Original Article

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Vitamin B-6 Reduces Nitrite Oxide Levels, Interleukin-6, and Plasminogen Activator Inhibitor-1 in the Small Intestines and Reduces the Incidence of Post-Laparotomy Intraperitoneal Adhesions in Wistar Rats: A Randomized Trial

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Abstract:

Objective: Intraperitoneal adhesions occur in approximately 95% of cases after laparotomy. Vitamin B6 has a few effects, including the inhibition of nitric oxide (NO), suppression of Interleukin-6 (IL-6) production, and decreased fibrinolytic capacity. This study was conducted to determine whether intramuscular administration of Vitamin B6 reduces levels of NO, IL-6, and plasminogen activator inhibitor-1 (PAI-1) and the incidence of intraperitoneal adhesions.

Material and Methods: This experimental study was conducted with a Randomized Post-Test Only Control Group Design. This study used 20 male Wistar rats divided into 2 groups (treatment and control groups). The treatment group received an intramuscular injection of 10 mg/kg/day of Vitamin B6. NO, IL-6, and PAI-1 were measured using the Enzyme-linked Immunosorbent Assay (ELISA) method. Intraperitoneal adhesions were determined based on the Zuhkle criteria.

Results: The incidence of adhesion was 30% in the treatment group and 100% in the control group. NO, IL-6, PAI-1 levels, and the incidence of adhesion in the treatment group were significantly lower in the treatment group (p-value 0.002; <0.001; <0.001; 0.002, respectively). Furthermore, NO, IL-6, and PAI-1 levels were significantly lower in subjects without adhesions compared with those with adhesions (p-value<0.001, respectively). Vitamin B6 also decreased the risk of adhesions 3.3 times compared to the control group (HR 3.3; 95% CI: 1.29-8.59).

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Conclusion: There was a significant reduction in NO, IL-6, and PAI-1 levels after intramuscular administration of Vitamin B6, which contributed to a lower incidence of intraperitoneal adhesions.

Keywords: IL-6, intraperitoneal adhesion, NO, PAI-1, Vitamin B6

Introduction

The development of postoperative intraperitoneal adhesions is one of the most common complications after abdominal surgery[1]. Adhesions are a difficult problem for surgeons and a high economic burden for public health[2]. Although there has been a lot of research in this area, there is still no clinical standard for prevention, either surgically or pharmacologically, to control adhesion formation postoperatively[3].

The process of formation of intraperitoneal adhesions involves inflammatory mediators, fibrinolytic homeostasis, and oxidative stress reaction, and all of them work together to restore network integrity. Fibrinolysis is the key to understanding the pathogenesis of adhesions; fibrinolysis plays a role in the process of adhesion for breaking down the fibrin clot that forms during the healing process. Plasminogen will be converted to plasmin by the tissue plasminogen activator (tPA) and urokinase-like plasminogen activator (uPA). The main role of plasmin is fibrin degradation, whereas this process is inhibited by plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2). If plasminogen activator inhibitor (PAI) increases, the fibrinolysis process will be disturbed, leading to the formation of adhesions[4].

Vitamin B6 has been shown to modulate hypoxic and inflammatory effects, although its local effect on the peritoneum has not yet been confirmed[5]. Vitamin B6 was found to neutralize nitric oxide (NO) dysfunction by phosphorylation of endothelial nitric oxide synthase eNOS Thr495 through the mediation of protein kinase C alpha. Vitamin B6 can also reduce IL-6, which reduces inflammation significantly[6].

There is no direct study about the effect of Vitamin B6 in preventing intraperitoneal adhesion after surgery, in vivo or in vitro. Therefore, this study was conducted to determine the effect of Vitamin B6 administration in lowering the incidence of intraperitoneal adhesion.

Material and Methods

This experimental study used a randomized post-test-only control group design and was conducted in the Pharmacology Laboratory, Udayana University, Denpasar. The sample size was determined using the Federer formula, which resulted in a minimum of 10 subjects in each arm.

Subjects were male Wistar rats aged 6-8 weeks with a body weight of 200-250 grams. Twenty male rats (*Rattus norvegicus* Wistar strain) were prepared one week before for adaptation. Anesthesia was conducted using a ketamine 25 mg/kgBW intramuscular injection in the thigh muscle of the rats. Subsequently, the skin was disinfected using povidone iodine 10%. After anesthesia and disinfection, a 4-cm midline laparotomy was performed with caecal abrasion (CA) and gauze scrapping. Caecum abrasion and parietal peritoneum were conducted by scraping with gauze until wounds were present in the serous layer (bleeding spots for 1 cm). The caecum was then returned to the normal anatomical position inside the abdomen. The abdomen was subsequently closed, and the rats were monitored for 6 days. The rats were randomly assigned to the treatment group (receiving intramuscular injection of Vitamin B6 10 mg/kg/day) and the control group (no injection of Vitamin B6) for 10 rats, respectively. A simple randomization technique was used by researchers who

were blinded to the sample allocation. Relaparotomy was performed on the 6th day: the small intestine tissue of the rats was obtained for examination of NO, IL-6, and PAI-1 levels by Enzyme-Linked Immunosorbent Assay (ELISA). Assessment of intraperitoneal adhesions was performed according to the Zuhkle criteria. Rats that died or were severely ill during the study period were excluded from the study.

NO was measured using the ELISA method, with a normal value of 25–45 µmol/L. NO was categorized into low (<25 µmol/L), normal (25–45 µmol/L), and high (>45 µmol/L). Interleukin-6 (IL-6) was also measured using the ELISA method. The PAI-1 was obtained from the post-laparotomy intestine samples and measured using the ELISA method. Intraperitoneal adhesion was determined based on the Zuhkle criteria. Data in this study were analyzed using the SPSS program version 26. Outcomes were measured using the Mann-Whitney U test and Fisher Exact test. This study was approved by the Ethics Committee of Udayana University, Indonesia, with a protocol number of 2021.03.1.1292.

Results

All subjects were included in the final analysis, and no subjects died in the final analysis. The mean and median levels of NO, IL-6, and PAI-1 in the treatment group were much lower than in the control group (Table 1). Based on the incidence and intraperitoneal adhesion grade, the incidence of adhesion in the treatment group was 30%, while the control group had an incidence of 100% (Table 2).

The normality test with Shapiro-Wilk showed non-normally distributed data at the NO, IL-6, and PAI-1 levels (p-value 0.002, <0.001, <0.001, respectively) for the treatment group, while the control group had normally distributed data (p-value 0.111, <0.276, <0.228, respectively). Table 3 shows that there were statistically significant differences in the NO, IL-6, and PAI-1 levels between treatment and control groups (p-value 0.002; <0.001; <0.001, respectively) based on the Mann-Whitney U test.

The box plot for NO levels shows that NO levels in the treatment group were distributed within lower values, with most values under 20 and only 2 values deviating extremely above 40. Moreover, NO levels in the control group were distributed within higher values, even higher than the 2 extremely deviating values in the treatment group (Figure 1). The box plot for IL-6 levels in the treatment group also shows that IL-6 levels were distributed within lower values, with most values under 1000, while the IL-6 levels in the control group were distributed within higher values, reaching up to over 600 (Figure 2). Similarly, the box plot for PAI-1 levels shows that PAI-1 levels in the

Table 1 Distribution of nitric oxide (NO), interleukin-6 (IL-6), PAI-1 intestinal levels between groups

Variable	Treatment group				Control group			
	Mean (S.D.)	Median (IQR)	Range (min-max)	Normality test	Mean (S.D.)	Median (IQR)	Range (min-max)	Normality test
NO (µg/mL)	16.5 (18.7)	9.9 (15.9)	1.9-57.8	0.002	81.9 (69.4)	71.0 (127.3)	12.3-195	0.111
IL-6 (µg/mL)	44.6 (10.8)	47.6 (4.2)	14.3-51.8	<0.001	393.7 (238.3)	409.1 (489.7)	53.8-685.9	0.276
PAI-1 (ng/L)	1.2 (1.3)	0.6 (1.0)	0.4-3.8	<0.001	9.2 (7.0)	6.5 (11.5)	1.4-21.7	0.228

NO=nitric oxide, IL-6=interleukin 6, PAI-1=plasminogen activator inhibitor-1, S.D.=standard deviation, IQR=Interquartile range, Min=minimum, Max=maximum

treatment group were also distributed within lower values, with most values under 2 and only 2 values deviating to the range of 2.5–5. In the control group, the PAI-1 levels were distributed within higher values, even higher than the 2 deviating extreme values within the treatment group (Figure 3).

Based on the Fisher Exact test, we found a statistically significant difference in the incidence of intraperitoneal adhesion between the treatment and control group (p-value 0.002) (Table 4). Furthermore, Vitamin B6 intake decreased the risk of adhesion 3.3 times compared to the control group (HR=3.3; 95% CI: 1.29–8.59).

Table 2 Distribution and incidence of intraperitoneal adhesion between groups

Grade of adhesion	Treatment group		Control group	
	n	%	n	%
Without adhesion (Grade 0)	7	70	0	0
With adhesion				
Slight (Grade 1)	1	10	2	20
Mild (Grade 2)	0	0	1	10
Moderate (Grade 3)	2	20	5	50
Heavy (Grade 4)	0	0	2	20

Table 3 The difference of NO, IL-6, and PAI-1 between groups

Variable	Mean rank		Mann-whitney U value	p-value
	Treatment group	Control group		
NO	6.5	14.5	10.0	0.002*
IL-6	5.5	15.5	0	<0.001*
PAI-1	5.9	15.1	4.0	<0.001*

NO=nitric oxyde, IL-6=interleukin-6, PAI-1=plasminogen activator inhibitor, *Statistically significant

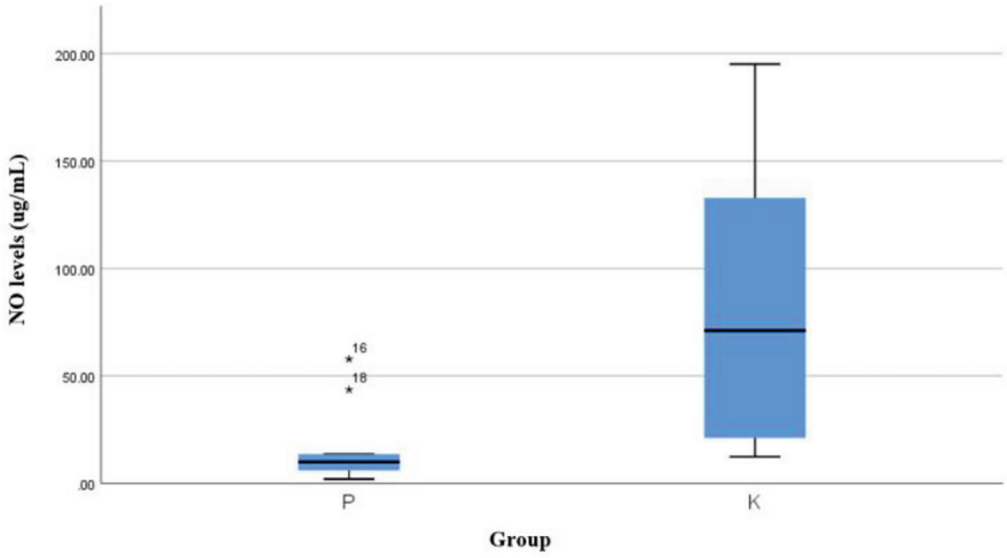

Figure 1 Box plot of the nitric oxide levels

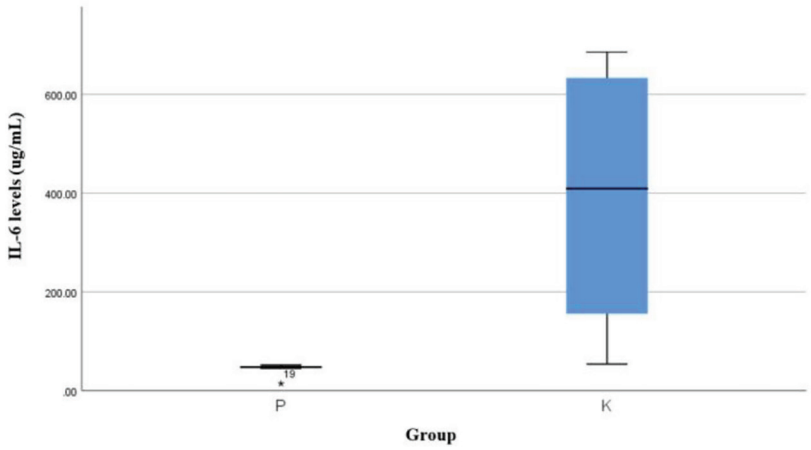

Figure 2 Box plot of the interleukin-6 levels

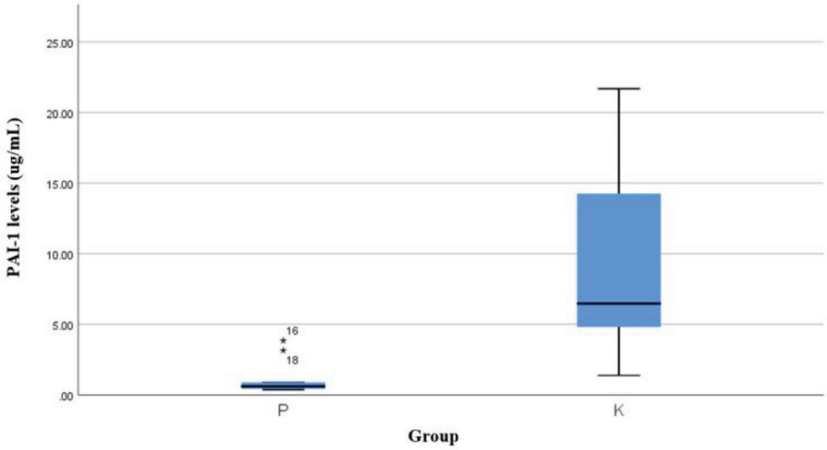

Figure 3 Box plot of the plasminogen activator inhibitor levels

Table 4 The difference of intraperitoneal adhesion between groups

Group	Adhesion		Total	Hazard ratio (HR) (95% CI)	p-value
	Yes	No			
Treatment	3 (30.0)	7 (70)	10	3.3 (1.293-8.591)	0.002*
Control	10 (100)	0 (0.0)	10		
Total	13 (65%)	7 (35%)	20		

*Statistically significant

Discussion

Peritoneal adhesion is reported to be the cause of 65-75% of cases of ileus obstruction, in which half of the cases are in acute conditions[7]. Further understanding regarding the pathophysiology of the development of intraperitoneal adhesion is necessary in order to bridge the clinical application of choosing the safest and most effective therapy in preventing intraperitoneal adhesion.

Evidence showed that lipid peroxidation resulting in oxidative stress is also responsible for the development of intraperitoneal adhesion besides NO and O2. Studies have shown that hypoxia, increasing the level of iNOS with a decrease in nNOS and eNOS levels, triggers the growth of adhesion after surgery[8].

This study found that intestinal NO levels were significantly lower in the Vitamin B6 supplementation group compared to the control group. A study by Wittman et al. also found that an administration of L-arginine decreased the level of hydroxyproline in the wound, leading to increased type 1 collagen deposition[9].

Vitamin B6 also hinders iNOS and cyclooxygenase 2 (COX2) induced by lipopolysaccharide due to the suppression of Nf-κB activation and pro-inflammatory transcription factors[10]. Ji et al. found that Vitamin B6 disrupts the formation of NO in endothelial cells and has preventive effects on endothelial dysfunction, although it requires a higher dose of 200 mg[11,12].

IL-6 was found to participate in modulating the cellular response to peritoneal injury in a series of inflammatory pathways. Saba et al. (1996) demonstrated that IL-6 plays a major role in the formation of peritoneal adhesions. The score of adhesion formation increased significantly in the IL-6 group (2.78±0.44, Mean±S.D.) and decreased in the anti-IL-6 group (1.40±0.52) compared to the control group (2.00±0.50) (p-value<0.03)[13].

In this study, it was shown that intestinal IL-6 levels were significantly lower in the Vitamin B6 group compared to the control group. Consistent with these results, Vitamin B6 was found to reduce the expression of pro-inflammatory cytokines through the suppression of the NF-kB, MAPK, and Toll-like receptor-1 (TLR-1) signalling pathways, thereby decreasing pro-inflammatory interleukins, especially IL-6[14]. In addition, pyridoxine is known to induce monocyte macrophages to undergo apoptosis, thereby decreasing proinflammatory cytokines[15].

Huang et al. (2010) showed that large doses of Vitamin B6 supplementation (100 mg/day) suppressed pro-inflammatory cytokines (IL-6 and Tumor necrosis factor-alpha (TNF-alpha)) in patients with rheumatoid arthritis at week 12. Du et al. also found that mRNA expression of IL-1β, TNF-α, IL-6, and iNOS was reduced in the Vitamin B6 group compared to the control group. In the study of Shan et al. (2020), the dose-dependent administration of Vitamin B6 was shown to prevent increases in TNF-α, IL-1β, and IL-6 in the macrophage culture media, given an LPS-induced inflammatory gene expression of 100 ng/mL per 24 hours[16,17].

The physiological fibrinolytic cascade is initiated by plasmin. Tissue plasminogen activator (tPA) is responsible for producing 95% of the plasmin produced in response to peritoneal injury[18]. Plasminogen is converted to plasmin by the PA and the uPA. The main role of plasmin is the degradation of fibrin, whereas PAI-1 inhibits this process. Surgery dramatically reduces fibrinolytic activity by increasing PAI levels and reducing tissue oxygenation[19].

In this study, it was shown that intestinal PAI-1 levels were significantly lower in the Vitamin B6 group compared to the control group. The direct effect of Vitamin B6 on fibrinolysis has not yet been confirmed, but a Vitamin B6 derivative, pyridoxal phosphate, significantly stimulated fibrinolytic activity in both in vivo and in vitro studies. A study by Dusitanond et al. in 2004 showed that Vitamin B6, in combination with Vitamin B12 and folic acid, increased the effect of fibrinolysis on hemostasis. Another study by Rodrigues et al. in 2006 showed that Vitamin B6 supplementation contributed to the decrease in PAI-1 to an unknown extent. In vitro, homocysteine impairs the intrinsic fibrinolytic system of endothelial cells by forming a Cys9 covalent derivative to prevent any interaction with t-PA and increase PAI-1[20,21].

The formation of intraperitoneal adhesions occurs in response to peritoneal trauma from tissue manipulation, ischemia (hypoxia), and inflammation. Theoretically, Vitamin B6 has interesting biological properties and actions in preventing peritoneal adhesions. In this study, it was shown that the incidence of intraperitoneal adhesions in the treatment group was significantly lower than in the control group, in which no non-adhesion events were found in Wistar rats that did not receive Vitamin B6 supplementation. In this regard, previous studies have shown that the administration of Vitamin B6 is not only proven to reduce the levels of the 3 previous biomarkers, but Vitamin B6 is also macroscopically proven to prevent intraperitoneal adhesions[3].

Furthermore, this study showed that the levels of NO, IL-6, and PAI-1 were significantly lower in the adhesion group than in the non-adhesion group. These results support the theory that the role of Vitamin B6 supplementation in preventing adhesion is based on the pathways of free radical (NO) prevention, inflammation prevention (IL-6), and fibrinolysis (PAI-1). In the risk analysis, an administration of intramuscular Vitamin B6 was found to reduce the risk of developing adhesions 3.33 times compared to the group without Vitamin B6. This study has shown that Vitamin B6 reduces oxidative stress via the Nom pathway, reduces inflammation via the IL-6 pathway, and reduces fibrinolysis via the PAI-1 pathway, which bridges the potential effect for adhesion prevention. However, this study has several limitations, including the small sample size, short period of follow-up, and the presence of potential confounding factors such as wound healing and overall inflammatory response, and the lack of a sham group. A greater sample size would improve the statistical power of the study, while the addition of a sham group would increase the validity of the results. Future studies should also consider the side effects of Vitamin B6 in order to assess its safety.

Conclusion

NO, IL-6, and PAI-1 levels in the small intestine tissue of rats on day 6 following Vitamin B6 administration were significantly lower compared to the control group. Furthermore, the incidence of intraperitoneal adhesion was also significantly lower in the group with Vitamin B6 compared to the control group.

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Conflict of interest

The authors report no conflict of interest.

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