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Effects of antibiotic residue pollution released into the Persian Gulf's coastal environment on the living organisms: A laboratory study on the pacific white shrimp (*Penaeus vannamei*)

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Abstract

Background: Antibiotic residues discharged into the Persian Gulf pose a high health risk to the ecosystem and the public. This study aimed to investigate the toxicity effects of amoxicillin (AMX), the most common antibiotic residue released by municipal sewage into the marine environment of the northern Persian Gulf, on the growth performance and hematological parameters of Pacific white shrimp (*Penaeus vannamei*) juveniles.

Methods: For a 60-day experimental trial, shrimps were exposed to different doses (100, 300, and 500 µg/L) within the actual concentration range of AMX discharged into nature. Total length (TL; cm), carapace length (CL in mm), and body weight of shrimps were measured at the beginning and end of the survey. Haemolymph samples were taken to analyze the immunological parameters.

Results: At the end of a 60-day experimental trial, the growth performance indices (WGR%, SGR%, TLI in cm, CLI in mm, and SR%) were not significantly different among the groups ($P > 0.05$). Although all examined hematological parameters (AST, ALT, ALP, TP, ALB, and IgG) increased in shrimp exposed to AMX residues, only TP showed a significant difference ($P < 0.05$). The total haemocyte count (THC) and differential haemocyte count (DHC) of shrimp exposed to AMX were significantly different from the control ($P < 0.05$).

Conclusion: Detecting the ecotoxicological effects of pharmaceuticals on non-target species is essential for maintaining a healthy aquatic ecosystem. These findings show that chronic exposure to these compounds may interfere with the species' metabolism, offering valuable insights into marine pollution in the Persian Gulf and beyond.

Keywords: Ecosystem, Anti-bacterial agents, Amoxicillin, Sewage

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Introduction

Understanding the effects of pharmaceutical residues on different environments and their toxicity to living organisms is crucial for assessing environmental risks and predicting potential consequences. Antibiotics are the most widely prescribed medicines worldwide after drugs for high blood pressure and diabetes treatment (1). Antibiotics are widely used for treating human infectious diseases and are also employed in livestock and aquaculture industries to promote growth, prevent infections, and reduce mortality (2-4). The residues of antibiotics enter the aquatic environment through several pathways, such as the manufacturing industry, municipal sewage, animal husbandry, aquaculture fields, and runoff of agricultural farms containing animal fertilizer (3,5). Since conventional wastewater treatment plants (WWTPs) are ineffective at completely removing antibiotics, their effluents serve as a major source of antibiotic pollution in aquatic environments (6-8). Much literature has been devoted to illustrating the ecological hazard of these emerging contaminants (ECs) on the aquatic environment and its organisms from different trophic levels (9-11). However, there are still unknown aspects in this direction. For example, Pan et al examined the toxic effects of

amoxicillin (AMX) on the photosynthesis performance of *Synechocystis* sp., freshwater cyanobacteria, and concluded that exposure to amoxicillin deteriorated photosynthesis (12). Matozzo et al also investigated AMX impacts on the haemocyte parameters of two bivalve species, namely *Uditapes philippinarum* and *Mytilus galloprovincialis*. They argued that total haemocyte count (THC) and haemolymph pH decreased depending on exposure duration and concentration (13). Chowdhury et al examined the genotoxic effects of AMX residues on embryos of *Danio rerio*, a freshwater fish belonging to the Danionidae family, and demonstrated DNA damage in specimens exposed to AMX (14).

The Persian Gulf, a waterbody with an area of ~239,000 km and an average depth of 36 m (15), is located in western Asia and separates the Iranian plateau and the Arabian Peninsula. The biodiversity and species richness of the Persian Gulf are threatened by anthropogenic activities such as overfishing (16,17), different kinds of pollution (18-22), and climatic changes (23). Discharging untreated municipal wastewater into the coastal environment is one of the primary sources of pollution in the Persian Gulf, particularly on the northern side. The WWTP effluent of Bandar Abbas city, the crowded beach city in southern Iran, is a point source for antibiotic residues entering the Persian Gulf (24,25). Daliri et al reported that the WWTP outlets of Bandar Abbas are released at about 500 to 700 L/s into the marine ecosystem, which contains 335.17 ± 105.11 and 288.17 ± 37.94 µg/L of AMX and azithromycin (AZM) residues. They assessed the potential ecological risk of these substances and categorized them as high-risk contaminants for the Persian Gulf ecosystem and the surrounding human society (25). Considering the high environmental risk of AMX residues for the Persian Gulf marine ecosystem, to address this knowledge gap, this study aimed to investigate the ecotoxicological effects of AMX on *Penaeus vannamei*, a key species in aquaculture. Specifically, we (i) selected AMX concentrations based on real-world pollution levels reported in the Persian Gulf (25) and (ii) conducted a long-term exposure experiment using a completely randomized design (CRD) to evaluate its impact on growth performance and hematological parameters.

Materials and Methods

Experimental animals and culture conditions

The shrimp used in this experiment were obtained from a private farm in Tiab Shomali, Hormozgan, Iran. The two-month experiment was conducted at the Persian Gulf Breeding Center (PGBC) in Kolahi, Hormozgan, Iran. Six hundred healthy Pacific white shrimp juveniles (*P. vannamei*) with a mean (± SD) body weight and total length of 9.28 ± 0.37 g and 9.23 ± 1.77 cm, respectively, were randomly distributed in 12 static indoor circular polypropylene tanks (300 L). Table 1 shows the initial values of these morphometric characteristics in detail, which were not significantly different (α = 0.01). They were acclimated for at least three days before the experiments. Shrimp were maintained in aerated, filtered seawater with the following parameters: salinity (36.5 ± 0.5 g/L), temperature (32 ± 2°C), pH (8.0 ± 1.1), and dissolved oxygen (5.8 ± 0.4 mg/L). Shrimp fed four times per day (7:00, 11:00, 15:00, and 19:00) with a proportion of 3% experimental shrimp body by Faradaneh company's commercial shrimp feed (protein ≥ 41-43%, fat ≥ 8%, fiber ≤ 3). The rearing water was changed every three days by about 75%. Morphometric features of the shrimps were measured biweekly, and accordingly, the feeding ratio was recomputed.

Antibiotic exposure

Amoxicillin trihydrate suspension (made in Kausar Pharmaceutical Company, Iran, CAS number: 32821) was purchased for the experiment and kept at 5°C until used. Shrimp were exposed to three concentrations of AMX (100, 300, and 500 µg/L), along with a control group (no antibiotic exposure). Each treatment was conducted in triplicate.

Growth performance and survival rate

The mean weight gain rate (WGR%), specific growth rate (SGR%), total length (TL; cm), carapace length (CL in mm) increase, and survival rate (SR%) for all groups were obtained by the following formulas:

$$\text{WGR \%} = \left[\left(\text{final body weight} - \text{initial body weight} \right) \Big/ \text{initial body weight} \right] \times 100 \quad (1)$$

$$\text{SGR \%} = \left(\text{Ln final body weight} - \text{Ln initial body weight} \right) \Big/ \text{days} \times 100 \quad (2)$$

$$\text{TLI}\left(\text{mm}\right) = \text{final total length} - \text{initial total length} \quad (3)$$

$$\text{CLI}\left(\text{mm}\right) = \text{final carapace length} - \text{initial carapace length} \quad (4)$$

$$\text{SR \%} = \text{final number of shrimp} \Big/ \text{initial number of shrimp} \times 100 \quad (5)$$

Immunological parameters

Shrimp feeding was stopped 24 hours before the sampling of haemolymph. Haemolymph was taken from 6 specimens per treatment. The samples were drawn using a 1-ml sterile syringe along with a 25-gauge needle containing 0.3 ml precooled anticoagulant (10 mM trisodium citrate, 100 mM sodium chloride, and 250 mM sucrose, pH 7.6, 4°C) (26). Haemolymph samples were divided into two portions:

Part I: Used for measuring total haemocyte count (THC) and differential haemocyte count (DHC). Part II: Centrifuged at 4600 g for 10 min at 4°C. The supernatant was collected in a sterile tube and stored at −20°C for further biochemical analysis in a clinical laboratory. The COBAS INTEGRA 400 plus automated chemistry

Table 1. Initial weight, total length (TL), and carapace length (CL) of individuals distributed in experimental treatments.

Features	AMX concentrations (µg/L)			
	0	100	300	500
Initial weight (g)	9.25±0.31	9.35±0.28	9.34±0.30	9.07±0.21
Initial TL (cm)	9.14±1.31	9.29±1.54	9.41±1.29	9.07±1.07
Initial CL (mm)	35.90±0.28	35.53±0.34	36.50±0.49	34.96±0.33

analyzer was utilized for analyzing the plasma samples and determining aspartate transaminase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) enzymes, and total protein (TP), albumin (ALB), and immunoglobulin G (IgG) parameters.

Statistical analysis

The normality of distributions and homogeneity of variances were tested by the Shapiro-Wilk and Levene tests, respectively. For non-normally distributed data, logarithmic transformations were applied before rechecking normality. The groups' means (±SE) were compared by One-way ANOVA and Duncan's post-hoc tests. The Kruskal-Wallis non-parametric test was also used to compare ALB data. All statistical analyses were performed using SPSS 23 and Excel 2016, with a significance threshold of $\alpha = 0.05$.

Results

Growth parameters

Table 2 presents the growth and survival rates of shrimp exposed to different AMX concentrations. No significant differences were observed among treatments ($P > 0.05$). The highest WGR (26.17±4.49%) and SGR (0.23±0.11%) were observed in shrimp exposed to 100 µg/L AMX, whereas the lowest WGR (17.15±8.57%) and SGR (0.06±0.01%) occurred at 500 µg/L AMX. Shrimp exposed to 300 µg/L AMX exhibited the highest total length (TL) and carapace length (CL) increases, whereas the control group (0 µg/L) showed the lowest. However, these differences were not statistically significant ($P > 0.05$). Although shrimp exposed to AMX exhibited a slight decline in survival rates compared to the control, the differences were not statistically significant ($P > 0.05$).

Plasma immunological parameters

Figure 1 illustrates the plasma immunological parameters of shrimp across different AMX concentrations. All measured plasma immunological parameters were higher in AMX-treated groups compared to the control. However, ANOVA revealed a significant difference only in total protein (TP) levels ($P = 0.04$, $F = 3.23$). Post-hoc analysis indicated that TP at 100 µg/L AMX was not significantly different from other treatments.

Table 3 indicates the result of the total haemocyte count (THC) and differential haemocyte count (DHC) of shrimps cultured in experimental treatments. The THC of shrimp exposed to AMX concentrations increased compared to the control ($P < 0.05$). DHC analysis showed a significant increase in granular cells (GC) and semi-granular cells (SGC) in AMX-treated shrimp, while hyaline cells (HC) were significantly lower compared to the control ($P < 0.05$, Table 3).

Discussion

Amoxicillin is typically detected in ng/L and µg/L in aquatic ecosystems (10,27). Even though the development of antibiotic-resistant bacteria is the primary concern regarding the discharge of antibiotic residues into these environments, it may have potential risks for non-target organisms (3,13). In the present research, the actual concentration of AMX, which is discharged into the Persian Gulf's marine environment, was considered to simulate its toxic effects on aquatic organisms. The Pacific white shrimp (*P. vannamei*) was chosen to test the organism because of (i) adaptability to laboratory conditions, (ii) the simple hematopoietic system of crustaceans, which is more affected by environmental changes than fish (28), and finally, (iii) easy accessibility.

Here, we found that examined AMX concentrations did not affect the growth indices (WGR, SGR, TLI, and CLI) of the *P. vannamei* studied in the present paper. However, these AMX concentrations may affect larval development or mature reproduction, and it is necessary to design similar experiments to measure the effects of the AMX concentration on shrimp's growth at different life stages. The toxic effects of antibiotics on the growth of some aquatic invertebrates (mostly zooplanktons) have also been investigated, and it has been concluded that the growth of organisms is inhibited mainly at concentrations two or three times higher than those found in aquatic environments (29), which leads to an underestimation of antibiotic hazards. The survival means of shrimps exposed to AMX concentrations were also decreased compared to the control, but it was not statistically significant. The combined toxicity of AMX and other antibiotics/pharmaceuticals may affect the growth of aquatic organisms. As Flaherty and Dodson (2005) reported, exposure to a single pharmaceutical compound (fluoxetine and clofibric acid) did not significantly affect the growth of *Daphnia magna*, but mixtures of these substances affected growth and reproduction (30). There is also much persuasive evidence that the chronic toxicity of antibiotics mainly appears as an immune response and DNA damage (14,31,32).

As stated in the plasma immunological result section,

Table 2. Growth performance and survival rate of *P. vannamei* exposed to different AMX concentrations (µg/L).

Indices	AMX concentrations (µg/L)				Sig. statistic	
	0	100	300	500	F	*P*
WGR (%)	22.04±3.43	26.17±4.49	23.93±5.13	17.15±8.57	0.45	0.73
SGR (%)	0.22±0.06	0.23±0.11	0.11±0.06	0.06±0.01	1.42	0.31
TLI (cm)	1.10±0.46	2.57±1.45	2.97±1.33	2.07±0.60	0.58	0.64
CLI (mm)	0.77±0.18	1.54±0.19	0.67±0.27	1.00±0.57	1.31	0.33
SR (%)	94.00±1.15	91.33±2.91	86.67±2.40	89.33±5.93	0.76	0.55

WGR: Weight gain rate; SGR: Specific growth rate; TLI: Total length increase; CLI: Carapace length increase; Sr: Survival rate.

Table 3. Mean (±SE) values of THC (total haemocyte count), GC (granular cells), SGC (semi-granular cells), and HC (hyaline cells) in shrimp exposed to different AMX concentrations

Parameters	AMX concentrations (µg/L)				Sig. statistic	
	0	100	300	500	F	*P*
THC ($\times 10^6$ ml^{-1})	0.69±0.09^a	1.83±0.58^b	2.15±0.32^b	1.58±0.21^b	3.18	0.03
GC (%)	7.00±1.19^a	34.55±5.79b	37.33±5.40^b	29.44±2.60^b	10.73	0.00
SGC (%)	38.44±5.63^a	53.89±4.37^b	56.33±4.17^b	62.44±2.07^b	4.85	0.02
HC (%)	54.55±5.86^a	11.56±2.54^b	6.33±1.62^b	8.11±1.28^b	20.24	0.00

Values with different letters in the same row are significantly different ($P<0.05$).

all examined parameters of shrimp exposed to AMX increased compared to the control, while a significant difference was mainly observed for TP. The response of the shrimp's immune system to environmental changes and stresses is mainly based on proteins (33). Hence, TP is a standard indicator to evaluate these physiological effects (34). Our findings showed that the level of TP increases with increasing AMX concentration. Given that haemocyanin (the respiratory protein) constitutes 80 to 95% of the total protein concentration in decapod haemolymph (35,36), it can be concluded that AMX, as an environmental stressor, increased the oxygen demands or antioxidant mechanisms in shrimp. This is consistence with the result of Hagerman (1986), who kept the Brown shrimp (*Crangon crangon*) under normoxia and moderate (40% saturation) hypoxia and reported an increase in haemocyanin concentrations (37). Zhou et al investigated the toxicity of tributyltin (TBT) on the haemolymph protein of marine gastropods (*Haliotis diversicolor supertexta*) and argued that exposure to different doses of TBT (0, 2, 10, and 50 ng/L) caused an increase in the protein of haemolymph (38). Jamshidizadeh et al also examined the immune responses of *P. vannamei* to different levels of aflatoxin in food, and reported an increase in TP in haemolymph of shrimp (39). On the contrary, Chang et al asserted that haemocyanin and protein levels in the haemolymph of the Kuruma prawn (*Penaeus japonicus*) decreased when exposed to ambient ammonia (40). While Chen and Kou reported that haemolymph haemocyanin of the Chinese white shrimp juveniles (*Penaeus chinensis*) increased with an increase in ambient ammonia up to 9.9 mg/L concentration (41).

For other immunological parameters (AST, ALT, ALP, etc.) where the increasing trends were not statistically significant, it is important to take the concept of biological relevance into account for a more careful interpretation. Martínez-Abraín argued extensively that many researchers in quantitative ecological studies consider "statistically significant" as the only strong evidence for interpreting the null hypothesis and the biological relevance of an effect, but it is wrong (42). He asserted that in statistics, the term "significant" has a distinct meaning and does not convey any value judgment regarding magnitude. AST and ALT enzymes are biomarkers of shrimp hepatopancreas damage or disease at high concentrations in the haemolymph. These enzymes are mainly present in the hepatopancreas, but AST is also produced in the heart, kidney, etc. Because AST and ALT are commonly responsible for the catalysis of transamination between amino acids and ketonic acid, their increase (particularly ALT) means poor hepatopancreas performance (43,44). In our results, the deviation of AST and ALT mean levels was about 14 and 19 units between the control and the AMX-treated groups. Although there is no standard upper limit for ALT and AST in crustaceans, it can be assumed that hepatopancreas function deteriorated in shrimps exposed to AMX. ALP was another immune enzyme analyzed in serum, playing a role in phosphate activity by removing phosphate groups from various molecules, including proteins, nucleotides, and alkaloids. ALP level is increased chiefly in the haemolymph of shrimp following hepatic damage (45). In similar research, Duan et al investigated the immune responses of *P. vannamei* exposed to acute sulfide stress and reported that ALP activity increased after sulfide exposure for 12 and 24 h (46).

Serum albumin concentration also indicates the metabolic and nutritional condition of the body (47), which hypo-albuminemia mostly implies liver damage

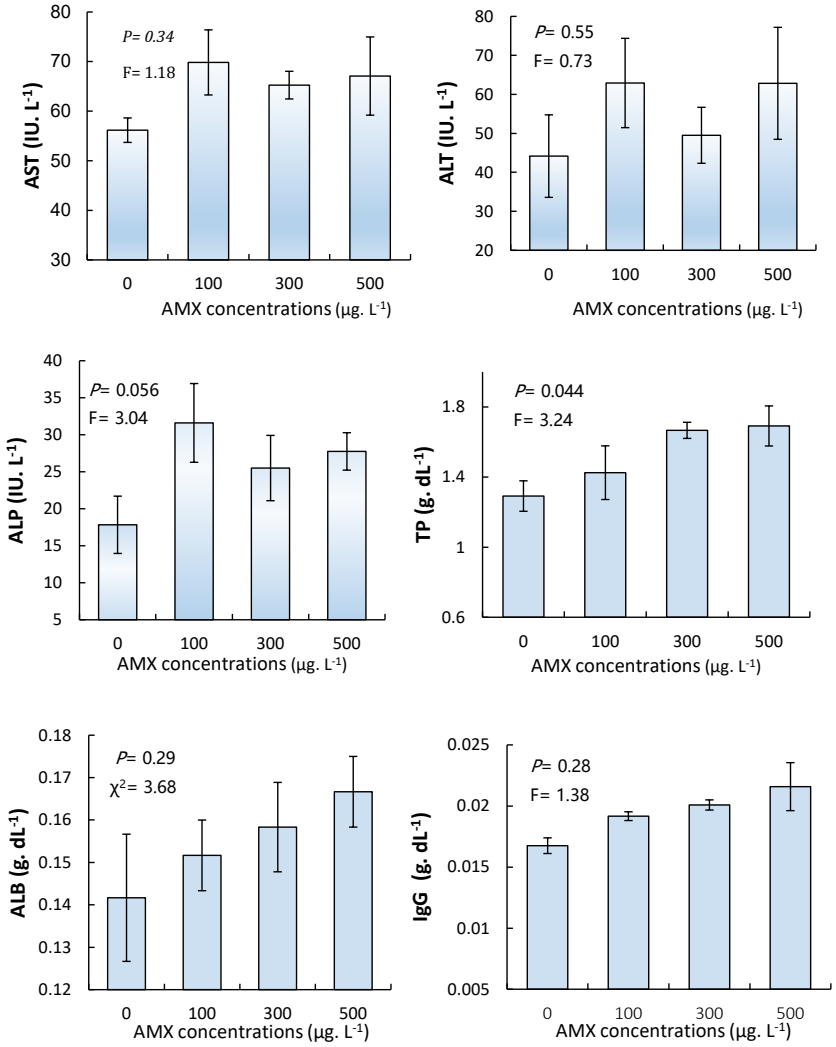

Figure 1. Mean (±SE) values of plasma biochemical parameters of shrimp exposed to experimental AMX concentrations for 60 days (AST: aspartate transaminase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; TP: total protein; ALB: albumin, IgG: immunoglobulin G). Different letters indicate statistically significant differences between experimental treatments ($P<0.05$)

or disease in many species (48). Here, it was found that the albumin content of haemolymph slightly increased in the AMX-exposed groups, which could be due to hepatic damage caused by AMX contamination. However, Qiu et al explored the effects of T-2 toxin, a trichothecene mycotoxin produced by *Fusarium* spp., on the immune function of *P. vannamei* and stated that the albumin content declined in the haemolymph of shrimps exposed to this toxin (49). They suggested that this reduction might be caused by protein inhibition through T-2 binding to the eukaryotic 60S ribosomal subunit, a decrease in dietary protein utilization efficiency due to intestinal mucosal damage, and other related factors. Moreover, immunoglobulin G (IgG) was the latest examined immune parameter in serum, which increased faintly with increasing AMX concentration. Immunoglobulins, known as antibodies, are made by plasma cells when the body feels a foreign substance (bacteria, viruses, and other germs) entering the body. Immunoglobulins include five major types in the body, namely Ig A, IgG, Ig M, Ig D, and Ig E, of which IgG is the most common type and allergic disease researchers are particularly focused on (50-52).

Our obtained result also showed that exposure to AMX contamination increased the THC, GC, and SGC levels and conversely decreased HC in shrimp haemolymph. Decapod hemocytes are mainly divided into two groups, granulocytes and hyaline hemocytes. Granulocytes are responsible for defense against foreign irritants such as stress and disease (53,54), but hyaline cells are important in hemolymph coagulation and exoskeleton hardening after molting (55,56). Therefore, it can be concluded that AMX as a stressor caused the production of granulocytes in shrimps, and subsequently THC also increased. An increase in THC of aquatic organisms exposed to antibiotics has been reported in many studies. For example, Matozzo et al argued that exposure to trimethoprim increased THC in edible species of saltwater clam *Ruditapes philippinarum* (13). Munari et al also demonstrated that the effects of fluoxetine (FXT) on THC in bivalve *Venerupis philippinarum* depended on the FXT

concentrations, in such a way that THC increased at low concentrations (57). In contrast, Matozzo et al showed that AMX exposure (100 to 400 µg/L) slightly decreased the THC in *R. philippinarum* and *M. galloprovincialis*, and these different effects can be due to species type and contaminant levels (13).

Conclusion

In the past two decades, antibiotic residues in aquatic environments and their effects on non-target organisms have received chiefly attention from scientific researchers. AMX, a widely used antibiotic worldwide, is classified as a high-priority contaminant for environmental monitoring and assessment. According to the report by Daliri et al, AMX has extensively entered the coastal environment of the northern Persian Gulf through urban WWTP, which has a high health risk for the marine ecosystem and society (25). This study evaluated the toxicity effects of AMX concentration discharged into the environment on a non-target organism (*P. vannamei*). Based on the present results, selected hematological parameters were changed in shrimp exposed to AMX (especially TP, THC, and DHC) compared to the control. Therefore, we can claim that the examined doses of AMX residues could be hazardous for the immune system of *P. vannamei*. Given that there is little information about this type of pollution in the Persian Gulf region, researchers should pay more attention to this emerging contaminant and its effect on non-target organisms. They can research on (i) the chronic toxicity and potentially subtle effects of AMX on other organisms such as planktons and nektons, (ii) bioaccumulation of AMX residues in the tissues of organisms and transfer efficiency to upper trophic levels, and (iii) combined toxicity of AMX and other antibiotics such as Azithromycin, heavy metals, oil pollution, and environmental factors such as temperature, salinity, and pH.

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Authors' contributions

Conceptualization: Moslem Daliri.
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Visualization: Moslem Daliri Moslem Sharifinia.
Writing – original draft: Moslem Daliri.
Writing – review & editing: Moslem Daliri, Moslem Sharifinia.

Competing interests

The authors declare no competing interests.

Ethical issues

The research methodology was approved by the Research Ethics Committees of Hormozgan University of Medical Sciences, with the approval ID IR.HUMS.REC.1401.318.

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