

ORIGINAL RESEARCH

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Effect of caffeic acid phenethyl ester (CAPE) on vascular endothelial growth factor a (VEGF-A) gene expression in gentamicin-induced acute renal nephrotoxicity**Zeynal Mete Karaca¹, Hasan Ozen², Muslum Akgoz³, Yilmaz Cigremis¹**¹*Inonu University, Faculty of Medicine, Department of Medical Biology and Genetics, Malatya, Turkey*²*Kafkas University, Faculty of Veterinary Medicine, Department of Pathology, Kars, Turkey*³*TUBITAK Ulusal Metroloji Enstitüsü (UME), Bioanalysis Laboratory, Gebze, Kocaeli, Turkey*

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Abstract

Vascular endothelial growth factor-A (VEGF-A) gene expression in an experimental gentamicin-induced nephrotoxicity and ameliorative effect of caffeic acid phenethyl ester (CAPE) was investigated in rats. Animals were divided into four groups (n=8); control (C) group animals were given 10% dimethylsulfoxide (DMSO); gentamicin (G) group animals were given 100 mg/kg/day gentamicin; CAPE group animals were given 30 mg/kg/day CAPE and CAPE+G group animals were given 100 mg/kg/day gentamicin plus 30 mg/kg/day CAPE. Serum creatinine and BUN levels significantly increased in gentamicin group as compared to the control group ($p<0.05$) while CAPE treatment did not significantly lower the levels of either BUN or creatinine ($p>0.05$). Gene expression level of VEGF-A in gentamicin group significantly decreased as compared to the control group, however, CAPE treatment did not have any increasing effect on the gene expression level. According to histopathological investigation, gentamicin treatment caused prominent degeneration in kidney tissue and CAPE treatment had only slight beneficial effect on lowering the tissue degeneration. The results showed that gentamicin decreases VEGF-A gene expression and this might be related to the tissue degeneration at cellular level. However, CAPE treatment did not have significant ameliorative effect in lowering the gentamicin induced nephrotoxicity.

Keywords: VEGF, CAPE, gentamicin, nephrotoxicity, rat**Introduction**

Amino glycosides are commonly used in treatment of infections caused by gram (-) bacteria and gentamicin is the most frequently used agent in this group of medicine. However, nephrotoxicity induced by amino glycosides limits their safe usage [1]. Gentamicin impacts on kidney by causing degeneration in both proximal tubules and blood circulation in kidney, and hence, tissue degeneration results in renal failure [2]. It has been reported that 10 to 20% of patients treated by gentamicin develop acute renal failure [3], and 30% of patients receiving gentamicin more than seven days exhibit signs of nephrotoxicity [4]. Gentamicin nephrotoxicity is characterized by typical tubular epithelial cytotoxicity [3]. However, after stopping the use of gentamicin, nephrotoxicity can diminish and kidney tissue may return to normal functioning [5].

Vascular endothelial growth factor (VEGF) is a specific mitogen for vascular endothelial cells and regulates vasculogenesis and angiogenesis [6]. It is a heparin binding glycoprotein, and synthesized by macrophages as well as fibroblasts, endothelial and epidermal cells. It has been reported that VEGF is normally present in renal proximal tubule cells [7]. Members of VEGF function by

binding to their specific receptors. VEGF mediates microvascular tube formation of endothelial cells by modulating their migration and proliferation [8]. Therefore, VEGF can play roles in neovascularization, wound healing, vascular permeabilization, embryogenesis and regulation of menstrual cycle [9].

Caffeic acid phenethyl ester (CAPE) is a bioactive component of honeybee propolis. It is a strong antioxidant and inhibitor of nuclear factor kappa B (NF- κ B). Besides being a strong potent antioxidant, CAPE has anti-inflammatory, mitogenic, anti-carcinogenic, anti-virogenic, and immunomodulatory functions [10]. Its antioxidant effect was previously shown in a gentamicin induced acute renal toxicity model, in that CAPE normalized the altered levels of superoxide dismutase, catalase, nitric oxide and malondialdehyde upon gentamicin treatment [11].

In the current study, ameliorative potential of CAPE in gentamicin induced acute renal degeneration was evaluated by investigating specifically the gene expression of VEGF and kidney tissue through histopathological examination.

Material and Methods**Animals and treatments**

All animal treatments were carried out in compliance with guidelines of the Institutional Animal Ethics Committee and

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approved by the Local Ethics Committee at Inonu University (2012/A-74). Wistar albino rats weighing 200-230 gr at 8-10 week-old were used in this study. Animals were kept at 21°C, with 50%-60% relative humidity and a 12 h/12 h light/dark cycle. The animals were given standard pellet food, and water was provided ad libitum.

Nephrotoxicity was developed by the method of Parlakpınar et al. [11] with minor modifications. Total of 32 rats were equally divided into 4 groups as follows; Control group (C): 2 ml/kg/day dimethyl sulfoxide (DMSO) was given intraperitoneally (ip.) for 10 days; CAPE group (CAPE): 30 mg/kg/day CAPE diluted in DMSO was given ip. for 10 days; CAPE + Gentamicin group (CAPE+G): 30 mg/kg/day [12] CAPE diluted in DMSO was given ip. for 10 days plus 100 mg/kg/day gentamicin was injected ip. 15 min after CAPE treatment, starting at day 3; and Gentamicin group (G): 100 mg/kg/day gentamicin was given ip. for 8 days. At the end of the experimental period, blood was collected and then the rats were sacrificed for collection of kidney tissues.

Serum urea and creatinine measurements

Blood samples were left in the room for 20 minutes and serum was separated by centrifugation at 5000 rpm for 15 minutes. Serum urea nitrogen and creatinine levels were determined using in the Abbott Architects c8000 auto analyser (Abbott Laboratories, Abbott Park, Illinois, U.S.A.) at Inonu University Medical School, Medical Biochemistry Laboratory.

RT-qPCR for VEGF mRNA expression

Kidney samples collected from the groups were minced on ice, placed in RNA saving solution and kept until analysis in -80 °C freezer. RNA was extracted with Pure RNA Tissue kit (Roche, Lot no: 14289400, ref no: 12033674001). cDNA synthesis was performed by Transcriptor First Strand cDNA Synthesis Kit (Roche, Lot no:14797225, Ref no: 04 897 030 001). Real-time PCR was performed in a Light Cycler Instrument (Roche Applied Science) using Start Essential DNA Probes Master Kit (Lot no: 14554900, Ref no: 06402682001)” and “Real Time Ready Assay (*β-Actin* lot no: 90014990, config no: 100072217; *VEGF-A* lot no: 90014991, config no: 100172226) with the primer pairs listed in Table 1. Reaction volumes were set at 10 µl. 5 µl master mix containing 0.5 µl real time ready mix, 2 µl PCR grade water and 2.5 µl cDNA was prepared. Samples were run as triplicate. The cycling protocol was set as the following; an initial 10-min denaturation step at 95°C, followed by 55 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 1s.

To determine the change in *VEGF-A* gene expression among the groups, *β-Actin* gene was selected as housekeeping gene and relative mRNA expression levels were calculated according to housekeeping genes using the $2^{-\Delta\Delta Ct}$ method. PCR products were also run in DNA agarose gels and expected PCR products were obtained as 76 bp and 73 bp for *β-Actin* and *VEGF-A* genes, respectively [13] (Figure 1A, Figure 1B).

Table 1. Primer sequences and expected product size for VEGF-A and β-Actin

Genes	Primer sequences	NCBI Reference Sequence	PCR Product size (bp)
β-Actin	F:5'-CTGGCTCCTAGCACCATGA-3' R:5'-TAGAGCCACCAATCCACACA-3	NM_031144.3	76
VEGF-A	F:5'-AAAAACGAAAGCGCAAGAAA-3' R:5'-TTTCTCCGCTCTGAACAAGG-3'	NM_001110335.1	73

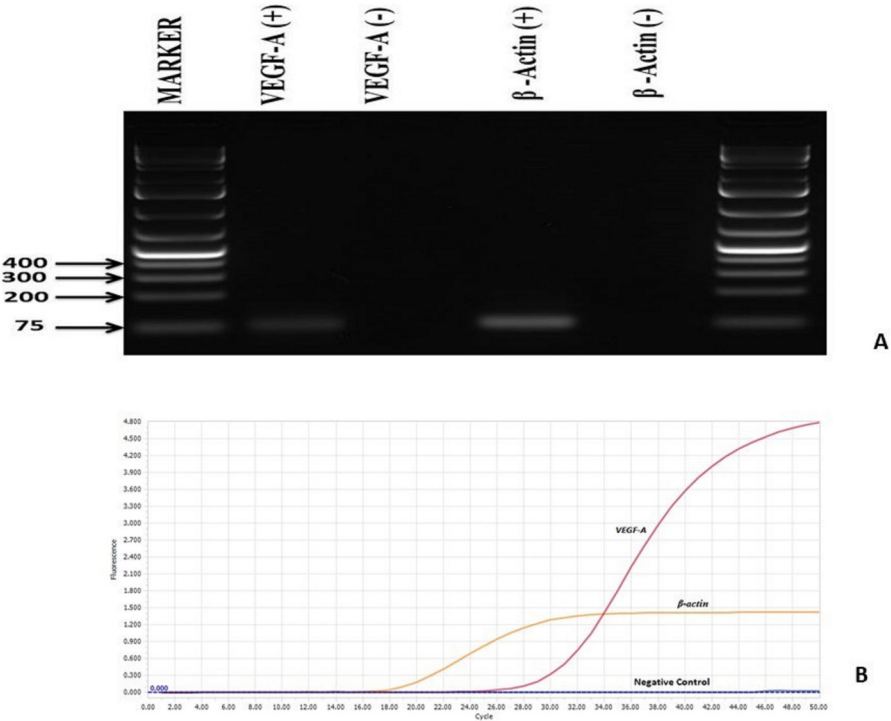


Figure 1. Agarose gel electrophoresis (A) and amplification curves (B) of the RT-qPCR results of VEGF-A and β-actin mRNA. After total mRNA extraction of rat kidney, cDNA was obtained and PCRs were performed with primers for β-actin and VEGF-A (Table 1) and PCR products were run in an agarose gel (2%). Fermentas 75 bp DNA ladder was used as DNA marker

Histopathology

Samples of kidney tissues were fixed in 10% neutral buffered formalin and then embedded in paraffin blocks. Sections cut at 5 μ m thicknesses from the paraffin blocks were routinely processed for hematoxylin and eosin (HE) staining and observed under a light microscope for evaluation of pathological changes.

Statistical Analysis

Statistical analysis of the data was performed by MedCalc® Software (11.4.2.0). The data was presented as media (min-max). Normal distribution of groups was determined by Shapiro-Wilk test. The data for serum creatinine, BUN and tissue VEGF gene expression presented non-normal distribution ($p < 0.05$). Data for creatinine, BUN and VEGF-A gene expression was evaluated by Kruskal-Wallis analysis, and compared among the groups by

Conover test. Differences were considered significant if the p value was less than 0.05.

Results

Biochemical analysis

Biochemical analysis showed that serum BUN level increased significantly upon gentamicin treatment in group G as compared to the others ($p < 0.05$). Serum BUN level in CAPE+G group was not different than group G (Figure 2A).

Serum creatinine level was also significantly higher in group G than those of the others ($p < 0.05$). As compared to group G, the level of serum creatinine in group CAPE+G did not significantly differ ($p > 0.05$) (Figure 2B).

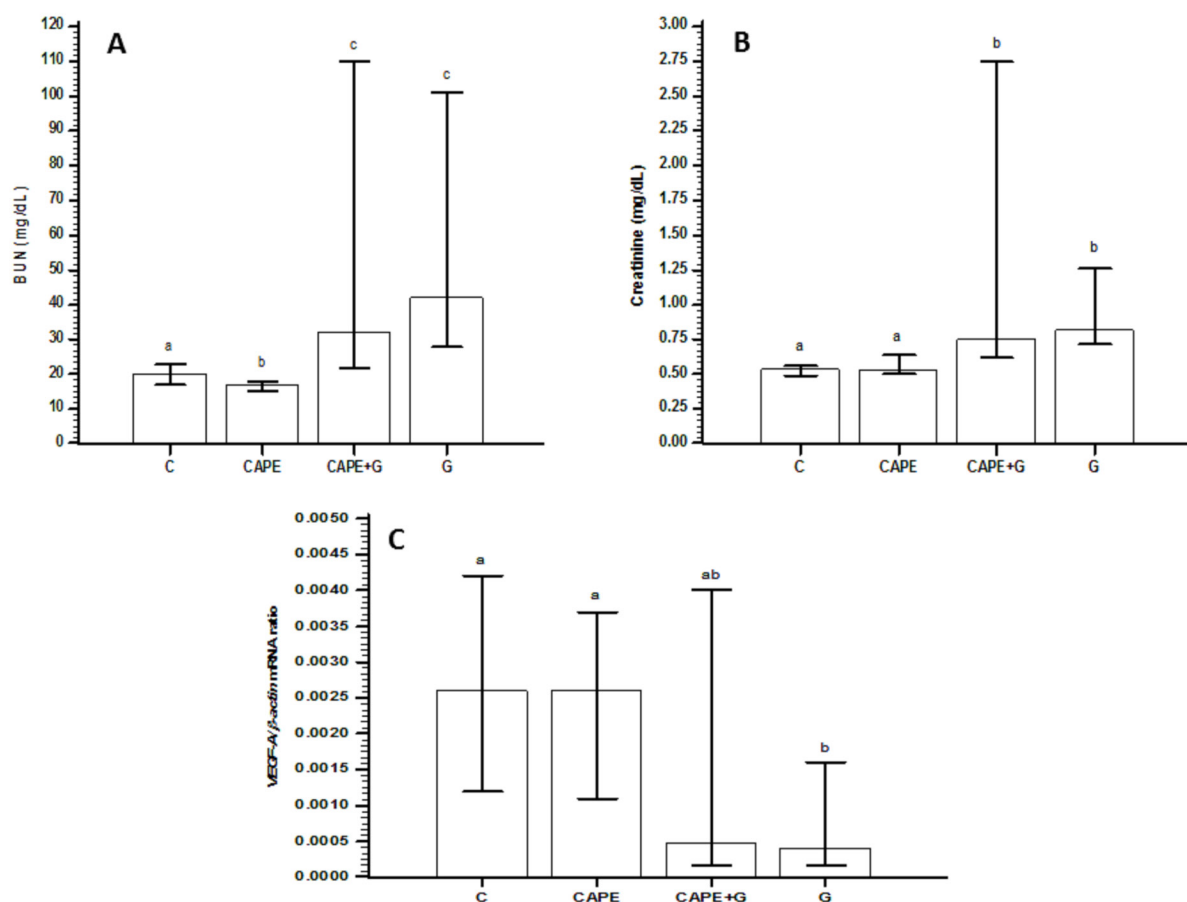


Figure 2. Statistical comparison the levels of serum BUN (A), creatinine (B) and gene expression of VEGF-A (C) among groups; Control (C), Caffeic acid phenethyl ester (CAPE), Caffeic acid phenethyl ester + Gentamicin (CAPE+G), Gentamicin (G). Data was presented as median (min-max). Values marked with different letters differ significantly ($p < 0.05$)

VEGF-A mRNA expression

VEGF/ β -actin mRNA expression levels were determined and compared among the groups and the results were shown in Figure 2C. The expression level of *VEGF-A mRNA* in group G was significantly lower than those of C and CAPE groups ($p < 0.05$). However, there was no statistical difference between group G and group CAPE+G ($p > 0.05$).

Histopathology

In microscopic examination, normal histomorphology of kidney

was observed in C group (Figure 3A). Moderate to severe damage characterized by cellular degeneration and necrosis in proximal tubule epithelia was noted in group G (Figure 3D). Glomerular degeneration was quite evident in some animals. Mononuclear inflammatory cellular infiltration was also noted in some cases. Similar to group C, no pathological changes was observed in tissues of group CAPE (Figure 3B). In CAPE+G group, though individual differences were present, generally, limited number of degenerated tubules with no or little mononuclear cellular infiltration was detected (Figure 3C).

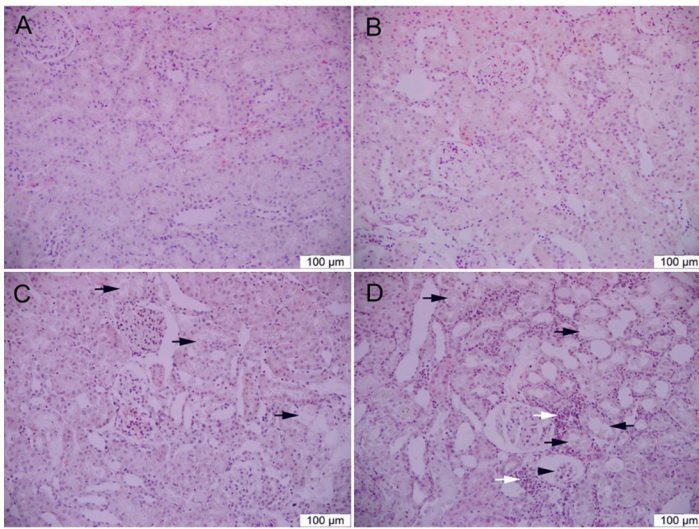


Figure 3. Histopathological evaluation of kidney tissues. A) Normal histomorphology of kidney in control group; B) Normal histomorphology of kidney in CAPE group; C) Moderate renal tubular degeneration in CAPE+G group (black arrows); and D) Severe renal tubular degeneration and necrosis (black arrows), glomerular degeneration (arrowhead) and mononuclear cellular infiltration (white arrows) in group G

Discussion

In the present study, ameliorative effect of CAPE in acute renal toxicity was investigated by evaluating the expression of VEGF-A gene. Gentamicin treatment was shown to decrease the expression of VEGF-A gene as compared to the control group, and the CAPE treatment in gentamicin given mice did not have any increasing effect. Similarly, increased serum creatinine and BUN levels in gentamicin group was not affected significantly by CAPE treatment. In histopathological investigation, severe degeneration detected in kidney tissue of gentamicin given animals was observed to show limited amelioration with CAPE treatment.

VEGF expression was shown to be high in human kidney visceral epithelial cells by immunohistochemistry and in situ hybridizations techniques [14]. VEGF was suggested to involve in regulation of glomerular vascular permeability [15], saving of renal tubular cells [16] and glomerular basement membrane structure [17], calcium homeostasis and podocyte survival [18] in humans. Several roles in normal kidney functioning and presence in many locations suggests that VEGF may involve in regulation of endothelial cells. Many glomerular diseases are characterized by changes in the expression of VEGF, and hence suggested to be related to its expression in the kidney tissue [19].

VEGF gene expression was shown to change depending on the various pathological conditions and disease states. Decreased gene expression was described in psoriasis, highly vascularized tumors, leukemia, endometriosis, and on various steps of reproduction [20]. In kidney biopsy samples collected from patients with minimal change disease, VEGF expression was detected to be lower than normal while no change was reported in the VEGF receptor gene expression levels [21]. In another study conducted with 47 patients having glomerular diseases, VEGF gene expression was shown to decrease in focal and global glomerular sclerosis, amyloidosis, diabetes, crescentic glomerulonephritis, and diffuse endocapillary proliferative glomerulonephritis associated with systemic lupus erythematosus [19].

According to several studies, VEGF gene expression did not decrease after nephrotoxicity. In an experimental gentamicin induced nephrotoxicity model where 80 mg/kg gentamicin was given intramuscularly for 7 days, no changes of VEGF gene expression was observed in rats [22]. On the other hand, increased gene expression was recorded in kidney tissues of streptozotocin given rats [23]. Although a relationship between the increased VEGF gene expression and glomerular degeneration exist, it is not known for sure that this increase is the cause or the result of pathological changes.

It has been suggested that VEGF might act as a protector in some renal degeneration cases [24]. Therefore, we may postulate that VEGF expression increases initially during renal degeneration. However, increased VEGF might further affect on the endothelial cells, which are capable of producing VEGF itself, and hence cause the degeneration and death of these cells. In turn, degenerated endothelial cells could not produce enough VEGF in response [19]. Therefore, the reason of decreased gene expression seen in our study may be explained by the degeneration of cells normally producing VEGF. Increased free radicals, which are the main cause of cellular degeneration, might be involved in the decreased VEGF gene expression. Such a relationship was previously shown in hypertrophied rat heart where free radicals were detected to be increased [25]. Gentamicin might act in a similar way by involving in the production of free radical species [26]. Although the exact mechanism of how gentamicin induced nephrotoxicity develops is not known, free oxygen species were indicated to be involved, and hence cause lipid peroxidation by increasing mitochondrial hydrogen peroxide and nitric oxide [27].

Histopathological changes in gentamicin-induced nephrotoxicity were previously described [27,28]. In these investigations, increased serum creatinine and BUN levels was also reported. These findings comply with the results of the current study. The decrease in VEGF gene expression might be the probable cause of pathologies observed. It has been stated that VEGF might be involved in the glomerular regeneration in mesangio proliferative nephritis, and the decreased VEGF as a result of podocyte degeneration triggers endothelial cell loss and development of glomerulosclerosis [29]. Therefore, it might be speculated that the histopathological changes seen in the present study might be the result of decreased gene expression of VEGF. Supporting this assumption, it has been shown that anti-VEGF treatment causes renal degeneration indicating that VEGF is an important mediator in renal homeostasis [30-32].

In the present investigation, CAPE treatment was shown to have no effect on the VEGF-A gene expression as well as serum creatinine and BUN levels. However, only limited recovery in kidney histology was observed. This histopathological result parallels the findings of Vardi et al.[33] in that necrosis developed during the nephrotoxicity induced by 100 mg/kg gentamicin in rats were ameliorated by CAPE treatment. It has been suggested that this ameliorative effect might be the result of free radical scavenging activity of CAPE. A similar conclusion has also been made by Parlakpinar et al. [11] for CAPE use in gentamicin nephrotoxicity. CAPE induced restorative effects was also described not only histologically but also biochemically in gentamicin and cisplatin toxicities [34].

Conclusion

In conclusion, we have shown that VEGF might be one of the most important mediators in development of nephrotoxicity. Decreased expression of VEGF in kidney tissue might be linked to insufficiencies or irregularities in podocyte survival, glomerular vascular permeability, glomerular membrane structure deformation, and calcium homeostasis, which all help to the development of kidney degeneration.

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Competing interests

The authors declare that they have no competing interest

Financial Disclosure

The financial support for this study was provided by the investigators themselves.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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