

Effects of NSAIDs and environmental oxygen pressure on bone regeneration.

Efecto de los AINEs y la presión de oxígeno ambiental sobre la regeneración ósea.

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Abstract: Objective: To evaluate the effects of administering diclofenac and ketoprofen, as well as the effects of environmental oxygen pressure variation on mandibular bone regeneration. Methods: Thirty-six guinea pigs were distributed into two equal groups. Mandibular bone defects were performed on both groups. Group A was monitored under oxygen pressure at altitude (3320msl, 107mm Hg). Group B was monitored at sea level oxygen pressure (150msl, 157mm Hg). Each group was subdivided into 3 equal groups (A1, A2, A3 and B1, B2, B3). Subgroups A1 and B1 were given diclofenac; subgroups A2 and B2 ketoprofen; subgroups A3 and B3 NaCl. Bone regeneration was evaluated histologically on days 15 and 30. Results: After 15 days in the group controlled at sea level, the level of osteoblasts presented by the control subgroup was significantly higher (28.00±2.65) compared to the diclofenac subgroup (16.00±6.25) and to the ketoprofen subgroup (18.00±4.36); ($p=0.041$). After 15 days in the group controlled at altitude, the level of osteoblasts was significantly higher in the control subgroup (38.00±5.29) compared to the diclofenac subgroup (21.67±6.35) and to the ketoprofen subgroup (19.33±2.52); $p=0.007$. After 30 days in the group at sea level there was no difference found in the cell counting; $p>0.05$. After 30 days in the group controlled at altitude, the level of osteoblast was significantly higher in the control subgroup (58.00±4.58) compared to the diclofenac subgroup (34.33±4.73) and the ketoprofen subgroup (34.00±11.14); ($p=0.003$). Conclusion: The administration of diclofenac and ketoprofen produced lower mandibular bone regeneration, the effect being significantly more negative at sea level.

Keywords: Bone Regeneration; atmospheric pressure; anti-Inflammatory agents, non-steroidal; hypoxia; hypoxia-inducible factor 1.

Resumen: Objetivo: Evaluar el efecto de la administración de diclofenaco y ketoprofeno y de la variación de la presión de oxígeno ambiental sobre la regeneración ósea mandibular. Métodos: Participaron 36 cobayos distribuidos en dos grupos iguales. A ambos grupos se les realizaron defectos óseos mandibulares. El Grupo A fue controlado bajo presión de oxígeno en altura (3320msnm, 107mm Hg). El Grupo B fue controlado bajo presión de oxígeno a nivel del mar (150msnm, 157mm Hg). Cada grupo fue dividido en 3 subgrupos iguales (A1, A2, A3 y B1, B2, B3). Los subgrupos A1 y B1 recibieron diclofenaco; A2 y B2, ketoprofeno; A3 y B3, NaCl. La regeneración ósea fue evaluada histológicamente a los 15 y 30 días. Resultados: A nivel del mar, a los 15 días, hubo una significativa mayor cantidad de osteoblastos en el subgrupo control (28,00±2,65) comparado con el subgrupo diclofenaco (16,00±6,25) y ketoprofeno (18,00±4,36); ($p=0,041$). En altura, a los 15 días, hubo una significativa mayor cantidad de osteoblastos en el subgrupo control (38,00±5,29) comparado con el subgrupo diclofenaco (21,67±6,35) y ketoprofeno (19,33±2,52); $p=0,007$. A nivel del mar, a los 30 días, no se encontró diferencia en el conteo celular; $p>0,05$. En altura, a los 30 días, se encontró una significativa mayor cantidad de osteoblastos en el subgrupo control (58,00±4,58) comparado con el subgrupo diclofenaco (34,33±4,73) y ketoprofeno (34,00±11,14); ($p=0,003$). Conclusión: La administración de diclofenaco y ketoprofeno produjeron una menor regeneración ósea mandibular, siendo este efecto significativamente más negativo a nivel del mar.

Palabras Clave: Regeneración ósea; presión atmosférica; antiinflamatorios no esteroideos; hipoxia; factor 1 inducible por la hipoxia.

INTRODUCTION.

Prostaglandins are inflammatory molecules that can favor bone regeneration by boosting angiogenesis and stimulating the activity of osteoclasts and osteoblasts.¹ Nonsteroidal anti-inflammatory drugs (NSAIDs), which are administered regularly for treatment of musculoskeletal-related pathologies, are strong cyclooxygenase (COX) inhibitors. Many studies have demonstrated on animal models² that administering COX inhibitors could delay the regeneration process of bone fractures. The administration of acetylsalicylic acid (ASA) has been shown to inhibit inflammatory response in a tibia fracture model in mice, resulting in a lower echogenicity on ultrasound images of fractures compared to the group that received no treatment.

ASA has also been shown to inhibit cell death, as well as the expression of COX-2 and leukotriene B₄ receptor 1 (BLT1). Nevertheless, treatment with indomethacin did not reduce echogenicity in a fracture model, as confirmed by the levels of cell death, as well as COX-2 or BLT1³⁻⁵ expression levels.

Moreover, previous reports have evaluated the relationship between variations of environmental oxygen pressure and bone regeneration after osteotomy in animal models, which resulted in a positive relationship between lower environmental oxygen availability and a higher bone formation.⁶⁻⁸ A decrease of environmental oxygen favors the generation of diverse hypoxia-dependent molecules, especially hypoxia-inducible factors (HIFs), vascular endothelial growth factor (VEGF) and bone morphogenetic protein type 2 (BMP-2).⁸

As of today, there exist many analgesic and anti-inflammatory alternatives for various oral pathologies; however, there are still numerous voids of information about their effects on the regenerative process of the stomatognathic system, especially regarding their possible effects on the deeper tissues of the attachment periodontium (alveolar bone and periodontal ligament). NSAIDs are the most prescribed drugs worldwide for the treatment of pain and acute inflammation, and are especially common during the post operative period following many dental procedures. The present study aims to present new evidence for the effects of NSAIDs on the biology of the alveolar bone, taking into account the

effects that variability of environmental oxygen pressure may have on the process as a whole.

Thus, the objective of this study was to evaluate the effects of administering diclofenac and ketoprofen, as well as the effects of environmental oxygen pressure on the regeneration of mandibular bone defects in guinea pigs.

MATERIALS AND METHODS.

Ethical considerations

The animals used in this study were treated in compliance with all WMA Statement on Animal use in Biomedical Research standards.⁹ All procedures took place as established by the ethics committee of the Universidad Nacional Mayor de San Marcos.

Animals

The present study was of experimental type. Thirty-six male Hartley guinea pigs 1000g (+/- 100g) of body mass were provided by the Veterinary Medicine School of the Universidad Nacional Agraria La Molina in Lima, Peru.

The subjects were randomly distributed into two groups of 18 guinea pigs each (Group A and Group B). Guinea pigs from group A were conditioned and acclimated for 7 days at the Animal center of IVITA (Instituto Veterinario de Investigaciones Tropicales y de Altura) – Mantaro, National University of San Marcos (3320 m a.s.l., 107mm Hg). Guinea pigs from group B were conditioned and acclimated for 7 days on the Animal center of the Faculty of Medicine from the same institution (150 m a.s.l., 157mm Hg). All guinea pigs were kept at room temperature on an alternating 12h light–dark cycle, beginning at 8 am, and had access to water and a balanced diet ad libitum.

Treatment protocol and creation of bone defects

The 36 guinea pigs were administered general anesthesia with a dose of 30 mg/kg of Ketamine (Ketalar®) via intramuscular injection. For local anesthetic starting from a right mandibular angle and advancing 15mm through the mandibular body, 1mL of lidocaine 2% with epinephrine 1:80000 (Xylestesin®) was injected. Afterwards, the fur from the right mandibular zone was removed with depilatory cream, and the area was then disinfected with povidone-iodine. Following this procedure a 10mm extraoral incision was performed on the center of the mandibular body with a No.15 scalpel (1mm over the lower edge of the mandible). The incision

cut through the skin, subcutaneous tissue, muscle tissue and periosteum until the mandibular bone was exposed, then two bone defects were performed on the mandible using 2mm tungsten carbide drills (Maillefer®). Finally, the affected tissues were repaired and closed with absorbable sutures.

Group A was controlled under environmental oxygen pressure conditions at altitude (3320 m.a.s.l., 107 mmHg), while group B was controlled under environmental oxygen pressure conditions at sea level (150 m.a.s.l., 157 mmHg). Both groups were divided into three subgroups of six guinea pigs each (Group A: subgroups A1, A2, A3; Group B: subgroups B1, B2, B3). The guinea pigs from subgroups A1 and B1 were administered 1mg/Kg of diclofenac; A2 and B2 1mg/Kg of ketoprofen; and A3 and B3 1mg/Kg of NaCl (the last two being the control subgroups). All treatments were administered intramuscularly every 12 hours for 3 days. After 15 and 30 days following the interventions, the subjects were euthanized with a pentobarbital overdose (1mL per 250g of weight via intraperitoneal injection).

Histopathological analysis

Samples were taken from the mandible and dissected with a 6mm margin from the surgically intervened region. The samples were fixed in saline solution buffered with 10% phosphate for 24 hours, then underwent decalcification with 14.5% ethylenediaminetetraacetic acid at room temperature for 5 to 6 weeks. Afterwards, the samples

were dehydrated with ethanol and embedded in paraffin, then cut into 5mm sections with a microtome (Kedee KD-2258) and stained with hematoxylin and eosin. The digital images of the histological sections were captured with an optical microscope (Micros MCX100 LCD Crocus II).

The histopathological analysis was carried out by a pathologist, who was not informed of each sample's subgroup and focused instead on the cell counting of osteoblasts and osteocytes. The intervened zone was identified using a microscope, and four areas were randomly chosen and analyzed, and the cell counting of osteoblasts and osteocytes was obtained in a clock-wise manner. A mean was determined from the observation of the four areas, which was the final figure of osteoblasts and osteocytes for each guinea pig. Each microscopic area was 0.458mm² at a total magnification of 400X. This procedure was carried out 15 and 30 days after all the guinea pigs had been intervened. All values from all groups and subgroups involved in the study were compared.

Statistical analysis

Data were collected using SPSS 21.0 statistical package. The data from the counts presented a normal distribution. ANOVA was used in order to evaluate the cell counting differences between the groups at sea level and those at altitude after 15 and 30 days. A value of $p < 0.05$ was considered as statistically significant. All cell counting data are presented as mean ± standard deviation.

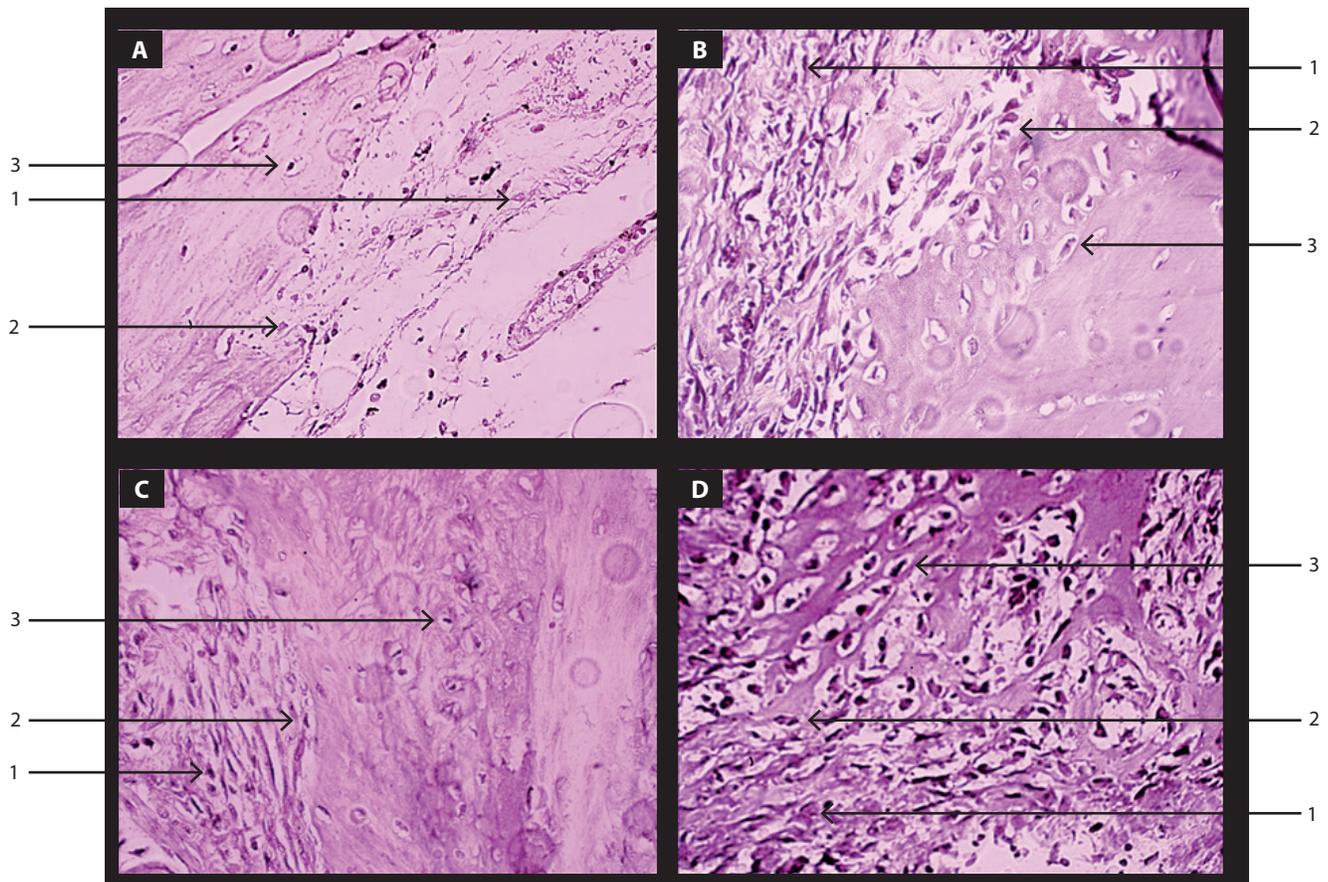
Table 1. Cell counts in the studied groups after 15 days.

	At higher altitude (3320 m a.s.l)				At sea level			
	Osteoblasts Mean±SD	<i>p</i>	Osteocytes Mean±SD	<i>p</i>	Osteoblasts Mean±SD	<i>p</i>	Osteocytes Mean±SD	<i>p</i>
Control	38.00±5.29	0.007	24.67±4.51	0.648	28.00±2.65	0.041	18.00±3.61	0.47
Diclofenac	21.67±6.35		22.33±4.16		16.00±6.25		16.33±2.52	
Ketoprofen	19.33±2.52		25.00±1.73		18.00±4.36		19.33±2.08	

Table 2. Cell counts in the studied groups after 30 days.

	At higher altitude (3320 m a.s.l)				At sea level			
	Osteoblasts Mean±SD	<i>p</i>	Osteocytes Mean ± SD	<i>p</i>	Osteoblasts Mean±SD	<i>p</i>	Osteocytes Mean±SD	<i>p</i>
Control	58.00±4.58	0.003	34.00±11.14	0.524	53.02±2.35	0.10	33.07±3.61	0.54
Diclofenac	34.33±4.73		37.33±5.51		35.08±3.64		35.23±2.52	
Ketoprofen	34.00±11.14		41.67±5.51		36.84±8.65		40.35±2.67	

Figure 1. The histopathological results (osteoblasts and osteocytes) show that administration of diclofenac and ketoprofen decreased cell counting at days 15 and 30.



A: Micrograph of representative zone of the diclofenac group at sea level at 15 days. 400x magnification and colored with hematoxylin and eosin. The presence of granulation connective tissue is observed with the presence of 1: fibroblasts, 2: osteoblasts and 3: osteocytes.
B: Micrograph of representative zone of the diclofenac group at altitude at 15 days. 400x magnification and colored with H-E. The presence of granulation connective tissue is observed with the presence of 1: fibroblasts, 2: osteoblasts and 3: osteocytes.
C: Micrograph of representative zone of the diclofenac group at sea level at 30 days. 400x magnification and colored with H-E. The presence of granulation connective tissue is observed with the presence of 1: fibroblasts, 2: osteoblasts and 3: osteocytes.
D: Micrograph of representative zone of the diclofenac group at altitude at 30 days. 400x magnification and colored with H-E. The presence of granulation connective tissue is observed with the presence of 1: fibroblasts, 2: osteoblasts and 3: osteocytes.

RESULTS.

Osteoblast and osteocyte counts after 15 days

At higher altitude, the number of osteoblasts was 38 ± 5.29 for the control subgroup; 21.67 ± 6.35 for the diclofenac subgroup; and 19.33 ± 2.52 for the ketoprofen subgroup. ($p=0.007$) At sea level, the number of osteoblasts was 28 ± 2.65 for the control subgroup; 16 ± 6.25 for the diclofenac subgroup; and 18.00 ± 4.36 for the ketoprofen subgroup. ($p=0.041$)

The Tukey test showed no significant difference between the diclofenac and the ketoprofen subgroups. However, both groups differ significantly from the control group. No difference was observable between sea level and at higher altitude concerning osteocyte counts ($p=0.648$ and $p=0.47$; respectively). (Table 1)

Osteoblast and osteocyte counts after 30 days

At higher altitude, the number of osteoblasts was 58 ± 4.58 for the control subgroup; 34.33 ± 4.73 for the diclofenac subgroup; and 34 ± 11.14 for the ketoprofen subgroup. ($p=0.003$)

At sea level, the number of osteoblasts was 53.02 ± 2.35 for the control subgroup; 35.08 ± 3.64 for the diclofenac subgroup; and ketoprofen subgroup was 36.84 ± 8.65 . ($p=0.10$)

The Tukey test showed no significant difference between the diclofenac and the ketoprofen subgroups. However, both groups differ significantly from the control group. No difference was observable between sea level and higher altitude concerning osteocyte counts ($p=0.524$ and $p=0.54$; respectively).

Histopathological analysis

The histopathological results (osteoblasts and osteocytes) show that administration of diclofenac and ketoprofen decreased cell counting at days 15 and 30. (Figure 1)

Likewise, it was observed that on day 15 the number of osteoblasts and osteocytes was higher on the diclofenac group at higher altitude (Figure 1B) compared to the diclofenac group at sea level (Figure 1A); $p < 0.05$. However, the ketoprofen subgroup presented no significant difference between cell counts regarding environmental oxygen pressure. The results after 30 days showed no significant difference in cell counts between Group A (Figure 1D) and group B (Figure 1C); $p > 0.05$.

DISCUSSION.

The present study shows the negative effect that inhibiting COX (via diclofenac and ketoprofen administration) can have on bone regeneration. A lower number of osteoblasts and osteocytes was observed in these treatment groups compared to the control groups at days 15 and 30. This is due to prostaglandins playing an important role on bone resorption and apposition, as mentioned by Boyan *et al.*,¹⁰ who demonstrated that both COX-1 and COX-2 are involved in the production by osteoblasts of osteocalcin, PGE2 and TGF- β 1. Moreover, their study suggests that PGE2 is important on the proliferation and differentiation of osteoblasts, and that its inhibition may be hindered by NSAIDs.

In this study at higher altitude evidenced a significant decrease in the osteoblast number in the diclofenac group compared to the control group ($p = 0.01$). Karakawa *et al.*,¹¹ demonstrated that administering diclofenac negatively regulates the differentiation and activation of osteoclasts via the inhibition of nuclear factor kappa B (NF κ B), thus reducing bone regeneration.

Similar results were found by Lumawig *et al.*,¹² who aimed to determine whether diclofenac could play a role on delayed or nonunion spine fusion in 273 patients who underwent posterior lumbar interbody fusion. Results showed that diclofenac showed a dose-dependent inhibitory effect in the spinal fusion ($r = 0.271$; $p < 0.001$). In contrast, Inal *et al.*,¹³ found that diclofenac had no negative effect on osteoblast counts in peroneal fracture healing (67.14 ± 14.96) compared with a control group (67.15 ± 7.55); $p = 1.01$.

They also reported that ketoprofen had a significantly negative effect on bone healing (57.14 ± 12.14) compared to the control group (67.14 ± 7.55); $p = 0.003$. A similar result was obtained in the present study, as ketoprofen administration reduced the osteoblast counts (18 ± 4.36) in comparison with the control group (28.00 ± 2.65); $p = 0.028$. However, Sevimli *et al.*,¹⁴ found in the histopathological analysis (scale from 1 to 10 recommended by Huo *et al.*,¹⁵ according to the proportion of fibrous tissue, cartilage, new and mature bone) that at the fourth week, dexketoprofen had no negative effect on the healing of closed fractures in a tibia model fixed with intramedullary rods (8.8 ± 0.64) compared to the control group (9.33 ± 0.50); $p = 0.548$.

Recently published investigations report that most NSAIDs (indomethacin, diclofenac, ketoprofen and others) have a negative effect on osteoblast development, by hindering the cellular cycle and inducing apoptosis. The effect on the osteoblastic differentiation varies depending on the drug, dose and duration of treatment.¹⁰⁻¹²

Williams *et al.*,¹⁶ indicated that paracetamol is a risk factor in fractures when administered chronically since it inhibits cyclooxygenase and endocannabinoids, which may negatively affect bone cells signals. Zhang *et al.*,¹⁷ reported that, at high doses, indomethacin inhibited collagen formation in primary tenocytes, delaying healing and bone formation.

Hatipoglu *et al.*,¹⁸ evidenced that the parenteral administration of NSAIDs even at low doses (ketoprofen 0.98 mg/kg, meloxicam 0.12mg/kg, diclofenac 1mg/kg), produced a decrease in the density of the trabecular bone and an increase on the osteoclastic density in peroneal close fractures in mice. Cai *et al.*,¹⁹ however, found that diclofenac (2mg/Kg/day) when administered for 7 days did not affect osteointegration of dental implants (bone-implant contact = 89.3 ± 3.1) compared to the control group (bone-implant contact = 79.3 ± 6.7) in a rabbit skull Calvarial model, $p = 0.088$. Hou *et al.*,¹⁵ showed that administering ibuprofen (30mg/Kg/day), there were no alterations on the healing of tibial fractures in rats for 12 weeks. Histopathology, levels of osteocalcin and histomorphometric parameters on bone remodeling (bone volume, rate of bone formation) were similar in both groups of the study. Nyangoga *et al.*,²⁰ reported that the administration of ketoprofen at small doses (2mg/Kg/

day subcutaneous) had no effect on the amount of bone formation nor on the number of macrophage. Therefore, it can be inferred that small doses of NSAIDs administered during a short period may present no deleterious effects on healing, remodeling and bone apposition. These results differ from those obtained in the present study, where small doses of ketoprofen (1mg/kg) were administered every 12 hours for 3 day, and a negative effect on bone healing was observed. This means that there could be a significant variation in the inhibitory effect according to the NSAID and dosage.

Another study carried out by Wada *et al.*,²¹ showed that the administration of acetylsalicylic acid (ASA) favors bone regeneration in diabetic and normoglycemic mice. Vestergaard *et al.*,²² found that small doses of ASA were generally associated with a heightened fracture risk and specifically with hip fractures OR=1.22 (1.19–1.25). However, further studies are recommended. In the present study, guinea pigs at higher altitude showed a higher number of osteoblasts and osteocytes in comparison with the group evaluated at sea level. The variation in oxygen pressure in the environment is an important factor in bone regeneration.

Similar findings were registered by Jiang *et al.*,²³ who observed that the administration of hypoxia-inducible factor (HIF) -1 α accelerated osteogenesis in a mandibular distraction model in rabbits, which was dose-dependent; $p < 0.001$. Also, Oishi *et al.*,²⁴ found an increase in the expression of HIF-1 α , VEGF, alkaline phosphatase and bone morphogenetic protein-2 (BMP-2) after intermittent exposure to hypoxia. However, Wang *et al.*,²⁵ conducted an *in vitro* study to evaluate the effect of hypoxia on mesenchymal stem cells from the bone marrow of rats during osteogenesis. The results showed that exposure to hypoxia for seven days (2% oxygen) produced a marked

decrease in the activity of alkaline phosphatase, the expression of osteocalcin and the transcription factor Runx2/Cbfa1. Numerous studies have analyzed bone metabolic processes, both in normoxia and hypoxia, not without controversy. Some investigations conclude that the processes involved in bone formation and regeneration are increased in tissues under normoxia, ~21% O₂; while in other studies the same processes are increased in tissues under hypoxia, ~1-5% O₂.²⁵⁻²⁸

CONCLUSION.

The administration of diclofenac and ketoprofen produced a negative effect on mandibular bone regeneration. It was also shown that there is a positive influence of hypoxia on mandibular bone regeneration.

Conflict of interests: The authors declare no conflict of interest.

Ethics approval: Approved by the Ethics Committee of the Universidad Nacional Mayor de San Marcos

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Authors' contributions: Víctor Chumpitaz-Cerrate, Elías Aguirre-Siancas and Lesly Chávez-Rimache wrote the manuscript and all the coauthors read and edited it. César Franco-Quino and Eliberto Ruiz-Ramirez carried out the analysis and interpretation of the data. Victoria Caldas-Cueva provided relevant information regarding the bibliographic review.

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