Original Article

The Effect of Nefopam Hydrochloride on the Liver, Heart, and Brain of Rats: Acute Toxicity and Mechanisms of Nefopam Toxicity

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Abstract

Nefopam hydrochloride (Acupan) is commonly used to relieve moderate to severe postoperative pain. This drug is generally well tolerated; however, it has been associated with cardiac conduction problems, cerebral edema, fever, and renal failure when taken in excess. The aim is to investigate the effect of toxic doses of nefopam on the liver, heart, and brain of rats after exposure (acute toxicity of nefopam and concentration in these organs. Demonstrate whether TNF-α and NF-κB, and some selected interleukins (IL1β and IL-10) have a role in the mechanism of organ injury induced by nefopam Methods: Fourteen(14) adult rats, Group I controls n = 7: Rats were administered a single intraperitoneal injection (IP) with normal saline Group II Nefopam-treated animals: each rat injected with a one toxic dose IDP (40 mg/kg) Nefopam and then sacrificed 24 hours after drug administration, and whole blood and organs were collected. Results: After injection of a toxic dose of nefopam showing a decrease in serum level of TNF-α (17.96 Pg per ml to 14.53 Pg per ml) and a decrease in IL 10 (from 36.26 to 23.22) and IL from (6.0 to 5.3 pg/ml) and an increase in NF-κB (396.314 TO 416.266 Pg/ml), the cardiac marker also changed CK-MB. Conclusion: Nefopam(acute toxic model) produced various effects on serum levels of cytokines (whether inflammatory or anti-inflammatory markers), in addition, it did not have significant effects on CK-MB, ALT, AST, and ALP; this may indicate that this acute toxic dose of Nefopam did not have significant effects on heart and liver tissues.

Keywords: nefopam toxicity; inflammatory markers; TNF-α; cardiac markers, HPLC analysis.

Introduction

The term toxicity indicates the extent to which a chemicals (toxin or poison) can cause harmfull effect to human (1), Various mechanisms of harmful effects of drugs and chemicals were demonstrated, including: I- development of reactive oxygen and nitrogen species (ROS and RNS) that can make injury to target molecule damage (2) ; II-dysregulation of gene expression and transcription (3); III-dysregulation of electrically excitable cells such as nerve cell, skeletal, cardiac, and smooth muscle cells; IV-alteration of the immune response; V-apoptosis; and VI-sustained elevation of intracellular Ca2+ that has been reported to produce harmful consequences (4,5).

Nefopam is a analgesic act on pain centers of the brain however its nonopioid pain killing drug belongs to benzoazocine group . It is frequently used as an alternative to opioid analgesic medications for the alleviation of moderate to severe pain (6).

The exact mechanism-of-action of its analgesic action is not well known , although inhibition of reuptake of various neurotransmitters, including serotonin, dopamine, and noradrenaline (NE), is thought to be involved.

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Furthermore, antinociception induced by nefopam in mice models that can be mediated by the H3 receptor. Furthermore, the drug has been reported to have some antimuscarinic effect (6) .

Inflammatory Markers

Tumor Necrosis Factor alpha (TNF-α) is a protein that regulates cell signaling. Its primary function is to regulate immune cells, but it can also play a role in systemic inflammation (7). TNF- α has also been associated with a variety of human diseases, as well as inducing fever, apoptosis, inflammation, and inhibiting tumorigenesis and viral replication. TNF-α is also believed to be the most essential mediator of cardiovascular disease (8).

Similarly, both the transcriptional and translational processes can be intricate in the rule of TNF-α synthesis. At the transcriptional level, nuclear factor kappa B (NFκB) considered the major redox-sensitive transcriptional factor associated with TNF-α production (9).

The nuclear factor kappa light chain enhancer of activated B cells (NFκB), a protein complex that regulates DNA transcription, cytokine synthesis, and cell survival, has been discovered. NFκB misregulation that has led to an increase in cancer, inflammatory disease, and some immunity diseases, as well as to poor immunological development. It can be found in almost all types of animal cells and can play a role in cell response to extrinsic or intrinsic effect such as stress, cytokine, oxidized radical's , ultraviolet (UV) irradiation, oxidize LDL, and microbus antigens, or other stimuli(10).

Increase in exposure to various stimuli such as free radicals and ROS , stress conditions , cytokines , heavy metals, UV-UV ways, LDL (Oxidized) and bacterial or viral proteins, among many other stimuli (11).

Interleukin 1 beta (IL1β) and Interleukin 10 (IL-10) can be encoded by the IL1B gene. Generally, IL-1 has been reported to be an important mediator of inflammatory reactions. Furthermore, two forms of the intended mediator were noted; namely, IL-α and IL-β. The latter has been the subject of experimental investigation since it is easily identified in blood (12,13).

Interleukin-10 (IL-10) is inflammatory substance that produced mainly from monocytes and, maybe (in less amount) from lymphocytes. It may suppress inflammation through a variety of mechanisms, including lowering HLA class II expression, decreasing T cell IL-2 secretion, and decreasing IL-1α, IL-1β, TNF-α and IL-8 production by activated monocytes/macrophages. It may also help B cells survive, proliferate, and produce antibodies (12).

The Aim of the study

To identify the effect of toxic doses of Nefopam on the liver, heart and brain of rats using different duration of exposure (acute for Nefopam IP injected) and its concentration in these organs. Demonstrate whether TNF-α and NF-κB, and some selected interleukins (IL1β, and IL-10) play a role in the mechanism of organ injury induced by Nefopam.

Materials and Methods:

A: Laboratory Animals

Fourteen (14) adult Wistar Albino male rats; their age range (3-5) Months, In this study, rats weighed between 200 and 250 grams. They were collected from the College of Pharmacy's Animal House at Baghdad University. They were housed in polypropylene plastic cages (three animals per cage) under carefully controlled circumstances (temperature, humidity, and light/dark cycles) (7). The animals were given unlimited access to commercial pellet diet and water. Rats were randomly assigned to 2 groups (7 animals each) as follows:

Groups

I- Controls: Rats were administered a single intraperitoneal injection (IP) with normal saline (NS (0.5 ml / 250 g body weight) and then the animals were euthanized with anesthetic ether 24 hours after NS giving (such as ketamine) (2) .

II- Nefopam -treated animals: Rats were injected with a one toxic amount of IPP (40 mg/kg) Nefopam and then the experimental rats were sacrificed with anesthetic ether 24 hours after drug administration (7,25).

B. Collection and preparation of blood samples. Rats were sacrificed with anesthetic diethyl ether after 24 hours of Nefopam treatment and underwent laparotomy. Whole blood was taken by heart puncture and divided into two aliquots:

Part 1: Whole blood was placed in a polyethylene gel tube and allowed to clot before being centrifuged for 15 minutes at 3500 rpm to obtain serum, which was separated and stored at -40 C until analysis. Serum was used for the estimation of TNF-α, NF-κB, IL-10, IL-1β, CK-MB, ALT, and AST (13,14,23).

Furthermore, after using anesthetic diethyl ether to euthanize rats, rats treated with Nefopam were laparotomy and blood was taken through heart puncture and placed in a polyethylene gel tube. Blood samples were placed in room temperature and then placed on centrifuge at 3500 rpm for 15 minutes to obtain serum, which was separated and frizzed at -40 C° till the time of evaluation. Serum TNF-α, -, NF-κB, IL-10, IL-1, CK-MB, ALT, and AST were estimated using a sandwich enzyme-linked immunosorbent test (ELISA) and Nefopam concentrations were also determined (7,16).

C. Measurements of serum Nefopam concentrations

The concentrations of Nefopam in serum by using the Knauer HPLC system and the C18 reversed chromatography was used to evaluate and separated the content and concentrations of substances. For Nefopam , the mobile phase contained buffer and acetonitrile. The mobile phase was filtered from a 0.45 µm nylon film filter(15,17) .

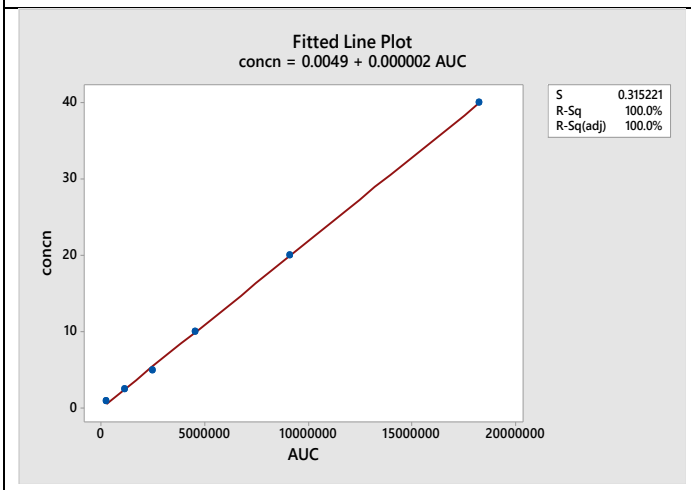
Preparation of Standards and Calibration Curve of Nefopam

Each stock solution of Nefopam 200 g/ml was made by liquifying 10 mg in 50 ml of dilutant, that were made by combining one hundred ml of acetonitrile with one hundred ml of water in a volumetric flask, then diluted to reach two hundred ml (17, 24) . A calibration curve was produced utilizing the area under the curve (AUC) versus the concentration (Figures 2-1), and HPLC was used to separate several dilutions from the stock solution to produce final concentrations (1.0, 2.5, 5.0, 10, 20 and 40 g/0.5ml) for Nefopam (Tables 2-4). (Figure 2.2) .

Concentration (mcg)	AUC
1	259,989.00
2.5	1,172,547.00
5	2,488,871.00
10	4,558,464.00
20	9,105,128.00
40	18,253,992.00

$$concentration = 0.0049 + 0.000002\ AUC$$

Table 2-4. Final concentrations of Nefopam with the area under the curve (AUC)



Figures 2-1: Standard curve of the Nefopam standard according to the concentration *vs. the* area under the curve (AUC).

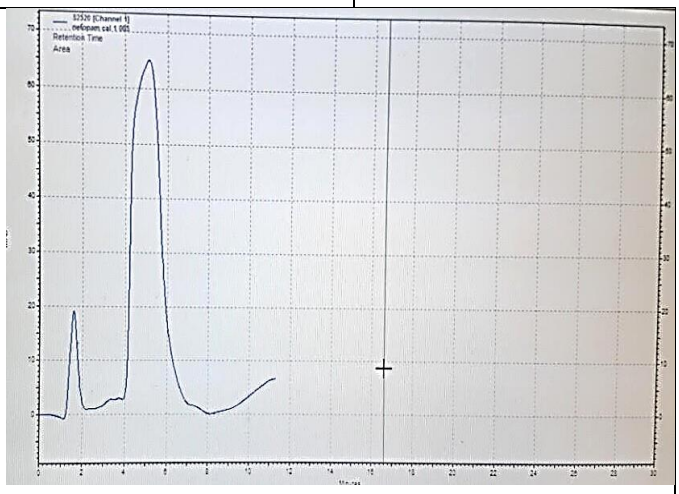


Figure 2-2. High Performance Liquid Chromatography (HPLC) chromatogram for Nefopam standard solution.

D. Collection and preparation of liver, heart, and brain tissues homogenates

After authorization of the animals using anesthetic diethyl ether, the rats underwent laparotomy to obtain livers and hearts, then the intended organs were rapidly separated and perfused with normal-saline (4°C) to successfully remove the blood and then each of the liver and heart tissue was weighed and minced into small pieces. Concerning the collection and

preparation of brain tissue homogenate of each rat utilized in this study, The skin of the animal's head was removed, and the muscles and fascia of the dorsal and posterior parts of the skull were tugged / scraped away using small surgical scissors. The arachnoid membrane was carefully removed and digested with a tissue homogenizer after the bone was properly sliced and the brain was slowly removed, cleaned in saline at 4°C, and the arachnoid membrane was carefully removed and digested. The digested tissue samples were stored at -80°C for HPLC and GC-FID analysis to determine the amounts of Nefopam in each of the tissues listed above (18).

Measurement of nefopam tissue contents in the liver, heart, and brain of male rats Five hundred (500) milligrams of liver, heart, and brain tissue were obtained from each male rat and then each was homogenized and deproteinized with 400µl of acetonitrile and 500µl of methanol. The resulting homogenate was vortexed for 1 minute before being centrifuged, filtered, and evaporated to dryness in a vacuum oven at 45 ° C. The dried substantial was then re-formed with one hundred µl of the mobile phase and centrifuged once more. The resulting supernatant was then directly fed into the HPLC, yielding 30 µl. Before HPLC analysis, the samples were not diluted (18,22).

E. Statistical analysis

Statistical Package for the Social Sciences (SPSS) 20.0.0, Minitab 17.1.0, and Graph Pad Prism 7.0 software package were used to perform the statistical analysis.

Data were expressed as mean values, mean± standard deviation (SD) of samples. Furthermore, one-way analysis of variance(ANOVA) was used to establish the statistical significance of the differences between the groups. P-values less than 0.05 were considered statistically significant differences.

Results

Effects on serum inflammatory mediators (tumor necrosis factor-alpha (TNF-α) level ,(NF-κB) level , IL- 10 and IL 1 B)

Table 3-1 and Figure 3-1 showed that the serum TNF-level of TNF-α in IP rats injected with a single toxic dose (40 mg/kg) of Nefopam (acute toxicity) was significantly decreased (P<0.05) compared to controls; the levels were, respectively, 14.530 ± 2.780 and 17.961 ± 2.816.

Regarding serum levels of NF-κB in rats, I.P injected with a one toxic dose (40 mg/kg) of Nefopam weren't significantly different (P>0.05) compared to control rats. Serum content of NF-κB were, respectively, 416.266 ± 48.806 and 396.314 ± 20.372.

Furthermore, the results of Table 3-1 showed that rats IPP injected with a single toxic dose (40 mg/kg) of Nefopam , the serum level of IL-10 was significantly reduced (P<0.05) compared to control animals. The level being respectively, 23.226 ± 7.038 and 36.265 ± 9.506; compared to control rats. Serum IL10 levels were 51.530 ± 7.680 and 36.265 ± 9.506, respectively, however serum IL-1β levels were not significantly different (P>0.05) , The serum levels of serum IL-1β were respectively 5.301 ± 4.257 and 6.008 ± 3.083, Nefopam was not significantly different in (P>0.05) compared to control animals; the levels were respectively 6.012 ± 1.605 and 7.313 ± 1.009

Table 3-1. Effects of single toxic dose of Nefopam (40mg/kg) on serum TNF-α, NF-κB, IL-10 and IL-1β level in rats.

Groups (n=7)	**Tumor necrosis factor-alpha (TNF-α) Pg/ml**	**NF-κB Pg/ml**	**IL-10 levels**	**IL-1β levels**
Control (n=7)	17.961 ± 2.816	396.314 ± 20.372	36.265 ± 9.506	6.008 ± 3.083
Nefopam -treated (40 mg/kg)	14.530 ± 2.780 * a	416.266 ± 48.806 a	23.226 ± 7.038 * a	5.301 ± 4.257 a

- *: Significant variance compared to the control group (P<0.05).

- Values with nonidentical superscripts (a and b) are significantly different (P<0.05).

- n = number of animals for each group n=7 .

Effects on serum creatine kinase myocardial band isoenzyme (CK-MB) activity.

Serum levels of CK-MB in IP rats injected with a single toxic dose (40 mg/kg) of Nefopam were not significantly different (P>0.05) compared to the control group of animals; the levels were, respectively, 0.948 ± 0.548 and 1.451 ± 0.671, (Table 3-2)

Tables 3-2. Effects of a single toxic dose (acute toxicity) of Nefopam (40mg/kg)on creatine kinase myocardial band isoenzyme (CK-MB) activity levels in rats.

Groups	CK-MB activity levels
Control (n=7)	1.451 ± 0.671
Nefopam (40mg/kg) (n=7)	0.948 ± 0.548 a

- *: Significant variance (P<0.05) compared to control group.

- Values with nonidentical superscripts (a and b) are significantly different (P<0.05).

- n = number of animals.

Effects on Serum Alanine Aminotransferase (ALT) hepatic marker, Activity of Aspartate Aminotransferase (AST), and Alkaline Phosphatase (ALP)

Table 3-3 showed that serum ALT in IP rats injected with a single toxic dose (40 mg/kg) of Nefopam was non-significantly (P>0.05) different compared to the control group of animals; the levels were, respectively, 8.549 ± 2.388 and 10.166 ± 1.914

Nefopam was not significantly different compared with the corresponding activity levels of the control rats (P>0.05); the activity levels were respectively, 2.044 ± 0.514 and 2.475 ± 0.428.

Table 3-3 showed that the serum ALP activity level in IP rats injected with the toxic dose (40 mg/kg) of Nefopam was not significantly different (P>0.05) compared to the corresponding serum activity in control rats; The levels were, respectively, 30.827 ± 3.171 and 32.696 ± 3.137.

Table 3-3. Effects of a single toxic dose (acute toxicity) of Nefopam (40mg/kg) on serum levels of alanine aminotransferase (ALT) , aspartate aminotransferase (AST), and alkaline phosphatase (ALP) activity in rats.

Groups	ALT activity levels Pg/ml	AST activity levels Pg/ml	Alkaline phosphatase (ALP) Pg/ml
Control (n=7)	10.166 ± 1.914	2.475 ± 0.428	32.696 ± 3.137
Nefopam (40mg/kg) (n=7)	8.549 ± 2.388 [a]	2.044 ± 0.514 [a]	30.827 ± 3.171 [a]

- *: Significant variance (P<0.05) as compared to the control group.

- Values with nonidentical superscript (a and b) are significantly different (P<0.05).

- N= Number of animals.

Concentrations of acute toxic doses of nefopam in (serum), and each drug in the brain, liver and heart

Nefopam Concentrations in Serum, Brain, Liver and Heart

Table 3-4 showed that after 24 hours of injection of IP with 40mg/kg Nefopam into rats, the concentration of the intended drug in serum was 20.600±4.824µg/500ml; while, its concentration in the brain was 28.451± 1.935µg/500mg; besides, the concentration of the toxic IP dose (40mg/kg) of Nefopam in the liver was 29.148 ± 5.316 µg/500mg; additionally in the heart, the concentration of the toxic IP dose of Nefopam is 30.287 ± 4.468µg/500mg.

Furthermore, there was a significant reduction (P<0.05) in the serum concentration of Nefopam IP injected as a single toxic dose (40 mg / kg) in rats compared to its concentration in brain, liver and heart tissues; Furthermore, non-significant differences (P>0.05) among the concentrations of Nefopam IP injected as a single toxic dose (40mg/kg) in brain tissue compared to liver and heart tissues. Table (3-4) and Figure (3-1).

Table 3-4: Concentrations of acute IP dose of Nefopam 40mg/kg in serum, brain, liver, and heart.

Number of animals N=7	Concentrations of acute IP dose of Nefopam			
	Serum µg/ml	Brain µg/mg	Liver µg/mg	Heart µg/mg
Nefopam 40mg/kg	20.600 ± 4.824	28.451 ± 1.935 * a	29.148 ± 5.316 * a	30.287 ± 4.468 * a

* Significant difference (P<0.05) compared to the Mean±SD concentration in serum.

Values with identical values of small letters (a) are not significantly different (P>0.05).

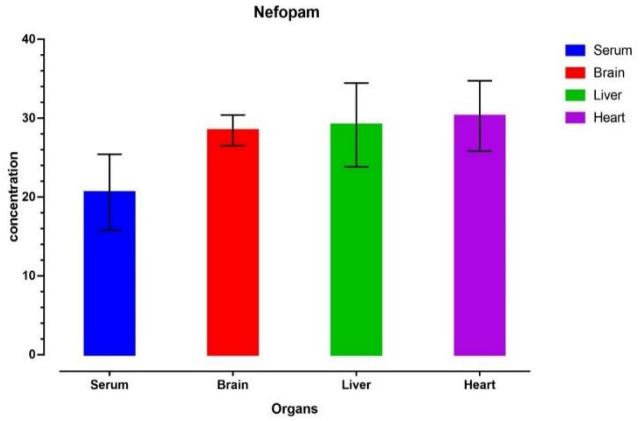


Figure 3-1. Histogram of the mean concentration of a single toxic dose of Nefopam (40mg/kg) in rats' serum, brain, liver, and heart.

Discussion

1- Effects of acute toxic doses of nefopam on various parameters selected.

A- Effects on the serum level of tumor necrosis factor-alpha (TNF-α) and (NF-κB) level.

A single toxic dose of Nefopam (40mg/kg) (acute toxicity model) significantly reduced serum TNF-α levels compared to the control (P0.05), which could be related to the immune suppressor effect that the targeted toxic dose of Nefopam can cause. There's limited research on the effect of nefopam on TNF-α levels, thus the findings of this study cannot be compared with those of others.

Nefopam produced a non-significant difference (P>0.05) in the serum level of NF-κB compared to control animals. The proposition could be explained that Nefopam may not have an effect on NF-κB as does other immunological mediators such as IL-10 and TNF-α. Until now, no studies have been reported on the effect of a single toxic dose (40 mg / kg) of Nefopam on NF-κB level.

B- Effects on serum level of interleukin 10 (IL-10) and serum interleukin-1beta (IL-1β) .

Nefopam significantly reduced the serum level of IL-10 (an anti-inflammatory cytokine) compared to the control (P<0.05). Until now, no studies have been reported on the effect of a single toxic dose (40 mg / kg) of Nefopam on the effect of Nefopam on the serum level of IL-10.

However, this result is expected because Nefopam can decrease the level of interleukin as an expected mechanism of its pain management (19,20).

However , Nefopam were non-significantly different (P>0.05) compared to the corresponding serum level in control animals. No studies have yet been reported on the effect of acute toxic dose of Nefopam on this respect.

However, this finding may be contrary to Moini et al. that the proposed IL-1β can affect significantly after taking opioid or Nefopam (20-23), but more research must be done.

C- Effects on serum creatine kinase myocardial band (CK-MB) isoenzyme activity.

In the present study, there were no significant difference (P>0.05) in serum CK-MB level in rats injected with an acute toxic dose of IP Nefopam (40mg/kg) compared to control animals. It can be suggested that acute intoxication with nefopam may not affect CK-MB in myocytes; as no study has yet been reported to assess this effect, however, a case report on nefopam toxicity proposed that nefopam may cause death due to atherosclerosis (22,24).

D- Effects on serum -alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline enzyme activity of ALP.

In the current study, a single toxic dose of Nefopam (40mg/kg) produced non- significant differences (P>0.05) on serum ALT, AST and ALP activity compared to the control. There was a previous study on the effect of acute toxic dose of nefopam on liver enzyme that has been conducted in kitchens that shows toxicity at dose (18 mg / kg); however, no other result has been obtained (25-27).

2- Concentrations of acute toxic doses of nefopam in (serum) and each drug in brain, liver, and heart

In the current study, the concentration of acute toxic dose (40mg/kg) of Nefopam in serum was significantly lowered (P<0.05) compared to its concentration in each homogenate of heart, liver, and brain tissue homogenate; Furthermore, despite the fact that the concentration of acute toxic dose of Nefopam was higher in the liver than in the heart than in the liver than in the brain; but the concentration of Nefopam did not show significantly different (P>0.05) among the tissue homogenates mentioned above. There is no study reported yet to support the findings of this study although the identification test developed every day(28-30).

Conclusions

It is possible to conclude, based on the findings of this research, that A single toxic dose (40mg/kg) of Nefopam IP injected into rats (acute toxic model) produced various effects on serum levels of cytokines (whether inflammatory or anti-inflammatory markers); besides, it did not have effects on different enzyme activity measured in serum (CK-MB, as a marker that may be elevated in heart injury; ALT, AST and ALP, elevated markers in liver injury); Despite the above, we cannot be certain that there is no toxicity to the liver or heart due to the apparent change and high levels of liver enzymes, but they are not sufficient to prove toxicity.

Recommendation for future work

1. Continuous research could be performed to assess the availability of chronically used drugs in tissues and its

correlation with the possible unwanted effects of drugs.

2. Continuous research could be performed to assess histopathological changes in acute and chronic toxicity

3. Further in vivo studies using large-scale animals and various toxicity models to clarify the roles of pro-inflammatory cytokines (31), markers of lipid peroxidation (32), leptin level (29), Oxidative Stress such as Al-Hussaniy et al (34) but with nefopam , and apoptosis (33) in Nefopam-related multiorgan damage in rats such as what have done on .

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• We have received ethical approval to conduct laboratory tests on experimental animals by the University of Baghdad and by the Iraqi Ministry of Health thesis approval Conflict of Interest The authors declare that there is no conflict of interest.

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