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Development of hyperimmune serum for diagnosis and therapy against Jembrana disease in Bali cattle

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Abstract
Bali cattle are indigenous Bali that has been widely distributed throughout Indonesia, form about 27% of the total cattle population, and they make the highest contribution to beef production in Indonesia. The animals possess many superiorities compared to other breeds but they are very susceptible to Jembrana disease virus (JDV) infection which is classified as one of the strategic-infectious animal diseases. The purpose of this study was to provide hyperimmune serum appropriate for the treatment of JDV infection and routinely serological diagnosis. The hyperimmune serum against JDV infection was provided using the method of Mhoma method and hyperimmune was developed using a different types of vaccines. firstly immunized intramuscularly with vaccine containing the CFA adjuvants and the second were prepare with the commercial vaccine twice at one monthly interval. Prophylaxis with these hyperimmune after infection was failed in challenge experiments.

Keywords: Bali cattle, JDV, hyperimmune serum, serological diagnosis

1. Introduction

The first outbreak of Jembrana disease, a highly infectious disease affecting Bali cattle (*Bos javanicus*) was reported in the Jembrana district of Bali, Indonesia, in December 1964 (Adiwinata, 1967) [1]. It was reported that an estimated 60% of Bali cattle were affected with a mortality rate of 98.9%, during the outbreak (Ramachandran, 1996) [2]. The most easily observed and striking clinical sign that can be a useful signature of Jembrana disease under field conditions is an enlargement of the superficial lymph nodes (Dharma *et al*., 1994) [3]. Lest then a year, the disease spread to all 8 districts of Bali with a mortality rate of about 20% within a total Bali cattle population of approximately 300,000 cattle on the island (Pranoto and Pudjiastono, 1967) [4]. Although further smaller outbreaks were reported in other districts of Bali in the following years, these later outbreaks were considered milder, probably due to a level of natural immunity among the local cattle population (Ramachandran, 1996) [2].

The first outbreak of JDV infection outside Bali was in 1976 in Lampung province of the island of Sumatra, and followed by a number of outbreaks in many other regions such in East Java in 1978, West Sumatra in 1992 and South Kalimantan in 1993 (Prabowo, 1996, Tembok and Erinaldi, 1996, Hartaningsih *et al.,* 1993) [5, 6, 7]. Interestingly, during the outbreack, only Bali cattle were affected but not crossbred Bali cattle (*Bos javanicus x Bos indicus*), *Bos indicus* cattle, buffalo, goats and sheep that were also present in the affected locations. The spread of the spread of JDV to the infected islands was previously assumed due to the illegal transportation of JDV-infected cattle (Hartaningsih *et al*., 1993; Soeharsono *et al.,* 1995) [7, 8]. This hypothesis was finally confirmed by genetic analysis that of JDV strains obtained from those infected islands were very similar, with 97-100% nucleotide homology in *gag* sequences, suggesting JDV-carrier animals were previously transported from JDV-infected islands (Desport *et al.,* 2007) [9].

The causative agent of Jembrana disease was identified as a bovine lentivirus and designated *Jembrana disease virus* (JDV). This atypical lentivirus causes an acutely pathogenic disease that is associated with clinical signs and pathological lesions attributable to a disease primarily affecting the lymphoid system (Dharma *et al.,*1994) [3].

Immuno-histochemical studies showed that the tropism of JDV was B-cells but not T-cells which explained the absent of humoral antibodies during the critical stages of the disease progress (Tenaya *et al*., 2012) [10]. So far, vaccination against the JDV infection using inactivated vaccine is considered less effective, not always available, it is made from JDV-infected animals and is contrary to the principle of animal welfare. The availability of hyperimmune serum for therapy purposes and routine serological diagnoses during the critical stages of JDV infection are crucial. This reagent is not available commercially and therefore this is the responsibility of DIC Denpasar as the reference laboratory for Jembarana disease in Indonesia to provide it. The aims of the study reported in this paper were to prepare hyperimmune serum for the diagnosis and immunotherapy to JDV infection in Indonesia.

Material and Methods

Experimental animals

A total of seven Bali cattle used in this study were obtained from Nusa Penida, a small island adjacent to Bali, where Jembrana disease has never been reported and where antibodies to JDV have not been detected. All cattle were female, approximately 12 months of age, and weighed 80-100 kg, Animals for these experiments were transported to Bali Island to the Disease Investigation Centre Region VI Denpasar Bali where the research was conducted. Before use, they were pre-treated with oxytetracycline at a dose rate of 5 mg/kg body weight for 3 consecutive days, a broad spectrum anthelmintic, and vaccinated against hemorrhagic septicemia. The absence of antibody to JDV before the animals used was confirmed by an ELISA test using a JDV recombinant CA antigen (Burkala *et al*., 1998) [11]. The use of these cattle in this study was approved by the Animal Ethics Committee, Faculty of Veterinary Medicine, Udayana University.

Hyperimmune serum preparations

The hyperimmune serum against JDV infection was provided using the method of Mhoma (1992) in Tenaya *et al.* (1998) [12]. The antigen used as immunogen was a 15 % of spleen suspension (Strain Tabanan) prepared from an experimentally-JDV infected cattle (Hartaningsih *et al.,* 2001) [13]. The spleen suspension was firstly inactivated with 0.1% Triton X100 and emulsified using complete-Freund adjuvant (CFA), and a similar suspension was also prepared but emulsified with Incomplete -Freund adjuvant (ICFA). The emulsified suspensions were added penicillin-streptomycin 10 IU penicillin, 10 mg streptomycin, and Fungizone 5 µg/ml, to prevent microbial contamination. As control vaccine, a commercial inactivated-JDV vaccine (Produced by Pusvetma Lab), was also provided.

Two experimental cattle (CB2 and CB3) were used to prepare hyperimmune serum, although using a different type of vaccines. Cattle CB2 was firstly immunized intramuscularly with 3 ml of vaccine containing the CFA adjuvants, and repeated with 3 ml of vaccine containing the ICFA at 2 weeks interval for a further 5 times. Meanwhile CB, 3 was only immunized intramuscularly with 3 ml of the commercial vaccine twice at one monthly interval. Daily clinical observations were noted and serum was collected every two weeks to check the progression of antibody production and harvested two months after the last immunization when the highest titer of the antibody was detected. The serum samples were aliquoted and kept at -20 °C after being tested using ELISA test and heat-inactivated at 56 °C for 30 minutes.

Inoculation of a JVD-donor

For further research, a donor animal was prepared by infecting cattle (CB4) with 1 ml of a 10% homogenate of spleen in DMEM which had previously been prepared from an animal experimentally infected with $JDV_{Tab/87}$ (Soeharsono *et al.,* 1995) [8]. Daily rectal temperatures and other clinical signs of JD were monitored after the initial infection. On the second day of fever (5-6 days), a blood sample was collected for plasma and stored at -80 °C before being used after being confirmed with RT-PCR.

Preparation of infected cattle for hyperimmune therapy

Four cattle (CB01, CB02, CB03, and CB05) were inoculated intravenously with 1 ml of 10^{-3}/ml plasma collected from the donor animal, containing approximately 10^5/ml of JDV-ID50 infectious dose. Daily clinical symptoms were again recorded, and on the second fever, CB02 and CB05 were intravenously treated with 50 ml heat-inactivated hyperimmune serum prepared from CB2, immunized with a non-commercial vaccine. Meanwhile, CB 01 and CB03 were treated in a similar way to those CB02 and CB05 but using 50 ml heat-inactivated hyperimmune serum prepared from CB3, immunized with the commercial vaccine (Pusvetma Laboratory). Following the serum treatment, all experimental cattle were observed daily for recording any clinical symptoms, and blood samples were collected for hematological studies and RNA analysis with RT-PCR.

Results.

Analysis of hyperimmune serum.

Five days after immunization, cattle CB2 that had just been immunized with CFA emulsified vaccine, developed two days fever but not typical classical signs of JDV infection which is consistently five days. Meanwhile, cattle CB3 immunized with the commercial vaccine remained normal until the experiment was terminated (Fig.1). All immunized cattle released adequate antibody titer as measured with the ELISA test, but cattle CB2 immunized with the vaccine emulsified with the CFA and ICFA, as it expected, produced significantly higher antibody titer than cattle CB3 immunized with the commercial vaccine. Consecutive in time progression of vaccination and antibody titer of CB2 and CB3 are presented in Figures 2 and Figure 3 respectively.

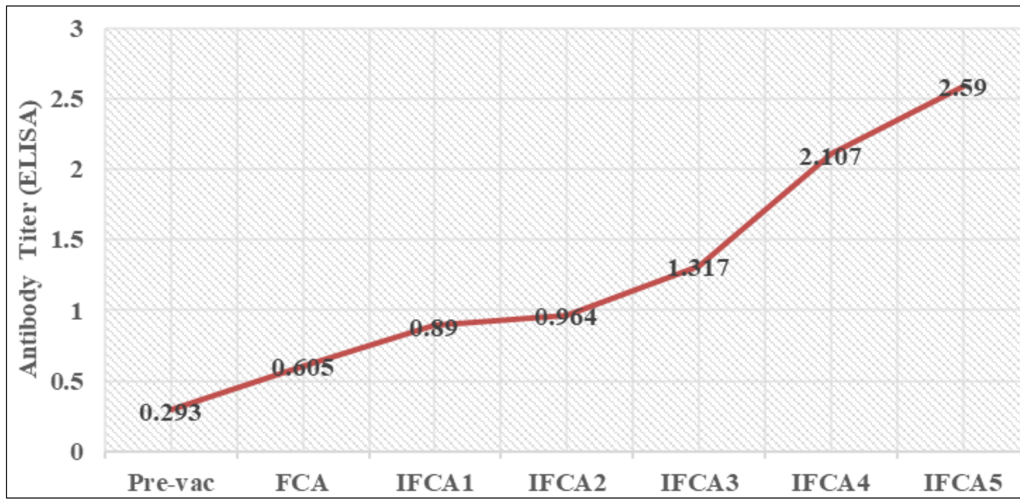

Fig 1: Daily rectal temperature of two immunized cattle with two types of difference vaccines. Note: temperature ≥ 39.5 °C was considered fever. Cattle CB2 experienced two consecutive days of fever, day 5 and day 6 (line blue) but CB3 remained normal until 14 days after the initial immunization (line green).

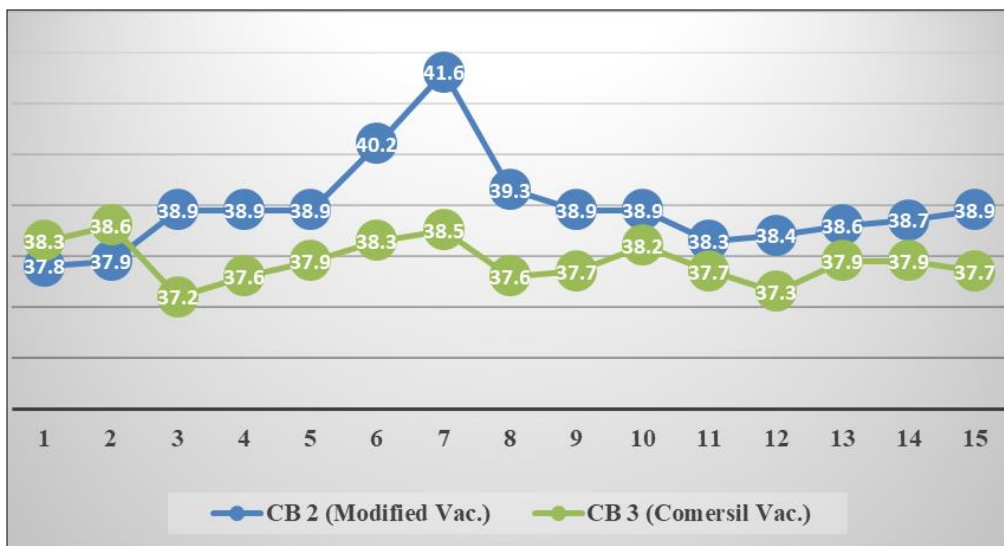

Fig 2: Progression of antibody titer of CB2 immunized with CFA and ICFA detected every two weeks together with the time of vaccination. Note: Before vaccination, the baseline antibody titer was only 0.293 but was then progressively increased to a peak of 2.59

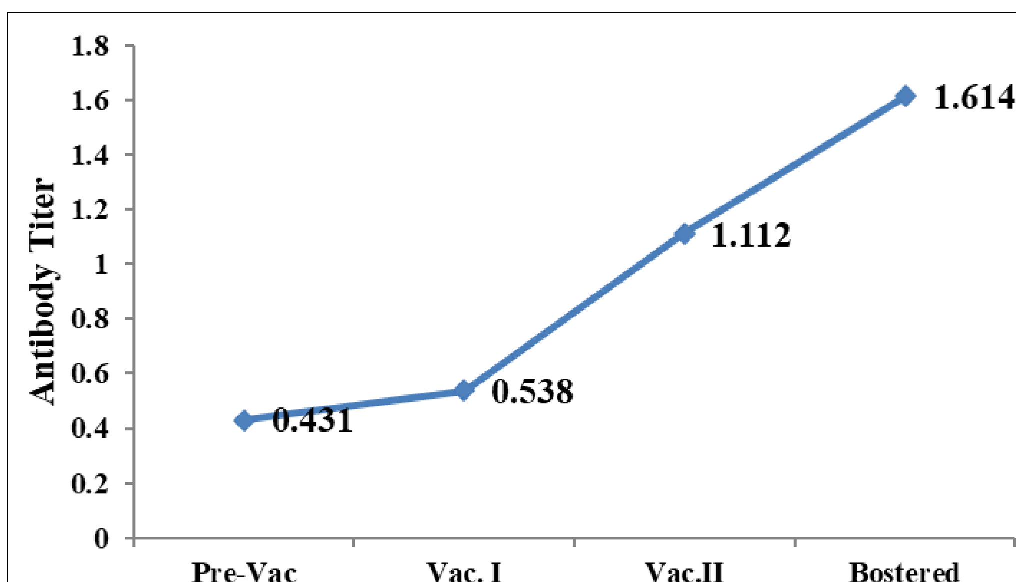

Fig 3: Progression of antibody titer of CB3 immunized with commercial vaccine detected every month together with the time of vaccination. Note: Before vaccination, the base line antibody titer was only 0.431 but was then progressively increased to a peak of 1.614

Confirmation of donor persistently showed typical signs of JDV infection

A donor animal CB4 inoculated with a 10% homogenate of spleen consistently showed typical clinical signs of JDV infection, including 5 days of fever after the initial infection (Fig. 4), and typical hematological changes (Fig.5). The donor animal was required for infecting four cattle with a standard viral load titer of about 10^{-3}/ml that then treated with hyperimmune serum produced in this study.

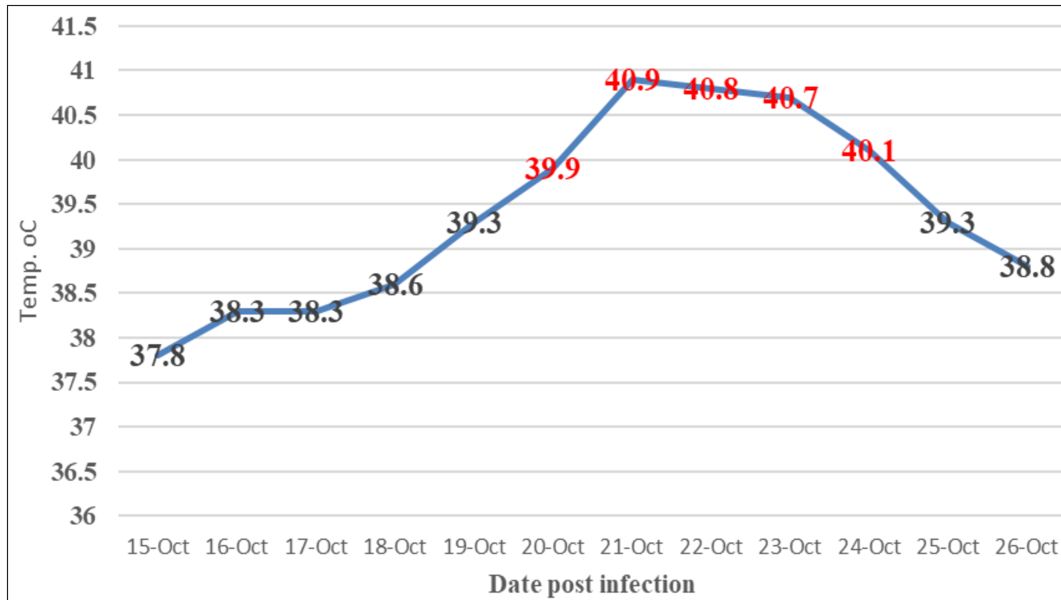

Fig 4: An elevated rectal temperature of donor CB4 for five days started at day five after the initial infection suggested a typical clinical sign of JDV infection. Note: temperature ≥ 39.5 °C was considered fever (red color).

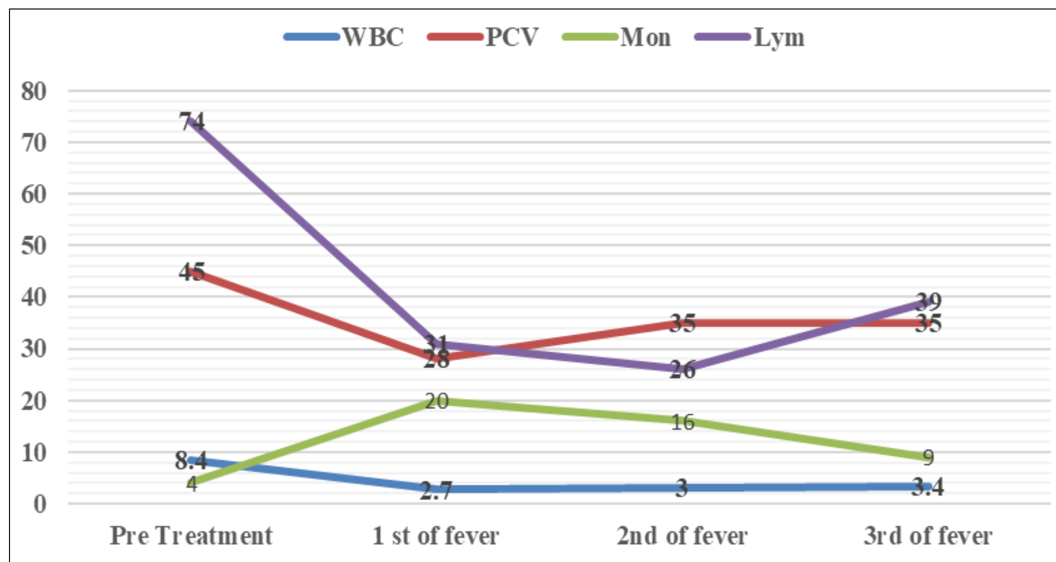

Fig 5: Hematological data of donor CB4 showed typical hematological changes of JDV infection including significant reduction of white blood cell (WBC), packed cell volume (PCV) and lymphocyte detected during 1st, 2nd, 3rd of fever indicated by purple, red and blue colors respectively, but monocytes (Mo) slightly increased associated with bacterial infection during the acute stages of disease progression. Note: values of the blood components tested are indicated.

The implementation of hyperimmune serum in JDV infected cattle

All of the four experimental cattle (CB01, CB02, CB03, and CB05) infected with 1 ml of 10^{-3}/ml plasma prepared from the donor animal experienced fever, a typical clinical sign of JDV infection (Tabel 1). During the second day of fever, CB02 and CB05 were intravenously treated with 20 ml of heat inactivated-hyperimmune serum originating from CB2 immunized with the vaccine emulsified with CFA and ICFA. In contrast, CB01 and 05 were treated with the same volume using NaCl physiologic as control treatment.

Table 1: Rectal temperature of experimental cattle inoculated with JDV-infected plasma originated from the donor animal. Cattle CB02 and CB05 were treated with heat-inactivated hyperimmune serum but cattle CB01 and CB03 were treated with NaCl physiologic on the second day of fever.

Day post infection	Temperature (°C) body condition							
	CB 01		CB 02		CB 03		CB 05	
0	38.5	fine	38.2	fine	38.3	fine	38.4	fine
1	38.2	fine	37.9	fine	38.2	fine	38.2	fine
2	38.5	fine	38.0	fine	38.3	fine	38.5	fine
3	38.4	fine	38.1	fine	38.3	fine	38.3	fine
4	38.3	fine	37.9	fine	38.2	fine	38.4	fine
5	38.7	fine	38.3	fine	38.5	fine	38.6	fine
6	39.5	fine	40.1	fine	39.2	fine	40.3	fine
7	40.5*	fine	40.1*	fine	40.6	fine	40.6*	fine
8	40.8	fine	40.9	fine	40.9*	fine	41.0	fine
9	38.6	fine	40.1	fine	40.9	fine	40.3	fine
10	38.3	fine	40.5	fine	40.7	fine	39.1	fine
11	38.2	fine	39.9	fine	39.6	fine	39.1	fine
12	38.3	fine	38.6	fine	38.7	fine	38.6	fine
13	38.2	fine	38.3	fine	38.6	fine	38.3	fine
14	38.3	fine	38.3	fine	38.8	fine	38.6	fine
15	38.2	fine	38.3	fine	38.6	fine	38.1	fine
16	38.2	fine	38.1	fine	38.7	fine	37.8	fine
17	38.1	fine	38.1	fine	38.5	fine	38.4	fine

Cattle CB02 and CB05 did not show a significant reduction in fever after hyperimmune therapy which was similar to those of control animals (CB01 and CB03). Although all the experimental animals experienced fever, they all lived in good body condition until the experiment was terminated. All animals experienced leucopenia, a typical feature of JDV infection (data not shown), and all were PCR positive. Based on clinical signs noted, typical hematological changes and PCR analyses have clearly demonstrated that the hyperimmune serum gave failed to neutralize JDV replication during the course of the disease progression.

Discussion
This was the first study to provide hyperimmune serum as a positive control serum for detecting JDV infection in Indonesia. The provided serum was intended to use for immune therapy *in vivo*, to know if the serum has the potency to neutralize circulating JDV in recently JD-infected cattle. Previously, serum positive control for the serological assays was provided from naturally JDV-infected cattle which has recently been quite difficult to find.
CB2 vaccinated with 15% of inactivated spleen suspension emulsified with CFA experienced a day of fever started on day five after vaccination but no other JDV-clinical symptoms were noted and PCR test for the animal was negative (data not shown). This condition may be associated with the inactivation process of the virus in the spleen was not adequate, although the virus did replicate further. Moreover, the presence of CFA used to emulsify the antigen may be responsible to weaken cattle condition as it contained mycobacteria particles (Tenaya *et al.,* 1991). However, CB2 after being further immunized using an ICFA-emulsified vaccine demonstrated a gradual increase of antibody titer up to a peak of 2.6 than those of using a commercial vaccine which was only 1.6, detected using a standard ELISA test (Hartaningsih *et al*., 2001; Tenaya *et al.,* 1991) [13, 14]. In order to get a standard optical density of 1.2-1.5, the hyperimmune serum produced using CFA and ICFA oils should be diluted at 1:200 but only 1:100 for those produced with the commercial vaccine. The use of combined CFA and IFCA emulsified vaccines contributed to a significant efficiency of using the hyperimmune serum by almost 100%.

The administration of hyperimmune serum provided by the use of CFA and ICFA emulsified vaccine failed to hamper JDV replication in acutely JDV-infected cattle. All of the two animals treated with the serum showed typical clinical signs of JD and PCR-reactive, suggesting a lack of neutralizing potencies. A similar feature was also observed in control animals, treated with saline solution. The failure of the protection was associated with a very low volume of serum (20 ml) given and a very low IgG titer in the serum compared with a very high viral load on a sick animal. The absence of humoral antibodies during the acute stage of JDV infection was due to the infection of B-cells as the target cell of the virus (Tenaya *et al*, 2012) [10]. If a high volume of hyperimmune serum that contained a high titer of specific IgG was administered at the acute stage, it was believed that JDV-infected cattle could be cured. Primary studies reported here, provided early data that high titer of IgG should be prepared before conducting *in vivo* studies, to determine the appropriate IgG dose for immune therapy against JDV infection as an effort to practice animal welfare.

Conclusion
This study succeeded to provide a high titer of hyperimmune serum with was considered valuable for serological works. However, the recently provided hyperimmune failed to combat JDV during the acute phase of the disease which was strongly associated with a low volume of the hyperimmune serum given, together with a low titer of specific IgG in the serum. Further work is required to perfect the current results by preparing hyperimmune serum that contains good quality and quantity of specific IgG for the treatment of JDV infection.

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Conflict of Interest
The authors declare that there is no conflict of interest with regard to the publication of this manuscript.

Author contribution

All authors were actively involved in the early planning of research, experimentation, collecting and analysis of data research, preparation, and completion of the manuscript.

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