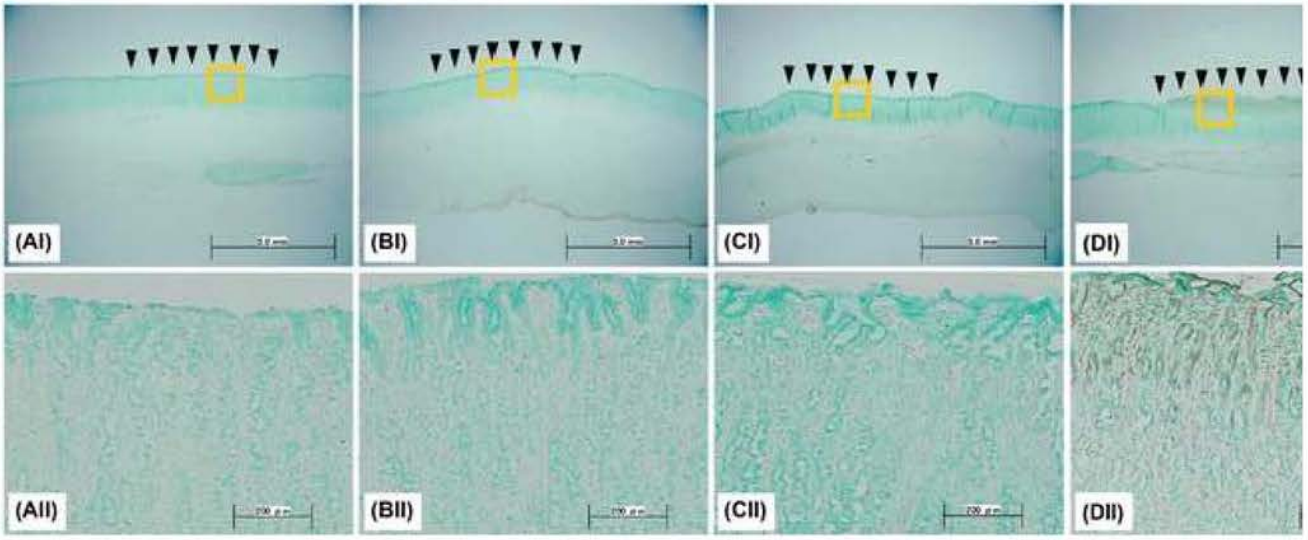

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