Research Article

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KIM-1 and GADDI-153 gene expression in paracetamol-induced acute kidney injury: effects of N-acetylcysteine, N-acetylmethionine, and N-acetylglucosamine

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Abstract

Objectives: Acute kidney injury (AKI) is a critical clinical event characterized by a reduction in the excretory function of the kidneys. N-acetylcysteine (NAC), N-acetylmethionine (NAM) and N-acetylglucosamine (NAG) are antioxidants with scanty known genetic mechanisms. We aimed to assess both kidney injury molecule-1 (KIM-1) and growth-arrested DNA damage-inducible gene-153 (GADD-153) genes expression in paracetamol (PA) induced AKI. Also, to recognize whether NAC, NAM and/or NAG have roles in altering the expression of these genes for ameliorating the AKI induced by PA.

Methods: The present preliminary study achieved the AKI model by oral administration of PA therapeutic dose for 15 days in experimental male rats. Serum urea, creatinine, and renal oxidative stress parameters were analyzed. Genetic expression of KIM-1 and GADD-153 were determined using real time-PCR.

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Results: Significant elevations of serum urea, creatinine and nitric oxide in renal tissue after PA administration; however, total thiol content was reduced. In addition, both KIM-1 and GADD-153 were upregulated. These biochemical alterations were improved after using NAC and partially after NAM; however, NAG had little effect.

Conclusions: Up-regulation of both KIM-1 and GADD-153 occur in AKI induced by PA, which was significantly reversed by NAC.

Keywords: acute kidney injury; growth arrested DNA damage-inducible gene-153; kidney injury molecule-1; N-acetylcysteine; paracetamol.

Introduction

Acute kidney injury (AKI) is a sudden onset of renal damage or dysfunction and it occurs due to either pre-renal, renal, or post renal causes [1].

Paracetamol (PA) is a non-steroidal analgesic and antipyretic drug. In the kidney, PA can be deacetylated and

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Ahmed Alamir Mahmoud Abdallah, Department of Medical Biochemistry, Faculty of Medicine, Sohag University, Sohag, Egypt. https://orcid.org/0000-0002-9111-6812 changed to the nephrotoxic component, p-aminophenol (PAP), which is similar to N-acetyl-p-benzoquinone imine (NAPQI) in its way of excretion; hence in case of reduction of reduced glutathione (GSH), PAP causes cytotoxicity to the kidney [2]. Additionally, AKI was occurred as a consequence of liver damage in PA cumulative dose due to the presence of CYP450 enzyme in the cortex of renal tissue [3].

N-acetylcysteine (NAC), a thiol, is the acetylated compound of the amino acid L-cysteine [4], and it is also a reduced glutathione precursor [5]. N-acetylmethionine (NAM) is an essential amino acid formed as a result of the acetylation process with methionine, and it has two isomers; N-acetyl-L-methionine and N-acetyl-D-methionine [6]. N-acetylglucosamine (NAG), an amino sugar, is a prominent precursor of glycosaminoglycans, proteoglycans and glycoproteins, and it is chiefly obtained as a result of acid hydrolysis of chitin in shrimp shells [7]. Kidney injury molecule-1 (KIM-1) is a type I membrane glycoprotein, consists of an extracellular and a cytoplasmic compartment, and it has a vital role as an indicator of AKI [8]. Growth arrested DNA damage-inducible gene-153 (GADD-153) is one of the proteins that are affected by endoplasmic reticulum stress that is produced from AKI [9].

Previously published work was performed on the antioxidant activity of NAC, NAM, and NAG for reversing PA toxicity in liver and was attributed to their abilities to regulate the intracellular level of GSH [10]. In light of these information, the current study aimed to explore the expression profiles of both kidney injury molecule-1 (KIM-1) and growth-arrested DNA damage inducible gene-153 (GADDI-153) genes in AKI and compared the efficacy of the three antioxidant drugs (NAC, NAM or NAG), on PA therapeutic dose-induced AKI to provide the possible genetic mechanisms of action of such drugs.

Materials and methods

Chemicals, reagents, and kits

PA, NAC, NAM and NAG were purchased from Sigma-Aldrich (USA) and their catalog numbers were (103-90-2), (616-91-1), (65-82-7) and (7512-17-6) respectively. On the other hand, dimethylsulphoxide (DMSO), sodium carbonate, sodium hydroxide, copper sulphate, folin-Ciocateau phenol reagent, albumin, sulphanilamide, N-(1naphthyle) ethylene di-amine dihydrochloride, phosphoric acid, sodium nitrite, 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma-Aldrich (USA). However, disodium hydrogen phosphate and sodium dihydrogen phosphate were bought from (LOBA-Chemie). Kits of urea, creatinine, and total protein (TP) were supplied from Lab. Essentials Inc.

Further, Direct-zol $^{\text{TM}}$ RNA Micro Prep (R2063) was obtained from Zymo research. While cDNA Reverse Transcription Kit (4374967) and

rattus norvegicus primers of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), KIM-1 and GADD-153 were supplied from Thermo Fisher Scientific (USA). Further, isoflurane was obtained from the pharmacy.

Animals and treatments

Fifty male albino *Wistar* rats of Bwt of 120–140 g and 8–10 weeks old age were got from the animal house in Faculty of Medicine, Assiut University, Assiut; Egypt. Rats were housed one week for acclimatization in polycarbonate cages in controlled environmental conditions of temperature $(23 \pm 2\,^{\circ}\text{C})$, relative humidity (20%), and $12\,\text{h}/12\,\text{h}\,\text{light}/\text{dark}$ cycle. Food and water were provided *ad-libitum*. The protocol was approved according to the Ethics Committee on Animal Experimentation of Assiut University, Faculty of Veterinary Medicine, and the Guide for the Care and Use of Laboratory Animals (National Institute of Health publication No. 8023, revised 1978). The experimental period was conducted for 15 days. Rats were randomly divided into five groups of 10 animals in each group that were categorized as follows:

- Group 1: Control vehicle (DMSO): Rats were orally administrated
 1% of DMSO for 15 days.
- Group 2: AKI-induced group (AKI): Rats have orally administrated PA at dose 42.84 mg/kg body weight (Bwt) dissolved in DMSO 1% for 15 days.
- Group 3: AKI-treated NAC group (AKI-NAC): Rats were orally administrated both PA at dose 42.84 mg/kg Bwt and NAC at dose 15.38 mg/kg Bwt and both were dissolved in DMSO 1% for 15 days.
- Group 4: AKI-treated NAM group (AKI-NAM): Rats were orally administrated both PA at dose 42.84 mg/kg Bwt and NAM at dose 18.07 mg/kg Bwt and both were dissolved in DMSO 1% for 15 days.
- Group 5: AKI-treated NAG group (AKI-NAG): Rats were orally administrated both PA at dose 42.84 mg/kg Bwt and NAG at dose 20.9 mg/kg Bwt and both were dissolved in DMSO 1% for 15 days.

The PA dose in the present study was selected according to El-Boshy, BaSalamah [11] who exhibited that 4 gm/day was the maximum safe dose. However, NAC, NAM and NAG doses were calculated according to [12].

Sample preparation

Rats' scarification and blood and tissue sampling were accomplished via inhalation of 1% isoflurane, and all efforts were done to decrease the animal suffering [13]. After 15 days from starting the experiment, rats were overnight fasted for 8 h, blood samples were gathered in Wisterman tubes, centrifuged at 4,000 rpm for 10 min using table centrifuge (2002, Germany) and the obtained sera were kept at -80 °C until analyzed. Renal tissues from sacrificed rats were directly removed, washed with ice-cold saline, and divided into three parts. One part was used for homogenization, second part was preserved in liquid nitrogen and stored at -80 °C for molecular study and the third part was conserved in 10% neutral buffered formalin for histological investigations. Homogenization of renal tissue was performed in 0.1 M phosphate buffer saline (PBS), pH 7.4, using a homogenizer (099C K4424, USA). After that, the homogenates were centrifuged at 6,000 rpm for 10 min. At 4 °C via cooling centrifuge (Mikro 220R, Germany) and finally, supernatants were collected and kept at -80 °C until analysis time.

Kidney function tests assay: Serum urea and creatinine concentrations were determined using colorimetric commercial assay kits supplied by spectrum diagnostic, Egypt, and the data were expressed as mg/dL.

Determination of TP concentration in renal homogenate: TP content in renal tissue was estimated by Lowery technique which based on reaction of phenolic group of aromatic amino acid such as tyrosine and tryptophan with Folin-Ciocalteau reagent at alkaline media producing a blue purple color at 750 nm and results were expressed as g/total weight renal tissue [14].

Determination of oxidative stress parameters in renal tissue

Determination of nitric oxide (NO) concentration: NO concentration was assayed according to Paya, Maupoil [15]. Briefly different concentrations of sodium nitrite standard (100, 50, 25, 10 and 5 mg/dL) were prepared, then using a 96-well ELISA plate, 100 μL of each standard concentration and renal tissue homogenate were added in standard and sample wells, respectively to 100 µL of Griess reagent (1% sulphanilamide and 0.1% N-(1naphthyle) ethylene di-amine dihydrochloride in 2.5% phosphoric acid). Plate content was incubated at room temperature for 10 min. Then the absorbance of the produced reddish-purple compound was read via ELISA reader (HEEPF D-080-HO, Biotec, USA) at 550 nm. The concentrations of samples were calculated from the standard curve and expressed as nM/mg renal tissue protein.

Determination of total thiol content (THC): THC was estimated as designated by Ellman [16] technique with slight modification where $25~\mu L$ of renal tissue homogenate (sample) and water (blank) were added to 25 μL of phosphate buffer, 0.1 M, pH 8.0 in ELISA plate. Gentle shaking of the plate was done and 250 μL of DTNB 0.4% was added in wells containing samples only. After 1 h of incubation of the plate at room temperature, the absorbance of yielded color was measured using ELISA reader at 410 nm. THC in the samples was calculated by subtracting the absorbance of sample from sample blank and the result divided by the molar extinction coefficient of 13,600 M⁻¹ cm⁻¹, and the final result was expressed as nM/mg tissue protein.

Quantitative real-time PCR (qRT-PCR): qRT-PCR was performed according to Varma, Field [17] with slight modifications. Firstly, the total RNA of renal tissue samples was isolated using Direct-zol[™] RNA MicroPrepin in accordance with the manufacturer's instructions and was converted to cDNA. After that, qRT-PCR was done on 7,500 fast real-time PCR (Applied Biosystems, CA, USA) under the following conditions, hot start step at 95 $^{\circ}$ C for 10 s, initial denaturation for 25 s at 95 °C, annealing and extension for 70 s at 60 °C for 40 cycles. The

Table 1: Primer sequences used for gRT-PCR in the study.

	Primer sequence (5'3')
GAPDH	F- ATCACCATCTTCCAGGAGCG
	R- GATGGCATGGACTGTGGTCA
KIM-1	F- GGTCACCCTGTCACAATTCC
	R- CTCGGCAACAATACAGACCA
GADDI-153	F- TCTGCCTTTCGCCTTTGAG
	R- GCTTTGGGAGGTGCTTGTG

relative expression levels were calculated using the equation of fold change=2^-\Deltact method. Target genes expression levels were normalized to the housekeeping gene, GAPDH, and all PCR primers were presented in Table 1.

Histological study: All histological procedures were performed in Histology Department, Faculty of Medicine, Assiut University, Egypt, according to Bancroft and Gamble [18] method. In brief, after rat's scarified part of renal tissue was taken directly from all groups and bathed with saline and small specimens were finally fixed in 10% buffered formalin solution for two days at the room temperature, then, processed to perform paraffin sections. Several sections of 7 µm diameter were made and stained by hematoxylin and eosin (H&E) stain to detect any abnormalities.

Statistical analysis

Statistical analysis was done to detect a significant difference (p<0.05) in obtained results. Statistical data analysis was performed using GraphPad Prism version 7.0 b software (Graph Pad Software Inc., San Diego, CA, USA). All notified values were displayed as mean \pm standard error (SE). Statistical significance was made out by one-way analysis of variance, followed by Tukey's multiple comparison tests.

Results

Serum urea and creatinine concentrations in AKI group compared to AKI-NAC, AKI-NAM and AKI-NAG groups

Experimental AKI rats showed an increase of serum urea and creatinine by (86.4 and 30.2%, respectively) compared to DMSO group. On the other hand, using NAC, NAM and NAG for 15 days after AKI induction caused reduction of serum urea by 16.0% in AKI-NAC, 17.9% in AKI-NAM and 12.7% AKI-NAG groups. Also, serum creatinine was decreased by (17.9, 18.3, and 21.8%) in AKI-NAC, AKI-NAM and AKI-NAG, respectively in comparison to AKI (Figure 1).

Variations of renal TP, NO, and THC in AKI group compared to AKI-NAC, AKI-NAM, and AKI-NAG groups

Data in Figure 2 exhibited a reduction of renal TP concentration by 7.8% in AKI compared to DMSO, and such reduction was improved in AKI-NAC, AKI-NAM, and AKI-NAG groups by (8.4, 7.2, and 6.7%, respectively). Further, results in Figure 2 exhibited an elevation of NO concentration in AKI (276.9 ± 1.9) in comparison to DMSO (245.4 ± 0.5) and an oral administration of NAC, AAM, and

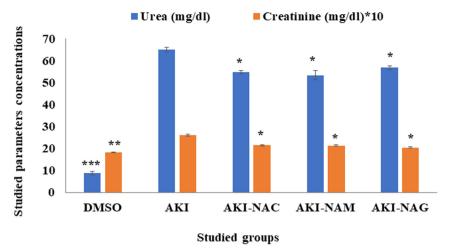


Figure 1: Serum urea and creatinine concentrations of DMSO, AKI, AKI-NAC, AKI-NAM and AKI-NAG groups.

Values were expressed as mean ± SE (n=10). Significance: ***p<0.001, **p<0.01, *p<0.05 comparing to AKI.

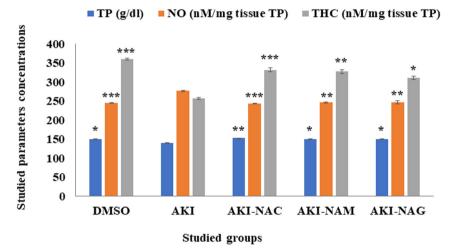


Figure 2: TP and NO concentrations and THC in renal tissue of DMSO, AKI, AKI-NAC, AKI-NAM and AKI-NAG groups. Values were expressed as mean \pm SE (n=10). Significance: ***p<0.001, **p<0.01, *p<0.05 comparing to AKI.

NAG for 15 days led to decline the NO to (243.7 ± 0.4) , (246.4 ± 1.1) and (247.2 ± 1.7) , respectively. On the other hand, THC showed diminishing in the case of AKI in comparison to DMSO by 40.4%, and such was restored by (22.7, 21.6, and 17.4%) in AKI-NAC, AKI-NAM and AKI-NAG, respectively.

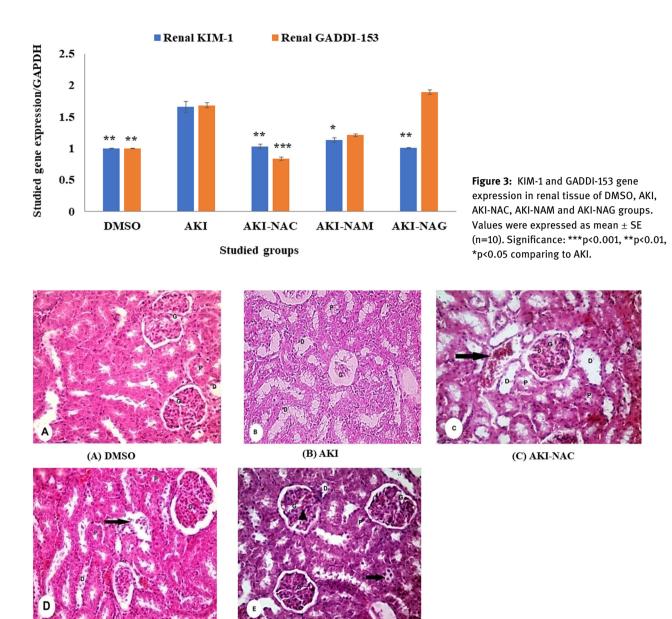
Renal KIM-1 and GADDI-153 gene expression in AKI group compared to AKI-NAC, AKI-NAM and AKI-NAG groups

Gene expression of both KIM-1 and GADDI-153 revealed upregulation in AKI by (39.8 and 40.5%) in AKI more than DMSO. However, KIM-1 was down-regulated in AKI-NAC, AKI-NAM and AKI-NAG by (61.2, 46.9 and 64.4%, respectively), but GADDI-153 expression was reduced only in case of using of NAC and NAM nearly by (1.0 and 0.4-folds, respectively) (Figure 3).

Histological variations of renal tissue in AKI group compared to AKI-NAC, AKI-NAM and AKI-NAG groups

The results in Figure 4A showed normal renal cortex that was characterized by the presence of renal corpuscles, proximal convoluted tubules (PCT), and distal convoluted tubules (DCT). PCT was lined by a high cuboidal epithelium with highly acidophilic cytoplasm and a narrow lumen. DCT was lined by cuboidal cells with less acidophilic cytoplasm and wider lumen than PCT. Also, each renal corpuscle was formed from a glomerular capillary tuft surrounded by capsular space and Bowman's capsule.

On the other side, data of AKI showed severe damage in the renal tissue that involved structural alterations of the renal corpuscle with PCT and DCT and some renal corpuscles showed atrophic glomeruli. Additionally, various renal tubules were noticed with vacuolated cytoplasm with



(E) AKI-NAG

Figure 4: Photomicrographs of renal tissue in different rat's groups stained with H&E. (A) DMSO, (B) AKI, (C) AKI-NAC, (D) AKI-NAM and (E) AKI-NAG (X=400).

loss of integrity of their brush border and loss of basal striations (Figure 4B).

(D) AKI-NAM

After NAC administration, the histological examination of renal tissue showed nearly normal glomeruli with mild tubular degeneration, and also congestion was observed in some blood vessels (Figure 4C). Further, NAM caused normal glomeruli, but the congestion of blood vessels and extravasation of blood was markedly observed (Figure 4C). Finally, NAG showed normal glomeruli except for congestion of glomerular capillaries. The kidney tubules appeared nearly normal. Focal cellular infiltration in

between the tubules was observed (Figure 4D). Notably, all the histological results exposed the alterations in the renal cortex, while the renal medulla was not affected.

Discussion

The current study was noted that PA administration at its therapeutic dose for 15 day caused AKI, which was explained by its cumulative adverse effect. Our results exhibited an elevation of urea and creatinine in the AKI

group, which may be attributed to a lower rate of their excretion outside the body as a consequence of glomerular atrophy, and this was in accordance with Eraky and Abo El-Magd [19]. Moreover, the data in this study revealed an elevation of NO and reduction of THC in the AKI group, which indicate that oxidative stress plays an essential role in renal toxicity induced by PA pathway where NAPQI accumulated and reacted with cellular proteins producing ROS and life-threatening free radicals, which hinders the endogenous antioxidant enzymes that responsible for NAPQI scavenging [20].

In the present study, overexpression of KIM-1 was a consequence of AKI. The proposed mechanism was correlated to the chemical structure of KIM-1, where following AKI, the extracellular part of KIM-1 shed into extracellular space of renal tubules, and hence it released in urine in excess amount compared to the normal condition [21], and such pathway is regulated via mitogen-activated protein kinase (MAPK) pathway. Another hypothesis illustrated the reason of renal tissue KIM-1 upregulation was attributed to extracellular regulated kinase 1/2 (ERK11/2) and signal transducer and activator of transcription 3 (STAT3) phosphorylated pathway where STAT3 bounded to the promotor of KIM-1 and increased its expression at mRNA and protein levels [22]. Our findings were in line with Colombo, Looker [23], who reported a higher concentration of serum KIM-1 in patients with AKI.

Overexpression of GADD-153 during AKI is related to some theories, one of them based on autophosphorylation of protein kinase RNA-like ER kinase (PERK), which phosphorylates eukaryotic translation initiation factor-2α (EIF-2α) and finally activates transcription factor 4 (ATF4) [24]. Further ROS upregulates the GADD-153 through the activator protein-1 (AP-1) [25] and MAPKs signaling pathways [26]. Similar to our results, Dai, Liu [27] elucidated the role of ROS in enhancing the GADD-153 expression at the mRNA level.

NAC is considered one of the accepted antidotes for reducing the adverse effect of PA-induced AKI via its capability for NAPQI detoxification that is produced from PA metabolism [28]. Such detoxified role of NAC was credited to its antioxidant and anti-inflammatory effects [29]. On the same hand, its antioxidant efficacy was mainly related to i: biochemical conversion of NAC to L-cysteine, one of sulfur-containing amino acid, and ii: biosynthesis of non-enzymatic antioxidant GSH, which conjugated with NAPQI [30]. Additionally, The current results revealed a lower level of NO and elevation of THC in AKI-NAC group, and this attributed to the presence of the thiol group in NAC that scavenges the produced ROS. Moreover, a direct antioxidant effect of NAC was reported through its

interaction with ROS, such as hydrogen peroxide, hydroxyl radicals, superoxide anions, and hypochlorous acid produced from renal tissue damage induced by PA [31].

Down-regulation of KIM-1 and GADD-153 in our study was achieved through NAC administration and that attributed to its antioxidant and anti-inflammatory effect. It was reported that NAC possessed anti-inflammatory strength via several pathways. It inhibits the initiation of MAPK, AP-1 and nuclear factor kappa-B transcription factor [32] and adjusts the redox status via ERK- MAPK signaling pathway [33]. Additionally, NAC reduces the mitochondrial membrane permeability, which is the main source of ROS [34] and inhibits renal apoptosis [35]. The results demonstrated that NAC role in down-regulation of GADD-153 expression was related to elevation of GSH content inside the mitochondria, which reduces the ER stress [36]. Of interest, in the hippocampus of rats subjected to stress, NAC corrects the elevated expression of GADD-153 that was an indicator of ER stress restoration [37].

The second therapy was used in our study against AKI was NAM. NAM is an acetylated form of methionine that is a crucial methyl donor amino acid and acts as a scavenger for multiple oxidizing fragments and ROS [38]. The current study showed a reduction of NO concentration and promotion of THC after oral administration of NAM in AKI induced by PA and this may be related to the protective function of NAM via its role in the biosynthesis of i: S-adenosylmethionine which is another source of methyl group [39] and ii: cysteine which is rate-limiting precursor of GSH to compensate its depletion during AKI [40]. Our findings were in agreement with Lertratanangkoon, Scimeca [41] in hamsters where NAM achieved protection against bromobenzene toxicity via its ability to raise the GSH level. Regarding the ameliorative effect of NAM to downregulate both KIM-1 and GADD-153 expressions may be explained by its antioxidant activity, but till now, no previous studies could be traced in literature illustrating the reasons, and our study is the first research in this point. Hence further studies and molecular analysis of various possible pathways are recommended to give a full explanation.

On the other hand, NAG in current data showed less improvement of NO level and THC in comparison to NAC and NAM. Also, NAG was significant only in the regulation of KIM-1 and non-significant to GADD-153 when compared to AKI.

Conclusions

Therapeutic dose of paracetamol for 15-days leads to acute kidney injury and such injury was ameliorated via N-acetylcysteine possibly through down regulation of both KIM-1 and GADD-153 gene expressions in the kidney.

Study's limitations

The present research could be considered as a preliminary study due to the lack of confirmation of the results with corresponding protein expressions assays due to limited funding resources which could be performed in future researches.

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Author contributions: Study concept and design: MHH, THS, NAM and ASH; Experimental procedures: NAM, MHH, ME-Z, EAA, ASH and NAMM; Blood samples collection, biochemical and genetic assays: NAM, MHH, HFH, ME-Z, ASH and AAMA; Histological examinations: EAA and NAMM; Statistical analysis: NAM, MHH, HFH, ME-Z, ASH, SAM and AAMA; literature search: NAM, MHH, HFH, ME-Z, SAM, EAA, ASH and AAMA; First manuscript draft: MHH, AAMA and ME-Z. NAM and MHH contributed equally in this research. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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