The effect of sulbutiamine, thiamine, riboflavin and their combinations on apoptotic biomarkers (CASP-3, CASP-9) and neutrophil gelatinase-associated lipocalin in vancomycin-induced acute renal failure in male rats

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Abstract:

Vancomycin is a glycopeptide antibiotic used to treat anaerobic and aerobic gram-positive bacteria, for example, methicillin-resistant Staphylococcus aureus (MRSA) and Staphylococcus epidermidis. Vancomycin had some side effects, such as nephrotoxicity, ototoxicity, hypersensitivity reactions, and more. The present study aimed to evaluate the potential apoptotic effect of vancomycin on renal tissue and also evaluate the reno-protective effects of sulbutiamine, thiamine, riboflavin, and their combinations through inhibiting apoptosis.

Forty-two male rats were employed in this study, divided randomly into seven groups: the first group was the control group, the second group (the vancomycin group received 200 mg/ml twice daily) from the 15^{th}-21^{st} day of the study, and the other three groups were pretreated with sulbutiamine (50 mg/kg, orally once daily), thiamine (100 mg/kg, orally once daily), and riboflavin (100 mg/kg, orally once daily), respectively, for 21 days of the study. The sixth group was a combination group that received a mix of sulbutiamine and riboflavin, and the seventh group received a mix of thiamine and riboflavin for 21 days. The last five groups received vancomycin 200 mg/ml twice daily from the 15^{th}-21^{st} day of the study. The result of this study showed a significant increase $p<0.05$ in caspase-3, caspase-9, and NGAL in the induction group, while in the other group that was pretreated with sulbutiamine, thiamine, riboflavin, and their combinations, the mean tissue CASP-3, CASP-9, and concentration were significantly decreased. We concluded that the sulbutiamine, thiamine, and riboflavin-treated groups showed a significant decrease in caspase 3, caspase 9, and NGAL levels compared to the vancomycin-treated group. The combination group (sulbutiamine and riboflavin) showed the most significant decrease in mean tissue concentrations of CASP3, CASP9, and NGAL due to the additive effects of both treatments.

Key words: Vancomycin, ARF, Sulbutiamine, Apoptosis, NGAL.

تأثير السلبوتيامين والثيامين والريبوفلافين ومزيجهما على المؤشرات الحيوية للموت المبرمج في الفشل الكلوي الحاد الناجم عن الفانكومايسين في ذكور الجرذان

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خلاصة

الفانكومايسين هو مضاد حيوي جلايكوببتايدي، يستخدم لعلاج البكتيريا اللاهوائية والهوائية موجبة الجرام، على سبيل المثال، المكورات العنقودية الذهبية المقاومة للميثيسيلين (MRSA) والمكورات العنقودية الجلدية. للفانكومايسين بعض الآثار الجانبية مثل السمية الكلوية، السمية الأذنية، تفاعلات فرط الحساسية، وغيرها.

تهدف هذه الدراسة إلى تقييم تأثير الموت المبرمج المحتمل للفانكومايسين على أنسجة الكلى وكذلك تقييم التأثير الوقائي للكلى لكل من السولبوتيامين والثيامين والريبوفلافين ومزيجهما من خلال تثبيط موت الخلايا المبرمج.

تم إستخدام إثنان وأربعون من ذكور الفئران في هذه الدراسة، وقد تم تقسيمهم عشوائياً إلى سبع مجموعات؛ المجموعة الأولى مجموعة السيطرة، المجموعة الثانية مجموعة إستحداث الفشل الكلوي الحاد عن طريق إعطاء الفانكومايسين بجرعة (200 ملغم/مل مرتين يومياً) من اليوم الخامس عشر الى اليوم الواحد والعشرون من الدراسة. المجموعات الثلاث الأخرى أعطيت السلبيوتامين (50 ملغ/كغم عن طريق الفم مرة يوميا) والثيامين (100 ملغ/كغم عن طريق الفم مرة يوميا) والريبوفلافين (100 ملغ/كغم عن طريق الفم مرة يوميا) على التوالي لمدة 21 يوما من الدراسة، والمجموعة السادسة أعطيت مزيج من (سلبيوتامين + ريبوفلافين) والمجموعة السابعة أعطي (ثيامين + ريبوفلافين) لمدة 21 يوماً، وتم إعطاء هذه المجموعات الخمس الفانكومايسين بجرعة (200 ملغم/مل مرتين يومياً) من اليوم الخامس عشر الى اليوم الواحد والعشرون من الدراسة.

أظهرت نتائج هذه الدراسة زيادة معنوية $p<0.0$ في الكاسبيز 3 والكاسبيز 9 وNGAL في المجموعة الثانية (مجمعة الإستحداث)، بينما أظهر متوسط تركيز الكاسبيز 3 والكاسبيز 9 وNGAL في الأنسجة إنخفاضاً بشكل ملحوظ في المجموعات الأخرى التي تم معاملتها بالسلبيوتيامين والثيامين والريبوفلافين. استنتجنا إلى أن المجموعات المعالجة بالسلبيوتامين والثيامين والريبوفلافين أظهرت انخفاضًا كبيرًا في مستويات كاسبيز 3 وكاسبيز 9 وNGAL مقارنة بالمجموعة المعالجة بالفانكومايسين. أظهرت المجموعة المركبة (السلبيوتامين والريبوفلافين) الانخفاض الأكثر أهمية في متوسط تركيزات للكاسبيز 3 وكاسبيز 9 وNGAL بسبب التأثيرات الإضافية لكلا العلاجين.

الكلمات المفتاحية: فانكومايسين, الفشل الكلوي الحاد، سلبيوتامين, موت الخلايا المبرمج، NGAL.

Introduction

Acute kidney injury (AKI), also referred to acute renal failure (ARF), is a complicated medical illness associated with a high death and morbidity rate. Nephrotoxic medications are associated with 19–33% of AKI incidents among hospitalized patients [1]. The typical biochemical markers of acute kidney injury (AKI), such as serum creatinine and BUN, are undetectable until permanent damage to the kidneys occurs. Because of this, it becomes more difficult to diagnose AKI early, and the death rate increases. New early markers for AKI are needed to enhance outcome prediction, diagnosis, therapy, and prevention. Neutrophil gelatinase-associated lipocalin (NGAL) has been shown to have a close relationship with AKI in recent research. Urine and serum NGAL expression is greatly elevated in AKI, according to several experimental and clinical studies. Specifically, the level of NGAL in the urine is directly linked to the severity of kidney damage and can be identified earlier than other markers of AKI [2]. Apoptosis is a crucial process in ischemic acute kidney injury (AKI), yet there is debate over its significance in septic AKI. Apoptotic biomarkers have the ability to identify AKI in progress before a clinical diagnosis is made [3].

Vancomycin is a glycopeptide antibiotic that is extensively used to treat anaerobic and aerobic gram-positive bacteria, for example, methicillin-resistant Staphylococcus aureus (MRSA) and Staphylococcus epidermidis [4]. Despite being allowed for clinical use since 1958, VCM has some side effects, such as

nephrotoxicity [5]. It's unclear exactly how VCM causes nephrotoxicity [6],[7]. Oxidative stress, apoptosis, and inflammation have all been linked to its pathophysiology, according to many studies, which impacts renal proximal tubular epithelial cells and results in acute tubulointerstitial injury and renal tubular ischemia [8],[9],[10]. Oxidative stress upregulates the caspase family, which is responsible for caspase-dependent apoptosis [11]. After exposure to acute ischemia or nephrotoxic compounds in the kidney, renal tubular cells are fatally killed by apoptosis [12]. Numerous investigations have demonstrated that free radicals contribute to VCM-induced kidney failure by altering the innate antioxidant defense mechanism of cells [13]. Preserving the kidneys from unfavorable side effects is one strategy to increase the beneficial effects of VCM in clinical settings. Because of this, natural substances that include antioxidant and anti-apoptotic activities and have the potential to mitigate or prevent renal impairment caused by VCM may prove useful in clinical settings [14].

Sulbutiamine (the synthetic substance) is made up of two thiamine (vitamin B1) molecules joined by a disulfide link, which gives the chemical its lipophilic characteristics. As a result, it boosts the amounts of thiamine and thiamine phosphate esters in the brain and passes the blood-brain barrier more easily than thiamine [15]. It is hypothesized that sulbutiamine can effectively treat chronic fatigue and asthenia. The previous retrospective study set out to assess how sulbutiamine affected the degree of fatigue experienced by multiple sclerosis patients [16]. Serum deprivation-induced apoptotic cell death is attenuated by sulbutiamine, which also dose-dependently increases reduced glutathione (GSH) and glutathione S-transferase (GST) activity. Moreover, sulbutiamine reduces the expression of apoptosis-inducing factor (AIF) and cleaved caspase-3 [17].

Thiamine, often known as vitamin B1, is a helpful cofactor for various enzymes, particularly those involved in mitochondrial localization, which is crucial in avoiding apoptosis [18]. Because thiamin is in the cationic form (T+) at physiological pH, it has an impact on neuronal membrane potential as well as nerve message transmission and conduction [19].Triphosphate (TTP), pyrophosphate (TPP), and monophosphate (TMP) are the products of phosphorylating thiamine [20]. The primary and most important function of T+ is as an antioxidant, in contrast to TTP and TPP, which are involved in energy metabolism reactions. Thiamine is oxidized to produce thiamine disulfide and thiochrome compounds, which function as antioxidants and can control inflammatory responses and apoptotic events [21].

Riboflavin, sometimes referred to as vitamin B2, is a vital element that is essential for human health maintenance. Being a precursor to the electron-carrying molecules flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), it is crucial for a variety of redox processes, the synthesis of mitochondrial energy, and cellular activity[22]. According to reports, riboflavin saves cells from the damaging effects of reactive ROS, which can degrade proteins, lipids, and DNA and induce cell stress and trigger a rise in apoptosis [23],[24]. Riboflavin functions as an antioxidant. Additionally, riboflavin has demonstrated anti-inflammatory properties in a number of animal models[25]. Riboflavin deficiency also causes apoptosis and suppresses cell division in vivo [26].

Methods

In this study, 42 male Wister rats weighing 160-200 grams were used. Rats were housed in large, comfortable cages in the “Iraqi Center for Cancer Research” after being

obtained from this center. Animal Ethical Committee of the Pharmacology and Toxicology Department College of Pharmacy at Mustansiriyah University gave its approval before this study had begun. Rats were placed in a controlled environment with (24±2°C) temperature, 40-50% humidity, and a 12-hour light–dark cycle for one week in order to allow them to acclimatize**.** Their access to food and drink was unrestricted [27].

Experimental design

Rats were divided into seven groups, with six rats in each group.

1. Control group: rats received PBS orally for 21 days, then received intraperitoneal injections of normal saline (5 ml/kg) twice daily from the 15th-21st day of the study.
2. Induction group: rats were administered PBS orally for 21 days, then received intraperitoneal injections of vancomycin (200 mg/kg i.p.) twice daily from the 15th-21st day of the study.
3. Sulbutiamine group: rats received sulutiamine (50 mg/kg p.o.) for 21 days, then received intraperitoneal injections of vancomycin (200 mg/kg i.p.) twice daily from the 15th-21st day of the study.
4. Thiamine group: rats received thiamine (100 mg/kg p.o.) for 21 days, then received intraperitoneal injections of vancomycin (200 mg/kg i.p.) twice daily from the 15th-21st day.
5. Riboflavin group: rats received riboflavin (100 mg/kg p.o.) for 21 days, then received intraperitoneal injections of vancomycin (200 mg/kg i.p.) twice daily from the 15th-21st day of the study.
6. Combination group (sulbutiamine+riboflavin): rats received a combination of sulbutiamine (50 mg/kg p.o.) and riboflavin (100 mg/kg p.o.) for 21 days, then received intraperitoneal injections of vancomycin (200 mg/kg i.p.) twice daily from the 15th-21st day of the study.
7. Combination group (thiamine+riboflavin): rats received a combination of thiamine (100 mg/kg p.o.) and riboflavin (100 mg/kg p.o.) for 21 days, then received intraperitoneal injections of vancomycin (200 mg/kg i.p.) twice daily from the 15th-21st day of the study.

The rats were given xylazine (VMD-inovet) (10 mg/kg) and ketamine (Bremer-pharma) at 90 mg/kg to induce unconsciousness on the twenty-second day. Using forceps and scissors, the rat's abdominal cavity was opened to remove the kidney. As directed by the manufacturer for ELISA analysis, the kidney was homogenized using a tissue homogenizer with 10 ml of phosphate buffered saline (PBS) and kept at -20 °C for the biochemical experiment.

Determination of Caspase 3 in renal tissue by ELISA technique

A biotinylated polyclonal antibody was used as the detection antibody, and an anti-Human Caspase-3 monoclonal antibody served as the pre-coated antibody. According to the manufacturers' instructions, the steps were as follows: Antibodies had already been pre-coated on the plate. Antibodies that failed to stick to the plate were all removed by washing. The leftover antibodies on the plate were blocked using irrelevant proteins. After that, the sample containing the caspase-3 target analyte was used, leading to the creation of an antigen-antibody combination by immobilizing the target analyte with analyte-specific capture antibodies. Then, by cleaning the wells, all suspended particles and possible contaminants were eliminated. An antibody, antigen, and antibody complex were created by filling the wells with a biotin-labeled antibody that was specific for the target analyte. The plate was then cleaned to get rid of any unbound antibodies. Following that, the wells were filled with horseradish peroxidase and avidin, which were then bound by the biotin-labeled antibodies. The quantity of target analyte in

the sample was positively correlated with the amount of reporter enzyme present. Then wash again. At the end, substrates for the HRP reaction were added. By adding sulfuric acid solution, the enzyme-substrate process was halted, and the Huma-reader device was used to estimate the sample concentrations based on color changes.

Determination of Caspase 9 in renal tissue by ELISA technique

By using the same technique and principle explained previously, caspase-3 was detected. The plate had been pre-coated with a monoclonal antibody that was specific to caspase-9. The wells of the plates were filled with standards and homogenized tissue samples. The antibodies were pre-coated wells bound to caspase-9. For caspase-9 on the plate, a polyclonal antibody specific for caspase-9 coupled with horseradish peroxidase (HRP) was applied to each well. The procedure was employed according to the manufacturers' instructions. Following the plate incubation, all unbound components were removed from the wells by giving them a thorough wash. Subsequently, substrate solutions were applied to each well. The colour shift was observed spectrophotometrically at 450 nm by the Huma-reader subsequent to the addition of a sulfuric acid solution, which interrupted the enzyme-substrate process.

Determination of NGAL in serum by ELISA technique:

According to the manufacturers' instructions, the steps were as follows: The serum sample and standers are pipetted into ELISA plate wells that are precoated with NGAL-specific antibodies. Then wash the plate with PBS or TBS to remove uncombined antibodies and impurities. Adding avidin-peroxidase conjugated to the wells was followed by another wash. Adding TMP substrate, according to how much NGAL was present, a blue color appeared from peroxidase activity. Then we ceased the color development with the addition of a stooping reagent, which turns the blue color to yellow, and the color intensity was measured spectrophotometrically at 450nm.

Results

The effect of sulbutiamine, thiamine, riboflavin and their combinations on tissue apoptotic marker CASP3, CASP9:

The table below shows the effect of sulbutiamine, thiamine, and riboflavin and their combinations on tissue apoptotic markers in vancomycin-induced acute renal failure in male rats.

Table 1: The effect of sulbutiamine, thiamine, riboflavin and their combinations on apoptotic biomarker in vancomycin-induced acute renal failure in male rats.

Groups	Caspase3 ng/ml	Caspase9 ng/ml
Control	18.19 ± 0.44^{cd}	194.73 ± 9.94^{c}
Induction	39.09 ± 0.23^{a}	631.61 ± 141.82^{a}
Sulbutiamine	18.65 ± 0.38^{c}	275.63 ± 20.06^{bc}
Thiamin	19.71 ± 0.35^{b}	360.91 ± 21.85^{b}
Riboflavin	19.77 ± 0.44^{b}	367.51 ± 17.44^{b}
Sulbutiamine+ Riboflavin	17.52 ± 0.11^{d}	184.06 ± 9.94^{c}
Thiamin + Riboflavin	18.67 ± 0.40^{c}	210.13 ± 10.68^{bc}

Data are represented as mean±SD (standard deviations).
Different lowercase letters (a,b,c,d) referred to significant differences $p<0.05$ among groups.

A. The effect of the sulbutiamine, thiamine, riboflavin and their combinations on the CASP3 level:

The induction group had a mean tissue caspase-3 concentration of (39.09±0.23 ng/ml), which was considerably greater *p<0.05* than the control group's value of (18.19±0.44 ng/ml). Following 21 days of sulbutiamine, thiamine, riboflavin, sulbutiamine+ Riboflavin, and thiamin + Riboflavin therapy, the mean tissue concentration of CASP-3 was significantly lower *p<0.05* in the aforementioned groups (18.65±0.38 ng/ml), (19.71±0.35 ng/ml), (19.77±0.44 ng/ml), (17.52±0.11 ng/ml), and (18.67±0.40 ng/ml) respectively, when compared to the induction group. As shown in figure (1).

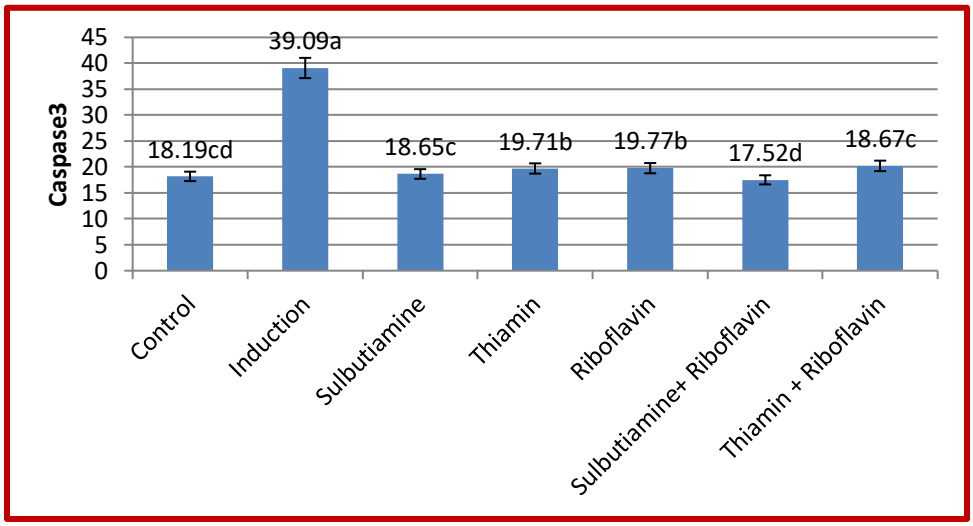

Figure (1): The effect of sulbutiamine, thiamine, riboflavin and their combinations on CASP-3.

B. The effect of the sulbutiamine, thiamine, riboflavin and their combinations on the CASP9 level:

The findings showed that the induction group's mean tissue CASP-9 concentration (631.61±141.82 ng/ml) was considerably greater than that of the control group (194.73±9.94 ng/ml). Following a 21-day course of sulbutiamine, thiamine, riboflavin, sulbutiamine+ Riboflavin, and thiamin + Riboflavin therapy, the mean tissue concentration of CASP9 was significantly lower than that of the induction group at *p<0.05* (275.63±20.06 ng/ml), (360.91±21.85 ng/ml), (367.51±17.44 ng/ml), (184.06±9.94 ng/ml), and (210.13±10.68 ng/ml), respectively. The combination group (sulbutiamine + riboflavin) had the best findings, with the mean tissue CASP-9 concentration falling nearly to the control group's level. As shown in figure (2).

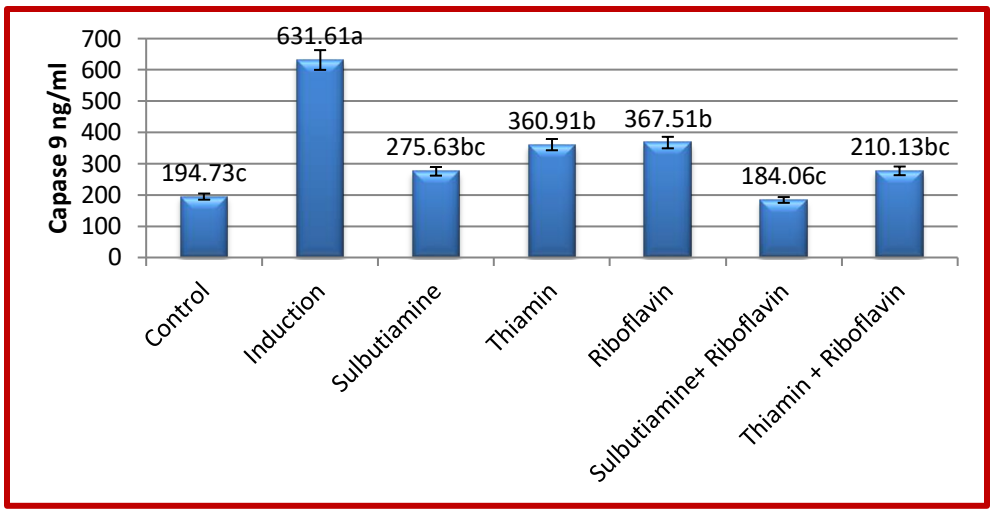

Figure (2): The effect of sulbutiamine, thiamine, riboflavin and their combinations on CASP-9.

The effect of sulbutiamine, thiamine, riboflavin and their combinations on serum NGAL:

Table 2 shows the effect of sulbutiamine, thiamine, riboflavin and their combinations on neutrophil gelatinase associated-lipocalin marker in vancomycin-induced acute renal failure in male rats.

The study found that the induction group had considerably higher mean serum NGAL levels (1788.23±31.74 ng/ml) compared to the control group (660.87±29.84 ng/ml). Treatment with sulbutiamine, thiamine, riboflavin, sulbutiamine+riboflavin, and Thiamin+riboflavin resulted in significant decreases $p<0.05$ in mean serum NGAL concentration (754.04±43.61 ng/ml), (924.53±20.79 ng/ml), (820.50±51.81 ng/ml), (570.79±18.27 ng/ml), and (733.99±18.51 ng/ml) when compared to the induction group.

A better result was shown with the combination group, which significantly reduced $p<0.05$ in the mean concentration of NGAL level (570.79±18.27 ng/ml) compared to the induction group (1788.23±31.74 ng/ml) but showed no significant differences with the control group. As shown in figure (3).

Table 2: The effect of sulbutiamine, thiamine, riboflavin and their combinations on NGAL marker in vancomycin-induced acute renal failure in male rats.

Groups	NGAL ng/ml
Control	660.87±29.84^{de}
Induction	1788.23±31.74^{a}
Sulbutiamine	754.04±43.61^{cd}
Thiamin	924.53±20.79^{b}
Riboflavin	820.50±51.81^{c}
Sulbutiamine+ Riboflavin	570.79±18.27^{e}
Thiamin + Riboflavin	733.99±18.51^{cd}
LSD	94.56

Data are represented as mean±SD (standard deviations).

Different lowercase letters (a,b,c,d) referred to significant differences p<0.05 among groups.

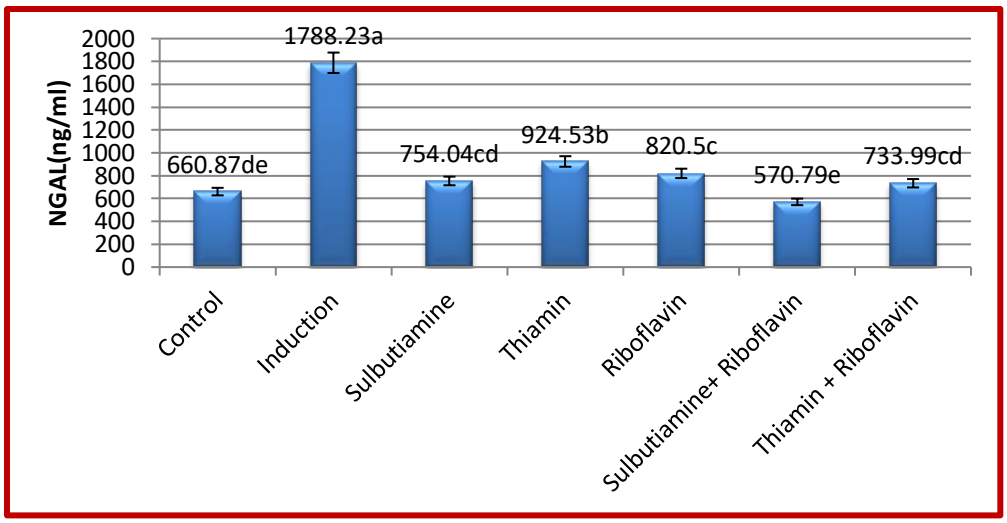

Figure (3): The effect of sulbutiamine, thiamine, riboflavin and their combinations on the serum NGAL level.

Discussion

Vancomycin is one of the most common antibiotics used to treat methicillin-resistant Staphylococcus aureus (MRSA). However, because of the possibility of negative effects, vancomycin use is closely restricted [28]. Vancomycin's primary side effects include nephrotoxicity, ototoxicity, hypersensitivity reactions, and more [29].

In cultured renal cells, vancomycin increases oxygen consumption, suggesting that it can increase mitochondrial oxidative phosphorylation. Consuming oxygen can result in the production of reactive oxygen species [30].

ROS cause lipid peroxidation, which alters cardiolipin in cell membranes, resulting in mitochondrial membrane depolarization. Damage to the mitochondrial membrane causes the release of cytochrome C, the activation of caspases, and apoptosis [31].

To conduct this study, sulbutiamine, thiamine, riboflavin, and their combination were used to prevent kidney injury caused by the treatment with vancomycin. Analyzing the apoptotic pathway was done. In this study, the mean tissue caspase-3 and caspase-9 concentrations were higher in the induction group (vancomycin group) as compared to the control group, and this is consistent with a previous study that revealed VCM caused the LLC-PK1 cells to undergo apoptosis by depolarizing the mitochondrial membrane, generating more intracellular reactive oxygen species, and activating caspase-9 and −3/7 [9].

Sulbutiamine is a lipophilic compound; in comparison to thiamine, sulbutiamine has a greater ability to pass through cellular membranes. Thioesterase activity quickly breaks it down into individual thiamine molecules inside the cell. The main reason sulbutiamine has an antioxidant impact is because of its thiol content, which has the ability to control the antioxidant environment inside cells [15].

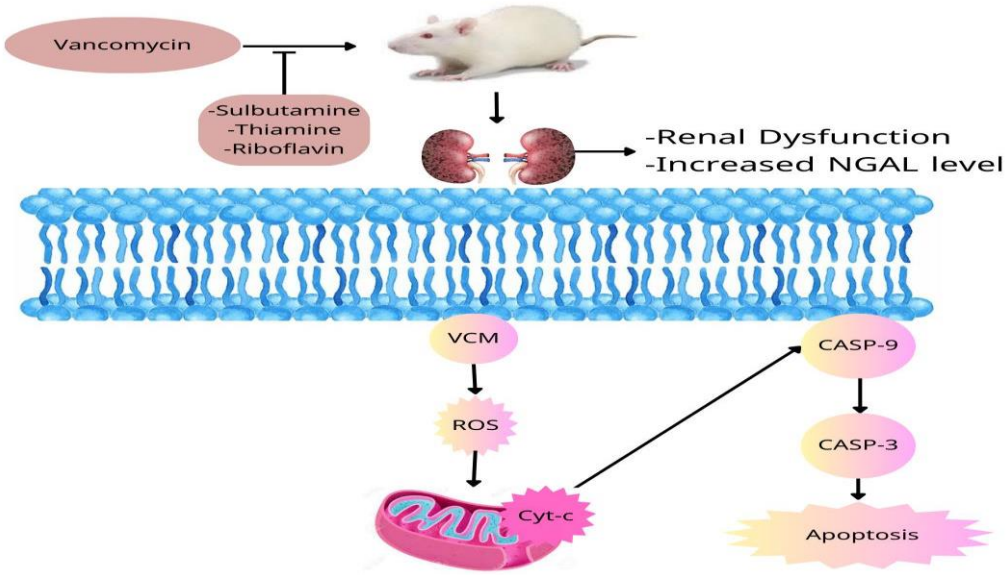

Figure (4): The anti-apoptotic mechanism of sulbutiamine, thiamine and riboflavin on vancomycin-induced acute renal failure in male rats.

Thiamine is metabolized to the active product thiamine pyrophosphate. Thiamine pyrophosphate's antioxidant activity may be responsible for the protective mechanism it is thought to have against cisplatin-induced testicular damage. Sulbutiamine, thiamine, and riboflavin are expressed as antioxidant supplements. These vitamins lead to scavenging free radicals, preventing apoptosis, and resolving renal injury[32],[33],[34].

Our study demonstrated that after 21 treatments with sulbutiamine, thiamine, and riboflavin, the mean tissue caspase-3 and caspase-9 decreased significantly as compared with the induction group, and this is consistent with previous studies [17],[35],[36]. The most promising novel marker of renal epithelial injury is neutrophil gelatinase-associated lipocalin (NGAL), a 25-kDa protein generated by injured nephron epithelia. Unlike urine output and serum creatinine, which are indicators of kidney function, NGAL is released into the bloodstream and urine after being selectively stimulated in the injured nephron, making it easy to assess [37]. Kidney damage causes an increase in the synthesis of NGAL, which is generated in the distal nephron. NGAL is a substance that transports ions. Actually, damage markers are preferable than functional markers [38]. A previous study [39] revealed that in order to investigate the genetic relationship between NGAL and caspase 3 in human proximal renal tubular epithelial cells and HK-2 cells, they used LPS stimulation. They discovered that 1-3 hours after treatment, caspase-3 mRNA was significantly up-regulated, and 1-6 hours after LPS treatment, NGAL mRNA was upregulated. These findings suggest that apoptosis in HK-2 cells was initiated in a manner similar to that in rats with septic AKI and that NGAL and caspase-3 are linked together. When nephrotoxic compounds damage kidney cells, NGAL expression is increased and expressed in the tubular epithelium. According to previous investigations, VCM-induced kidney injury can be detected by the sensitive biomarker NGAL[40],[41]. In this study, we also found that NGAL expression was significantly enhanced after kidney injury by vancomycin. The current study revealed that after three

weeks of treatment with sulbutiamine, thiamine, and riboflavin, the kidney injury will be resolved, and the mean concentration of NGAL was significantly decreased as compared with the induction group. The best results were shown with the combination group (sulbutiamine+riboflavin) due to the additive action of riboflavin with sulbutiamine.

Conclusion

According to the findings of this study, downregulating caspase 3 and caspase 9 within the apoptotic pathway in renal tissues and decreasing the mean serum concentration of NGAL can be achieved by pretreatment with sulbutiamine, thiamine, and riboflavin alone or in combination to mitigate vancomycin-induced acute renal failure. Because riboflavin and sulbutiamine have an additive effect, the combination group (sulbutiamine and riboflavin) had the best results.

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