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Optimal Dose of 5-Fluorouracil in Inducing Oral Mucositis in Rats

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ABSTRACT

Oral mucositis is a side effect of chemotherapy, characterized by deep ulceration that causes white spots in the mouth. This research aimed to determine the optimal dose of 5-Fluorouracil in causing oral mucositis in rats by injecting a single dose intraperitoneally (IP) with and without chemical stimulus. The research design was a post-test control group with 24 male Wistar rats divided into eight groups. Six groups were injected with 5-Fluorouracil without chemical stimulus at a dose of 200 mg/kg, 175 mg/kg, 150 mg/kg, 125 mg/kg, 75 mg/kg, and 70 mg/kg. Meanwhile, two groups were given chemical stimulus with a dose of 5-fluorouracil 100 mg/kg (D0: 60 mg/kg, D2: 40 mg/kg), and 60 mg/kg. Observation was carried out for 21 days by paying attention to the appearance of oral mucositis and how long the rats survived. The results showed that rats injected with 5-Fluorouracil at a dose of 175 mg/kg, 150 mg/kg, 125 mg/kg, 75 mg/kg, and 70 mg/kg died before 21 days without the appearance of oral mucositis. Furthermore, rats injected with a dose of 100 mg/kg survived until an average of 13.34 days and oral mucositis appeared on day 7. Rats injected with a dose of 60 mg/kg survived more than 21 days with oral mucositis appearing on day 5. In conclusion, the optimal dose to cause oral mucositis in rats was 60 mg/kg and added a chemical stimulus in the form of 50% acetic acid.

Keywords: Oral Mucositis, 5-Fluorouracil, Chemotherapeutic Agent, Optimal Dose

ABSTRAK

Mukositis oral merupakan efek samping dari pemberian kemoterapi, dengan ciri yaitu ulserasi dalam hingga menimbulkan bercak putih di mulut. Tujuan penelitian ini untuk mengetahui dosis optimal 5-Fluorouracil dalam memunculkan mukositis oral pada tikus, dengan penginjeksian dosis tunggal secara intraperitoneal (IP) tanpa stimulus kimia dan dengan stimulus kimia. Desain penelitian adalah *post test control group*. Subjek 24 tikus Wistar jantan yang dibagi menjadi 8 kelompok, 6 kelompok diinjeksi 5-Fluorouracil tanpa stimulus kimia dengan dosis 200 mg/kg, 175 mg/kg, 150 mg/kg, 125 mg/kg, 75 mg/kg, 70 mg/kg, 2 kelompok diberikan stimulus kimia dengan dosis 5-fluoruracil 100 mg/kg (D0: 60 mg/kg, D2: 40 mg/kg), dan 60 mg/kg. Pengamatan dalam 21 hari dengan memperhatikan munculnya mukositis oral dan berapa lama tikus bertahan hidup. Hasil penelitian mendapatkan tikus yang diinjeksikan 5-Fluorouracil dengan dosis 175 mg/kg, 150 mg/kg, 125 mg/kg, 75 mg/kg, dan 70 mg/kg mengalami kematian sebelum 21 hari tanpa munculnya mukositis oral, tikus yang diinjeksikan dosis 100 mg/kg bertahan hingga rerata 13,34 hari dan mukositis oral muncul pada hari ke-7, tikus yang diinjeksikan dosis 60 mg/kg bertahan lebih dari 21 hari dengan mukositis oral muncul pada hari ke-5. Disimpulkan dosis optimal memunculkan mukositis oral pada tikus adalah 60 mg/kg dan ditambah stimulus kimia berupa asam asetat 50%.

Kata kunci: Mukositis Oral, 5-Fluoruracil, Agent Kemoterapi, Dosis Optimal

1. Introduction

Cancer is a severe condition characterized by the uncontrolled and abnormal proliferation of cells within body tissues. According to WHO, there were a total of 18.1 million cancer cases globally in 2020 with 9.3 and 8.8 million cases found in males and females, respectively [1,2]. The International Agency for Research on Cancer (IARC) reported that the disease caused 9.7 million deaths in 2022. This number was projected to increase by approximately 77% in 2050, mainly due to population growth and the widespread of cancer risk factors, such as tobacco use, obesity, insufficient physical activity, poor diet, and air pollution [3,4]. According to the Indonesian Ministry of Health, the incidence of cancer in Indonesia is 136 people per 100,000 population and ranks 8th in Southeast Asia.[5]

In the field of Dentistry, oral cavity cancer is the most prevalent type of malignant tumor. This cancer causes the aggressive growth of epithelial cells that line the lower lip, the front two-thirds of the tongue, the floor of the mouth, and the alveolar ridges of both the maxilla and mandible [6]. The incidence of lip and oral cancer was estimated at 377,713 new cases and 177,757 deaths in 2020. Furthermore, oral cancer is more common in males and the elderly between the ages of 50 and 60 years. This disease is more lethal in males compared to females and varies significantly according to socioeconomic circumstances [7,8].

The management of cancer can include surgery, radiotherapy, chemotherapy, immunotherapy (biotherapy), and hormone therapy [8]. Chemotherapy uses antineoplastic preparations to kill tumor cells by disrupting cellular function and reproduction [9]. Cancer treatment with chemotherapy has a cure rate of up to 80% [10]. The success of chemotherapy treatment was considered more effective because the drug was directly administered through the blood vessels, thereby reaching cancer cells that have metastasized to other tissues [11]. Kim HS et al. (2016) reported that chemotherapy effectively prevents cancer cell metastases to other organs [12]. This therapy also has side effects on the oral cavity in the form of xerostomia, periodontal infection, fungal infection (candidiasis), gingivitis, impaired taste, and oral mucositis. The most common lesion is oral mucositis due to chemotherapy in the form of damage to the mucosal layer. This lesion has an incidence rate of more than 60% of the various complications in the oral cavity due to chemotherapy [13,14].

Oral mucositis is an inflammation of the oral mucosa, which includes the cheeks, lips, gingiva, tongue, palate, pharynx, and floor of the mouth [15,16]. Mucositis has signs ranging from redness, swelling, and deep ulceration to white spots in the mouth and tongue [15]. The size of ulcers varies from <1 cm^2 to >3 cm^2 [17]. According to Treister (2015), oral mucositis is a common complication of chemotherapy that begins 5-10 days after administration and can last 7-14 days. The incidence in children is higher than in adults and occurs in about 40% of patients undergoing cancer chemotherapy and 75% without radiation [18]. Oral mucositis affects 30-80% of cancer patients worldwide, with specific research in Yogyakarta, Indonesia, reporting a 42.4% incidence rate among pediatric cancer patients [16].

Chemotherapeutic agents include antimetabolites (methotrexate), DNA interactive compounds (cisplatin, doxorubicin), anti-tubulin compounds (taxanes), hormones, and molecular targeting compounds, with 5-Fluorouracil being the most commonly used chemotherapy agents. Furthermore, 5-Fluorouracil (5-FU) is the primary chemotherapeutic agent used for cancer therapy and is an antimetabolite that acts antagonistically with thymine on the activity of the enzyme thymidylate synthetase [19,20]. The chemotherapeutic agents that often cause mucositis are 5-Fluorouracil, Irinotecan, and *Methotrexate* [17,21]. Using 5-Fluorouracil at high or low doses can cause oral mucositis, with different onset times [22,23]. This chemotherapeutic agent can interfere with nucleoside metabolism, causing cytotoxicity and cell death in humans [24]. In addition, irinotecan is a drug used to treat cancer, and the active metabolite, SN-38, mainly causes side effects. Some common side effects of this chemotherapeutic agent are neutropenia, diarrhea, nausea, vomiting, alopecia, mucositis, and fatigue [25,26]. Using irinotecan decreased body weight and food intake in test animals [27]. Another chemotherapy option for various cancers is methotrexate. Some side effects of low-dose methotrexate include gastrointestinal manifestations such as nausea, vomiting, mucositis, and loss of appetite [28]. The side effects of methotrexate are associated with the therapeutic dose during use as well as the duration of therapy. A previous research reported that individual side effect tolerance may also be affected by age [29].

Understanding the development and healing of mucositis caused by chemotherapeutic agents can be done using various animal models, allowing preclinical research to trial treatments in experimental animals. In general, the presence of oral mucositis depends on the dose of a chemotherapeutic agent, the number of

administration cycles, gender, weight, and height of the patient in humans, and genetic susceptibility are risk factors for mucositis. Intraperitoneal (IP) administration of single high or multiple low doses of 5-Fluorouracil is the most widely used and simple method to induce mucositis and disrupt epithelial proliferation [30].

Chang et al (2015) used a dose of 100 mg/kg 5-fluorouracil by IP injection without any chemical stimulus or mechanical trauma to the oral cavity of rats to induce oral mucositis. Macro analysis conducted after two weeks of 5-fluorouracil administration found severe erythema and histopathological observations in the fifth week of rat oral mucosa showed the prevalence of neutrophils, hemorrhagic areas, edema, and extensive ulcers [22]. Research shows that oral mucositis is hard to induce in experimental animals using only chemotherapy, or sans radiation, partly due to the protective stratified squamous epithelium of rats in the oral cavity. Therefore, additional mechanical or chemical trauma to the oral mucosa of rats was generally required to develop chemotherapeutic agent-induced mucositis [31].

Research by Kim DH et al. (2023) injected 5-fluorouracil in rats with three different doses, namely doses of 160, 200, and 240 mg/kg. Dose of 160 and 200 mg/kg caused rats to live until day 7 and given 25% acetic acid as a stimulus, while two out of four rats injected with a dose of 240 mg/kg experienced death on days 2 and 3 [32]. Thieme et al. (2020) used a dose of 100 mg/kg, given in two stages of injection, namely 60 mg/kg and 40 mg/kg for days 1 and 2, respectively. The results reported oral mucositis appeared on day 5 and without death until day 14 observation [33]. Takauchi et al. (2020) administered three doses of 5-fluorouracil to rats, showing that 40 mg/kg was the optimal dose to induce oral mucositis. However, at 50 mg/kg, mortality reached 40% within days, and all rats exhibited severe weight loss (95-98%) by the 5th day of observation [31]. The chemotherapeutic agent is known to induce mucositis with severe diarrhea, leading to anorexia, dehydration, and various systemic toxicities that cause weight loss, causing death to rats due to stress [30]. The optimal dose of 5-fluorouracil in inducing oral mucositis is still uncertain, as previous research showed many differences. This research investigated the optimal dose of 5-fluorouracil, administered intraperitoneally, to induce oral mucositis in rats, with and without 50% acetic acid as a chemical stimulus. The aim was to establish a preclinical rat model for evaluating the efficacy of interventions to prevent and treat chemotherapy-induced oral mucositis.

2. Materials and Methods

This preliminary research was conducted on Wistar rats to determine the correct dose for administering 5-fluorouracil chemotherapy drugs intraperitoneally. The dose of chemotherapy drugs was used to administer herbal medicine to rats as a drug to accelerate the healing of mucositis without mortality due to the anticancer drug. This research obtained a permit from the Animal Research Ethics Committees Faculty of Mathematics and Natural Sciences - University of North Sumatra (AREC) with No. 0580/KEPH-FMIPA/20. An *in vivo* laboratory experiment was carried out on male Wistar rats (*Rattus norvegicus*) at the Scholar Research Laboratory. A total of 24 male Wistar rats were used, which were divided into eight test groups. Six groups were given 5-fluorouracil without giving chemical stimulus (acetic acid), namely rats that received doses of 200 mg/kg, 175 mg/kg, 150 mg/kg, 125 mg/kg, 75 mg/kg, and 70 mg/kg. The other two groups were given 5-fluorouracil injection plus chemical stimulus (50% acetic acid), namely rats that received a dose of 100 mg/kg (D0: 60 mg/kg, D2: 40 mg/kg), 60 mg/kg.

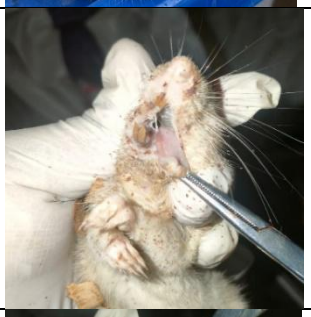
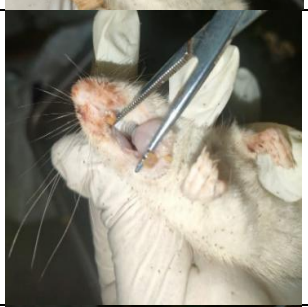
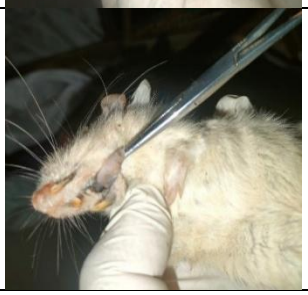
The simple random sampling method was used for each test group. The inclusion criteria were male Wistar rats with a body weight of 150-200 grams, 8-12 weeks old, and healthy with marked active movement, while the exclusion criteria were rats with a weight loss of > 10% or < 150 grams.

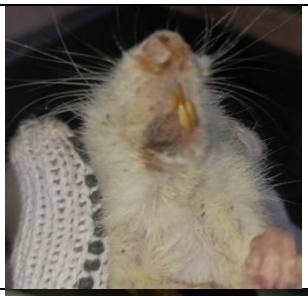
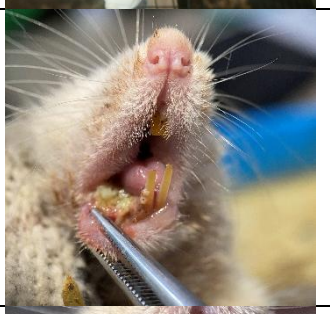
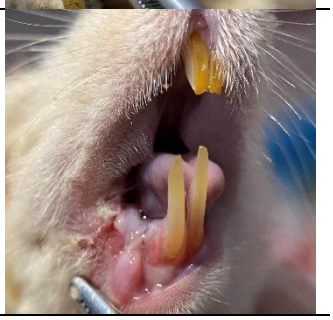
The experimental animals were divided into eight test groups and acclimatized for seven days. Induction of oral mucositis in rats was carried out by giving general anaesthesia through IP injection using ketamine HCL (0.2 ml/injection) with a 1 ml syringe. Rat was then injected with chemotherapy drugs 5-Fluorouracil intraperitoneally at a dose of 200 mg/kg, 175 mg/kg, 150 mg/kg, 125 mg/kg, 100 mg with two stages of injection (D0: 60 mg/kg, D2: 40 mg/kg), 75 mg/kg, 70 mg/kg and 60 mg/kg. On the second day, rats receiving 5-fluorouracil injections (100 mg/kg and 60 mg/kg) were subjected to a chemical stimulus. A 3x3 mm Whatman paper no. 1 soaked in 50% acetic acid (H3COOH) was applied to the buccal mucosa for 30 seconds, inducing damage to the oral mucosal layer. Subsequently, the rat buccal mucosa was observed for 21 days to determine the appearance of oral mucositis and the death of the rat.

3. Results

The results of the optimal dose of 5-Fluorouracil in triggering oral mucositis were tested in 21 days. Ulcers on the oral part were observed in all rats that survived after the administration of chemotherapeutic agents. Rats given 5-Fluorouracil at a dose of 200 mg/kg died on average on day 8.33 (days 8, 8, 9) with oral mucositis appearing on day 5, but on day 6 cheek necrosis occurred (Table 1). Rat injected with 5-Fluorouracil at doses of 175 mg/kg, 150 mg/kg, 125 mg/kg, 75 mg/kg, and 70 mg/kg experienced average mortality on days 8.67 (D-8, 9, 9), 10.33 days (D-10, 10, 11), 10,67 days (D-10, 11, 11), 11.33 days (D-11, 11, 12), 13.67 days (D-15, 13, 13) without any oral mucositis. Furthermore, rats injected with a dose of 100 mg/kg at two stages (D0: 60 mg/kg, D2: 40 mg/kg) survived on an average of 13.34 days (D-15, 13, 13) and oral mucositis appeared on day 7. Rats injected with a dose of 60 mg/kg survived for more than 21 days with oral mucositis appearing on day 5, as shown in Table 1.

Table 1. Optimal dose of 5-Fluorouracil-induced oral mucositis in rats

No.	5-Fluorouracil dosage	Giving stimulus (50% acetic acid)	Average Length of Life of Rats	Time Oral mucositis appeared	Image
1.	200 mg/kg	-	8.33 days	5 days	
2.	175 mg/kg	-	8.67 days	-	
3.	150 mg/kg	-	10.33 days	-	
4.	125 mg/kg	-	10.67 days	-	

5.	75 mg/kg	-	11.33 days	-	
6.	70 mg/kg	-	13.67 days	-	
7.	100 mg/kg two-stage dosing. D0: 60 mg/kg, D2: 40 mg/kg	+	13.34 days	7 days	
8.	60 mg/kg	+	>21 days	5 days	

From the observations, it was found that the optimal dose of oral mucositis in rats was 60 mg/kg with the *chemical stimulus* using 50% acetic acid (Figure 1), where erythema, hyperemia, bleeding areas, ulcers, and abscesses were seen.

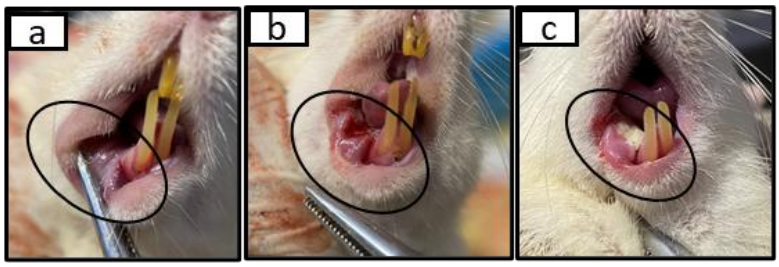


Figure 1. Development of oral mucositis in rats using a dose of 5-Fluorouracil 60 mg/kg and 50% acetic acid. (A) Healthy oral mucositis was observed on the first day after the rats were injected with 5-fluorouracil; (B) Ulcers appearing on the 4th day after 50% acetic acid administration; (C) Oral mucositis worsening on the 6th day.

4. Discussion

Chemotherapy-induced oral mucositis is an inflammatory condition of mucosal cells in the oral cavity characterized by erythema and atrophy/ulceration. It is experienced by patients undergoing cancer treatment

using chemotherapy. Some studies show that around 80% of patients undergoing high-dose chemotherapy will experience oral mucositis, as well as around 20-40% of patients undergoing conventional chemotherapy.[34,35] Oral mucositis occurs depending on the dose, the number of cycles of chemotherapy, the anticancer regimen given, and the patient's characteristics, such as gender, weight, height of the patient, and genetic susceptibility, are risk factors for mucositis.[33]

The most common chemotherapeutic agents that cause mucositis are 5-FU (5-Fluorouracil), Irinotecan, and *Methotrexate*.[17,21] 5-Fluorouracil at high or low doses can cause oral mucositis, but only at different times.[23,36] In some literature, the dose of 5-Fluorouracil and the method to cause oral mucositis differ. After conducting macro analysis, Chang et al. (2015) found erythema in the second week without bleeding, extensive ulcers and abscesses, in a dead rat during the research period, after dosing 100 mg/kg-fluorouracil by intraperitoneal injection.[22] Takeuchi et al. (2020) found that the optimal dose for inducing mucositis was 40 mg/kg. A dose of 50 mg/kg, however, caused 40% mortality of the entire number of experimental rats. In contrast, according to research conducted by Kim DH et al. (2023), the optimal dose to cause oral mucositis is 200 mg/kg. Still, this dose can cause immune-suppressive status, failure of weight gain, and impairment of epithelium regeneration in rats.[32] Miyano (2020) used a 40 mg/kg dose with three injections.[37]

In this research, tests were carried out to bring up oral mucositis using only 5-fluorouracil without being given a chemical stimulus (acetic acid) at a dose of 200 mg/kg, 175 mg/kg, 150 mg/kg, 125 mg/kg, 75 mg/kg, 70 mg/kg, then using 5-fluorouracil and given a chemical stimulus (acetic acid on the second day at a dose of 100 mg with two stages of injection (D0: 60 mg/kg, D2: 40 mg/kg), and 60 mg/kg. From the preliminary study conducted, it was found that the dose that did not cause mortality in rats up to day 14 was 100 mg with two stages of injection (D0: 60 mg/kg, D2: 40 mg/kg) and as well as the lowest dose of 60 mg, so in this study, these two doses were administered along with a chemical stimulus in the form of 50% acetic acid.

In rats given 5-fluorouracil at 200 mg/kg dose without chemical stimulus, mucositis appeared on the day 5. However, the rats had tissue necrosis on the outer cheek, a drastic decrease in body weight, and died on an average of day 8.33 (D-8, 8, 9). Rats given 5-fluorouracil without chemical stimulus at doses of 175 mg/kg, 150 mg/kg, 125 mg/kg, 75 mg/kg, and 70 mg/kg experienced death on the average of day 8.67 (D-8, 9, 9), 10.33 days (D-10, 10, 11), 10.67 days (D-10, 11, 11), 11.33 days (D-11, 11, 12), 13.67 days (D-15, 13, 13) without oral mucositis appearing in rats. Similarly, rats given 5-fluorouracil at a dose of 100 mg/kg with two stages of injection (D0: 60 mg/kg, D2: 40 mg/kg) experienced oral mucositis on the buccal mucosa on the day 7. Furthermore, in rats given 5-fluorouracil at a dose of 60 mg/kg, oral mucositis appeared in the buccal mucosa of rats on the day 5 after 5-fluorouracil administration where erythema, hyperemia, bleeding areas, ulcers, and abscesses were seen in the rat mucosa, and rats survived for more than 21 days.

In this observation, the optimal dose to induce oral mucositis was 60 mg/kg, lower than 200 mg/kg of previous research [30,32]. The administration of a high dose may affect the severity and duration of oral mucositis. Furthermore, the chemotherapeutic agent 5-fluorouracil is highly cytotoxic due to its conversion to *fluorodeoxyuridine monophosphate* (FdUMP) in the cell. This forms a stable complex with *thymidylate synthase* (TS), which will then inhibit the production of *deoxythymidine monophosphate* (dTMP). Furthermore, 5-Fluorouracil can be directly connected to DNA, resulting in nucleotide damage, and can affect RNA synthesis, interfering with cellular protein formation and causing apoptosis or cell death [38]. Potential factors contributing to the observed effects include stress induced by injection in rats, even at low doses. Additionally, 5-fluorouracil is known to trigger mucositis in intestinal organs, leading to severe diarrhea, anorexia, dehydration, and systemic toxicities. These factors result in significant weight loss in rats. Furthermore, the direct toxicity of 5-Fluorouracil to the epithelium, particularly the basal and supra-basal layers, can induce cell death during the initiation phase, impairing healing processes and leading to prolonged wound formation [31,32].

In contrast to other research, Chang et al. (2015) and Mohammed et al. (2023) induced oral mucositis using solely 5-fluorouracil as the chemotherapeutic agent, without chemical or mechanical stimuli [22,36]. However, oral mucositis did not appear until all rats died, and 50% acetic acid was used as a chemical stimulus agent to destroy the basal layer of the epithelium. This was applied using *Whatman* paper no.1 size 3x3 mm, soaked in acetic acid. Furthermore, this research used 50% acetic acid according to Miyano (2020), Yamaguchi, et al (2016), and Katagiri, et al (2018) [37-40]. Tancharoen, et al (2018) used mechanical trauma,

namely shallow scratching with an 18-gauge needle tip to irritate locally and induce mucosal ulceration [41]. However, this method experiences difficulty in determining the depth and area of scratching to be made on the rat mucosa. This research used acetic acid because the dose and area of paper used during application could be determined. Acetic acid is needed to destroy the surface layer of the oral mucosa as a chemical stimulus, which will then cause oral mucositis with 5-fluorouracil as an inducer. This acid is important as a stimulus in the formation of oral mucositis due to chemotherapy, while Yoshino et al. (2013) found that 5-Fluorouracil treatment did not induce oral mucositis [42]. The oral mucosal tissue of the rats became perforated with the administration of 5-Fluoruracil. Acetic acid damaged the surface layer and excessive cell proliferation leading to ulcers with a large area and duration sufficient for further research [31,37].

The limitation of this research is the absence of histological tests performed on the injured rat mucosa and the lack of observation on weight loss in the rat after chemotherapy injection. Future research should include histological tests to assess the condition of the mucosal epithelial cells and record the weight loss in the rat.

5. Conclusion

In conclusion, the optimal dose to induce oral mucositis was 60 mg/kg. The combination with 50% acetic acid as a chemical stimulus agent, could induce wounds on the buccal mucosa of rats on day 5.

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7. Conflict of Interest

The authors declare that there are no conflicts of interest to disclose concerning this research.

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