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Can elevated prolidase activity predict the duration of ischemic exposure in different types of ischemia?

Yükselmiş prolidaz aktivitesi farklı iskemi tiplerinde iskemi maruziyet süresini belirleyebilir mi?

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Background: This study aims to determine the relationship between serum prolidase activity and ischemia duration in different ischemia types.

Methods: Forty male Sprague Dawley rats were divided into five equal groups. The rats were sacrificed and blood samples were obtained to determine the basal serum prolidase levels in group 1 (control group) without any intervention. In groups 2 and 3, the superior mesenteric arteries were clamped with simple laparotomy to induce mesenteric ischemia. In groups 4 and 5, the right common femoral artery was clamped to induce peripheral ischemia and blood samples were taken at 120 and at 360 minutes, respectively. The serum prolidase levels were measured using the samples obtained from each group.

Results: The basal prolidase level in rats was found to be 266.8±20.5 U/L. The serum prolidase levels increased after two-hours of peripheral (404.0±105.6 U/L) and mesenteric ischemia (317.1±121.4 U/L). However, the serum prolidase levels decreased after six-hours of peripheral (346.1±104.9 U/L) and mesenteric ischemia (233.4±36.6 U/L). Although the serum prolidase levels were elevated in the second hour of mesenteric ischemia, they were lower than the enzyme levels obtained after two-hours of peripheral ischemia (p=0.006).

Conclusion: The serum prolidase level may be an important predictive biomarker for identifying the duration of ischemia.

Key words: Biomarker; ischemia duration; mesenteric ischemia; peripheral ischemia; prolidase level.

Amaç: Bu çalışmada farklı iskemi tiplerinde serum prolidaz aktivitesi ve iskemi süresi arasındaki ilişkinin belirlenmesi amaçlandı.

Çalışma planı: Kırk erkek Sprague-Dawley cinsi sıçan beş eşit gruba bölündü. Grup 1'deki sıçanlar (kontrol grubu) bazal serum prolidaz düzeylerinin belirlenmesi için herhangi bir işlem uygulanmadan sakrifiye edildi ve kan örnekleri alındı. Grup 2 ve 3'de mezenterik iskemi oluşturmak için basit laparotomi yapılarak, superior mezenterik arterler klemplendi. Grup 4 ve 5'de periferik iskemi oluşturmak için sağ ana femoral arter klemplenerek sırasıyla 120. ve 360. dakikalarda kan örnekleri alındı. Tüm gruplardan elde edilen örneklerden serum prolidaz düzeyi ölçüldü.

Bulgular: Sıçanlardaki bazal prolidaz düzeyleri 266.8±20.5 U/L olarak bulundu. Periferik (404.0±105.6 U/L) ve mezenterik iskeminin (317.1±121.4 U/L) iki saat sonrasında serum prolidaz aktiviteleri arttı. Ancak, periferik (346.1±104.9 U/L) ve mezenterik iskeminin (233.4±36.6 U/L) altı saat sonrasında serum prolidaz düzeyleri azaldı. Serum prolidaz düzeyleri mezenterik iskemide artmasında rağmen, bu periferik iskemide iki saat sonra elde edilen enzim düzeylerinden daha düşüktü (p=0.006).

Sonuç: Serum prolidaz aktivitesi, iskemi süresinin belirlenmesinde önemli bir öngördürücü biyobelirteç olabilir.

Anahtar sözcükler: Biyobelirteç; iskemi süresi; mezenterik iskemi; periferik iskemi; prolidaz düzeyi.

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Tissue ischemia is defined as an insufficient supply of blood to distal areas, and it commonly arises from occlusion, vasospasms, and/or hypoperfusion of the vasculature. Ischemic duration is an important determinant for tissue recovery or end-organ damage.[1,2] For example, cardiac ischemia of 30 minutes or longer usually results in fatal cardiac arrest.[1] Thus, early reperfusion is important for tissue salvage.[1,2] A delayed response to ischemic events can cause reperfusion syndromes such as myonephropathic metabolic syndrome (MNMS), which Haimovici previously described.[3] Therefore, the duration of tissue ischemia is also an important determinant of the clinical outcome.

Previous studies have investigated many biomarkers for determining ischemic duration, and troponin and myoglobin play a part in myocardial ischemia (MI).[4,5] However, recent studies have focused on determining the tissue-specific biomarkers for ischemia.[6]

Prolidase is a type of unique peptidase that has a major role in collagen metabolism.[7] Previously, the efficacy of collagen metabolism was demonstrated with regard to vascular thrombotic disorders and atherosclerosis,[8-10] and recent studies that have focused on this relationship claim that prolidase levels may have a predictive value for vascular disorders such as coronary events.[11] However, up to this point, none of the findings have been able to adequately describe the variations in serum prolidase levels that occur with different ischemic conditions.

In this study, we aimed to determine the predictive value of serum prolidase levels for the duration of ischemia in rat models with mesenteric and peripheral ischemia.

MATERIALS AND METHODS

Study design

This study was designed as a randomized, controlled, single-blinded, interventional animal study. The study protocol was approved by the local animal ethics committee and conducted in accordance with the "Animal Welfare Act and Guide for the Care and Use of Laboratory Animals" prepared by that committee.

Animal subjects

Forty male Sprague-Dawley rats (aged 8-12 weeks) weighing 230±30 grams (mean ± standard deviation) were obtained from the laboratory animal production unit, a facility for breeding rats, mice, and other animals for experimental purposes. For one week before the experiment was initiated, the rats were placed in a room with a controlled temperature (22±2 °C) and humidity (50±5%) as well as a 12-hour light/dark cycle. A standard diet and tap water were provided ad libitum, but the rats were given only water for the 12-hours prior to the initiation of the experimental procedure.

Study protocol

The rats were randomized into four different groups of eight animals each, and all operations were performed simultaneously for sample standardization. All of the subjects were anesthetized with 130 mg/kg ketamine (Ketalar, JHP Pharmaceuticals, LLC, Parsippany, NJ, USA) and 20 mg/kg xylasine (Rompun®, Bayer Healthcare AG, Leverkusen, Germany) via an intraperitoneal line, and ketamine hydrochloride (HCL) (50 mg/kg) (Ketalar®; Parke Davis, Eczacıbaşı, İstanbul, Turkey) was used to maintain anesthesia. The breathing rate, pulse, oxygen saturation (sO_2), and body temperatures were continuously monitored, and a heating pad was applied during anesthesia to maintain the appropriate body temperature in all of the rats.

The first group (group 1) served as the control group and was used to determine the basal values and normal prolidase range in the rat models. With this in mind, blood samples were obtained from these animals at the beginning of the study. In the rats in groups 2 and 3, the superior mesenteric artery (SMA) was clamped via a simple laparotomy to induce mesenteric ischemia, and blood samples were obtained at 120 and 360 minutes after ischemia had been induced. In groups 4 and 5, the right common femoral artery was clamped to induce peripheral ischemia, and blood samples were obtained at the same times as groups 2 and 3. All of the blood samples were gathered during the critical six-hour period after the induction of ischemia.

Intracardiac blood samples (3 ml) were taken from each rat and stored in citrate tubes. The intestines were then macroscopically examined, and 1 cm intestinal segments were removed for histopathological examination. Finally, ischemia was confirmed following a microscopic examination.

The primary endpoint was interventional mortality, and the secondary endpoints were auto-mutilation, additional injury, and unconfirmed ischemia.

Laboratory analysis

Each sample was immediately centrifuged at 4,000 rpm at 4 C° for 10 minutes and then transferred into an Eppendorf tube (New England BioLabs, USA).

Table 1. Comparison of the groups according to ANOVA and Bonferroni tests

	Mean±SD	ANOVA F	ANOVA p*	Post-hoc Bonferroni test	Bonferroni p**
Control	266.8±20.5				
M2	317.1±121.4				
M6	233.4±36.6	4.367	0.006	Normal vs. two hours of peripheral ischemia	0.006
P2	404.0±105.6				
P6	346.1±104.9				

SD: Standard deviation; F: Test statistics of ANOVA; p*: Significance obtained by ANOVA; p**: Significance obtained by post-hoc Bonferroni test; M2: Two hours after mesenteric ischemia; M6: Six hours after mesenteric ischemia; P2: Two hours after peripheral ischemia; P6: Six hours after peripheral ischemia.

The samples were stored on ice and maintained at -70 C° until the end of the study, which was completed in one week.

Prolidase measurement

The plasma prolidase levels were measured in all of the blood samples by a biochemist who was blinded to the groups using spectrophotometry, and prolidase activity was determined by measuring the proline levels produced by the prolidase. The supernatant was diluted two-fold with the physiological serum, and the mixture (25 µl) was preincubated with 75 µL of a preincubation solution [50 mmol/L tris (hydroxymethyl) aminomethane hydrochloride (TrisHCl) buffer (pH 7) containing 1 mmol/L glutathione and 50 mmol/L manganese(II) chloride (MnCl2)] at 370 ºC for 30 minutes, and the reaction mixture, which contained 144 mmol/L Gly-Pro (glycyl-proline, Sigma-Aldrich Co., St. Louis, MO, USA) (pH 7.8) (100 L), was incubated with 100 µL of the preincubated sample at 370 ºC for five minutes. To stop the incubation reaction, 1 mL of glacial acetic acid was added. After adding 300 µL of the TrisHCl buffer (pH 7.8) and 1 mL of a ninhydrin solution (3 g/dL ninhydrin melted in 0.5 mol/L orthophosphoric acid), the mixture was incubated at 900 ºC for 20 minutes and cooled on ice. The proline levels were then determined by measuring the absorbance of the mixture at 515 nm according to the method proposed by Myara et al.[12]

Statistical analysis

The results were expressed as mean ± standard deviation (SD). The normal distribution was then tested using the Kolmogorov-Smirnov test, and analysis of variance (ANOVA) and Bonferroni post-hoc tests were utilized to compare the groups. All statistical procedures were performed using the SPSS version 15.0 for Windows (SPSS Inc., Chicago, IL, USA) software program, and a p value of 0.05 was considered to be statistically significant.

RESULTS

The mean serum prolidase levels in groups 1, 2, 3, 4, and 5 were found to be 266.8±20.5, 317.1±121.4, 233.4±36.6, 404.0±105.6, and 346.1±104.9, respectively (Table 1).

In addition, the prolidase levels were elevated in groups 2, 3, 4, and 5 during the initial hours, with the highest values being obtained after two-hours of ischemia (Figure 1). The prolidase levels were also significantly higher than the baseline levels after the second hour of ischemia in groups 3 and 4 (p=0.045), whereas they were not significantly increased in groups 2 and 3 (p=0.72), although these values were higher than both the basal and six-hour values in these groups. Furthermore, a significant reduction was observed after six-hours of mesenteric ischemia. A comparison of the prolidase values, with the highest being in the second hour of peripheral ischemia and lowest being in the sixth

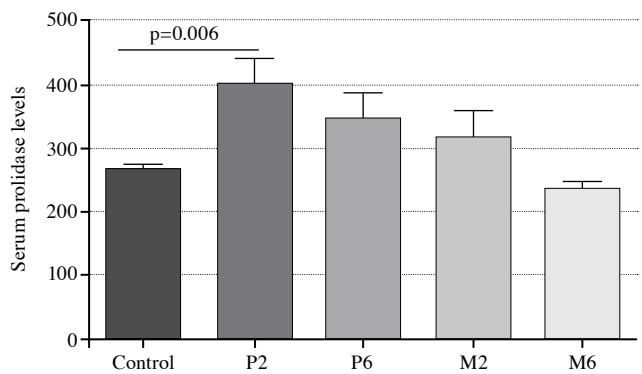

Figure 1. Serum prolidase levels in each group. P2: Two hours after peripheral ischemia; P6: Six hours after peripheral ischemia; M2: Two hours after mesenteric ischemia; M6: Six hours after mesenteric ischemia; * A *p* value of <0.05 was considered to be significant.

hour of mesenteric ischemia, revealed a statistically significant difference (p=0.006). A comparison of the mean values of the groups according to the ANOVA and Bonferroni post-hoc tests is presented in Table 1.

The increases were higher in all of the groups except the controls after two-hours of ischemia. However, the most significant elevations were obtained after two-hours of peripheral ischemia (p=0.006). We also found that the serum plasma levels decreased progressively as the exposure to ischemia increased. Additionally, the prolidase levels fell to levels that were close to the baseline values after six-hours of mesenteric ischemia (Figure 1). Our results also showed that the prolidase levels increased in the early stages (second hour) of ischemia, and then decreased in the advanced stages (6^{th} hour) of ischemia.

DISCUSSION

Early diagnosis and appropriate management are the essential determinants for ameliorating the clinical outcome of acute ischemic events. Despite the need for the timely, safe, and effective treatment of ischemic pathologies, this often occurs too late because of the lack of reliable and specific determinants, as is common with cases of mesenteric ischemia.[13] Although ischemia can occur in many tissues with vital clinical outcomes, most studies have focused on MI conditions. For example, acute mesenteric ischemia progresses with mortality rates of 50-70%. On the other hand, critical limb ischemia is fatal in only 15-25% of all cases and is the most common reason for the loss of limbs.[14-16] Early diagnosis within the first four to six-hours along with collaborative management resulting in successful treatment are required to provide optimum health restoration.[15,16] Traditionally, the biomarkers that were invented for detecting early ischemia in a timely manner and for determining, with adequate sensitivity and specificity, the impact of the ischemic size and providing a prognosis are inadequate when used alone.[17] Therefore, new biomarkers should be developed to improve the efficacy of novel markers and allow for new options in the diagnosis and management of patients.

Prolidase is a kind of exopeptidase that plays an important role in collagen metabolism, and the activity of this enzyme can be evaluated in hemolysates, leukocytes, and fibroblasts. Collagen is also a crucial component in the development of atherosclerotic lesions.[7-10] It is still unclear how prolidase is regulated metabolically. Previous studies have mentioned that this occurs via the interaction of a type 1 collagen with the β1-integrin receptor in human skin,[7] Savaş et al.[18] designed a similar study in which they measured the serum prolidase levels in patients with erectile dysfunction and obtained significant results as elevated serum prolidase activity is related with erectile dysfunction. In addition, Akçakoyun et al.[19] reported low serum prolidase levels in patients with ascending aortic dilatation. A study by Surazynski et al.[20] claimed that prolidase may have an unrecognized role in angiogenic signaling, and they determined that the overexpression of prolidase is related to increased levels of nuclear hypoxia-inducible factor-1 alpha (HIF-1).[20] Furthermore, in a particularly intriguing study, Sezen et al.[21] reported lower serum prolidase activity in patients with ischemic cardiomyopathy. Additionally, Yıldız et al.[11] found a correlation between plasma prolidase activity and the severity of coronary artery disease (CAD), and also claimed that increased plasma prolidase activity might be an independent predictor of CAD. In our study, we obtained similar elevated serum prolidase levels in both the mesenteric and peripheral ischemia groups. Nevertheless, the serum prolidase levels were elevated with mesenteric ischemia, but they were lower than the enzyme levels obtained after two-hours of peripheral ischemia (p=0.006).

Study limitations

Our study contained two major limitations. The multi-systemic nature of serum prolidase activity means that other systemic events besides acute ischemia can also lead to various changes in serum prolidase activity, Because of this, the specificity of the serum prolidase activity is also decreased. In addition, the small number of samples along with their timing were too limited to allow for broad generalizations. Therefore, to obtain more definitive results, serial blood sampling is required.

Conclusion

In this study, we determined that the serum prolidase levels reached their highest values after two-hours of ischemia and then decreased after six-hours of ischemia. Notably, the values obtained after six-hours of mesenteric ischemia were lower than the basal values (233.4±36.6 vs. 266.8±20.5). Therefore, monitoring these changes might help determine which population is at risk for post-perfusion syndrome in patients with limb ischemia.

Declaration of conflicting interests

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