

Effect of lansoprazole on human sperm motility, sperm viability, seminal nitric oxide production, and seminal calcium chelation

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Abstract

Lansoprazole is a proton-pump inhibitor that is commonly used to treat many gastric illnesses. However, little is known about its effect on sperm function. Here, we investigated the *in vitro* effect of LP on human sperm motility, viability, nitric oxide (NO) production, and the ability of LP to chelate seminal calcium. Seventy-two semen samples from normozoospermic men were tested in this study. The effects of LP at 0.375, 0.75, 1.5, and 3 µg/mL on sperm motility and viability as well as at 3 µg/mL on NO production and calcium chelation in semen were assessed. Lansoprazole at 3 µg/mL significantly decreased total and progressive sperm motility ($P = 0.0021$, $P = 0.0256$, respectively), but not sperm viability ($P = 0.8763$). In addition, semen samples supplemented with 3 µg/mL LP had insignificant changes ($P = 0.9085$) in nitrite concentrations. Moreover, LP exhibited a significant ($P < 0.0001$) calcium chelation effect in semen. In conclusion, LP reduced sperm motility, but not viability. The reduction in sperm motility may be due to the calcium chelating effect of LP and/or decreased Na^+/K^+ -ATPase activity, but not an alteration in NO production. Besides, none of the tested parameters was found to be correlated with male age.

Keywords: Human semen; Lansoprazole; Nitric oxide; Sperm motility; Sperm viability.

INTRODUCTION

Lansoprazole (LP) is one of the most beneficial and well-tolerated drugs that is used to treat many gastric and esophageal conditions such as gastroesophageal reflux disease, dyspepsia, gastrinomas, reflux esophagitis, and peptic ulcer disease (1). In addition, LP is used to decrease the risk of NSAID-induced gastric bleeding (2). Chemically, LP consists of a substituted benzimidazole ring and pyridine ring connected by a sulfoxide-containing chain (3). Mechanistically, this drug is a proton pump inhibitor in the same pharmacologic class as omeprazole (4). It inhibits the activity of H^+/K^+ -ATPase pumps located at the secretory surface of gastric parietal cells, which reduces the amount of gastric acid production (4,5).

There is evidence that LP can enter the blood-testicle barrier in rats, a finding which can be applied to all mammals including humans (6). This evidence suggests that the use of LP to treat gastric disorders may inadvertently cause the production of nitric oxide (NO) and reactive oxygen species inside the testicle leading to sperm injury.

Due to the widespread use of LP, it is highly beneficial to investigate the possible link between frequent and sustained use of this drug and potential infertility. This study is the first of its type to determine whether LP has an adverse effect on sperm function and yields valuable results for physicians, as well as patients with poor semen quality (i.e., oligozoospermia, or asthenozoospermia) (4).

Therefore, we hypothesized that the presence of LP in semen samples, at standard concentrations that are compatible to those in the human body, may negatively affect sperm parameters. Accordingly, our goal was to measure the effect of LP on the main sperm parameters such as motility and viability. In addition, specific markers such as seminal NO, a free radical gas, were measured to explain the significant findings. Furthermore, the observed effect of LP on the tested sperm parameters was correlated with male age.

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MATERIALS AND METHODS

Materials

Ethanol was purchased from Fischer Chemical (HK) Limited (Hong Kong). Phosphate Buffered Saline (PBS) was purchased from BIOWEST SAS (France). Lansoprazole (C₁₆H₁₄F₃N₃O₂S) was purchased from Santa Cruz Biotechnology, Inc (Dallas, Texas, USA). Eosin (C₂₀H₆Br₄Na₂O₅) was purchased from Hunter Scientific Limited (Widdington Saffron Walden Essex, UK).

Sample collection

Normozoospermic samples (n = 63) were collected from males who attended the *in vitro* fertilization center of King Abdullah University Hospital in Irbid (Jordan) during the period from May 2017 to January 2018. The samples were obtained after at least 72 h of sexual abstinence. Each sample was analyzed immediately after collection for volume, viscosity, sperm concentration, motility, and morphology, according to the world health organization (WHO) guidelines (2010). Men with a sperm count equal or greater than 15 million/mL and a total sperm motility equal or greater than 40% were considered normozoospermic (7).

The study was approved by the Institutional Board Committee of Jordan University of Science and Technology (Irbid, Jordan). Each subject signed a consent form prior to sample collection.

Effect of lansoprazole on sperm motility and viability

Lansoprazole solution preparation and standardization

Considering the limited solubility of LP in different solvents and the toxic effect of these solvents on sperm, a method was standardized to solubilize LP powder in a suitable non-toxic solvent. A 50% ethanol solution was used as the solvent after it was determined that this concentration had a measured effect on sperm motility. Given that, the solubility of LP in absolute ethanol, at 25 °C, equals 14 mg/mL. A stock solution of 0.1 mg/mL LP was prepared by weighing 0.1 mg LP powder using a digital balance completed by 50% ethanol to

reach a volume of 1 mL, which was then followed by a serial dilution of three other concentrations at 0.05 mg/mL, 0.025 mg/mL, and 0.0125 mg/mL. Each of these solutions was freshly prepared for each batch of sperm samples.

Sperm preparation and treatment

Sixty-three normozoospermic samples were utilized to assess the effect of LP at four different concentrations (0.375, 0.75, 1.5, and 3 µg/mL). Each semen sample (> 1.8 mL) was gently mixed and divided into six aliquots of equal volume (300 µL each). The first aliquot was treated with 10 µL of PBS (plain control), and the second aliquot was treated with 10 µL of 50% alcohol dissolved in PBS (control). The remaining four aliquots were each treated with 10 µL of LP (dissolved in 50% ethanol) to reach the final concentrations mentioned above. All aliquots were then incubated at 37 °C for 1 h and subsequently assessed for sperm motility and viability.

Measurement of sperm motility

A total amount of 10 µL of each aliquot was used to evaluate sperm motility using a Makler counting chamber (Irvine Scientific 2511 Daimler St. Santa Ana, CA 92705). Phase-contrast optics at 200 × magnification was used to measure sperm motility. Sperm moving actively, either linearly or in a large circle were considered as progressive motile. Approximately 200 spermatozoa per replicate, for the percentage of both total and progressive motility, were assessed.

Measurement of sperm viability

Each aliquot was assessed for semen viability by mixing 10 µL of 1% eosin red stain using a wooden stirrer with 10 µL of the sample for 15 seconds. Next, 20 µL of 10% aqueous nigrosin were added and mixed well. A thin smear was prepared by pipetting 10 µL from the sample/eosin-nigrosin mixture onto a slide and allowed to air-dry. Smear slides were then mounted with a coverslip using Accu-mount media (Olympus America Inc., Center Valley, PA). Sperm viability measurements were performed on 200 spermatozoa counted on each slide in duplicate

sets using the 100 × objective lens (8). If the spermatozoa mugged the eosin-stain (pink) it was considered dead (non-viable), while if it did not mug the stain (white) it was considered alive (viable). Percentage of unstained sperm to total sperm (stained + unstained) was calculated for each sample.

Effect of lansoprazole on the level of nitric oxide produced by human sperm

A spectrophotometric method was used to assess the effect of LP at a concentration of 3 mg/mL on the level of NO produced by human sperm. We tested this concentration as it revealed a significant effect on sperm motility, given that NO is very crucial for adequate sperm motility (9).

This study examined 39 seminal plasma samples taken from different normospermic males with different sperm concentrations. The samples were collected from males who visited the assisted conception center at King Abdullah University Hospital in Irbid, Jordan.

Each sample (1 mL) was washed with human tubal fluid at 1:1 volume, and then centrifuged at 300 g for 5 min. After centrifugation, the supernatant was gently discarded and another 1 mL of human tubal fluid was added and the test tube was softly vortexed to re-suspend the washed sperm. These steps were principally performed to eliminate the effect of seminal plasma proteins that may interfere with NO assessment. After that, each sample was divided into 3 aliquots (300 µL each). The first aliquot was supplemented with 10 µL of 3 mg/mL of LP, while the two remaining aliquots were used as controls. The first control contained only the sample, whereas 10 µL of 50% ethanol was added to the other. Samples were mixed well and incubated at 37 °C for 1 h. Nitrite level; which is, an indirect measure of NO, was measured in all samples and controls using nitrite colorimetric assay (Molecular Probes Inc., Eugene, Oregon, USA).

Nitrite colorimetric assay, also known as the Griess assay, is a valid microspectrophotometric method to assess the level of NO in biological samples, including semen (9-11). In this assay, NO was measured indirectly by measuring its breakdown product

nitrite (NO₂⁻), which results from the oxidation of NO by nitric oxide synthase in human spermatozoa (12,13). Nitrite concentration is measured in a 2-step chemical reaction. In the first, sulfanilic acid reacts with NO₂⁻ in the sample to produce an intermediate product called diazonium which, in the second, reacts with N-(1-naphthyl) ethylenediamine to form a stable azo dye (14). The absorbance of this dye is directly proportional to nitrite concentration, and therefore with the NO produced, and can be measured spectrophotometrically at 540 nm.

Assessment of calcium chelating effect of lansoprazole in semen

This experiment was conducted to investigate the effect of LP on the level of seminal free calcium, which is a very crucial factor for sperm motion. Semen samples (n = 9) from different men who attended the andrology laboratory at King Abdullah University Hospital in the north of Jordan were collected over a 2-week period and included in this experiment. Each collected sample was centrifuged at 300 g for 5 min and the supernatant was carefully aspirated, transferred into another storage tube, and frozen at -20 °C for use. On the day of measurement, each sample was thawed at room temperature and gently vortexed. Three aliquots (each containing 250 µL) were prepared from each sample. The first aliquot was treated with 10 µL PBS, the second and third aliquots were supplemented with 10 µL LP and 10 µL ethylenediaminetetraacetic acid (EDTA) standardized-stock solutions to reach the final concentrations of 3 µg/mL of LP, and 3 µg/mL EDTA, respectively.

The binding of calcium ions by LP was assessed by a decrease in the maximum absorbance of the o-cresolphthalein complexone-Ca⁺² (CPC-Ca) complex (15,16). Seminal calcium ions were measured spectrophotometrically at 575 nm based on the formation of the purple-colored complex with the CPC using the commercially available kit (Linear Chemicals Inc., Barcelona, Spain). The intensity of the purple-colored complex (CPC-Ca) formed is proportional to the calcium concentration in the sample.

Statistical analysis

Data were expressed as the mean \pm standard error of the mean. Analysis of two variables was achieved by student's t-test, while multiple parallels were carried out using ordinary one-way ANOVA, followed Dunnett's multiple comparisons test. Also, analyses of relationships between variables and the male ages were performed using Spearman's nonparametric correlation analysis. The GraphPad Prism 5.01 computer software (GraphPad Software Inc., San Diego, CA, USA) was used for all statistical analysis. *P*-values of less than 0.05 were considered significant.

RESULTS

Figure 1 demonstrates the effect of LP at 0.375, 0.75, 1.5, and 3 $\mu\text{g}/\text{mL}$ on total

(Fig. 1A) and progressive (Fig. 1B) motility of human sperm. Lansoprazole at 3 $\mu\text{g}/\text{mL}$ significantly decreased both total and progressive motility of sperm ($P = 0.0021$, $P = 0.0256$, respectively). Figure 2 illustrates the effect of LP at 0.375, 0.75, 1.5, and 3 $\mu\text{g}/\text{mL}$ on human sperm viability as evaluated by Eosin test. None of these tested LP concentrations induced a significant effect on sperm viability.

Figure 3 illustrates the effect of LP at 3 $\mu\text{g}/\text{mL}$ on nitrite concentration, and hence on NO produced by human sperm, as evaluated by nitrite colorimetric assay. Washed sperm samples supplemented with LP at 3 $\mu\text{g}/\text{mL}$, at 37 $^{\circ}\text{C}$ for 1 h, had an insignificant increase in their nitrite concentration ($P = 0.9085$), and hence in the level of NO produced.

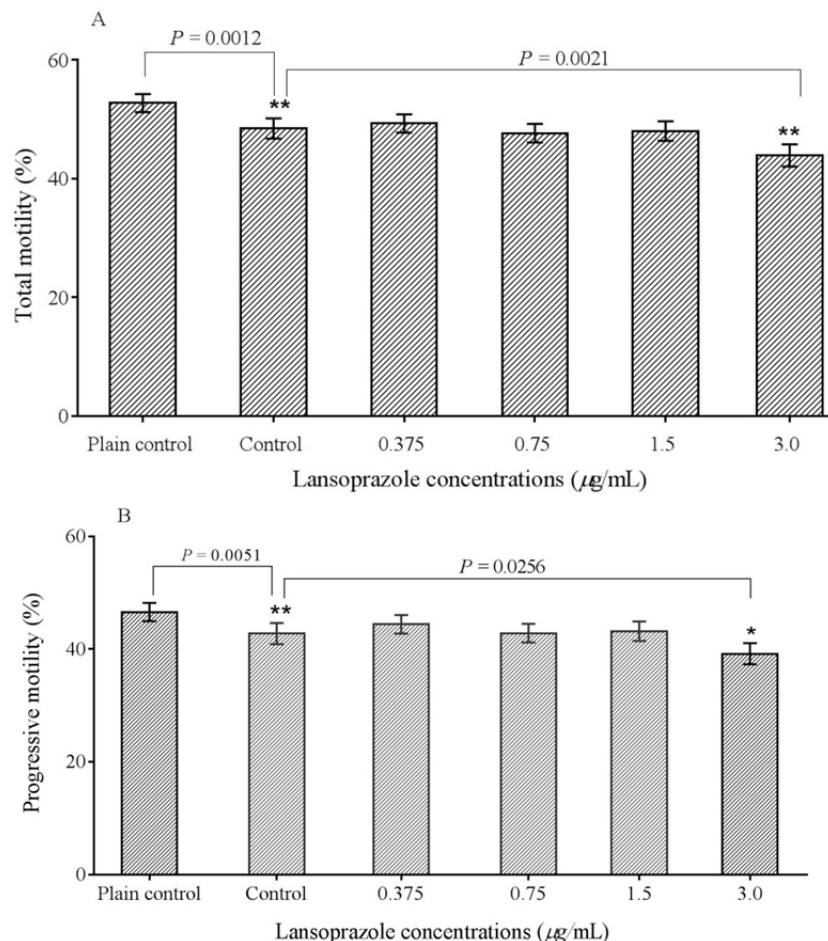


Fig. 1. Effect of lansoprazole (LP) at 0.375, 0.75, 1.5, and 3 $\mu\text{g}/\text{mL}$ on (A) total and (B) progressive motility of human sperm. Data are means \pm SEM ($n = 63$). Both total and progressive motility were significantly decreased in the presence of 3 $\mu\text{g}/\text{mL}$ of LP compared to control.

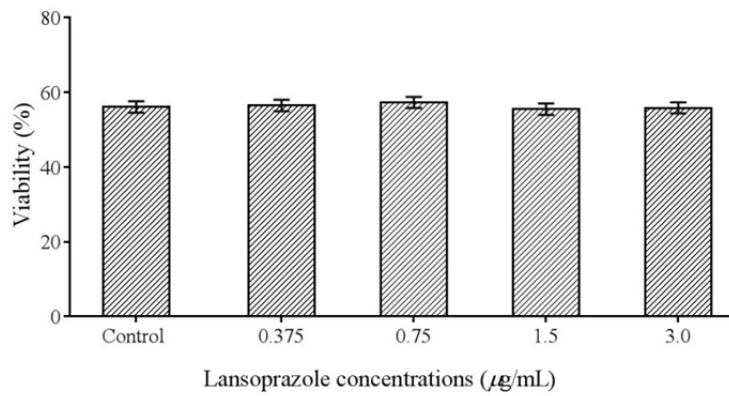


Fig. 2. Effect of lansoprazole (LP) at 0.375, 0.75, 1.5, and 3 µg/mL on human sperm viability. Data are means ± SEM (n = 63). There was no significant difference ($P > 0.05$) in sperm viability compared to control at any of the tested LP concentrations.

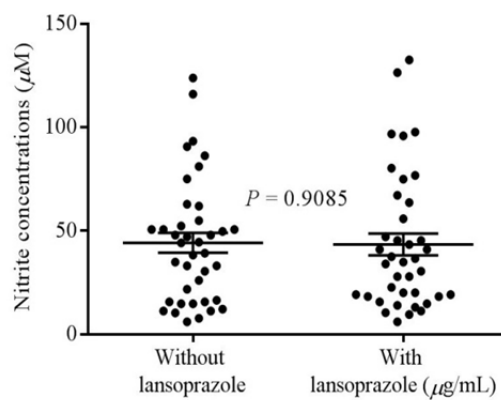


Fig. 3. Effect of lansoprazole (LP) at 3 µg/mL on nitrite concentration in human sperm samples (n = 39) compared to control (without LP) as evaluated by nitrite colorimetric assay. Data are means ± SEM. Lansoprazole at 3 µg/mL did not significantly alter the concentration of nitrite in sperm samples.

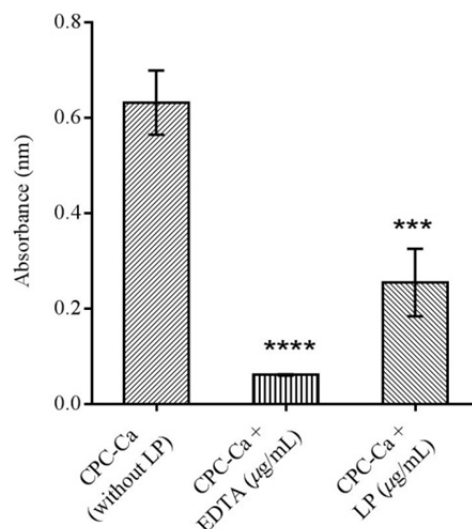


Fig. 4. Effect of lansoprazole (LP) at 3 µg/mL in reducing o-cresolphthalein complexone- Ca^{+2} (CPC-Ca) absorbance resulted from reaction between seminal calcium and CPC. Ethylenediaminetetraacetic acid (EDTA) (a known calcium chelator) was used as a positive control. Data are means ± SEM; n = 9. All means are significantly different vs. control group (CPC-Ca, without LP); (***) $P < 0.001$, (****) $P < 0.0001$.

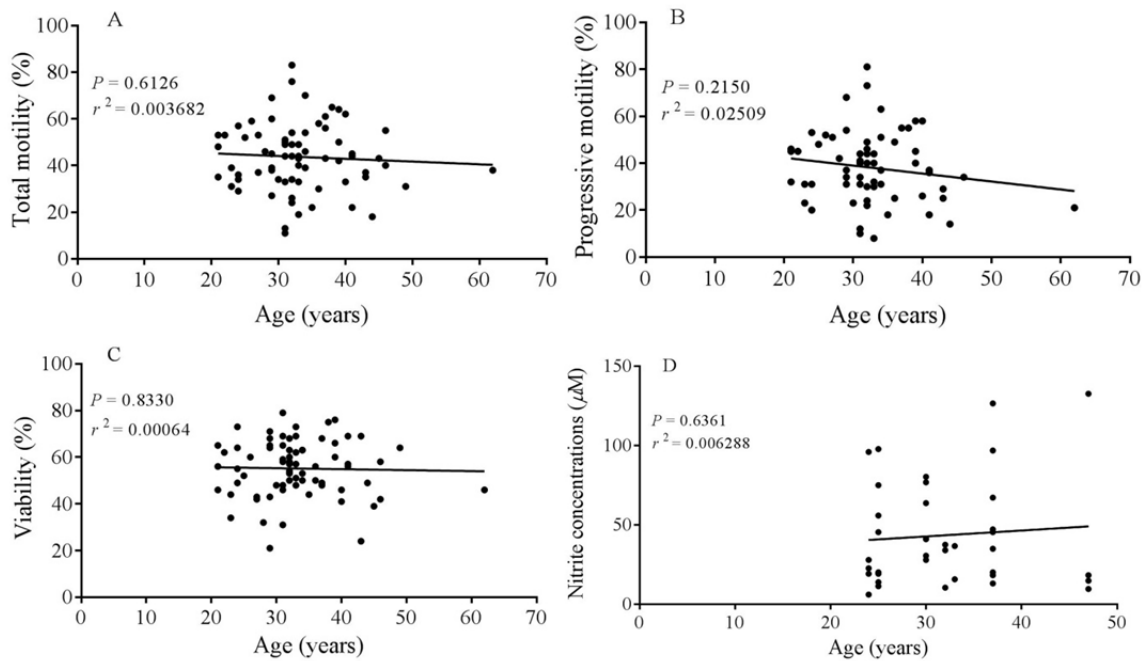


Fig. 5. Correlation between male age and the effect of lansoprazole (LP) at 3 $\mu\text{g}/\text{mL}$ on (A) progressive sperm motility, (B) total sperm motility, (C) sperm viability, and (D) nitrite concentration produced by human sperm. All of these correlations are statistically insignificant ($P < 0.05$).

Figure 4 demonstrates the calcium chelating effect of LP at 3 $\mu\text{g}/\text{mL}$ in human seminal plasma. Lansoprazole significantly ($P < 0.0001$) reduced the absorbance of the Ca-CPC colored-complex, indicating the ability of LP to chelate calcium ions. Ethylenediaminetetraacetic acid, a known calcium chelator, was used in this experiment as a control.

Figure 5 demonstrates the correlation between male age and the effect of LP at 3 $\mu\text{g}/\text{mL}$ on (A) progressive sperm motility, (B) total sperm motility, (C) sperm viability, and (D) nitrite concentration produced by sperm. As indicated in the figure, there was no significant correlation between any of these parameters and male age ($P = 0.6126$, $r^2 = 0.0037$; $P = 0.2150$, $r^2 = 0.0251$; $P = 0.8330$, $r^2 = 0.0006$; $P = 0.6361$, $r^2 = 0.0062$, respectively).

DISCUSSION

Although LP is extensively used to treat acid-related gastrointestinal diseases, its effect on human semen quality and hence on male infertility is still undetermined. Supporting *in vitro* studies on the safety of LP is of great importance for physicians as well as men with poor semen quality (e.g., men with

asthenozoospermia or oligozoospermia). In addition, investigations should be conducted in cases in which semen quality is threatened such as testicular cancer, testicular surgery, and chemotherapy treatments. To the best of our knowledge, this study is the first of its type to directly link LP and sperm parameters.

As a supporting study in the post-marketing surveillance or phase VI studies on LP, we asked whether the presence of LP in ejaculated semen would negatively affect sperm parameters, such as motility and viability. We found that LP at 3 $\mu\text{g}/\text{mL}$ decreased total and progressive motility, but not viability, of human sperm.

A previous case-control study by Huijgen *et al.* revealed that the long-term use of proton pump inhibitors, including LP, is associated with poor semen quality, particularly low sperm motility, in subfertile couples (17). These observations are to some extent in line with our observed effect of LP on sperm motility. Alternatively, in our previous systemic review, we concluded that omeprazole (another proton pump inhibitor) did not appear to change semen quality. However, this research is still immature and further studies are necessary to confirm this effect (4).

To explain the negative effect of LP on sperm motility, we measured the nitrite concentration as an indirect measure of the level of NO produced by sperm after adding LP. We suggested that the change in sperm motility after adding LP is modulated by alterations in the NO level. As a matter of fact, this suggestion is due to the evidence that NO is a double-edged sword in sperm motility; a certain amount of NO is crucial for adequate sperm motility (18), while higher (19-22), or lower levels of NO significantly reduces sperm motility (23). Studies by Lewis *et al.* and Wang *et al.* showed that endogenous NO production is critical for sperm motion (24, 25). In fact, NO enhances sperm motility by activating the soluble guanylate cyclase (sGC)/cGMP pathway (18). In this pathway, sGC catalyzes the conversion of guanosine 5'-triphosphate (GTP) to cyclic guanosine 3', 5'-monophosphate (cGMP) since it is the only receptor found to bind NO (18). Conversely, Balercia *et al.* showed that the concentration of NO in asthenozoospermic patients is much higher than in normozoospermic patients due to the overproduction of NO (26). This high NO level leads to oxidative damage, which lowers sperm motility (27). In the present study, we did not find a significant change in the nitrite concentration and hence in the NO level after adding LP to sperm. Therefore, it is unlikely that NO modulates the observed negative effect of LP on sperm motility at 3 $\mu\text{g/mL}$.

Free calcium is recognized as a key factor that regulates human sperm motility (28,29). Many synthetic as well as biological effectors may alter sperm function, and hence reproductive outcomes indirectly through a calcium-dependent mechanisms (30,31). Therefore, we suggest that the decrease in human sperm motility after adding LP to semen is due to the calcium chelation effect of LP. Indeed, the results from the intended spectrophotometric experiment supported our suggestion and indicated a calcium-chelating effect with LP. In this experiment, the decrease in the CPC-Ca absorbance suggested a competition between LP and the chromogen CPC to bind free calcium ions in the seminal plasma.

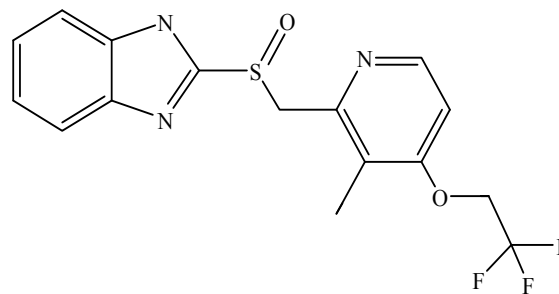


Fig. 6. The chemical structure of lansoprazol.

Indeed, chemical compounds with structures containing two or more of the following organic functional groups: $-\text{COOH}$, $-\text{SH}$, $-\text{NH}_2$, $-\text{NH}-$, $-\text{N}=\text{}$, $-\text{OH}$, $-\text{S}-$, $\text{C}=\text{O}$, $\text{S}=\text{O}$, $-\text{O}-$ are known to exhibit metal chelating effect (32,33). In this regard, LP with $-\text{NH}-$, $-\text{N}=\text{}$, $-\text{O}-$, and $\text{S}=\text{O}$ groups, is not an exception and is expected to act as a calcium chelator (Fig. 6).

Sodium-potassium adenosine triphosphatase (Na^+/K^+ -ATPase), also known as sodium-potassium pump, is an enzyme that is found in the plasma membrane of almost all animal cells (32,34). Na^+/K^+ -ATPase actively pumps sodium (out of cell) and potassium (into cell) against their concentration gradients to regulate the cell volume, maintain the cell resting potential, enhance cellular transport, affect signal transduction, and control neural activity states (35). It has been shown that Na^+/K^+ -ATPase is expressed in the midpiece of the spermatozoa and has been found to be very important for sperm motility (36). Lansoprazole at 10-100 μM was found to inhibit Na^+/K^+ -ATPase, and the maximum inhibition at 100 μM was approximately 86% (37). Accordingly, in the current study, the observed effect of LP on sperm motility may be partially due to decreased Na^+/K^+ -ATPase activity.

Independently, in this study, we presented the correlation between male age and the effect of LP at 3 $\mu\text{g/mL}$ on total and progressive sperm motility, sperm viability, and the nitrite concentration. We expected to find a correlation between male age and some of the tested parameters, especially sperm motility; however, against our expectation, none of the tested parameters were correlated with male age.

CONCLUSION

In conclusion, *in vitro*, LP reduced both total and progressive motility of human sperm, but not sperm viability. The reduction in sperm motility may be due to the calcium chelating effect of LP, but not an alteration in NO production. Besides, the observed effect of LP on sperm motility, viability, and NO production was not correlated with male age.

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