

Study on development and tissue permeation of different formulations of Fasudil

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Abstract. – OBJECTIVE: The study is intended to formulate Fasudil loaded vesicular system for application in the management of angina.

MATERIALS AND METHODS: Fasudil was made into a complex with phospholipid, and other different formulations were made, including Fasudil solution, liposomal form, and Fasudil loaded into the gel. A drug characterization study was conducted and noted. Drug release was quantified and analyzed and, finally, inoculated in Sprague-Dawley rats. These rats underwent anginal induction, and each formulation's effect on angina was evaluated.

RESULTS: Drug solution (F-Phos) and F-Phos-Lipo (liposomal dispersion form of the drug) have shown that more than half percent of them have been released within 1.5 hours, and the rapid release occurred from liposomal dispersion in the first hour. The study determined the viscosity of the different formulations, which was significantly ($p < 0.05$) higher than the theoretical sum of the viscosity of each formulation. The study found that the F-Phos-Lipo+P-407HMS formulation is the most effective as its application has the minimum infarct area percentage compared to the other formulations and can also reduce creatine kinase levels significantly as compared to the different formulations ($p < 0.05$).

CONCLUSIONS: The study concluded that the typical gel formulation (liposomal Fasudil dispersed in hydroxypropyl methylcellulose

solution, which is added to blank poloxamer 407) had been shown to have significantly anti-anginal properties, including easy administration, its application on the infarct area percentage and subsequently its pharmacological effect on the cardiac tissue.

Key Words:

Fasudil, Vasodilator, Angina, Infarct, Liposome.

Introduction

Around 50% of people with coronary artery disease have signs of chronic stable angina¹, which can be caused by either emotional or physical pressure. The current treatment guidelines, include drug treatment to decrease heart rate and blood pressure and increase heart muscle venous return, clinical or vertebral artery dissection cardiac failure, and several alternative procedures, such as transmural surgical intervention and enhanced outer counterpulsation. Clinical or incision revascularization processes are also obtainable. Furthermore, even after obtaining treatment strategies or undergoing cardiac surgery operations, most people continue to demonstrate signs of their disease.

Therefore, more current therapies are needed. An intracellular signaling material identified as the Rho transcription factor has been suggested as a potential medicinal target in treating stable angina². Rho kinase is concerned with the vascular smooth muscle contractile response to agonists like acetylcholine, angiotensin II, nuclear factor, levels of serotonin, platelet-derived nuclear factor, and serotonin. The movement of smooth muscle cells is driven by G-protein-coupled proteins, which stimulate Rho, which again stimulates Rho kinases. The Rho transcription factor is ultimately responsible for the contract. Myosin regulatory light chain (MRLC) phosphatase is activated and increased when it is linked to Rho kinase.

Due to the shortage of MRLC solid phosphatase, MRLC decides to stay in its activated state. Consequently, the experience of actin and myosin leads to contractions that can last for a greater amount of time. Rho kinase drugs have been demonstrated in mice of vascular tension to be an efficient means of preventing blood flow from developing³. *In vivo* experiments on human nerves have produced findings similar to these theories. A central venous version of Fasudil, a specific Rho medication that has been approved to be used in Japan to reduce cerebral vasospasm during subarachnoid hemorrhage.

Furthermore, this medicine is already under evaluation for use in angina therapy. Initial phase studies were conducted on people with chronic stable angina whose clinical and laboratory signs

of ischemia were noted after giving choline to them were involved in various initiation phase studies. Based on the research⁴, a cardiac infusion of 4.5 milligrams of Fasudil provided for fifteen minutes was able to avoid acetylcholine-induced ischemia in 85% of the people. Following oral administration, the body can process Fasudil with a half-life of 5.5 to 0.87 hours when taken in capsule form.

Fasudil pharmacological intervention at concentrations ranging from 5 mg three times a day to 40 mg three times daily enhanced total time to the start of 1 mm ST-segment disorder similar to baseline levels in initial statin experiments carried out in Japanese patients with moderate effort angina^{2,3}. The above processes were carried out in patient populations in Japan who had similar indications. Fasudil was well received, and its impacts on heart rate and blood pressure, both at rest and during walking, were negligible^{4,5}.

Fasudil is an effective vasodilator and a Rho-kinase inhibitor^{1,5}. It is a Bio Pharmaceutics Classification System (BCS) class IV medication. The drug's reported bioavailability was 15%^{1,2}. Since its discovery, it has been used to treat cerebral vasospasm, which is frequently caused by subarachnoid hemorrhage, and improve stroke patients' cognitive decline. It controls pulmonary hypertension effectively³. Fasudil has been shown to enhance memory in normal mice, indicating that it could treat age-related or neurodegenerative memory loss^{4,6}. Figure 1 shows the 3D and 2D structure of the Fasudil.

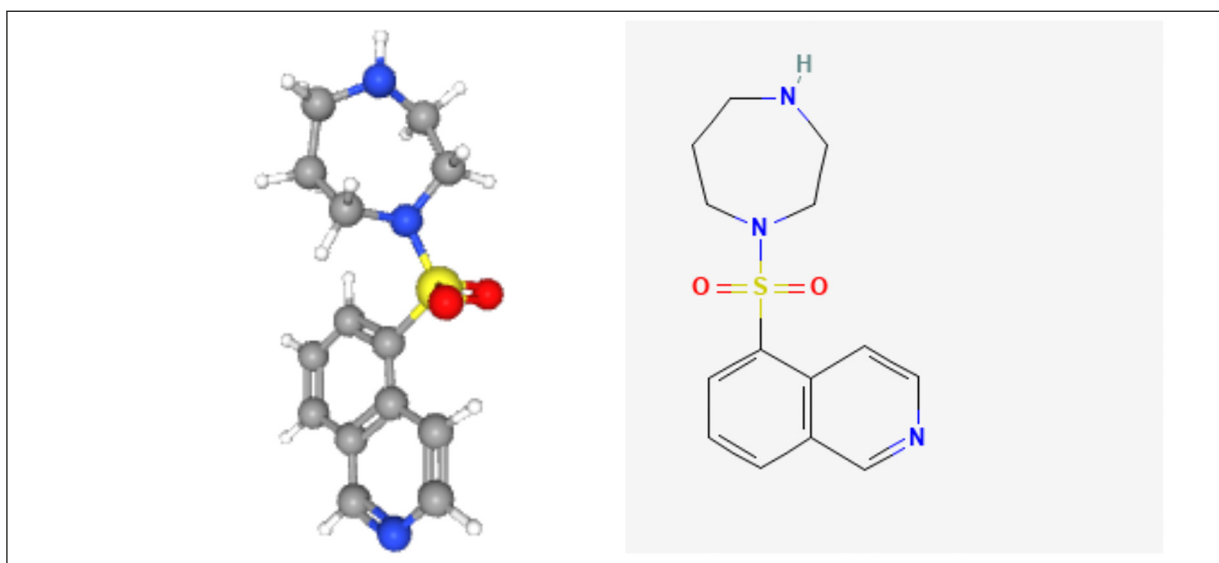


Figure 1. 3D and 2D structure of fasudil (C₁₄H₁₈CIN₃O₂S) Source: National Center for Biotechnology Information (2022).

Pulmonary Hypertension (PH) has recently received much attention because it has a much higher morbidity and mortality rate than previously thought^{7,8}. PH affects about 1% of the global population and is especially common (up to 10%) in people over 65⁷⁻⁹. As a pulmonary vascular disease, PH is distinguished by progressive increases in pulmonary vascular resistance (PVR) and pulmonary artery pressure (PAP) due to pulmonary vasoconstriction and remodelling¹⁰. The clinical diagnostic criteria for PH were mean pulmonary arterial pressure (mPAP) more significant than 20 mmHg at rest (greater than 30 mmHg during exercise), pulmonary arterial wedge pressure (PAWP) greater than 15 mmHg, and PVR greater than 3 Wood units^{10,11}.

A hemorrhagic stroke occurs when a blood vessel ruptures, resulting in brain bleeding. It is classified into two types: intracerebral hemorrhage (ICH), which occurs within the brain parenchyma, and subarachnoid hemorrhage (SAH), which occurs within the subarachnoid space¹¹. Hemorrhagic stroke accounts for 10-20% of all strokes and 40% of stroke-related deaths, which piqued researchers' interest due to its high mortality rate¹². Hemorrhagic stroke mortality rates range from 25-30% in high-income countries to 30-48% in low-to middle-income countries¹³.

Materials and Methods

The study was conducted in the Department of Pharmaceutics, University of Hail, Hail, Kingdom of Saudi Arabia, from November 2021 to October 2022.

Research Design and Sample Collection

Materials collected or purchased for this study were Fasudil hydrochloride (molecular formula: $C_{14}H_{18}ClN_3O_2S$), 100% soybean phosphatidylcholine, hydroxypropyl methylcellulose (HPMC K₄M), cholesterol and porcine mucin. Firstly, Fasudil is made into a complex with phospholipid (F-Phos) by employing solvent evaporation. They were prepared into a complex in various molar ratios at storage temperature (25°C) and the upper limit of body temperature (42°C). The time was maintained for 2 hours. The mixture of Fasudil and phospholipid was made, and then an evaporator was used to obtain the dry residue for further analysis.

A methanol injection procedure was employed to prepare the liposome dispersion loaded into

F-Phos, indicated as "F-Phos-Lipo". The authors added 3 ml of methanol into 80 mg of F-Phos, and deionized water was added to this mixture. The organic solvent was again evaporated at 55°C. The residue was refrigerated at 5°C to 8°C. Following the same technique, a blank liposome was prepared without adding Fasudil.

Different concentrations of blank poloxamer 407 (P-407), ranging from 17.5 to 25% w/w, were considered for the preparation of gels. Cold deionized water (4-8°C) was used for dissolving P-407, and the gel preparation was made by refrigeration overnight. For making the final gel preparation, Fasudil complex-loaded liposome-P407 was dispersed at a cold temperature. Hydroxypropyl methylcellulose solution was added to the liposomal dispersion in the same ratio of 1:1 and refrigerated overnight. Therefore, there were two gel preparation types: blank poloxamer 407 (P-407) and Hydroxypropyl methylcellulose solution added to P-407 (P-407HMS).

The entrapment efficiency (EE%) for F-Phos-Lipo and P-407HMS was determined by ultrafiltration/centrifugation for 30 minutes at 4,000 rpm at 4°C. Free Fasudil concentration was determined by ultraviolet-visible (UV) spectrophotometry, maintained at the coefficient of 0.999; limits of detection and quantitation were set at 2.023 and 6.131 µg/mL, respectively. Characterization studies were conducted for each emulsion. Sprague-Dawley rats were used for animal experimentation. Cardiac ischemia was induced, and the formulations prepared in this study were assessed for efficacy by determining the infarct area, left ventricular room, and creatinine kinase.

Animal Experimentation

The study considered Sprague-Dawley rats of 7-8 weeks, with an average weight of 260.42±10.5 grams. In total, there were 80 rats, and they were classified into four groups based on the formulation they received. The study protocol was conducted by "The Guide for the Care of Use of Laboratory Animals", formulated by the National Institute of Health (NIH). At first, the rats were given 2 weeks to adapt to the environment before the experiments. Each formulation was equivalent to 2.5 mg of Fasudil and was inoculated into the rats as classified.

Then, the rats were inoculated with sodium heparin at a dosage of 150 U/kg, and then left coronary artery occlusion was triggered. The animals were anesthetized using pentobarbital

intraperitoneally at 50 mg/kg. Then, they were kept in a ventilated room, maintaining a body temperature of 37°C, and a left thoracotomy was done. Then, the left coronary artery (LCA) was encircled by a 6-0 prolene suture just distal to its first branch, and its ends were tied with polyethylene tubing forming a snare, post-pericardiomy. The cardiac ischemia was confirmed by its pale color, cyanosis, and ST-T elevation in ECG.

Statistical Analysis

The study used SPSS v. 25 (IBM Corp., Armonk, NY, USA) and MS Excel software for practical statistical analysis. The statistical analysis was conducted using ANOVA, considering $p < 0.05$ as the significance level. The continuous variables were expressed as mean \pm standard deviation. The study process was performed following the Declaration of Helsinki guidelines.

Results

The study found complexation efficiency by combining Fasudil and Phospholipid in the ratio of 1:1 and 1:2. Table I shows the complexation efficiency in each ratio of the drug.

The study prepared various formulations of liposomes. Based on the particle size and Poly-Dispersity Index (PDI), the liposomal formulation F2 was chosen for further research and to consider as the dispersion medium for preparing F-Phos-Lipo. Table II shows the detailed properties of liposomal formulations.

The storage of formulations for three months has shown that the increase in particle size of F-Phos-Lipo is statistically insignificant ($p > 0.05$). Again, P-407HMS is shown to significantly change particle size and PDI ($p < 0.05$), implying that the storage stability is lower than F-Phos-Li-

po. Table III shows the detailed findings of the storage efficacy of F-Phos-Lipo and P-407HMS.

Each drug formulation was characterized in terms of differential scanning calorimetry (DSC), Fourier-transform infrared spectroscopy (FTIR), X-Ray diffraction (XRD), and differential thermal Analysis (DTA). Table IV shows the detailed characteristics of the drug formulations.

The study also analyzed the *in vitro* release of Fasudil from each formulation to compare the release of encapsulated drug forms with that of the normal solution. Drug diffusion compatibility influences the drug release mechanism through the cell membrane's lipid bilayer (Figure 2). The study found that the drug solution was released ultimately within 2.5 to 3 hours, while the controlled release was noted in each formulation. Drug solution (F-Phos) and F-Phos-Lipo (liposomal dispersion form of the drug) have shown that more than half percent of them have been released within 1.5 hours, and the rapid release occurred from liposomal dispersion in the first hour. Following the first hour, the drug was slowly released in the liposomal form. P-407HMS was found to have the slowest or most sustained release in the initial hours. After 4 hours, it steadily increased the highest capability to release the drug compared to the other formulations.

The study determined the viscosity of the different formulations, which was significantly ($p < 0.05$) higher than the theoretical sum of the viscosity of each formulation individually, given that the dilution ratio is maintained. Table V shows the mean viscosity of different preparations.

The study found that the F-Phos-Lipo+P-407HMS formulation is the most effective as its application has the minimum infarct area percentage compared to the other formulations and can also reduce creatine kinase levels sig-

Table I. Complexation efficiency in each ratio of drug and phospholipid ratios.

At 25°C at 2 hours reaction time	
Drug and phospholipid ratio	Complexation efficiency (%)
1:1	48.58 \pm 0.22
1:2	56.32 \pm 0.22
At 42°C at 2 hours reaction time	
Drug and phospholipid ratio	Complexation efficiency (%)
1:1	81.41 \pm 0.24
1:2	88.51 \pm 0.32

Table II. The findings of various formulations of Liposomes used in this study with their characteristics found.

Formulation	Particle size (nm)	PDI	Zeta potential (mV)
F1	157.77 ± 4.31	0.1 ± 0.001	-21.8 ± 0.8
F2	247.7 ± 6.45	0.3 ± 0.07	-22.25 ± 0.7
F3	259.66 ± 4.36	0.4 ± 0.038	-22.91 ± 0.9

Table III. Findings of storage efficacy of F-Phos-Lipo and P-407HMS.

Formulations	Point of time	Particle size (nm)	Zeta potential (mV)	PDI	Entrapment efficiency (%)
F-Phos-Lipo	After preparation	133.58 ± 1.7	-22.1 ± 0.96	0.3 ± 0.01	77.42 ± 0.26
	After six months	140.11 ± 2.2	-25.74 ± 0.8	0.3 ± 0.01	71.19 ± 0.28
P-407HMS	After preparation	239.74 ± 3.7	26.85 ± 1.6	0.3 ± 0.2	78.41 ± 0.6
	After six months	275.45 ± 5.1	23.97 ± 1.2	0.3 ± 0.0012	72.57 ± 0.9

PDI = Polydispersity Index.

Table IV. Characterization of the drug formulations developed in this study.

Parameters	F-Phos	F-Phos-Lipo	F-Phos+P-407HMS	F-Phos-Lipo+P-407HMS
DSC	30.11 ± 2.84°C	31.57 ± 3.18°C	33.47 ± 2.85°C	36.18 ± 0.98°C
FTIR (cm ⁻¹)	3,845 ± 1,231	3,745 ± 1,329	3,589 ± 1,147	3,294 ± 1,448
XRD	421.21 ± 224.3	433.52 ± 238.4	445.12 ± 312.2	422.95 ± 265.14
DTA (uV)	12.54 ± 8.44	15.75 ± 10.47	18.71 ± 9.58	17.58 ± 6.77

DSC: Differential Scanning Calorimetry; FTIR (cm⁻¹): Fourier Transform Infrared Spectroscopy (wavenumber in centimeters inverse); XRD: X-ray Diffraction; DTA (uV): Differential Thermal Analysis (microvolts); F-Phos: Fasudil-Phospholipid Complex; F-Phos-Lipo: Fasudil-Phospholipid Complex loaded into Liposomes; F-Phos+P-407HMS: Fasudil-Phospholipid Complex loaded into Liposomes and Poloxamer 407 Hydroxypropyl Methylcellulose Solution; F-Phos-Lipo+P-407HMS: Fasudil-Phospholipid Complex loaded into Liposomes and Poloxamer 407 Hydroxypropyl Methylcellulose Solution.

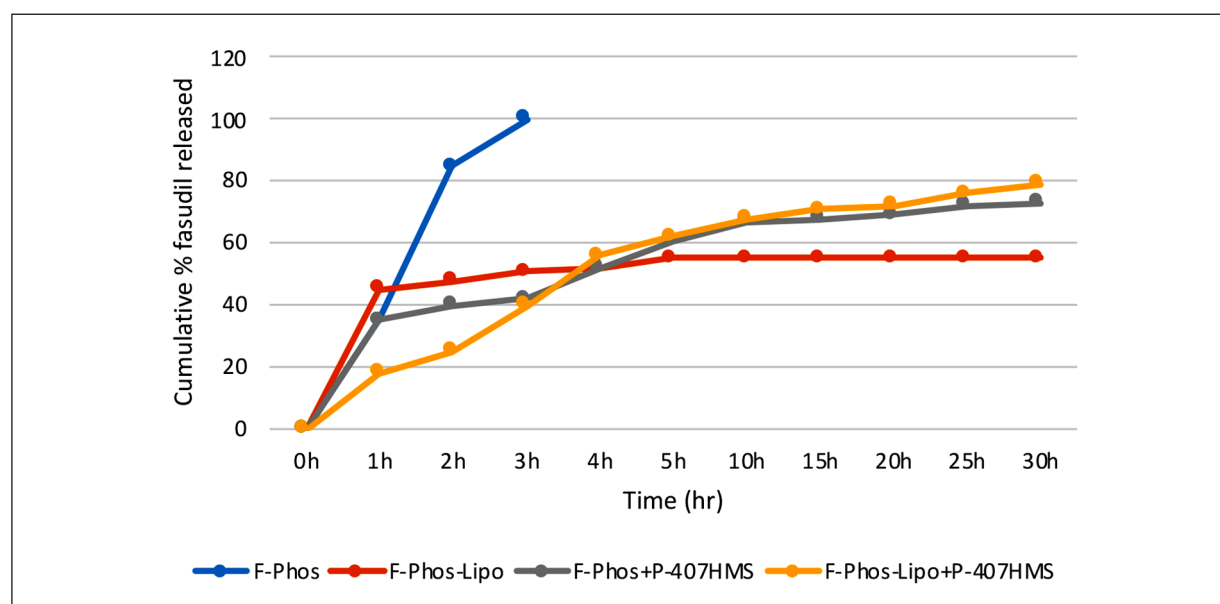

Figure 2. Release of fasudil formulations over 30 hours at a constant pH of 7.4 maintained at 50 pm and 37°C.

Table V. Effect of various formulations on infarct area and creatinine kinase.

Preparation	Mean viscosity (Cp)			
	Viscosity of water/mucin	Theoretical sum of 2 viscosities	Viscosity of formula/water	Viscosity of the mucoadhesion mixture
F-Phos	4.24 ± 0.21	5.14 ± 0.85	1.97 ± 0.01	9.12 ± 1.20
F-Phos-Lipo	4.17 ± 0.13	9.14 ± 0.94	6.29 ± 0.34	35.25 ± 1.95
F-Phos+P-407HMS	4.18 ± 0.22	10.12 ± 0.74	5.78 ± 0.75	39.67 ± 3.62
F-Phos-Lipo+P-407HMS	5.29 ± 0.32	12.84 ± 0.47	8.28 ± 0.55	55.45 ± 2.59

C p= Centipoise.

nificantly as compared to the other formulations ($p < 0.05$). Table VI details the effect of different formulations used in this study on the myocardial parameters.

Discussion

In a study group of people with stable angina, Fasudil at a dose of 80 mg three times daily over eight weeks was indicated to enhance workout time compared to baseline¹⁴. Therefore, consuming 80 mg of Fasudil three times a day increased 1 mm of ST-segment depression, which was statistically significant and about four times greater than that seen in the control group. The impacts of Fasudil on myocardial injury are at their lowest 4 hours after the last dose was provided¹⁵. Another study¹⁶ reviewed apoptosis of cardiomyocytes via Rho-Associated Protein Kinase (ROCK) signaling pathway, showing that Fasudil inhibits this pathway significantly and leads to the release of different types of ROCK. This results in the inhibition of oxidative stress, and hence, improvement of cardiac function occurs. Again, ROCK release allows the prevention of apoptosis.

Studies reported that Compared with a placebo, the use of Fasudil was linked with major significant improvements in mental restriction levels on the Safety Attitudes Questionnaire (SAQ) and therapy satisfaction scores. Moreover, a

positive pattern was noticed in the CCS group. Between the different treatments, there were no substantial differences in the duration of time before the development of angina, the number of times each week when angina occurred, or the amount of nitroglycerin consumed. 2 subjects from control group had similar rates of withdrawal from the research due to adverse events or severe circumstances leading to withdrawal^{17,18}. Most negative impacts were evaluated to be moderate and were not thought to be connected to the new medication, the findings that have been gathered from the systematic review performed by Prajapati et al¹⁹ were optimistic. The amount of the intended impact was evaluated in the calculated results in this systematic review, while the fundamental change in patient training duration was not taken into consideration. Therefore, more analyses are required to determine whether medication with Fasudil is related to increased exercise levels. However, there has been substantial strength in the research to identify differences in the time it has taken for ST-segment decline to achieve 1 mm, which was the primary final stage. They are also associated with a poor prognosis. In addition, the impact of Fasudil on a 1 mm ST-segment elevation is equal to the strength of information increases in current investigations²⁰⁻²² with the beta-blocker propranolol and nine calcium-channel blockers relative to placebo.

Table VI. Effect of various formulations on infarct area and creatinine kinase.

Parameters	F-Phos	F-Phos-Lipo	F-Phos+P-407HMS	F-Phos-Lipo+P-407HMS	p-value
Infarct area/left ventricular area (%)	26.21 ± 3.66	19.22 ± 4.25	13.74 ± 2.69	8.86 ± 2.85	0.0257
Infarct area/area at risk (%)	22.45 ± 1.32	14.31 ± 2.58	11.36 ± 3.21	7.19 ± 2.15	0.0314
Creatine kinase (IU/L)	2,134.4 ± 324.1	1,854.2 ± 365.2	1,741.2 ± 256.5	1,211.5 ± 127.5	0.0491

Several tried-and-true treatment strategies for angina reduced the period before myocardial ischemia started by 1.4 ± 2.7 minutes. The increase of 2.1 minutes noted with Fasudil is within this area and was obtained with the continuous usage of other medications as a context. In the way of comparison, a limited fatty acid oxidation drug that is under development for the therapies of symptoms increased the duration of myocardial ischemia at maximum when compared to placebo by 55.6 ± 8.2 seconds as statin therapy and by 34.5 ± 11.9 seconds when provided in regard to atenolol, amlodipine, or diltiazem^{22,23}. These results were obtained when comparing Ranolazine to the treatment group.

Conclusions

The study used liposomes for enhancement of the pharmacological use of Fasudil. Although Fasudil is regarded as a novel ROCK inhibitor, low entrapment and lower release capacity have restricted its practical application in the medical field. Hence, Fasudil was made into a complex with phospholipid, and various formulations were analyzed. The study concluded that the typical gel formulation (liposomal Fasudil dispersed in Hydroxypropyl methylcellulose solution, which is added to blank poloxamer 407) had been shown to have significantly anti-anginal properties, including easy administration, its application on the infarct area percentage and subsequently its pharmacological effect on the cardiac tissue. The study is limited by its lack of experimentation on various conditions, and different pharmacological properties could not be analyzed due to a lack of resources. The tolerability by the tissues and human trial needs to be conducted for effective formulation to be used as final.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Ethics Approval

The study was approved by the Research Ethics Committee (REC) at the University of Hail with the number H-2021-195.

Informed Consent

Not applicable.

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Authors' Contribution

We declare that this work was done with the equal contribution of all the authors of this article.

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