^{68}Ga-citrate positron emission tomography/computed tomography findings in an experimental model of acute appendicitis in rabbits

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Abstract

Objective: Acute appendicitis (AA) is a common urgent surgical situation of the gastrointestinal tract. Gal-lium-68 (^{68}Ga)-citrate has been recently investigated as a radiopharmaceutical for infection and inflammation imaging. Aim of the study was to determine the effectiveness of ^{68}Ga-citrate positron emission tomography/computed tomography (PET/CT) imaging in rabbits with experimentally induced AA. **Materials and Methods:** In the AA group (n=6), the appendices of the rabbits were surgically ligated. The sham group (n=6) was used as control. Gallium-68-citrate was synthesized. All rabbits were imaged using ^{68}Ga-citrate PET/CT at 36th h following the establishment of experimental models, and at 36th h, all rabbits were appendectomised. Appendices were examined histopathologically. Blood samples were drawn from all rabbits at the beginning and end of the experimental process. Interleukin-6 (IL-6) and procalcitonin (Pct) levels were measured. Acute appendicitis was confirmed histopathologically and biochemically. **Results:** Gallium-68-citrate PET/CT showed acute appendicitis in all rabbits. The sensitivity, specificity and accuracy of ^{68}Ga-citrate PET/CT in AA were 100%, 83.3% and 91.7%, respectively. **Conclusion:** Acute appendicitis is accurately imaged in an experimental rabbit model by using ^{68}Ga-citrate with PET/CT.

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Introduction

Acute appendicitis (AA) is a common urgent surgical situation. The incidence of AA in developed countries is 100-140 per 100,000 individuals annually [1]. The lifetime risk of AA is approximately 6.7%-8.6% [2]. Clinical characteristics of AA may range from mild symptoms to fatal conditions such as peritonitis and sepsis. Occasionally, the diagnosis can be very difficult. The primary treatment approach is surgery [3]. Although computed tomography (CT) is successful in diagnosis, sometimes it may be insufficient [4, 5]. The diagnostic methods used in AA aim to reduce hospital costs by decreasing the rate of negative laparotomy or appendiceal perforation [6]. Currently, there is no ideal diagnostic tool that can be used alone to provide a definite and accurate diagnosis before surgery. Acute appendicitis is an infectious and inflammatory disease. Thus, nuclear medicine infection and inflammation imaging methods might be performed for AA. Until now, these methods can be used in some cases that are suspicious for AA. Medical treatment has been discussed in AA cases in recent years [7]. Nuclear imaging methods may be helpful in evaluating the response to treatment in AA. Previous studies have reported the use of indium-111 (^{111}In) and technetium-99m-D,L-hexamethylene-propyleneamine oxime (^{99m}Tc-HMPAO)-labelled leukocyte scintigraphy, ^{99m}Tc-labelled human immune globulin and anti-granulocyte antibodies in AA cases [8, 9]. In recent years, incidental cases of AA have been identified during fluorine-18-fluorodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT) scans for oncologic screening [10-12]. One of the most commonly used radiopharmaceuticals for imaging infection and inflammation in the past 40 years is gallium-67 (^{67}Ga)-citrate [13]. However, due to long half-life, long imaging times of ^{67}Ga and higher patients' radiation exposure, it is not commercially available quickly [14]. This suggests that gallium-68 (^{68}Ga)-citrate can be used for infection and inflammation imaging instead of ^{67}Ga-citrate, since it can be easily available. Gallium-68-citrate has been used for infection and inflammation in preclinical and clinical trials [15, 16]. As far as we know, there are not any studies in the literature about the ^{68}Ga-citrate PET/CT findings in AA. In addition, there is limited number of

preclinical and clinical studies about the ^{68}Ga-citrate-like molecules such as ^{67}Ga and ^{99m}Tc-citrate. These radiopharmaceuticals have been reported to show high uptake in AA [17, 18]. In one case with intra-abdominal infection, focal severe ^{68}Ga-citrate uptake was shown [19]. The results of these studies suggest that ^{68}Ga-citrate may be a potential imaging agent for AA or intraabdominal infections. Some biochemical markers increase with the severity of inflammation. In addition to imaging methods, these biochemical markers are used in the diagnosis of AA. The most useful tests are leukocyte count and C-reactive protein level measurement [20]. However, the contribution of other acute-phase reactants, such as D-dimer, IL-2, IL-6 and Pct in AA diagnosis has also been investigated. These markers were reported to increase in AA [21-23].

The aim of our study was to investigate the diagnostic ability of ^{68}Ga-citrate PET/CT in experimentally induced AA rabbits.

Materials and Methods

The flow-chart of this prospective experimental study is shown in Figure 1.

^{68}Ga-citrate labelling and quality control

Gallium-68-citrate synthesis and quality control was performed with a cationic method using the Scintomics automated synthesis system (Scintomics GmbH GRP module 4V). The definition of chemical and radiochemical purity of ^{68}Ga-citrate was carried out according to the ICH Q2(R1) guideline. The standard quality control (QC) tests were analysed with Scintomics 8100 radio-HPLC system equipped with a radioactivity detector. Gallium-68-citrate synthesis was performed by a cationic method without using organic solvents. [24].

Animal experiments

This study was conducted after obtaining ethical approval from the Animal Experiments Ethics Committee (Jan 11, 2019-60758568-020/2717). European Union directives were complied with. The gender of the animal was not considered. All rabbits were cared for, kept in separate cages with 12-h day and night cycles at 25°C during all procedures and fed ad libitum. Twelve New Zealand rabbits (Oryctolagus cuniculus) that weighed 2100-2950g were divided into two groups: AA (n=6) and sham (n=6). In all rabbits, ketamine 35mg/kg (Ketasol 10mL, Richter Pharma AG, Wels -Australia) and xylazine 5mg/kg (Rompun 25mL, Bayer, Kansas, USA) were injected intramuscularly to induce general anaesthesia. The AA and sham models were established and prepared according to the study performed by Şimşek et al. (2016) (7) (Figure 2). All surgical procedures were performed by an experienced pediatric surgeon with a certificate of experimental animal use.

^{68}Ga-citrate PET/CT imaging and evaluation

All rabbits were anaesthetized. Gallium-68-citrate was injected into the ear vein of rabbits at a dose of 18MBq/kg. Following an uptake time of approximately 60min, acquisitions were conducted in the prone position. In all cases, PET/CT imaging was performed 36 hours after the establishment of the experimental model. Rabbits were examined using a PET/CT scanner (Gemini TF TOF PET-CT; Philips, Cleveland, OH; 3D mode, slice thickness of 5mm, 4×4×22mm, 256×256 matrix, transverse FOV 576mm and axial FOV 180mm). Whole-body emission scans were acquired for a duration of 2 min per position without intravenous contrast injection. Transmission images were obtained using low-dose CT (90mA, 100kV, 16 CT detectors, and 5-mm slice thickness).

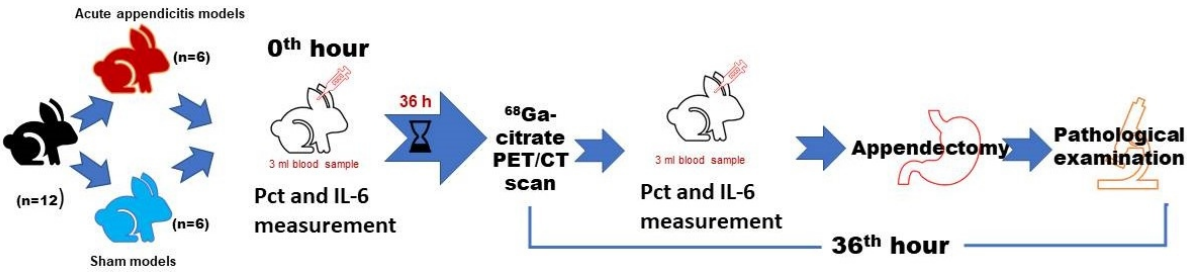

Figure 1. The flow-chart of experimental study.

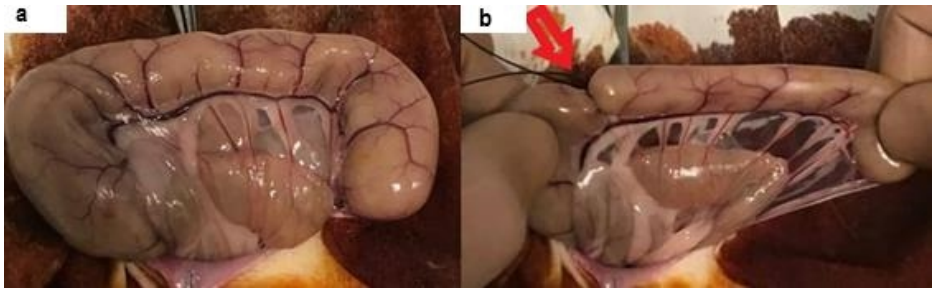

Figure 2. Establishment of the sham (a) and experimental models of AA (b). The arrow indicates the region where the appendix was ligated.).

Attenuation correction was performed for PET images using a CT map and ordered subsets-expectation maximization algorithm (33 subsets, 3 iterations). Transverse, sagittal and coronal sections (5-mm thickness) were generated from PET/CT fusion images and were evaluated using the Philips Fusion Viewer software (ver. 2.1; Philips Healthcare, Best, The Netherlands).

Gallium-68-citrate PET/CT images were blindly evaluated by a nuclear medicine physician both visually and semi-quantitatively. He decided whether acute appendicitis occurred or not. Findings were compared with histopathological results.

Procalcitonin and interleukin-6 examinations

A total of 3mL of blood was taken from the ear veins of AA and sham rabbits immediately following model establishment (0^{th} h) and just before performing appendectomy (36^{th} h). Consequently, blood samples were allowed to coagulate at a temperature of 4°C for 30 min. After centrifugation, serum samples were stored at -80°C until IL-6 and Pct serum levels were assessed. IL-6 and Pct levels were quantified using competitive inhibition, an enzyme immunoassay method using a commercial ELISA kit (USCN Life Science Inc., Wuhan, China). Intra- and inter-assay were <5% for both tests.

Appendectomy of rabbits and histopathological examination

After PET/CT imaging, all rabbits underwent appendectomy under anesthesia. Tissues were fixed at 10% formaldehyde solution for 24h and embedded in paraffin blocks. Four micrometer thick tissue sections were taken from each block, stained with hematoxylin-eosin and examined under a light microscope. Histopathological examinations were performed blindly by a single pathologist.

Statistical analysis

Data was analysed using SPSS 24.0 package software (IBM, Armonk, NY, USA). Continuous variables were expressed as mean±standard deviation, whereas categorical variables were expressed as numbers and percentages. When parametric test assumptions were provided, the significance of the difference between the two means was used to compare independent group differences. On the other hand, when parametric test assumptions were not provided, the Mann-Whitney U test was used to compare independent group differences. Wilcoxon paired two samples tests were also used for intra-group comparisons. A value of $P<0.05$ was considered statistically significant.

Results

Labelling of citrate with ^{68}Ga in an automated synthesis module and quality control

Labelling efficiency of ^{68}Ga-citrate was found >98%. The pH of the final product was 4-5. Quality control was evaluated using HPLC. Under the chromatographic conditions defined in the experimental section, the average retention time of ^{68}Ga and of ^{68}Ga-citrate was found to be 2.36min and 3.83 min, respectively.

^{68}Ga-citrate PET/CT and histopathological findings

During appendectomy, the appendices were evaluated; two cases were suppurative, three cases were perforated and one case was gangrenous in the AA group. No appendicitis was detected in any rabbit in the sham group. The histopathological and ^{68}Ga-citrate PET/CT results of AA and sham rabbits are shown in Table 1.

Table 2 demonstrates the histopathological and ^{68}Ga-citrate PET/CT results. According to these results, the sensitivity, specificity and accuracy of ^{68}Ga-citrate was 100%, 83.3% and 91.7%, respectively.

Gallium-68-citrate PET/CT accurately demonstrated appendicitis in all rabbits diagnosed with appendicitis histopathologically. All rabbits in the sham group were considered normal histopathologically, whereas one rabbit in the sham group (sham1) was interpreted as having appendicitis in PET/CT (false positive).

When PET/CT images of experimentally induced AA rabbits were examined, fluid consolidation in the appendix lumen and thickening in the appendix wall were observed. In addition, there was a moderate increase the ^{68}Ga-citrate uptake in the appendix wall and periappendicular region (Figure 3). Physiological uptake of ^{68}Ga-citrate was observed in sham group rabbits. Gallium-68-citrate has physiological uptake in the liver, spleen, kidneys, and great vessels (Figure 4).

Maximum standardized uptake value (SUVmax) measurements were performed while evaluating PET/CT results. The SUVmax values measured in the appendix region of all rabbits in the AA group in PET/CT were at a moderate level. However, SUVmax values measured in the abdominal region of rabbits in the sham group were lower than those in the AA group. The mean SUVmax calculated in acute appendicitis cases was 2.95±0.28 (2.64-3.50), and the SUVmax calculated in sham cases was 1.36±0.15 (1.12-1.52). In the statistical analysis, while comparing the SUVmax values measured from the images we found statistically significant difference between AA and sham groups ($P=0.002$).

In the histopathological evaluation of the AA group, neutrophil infiltration and focal necrosis in all cases and complete necrosis in perforated and gangrenous cases were detected. Histopathologically, appendicitis findings were not observed in all rabbits in the sham group. Figure 5 shows the histopathological features of the sham and AA groups.

Procalcitonin and interleukin-6 levels

When comparing the results of the initial blood samples taken from the AA and the sham groups, no statistically significant differences in Pct were found. We found statistically significant differences in IL-6 levels between the two groups ($P<0.05$). In basal blood measurements, IL-6 levels in the AA group were significantly higher than those in the sham group. At 36h, Pct and IL-6 levels in the AA group were significantly higher than those in the sham group ($P<0.05$). In the AA group, Pct and IL-6 levels were significantly higher at 36 h than at baseline ($P<0.05$). There was no statistically signifi-

Table 1. *The histopathological and ^{68}Ga-citrate PET/CT results of AA and Sham rabbits.*

Rabbits	Macroscopic diagnosis	Exudate	FN	Complete necrosis, peritonitis	NI	Pathological Diagnosis Appendicitis	^{68}Ga-citrate PET/CT results Appendicitis
AA1	perforated	+	++	++	++	Yes	Yes
AA2	suppurative	-	++	-	++	Yes	Yes
AA3	suppurative	-	++	-	++	Yes	Yes
AA4	perforated	+	++	++	++	Yes	Yes
AA5	gangrenous	-	+++	+++	+++	Yes	Yes
AA6	perforated	+	++	++	++	Yes	Yes
Sham1	normal	-	-	-	-	***No***	***Yes***
Sham2	normal	-	-	-	-	No	No
Sham3	normal	-	+	-	+	No	No
Sham4	normal	-	+	-	+	No	No
Sham5	normal	-	-	-	+	No	No
Sham6	normal	-	-	-	-	No	No

AA: acute appendicitis; FN: focal necrosis; NI: neutrophilic infiltration; (-): no; (+): mild;(++): moderate; (+++): severe

Table 2. *Sensitivity, specificity, PPV and NPV values for ^{68}Ga-citrate PET/CT.*

Statistics	Results (%)	CI (95%)
Sensitivity	100	54.07-100
Specificity	83.3	35.55-99.58
PPV	85.7	21.09-78.91
NPV	100	50.06-97.29
Accuracy	91.7	61.52-99.79

PPV: positive predictive value; NPV: negative predictive value

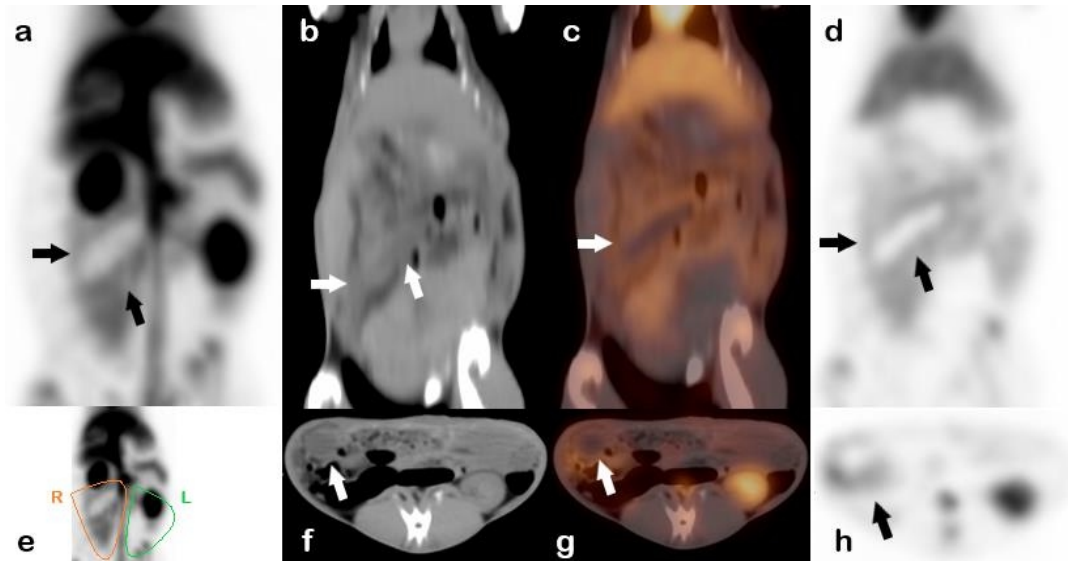

Figure 3. In ^{68}Ga-citrate PET/CT images of a rabbit (AA6) with experimental acute appendicitis, acute appendicitis is observed in the abdomen in the right quadrant (black and white arrows). MIP (a); PET/CT coronal CT image (b); coronal PET/CT fusion image (c), coronal PET image (d), transaxial CT fusion image (f), transaxial PET/CT fusion image (g), transaxial PET image (h). In the orange and green ROI show the right and left quadrants of the abdomen. Increased ^{68}Ga-citrate uptake is observed on the right upper side where the appendix is located (e). Increased ^{68}Ga-citrate uptake is observed in the appendix wall and the surrounding peritonitis area (a, d, h; black arrows).

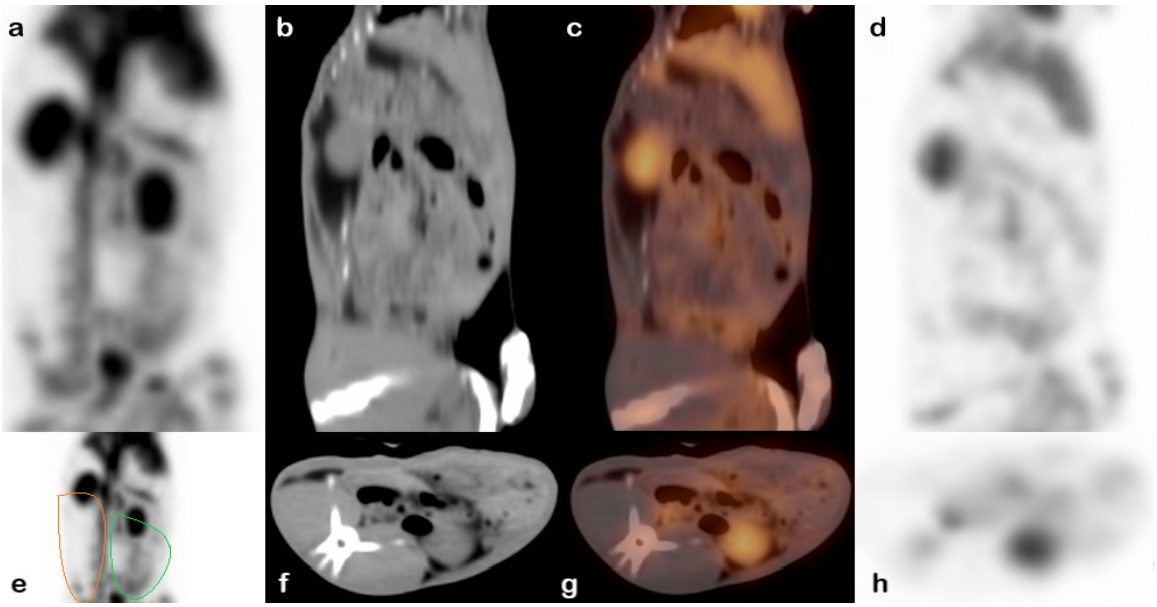

Figure 4. Gallium-68-citrate PET/CT images of a rabbit from a sham group (Sham 4). MIP (a); PET/CT coronal CT image (b); coronal PET/CT fusion image (c), coronal PET image (d), transaxial CT fusion image (f), transaxial PET/CT fusion image (g), transaxial PET image (h). Pathological ^{68}Ga-citrate uptake was not observed on the right side (orange ROI) of the abdomen (e).

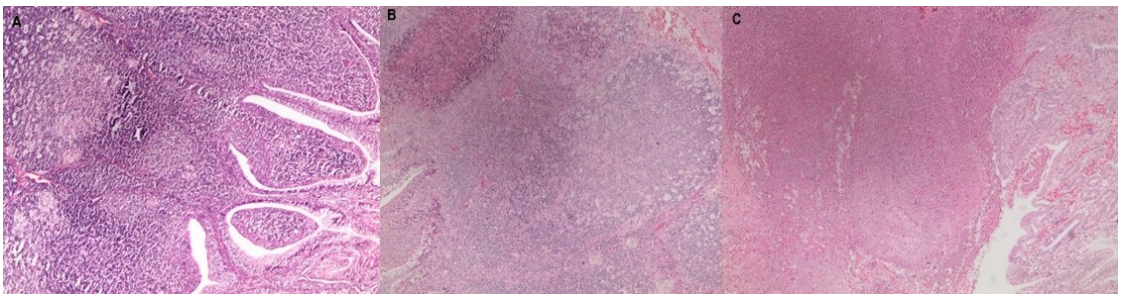

Figure 5. Histopathological view of the appendix of a rabbit in the sham group (a) neutrophil infiltration of the mucosa and appendix wall (b) Histopathological examination of another rabbit in the AA group showed full-layer necrosis (c) in the appendix wall (HEX 40).

cant change in these levels in the sham group at 36th h compared with those at baseline (Table 3).

Discussion

In acute appendicitis, the pathological process usually begins with the obstruction of the lumen. Intraluminal pressure increases and mucosal damage occurs. With mucosal damage, infection, necrosis and peritonitis develop on the appendix wall. Neutrophils quickly migrate to the site of infection. Theoretically, we can visualize this histopathological process with ^{68}Ga-citrate PET/CT.

In our study, we successfully synthesized ^{68}Ga-citrate by making changes in the automatic and closed synthesis unit. The labelling efficiency of the final product was >98%, and its pH was between 4 and 5. We correctly obtained the ^{68}Ga-citrate peak using HPLC and our findings are consistent with those of similar studies [15, 25-27].

As far as we know, this is the first rabbit study in the literature to evaluate an experimental AA model with ^{68}Ga-citrate PET/CT. Previously, many nuclear medicine imaging methods have been attempted in clinical and pre-clinical studies of AA [8-9]. However, none could achieve routine clinical use for AA. In acute appendicitis, molecular imaging methods may be needed in cases of unclear appendicitis and in evaluating the response to medical treatment. Long imaging times and complex procedures limit the routine use of these methods. Gallium-68-citrate's simple and short synthesis procedure, short imaging and evaluation time, superior diagnostic capability of PET/CT compared to other molecular imaging methods are its main advantages.

By considering histopathological findings as the gold standard, we calculated the sensitivity, specificity and accuracy of ^{68}Ga-citrate PET/CT in rabbit appendicitis to be 100%, 83.3% and 91.7%, respectively. The number of our cases was limited. To provide a fair idea, we calculated sensitivity, specificity, PPV, NPV, and accuracy. Turan et al. (1997) [17] investigated the appendicitis imaging efficacy of ^{99m}Tc-citrate and ^{67}Ga-citrate by establishing an AA model similar to that established in our study. In their study, ^{99m}Tc-citrate accurately detected 87.5% of AA cases. Furthermore, ^{67}Ga-citrate accurately detected AA in 75% of cases. Our results are better than those obtained using ^{99m}Tc-citrate and ^{67}Ga-citrate. In the experimental study by Turan et al. (1997) no anatomical correlations were made. Therefore, it is difficult to interpret which anatomical region is involved in the uptake of ^{99m}Tc-citrate and ^{67}Ga-citrate in the abdomen. In our study, the evaluation of CT images revealed that the appendix was swollen, with fluid consolidation in its lumen, thickening in the appendix wall and an increased density in the periappendicular areas. In PET images, moderate level of ^{68}Ga-citrate uptake was observed in the appendix wall and periappendicular region. As a result of histopathological evaluation of AA cases, neutrophil infiltration, peritonitis, and necrosis findings were detected in the appendix mucosa and wall. Gallium-68-citrate shows a moderate uptake in the inflammation area. This is due to increased blood flow, enhanced vascular permeability and migration of neutrophils to the inflammation site.

As is known, ^{68}Ga-citrate PET/CT is a hybrid imaging method. This method has a few drawbacks. First, ^{68}Ga-citrate uptake has moderate in AA. Second, because of the physiological ^{68}Ga uptake in abdomen, its evaluation was difficult. However, the successful anatomical evaluation capability of

Table 3. *Procalcitonin-Interleukin 6 results (pg/mL).*

		0 h		36 h		
	Groups	**Mean±SD**	**Median (min-max)**	**Mean±SD**	**Median (min-max)**	**P (in-group)**
Pct	AA	13.73±5.49	13.79 (4.45-19.56)	24±11.09	23.97 (5.45-39.56)	0.027#
	Sham	6.56±1.12	6.4 (5.47-8.52)	7.09±1.4	6.86 (5.25-9.52)	0.168
	P (inter-group)		0.065		0.041*	
IL-6	AA	4.59±1.47	4.8 (2.35-6.45)	10.62±1.14	10.73 (8.98-12.15)	0.028#
	Sham	1.97±0.18	2 (1.66-2.22)	2.29±0.44	2.07 (1.94-3.01)	0.344
	P (inter-group)		0.002*		0.002*	

*P<0.05, statistically significant difference; *Mann–Whitney U test; #Wilcoxon Paired test*

CT provides an important advantage in PET.

One of our cases (sham1) was also evaluated as false positive (FP). Histopathological examination revealed no evidence to explain this. Gallium-68-citrate has a high blood pool activity in the upper abdomen. In the ^{68}Ga-citrate PET/CT, the high blood pool in abdomen can be a pitfall for AA. In our previous study in which we examined the physiological distribution of ^{68}Ga-citrate in rabbit, we reported high blood pool activity in the liver, spleen, kidneys and large blood vessels in the abdomen [24]. These physiological uptakes in the atypical location of the appendicitis may make the evaluation difficult.

Acute-phase reactants are used as biochemical markers in the diagnosis of AA. In our study, Pct and IL-6 levels in rabbits in the AA group following appendectomy were significantly higher compared with those at baseline ($P<0.05$). Furthermore, there was not any significant difference between sham group and AAs at baseline. Basal IL-6 levels were higher in the AA group than in sham group and basal blood samples were taken immediately following the establishment of the AA and sham models. Hence, this demonstrates that IL-6 begins to increase rapidly with the onset of inflammation and our results are consistent with current literature findings [28]. Pct levels were not increased in patients with sterile inflammation or viral infection. Therefore, it remains a good biomarker in many inflammatory conditions such as AA, sepsis and meningitis. When both tests were concomitantly evaluated, the sensitivity and specificity values were 95% and 55%, respectively. It was reported that Pct and IL-6 are more efficient predictors of AA than IL-2 and D-dimer [23]. We proved that inflammation occurred using Pct and IL-6 elevation in rabbit AA models.

This study has several limitations. We were unable to use this imaging modality in real clinical cases because ^{68}Ga-citrate is not licensed for human use in our country. Also, this is an experimental animal study, so the number of animals used was small. Pre-clinical and clinical trials assessing larger populations in the future will more clearly reveal the value of ^{68}Ga citrate in AA.

In conclusion, ^{68}Ga-citrate PET/CT showed acute appendicitis in experimental rabbit models. Moderate ^{68}Ga-citrate uptake was observed in the appendicitis region. This study was conducted with a limited number of animal subjects and future researchers should use more subjects and real clinical cases.

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The authors declare that they have no conflicts of interest.

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