Histological characteristics of the myometrium in the postpartum hemorrhage of unknown etiology −A possible involvement of local immune reactions−

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Abstract: The aim of this study was to evaluate the histological characteristics of the myometrium obtained on postpartum hemorrhage (PPH) of unknown etiology secondary to uterine atony. They were selected among registered cases of clinically suspected amniotic fluid embolism (AFE) and classified as PPH of unknown etiology because of no obvious cause of PPH at Hamamatsu University School of Medicine, a registration center for clinical AFE in Japan. Immunohistochemical studies were done on myometrium using anti-mast cell tryptase, anti-neutrophil elastase, anti-CD68, anti-CD88, anti-CD3, and anti-ZnCP-1 antibodies. Massive infiltrations of inflammatory cells with mast cell degranulation within the myometrium secondary to complement activation were observed in PPH of unknown etiology (n=34), but not in control pregnant women (n=15) or after delivery women without PPH (n=18). The concomitant immunohistochemical detection of meconium in myometrium suggests that amniotic fluids or fetal materials are one of the candidates to induce maternal local immune activation in the PPH of unknown etiology. Postpartum acute myometritis in the absence of an infective etiology may be a histological characteristic of PPH of unknown etiology.

Suggested Reviewers:

Opposed Reviewers:

Professor. Shigeru Saito,

Dear Editor-in-Chief, 'Journal of Reproductive Immunology'

RE: JRI-S-14-00179, entitled “Histological characteristics of the myometrium in the postpartum hemorrhage of unknown etiology -A possible involvement of local immunereactions-” by Farhana et al.

Please find the enclosed manuscript entitled “Histological characteristics of the myometrium in the postpartum hemorrhage of unknown etiology -A possible involvement of local immunereactions-” revised by Farhana et al. On behalf of all the authors, I would like to ask you to consider our manuscript for publication in Journal of Reproductive Immunology as an original research article, again.

Postpartum hemorrhage (PPH) is the leading cause of maternal mortality in the world, and uterine atony is a major cause of PPH. Clinically, over-distended uterus and exhausted myometrium are considered as causes of uterine atony, however, most of cases presenting uterine atony have no explainable risk factors and no pathological evidence.

In this study, we found that acute massive infiltration of inflammatory cells and meconium derived components within the myometrium of the uterus in cases of ‘PPH of unknown etiology’ and would like to propose ‘Postpartum Acute Myometritis (PAM)’ as a concept of these cases. All study participants were provided informed consent, and an ethics review board approved this study design. I believe that the finding from this study could be of special interest not only to obstetrician but also to the readers of Journal of Reproductive Immunology.

Here, we deeply express appreciation for your excellent review process, appropriate reviewer’s comments to improve our manuscript and giving us one more opportunity to submit our revised manuscript. We have taken these comments and suggestions into account in the revised version of our paper. We have addressed all the comments by reviewers, as indicated on the attached pages, and we hope that our explanations and revisions are satisfactory.

We hope that revised version of our paper is now suitable for publication in “Journal of Reproductive Immunology” and we look forward to hearing from you at your earlist convenience.

This manuscript has not been published and is not under consideration for publication elsewhere. All the authors have read the manuscript and have approved this submission and report no conflicts of interest. Financial support for this study was provided by a grant from the Ministry of Education, Science, Culture and Sports, Japan (Grant Number: 24390379).

I very much hope that you share our finding. I am looking forward to hearing from you at your earliest convenience.

We would like to response to reviewers on our previous manuscript: JRI-S-14-00179.

<Reviewer # 1>

Specific comment # 1 (Materials and Methods)

Page 5: As control, please explain more such as gestational weeks and the number of previous pregnancies and deliveries about the specimens from myometrium of during pregnancy.

Response

In our recent paper, we wrote “the details about gestational weeks, number of previous pregnancies and deliveries about the control specimens in materials and methods.

Specific comment # 2 (Figure legends)

All figure legends need the explanation of figures, but authors mainly show staining methods. Please rewrite figure legends.

Response

In our recent paper, we rewrote “figure legends with explanation of figures”.

Minor points # 1

In immunohistochemistry, page 6, line 6: please add the following; (were fixed in) 10% (buffered?) formalin solution.

Response

In our recent paper, we rewrote “all specimens were fixed in 10% buffered formalin solution”.

Minor points # 2

Line 22-24, please indicate the name of the country of each company.

Response

We added the “name of the country of each company’’

Minor points # 3

In method for cell counts, page 6, line 51: please add the following; (in a total of 4 digital images) in each case.

Response

In our recent paper, we rewrote “numbers of muscle cells and positively stained cells in a total of 4 digital images in each case’’.

<Reviewer # 2>

Comment # 1

The authors presented CD4 positive cells in the myometrium are as T cells. However the antibody for CD4 is a marker for T cell and macrophage. The number of CD3 positive T cells should be provided. And they have to address how distinguish CD4 positive T cells from CD4 positive macrophages in the myometrium. Does they distinguish these cells by size or intensity of staining?

Response

In our recent paper, we provided the immunohistochemical data of anti-CD3 antibody but it showed equally negative value in all groups. So CD4 positive cells were probably macrophage.

Comment # 2

I think there is a possibility that the amnion or fetal components enter into the loose

postpartum myometrium and induce the acute inflammatory cells, for instance neutrophil and macrophage, infiltration. The authors present their concepts of PPH in p.12 line 38 and figure 4. They do not present the timing of occurring the some trigger. If they think the T cell infiltration is meaningful findings, such a chronic inflammatory cell infiltration should be occurred few days before. The authors should state these points in discussion.

Response

Immunostaining of CD3 and CD8 for T cell showed negative value in both control and PPH group. This suggested an acute phenomenon. Timing of triggering may be during or soon after delivery have mentioned in discussion in our recent paper.

Comment # 3

Regrettably, the quality of figure 1 is poor, especially the CD4 and CD8. These photos should be improved.

Response

In our recent paper, we have improved the quality of figures of CD4 and CD8, and they are sifted to supplemental Figure.2 to update our manuscript.

Comment # 4

In the table 1, first lane is named pregnant but in table 2 and figures, these lanes are named pregnancy. Which is written in correctly?

Response

In our recent paper, we corrected it to “pregnant” all through the manuscript.

Sincerely yours,

Naoaki Tamura, M.D., Ph.D.

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<Highlights>

- We study the pathologic features of the myometrium on postpartum hemorrhage (PPH) of unknown etiology.
- Massive infiltration of inflammatory and immune cells is significant in myometrium of PPH.
- Postpartum acute myometritis (PAM) could be a pathological concept of PPH of unknown etiology.
- Fetal components is a candidate of causes of PAM.

Histological characteristics of the myometrium in the postpartum hemorrhage of unknown etiology -A possible involvement of local immunereactions-

Mustari Farhana, Naoaki Tamura, Mari Mukai, Kotomi Ikuma, Yukiko Koumura, Naomi Furuta, Chizuko Yaguchi, Toshiyuki Uchida, Kazunao Suzuki, Kazuhiro Sugihara, Hiroaki Itoh, Naohiro Kanayama

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Running head: Myometritis in postpartum hemorrhage (PPH) of unknown etiology

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Abstract

The aim of this study was to evaluate the histological characteristics of the myometrium obtained on postpartum hemorrhage (PPH) of unknown etiology secondary to uterine atony. They were selected among registered cases of clinically suspected amniotic fluid embolism (AFE) and classified as PPH of unknown etiology because of no obvious cause of PPH at Hamamatsu University School of Medicine, a registration center for clinical AFE in Japan. Immunohistochemical studies were done on myometrium using anti-mast cell tryptase, anti-neutrophil elastase, anti-CD68, anti-CD88, anti-CD3, and anti-ZnCP-1 antibodies. Massive infiltrations of inflammatory cells with mast cell degranulation within the myometrium secondary to complement activation were observed in PPH of unknown etiology (n=34), but not in control pregnant women (n=15) or after delivery women without PPH (n=18). The concomitant immunohistochemical detection of meconium in myometrium suggests that amniotic fluids or fetal materials are one of the candidates to induce maternal local immune activation in the PPH of unknown etiology. Postpartum acute myometritis in the absence of an infective etiology may be a histological characteristic of PPH of unknown etiology.

Keywords: pregnancy; postpartum hemorrhage (PPH); uterine atony; myometritis; amniotic fluid embolism.

Introduction

Postpartum hemorrhage (PPH) is the leading cause of maternal mortality in the world, with an incidence estimated to be up to 10% (Oyelese et al., 2007, Mousa et al., 2014, Cunningham FG, 2010). Various conditions such as abnormal placentation, trauma to the genital tract, uterine atony, and coagulation defects are known causes of PPH (Cunningham FG, 2010). Among the above, uterine atony is a major cause of PPH, estimated to be responsible for 70% (Karoshi and Keith, 2009, Oyelese and Ananth, 2010); however, the etiology remains to be clarified in cases of PPH secondly to uterine atony. Over-distended uterus, and exhausted myometrium are considered as causes of uterine atony; however, it is yet to clarify why myometrium suddenly stops contraction after delivery, because most of cases presenting uterine atony have no explainable risk factors (Rouse et al., 2006).

We, a registration center for clinical amniotic fluid embolism (AFE) in Japan, and others have proposed clinical criteria for AFE with the main clinical symptom of massive PPH not explained by other diseases (Kanayama et al., 2011, Benson, 2012). Furthermore, from the accumulated data on registering cases of clinical AFE in Japan, massive PPH frequently accompanied by coagulopathy and uterine atony has been considered pathognomonic for clinical AFE (Benson, 2007). In other words, a number of cases of ‘PPH of unknown etiology’ have been regarded and managed as clinical AFE according to the criteria without any physical evidence.

Although the causative mechanism of clinical AFE is still unclear, a previous study demonstrating maternal complement activation in clinical AFE suggested that a pathological maternal immune reaction may be associated with the pathogenesis of 'PPH of unknown etiology' including clinical AFE (Kanayama and Tamura, 2014).

In the present study, we hypothesized that a maternal immune reaction localized in the myometrium deteriorates the myometrial function of contraction and causes PPH of unknown etiology secondary to uterine atony. To identify immunoreactive cells in myometrium with PPH having no etiology, we carried out immunohistochemical examinations.

Materials and Methods

Subjects

Cases of 'PPH with unknown etiology' were originally registered at Hamamatsu University School of Medicine from January 2011 to December 2012 as clinically suspected AFE according to the Japan consensus criteria for the diagnosis of AFE, whereby the major symptom was PPH except for cardiac arrest or respiratory failure (Tamura et al., 2014a). They were retrospectively and carefully selected as 'PPH with unknown etiology' secondary to uterine atony according to the reports of physicians in charge. Exclusion criteria were multiple fetuses, rupture of membranes, preterm labor, chorioamnionitis, uterine or cervical laceration, placenta previa, placenta accreta, preceding DIC such as sepsis, placental abruption, and preceding sudden maternal cardiac deterioration.

Tissues after abdominal hysterectomy after the onset of PPH were collected and stored at Hamamatsu University School of medicine. Mean time interval between deliveries and hysterectomy were 4.6 hours in PPH cases. Control tissues were obtained at Hamamatsu University Hospital after receiving written informed consent. The myometrial tissues were obtained by partial resection of the anterior wall of uterine body, 3-5 mm beneath serosa, during (n=15) or soon after (n=18) the delivery of neonates by cesarean section having uncomplicated pregnancy. 9 nulliparous and 6 multiparous pregnant women were selected for control with mean gestational age (37.8±0.7) mean parity (0.79±1.05) and mean gravida (1.14±1.10). Myometrial tissues from 6 nulliparous and

12 multiparous were also obtained after delivery of neonates as control having mean gestational age (38.3±1.5), mean parity (1.29±1.11) and mean gravida (1.57±0.98). All control cases were free from massive hemorrhage, hock, DIC, uterine atony, and any kind of allergic reaction. Patients' demographic data are shown in Table 1.

Immunohistochemistry

All specimens were fixed in 10% buffered formalin solution, embedded in paraffin, and cut into 3-μm-thick sections. Sections were stained with hematoxylin and eosin. For immunohistochemistry, the antigen was retrieved in a high pressure cooker for 20 min (temperature: 95˚C) using citrate buffer (pH 6) for tryptase and CD68 and Tris/EDTA buffer (pH 9) for CD88 and CD3. Endogenous peroxidase activity was blocked by H_2O_2 for 5 min. Primary antibody was applied at a ratio of 1:10,000 for tryptase (abcam®, UK), 1:200 for elastase (DakoCytomation, Denmark), 1:200 for CD68 (Thermo, UK), 1:200 for CD3 (Novocastra™ liquid, UK), 1:4000 for CD88(Cosmo Bio Co. Japan) with 120 ng/mL of mouse IgG for meconium-specific zinc coproporphyrin I (ZnCP-I) (Furuta et al., 2012), and incubated for 30 min. A positive reaction was visualized by 3,3-diaminobenzidine, counterstained with hematoxylin, coverslipped, and observed with an Olympus BX51 optical microscope. Halo patterns of the tryptase "golden reaction" around mast cells were considered to represent activated mast cells with degranulation (Fineschi et al., 2009, Tamura et al., 2014b).

Method for cell counts

Numbers of muscle cells and positively stained cells in a total of 4 digital images in each case under microscopic fields (50 mm^2) were counted and analyzed.

Statistical analysis

All values are presented as the median ± standard error (SE). Significant differences were assessed with the Mann-Whitney U test. A P-value of less than 0.05 was considered significant.

Approval

The Ethics Committee of Hamamatsu University School of Medicine approved all the procedures of this study (#24-130).

Results

Backgrounds of the subjects

Thirty-four cases were examined after being selected as 'PPH of unknown etiology', regarded as atonic uterine bleeding by excluding uterine or cervical laceration, placenta previa, placenta accreta, preceding DIC such as sepsis, placental abruption, and preceding sudden maternal cardiac deterioration.

Histological findings

Significant infiltration of inflammatory cells in myometiral stroma with sparse structure due to edema was observed in cases of PPH of unknown etiology (Fig.1C and 1F).

Immunostaining of C5aR (CD88) showed increased count of C5a receptor positive cells within the myometrium in PPH of unknown etiology than control (Fig. 1G-I)

Immunohistochemistry using anti-elastase antibody revealed a significantly higher infiltration of neutrophils within the uterine myometrium in cases of 'PPH of unknown etiology' (0.04979±0.01490 cells/one muscle cell) than in pregnant (0.00106±0.00033) and post-delivery (0.00618±0.00395) groups (Fig.2D-F, Fig.3C, and Table 2).

Significantly higher macrophage infiltration was shown using anti-CD68 antibody (0.15826±0.04183 cells/one muscle cell) in cases of 'PPH of unknown etiology' compared to

pregnant (0.00158±0.00042) and post-delivery (0.01234±0.00483) groups (Fig.2G-I, Fig.3D, and Table 2).

Immunohistochemistry using anti-CD3 antibody for T cells revealed equally negative values in all groups (Fig.2J-L and Table 2).

Total counts of mast cells were significantly higher in the 'PPH of unknown etiology' (0.03395±0.00242 cells/one muscle cell) group than in the pregnant (0.01485±0.00186) and post-delivery (0.02097±0.00556) groups (Fig.2A-C, Fig.3A, and Table 2). A halo of tryptase positivity around the mast cells (Fig.2C) indicated activated mast cell degranulation. Activated mast cell counts also increased (0.01775±0.00143 cells/one muscle cell: 56.5% of total mast cells) in the 'PPH of unknown etiology' group, but not significantly in the pregnant (0.00050±0.00015; 34.0% of total mast cells) and post-delivery (0.00444±0.00129; 2.4% of total mast cells) groups (Fig.3A, and Table 2).

Immunostaining of zinc coproporphyrin derived from meconium was detected in the uterine vessel of myometrium in 24 cases (70%) of PPH of unknown etiology (Supplemental Figure 1).

Discussion

PPH is the most common global cause of maternal death (Anderson, 2009, Mercier and Van de Velde, 2008). Seventy percent of PPH corresponds to uterine atony (Karoshi and Keith, 2009). This is the first histological and immunohistochemical study of uterine tissue in cases of 'PPH of unknown etiology' secondary to uterine atony. Our study showed the acute massive infiltration of inflammatory cells such as neutrophils, CD68-positive macrophages, within the myometrium of the uterus in cases of 'PPH of unknown etiology' (Fig.2F and 2I). It was suggested that an acute massive inflammatory reaction may lead to deterioration of the contractile function of myometrial cells, resulting in uterine atony with PPH. Since inflammatory cells are a major source of chemical mediators with a marked effect on vascular and uterine smooth muscle. The accumulation of fluid along with inflammatory cells within interstitial spaces resulted in stromal edema observed microscopically (Fig.1C and 1F). The occurrence of acute myometritis may provide a clue to understand how myometrial cells stop the synchronized contraction immediately after giving birth to the neonate and placenta by active labor.

It is generally believed that various populations of leukocytes are present not only in the decidua and endometrium, but also in the pregnant myometrium, where these cells are involved in pregnancy maintenance and uterine contraction during labor (Thomson et al., 1999, Ivanisevic et al., 2010, Osman et al., 2003). The present study demonstrated significantly higher number of

neutrophils, mast cells and CD68 macrophages are present in the myometrium of PPH compare to the control groups (Fig.3A-C). These cells were not originally resident but infiltrated from capillaries to interstitial spaces (Fig.1A-C). Positive findings for elastase, tryptase, and infiltration by activated macrophages suggested that inflammatory reactions were accelerated in the myometrium of 'PPH of unknown etiology'. Immunohistochemistry of CD3 and CD8 for T cells revealed equally negative values in all groups. This also suggested an acute onset during or soon after delivery. Cells detected by immunostaining of CD4 (supplemental Fig.2) probably infiltrated macrophages which was also confirm by CD68 staining (Fig.2G-I).

Based on the present histological evidence, we termed the condition 'Postpartum Acute Myometritis (PAM)' characterized by 'PPH of unknown etiology'. We propose the following histological criteria for PAM: massive infiltration of inflammatory cells as well as immune cells in the myometrium in the absence of an infective etiology. Complements activation may be the initial step of development of PAM by inflammatory cells infiltration and anaphylactoid reaction secondary to mast cell degranulation (Fig.4). Some researchers have reported evidence of complement involvement in AFE as well as mast cell involvement in uterine tissue (Benson et al., 2001, Tamura et al., 2014b). In the present study, the increased immunostaining of C5aR (Receptor for complement C5) is also supportive of complement activation locally in the myometrium of PPH of unknown etiology (Figure.1G-I).

We also observed significant activation of mast cells in cases of PPH of unknown etiology suggested the possibility that myometrial local immune activation might be initiated on encountering foreign substances. Interestingly, immunostaining of meconium-specific ZnCP-I was also observed, especially in relatively large numbers in 'PPH of unknown etiology' (Supplemental Figure.1), suggesting the possible involvement of the encounter with fetal substances probably originating from amniotic fluid in the local activation of maternal immune reactions in the myometrium. Although there could be several immunological stages during which normal physiological processes are disrupted and contribute to the development of PAM, as described in Figure. 4. We speculated that the presence of amniotic fluid or fetal material in the maternal circulation may stimulate the activation of complement system which might induce myometrial neutrophil and macrophage infiltration, mast cell degranulation and interstitial edema, leading to uterine atony and PPH. Thus, we provide supporting evidence of mast cell degranulation within the uterine myometrium and responsible for producing an anaphylactoid reaction. Of course, further investigations are necessary to clarify the association between myometrial immune activation and fetal components as well as to identify the substance that triggers the reaction.

In conclusion, "PAM", pathologically characterized by evidence of inflammatory cells infiltration and mast cell activation in the myometrium, is a common factor in 'PPH of unknown etiology'. Non-infective etiology such as fetal components is a candidate of triggers that causes

PAM secondary to complements activation. Future studies in this field will pave the way for the early diagnosis of pre-clinical stages related to ‘PPH of unknown etiology’ and the advancement of treatment.

Acknowledgements

The authors thank Mrs. Naoko Hakamada, Mrs. Yumiko Yamamoto, and Mrs. Naoko Kondo, for secretarial assistance. This work was supported in part by Grants-in-aid for Scientific Research from the Ministry of Education, Science, Culture and Sports, Japan (Grant number: 24390379).

Figure legends

Figure 1. A and D show representative myometrium during pregnancy without PPH. B and E from normal myometrium after delivery show slightly edematous interstitium without inflammatory cells. C and F from a PPH case show inflammatory cell infiltration and interstitial edema. (A-C: H&E staining. D-F: Alcian blue staining). Immunostaining of C5aR (CD88) in myometrium from pregnant (G), after delivery (H), and PPH (I) reveal CD88-positive cells are significantly higher in PPH group (I). Scale bars indicate 100 μm.

Figure 2. Immunostaining of mast cell tryptase (A-C) , neutrophil elastase (D-F), CD68 (G-I), and CD3(J-L) in the myometrium during pregnancy (A, D, G, J), after delivery (B, E, H, K) and with PPH (C, F, I, L) show significantly higher infiltration of mast cells (C), neutrophils (F) and macrophages (I) within the myometrium of PPH compare to the control groups. Golden reaction arround mast cells (C) indicate activated mast cells degranulation. Scale bars indicate 50 μm.

Figure 3. Columns indicating results for positive cell numbers in the myometrium during pregnancy (n=15), after delivery (n=18), and with PPH of unknown etiology (n=34). The number of tryptase-positive mast cells (A; white and black columns indicated total count of mast cells and

activated mast cells, respectively), elastase-positive neutrophils (B) and CD68-positive macrophages (C) were significantly higher in the PPH group among the three groups. *$P < 0.05$.

Figure 4. Scheme indicating the mechanism of pathogenesis in PPH of unknown etiology. *1: Anaphylatoxin (C3a, C5a), *2: IL-1, IL-8, TNF-α, *3: Degranulation of mast cells and *4: Matrix metalloproteinase and free radicals were possible components.

Table 1. Clinical characteristics of the patients in this study

Table 2. Exact cell counts are presented as the median ± SE. * indicates $p < 0.05$.

Supplemental Figure 1. Immunostaining of ZnCP-1-detected materials derived from meconium in amniotic fluid in the myometrium with PPH. Scale bars indicate 200 μm.

Supplemental Figure 2. Immunostaining of CD4 (A-C) shows significant infiltration of CD4 positive cells within the myometrium of PPH (C) than pregnant (A) and after delivery (B). But immunohistochemistry using anti-CD8 antibody (D-F) shows negative findings in all group. Scale bars indicate 50 μm.

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Table.1
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Table 1. Clinical characteristics of patients in this study

	Pregnant	After delivery	PPH of unknown etiology
No. of subjects	15	18	34
Age (years)	34.0 ± 4.0	37.0 ± 3.0	36.0 ± 4.7
Gravida	1.14 ± 1.10	1.57 ± 0.98	1.08 ± 0.99
Parity	0.79 ± 1.05	1.29 ± 1.11	0.88 ± 0.97
Nulliparous (%)	9 (60.0)	6 (33.3)	23 (67.6)
Multiparous (%)	6 (40.0)	12 (66.6)	11 (32.3)
Gastational age (weeks)	37.8 ± 0.7	38.3 ± 1.5	38.3 ± 2.1
Delivery methods			
Vaginal delivery (%)	0 (0.0)	0 (0.0)	15 (44.1)
Cesarean section (%)	15 (100.0)	18 (100.0)	19 (55.9)
Blood loss at delivery (mL)			
Vaginal delivery	---	---	8,723 ± 6,757
Cesarean section	---	716 ± 312	8,421 ± 6,230

Women without previous history of delivery was determined as parity 0.

Table.2
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Table 2. Summary of exact cell counts in this study

	Pregnant	After delivery	PPH of unknown etiology
Total mast cells	0.01485±0.00186	0.02097±0.00556	0.03395±0.00242*
Active mast cells	0.00050±0.00015	0.00444±0.00129	0.01775±0.00143*
Neutrophils	0.00106±0.00033	0.00618±0.00395	0.04979±0.01490*
CD68 positive cells (macrophages)	0.00158±0.00042	0.01234±0.00483	0.15826±0.04183*
CD3 positive cells (T cells)	0	0	0

Figure.1

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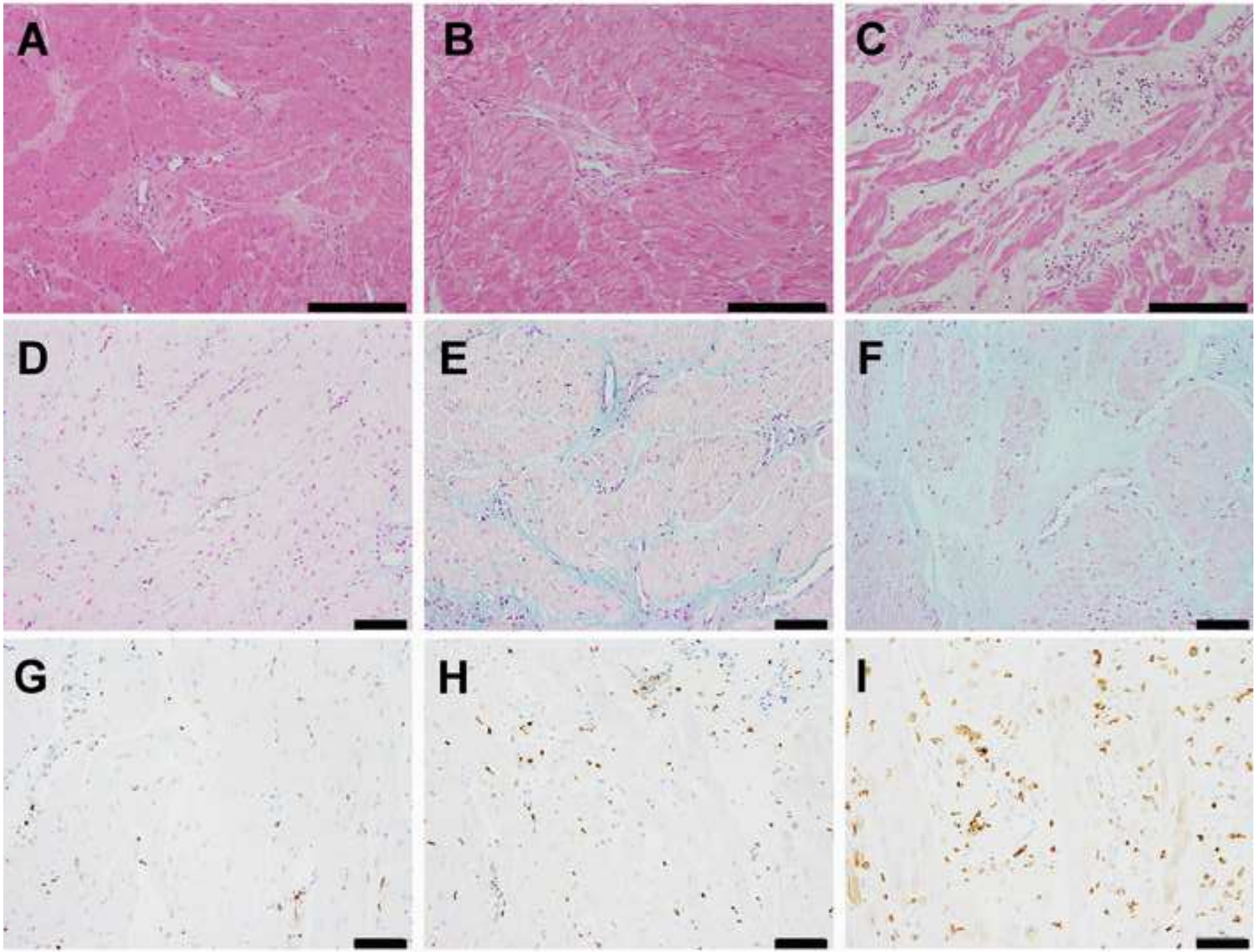

Figure.2

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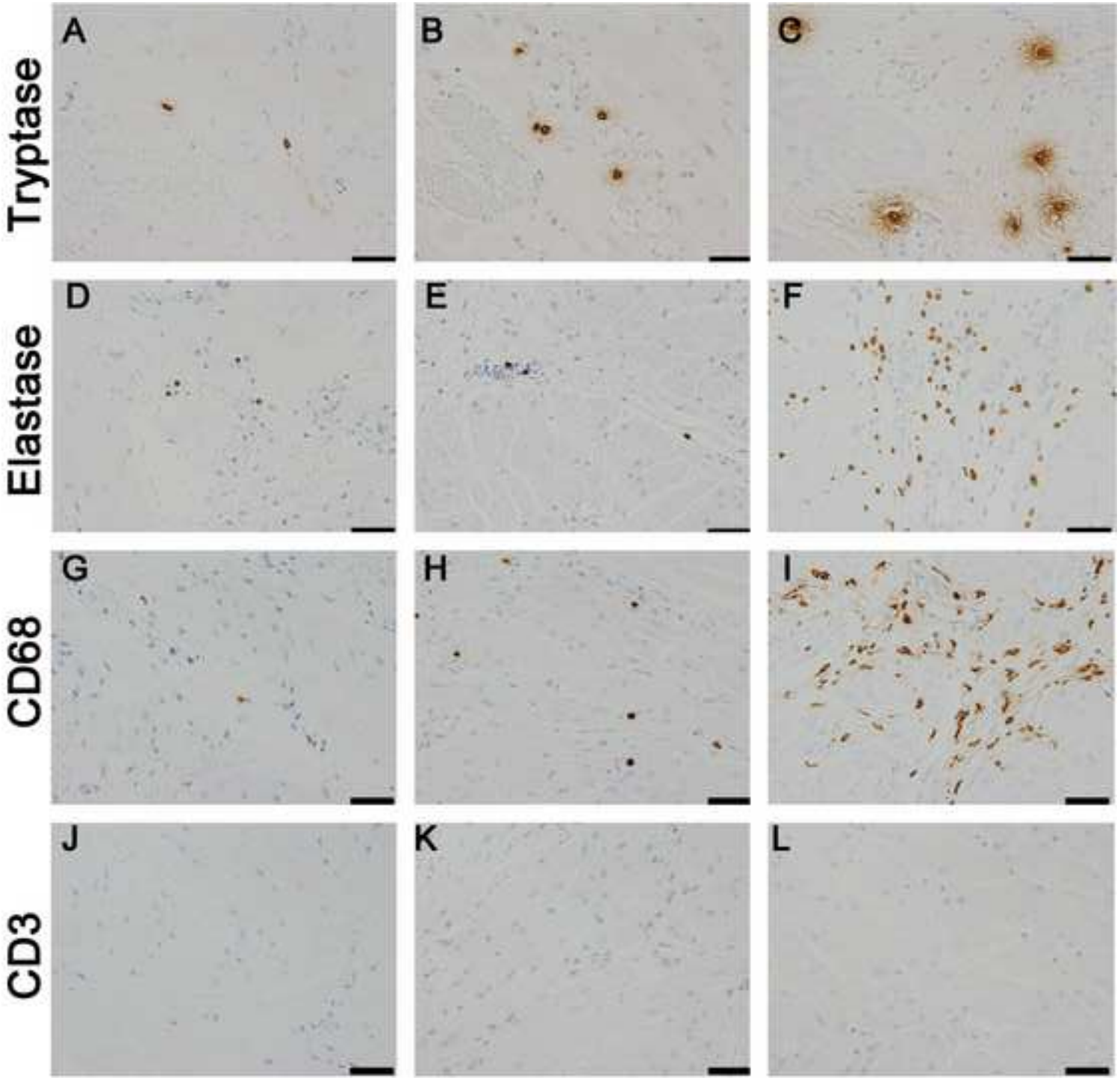

Figure.3

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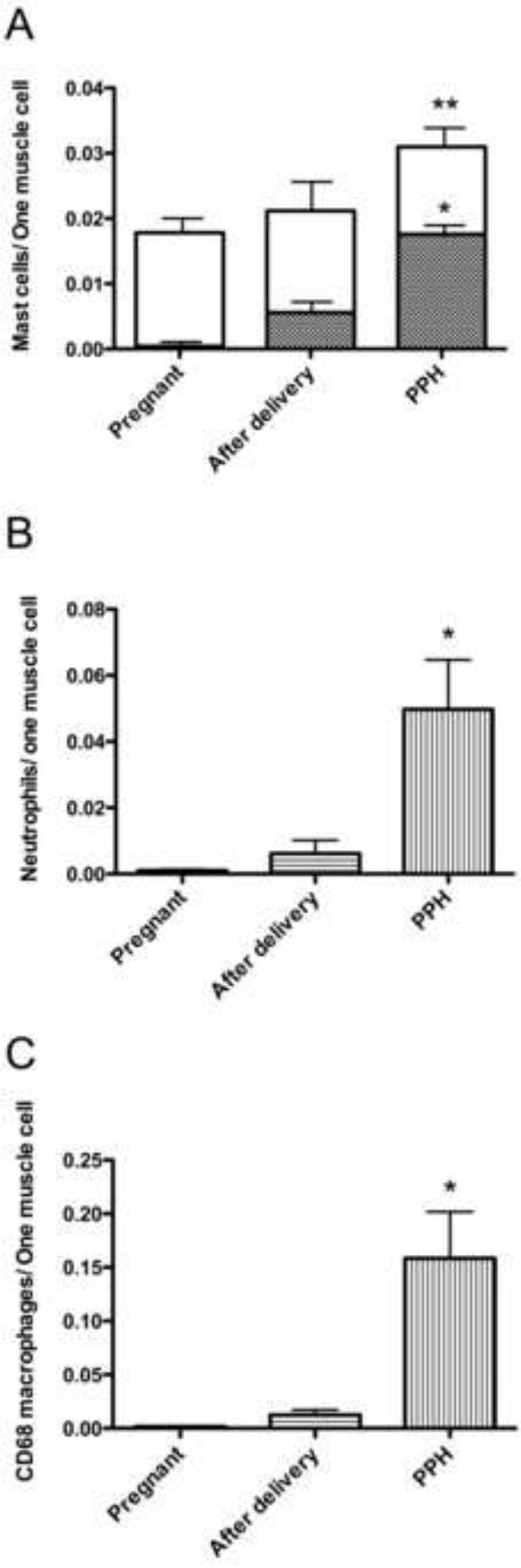

Figure.4
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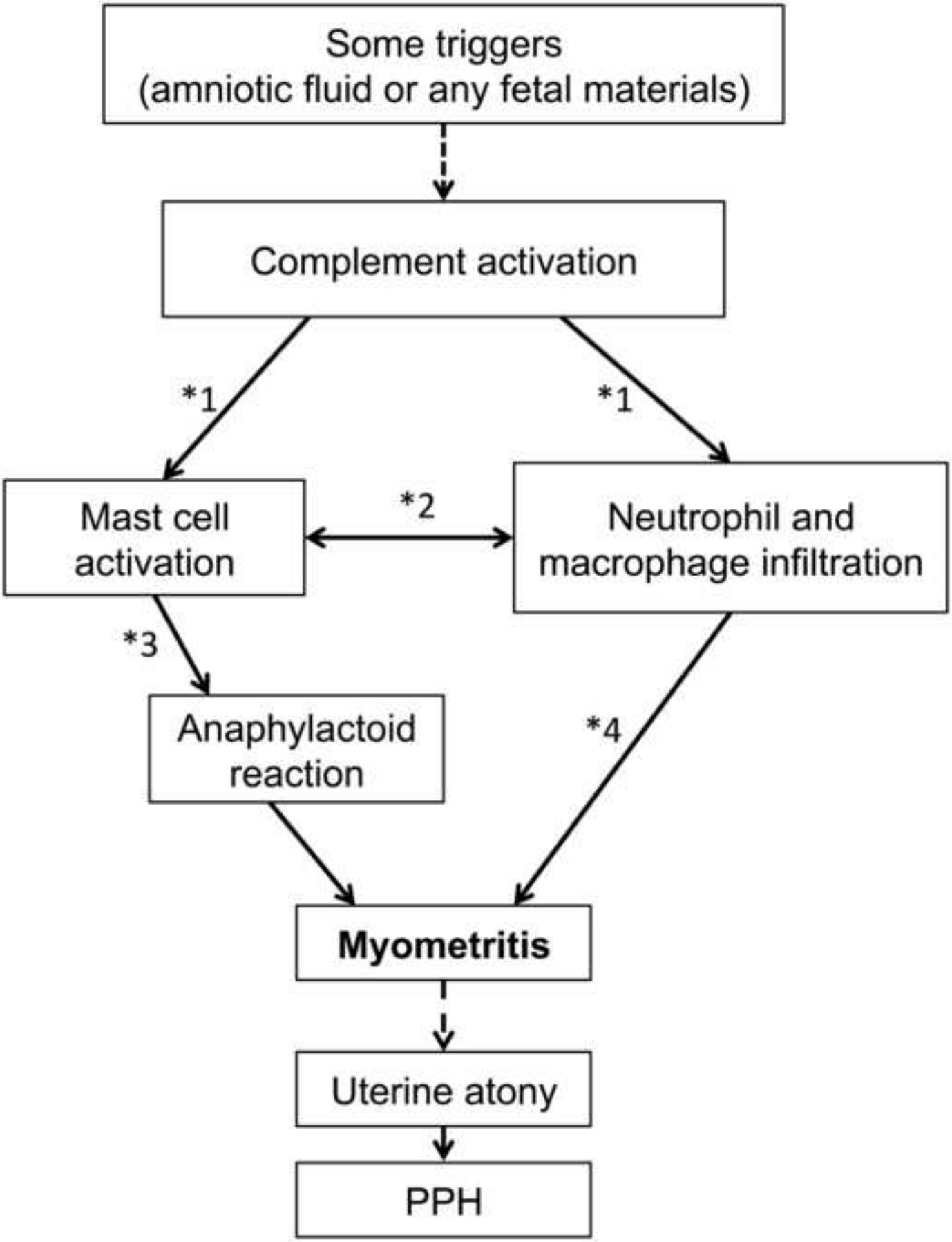

Conflict of Interest Statement

This manuscript has not been published and is not under consideration for publication elsewhere. All the authors have read the manuscript and have approved this submission and report no conflicts of interest. Financial support for this study was provided by a grant from the Ministry of Education, Science, Culture and Sports, Japan (Grant Number: 24390379).

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