The Effect of N-Acetylcysteine on Fibrosis in Experimental Rat Chronic Pancreatitis

Deneysel Kronik Pankreatitte N-Asetil Sistein Uygulamasinin Pankreatik Fibroz Üzerine Etkisi

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Abstract
In this study, we aimed to assess the effect of N-acetylcysteine on fibrosis, atrophy and oxidative stress with chronic pancreatitis in an animal model. Chronic pancreatitis is stimulated by injection of 0.4 ml, 2% trinitrobenzene sulfanic acid (TNBS) into major pancreatic ducts of the rats. 60 Sprague-Dawley rats were randomized into four group; Group I (n=15): saline (control), Grup II (n=15): fibrosis + N-acetylcysteine (NAC), Grup III (n=15): fibrosis (TNBS), Grup IV: ethanol. At the end of the 8th week, all rats were sacrificed, and rat pancreatic tissues, oxidative stress markers and progression of fibrosis and atrophy were examined. superoxide dismutase (SOD) and glutation peroxidase (GSH-Px) activities were significantly higher with respect to TNBS group (respectively $p<0.001$ and $p<0.001$); whereas tissue malondialdehyde (MDA) levels were found significantly lower in NAC group ($p<0.001$). Histopathological analysis of the tissues revealed that histopathological score of NAC group was significantly lower than TNBS group ($p<0.003$), and was found a significant relation between increase in oxidative stress and histopathological score (histopathological score/MDA: $p<0.03$, SOD: $p<0.04$, GSH-Px: $p<0,02$). In conclusion, administration of NAC decreases oxidative stress in chronic pancreatitis and has beneficial effects on fibrosis and atrophy.

Keywords: Animal Model, Experimental Chronic Pancreatitis, Fibrosis, N-Acetylcysteine

Öz
Bu çalışmada, N-asetilsisteinin, kronik pankreatit hayvan modelinde fibrozis, atrofi ve oksidatif stres üzerindeki etkisini değerlendirmeyi amaçladık. Kronik pankreatit, ratların ana pankreatik kanallarına 0.4 ml, %2'lik trinitrobenzen sülfanik asit (TNBS) enjeksiyonu ile uyarılır. 60 Sprague-Dawley sıçanı dört gruba randomize edildi; Grup I (n= 15): salin (sham), Grup II (n= 15): fibrozis + N-asetilsistein (NAC), Grup III (n= 15): fibrozis (TNBS), Grup IV: etanol. 8. haftanın sonunda tüm ratlar sakrefiye edildi ve pankreatik dokular, oksidatif stres belirteçleri, fibrozis ve atrofi progresyonları incelendi. NAC grubunda doku malondialdehid (MDA) düzeyleri anlamlı olarak düşük bulunurken ($p <0.001$); süperoksit dismutaz (SOD) ve glutation peroksidaz (GSH-Px) aktiviteleri TNBS grubuna göre anlamlı olarak yüksek bulundu (sırasıyla $p <0.001$ ve $p <0.001$). Dokulardaki histopatolojik incelemede NAC grubunun histopatolojik skorunun TNBS grubundan anlamlı derecede düşük olduğu izlenirken ($p<0.003$) oksidatif stres ile histopatolojik skordaki artış arasında anlamlı bir ilişki olduğu saptandı (histopatolojik skor/MDA: $p<0.03$, SOD: $p<0.04$, GSH-Px: $p<0.02$). Sonuç olarak, NAC uygulaması kronik pankreatitte oksidatif stresi azaltmakta olup fibrozis ve atrofi üzerinde yararlı etkilere sahiptir.

Anahtar Kelimeler: Deneysel Kronik Pankreatit, Fibrozis, Hayvan Modeli, N-Asetilsistein

Introduction

Chronic pancreatitis is a clinical condition characterized by irreversible exocrine and endocrine dysfunction resulting from progressive destruction as a result of persistent inflammation caused by various etiologies (1). The underlying histological findings independently of the underlying cause are defined as loss of acinar cells, inability of differentiation of acinar cells to tubul complexes, infiltration of immunocytes, degeneration of pancreatic nerves and irregular parenchymal fibrosis (2). Acute and chronic pancreatitis are separated by pathophysiological mechanisms. Whether recurrent acute pancreatitis attacks always lead to chronic pancreatitis is still controversial (3). Numerous growth factors, cytokines and chemokines have been shown to participate in the mechanism of chronic pancreatitis with their autocrine and paracrine effects such as local inflammation and collagen production. Functional and mechanical causes to initiate a number of events that lead to chronic inflammation and repair processes those mainly controlled by epidermal growth factor (EGF), platelet derived growth factor-beta (PDGF-β), transforming growth factor (TGF), tumor necrosis factor – alpha (TNF-α) and interleukin (IL) 1.6 and 10 activin-A, monocyte chemoattractant protein-1 (MCAP-1) and the renin angiotensin system. However, the primary mechanism of damage may vary depending on etiology (4-11).

Oxidative stress not only contributes to cell and tissue damage in acute pancreatitis but also

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participates in mechanism of chronic pancreatitis. For this reason, the effects of antioxidant treatment on chronic pancreatitis were studied by various researchers (12). There are no enough data that reactive oxygen species trigger the collagen accumulation in pancreas. However, molecules associated with oxidative stress and lipid peroxidation products, which are clearly associated with inflammation, are among the key players in the persistence of pathophysiological events leading to chronic pancreatitis fibrosis. After prolonged oxidative damage, natural antioxidant defense systems placed in the pancreas become insufficient to prevent damage and therefore antioxidant supplementation may reduce the development and degree of fibrosis, which is indicative of exocrine and endocrine pancreatic insufficiency.

N-acetyl cysteine (NAC) is a sulphydryl (SH) group source, SH group is required for defense system against reactive oxygen products. The major effect of NAC reduces the effects of oxidative stress (13, 14) and has been shown that especially when administered early, improving the experimental acute pancreatitis disease parameters (15-16). Choudhury et al. used bacterial lipopolysaccharide (LPS) to generate a chronic systemic inflammatory state in an experimental mice model that resulted with pancreatic cell injury and increased collagen content as indicative of fibrosis. They showed in presence of NAC, cell death was decreased in cultured isolated primary pancreatic cells with LPS (17).

This study, conducted with an animal model, was carried out with the aim of investigating whether NAC, a potent antioxidant, had the healing effects on chronic pancreatitis fibrogenesis.

Material and Method

Experimental Animals: 60 male Sprague Dawley rats weighing 240-270 gr were used in the study. Pre and post-surgical care of the rats, preparing the sterilized operating room conditions were carried out in cooperation with the surgical specialist veterinarians, anesthesia technician and technical personnel.

Working groups: 60 rats were randomized into four groups (n=15).

Group-I was the control group (sham) (n=15); 0.4 ml saline was infused into rat pancreatic duct. Group-II was pancreatic fibrosis + NAC treatment group (n=15); 0.4 ml of 2% trinitrobenzene sulphonic acid (TNBS) – in phosphate buffered saline (PBS) + 10% ethanol was injected into the rat pancreatic duct. Group-III was pancreatic fibrosis group (n=15); 0.4 ml of 2% TNBS-PBS + 10% ethanol was infused into the rat pancreatic duct. Group-IV was ethanol group (n=15); 0.4 ml of 10% ethanol was infused into the rat pancreatic duct. After 4 weeks from induction, for NAC treatment group 50 mg/kg/day NAC, for pancreatic fibrosis and ethanol group 10 ml/kg/day saline was administered intraperitoneally.

All rats were sacrificed at the end of 8 weeks. During this period, all rats were followed up with weekly weight gain.

Pancreatic fibrosis induction was achieved in male Sprague-Dawley rats by modifying the model described by Puig-Divi et al. (18, 19). Rats were subjected to anesthesia induction in sterile operating room conditions and at room temperature not causing hypothermia. The abdomen was opened with cutaneous and subcutaneous incision and the duodenum was taken out of the abdomen, then pancreatic duct was cannulated. The pancreatic biliary tract was closed with vascular microclemps to prevent the passage of the applied medication to the biliary system.

Pancreatic fibrosis induction was achieved by infusion of 2% TNBS-FTS (pH: 8) dissolved in 0.4 ml of 10% ethanol for approximately 60 min to avoid intracavitary pressure increase with slow infusion of this catheter. Pancreatic fibrosis induction was achieved by infusion of 2% TNBS-PBS (pH: 8) dissolved in 0.4 ml of 10% ethanol for approximately 60 minutes to avoid intracavitary pressure increase.

TNBS behaves as a hapten and reacts with lysine residues to produce an immunological response to the tissue. However, it also has direct toxic effects through superoxide and hydrogen peroxide radicals. The most important feature of acute inflammation created by TNBS is having ability to transform chronic inflammatory process (19).

After the induction, the pancreatic duct catheter and then, the vascular microclemp were pulled and final checks were performed and the sac was closed with 3.0 silk suture material. Rats were not given food or beverages for 24 hours postoperatively and were then placed in cages to allow free nutrition and fluid intake. The average time required for formation of chronic pancreatitis fibrosis by using TNBS administration into the pancreatic duct is approximately four weeks.

All rats were sacrificed eight weeks after induction. Gas and solid anesthesia were applied to the rats in preparation for sacrification. Anesthetized rats were sacrificed by administering anesthetic at lethal doses. Rats abdomen was reopened with anterior incision and pancreatic tissue specimens were taken as dry specimen for oxidative stress parameters study and into 10% formaldehyde for histopathological examination. All the samples were stored at -80 C.

In order to determine the oxidative stress status in the pancreatic tissue, MDA levels, GSH-Px and SOD enzyme activities which are known as indirect oxidative stress markers; were measured. Tissue MDA levels were expressed in nmol/gr, GSH-Px and SOD enzyme activities in U/gr (20, 21).

Sections from paraffin blocks prepared from pancreatic tissue specimens were taken with a microtome. Sections were stained with haemotoxylen-eosin (H-E), Masson's trichrome (for the purpose of demonstrating fibrosis) and immunohistochemical α-SMA stains (to demonstrate active stellate cells) (18). The sections were reviewed by an expert and experienced pathologist and clinician blindly. Every sample from each rat was evaluated twice at different times. A histopathological scoring system was prepared for statistical comparison between the groups, and the tissue samples prepared were scored according to this system (Table 1).

Table 1. Histopathological scoring of pancreatic tissue samples.

		Focal	Mild	Evident
Fibrosis	Pericellular	1	2	3
	Interlobular	3	5	7
Atrophy	Sublobular	1	3	5
	Lobular	3	5	7
Collagen		1	2	3

All of the statistical calculations were done with a package program with the help of microprocessor. In all statistical calculations, the alpha degree of freedom was assumed to be 0.05. p values less than 0.05 were considered as significant. Kruskal Wallis test was used in order to compare differences etween groups Mann-Whitney U and to compare more than two groups.

Results

All rats were monitored for eight weeks as two rats in a cage free for fluid and nutrient uptake. NAC was administered intraperitoneally to the treatment group at a daily dose of 50 mg/kg. In the other groups, sterile saline was injected into the peritoneum at a dose of 10 ml/kg every day. During the study period, two rat died as one in fibrosis and one in ethanol groups between 4-8 weeks.

Rats' weight follow-ups were done weekly. Weight loss in fibrosis and ethanol groups were significantly higher than the control group (fibrosis group p<0.002, ethanol group, p<0.003). In the saline and NAC group rats, while weight loss was observed within the first four weeks, in NAC group weight gain began to occur from the fourth week onwards. (NAC p>0.05, Saline p>0.05) (Figure 1).

Tissue MDA levels, SOD and GSH-Px enzyme activities were used as oxidative stress parameters. Tissue MDA level was measured in nmol/gr wet tissue, SOD and GSH-Px enzyme activities in U/gr wet tissue. (Table 2). In the subgroup analysis, tissue MDA levels were found to be significantly lower in the NAC group than in the pancreatic fibrosis (TNBS) group (p<0.001). SOD and GSH-Px enzyme activities in the pancreas tissue were significantly higher in the NAC group when compared to the pancreatic fibrosis group (p<0.001, p<0.001) (Table 3).

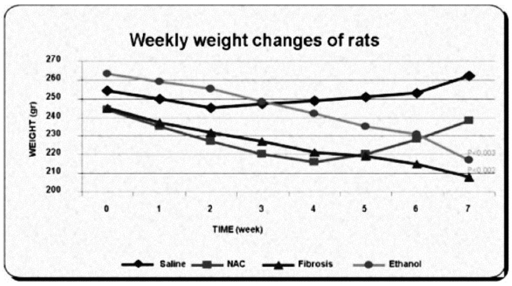

Figure 1. Weekly weight changes of rats. [Fibrosis and ethanol groups have statistically significant weight loss compared to baseline. (Fibrosis p <0.002, ethanol p <0.003).

Table 2. Comparison of oxidative stress markers between groups.

	Subgroup Analysis	Values	p
MDA (nmol/gr)	Fibrosis / Saline	37.6±0.8 / 4.3±0.2	***p<0.001**
	Fibrosis / NAC*	37.6±0.8 / 15.73±0.3*	
	NAC / Saline	15.73±0.3/ 4.3±0.2	
GSH-Px (U/gr)	Fibrosis / Saline	57.1±2.3 / 159.9±2.4	***p<0.001**
	Fibrosis / NAC*	57.1±2.3 / 185.3±1.8*	
	NAC / Saline	185.3±1.8 / 159.9±2.4	
SOD (U/gr)	Fibrosis / Saline	272.5±7.8 / 593.0±11.8	***p<0.001**
	Fibrosis / NAC*	272.5±7.8 / 381±5.5*	
	NAC / Saline	381±5.5 / 593.0±11.8	

Table 3. Histopathological pathology scores.

	Saline	NAC	Fibrosis	Ethanol
Pericellular Fibrosis	0.6±0.1	0.3±0.2	1.1±0.2	0.8±0.2
Interlobular Fibrosis	0.4±0.3	1.0±0.3	1.6±0.5	1.7±0.6
Collagen	1.0±0.1	0.9±0.2	1.5±0.2	1.6±0.3
Sublobular Atrophy	0.5±0.2	0.6±0.4	0.8±0.1	0.6±0.2
Lobular Atrophy	1.5±0.1	1.4±0.2	2.1±0.4	1.7±0.7
Mean score	0.8 ± 0.2	0.8 ± 0.3	1.4±0.3	1.3±0.4

We performed review and evaluations of pancreatic tissues in light microscopy with hematoxylin-eosin (HE), Masson's trichrome (MT) and α-SMA staining techniques (Figure 2.a).

Histopathological examination of the rats randomized to fibrosis and alcohol group showed significant; interstitial edema, inflammatory cell infiltration, aciner cell degradation, inter-/intralobular fibrosis and fatty degeneration were observed (Figure 2.b and 2.c). Basic histopathological findings in all experimental groups were lobular and sublobular localized segmental glandular atrophy, mononuclear inflammatory cell infiltration accompanied with pericellular and intralobular fibrosis. (Figure 2.c, 2.d, 2.e).

In the induction group with TNBS administration, fibrosis developed at advanced level after eight weeks. However, it was noted that the development of fibrosis in the group that started NAC application four weeks after induction was regressed. Severe sublobular and lobular involvement was demonstrated by using Masson's trichrome (Figure 2.f, 2.g, 2.h).

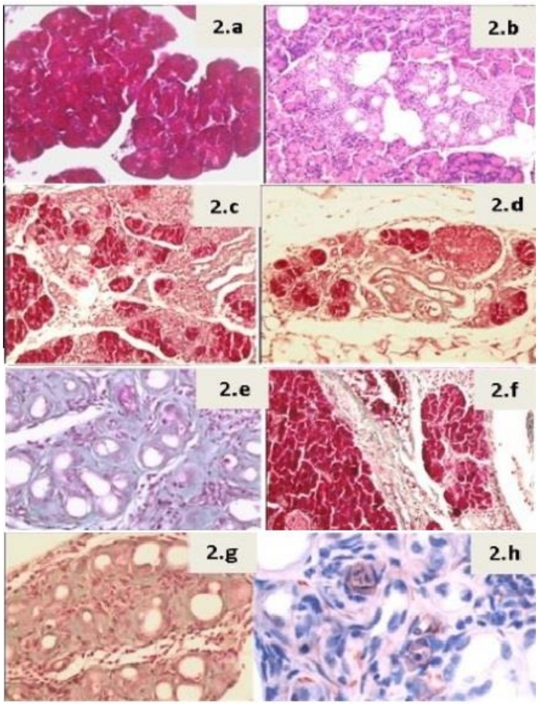

Figure 2. Histopathological findings.
2.a. Normal rat pancreatic tissue (Masson Trichrome x 200),
2.b. Preserved asine groups and sublobular atrophy (HEx100),
2.c. Diffuse sublobular atrophy (Masson Trichrome, x100),
2.d. Atrophic areas (Masson Trichrome, x100) with protected acinary clusters in pancreatic lobul,
2.e. Lobular atrophy and pericellular fibrosis (Masson Trichrome, x200),
2.f. Interlobular fibrosis and focal acinar atrophy (NAC group, Masson Trichrome, x100),
2.g. Lobular atrophy and pericellular fibrosis (NAC group, Masson Trichrome, x200),
2.h. Periacinar localized pancreatic stellate cells (A, α-SMA x200 B, α-SMA x 400)

The pathology scores obtained after examining pancreatic tissues according to the histopathological evaluation chart shown in Table 3 (Table 3). In all the parameters evaluated, the height in the fibrosis and ethanol groups was remarkable. Compared with the fibrosis group; improvement in pericellular/interlobular fibrosis and collagen accumulation was significant in the NAC group ($p<0.005$, $p<0.005$, $p<0.008$, respectively). When compared with the ethanol group, improvement in pericellular fibrosis was statistically significant in the NAC group ($p<0.001$).

In the analysis of subgroups of total pathology scores; The histopathological evaluation score of the NAC group was found to be statistically significantly lower than the fibrosis group ($p<0.003$). In addition, statistical comparison of pathological scores with oxidative stress parameters including all groups; (Pathology score / MDA: $p<0.03$, SOD: $p<0.04$, GSH-Px: $p <0.02$) (Figures 3 and 4) were found to be significantly related to the increase in tissue oxidative stress and high pathology score.

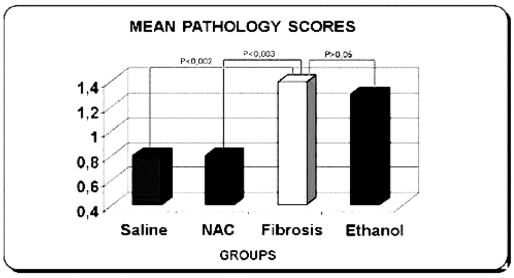

Figure 3. Histopathological fibrosis scores (Fibrosis/NAC; pericellular fibrosis $p<0.005$, interlobular fibrosis $p<0.005$, collagen $p<0.008$).

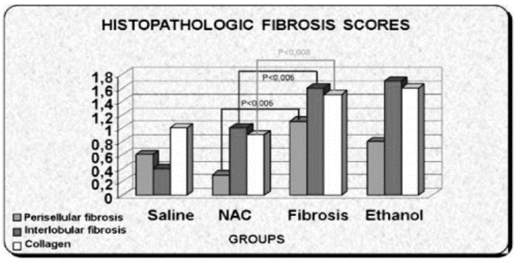

Figure 4. Histopathological evaluation mean pathology scores (Fibrosis/NAC $p<0.003$, Fibrosis/Saline $p<0.002$, Fibrosis/Ethanol $p>0.05$).

Discussion

The idea that oxidative stress played a role in the pathogenesis of chronic pancreatitis was first described by Braganza et al. Long-term studies have reached conclusions that support this theory (22-26).

As a result of our study, a significant correlation was found between oxidative stress and pathology scores obtained from pancreatic tissue. This relationship suggests that super oxide radicals (SOR) are effective during the chronic pancreatitis process. These findings are consistent with other studies in the literature (23, 25, 27). Increased oxidative stress due to various etiologic factors not only causes pancreatitis, but it also continues affecting the disease process. The role of increased tissue SOR in organ damage and fibrosis has led to the search for new therapeutic approaches. In an experimental study of rat model developed by Heras et al., chronic pancreatitis was developed with serulein and cyclosporin administration and the combination of methionine, beta-carotene, vitamin C, vitamin E and organic selenium were used for the purpose of treatment. Ultimately, antioxidant complex has been shown to reduce collagen accumulation (28). Mas et al. have constructed an experimental rat model using TNBS. They used taurine as treatment, a potent antioxidant, and found reduction in oxidative stress and statistically significant improvements in fibrosis and atrophy compared to the group without treatment (29). Interestingly, although there was a decrease in oxidative stress markers, there was no improvement in apoptosis and fibrosis with the use of Rapamycin, an mTOR inhibitor, in the chronic pancreatitis model of Ozturk and colleagues using TNBS (30).

By activating the nuclear transcription factor NF-κB, SOR regulates the gene expression of inflammatory cytokines such as TNF-α in acinar and stellate cells. TNF-α causes stellate cell proliferation and collagen synthesis (31-32). Blocking TNF-α increases survival in the pancreatitis process and reduces chronicity and sequelae (33, 34). Since increased oxidative stress in the tissue is present in all stages of pancreatitis process and is elevated during disease progression, successful results have been obtained with the studies hypothesing that if oxidative stress can be suppressed by an antioxidant therapy, NF-κB activation and TNF-α expression may be inhibited. Antioxidant therapy can prevent NF-κB activation in pancreatic stellate cells, aciner cells and neutrophils (17, 35-39).

The great majority of the studies done in the literature are about acute pancreatitis. In these studies, NAC is preferred to be used in combination therapy. There are no studies that test the NAC's ability to inhibit the development of chronic pancreatitis alone. So far, the antioxidant properties of NAC have been studied in other organs such as the liver and kidney.

NAC is a thiol compound and a reduced GSH source. The sulfhydryl compound can reduce intracellular cystine to cysteine, stimulate GSH synthesis, increase the glutathione-S-transferase activity and detoxification, and interact directly with SOR. SH is required for defense against SOR. So, NAC is also involved in the reduction of hydroxyl radicals and hydrogen peroxide (13, 17, 40-42).

In 1998, Gukovsky et al. indicated that in serulein induced rat acute pancreatitis, NAC treatment improved pancreatitis parameters by preventing NF-κB activation. In the same study, NAC also inhibited trypsin activation and IL-6 mRNA expression (146). Since 2000, the use of acute pancreatitis NAC has begun to be widely accepted due to the positive effects on oxidative stress and has been widely used as a single and / or combined study. Majority of these studies proved that NAC showed its protective and preventive effects by inhibiting NF- κB activation (16, 43-49).

After a series of experimental studies Sevillano et al. concluded that NAC treatment in early course of acute pancreatitis prevents associated pathological mechanisms in aciner, restores atrophy by protecting the aciner cell cycle, increases antioxidant defenses in acinar cells and reduces damages caused by pancreatitis (15, 50, 51).

Although studies with NAC in acute pancreatitis have provided definitive evidence of the benefits, there is no in vivo chronic pancreatitis study in the literature. Main targets were pancreatic stellate cells (PSC) and NF-κB in in vitro studies (17). In the cell culture medium Asaumi et al. studied PSC exposed to the pressure and concluded that SOR also plays a key role in pressure dependent PSC activation and extracellular matrix production, and antioxidants such as NAC can be effective against pancreatic fibrosis development (52).

In our study, oxidative stress parameters improved with NAC treatment, and pathology scores were correlated with this improvement. Using NAC with proven acute pancreatitis activity in chronic pancreatic rats, strengthens the antioxidant defense against SOR which are still active during the disease course through the SH group, and inhibits the inflammatory response by reducing NF-κB activation. On the other hand, today we know that PSCs are the primary cells that play a role in the development of chronic pancreatitis fibrosis. By being activated via NF-κB, PSCs synthase the extracellular matrix and express TGFβ. This inhibits matrix metalloproteinases and also inhibits the degradation of newly formed matrix (53, 54). Probably NAC suppresses the inflammatory response involved in the activation of these cells and induces apoptosis, thereby reduces the development of fibrosis and atrophy.

The time required for TNBS-induced chronic pancreatitis is approximately 4 weeks. NAC, which was started to be administered 4 weeks later, improved the histology of chronic pancreatitis. This result is a favorable finding that the antioxidant molecule which is used is not only for early course in acute pancreatitis, but also has a therapeutic feature on established fibrosis. There is not only a conclusive evidence for the prophylactic effects of NAC, but it has also been shown the healing effects in chronic pancreatitis related fibrosis and atrophy. There is a need for broader, comparative, in vivo and in vitro studies for the NAC molecule to take its place in the chronic pancreatitis treatment approach.

Ethics Committee Approval: Gülhane Military Medical Academy Local Ethics Committee Permission was obtained with the letter.

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