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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

The study of caseous lymphadenitis: dose dependent infection of *C. pseudotuberculosis* in mouse model via oral inoculation

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Abstract

The use of different dependent doses design offers unique opportunities to advance the study of diseases through the investigation of their respective outcomes at multi-disciplinary level. Such design can increase our understanding of the factors that shape disease establishment, course development and distribution of lesions in space and time. This paper utilizes three different doses of *C. pseudotuberculosis*, 10^3, 10^5 and 10^7 colony forming unit (CFU/ml) and illustrates their potential of disease induction at a range of histo-pathological and molecular scales. A mouse model is used to demonstrate the potential aspects of the doses in relation to the outcome of interest. Our approach is based on comparing the observed patterns of clinical features, postmortem and gross finding, as well as histo-pathological and molecular levels.

Keywords: Caseous lymphadenitis; dose dependent infections; mouse; oral inoculation; pathological features and PCR

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Introduction

Caseous lymphadenitis (CLA) is a disease of veterinary importance which is responsible for the economic losses of animal industry worldwide. Its loses include wasting, poor wool growth, decreased milk and meat production, reproductive disorders, premature culling, carcass condemnation and rarely death (Williamson, 2001; Arsenault et al., 2003; Paton et al., 2003). The disease is chronic in nature and appears subclinical in many cases of sheep and goats across the globe. Two forms of this disease have so far been recognized: external and internal forms of caseous lymphadenitis. The external form of CLA is characterized by formation of abscess in superficial lymph nodes such as the sub-mandibular, parotid, pre-scapular, sub-iliac, popliteal, supra-mammary lymph nodes and in subcutaneous tissues (Dorella et al., 2006). These abscesses also can develop internally in organs, such as lungs, liver, spleen, kidneys, uterus and internal lymph nodes, such as mediastinal and bronchial lymph nodes characterizing as visceral CLA (Merchant and Packer, 1967; Piontkowski and Shivvers, 1998). Transmission of CLA can occur through direct or indirect contact or through wound that come into contact with pus from the abscesses of infected animals (Nairn et al., 1974). Infection may also occur through the ingestion of contaminated food with *C. pseudotuberculosis* (Baird and Fontaine, 2007). Hence, this study was designed to prove the suggestion that the disease can orally be transmitted.

Molecular measures, as techniques of refinement, can offer high resolution answers in relation to questions on disease causation and, for infectious

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diseases, provide insight that is not available with traditional culture methods or species level identification of bacteria. In CLA settings, these techniques, in particular, DNA amplification by means of polymerase chain reaction (PCR) were very well suited for comparative analysis of isolates that were collected over short periods of time (Cetinkaya et al., 2002). In this paper, we present three different doses of infection of *C. pseudotuberculosis* in mouse model through oral route inoculation. In this study, we illustrated the potential of these doses in disease establishment, production of typical clinical signs and lesions at a range of histo-pathological scales, molecular resolution and analytical complexity.

Materials and Methods

Animals

Forty apparently healthy mice of approximately 2-3 weeks old were used in this study. The animals were placed in appropriate plastic cages with a stocking density of 5 mice per cage in an air-conditioned room and maintained in the isolation unit at the experimental animal house, Faculty of Veterinary Medicine, University Putra Malaysia. Their diet consisted of chaw pellets and tap water *ad libitum.* Prior to the experiment, all animals were kept for 2-3 weeks for acclimatization. All procedures and experiments described were taken under a project license approved by the Ethics committee of Faculty Veterinary Medicine, University Putra Malaysia (UPM).

Stock of *C. pseudotuberculosis*

The *C. pseudotuberculosis*, used in this experiment, was previously isolated from a naturally infected case of CLA at Agricultural University Park, University Putra Malaysia. The organism was then biochemically and molecularly identified as *C. pseudotuberculosis*. The bacteria were sub-cultured onto new blood agar plates for 48 h at 37°C. The cultures were then harvested and suspended in normal saline solution where the concentrations were estimated to the three different standard doses of 1×10^3 CFU/ml, 1×10^5 CFU/ml and 1×10^7 CFU/ml using the Mac-Farland technique.

Experimental procedure

The detailed information of the experimental design is outlined in Table 1. At the start of the experiment, the mice were divided into 4 major groups of 10 mice each (Table 1). Animals in Group A served as control group and were inoculated orally with 0.4 ml of sterile phosphate buffer saline (PBS), pH 7. Treated Groups B, C and D were challenged orally with 0.4 ml of 10^3, 10^5, 10^7 CFU/ml of *C. pseudotuberculosis* respectively. Thereafter, clinical signs were recorded and postmortem procedures were performed for gross and histo-pathological examination. All changes and abnormalities were recorded.

Clinical signs and scoring

The clinical observations presented by the diseased and non-diseased groups were regularly documented from the start of the study until the end point of the experiment. The information collected from the 4 groups (A, B, C & D) was based on the individual presentation of the clinical signs. In summary, the clinical signs of 4 groups were scored in scale of 0-3 based on the presence of following parameters: ruffled fur, eye discharge, movement and responsiveness. The score 0 represented no abnormality of clinical signs observed, 1 for mild, 2 for moderate, and 3 for severe abnormalities. The evaluation method of the scoring system is summarized in Table 2.

Histopathology and lesion scoring

Tissues were collected from the heart, lung, liver, spleen, small intestine, large intestine and brain for histo-pathological evaluation. The samples of these tissues were fixed with 10% formalin except for the brain tissue samples which were fixed with 40% formalin and processed in an automatic tissue processor. Tissues were processed into paraffin blocks and each section was routinely stained by haematoxylin and eosin. Microscopically, cellular changes were scored with 5 slides per organ. Within each slide, six microscopic fields were examined at different magnification. Lesion scoring was divided into 4 scores namely: score 0: normal, score 1: mild (less than 30% of field involved), score 2: moderate (30-60% of field involved) and score 3: severe (more than 60% of field involved).

DNA extraction method

For this study, DNA was extracted using boiling method. A few colonies from the cultures were transferred into an Eppendorf tube containing 50µl distilled water, and the suspension was boiled at 100°C for 15 minutes. After the boiling, the suspensions were cooled immediately on ice for 2 minutes. Then, the tubes were centrifuged at 13,000 rpm for 5 minutes. The upper phase that contains DNA as end product was carefully transferred into another Eppendorf tube to use as DNA template.

PCR condition

The PCR was performed in a touchdown thermocycler in a total reaction volume 10µl of PCR buffer, $MgCl_2$, 250µM of deoxynucleotide triphosphate, 2 U of Taq DNA polymerase, and 1µM of each forward and reverse primer and 5µl of template DNA. Amplification was performed with 30 cycles following an initial denaturating step at 94°C for 5 minutes. Each cycle involved denaturation at 94°C for 1 minutes, annealing at 56°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 5 minutes.

Primer design

The primer used in this study was targeted on the 16S rRNA gene *of C. pseudotuberculosis* and the sequence

Table 1: Experimental design

Group	Category	Description	No. of animals	Dose (CFU/ml)	Route of administration
A	Control	Uninfected but treated with sterile phosphate buffer saline (PBS)	10	0.4	Orally
B	Treated	Treated with *C. pseudotuberculosis*	10	$0.4x10^3$	Orally
C	Treated	Treated with *C. pseudotuberculosis*	10	$0.4x10^5$	Orally
D	Treated	Treated with *C. pseudotuberculosis*	10	$0.4x10^7$	Orally

Table 2: Clinical Observation Scoring System

Parameters	Clinical Score			
	0	1	2	3
Ruffled fur	Normal fur	Ruffled fur by 30% of the body	Ruffled fur by 60% of the body	Ruffled fur more than 60% of the body
Eye discharge	No discharge from the eyes	Eye discharge by 30%	Eye discharge by 60%	Eye discharge more than 60%
Movement	Normal movement and appetite	Reduced movement by 30%	Reduce movement by 60%	reduce movement more than 60%
Responsiveness	Normal responsiveness	Reduced responsiveness by 30%	reduce responsiveness by 60%	reduce responsiveness more than 60%

was selected from previously published work (Cetinkaya et al., 2002).

Agarose gel preparation

Of 1.5% agarose gel with volume of 100 ml was prepared by mixing 1.5g of agarose agar powder with a 100 ml 1% TAE buffer. The mixture was heated in microwave oven about 3-5 minutes and until all the precipitate was melted. After cooling to approximately 60°C, the mixture was poured into suitable size gel cassette and solidified (about 15 minutes) for PCR loading and electrophoresis.

Electrophoresis

Agarose gel was placed carefully in a gel holder tank and submerged with 1% of TAE buffer. About 5 µl of 100 bp dyed marker (Promega®) was then used as the ladder and 5 µl of PCR product was loaded into the well carefully. The electrophoresis of 1.5% agarose gel was run for about 45 minutes at 81V. Thereafter, the gel was stained with Gel Red stain solution and stirred for 20 minutes. The gel was placed under UV gel imaging capturing machine and the results was recorded.

Statistical analysis

The findings of the study were analyzed using statistical software R version 3.0.1 for Windows. The mean values obtained from the tested parameters of infected animals were compared with data from the control group using the Tukey-test. Differences were considered to be statistically significant with values of $P<0.05$.

Results

Pathological findings

The distribution of the pathological lesions, recorded on individual basis for the experimental units, is shown in Table 3. Gross and histo-pathological lesions were observed in different visceral organs of experimentally challenged mice with dose of 10^3, 10^5 and 10^7CFU /ml of *C. pseudotuberculosis*. In gross findings, there was no statistically significant difference between mice in group A & B throughout the experimental trial. However, animals challenged with dose 10^5 and 10^7CFU /ml of *C. pseudotuberculosis* showed significant gross and histo-pathological lesions in the liver and kidneys (Fig. 1-4). Macro abscess, enlargement, necrosis, congestion, and oedema were the most frequently recorded pathological findings in liver, kidney and spleen. However, some of the pathological findings (e.g. pneumonia, Brown-discoloration of intestines, watery contents in colon) were almost evenly distributed in the different dose infected mice, resulting in high presence of these lesions. The analysis of scoring system mentioned earlier revealed that doses of 10^7 and 10^5 were clinically and histo-pathologically significant in comparison to those infected with 10^3 of *C. pseudotuberculosis* and those served as a control group (data excluded).

Discussion

All mice from the treatment groups and control group (PBS) showed no significant ($P>0.05$) clinical signs of ruffled hair coats, eyes discharge, movement and responsiveness within 10 days after oral inoculation. These findings are in agreement with the findings of Jesse et al. (2013a) where no significant clinical signs presented for the similar parameters of the mice that were inoculated with 10^9CFU/ml of *C. pseudotuberculosis* via oral route and observed for 5 days. This may be due to shorter duration of post-infection and the progression of the disease might have developed if the duration of the study was sufficient enough. In contrast, these observations were not in agreement with results reported by Abdinasir et al. (2012). The explanation could be related to the different route of inoculations. Similarly, the

Table 3: Distribution of pathological findings recorded in mice experimentally induced with different doses of *C. pseudotuberculosis*

Pathological findings	Doses	Gross Observation	Culture and Isolation	Microscopic Observation	PCR detection
Liver macroabscess	10^7, 10^5	+	+	+	+
Renal macroabscess	10^7, 10^5	+	+	+	+
Splenic macroabscess	10^7, 10^5	+	+	+	+
Liver enlargement	10^7, 10^5	+	+	+	+
Renal enlargement	10^7, 10^5	+	+	+	+
Splenic enlargement	10^7, 10^5	+	+	+	+
Liver degeneration	10^7, 10^5, 10^3	+	+	+	+
Renal degeneration	10^7, 10^5, 10^3	+	+	+	+
Splenic degeneration	10^7, 10^5, 10^3	+	+	+	+
Liver (petechial) haemorrhages	10^7, 10^5, 10^3	+	+	+	+
Renal haemorrhages	10^7, 10^5, 10^3	NA	+	+	+
Splenic haemorrhages	10^7, 10^5, 10^3	NA	+	+	+
Lung haemorrhages	10^7, 10^5, 10^3	NA	+	+	+
Pneumonia	10^7, 10^5, 10^3	NA	+	+	+
Pulmonary oedema	10^7, 10^5, 10^3	NA	+	+	+
Liver oedema	10^7, 10^5, 10^3	NA	+	+	+
Splenic oedema	10^7, 10^5, 10^3	NA	+	+	+
Renal oedema	10^7, 10^5, 10^3	NA	+	+	+
Liver necrosis	10^7, 10^5, 10^3	NA	+	+	+
Renal necrosis	10^7, 10^5, 10^3	NA	+	+	+
Liver necrosis`	10^7, 10^5, 10^3	NA	+	+	+
Splenic necrosis	10^7, 10^5, 10^3	NA	+	+	+
Brown-discoloration of intestines	10^7, 10^5	+	+	+	+
Watery contents in colon	10^7, 10^5	+	+	+	+
Watery contents in jejunum	10^7, 10^5	+	+	+	+

NA = Not Applicable

findings of this study were not in agreement with those of Brown et al. (1987) and Jesse et al. (2011). The latter stated that the treatment group (10^9 CFU/ml) showed significant ($P<0.05$) clinical signs such as depression and ruffled hair coat. The difference between this study and the previous study may be due to route of infection where Jesse et al. (2011) used intraperitoneal route whereas in our study oral route was adopted**.**

Microscopically, the histo-pathological lesions observed in this study were similar to the ones found in CLA infected sheep and goats via different inoculation routes. The findings of this study were also similar to the findings of previous researches. Jesse et al. (2011) found similar histo-pathological changes in diseased mice in their study through intraperitoneally route. The histo-pathological lesions found in the lung, liver and kidney may be due to the two main virulence factors of *C. pseudotuberculosis* which are phospholipase D (PLD) and mycolic acid. The organism produces PLD which is a potent exotoxin that promotes the hydrolysis of ester bonds in mammalian cell membrane, resulting in the damage or destruction of host cell membranes, which may further lead to their dysfunction or disruption (Salyers and Witt, 1994). The spread of infection in the host may occur by the transfer of the bacterium via the blood or lymphatic system, resulting in the formation of lesions at other loci within the host (Williamson, 2001). Based on a study by Jennifer et al. (2010), mice that did not receive pilot vaccine and were inoculated with slow- and fast-growing *C. pseudotuberculosis* developed lesions in their liver, lungs, kidney and spleen. In this study, similar lesions were observed but not as severe due to the short period of this study. Oral route inoculation in mice infected with *C. pseudotuberculos* is able to produce similar pathological changes as with the natural cases of CLA in sheep and goats (Jesse, 2011). Organs of kidney, liver and lung were among the most affected organs that have been observed microscopically in dose $1x10^9$ CFU/ml via oral route inoculation in mice (Jesse et al., 2013b). Histo-pathological lesions observed in the affected organs are similar to the mice inoculated intraperitoneally (Jesse et al., 2011). Multiple abscess formation on the visceral organs were the features of *C.pseudotubeculosis* induced mice (Jesse et al., 2011).

Using PCR, The present study with different doses was able to detect the organism (*C.pseudotuberculosis*) in a wide range of organs, in particular, in the brain, intestine, stomach, heart and lungs. Of these organs, lymph nodes are the primary replication site of *C. pseudotuberculosis* in which our study was only able to detect the organism from group 3 with the concentration of 10^7CFU/ml of *C. pseudotuberculosis*. This is supported by Cetinkaya et al. (2002), where in their study encountered similar findings using PCR detection of the bacteria from the lymph nodes. The relatively rare detection could be attributed to the shorter of the

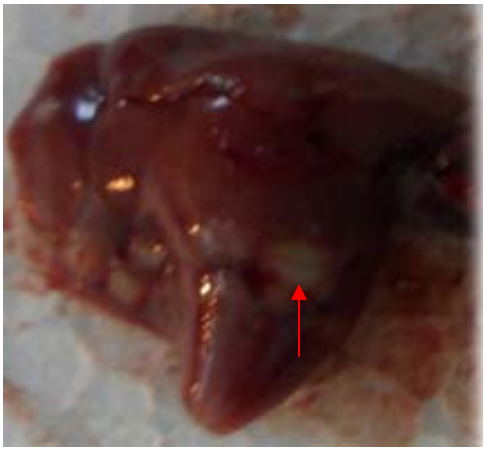

Fig. 1: Photomicrographic section of liver of mouse inoculated with dose of 10^5 *C. pseudotuberculosis.* Enlargement and presence of multiple abscesses on the surface (arrow)

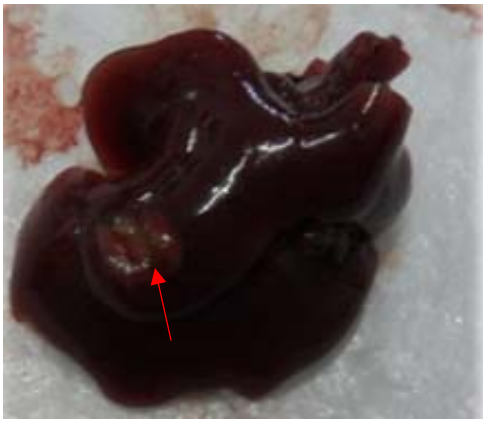

Fig. 2: Photomicrographic section of liver of mouse inoculated with dose of 10^7*C. pseudotuberculosis*: Focal area of abscess (arrow) and congestion.

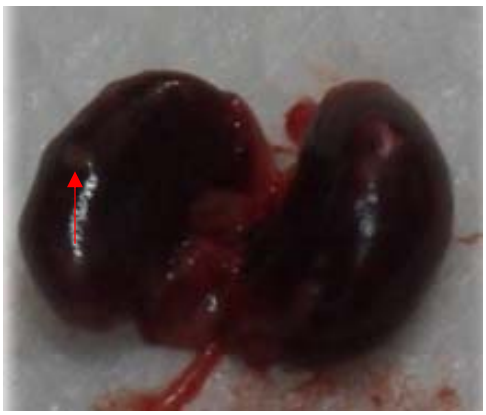

Fig. 3: Photomicrographic section of kidney of mouse inoculated with dose of 10^5 *C. pseudotuberculosis.* Multiple abscesses on the surface (arrow), and presence of congestion.

Fig. 4: Photomicrographic section of kidney of mouse inoculated with dose of 10^7*C. pseudotuberculosis*: Focal area of abscess (arrow).

experimental period allocated in our study. Another possible explanation could be that these classical mice might develop immunity against the organism as result of environmental contamination.

According to Fontaine et al. (2008), brain is the less commonly effected organ for internal form of CLA. The present study showed that there is presence of *C. pseudotuberculosis* in the brain from different doses of infection using PCR method. This finding is supported by Jesse et al. (2013b) where the author stated that the possible explanation could be in relation to the pathogenesis of CLA where the bacteria multiply in the superficial and visceral lymph nodes and spread to other organs via the lymphatic or haematogenous route (Glenn, 2000).

There were positive detection of *C. pseudotuberculosis* in stomach and intestine using PCR. This finding is not in agreement with previous study by Jesse et al. (2013b) that stated negative detection for stomach and intestine using PCR. This can be due to duration of this study where 10 days was chosen and it is longer duration compared to previous study which was only for 5 days (Jesse et al., 2013b).

Heart is less commonly affected organ in the internal form of CLA (Fontaine et al., 2008). However, in this study, there was positive detection of *C. pseudotuberculosis* in the heart using PCR. This may be due to the ability of the organism to spread through haematogenous route and cause septicaemia (Nairn and Robertson, 1974).

Fontaine et al. (2008) stated that the sites of visceral lesions are commonly found in the internal lymph nodes or lungs. Thus, in the present study, the positive detection of the *C. pseudotuberculosis* in the lungs is in agreement with Fontaine et al. (2008). Although experimental CLA infections within the lungs of sheep have been achieved by intra-tracheal administration of bacteria (Brown and Olander, 1987), more recent studies suggest that

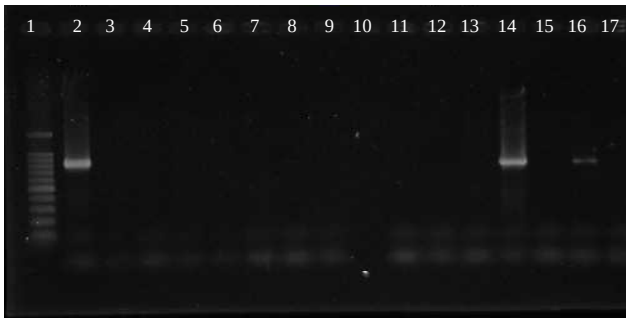

Fig. 5: PCR examination of tissue samples of mice No. 1 (3-10) & mice No. 2 (11-17) inoculated with dose 10^3 of *C. pseudotuberculosis*. Bands at 816 bp indicate *C. pseudotuberculosis.* 1: Ladder (100 bp), 2: Positive control (816), 3: Lymph nodes , 4: Intestine , 5: Stomach , 6: brain , 7: Heart, 8: Liver , 9: Lung , 10: spleen, 11: Lymph nodes , 12: Intestine, 13: Stomach, 14: Brain, 15: Heart , 16: Liver , 17: Lung.

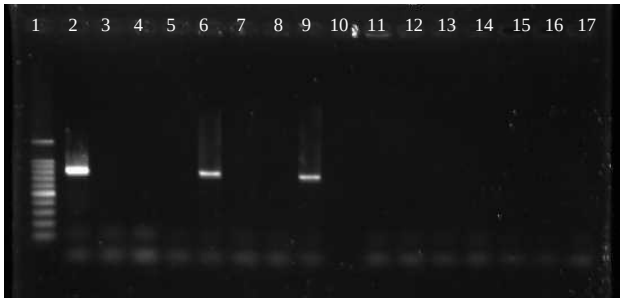

Fig. 6: PCR examination of tissue samples of mice No. 9 (3-10) & mice No. 10 (11-17) inoculated with dose 10^5 of *C. pseudotuberculosis*. Bands at 816bp indicate *C. pseudotuberculosis.* 1: Ladder (100 bp), 2: Positive control (816), 3: Lymph nodes , 4: Intestine , 5: Stomach , 6: brain , 7: Heart, 8: Liver , 9: Lung , 10: spleen, 11: Lymph nodes , 12: Intestine, 13: Stomach, 14: Brain, 15: Heart , 16: Liver , 17: Lung.

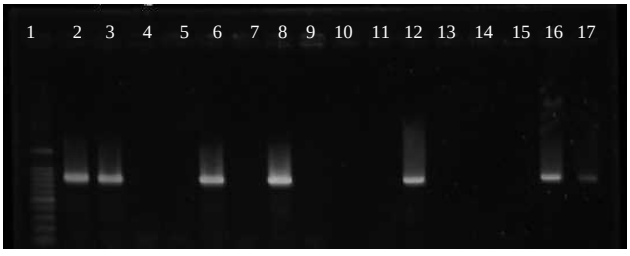

Fig. 7: PCR examination of tissue samples of mice No. 9 (3-10) & mice No. 10 (11-17) inoculated with dose 10^7 of *C. pseudotuberculosis*. Bands at 816 bp indicate *C. pseudotuberculosis.* 1: Ladder (100bp), 2: Positive control (816), 3: Lymph nodes , 4: Intestine , 5: Stomach , 6: brain , 7: Heart, 8: Liver , 9: Lung , 10: spleen, 11: Lymph nodes , 12: Intestine, 13: Stomach, 14: Brain, 15: Heart , 16: Liver , 17: Lung.

pulmonary lesions develop as parts of a systemic infection initiated elsewhere in the body. Therefore, the current study showed that oral route infection may cause lesion in the lung. There was positive detection of *C. pseudotuberculosis* in the liver using PCR. The findings was in agreement with Baird and Fontaine (2007) and Fontaine et al. (2008) who stated that the liver is one of the target organ in the internal form of CLA and perhaps this happens as a result of septicaemia and chronic state (Fontaine et al., 2008).

Thus, the present study showed that the oral inoculation of *C. pseudotuberculosis* with the different doses did cause the same effect as other natural route of transmission. Prolonged period is required for the onset of the infection in all organs. Detection of *C. pseudotuberculosis* with the minimal dose of bacteria using PCR can be efficient to facilitate the diagnosis based on the specificity and sensitivity in comparison to other diagnostic procedure.

Conclusion

From the result of the present study, all the mice experimentally inoculated with *C. pseudotuberculosis* revealed successful demonstration of infection with the bacteria. Further, we report that the doses 10^5 to10^7 are potential actors in terms of induction of pathological effects.

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