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**Activation of the alpha 7 nicotinic acetylcholine receptor by GTS-21 mitigates contrast nephropathy in a rat model**

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**Running title:** GTS-21 improves contrast nephropathy

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**Abbreviations**

CN: Contrast nephropathy
α7nAChR: α7 nicotinic acetylcholine receptor
AKI: Acute kidney injury
CKD: Chronic kidney disease
ROS: Reactive oxygen species
CAP: Cholinergic anti-inflammatory pathway
I/R: Ischemia/reperfusion
IP: Intaperitoneally
L_NAME: L-NG-Nitroarginine Methyl Ester
MDA: Malondialdehyde
GSH: Glutathione
MPO: Myeloperoxidase
BUN: Blood urea nitrogen
TUNEL: Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling
CRP: C-reactive protein

NAC: N-acetylcysteine

**Abstract**

**Introduction**: Contrast nephropathy (CN) is characterized by oxidative stress, vasoconstriction, tubular toxicity and hypoxia of the renal medulla. We aimed to test the therapeutic effects of an α7 nicotinic acetylcholine receptor (nAChR) agonist, GTS-21, in an experimental CN model.

**Methods**: Male Sprague–Dawley rats (n=40) were divided into 4 groups: saline-treated control, GTS-21-treated control, contrast, and GTS-21-treated contrast groups. Starting on the 1st day, GTS-21 (4 mg/kg, intraperitoneally) or saline was administered twice a day for 3 days. CN was induced on the second day by intravenous injection of indomethacin (10 mg/kg), L-NAME (10 mg/kg), and a contrast agent with high osmolarity (6 ml/kg; Urografin 76%). At the 72nd hour, blood and kidney samples were obtained for the determination of biochemical, histological, and gene expression parameters.

**Results:** Compared to those in control rats, the elevated serum BUN level in the contrast group decreased with GTS-21 treatment, while H&E staining and TUNEL assays showed that contrast-induced renal injury was improved by GTS-21. Moreover, GTS-21 treatment in the CN also increased the antioxidant glutathione level. In the contrast group, a significant increase in IL-6 expression and a decrease in TGF-β expression were observed; however, GTS-21 treatment decreased IL-6 expression and increased TGF-β expression.

**Conclusion**: GTS-21 significantly alleviated renal injury parameters through antioxidant, anti-inflammatory, and antiapoptotic mechanisms in the CN model.





## 1.Introduction

Contrast nephropathy (CN), which is a common cause of hospital-associated acute kidney injury (AKI), is characterized by elevated serum creatinine levels within 48-72 hours after intravenous or intraarterial injection of a contrast agent [1, 2]. Nonetheless, no definitive treatments are available for CN yet, except for extensively described preventive safety strategies that are regularly updated [3]. Currently, the main strategy for preventing the development of CN is prophylactic measures. As suggested by a meta-analysis, N-acetylcysteine administration and hydration are the main suggested pharmacological and nonpharmacological treatments [4]. The main risk factors for the development of CN are chronic kidney disease (CKD), diabetes mellitus, and congestive heart failure [4]. Renal medullary hypoxia, which includes a decrease in the regional microcirculation along with an increase in tubular cell oxygen demand, is involved in the development of cellular toxicity and CN [2, 5]. Following contrast agent administration, an imbalance arises between the vasoconstrictor and the vasodilatory mediators. Medullary hypoxia and acute tubular necrosis occur due to prolonged medullary vasoconstriction in afferent arterioles due to increases in viscosity, osmotic load, and the use of vasoconstrictors along with decreases in the use of vasodilators [5]. Therefore, the inhibition of vasodilators by indomethacin and nitric oxide synthase inhibitors before contrast agent administration is commonly used to create an experimental model of CN [5, 6]. Since increased generation of reactive oxygen species (ROS) and depletion of antioxidant enzyme activity are evident in the postischemic process, oxidative products are expected to play a prominent role in acute tubular necrosis and the development of CN [7, 8]. Furthermore, the highly regulated and controlled process of apoptosis is another crucial mechanism in the development of CN [9].

The cholinergic anti-inflammatory pathway (CAP) acts via the activation of alpha-7 nicotinic receptors (α7nAChR) [10-12], which are extensively expressed in epithelial cells of the tubules, endothelial and epithelial cells of vessels, macrophages and several immunocompetent cells and are related to the modulation and suppression of inflammation [11-13]. Immunostaining of normal renal tissues revealed that α7nAChRs were predominantly expressed in the endothelial cells of the peritubular capillaries and venules in the cortex, while renal ischemia–reperfusion (I/R) injury further increased α7nAChR staining [14]. In several inflammatory models,

a selective agonist of α7nACh GTS-21 (3-(2,4-dimethoxybenzylidene anabasine; DMXB-A) has been shown to reduce oxidative injury, inflammation and apoptosis [15, 16]. Furthermore, in models of cisplatin nephrotoxicity, sepsis-associated AKI, and ischemia/reperfusion (I/R) injury, which are all acute renal injury models, GTS-21 has been shown to exert protective effects on renal tissue. Following renal I/R injury, treatment with GTS-21 has also been shown to inhibit neutrophil infiltration with a concomitant reduction in the circulating levels of the proinflammatory cytokine IL-6 and lung chemokine ligand (CX-C motif) (CXCL) 1 and CXCL2 [12, 17-19]. However, no studies have evaluated the putative therapeutic effects of GTS-21 on CN.

Based on the aforementioned studies, GTS-21 may be a promising molecule for inhibiting inflammation and oxidative renal damage. In the present study, our aim was to investigate whether GTS-21 treatment could be effective in alleviating the severity of experimental CN.

## 2. Materials and Methods

All the experimental procedures were reviewed and approved by the Committee on Ethics on Animal Experiments, Marmara University School of Medicine, Istanbul, Turkey (Date: 5.03.2018, protocol code: 24. 2018.mar), and the experiments were performed in compliance with the ARRIVE guidelines, the New York Academy of Sciences guidelines and the Turkish law on the use of animals in experiments. Adult male Sprague–Dawley rats (250-300 g, 17-20 weeks old) were obtained from the Marmara University animal house (DEHAMER) and were then housed in standard cages with controlled temperature (22 ± 3°C), humidity (55-60%), and photocycle (12 h light/12 h dark). Rats were fed standard laboratory chow and had free access to water.

### 2.1. Experimental Design

Before the initiation of the experiments, an adaptation period of one week was allowed to minimize the stress caused by environmental changes. Saline-treated control (1 ml/kg, n=8), GTS-21-treated control (4 mg/kg; Cayman Chemical, Michigan, USA, n=8), contrast (saline 1 ml/kg, n=14), and GTS-21-treated contrast (4 mg/kg; n=10) groups were administered GTS-21 or saline intraperitoneally (i.p.) twice a day for 3 days. The rationale for choosing the dose of GTS-21 was based on the literature [19]. At 24 hours following the first two injections, under ketamine (100 mg/kg, i.p.) and chlorpromazine (3-5 mg/kg, i.p.) anesthesia, CN was induced by intravenous (tail vein) injection of indomethacin (10 mg/kg; Sigma–Aldrich Corp., MO, USA), L-NG-nitroarginine methyl ester (L-NAME, 10 mg/kg; Sigma–Aldrich Corp., MO, USA) and the high osmolar contrast agent meglumine/sodium diatrizoate (6 ml/kg; Urografin 76%, Bayer-Schering, Leverkusen, Germany) at 15-minute intervals (6). At the end of 72 hours, the rats were euthanized by cardiac puncture under anesthesia (ketamine 100 mg/kg and chlorpromazine 3-5 mg/kg; i.p.), and the collected blood samples were centrifuged at 1500 × g for 15 min at 4°C. Serum samples were isolated in accordance with standard processing protocols before aliquoting and were stored (at −80°C) for subsequent analysis of blood urea nitrogen (BUN) and creatinine levels. The left kidney was divided into 3 parts (upper, middle and lower poles), while the right kidney was divided into 6 pieces to study all parameters. Right kidney upper pole samples were placed in 10% formaldehyde for histopathological evaluation. The remaining samples were used to study all the biochemical parameters in identical segments of kidneys. All tissue samples were stored at −80°C for the measurement of malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO) activity and the gene expression of IL-6, IL-1β and TGF-β.

### 2.2. Biochemical analysis of serum

Serum levels of BUN and creatinine were measured by an automated analyzer (Cobas 8000, Roche Diagnostics, Basel, Switzerland).

#### 2.2.1. Measurement of Myeloperoxidase Activity in Renal Tissues

As an indicator of neutrophil infiltration into renal tissue, MPO activity was measured by using the modified Bradley method. The activity of MPO was determined by spectrophotometry at 460 nm through the measurement of $H_2O_2$-dependent oxidation of o-dianisidine. 2HCl. Enzyme activity was defined as the amount of MPO expressed as per gram of tissue weight [20].

#### 2.2.2. Malondialdehyde and Glutathione Assays in Renal Tissues

As a marker of tissue oxidation, MDA levels were assayed by monitoring thiobarbituric acid reactive substance formation using a spectrophotometer, and the results are expressed as nmol MDA/g tissue [20]. The antioxidant GSH levels were measured with a spectrophotometer using a modification of the Ellman procedure, and the results are expressed as μmol GSH/g tissue [20].

### 2.3. Histopathological Examination of Renal Tissues

Following euthanasia, the upper poles of the right kidneys were fixed in 10% formaldehyde solution. Light microscopic evaluations were performed under routine histological procedures. Paraffin sections of 4 μm were cut with a rotary microtome (Leica RM2125RT, Wetzlar, Deutchland) and stained with hematoxylin and eosin

(H&E) dye. The histological sections were photographed with a light microscope (Olympus BX51, Tokyo, Japan) attached to a digital camera (Olympus DP72, Tokyo, Japan) and evaluated by an experienced histologist (Ö.T.Ç. K) blinded to the experimental groups. Semiquantitative evaluations were performed in 5 different areas of each of the tissue sections at x200 magnification. Histopathological scoring was performed by grading tubular epithelial degeneration, tubular dilatation, edema/inflammation, congestion, and glomerular degeneration as *0: none, 1: mild, 2: moderate, and 3: severe*, respectively. The total histologic injury score was expressed as the sum of each of the parameters, with a maximum score of [20].

**2.4. TUNEL Assay in Renal Tissues**

Kidney samples were analyzed via terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) analysis (ApopTag® Plus Peroxidase *In Situ* Apoptosis Kit, Millipore, Cat No: S7101, USA) to detect apoptotic cells. According to the manufacturer's instructions, tissue sections were deparaffinized, rehydrated in alcohol and treated with proteinase K (20 µg/ml, at 37°C) for 20 minutes. $H_2O_2$ in methanol (3%) was used to block endogenous peroxidase activity. After rinsing with PBS, the sections were incubated with TdT equilibration buffer for 30 minutes; then, the slides were covered with TdT enzyme solution and incubated for 1 hour at 37°C. STOP solution was added to the sections for 5 minutes. Following washing in PBS, the sections were incubated with an anti-digoxigenin conjugate for 30 minutes in a humidified chamber. The slides were rinsed in PBS buffer, and the entire tissue sections were covered with DAB solution for 10 minutes. The nuclei were counterstained with Mayer's hematoxylin and examined with a light microscope (Olympus BX51, Japan) and photographed by an attached digital camera system (Olympus DP72, Japan). Semiquantitative scoring was performed as described previously [21], where 0: positive in <5% of the cells, 1: positive in 5−25% of the cells, 2: positive in 26−50% of the cells, and 3: positive in >50% of the cells/area.

**2.5. Gene expression of IL-6, IL-1β and TGF-β in renal tissue**

RNA isolation and cDNA synthesis were performed by using commercial kits (PureLink RNA Mini Kit, Cat. No. 12183018A and High-Capacity cDNA Reverse Transcription Kit, Cat. No. 4368814; Thermo Fisher Scientific, USA). The expression of the IL-6, IL-1β, TGF-β, and beta-actin genes was assayed by qPCR (TaqMan™ Gene Expression Assay (FAM), Thermo Fisher Scientific, USA) using LightCycler 480 software. Ct values were measured, and relative levels of IL-6, IL-1β and TGF-β were calculated via the $2^{-\Delta\Delta Ct}$ method, with beta-actin used as a housekeeping gene [22].

**2.6. Statistical analysis**

The statistical analyses were performed using the software GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). The data of control rats (n=8 in each) and contrast-induced rats that survived (n=8-9) were used for statistical analysis. All data in the present manuscript are expressed as the mean ± standard error of the mean (SEM). Analysis of the data was carried out by one-way ANOVA and the Mann–Whitney test where appropriate. Survival ratios of the experimental groups were compared using chi-square analysis. The statistical significance of the qPCR results was evaluated with ΔCt values. A gene expression level graph was generated with the $^{2-\Delta\Delta Ct}$ method. Differences were considered statistically significant at $p<0.05$.

**3. RESULTS**

**3.1. Effects of GTS-21 on Contrast-induced alterations in survival rate and renal function**

The survival rates in the saline-treated contrast group (64.3%; 9/14) and the GTS-21-treated contrast group (80.0%; 8/10) were similar (Chi-square 0.54; p=0.46). By the end of the experiments, the serum BUN and creatinine levels were similar in both the control and GTS-21-control groups, showing that GTS-21 treatment had no effect. However, the serum BUN and creatinine levels were significantly elevated by the induction of contrast nephropathy (Figure 1a; $p<0.001$ and b; $p<0.05$), verifying the presence of impaired renal function. In contrast, repeated treatment with GTS-21 significantly reduced the BUN level ($p<0.05$), while the creatinine level decreased to the control level.

**3.2. Effects of GTS-21 on Contrast-induced Alterations in Oxidative Parameters**

MDA, an indicator of oxidative damage, was significantly greater in the saline-treated group ($p<0.001$) and GTS-21-treated group ($p<0.01$) than in the control group (Figure 2a). Additionally, significantly elevated GSH levels were detected in the GTS-21-contrast group compared to the control group ($p<0.01$, Figure 2b), indicating an increase in antioxidant capacity. However, MPO activity did not significantly differ among the experimental groups ($p>0.05$; Figure 2c).

**3.3. Effects of GTS-21 on Contrast-induced Alterations in Histopathological Parameters**

The control and GTS-21 control groups demonstrated a normal renal morphology of parenchyma with well-organized tubules and glomeruli (Figure 3b). The tubular epithelial degeneration scores were similar between the

control (0.6 ± 0.1) and GTS-21-control (0.6 ± 0.1) groups. Contrast agent treatment caused remarkable tubular dilatation and atrophy along with glomerular degeneration (Figure 3b). The dilated tubules were fulfilled by epithelial desquamation, severe interstitial congestion, and edema/inflammation. The tubular injury scores also revealed that contrast media injection caused significant epithelial degeneration in the saline-treated contrast group (2.0 ± 0.2, $p<0.001$), while GTS-21 treatment ameliorated contrast-induced tubular degeneration (1.0 ± 0.1, $p<0.001$). Furthermore, the total histopathological injury score, as expressed by the sum of the scores of the five parameters (glomerular and tubular epithelial degeneration, tubular dilatation, edema/inflammation, and congestion), was significantly greater in the saline-treated group than in the control group ($p<0.01$, Figure 3a). Overall, the increase in the total histopathological injury score in the contrast group was attenuated by GTS-21 treatment ($p<0.01$, Figure 3a).

**3.4. Effects of GTS-21 on Contrast-induced Alterations in Apoptosis**

The number of TUNEL (+) cells was detected, and the semiquantitative TUNEL score was recorded. An increase in the number of TUNEL (+) cells in the saline-treated contrast group ($p<0.01$) revealed that the induction of CN resulted in renal tubular cell apoptosis, while GST-21 treatment significantly decreased the number of TUNEL (+) cells ($p <0.05$). (Figure 4a-b).

**3.5. Effects of GTS-21 on Contrast-induced Alterations in Cytokine Expression**

The gene expression level of the proinflammatory cytokine IL-6 significantly increased by 6-fold in the saline-treated control group compared to the control group, while GTS-21 treatment reversed this increase ($p<0.01$, Figure 5a). However, IL-1β expression did not significantly differ among the groups (Figure 5c). The gene expression levels of TGF-β were significantly decreased in the saline-treated group ($p<0.01$), while GTS-21 treatment ameliorated this suppression ($p<0.05$, Figure 5b).

**4. Discussion**

In the present study, we investigated the therapeutic effects of GTS-21, a selective α7nAChR agonist, on histopathological and functional alterations due to CN. To our knowledge, this is the first study demonstrating the effect of GTS-21 in an experimental CN model. In the CN model, rats demonstrated impaired kidney functions with elevated levels of IL-6, lipid peroxidation, and apoptosis, along with decreased TGF-β levels. These changes were supported by histological deterioration. However, GTS-21 treatment improved kidney function and alleviated histopathological changes, oxidative stress, apoptosis, and inflammation.

The imbalance between the vasoconstrictor and vasodilatory mediators as a result of contrast agent administration causes tubular injury, medullary hypoxia, increased oxidative stress, and inflammatory responses [5, 6]. Cholinergic mediators are known to exert anti-inflammatory effects via α7nAChR, which is expressed in several neurologic and nonneurological cells. The α7 nAChR agonist GTS-21 was shown to reduce TNF-α in an endotoxemia model, while the α7 nAChR antagonist CNI-1493 reduced alveolar hemorrhage and interstitial edema and attenuated the effects of renal I/R injury in the pulmonary vasculature [23, 24]. Yeboah et al. showed that GTS-21 treatment reduced I/R-induced renal dysfunction and ameliorated acute tubular injury by suppressing MPO activity and inhibiting TNF-α production [12]. Kwasa et al. [25] reported that patients with elevated CRP and IL-6 levels had a doubled risk of developing CN compared to patients with normal CRP levels, demonstrating that CN is common in patients with inflammation. CN induces inflammation by activating Toll-like receptor-2 and C-X-C chemokine receptor-4-dependent pathways, resulting in the release of IL-6 and monocyte chemotactic proteins [26]. In patients with multiple myeloma, malignancies, and sepsis, the risk of CN during contrast agent administration has been reported to increase due to the prothrombotic and vasoconstrictive effects of inflammatory mediators [27].

Cytokines can be triggered in many inflammatory models that affect the kidneys, such as renal I/R injury, cisplatin nephrotoxicity, and sepsis-associated AKI, which have indicated that the upregulation of IL-6 could be regarded as a predictor of CN [12, 18, 19, 28, 29]. Similarly, our data also demonstrated that IL-6 expression was significantly increased in the saline-treated group, which confirms that IL-6 is a key proinflammatory cytokine involved in the pathogenesis of contrast-induced renal injury. On the other hand, the activation of the 7nAChR α7nAChR by vagal nerve stimulation or α7nAChR agonists, such as nicotine or GTS-21, results in decreased levels of IL-6 [19]. A recent study of renal I/R injury also showed that GTS-21 reduced pulmonary neutrophil infiltration, vascular leakage, and circulating IL-6 levels by affecting splenic macrophages [17]. In parallel with these reports, GTS-21 treatment in the present study also reduced IL-6 expression. However, the expression levels of IL-1β were similar in all the experimental groups. The lack of any increase in IL-1β expression may be explained by the inhibition of prostaglandin synthesis by indomethacin, which was used to induce CN in this study. Nevertheless, the present

findings demonstrate that GTS-21 prevents contrast-induced histopathological injury but does not fully ameliorate biochemical and inflammatory markers of damage.

A link has recently been identified between the cytokines IL-6 and TGF-β [30]. Together, elevated IL-6 and TGF-β can lead to a profibrotic response within the kidney, which is driven by the activation of proximal tubular cells [31]. Although TGF-β may cause renal fibrosis in several renal diseases, it also possesses renoprotective effects by reducing inflammation and inducing autophagy [32]. In our study, the suppression of TGF-β gene expression due to CN induction was abolished by GTS-21 treatment, which may have contributed to the amelioration of contrast nephropathy. TGF-β has antifibrotic effects on models of unilateral urethral obstruction, diabetes, and hypertensive nephropathy [33-36]. In unilateral urethral obstruction and crescentic glomerulonephritis, the anti-inflammatory effect of TGF-β signaling was shown to act through the suppression of NF-κB [37, 38]. In our study, TGF-β gene expression was approximately lower in the saline-treated group than in the control group, and this reduction was significantly reversed by GTS-21 treatment. In parallel with our results, an increase in TGF-β was previously shown to be accompanied by accelerated skin wound healing [39]. Taken together, these findings indicate that the significant increase in TGF-β in the GTS-21 contrast group confirms the anti-inflammatory effects of GTS-21 [40]. In our study, the GTS-21-treated control group exhibited improved histopathological structure with reduced glomerular/tubular degeneration and tubular atrophy, which was accompanied by the suppression of IL-6 and maintenance of TGF-β expression in the kidneys.

Inoue et al. demonstrated that vagal nerve stimulation protects against renal I/R injury, which was suggested to occur via the infiltration of anti-inflammatory splenocytes into the kidney [41]. When stimulated in the spleen by vagal nerve stimulation, splenocytes act as important coordination centers of the cholinergic system. Renal I/R injury and inflammation in the kidneys are attenuated by nicotine and GTS-21, which act on acetylcholine receptors in epithelial cells of the proximal tubules [12]. Recently, GTS-21 was shown to inhibit inflammation in α7 nAChR-knockout mice, indicating that GTS-21 may also have anti-inflammatory effects independent of α7 nAChR receptors [42]. *In vivo* and *in vitro* laboratory studies have explored several antioxidants for their putative protective effects on CN. Melatonin, L-carnitine, vitamin E, recombinant manganese SOD, and N-acetylcysteine (NAC) were demonstrated to exert renoprotective effects by decreasing inflammation and apoptosis in conjunction with attenuating serum creatinine levels and alleviating renal damage [43-47]. These studies suggest that the imbalance between oxidative stress and antioxidants plays a central role in the development of renal injury, and reconstruction of the normal balance of oxidant and antioxidant systems could be critical for the treatment of CN [48]. Recently, a meta-analysis demonstrated that trimetazidine, an anti-ischemic agent with anti-oxidant properties, added to the hydration was associated with a reduced risk of CN in patients undergoing coronary angiography [49]. In an another study, nicorandil, an ATP-sensitive potassium channel activator and a nitric oxide donor. and ranolazine, that has cytoprotective effects by anti-oxidative stress and anti-apoptotic properties through calcium signaling stimulation, were shown to protect against the development of CN in patients with mild to moderate renal dysfunction [50]. In our study, despite any depletion of GSH by the induction of CN, the antioxidant GSH was significantly increased in the GTS-21-treated group compared with the control group. Administration of GTS-21 alone has previously been shown to increase renal GSH capacity by triggering a target role in the GSH pathway [51]. Taken together with these reports, the present findings suggest that the protective effect of GTS-21 on contrast nephropathy involves its antioxidant effect.

In many studies, renal MPO levels have been shown to increase in animal models of cisplatin nephrotoxicity, sepsis-associated AKI and I/R injury [12, 18, 19]. However, our results showed that renal MPO levels did not differ among the experimental groups, suggesting that contrast-related renal inflammation does not involve neutrophil infiltration into the kidneys. On the other hand, the levels of MDA, one of the best predictors of oxidative injury to the cell membrane, were elevated in the CN model and in some AKI models induced with nephrotoxic agents (vancomycin, cisplatin, doxorubicin, gentamicin) [52-56]. Although there are many studies evaluating the relationships between the nephrotoxic effects of many agents and oxidative stress on the levels of MDA in the literature, no studies have evaluated the effects of GTS-21 on MDA levels. In our study, significant increases in MDA levels were evident in the saline- and GTS-21-treated CN groups, revealing that our model successfully initiated oxidative renal damage; however, despite its antioxidant effects, GTS-21 treatment was not capable of fully reversing oxidative injury to renal tissue. On the other hand, apoptosis was significantly increased in the contrast group treated with saline, while elevated TUNEL staining in the CN was suppressed by GTS-21, confirming its antiapoptotic effect. Romano et al. reported that CA administration decreases cell viability, which is accompanied by increases in caspase 3, 8, 9, and 10 activity in distal tubular cells [57]. In parallel to our results, GTS-21 improved cisplatin-induced AKI by reducing inflammation and apoptosis, which was also measured by

TUNEL assay [19]. In addition, the antiapoptotic effects of GTS-21 have also been demonstrated in models of bleomycin-incubated cell culture and sepsis-associated myocardial and renal injury models [58-60]. Therefore, the protective effect of GTS-21 treatment against CN-induced renal dysfunction, lipid peroxidation, and tubular injury appears to involve the antiapoptotic effect of GTS-21 on kidney tissue.

A study by Kitagawa et al. investigated the safety and pharmacokinetics of GTS-21 in healthy male humans. They demonstrated that GTS-21 is well tolerated up to a dose of 450 mg/day and adverse effects were similar to placebo [61]. Based on safety profile of GTS-21 on humans and its mitigating effects on CN in our rodent model, phase 2 -3 studies should be planned to explore the effect of GTS-21 on the prevention of CN in patients with chronic kidney disease.

The limitations of our study include the lack of evaluation of the long-term effects of GTS-21 and the involvement of the SMAD2 and SMAD7 signaling pathways in apoptosis, inflammation, and renal fibrosis. Similarly, additional studies are needed to determine the effects of GTS-21 at earlier time points after CN induction. A single dose of GTS-21 was chosen based on a previous study [19], but a dose-response study could better reveal the effectiveness of GTS-21 for treating contrast-related renal injury. In addition to GSH, superoxide dismutase and catalase could be measured to further support the antioxidant effect of GTS-21, as described in previous studies [62]. Moreover, splenectomy could eliminate the endogenous activation of the CAP and natural immunity, which could provide evidence to explain the mechanisms of action of GTS-21.

## 5. Conclusion

In conclusion, our study provides the first evidence that the selective α7 nAChR agonist GTS-21 alleviates contrast-induced nephropathy through its anti-inflammatory, antioxidant and antiapoptotic mechanisms and improves functional and morphological deterioration induced by contrast nephropathy. These results suggest that stimulation of the anticholinergic pathway is a potential target for preventing CN. Further studies are warranted to determine the clinical utility of the selective α7 nAChR agonist GTS-21 in preventing CN.

## Statement of Ethics

The experimental procedures involving animals in this study were approved by the Animal Ethics Committee of Marmara University (Date: 5.03.2018, No: 24. 2018.mar), Turkey.


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## Patient consent for publication

Not applicable.

## Conflict of interest statement

The authors declare that they have no competing interests.

**Authors' contributions:** 1) conception or design, or analysis and interpretation of data; Seçkin Akçay 2) drafting the article or revising it; Zarife Nigar Özdemir-Kumral, Özlem Tuğçe Çilingir-Kaya, İrem Peker-Eyüboğlu, Mustafa Akkiprik 3) providing intellectual content of critical importance to the work described; Mehmet Koç 4) final approval of the version to be published; Berrak Yeğen and Mehmet Koç.

## Data availability statement

All the data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

**REFERENCES**


1. Nash, K., A. Hafeez, and S. Hou, *Hospital-acquired renal insufficiency.* Am J Kidney Dis, 2002. **39**(5): p. 930-6.
2. Mamoulakis, C., et al., *Contrast-induced nephropathy: Basic concepts, pathophysiological implications and prevention strategies.* Pharmacol Ther, 2017. **180**: p. 99-112.
3. Wichmann, J.L., et al., *Contrast-Induced Nephropathy.* Circulation, 2015. **132**(20): p. 1931-6.
4. Subramaniam, R.M., et al., *Effectiveness of Prevention Strategies for Contrast-Induced Nephropathy: A Systematic Review and Meta-analysis.* Ann Intern Med, 2016. **164**(6): p. 406-16.
5. Heyman, S.N., S. Rosen, and C. Rosenberger, *Renal parenchymal hypoxia, hypoxia adaptation, and the pathogenesis of radiocontrast nephropathy.* Clin J Am Soc Nephrol, 2008. **3**(1): p. 288-96.
6. Agmon, Y., et al., *Nitric oxide and prostanoids protect the renal outer medulla from radiocontrast toxicity in the rat.* J Clin Invest, 1994. **94**(3): p. 1069-75.
7. Yoshioka, T., A. Fogo, and J.K. Beckman, *Reduced activity of antioxidant enzymes underlies contrast media-induced renal injury in volume depletion.* Kidney Int, 1992. **41**(4): p. 1008-15.
8. Heyman, S.N., et al., *Reactive oxygen species and the pathogenesis of radiocontrast-induced nephropathy.* Invest Radiol, 2010. **45**(4): p. 188-95.
9. Haller, C. and I. Hizoh, *The cytotoxicity of iodinated radiocontrast agents on renal cells in vitro.* Invest Radiol, 2004. **39**(3): p. 149-54.
10. Sadis, C., et al., *Nicotine protects kidney from renal ischemia/reperfusion injury through the cholinergic anti-inflammatory pathway.* PLoS One, 2007. **2**(5): p. e469.
11. Wang, H., et al., *Nicotinic acetylcholine receptor alpha7 subunit is an essential regulator of inflammation.* Nature, 2003. **421**(6921): p. 384-8.
12. Yeboah, M.M., et al., *Cholinergic agonists attenuate renal ischemia-reperfusion injury in rats.* Kidney Int, 2008. **74**(1): p. 62-9.
13. Wang, Y., et al., *Human bronchial epithelial and endothelial cells express alpha7 nicotinic acetylcholine receptors.* Mol Pharmacol, 2001. **60**(6): p. 1201-9.
14. Yeboah, M.M., et al., *Nicotinic acetylcholine receptor expression and regulation in the rat kidney after ischemia-reperfusion injury.* Am J Physiol Renal Physiol, 2008. **295**(3): p. F654-61.
15. Chatterjee, P.K., et al., *Cholinergic agonists regulate JAK2/STAT3 signaling to suppress endothelial cell activation.* Am J Physiol Cell Physiol, 2009. **297**(5): p. C1294-306.
16. Nullens, S., et al., *Effect of Gts-21, an Alpha7 Nicotinic Acetylcholine Receptor Agonist, on Clp-Induced Inflammatory, Gastrointestinal Motility, and Colonic Permeability Changes in Mice.* Shock, 2016. **45**(4): p. 450-9.
17. Goto, D., et al., *Nicotinic acetylcholine receptor agonist reduces acute lung injury after renal ischemia-reperfusion injury by acting on splenic macrophages in mice.* Am J Physiol Renal Physiol, 2022. **322**(5): p. F540-F552.
18. Chatterjee, P.K., et al., *Nicotinic acetylcholine receptor agonists attenuate septic acute kidney injury in mice by suppressing inflammation and proteasome activity.* PLoS One, 2012. **7**(5): p. e35361.
19. Chatterjee, P.K., et al., *Activation of the cholinergic anti-inflammatory pathway by GTS-21 attenuates cisplatin-induced acute kidney injury in mice.* PLoS One, 2017. **12**(11): p. e0188797.
20. Tezcan, N., et al., *Nesfatin-1 treatment preserves antioxidant status and attenuates renal fibrosis in rats with unilateral ureteral obstruction.* Nephrol Dial Transplant, 2022. **37**(7): p. 1238-1248.
21. Sahin Ersoy, G., et al., *Mycophenolate mofetil attenuates uterine ischaemia/reperfusion injury in a rat model.* Reprod Biomed Online, 2017. **34**(2): p. 115-123.
22. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, 2001. **29**(9): p. e45.
23. Kramer, A.A., et al., *Renal ischemia/reperfusion leads to macrophage-mediated increase in pulmonary vascular permeability.* Kidney Int, 1999. **55**(6): p. 2362-7.
24. Pavlov, V.A., et al., *Selective alpha7-nicotinic acetylcholine receptor agonist GTS-21 improves survival in murine endotoxemia and severe sepsis.* Crit Care Med, 2007. **35**(4): p. 1139-44.
25. Kwasa, E.A., S. Vinayak, and R. Armstrong, *The role of inflammation in contrast-induced nephropathy.* Br J Radiol, 2014. **87**(1041): p. 20130738.
26. Guan, X.F., et al., *Contrast Media-Induced Renal Inflammation Is Mediated Through HMGB1 and Its Receptors in Human Tubular Cells.* DNA Cell Biol, 2017. **36**(1): p. 67-76.

27. Stouthard, J.M., et al., *Interleukin-6 stimulates coagulation, not fibrinolysis, in humans.* Thromb Haemost, 1996. **76**(5): p. 738-42.
28. Hudzik, B., et al., *Serum interleukin-6 concentration predicts contrast-induced nephropathy in patients undergoing percutaneous coronary intervention.* Eur Cytokine Netw, 2010. **21**(2): p. 129-35.
29. Faubel, S., et al., *Cisplatin-induced acute renal failure is associated with an increase in the cytokines interleukin (IL)-1beta, IL-18, IL-6, and neutrophil infiltration in the kidney.* J Pharmacol Exp Ther, 2007. **322**(1): p. 8-15.
30. Luckett-Chastain, L.R. and R.M. Gallucci, *Interleukin (IL)-6 modulates transforming growth factor-beta expression in skin and dermal fibroblasts from IL-6-deficient mice.* Br J Dermatol, 2009. **161**(2): p. 237-48.
31. Turner, M., D. Chantry, and M. Feldmann, *Transforming growth factor beta induces the production of interleukin 6 by human peripheral blood mononuclear cells.* Cytokine, 1990. **2**(3): p. 211-6.
32. Sureshbabu, A., S.A. Muhsin, and M.E. Choi, *TGF-beta signaling in the kidney: profibrotic and protective effects.* Am J Physiol Renal Physiol, 2016. **310**(7): p. F596-F606.
33. Liu, G.X., et al., *Disruption of Smad7 promotes ANG II-mediated renal inflammation and fibrosis via Sp1-TGF-beta/Smad3-NF.kappaB-dependent mechanisms in mice.* PLoS One, 2013. **8**(1): p. e53573.
34. Chung, A.C., et al., *Disruption of the Smad7 gene promotes renal fibrosis and inflammation in unilateral ureteral obstruction (UUO) in mice.* Nephrol Dial Transplant, 2009. **24**(5): p. 1443-54.
35. Meng, X.M., et al., *Smad2 protects against TGF-beta/Smad3-mediated renal fibrosis.* J Am Soc Nephrol, 2010. **21**(9): p. 1477-87.
36. Chen, H.Y., et al., *The protective role of Smad7 in diabetic kidney disease: mechanism and therapeutic potential.* Diabetes, 2011. **60**(2): p. 590-601.
37. Huang, X.R., et al., *Latent TGF-beta1 protects against crescentic glomerulonephritis.* J Am Soc Nephrol, 2008. **19**(2): p. 233-42.
38. Wang, W., et al., *Signaling mechanism of TGF-beta1 in prevention of renal inflammation: role of Smad7.* J Am Soc Nephrol, 2005. **16**(5): p. 1371-83.
39. Solmaz, A., et al., *Nesfatin-1 improves oxidative skin injury in normoglycemic or hyperglycemic rats.* Peptides, 2016. **78**: p. 1-10.
40. Wu, S.J., et al., *Activation of the Cholinergic Anti-inflammatory Pathway Attenuated Angiotension II-Dependent Hypertension and Renal Injury.* Front Pharmacol, 2021. **12**: p. 593682.
41. Inoue, T., et al., *Vagus nerve stimulation mediates protection from kidney ischemia-reperfusion injury through alpha7nAChR+ splenocytes.* J Clin Invest, 2016. **126**(5): p. 1939-52.
42. Garg, B.K. and R.H. Loring, *GTS-21 has cell-specific anti-inflammatory effects independent of alpha7 nicotinic acetylcholine receptors.* PLoS One, 2019. **14**(4): p. e0214942.
43. Gazi, S., A. Altun, and O. Erdogan, *Contrast-induced nephropathy: preventive and protective effects of melatonin.* J Pineal Res, 2006. **41**(1): p. 53-7.
44. Kunak, C.S., et al., *Nephroprotective potential of carnitine against glycerol and contrast-induced kidney injury in rats through modulation of oxidative stress, proinflammatory cytokines, and apoptosis.* Br J Radiol, 2016. **89**(1058): p. 20140724.
45. Kongkham, S., S. Sriwong, and A. Tasanarong, *Protective effect of alpha tocopherol on contrast-induced nephropathy in rats.* Nefrologia, 2013. **33**(1): p. 116-23.
46. Pisani, A., et al., *Effect of a recombinant manganese superoxide dismutase on prevention of contrast-induced acute kidney injury.* Clin Exp Nephrol, 2014. **18**(3): p. 424-31.
47. Ozturk, O., et al., *An experimental study on the preventive effects of N-acetyl cysteine and ozone treatment against contrast-induced nephropathy.* Acta Cir Bras, 2018. **33**(6): p. 508-517.
48. Li, Y. and J. Wang, *Contrast-induced acute kidney injury: a review of definition, pathogenesis, risk factors, prevention and treatment.* BMC Nephrol, 2024. **25**(1): p. 140.
49. Nair, T., et al., *Efficacy of Trimetazidine in the Prevention of Contrast-Induced Nephropathy in Patients Undergoing Contrast Coronary Intervention: A Systematic Review and Meta-Analysis (PRISMA).* J Clin Med, 2024. **13**(7).
50. Yusuf, J., et al., *Efficacy of nicorandil and ranolazine in prevention of contrast-induced nephropathy in patients with mild-to-moderate renal dysfunction: a randomized controlled trial.* Coronary artery disease, 2024. 35(3), 186–192.
51. Godin, D.V., et al., *Antioxidant enzyme alterations in experimental and clinical diabetes.* Mol Cell Biochem, 1988. **84**(2): p. 223-31.

52. Yagmurca, M., et al., *Caffeic acid phenethyl ester as a protective agent against doxorubicin nephrotoxicity in rats.* Clin Chim Acta, 2004. **348**(1-2): p. 27-34.
53. Vardi, N., et al., *Gentamicin-induced nephrotoxicity and protective effect of caffeic acid phenethyl ester in rats.* Fundam Clin Pharmacol, 2005. **19**(2): p. 173-7.
54. Oktem, F., et al., *In vivo evidences suggesting the role of oxidative stress in pathogenesis of vancomycin-induced nephrotoxicity: protection by erdosteine.* Toxicology, 2005. **215**(3): p. 227-33.
55. Ozen, S., et al., *Role of caffeic acid phenethyl ester, an active component of propolis, against cisplatin-induced nephrotoxicity in rats.* J Appl Toxicol, 2004. **24**(1): p. 27-35.
56. Aksu, F., et al., *Antioxidant and renoprotective effects of sphingosylphosphorylcholine on contrast-induced nephropathy in rats.* Ren Fail, 2016. **38**(7): p. 1089-98.
57. Romano, G., et al., *Contrast agents and renal cell apoptosis.* Eur Heart J, 2008. **29**(20): p. 2569-76.
58. Gimonet, D., et al., *Functional role of nicotinic acetylcholine receptors in apoptosis in HL-60 cell line.* Eur J Pharmacol, 2003. **482**(1-3): p. 25-9.
59. Kong, W., et al., *GTS-21 Protected Against LPS-Induced Sepsis Myocardial Injury in Mice Through alpha7nAChR.* Inflammation, 2018. **41**(3): p. 1073-1083.
60. Gao, Y., et al., *GTS-21 attenuates LPS-induced renal injury via the cholinergic anti-inflammatory pathway in mice.* Am J Transl Res, 2017. **9**(10): p. 4673-4681.
61. Kitagawa, H., et al., *Safety, pharmacokinetics, and effects on cognitive function of multiple doses of GTS-21 in healthy, male volunteers.* Neuropsychopharmacology, 2003. **28**(3): p. 542-51.
62. Wang, H., et al., *GTS-21 Promotes alpha7 nAChR to Alleviate Intestinal Ischemia-Reperfusion-Induced Apoptosis and Inflammation of Enterocytes.* Med Sci Monit, 2020. **26**: p. e921618.

**FIGURE LEGENDS**

**Figure 1:** Serum levels of (A) BUN and (B) creatinine in the control and contrast-induced nephropathy (CN) groups treated with either saline or GTS-21. * $p<0.05$, *** $p<0.001$ compared to the control group, + $p<0.05$ compared to the saline-treated CN group. The data are expressed as the mean ± SEM. n=5-8 for each group.

**Figure 2:** Renal tissue levels of (A) malondialdehyde (MDA), (B) glutathione (GSH) and (C) myeloperoxidase activity (MPO) in the control and contrast-induced nephropathy (CN) groups treated with either saline or GTS-21. ** $p<0.01$, *** $p<0.001$ compared to the respective control groups; ++ $p<0.01$ compared to the saline-treated CN group. The data are expressed as the mean ± SEM. n=5-8 for each group.

**Figure 3:** Histological scores were improved by GTS-21 treatment. A) Total histopathological injury score in the control and contrast-induced nephropathy (CN) groups treated with either saline or GTS-21. ** $p<0.01$ compared to the control group; ++ $p<0.01$ compared to the saline-treated CN group. The data are expressed as the mean ± SEM. n=5-8 for each group. B) Representative micrographs from all experimental groups stained with hematoxylin and eosin: a. Control group: regular morphology of both glomeruli (arrowhead) and tubules (arrow) with minimal interstitial congestion (*); b. GTS-21-control group: glomeruli (arrowhead) and tubules (arrow) with regular morphology, minimal interstitial edema (*) and irregular glomeruli (blue arrowhead); c. Saline-treated CN group: glomeruli (white arrowhead) and tubules (blue arrow) with irregular morphology; d. GTS-21-treated CN group: glomeruli (arrowhead) and tubules (arrow) with regular morphology. Original magnification: x100, upper inset: x200, lower inset: x400

**Figure 4:** A) Semiquantitative TUNEL staining in the control and contrast-induced nephropathy (CN) groups treated with either saline or GTS-21; B) Representative micrographs of TUNEL-stained kidney tissues. a. Control group. b. Saline-treated CN group. c. GTS-21-treated control group. d. GTS-21-treated CN group. TUNEL-positive cells are indicated by black arrows indicating apoptosis. ** $p<0.01$ compared to the control group; + $p<0.05$ compared to the GTS-21-CN group. The data are expressed as the mean ± SEM. n=5-8 for each group. Original magnification: 100×.

**Figure 5:** (A) TGF-β, (B) IL-6, (C) IL-1β and expression levels in renal tissues of the control and contrast-induced nephropathy (CN) groups treated with either saline or GTS-21. *$p<0.05$, ** $p<0.01$ compared to the respective control groups; + $p<0.05$, ++ $p<0.01$ compared to the saline-treated CN group. Statistical significance was evaluated with ΔCt values. A gene expression level graph was generated with the $^{2-\Delta\Delta Ct}$ method. The data are expressed as the mean ± SEM. n=5-8 for each group.