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Original Communication

The Protective Effect of Thiamine Pryophosphate Against Sugar-Induced Retinal Neovascularisation in Rats

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Abstract: The aim of this study was to investigate the effect of thiamine pyrophosphate (TPP), administered via sugar water, on retinal neovascularisation in rats. Animals were assigned to three groups, namely the TPP sugar-water group (TPSWG, n = 12), the control group (CG, n = 12) and the healthy group (HG, n = 12). The TPSWG was injected intraperitoneally with TPP once a day for 6 months. CG and HG rats were given distilled water in the same way. TPSWG and CG rats were left free to access an additional 0.292 mmol /ml of sugar water for 6 months. The fasting blood glucose (FBG) levels of the animals were measured monthly. After 6 months, biochemical, gene expression and histopathologic analyses were carried out in the retinal tissues removed from the animals after they were killed. The measured FBG levels were 6.96 ± 0.09 mmol/ml ($p < 0.0001$ vs. HG), 6.95 ± 0.06 mmol/ml ($p < 0.0001$ vs. HG) and 3.94 ± 0.10 mmol/ml in the CG, TPSWG and HG groups, respectively. The malondialdehyde (MDA) levels were found to be 2.82 ± 0.23 ($p < 0.0001$ vs. HG), 1.40 ± 0.32 ($p < 0.0001$ vs. HG) and 1.66 ± 0.17 in the CG, TPSWG and HG, respectively. Interleukin 1 beta (IL-1β) gene expression was increased (3.78 ± 0.29, $p < 0.0001$) and total glutathione (tGSH) was decreased (1.32 ± 0.25, $p < 0.0001$) in the retinal tissue of CG compared with TPSWG (1.92 ± 0.29 and 3.18 ± 0.46, respectively). Increased vascularisation and oedema were observed in the retinal tissue of CG, while the retinal tissues of TPSWG and HG rats had a normal histopathological appearance. A carbohydrate-rich diet may lead to pathological changes in the retina even in nondiabetics, but this may be overcome by TPP administration.

Keywords: Gene expression, retinopathy, rat, thiamine pyrophosphate

Introduction

In the literature, a glucose tolerance test performed without damaging pancreatic β cells is used to investigate diabetic complications [1]. However, it is known that most of patients develop hyperglycaemia even if they have no history of diabetes [2, 3]. It has been reported that nondiabetic hyperglycaemia may be due to nutrition [4], and it may lead to an increase in the functioning of the oxidant system and a decrease in the antioxidant system [5]. Oxidative stress is thought to be due to the overproduction of advanced glycation end products in hyperglycaemia, which can cause vascular injury [6]. Hyperglycaemia has been reported to increase inflammatory cytokines, causing endothelial dysfunction [7, 8]; chronic exposure to hyperglycaemia may initiate a number of changes that lead to microvascular damage and retinal dysfunction [9]. Known as the formation of new blood vessels, neovascularisation is the major finding related to proliferative diabetic retinopathy [10].

In the literature, neovascularisation is considered an important pathological condition leading to vision loss [11,

12]. It has been stated that neovascularisation may lead to 'retinopathy', which is manifested by complications [13]. The increased permeability due to neovascularisation causes retinal oedema and haemorrhage [14]. It has been reported that the malondialdehyde (MDA) and levels increase along with a decrease in the total glutathione (tGSH) level in retinal tissue that has developed neovascularisation [15]. Furthermore, it has been reported that interleukin 1 beta (IL-1β) has a role in the development of diabetic retinopathy [16].

Laser photocoagulation is a technical procedure that has shown good results in the treatment of neovascularisation. In this procedure, newly formed blood vessels are destroyed and vascular leakage is reduced. However, this procedure may damage the healthy portion of the retina and lead to local loss of the visual field [17]. Therefore, it is necessary to investigate the development of new drugs and new indications of existing medications to prevent neovascularisation associated with hyperglycaemia. Thiamine pyrophosphate (TPP), which is tested in this study, has a protective effect against retinal neovascularisation that may be enhanced with the administration of sugar water; in this study, the active metabolite of thiamine is employed [18]. Thiamine regulates intracellular glucose management through multiple mechanisms [19]. It has been reported that the correct high glucose-induced abnormalities occur via reducing reactive oxygen species production, which has been shown in both cellular studies [20, 21] and animal models [22]. Furthermore, thiamine has been shown to reduce the progression of retinopathy and nephropathy in animals with experimental diabetes [23]. Yapca et al. reported that hyperglycaemia led to infertility in rats, and antioxidant therapy failed to prevent this infertility; however, TPP was able to reverse this effect [24]. This indicates that more important mechanisms other than antioxidant activity may be involved in the treatment of diabetic complications. In our previous research, we demonstrated that TPP prevented oxidative retinal damage caused by ethambutol [25].. In addition, in a recent study, it was demonstrated in rats that hyperglycaemia induced with alloxan led to diabetic proliferative retinopathy; TPP was reported to reduce the severity of the retinopathy [15]. This information suggests that people prone to having a carbohydrate-rich diet may develop retinopathy, and the role of thiamine may be in diabetic endothelial micro and macroangiopathy [26]. Furthermore, it indicates that TPP may be beneficial in the prevention of retinal vascular complications that may develop in these nondiabetic persons.

No study was found in the literature search on the protective effect of TPP against retinal neovascularisation occurring in animals given sugar in addition to drinking water over a long period. Therefore, the objective of this study is to investigate the effect of TPP on neovascularisation that may occur in the retina of rats administered sugar water in addition to normal drinking water.

Material and Methods

Animals

Experimental animals were obtained from Ataturk University, Medical Experimental Applications and Research Center. A total of 36 Wistar albino male rats weighing 300–310 g were used in the experiment. To ensure environmental adaptation, rats were housed and fed over a week at normal room temperature (22 °C) in the pharmacology laboratory. This study's conformity to ethical principles was determined by the Ataturk University, Medical Experimental Applications and Research Center local ethics committee (date: 27.11.2015, number: 183).

Chemical agents

The thiopental sodium used in the experiment was obtained from IE Ulagay (Istanbul, Turkey). TPP was obtained from Biopharma (Moscow, Russia).

Experimental procedure

Animals used in the experiment were divided into three groups, as follows: the TPP sugar-water group (TPSWG; $n = 12$), the control group (CG; $n = 12$) and the healthy group (HG; $n = 12$). Six rats from each group were used in biochemical and gene expression analyses and another six rats were used for histopathological examination. Animals in the TPSWG were injected intraperitoneally with TPP at a dose of 20 mg/kg once a day for 6 months. CG and HG rats were intraperitoneally administered an identical volume (0.1 ml) of distilled water as a solvent once a day for 6 months. All rat groups were fed with a standard pellet diet and water *ad libitum* in the normal laboratory setting. However, TPSWG and CG rats were given both normal drinking water and 0.292 mmol/ml of sugar water, while HG rats were given only normal drinking water for 6 months.

Saccharose was used as the sugar in this study. Whether the animals drank the sugar water was assessed by comparing the difference between normal water and sugar water administered at an identical volume. The levels of fasting blood glucose (FBG) were measured before TPP and sugar-water administration from blood samples collected from the tail veins. Following this, FBG levels were meas-

ured once a month. A commercially available blood glucose meter was used to measure the FBG levels.

At the end of 6 months, all rats were sacrificed using a high dose of thiopental sodium anaesthesia, and the retinal layers of both eyes were removed under sterile conditions. Then MDA, tGSH, and IL-1β were studied, and gene expression and histopathologic analysis were carried out in the retinal tissues removed from the killed animals. The results obtained from TPSWG rats were assessed in comparison with those of the CG and HG.

Gene expression of MDA, tGSH and IL-1β

RNA isolation

RNA was isolated from homogenised retinal tissue samples using the Roche Magna Pure LC Instrument (Roche Diagnostics, Mannheim, Germany) with a MagNA Pure LC RNA Kit (Roche Diagnostics). The quantity and quality of the isolated RNA were assessed using a nucleic acid measurement device (MaestroNano, Hsinchu City, Taiwan). Fifty microliters (μl) of RNA samples were stored at –80 °C.

cDNA synthesis

cDNA was synthesised from the isolated RNA samples using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). For each subject, 1 μl of ddH_2O, 10 μl of RNA and 2 μl of random Primer were combined and incubated in a thermal cycler for 10 min at 65 °C. After incubation, 4 μl of reaction buffer, 0.5 μl of RNAase, 2 μl of deoxynucleotide mix, and 0.5 μl of reverse transcriptase were added; the reactions were incubated for 10 min at 25 °C, 30 min at 55 °C and 5 min at 85 °C, before being held at 4 °C.

Quantitative gene expression evaluation by real-time polymerase chain reaction (RT-qPCR)

For each cDNA sample, gene expressions of IL-1β (Roche, Assay ID 500587), and tumour necrosis factor alpha (TNF-α; Roche, Assay ID 502875) and the reference gene (G6PD; Roche, Assay ID 502302) were analysed using the Roche LightCycler 480 II Real-Time PCR instrument (Roche Diagnostics). PCR reactions occurred in a final volume of 20 μl with the following components: 5 μl of cDNA, 3 μl of distilled water, 10 μl of LightCycler 480 Probes Master (Roche Diagnostics) and 2 μl of primer-probe set (Real-Time Ready single assay; Roche Diagnostics). Cycle conditions of the relative quantitative polymerase chain reaction PCR (qPCR) were preincubation at 95 °C for 10 min followed by 45 amplification cycles of 95 °C for 10 s, 6 °C for 30 s and 72 °C for 1 s, followed by cooling at 40 °C for 30 s. qPCR analysis and calculation of quantification cycle (Cq) values for relative quantification were carried out using the LightCycler 480 Software, Version 1.5 (Roche Diagnostics). Relative quantitative amounts were calculated by dividing the target genes by the expression level of the reference gene. The reference gene was used for normalisation of target gene expression.

Histopathological examination

Eye enucleation material removed from the rats was fixed in 10 % (v/v) formalin; following routine tissue processes, 5-μm sections were obtained from the paraffin blocks. After deparaffinisation and rehydration, the sections were stained with hematoxylin and eosin (H&E). All sections were coded and evaluated under an optical microscope (Olympus BX 51, Tokyo, Japan) by the same pathologist, who was unaware of the treatment protocol; images were taken using a digital camera (Olympus DP 71, Tokyo, Japan). The Anti-CD34 antibody (1:50; Clone QBEnd/10, Novocastra Reagents, Leica Biosystems, Wetzlar, Germany) was used to define neovascularisation immunohistochemically. All sections were placed on positively charged slides and stained with Anti-CD34 via an automated immunoassay device (BOND-III Fully Automated IHC and ISH, Leica Microsystems, Wetzlar, Germany). Images were taken using a digital camera (Olympus DP 71, Tokyo, Japan).

Statistical analysis

Results obtained from the experiment are expressed as mean ± standard error of mean (x ± SEM). The significance level of the differences between the groups was determined using the one-way analysis of variance (ANOVA) test followed by Fisher's post hoc least significant differences (LSD) analysis. All statistical processes were performed using SPSS for Windows version 18.0 (IBM Analytics, New York, USA) statistical software and a p-value < 0.05 was considered statistically significant.

Results

Objective findings

Animals in the CG, TPSWG and HG were observed to consume normal drinking water at almost the same rate. However, the CG and TPSWG were found to consume sugar

water in addition to normal drinking water. Furthermore, rats in the CG and TPSWG were found to drink more sugar water than drinking water. Consumption of pellet feed was almost the same for all groups.

Biochemical findings

Amount of FBG

As shown in Table I, levels of FBG were significantly increased in the CG and TPSWG at months 1–6 after administration of sugar water began compared to the levels before sugar-water administration ($p < 0.0001$). However, the differences in the FBG levels between the CG and TPSWG in this period were not statistically significant ($p > 0.05$). The levels of FBG measured in this period in the HG were almost the same before and after sugar-water administration.

Results of MDA, tGSH and IL-1β gene expression

As shown in Figure 1, MDA gene expression was 1.66 ± 0.17 in the retinal tissue of the HG, while this value was found to be increased, at 2.81 ± 0.09 ($p < 0.001$ vs. HG) in the retinal tissue of the CG, which was administered 0.292 mmol /ml of sugar water. However, MDA gene expression was decreased at 1.40 ± 0.13 ($p > 0.05$ vs. HG) in the retinal tissue of the TPSWG, which was treated with TPP. Statistical analysis revealed a significant difference between the CG and TPSWG and an insignificant difference between the TPSWG and HG in terms of MDA.

tGSH gene expression was found to be 3.51 ± 0.15 in the retinal tissue of the HG. This value was found to be 1.31 ± 0.10 ($p < 0.001$ vs. HG) in the CG administered 0.1 0.292 mmol of sugar water and 3.18 ± 0.18 ($p > 0.05$ vs. HG) in the TPSWG. The difference in tGSH between the HG and TPSWG was not statistically significant, while the difference between the tGSH values of the HG and TPSWG and that of the CG was statistically insignificant.

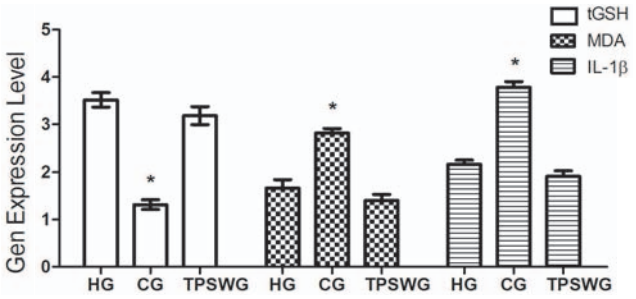

Figure 1. The effects of TPP and sugar water on tGSH, MDA and IL-1β gene expression levels.
*$p < 0.0001$ vs. TPSWG
TPP: Thiamine pyrophosphate
tGSH: Total glutathione
MDA: Malondialdehyde
IL-1β: Interleukin 1 beta
TPSWG: Thiamine pyrophosphate sugar-water group

IL-1β gene expression was also significantly higher in the CG (3.78 ± 0.12) compared with the HG (2.17 ± 0.89, $p < 0.001$) and TPSWG (1.91 ± 0.12, $p < 0.001$). Moreover, the IL-1β gene expression values were found to be extremely close to each other in the HG and TPSWG ($p > 0.05$; Figure 1).

Histopathological findings

In Figures 2a and 2b, the optic microscopic images show the normal histopathologic and immunohistochemical views of the retinal tissue of the HG, respectively. However, increased vascularisation (black arrow) and oedema

Table I. FBG levels of HG, CG and TPSWG groups in retinal tissue before and after sugar-water administration

Groups	Fasting Blood Glucose (μmol/ml)						
	Before the sugar water administration	After the sugar water administration					
		1 st month	2 nd month	3 rd month	4 th month	5 th month	6 th month
HG	4.27 ± 0.09	3.86 ± 0.12	4.18 ± 0.11	3.81 ± 0.15	3.97 ± 0.11	4.27 ± 0.07	3.94 ± 0.10
CG	4.06 ± 0.07	6.27 ± 0.16	6.52 ± 0.13	6.37 ± 0.12	6.86 ± 0.08	6.93 ± 0.05	6.96 ± 0.09
TPSWG	4.16 ± 0.16	6.33 ± 0.14	6.65 ± 0.06	6.40 ± 0.09	6.73 ± 10.08	6.87 ± 0.08	6.95 ± 0.06

FBG: Fasting blood glucose
HG: Healty group
CG: Control group
TPSWG: Thiamine pyrophosphate sugar-water group

(star) were observed histopathologically in the retinal tissue of the CG, which was administered 0.292 mmol /ml of sugar water for six months (Figure 2c). Increased vascularisation (arrows) was shown immunohistochemically (Figure 2d). In contrast, a close to normal appearance in the retina of the TPSWG, which was treated with TPP, was shown via histopathological (Figure 2e) and immunohistochemical (Figure 2f) studies.

Discussion

In this study, the levels of FBG were measured once a month in animals left free to access both normal drinking water and 0.292 mmol/ml sugar water; these were compared with the values of the animals given only drinking water. In addition, whether the retinal tissue of animals administered sugar water developed oxidative damage was assessed through the measurement of MDA, tGSH and IL-1β gene expressions and neovascularisation via histopathological analysis. Although level of FBG obtained in the experiment was significantly higher than that of the healthy animals, this is not considered to represent diabetes; in the literature, diabetics are defined as those with an FBG level above 13.88 μmol/ml [27].

It has been reported that hyperglycaemia as a result of nutrition may develop in persons with no history of diabetes [3, 4]. Hyperglycaemia is known to modulate oxidant/antioxidant balance in favour of oxidants, thereby creating tissue damage [5]. In the present study, glucose loading administered without damaging the pancreatic β cells in rats increased gene expression of MDA, which is an oxidant parameter, and decreased gene expression of tGSH, which is an antioxidant. Researchers have emphasised that chronic hyperglycaemia has an important role in decreased antioxidant capacity and the development of hyperglycaemic complications [28]. In another study, the final product of lipid peroxidation, MDA, was reported to have a role in the emergence of late diabetic complications [29]. However, MDA was found to be higher, while tGSH was lower, in the retinas of the rat group administered sugar water; we could not exactly consider this result to represent glycaemia, and it suggests that oxidative stress developed in the retina.

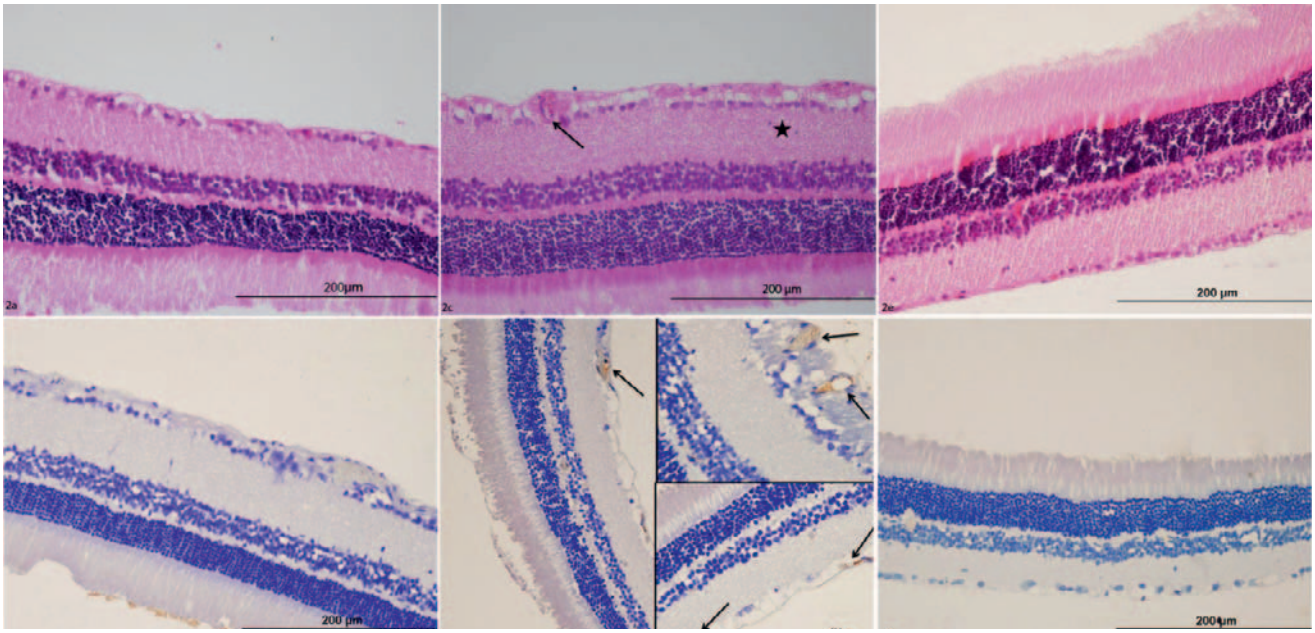

Figure 2a. Light microscope image of the retina of an HG rat. Normal histological appearance of retinal tissue was observed (haematoxylin and eosin [H&E], x40). HG: Healty group.

Figure 2b. Image of the retina of an HG rat. Neovascularisation was not observed with the Anti-CD 34 antibody, that was applied immunohistochemically (x40). HG: Healty group.

Figure 2c. Light microscope image of the retina of a CG rat (increased vascularisation [black arrow] and oedema [star] were observed; H&E, x40). CG: Control group.

Figure 2d. Immunohistochemical appearance of the retina of a CG rat. Neovascularisation [black arrows] was shown with the Anti-CD 34 antibody, that was applied immunohistochemically (x40). CG: Control group.

Figure 2e. Light microscope image of the retina of a TPSWG rat. Close to normal histological appearance in the retina was observed (H&E, x40). TPSWG: Thiamine pyrophosphate sugar-water group.

Figure 2 f. Image of the retina of a TPSWG rat Neovascularisation was not observed with the Anti-CD 34 antibody, that was applied immunohistochemically (x40 and x100). TPSWG: Thiamine pyrophosphate sugar-water group.

In this study, IL-1β gene expression was significantly increased in CG group, while increased MDA and decreased tGSH values were observed. In a study by Scuderi et al., hyperglycaemia was stated to increase the gene expressions of IL-1β and other proinflammatory cytokines in the retinal tissue [30]. It was argued in a study by Xie et al. that glucose and IL-1β may stimulate angiogenesis in the retina [31]. Known also as neovascularisation in the retina, this angiogenesis phenomenon results in vascular retinopathy due to secondary complications [13]. In our study, neovascularisation was also histopathologically detected in the retinal tissue of rats in the CG, with increases in the parameters reflecting oxidative stress and inflammation. This indicates that the gene expression outcomes were consistent with the histopathological findings.

Neovascularisation is a major finding used for diagnosis of diabetic retinopathy [10]. However, it is recognised as a pathological event not only in diabetes but also in all eye diseases and retinopathy models [25, 32]. In addition to neovascularisation, oedema was also developed in the retinal tissue of the CG. There are studies in the literature demonstrating that oedema in the retina results from the increased permeability due to neovascularisation [13, 14]. Oedema is reported to be observed at higher rates in proliferative as opposed to nonproliferative retinopathy [33].

As noted above, the difference in the levels of FBG between the CG and TPSWG administered sugar water was statistically insignificant. However, the oxidant/antioxidant balance changed in favour of oxidants in the CG and antioxidants in the TPSWG. Furthermore, while the CG developed neovascularisation and oedema, these findings were not observed in the TPSWG. In a recent study, TPP has been proposed to prevent the development of nondiabetic neovascularisation and oedema due to oxidative stress [25]. In their study, Park et al. argued that neovascularisation is associated with the production of reactive oxygen species and can be decreased with antioxidant therapy [34]. TPP has been reported to prevent an increase in MDA and a decrease in tGSH, which they lead to oxidative stress in the retinal tissue [25]. However, there are several studies in the literature arguing that TPP treats diabetic complications through an unknown mechanism other than antioxidant activity [24]. Thornalley reported that high cytosolic glucose concentrations in hyperglycaemia may lead to the accumulation of triose phosphates and the development of diabetic complications, while overproduction of triose phosphates and diabetic complications arising from excessive accumulation of triose phosphates may be prevented by stimulation of the reductive pentose phosphate pathway [35]. Gangolf et al. reported that reduction of the pentose phosphate pathway can be induced by cytosolic transketolase, which is dependent on TPP [36]. Thiamine was reported that prevent incipient retinopathy in diabetic rats. Thiamine increased the conversion of triosephosphates to ribose 5-phosphate through activation of transketolase, decreased protein kinase C activation, and reduced protein glycation [21, 22].

In conclusion, in the present study, the levels of FBG varied between 6.23 and 6.95 μmol/ml in rats given both normal drinking water and 0.292 mmol/ml of sugar water over a period of 6 months. Although this level of glucose is not considered sufficient to create a diabetic model, it produced neovascularisation and oedema in the retinal tissue of rats in the CG. Moreover, parameters reflecting oxidative stress from inflammation were increased and antioxidants were decreased in the retinal tissue of the CG. Although the levels of FBG in the animals treated with TPP were almost the same as those in the CG group, no pathological findings, such as neovascularisation and oedema, were observed in this group. These results indicate that persons without a history of diabetes but having a dietary habit of a carbohydrate-rich nutrition may develop vascular retinopathy. This suggests that TPP may be beneficial in the prophylaxis of nutritional retinal damage.

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Emine Cinici, Renad Mammadov and Durdu Altuner designed the experiments and wrote the paper, Renad Mammadov, Huseyin Findik, Bahadir Suleyman and Nihal Cetin conducted the animal experiments, Ilknur Calik and Hilal Balta performed histopathological studies, Ismail Hakki Tas analysed data. Ebru Sener performed immunohistochemical studies.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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