Original article

ACUTE PHASE RESPONSE AND OXIDATIVE STRESS PARAMETERS IN PNEUMONIC CAMEL CALVES (CAMELUS DROMEDARIUS)

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Summary

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Pneumonia is an emerging problem in dromedary camels, while no data concerning the diagnostic importance of acute phase proteins and oxidative stress markers in pneumonic camel calves are available. Twenty five spontaneously pneumonic camel calves were used in this study. In addition, 25 healthy camel calves were used as control group. Blood samples were collected from both groups for determination of blood picture and analysis of lipoprotein profile, fibrinogen (Fb), haptoglobin (Hp), serum amyloid A (SAA), tumor necrosis factor-alpha (TNF-α), interleukins (IL-6, IL-1α, IL1β, IL-10), interferon-gamma (IFN-γ) and malondialdehyde (MDA). Erythrocytic activity of superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT) were determined in erythrocyte haemolysate obtained immediately after sampling from anticoagulated blood. The results revealed significant elevations in total leukocytic and neutrophil counts in pneumonic camel calves compared to control values. The concentrations of Hp, SAA, Fb, IL-6, IL-1α, IL1β, IL-10, TNF-α and IFN-γ were significantly higher (1.6±0.1 g/L, 26.4±3.23 mg/L, 5.1±1.30 g/L, 19.7±1.6 pg/mL, 17.1±3.1 pg/mL, 36.0±1.5 pg/mL, 14.3±0.5 pg/mL, 23.3±1.7 ng/ml, 29.5±2.2 pg/mL respectively) in pneumonic camel calves than in controls. The study showed a significant increase in MDA (21.36±1.12 nmol/g protein) with significantly lower levels of erythrocytic GSH, SOD and CAT (4.42±0.65 mmol/g Hb, 3.1±0.22 U/mg Hb and 10.4±1.3 U/mg Hb, respectively) in the pneumonic camel calves vs healthy controls. The present study concluded that lipid profile, APP, pro-inflammatory cytokines and oxidative stress indices could serve as diagnostic parameters for pneumonia in camel calves.

Key words: camel calves, haptoglobin, interleukins, oxidative stress, pneumonia, serum amyloid A

INTRODUCTION

Pulmonary diseases are among the emerging problems of camels that are causing considerable losses in production and death (Kane *et al.*, 2005).

The lung is a multifunctional organ, which has a role in the exchange of gases while at the same time the mucosal surface interacts with external oxidants. Oxidative stress is an active field of research in veterinary medicine implicated in numerous disease processes including sepsis, mastitis, enteritis, pneumonia, and respiratory & joint diseases (Lykkesfeldt & Svendsen, 2007).

Acute phase proteins (AAPs) are positive (up-regulated; Hp, Fb and SAA) or negative (down-regulated; albumin, transferring and α-fetoproteins) depending on the response to the challenge. This response is accompanied by alterations in lipid metabolism in the form of higher serum triglycerides and lower high-density lipoprotein levels (Cabana *et al.*, 1989). Neutrophils in lungs respond to acute inflammation with a series of reactions ended by pathogen destruction (Soethout *et al.*, 2002). These reactions involve phagocytising pathogens, increasing antibodies, complement fixation and finally stimulate the production of reactive oxygen and nitrogen species (Segal, 2005). Moreover, monocytes and macrophages produce pro-inflammatory cytokines (TNF-α, IL1β, IL-12 and IFN-γ), mediating the effect of APPs, favouring T-helper cell differentiation that construct a bridge between innate resistance and adaptive immunity (Trinchieri, 2003).

In spite of the emerging interest in using APPs as health indicators, limited studies exist with respect to their use in dromedary camels. Also, only few conditions in camel medicine have been investigated in regard to the effects of oxidative stress.

The aim of the present study was to investigate the alterations in lipoprotein profile, acute phase response, pro-inflammatory, anti-inflammatory cytokines and oxidative stress parameters in pneumonic camel calves.

MATERIALS AND METHODS

Animals

Twenty-five camel calves (2–6 months of age, 18 male and 7 female) were referred to the Veterinary Teaching Hospital, King Faisal University, presenting clinical signs of pneumonia including fever (39.9 ± 0.52 ^{o}C), dyspnea (n=25), mouth breathing, nasal discharge (n=25), coughing (n=25), depression (n=25), weight loss, increased respiratory rate (21.4 ± 3.2 min^{-1}) and crackles sound (n=25) detected on lung auscultation. In addition, another 25 healthy camel calves (15 male and 10 female) of similar age used as a control group. They were admitted to the Teaching Hospital for periodical check and were active and apparently strong. The selected control calves were free from any signs of respiratory disorders or other health problems. All calves were clinically examined.

Sample collection

Two separate blood samples were collected from pneumonic and healthy camel calves. One sample was taken in Eppendorf tube anticoagulated with EDTA for haematological studies. The second blood sample was taken in plain tubes, centrifuged at 3000 rpm for 10 min and the clear serum was separated carefully and stored in Eppendorf tubes at –20 °C until serum biochemistry analysis. Moreover, nasal swabs, bronchoalveolar lavage were collected from pneumonic camel calves, and subjected to bacteriological examinations. In addition, heart blood and lung samples were collected from necropsied calves (4/25) and subjected to bacteriological examinations.

Cultural and biochemical identification of different pathogens

The different samples were subjected to microbiological culture on defibrinated blood agar (5%), MacConkey agar and Sabouraud agar, and incubated under aerobiosis at 37 $^{\circ}$C for 72 h. The same material was also incubated on blood agar (5%) under anaerobic conditions at 37 $^{\circ}$C for 120 h. The isolated microorganisms identified using VITEK2 Compact, Bio-mérieux, France.

Blood analysis

Haematological profile. Haematological examination was carried out using automatic cell counter (VetScan HM5 Hematology system, ABAXIS).

Blood biochemistry parameters. The levels of total protein, albumin, triglycerides, cholesterol, high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined in serum samples on a Beckman CX-7 autoanalyser using commercial kits (Sigma Chemical Co. Ltd., Poole, Dorset,UK).

Acute phase proteins, pro-inflammatory and anti-inflammatory cytokines. Serum Hp was determined using the haemoglobin-binding assay described by Makimura & Suzuki (1982). SAA was measured by a solid-phase sandwich ELISA with a commercially available ELISA kit (Phase SAA kit, Tridelta Ltd., Ireland), according to the manufacturer's instructions. IFN-γ, IL-6, IL-1α, IL-1β, IL-10 and TNF-α, levels were determined from undiluted serum samples using commercially available ELISA Kits (Biosource, Diagnostic Corporation, USA). The plates were read at 450 nm on a computerised automated microplate ELISA reader (Bio TEC, ELX800G, USA).

Oxidative stress parameters. Glutathione (GSH) concentration in the RBC haemolysate was measured using the method of Beutler *et al.* (1963) based on the development of a stable yellow colour when 2-nitrobenzoic acid is added to sulfhydryl compounds. The amount of reduced product, thionitrobenzene, was measured at 412 nm and expressed as mmol/g Hb. Superoxide dismutase (SOD) activity was estimated in the RBC haemolysate according to the method of Misra & Fridovich (1972) based on the ability of SOD to inhibit the autoxidation of epinephrine to adrenochrome in an alkaline medium (pH 10.2). The optical density was measured at 480 nm and expressed as U/mg Hb. Catalase (CAT) activity was measured in RBC haemolysate by the method of Beers & Sizer (1952). Decomposition of H_2O_2 was followed directly by decrease in absorbance at 240 nm, and the difference in absorbance per min/mg Hb was taken as a measure of the CAT activity.

Lipid peroxidation in serum was estimated as thiobarbituric acid reactive substances (TBARS) according to Placer *et al.* (1966). The method is based on formation of a colour complex between lipid peroxidation products and thiobarbituric acid (TBA). Briefly, 0.2 mL of serum was added to 1.3 mL of 0.2 mol/L Tris, 0.16 mol/L KCl buffer (pH 7.4). TBA (1.5 mL) was added and the mixture was heated in a boiling water bath for 10 min. After cooling, 3 mL of pyridine–butanol (3:1, v/v) and 1 mL of 1 mol/L NaOH were added. The absorbance was read at 548 nm against bi-distilled water as a blank. In this assay, 1,1,3,3-tetramethoxypropane was used as standard. Lipid peroxidation in serum was expressed as nmol serum malondialdehyde (sMDA)/g serum protein.

Statistical analysis

All data presented as means ± standard error of the means (SEM). The statistical significance between means was compared using Student's *t*-test; P≤0.05 was considered significant. All tests were performed using the computer package of the statistical analysis system (SAS, institute, Inc, 2002).

RESULTS

The bacteriological examination of pneumonic camel calves showed that the isolated microorganisms were *Staphylococcus* spp. (13/25), *Corynebacterium* spp. (7/25), *Streptococcus* spp., (2/25) *Pasteurella* spp. (1/25), *Klebsiella* spp. (1/25) and *Pseudomonas* (1/25). The mixed infection included *Staphylococcus* spp. *& Corynebacterium* spp. (4/25), *Staphylococcus* spp. *& Streptococcus* spp. (3/25), *Pasteurella* spp. *& Staphylococcus* spp. (1/25) and *Staphylococcus* spp. *& Klebsiella* spp. (1/25).

The haematological analysis of selected parameters demonstrated a significant elevation in total leukocyte and neutrophil counts in pneumonic camel calves compared to controls (Table 1).

The examined biochemical parameters were significantly (P≤0.05) increased in pneumonic camel calves except for total protein, albumin, albumin-globulin ratio, cholesterol and high density lipoprotein cholesterol (HDL-C) which were significantly (P≤0.05) lower compared to controls (Table 2).

Concerning the oxidative stress markers, the present study showed a significant (P≤0.05) increase in the levels of MDA with considerable (P≤0.05) decrease in the levels of erthrocytic GSH, SOD and CAT in the pneumonic camel calves vs healthy controls (Table 3). Serum concentrations of investigated APP and cytokines were significantly (P≤0.05) higher in pneumonic camel-calves (Tables 4 and 5).

There was a significant positive correlation between HP, SAA, fibrinogen,

Table 1. Haematological parameters in control (n=25) and pneumonic camel-calves (n=25). Data are presented as means ± SEM

Variables	Control camel calves	Pneumonic camel calves
RBCs (T/L)	10.64 ± 0.50	10.78 ± 0.44
Hb (g/L)	156.70 ± 15.20	154.70 ± 18.90
PCV (L/L)	0.29 ± 0.01	0.29 ± 0.01
MCV (fL)	24.52 ± 1.32	24.22 ± 1.42
MCHC (g/L)	564.20 ± 8.40	558.2 ± 9.40
WBCs (G/L)	15.43 ± 1.85	22.40 ± 1.40*
Neutrophils (G/L)	9.60 ± 2.56	18.60 ± 3.40*
Lymphocytes (G/L)	5.75 ± 2.50	5.66 ± 1.92
Monocytes (G/L)	0.02 ± 0.04	0.02 ± 0.03
Basophils (G/L)	0.18 ± 0.02	0.20 ± 0.03
Eosinophils (G/L)	0.24 ± 0.01	0.25 ± 0.01

RBC=red blood cell; Hb=haemoglobin; PCV=packed cell volume; MCV=mean corpuscular volume; MCHC=mean corpuscular haemoglobin concentration; WBC=white blood cell; * statistically significant difference between groups at P≤0.05.

Table 2. Protein, lipid and enzyme profiles in control (n=25) and pneumonic camel-calves (n=25). Data are presented as means ± SEM

Variables	Control camel calves	Pneumonic camel calves
Aspartate aminotransferase (U/L)	47.32 ± 1.22	111.32 ± 5.63*
Alanine aminotransferase (U/L)	16.56 ± 1.83	88.32 ± 4.32*
Alkaline phosphatase (U/L)	38.63 ± 1.56	90.32 ± 6.3*
HDL-cholesterol (mmol/L)	0.52 ± 0.02	0.24 ± 0.01*
LDL-cholesterol (mmol/L)	0.15 ± 0.01	0.28 ± 0.03*
Cholesterol (mmol/L)	0.86 ± 0.12	0.33 ± 0.03 *
Triglycerides (mmol/L)	0.47 ± 0.13	0.61 ± 0.21*
Total protein (g/L)	64.30 ± 5.30	56.20 ± 3.20*

* statistically significant difference between groups at P≤0.05.

Table 3. The levels of oxidative stress biomarkers and lipid peroxidation in control (n=25), and pneumonic camel-calves (n=25). Data are presented as means ± SEM

Variables	Control camel calves	Pneumonic camel calves
Erythrocytic SOD (U/mg Hb)	4.72 ± 0.42	3.10 ± 0.22*
Erythrocytic GSH (mmol/g Hb)	7.15 ± 0.33	4.42 ± 0.65*
Erythrocytic catalase (U/mg Hb)	15.70 ± 0.40	10.40 ± 1.30*
Serum MDA (nmol/g protein)	12.36 ± 0.85	21.36 ± 1.12*

SOD=superoxide dismutase; GSH=reduced glutathione; MDA=malondialdehyde; * statistically significant difference between groups at P≤0.05.

Table 4. Acute phase proteins (APPs) in control (n=25) and pneumonic camel calves (n=25). Data are presented as means ± SEM

Variables	Control camel calves	Pneumonic camel calves
Positive APPs		
Fibrinogen (g/L)	3.3 ± 0.30	5.1 ± 1.30*
Serum amyloid A (mg/L)	9.5 ± 0.40	26.4 ± 3.23*
Haptoglobin (g/L)	0.3 ± 0.03	1.6 ± 0.1*
Negative APPs		
Albumin (g/L)	34.5 ± 1.3	21.1 ± 1.2*
Globulins (g/L)	22.3 ± 2.3	44.1 ± 3.3*
Albumin/globulin ratio	1.62 ± 0.22	0.46 ± 0.12*

* statistically significant difference between groups at P≤0.05.

leukocyte and neurtophil counts in camel calves under investigation as shown in Table 6.

DISCUSSION

The present investigation is considered as one of the first in the field on camel calves' bronchopneumonia by using alte-

Table 5. The examined pro-inflammatory and anti-inflammatory cytokines in healthy (n=25), and *pneumonic* camel-calves (n=25). Data are presented as means ± SEM

Variables	Control camel calves	Pneumonic camel calves
Pro-inflammatory cytokines		
Interleukin-1α (pg/mL)	9.3 ± 0.3	17.1 ± 3.1*
Interleukin-1β (pg/mL)	22.1 ± 0.9	36.0 ± 1.5*
Tumour necrosis factor-α (ng/mL)	15.3 ± 0.5	23.3 ± 1.7*
Interferon-γ (pg/mL)	19.0 ± 0.5	29.5 ± 2.2*
Anti-inflammatory cytokines		
Interleukin-10 (pg/mL)	10.2 ± 0.4	14.3 ± 0.5*
Interleukin-6 (pg/mL)	13.9 ± 0.5	19.7 ± 1.6*

* statistically significant difference between groups at P≤0.05.

Table 6. Pearson's correlation coefficients of selected variables in camel calves under investigation

Variables	Hp (g/L)	SAA (mg/L)	Fb (g/L)	WBCs (G/L)
SAA (mg/L)	0.670***			
Fb (g/L)	0.688***	0.530**		
WBCs (G/L)	0.430*	0.406*	0.390*	
Neutrophils (G/L)	0.274*	0.318*	0.236*	0.796***

Hp=haptoglobin, SAA=serum amyloid A, Fb=fibrinogen; WBCs=white blood cells; *** very highly significant (P<0.0001), ** highly significant (P<0.001), * significant (P<0.05).

rations in lipid profile, APP, pro-inflammatory and anti-inflammatory cytokines and oxidative stress parameters. The significant (P≤0.05) elevation of total leukocyte and neutrophils counts in pneumonic camel calves (Table 1) may be attributed to a variety of immunomodulatory effects during lung inflammation (El-Ghmati *et al.*, 1996). Previous studies (Soethout *et al.*, 2002; Civelek *et al.*, 2007, Smith, 2009) reported such increase in leukocytes in pneumonic calves. Moreover, the pathological leukocyte range was observed in numerous infectious diseases (Civelek *et al.*, 2007, El-Deeb, 2011). The significant (P≤0.05) increase in ALT, AST and ALP activities and diminished liver albumin synthesis in pneumonic camel calves may be coupled with probable hepatocellular injury stimulated by inflammation (bronchopneumonia). Comparable elevated levels of ALT, AST and ALP were reported by Nikolic *et al.* (2006) in rats, Civelek *et al.* (2007) in neonatal calves and El-Deeb (2011) in buffalo calves.

The consequence of inflammation on hepatic albumin biosynthesis remains controversial (O'Leary *et al.*, 2003). However, decreased albumin level observed in the current study agrees with previous findings reported by Civelek *et al.* (2007) in calves and and El-Deeb (2011) in buffalo calves. Marked reduction (P≤0.05) of total cholesterol and HDL-C levels, accompanied by significant elevation of VLDL-C and triglycerides of bronchopneumonic camel calves were similar to

findings in septic patients (Amersfoort *et al.*, 2003), neonatal calves (Civelek *et al.*, 2007) and buffalo calves (El-Deeb, 2011). The reduction in serum cholesterol in pneumonic camel calves may be attributed either to inflammatory processes and subsequent changes in lipoprotein metabolism or liver dysfunction (Civelek *et al.*, 2007; El-Deeb, 2011). Lower HDL-C perhaps reflects its protective effects against inflammation, mediated via bacterial endotoxins binding and subsequent neutralisation (Wu *et al.*, 2004). It was confirmed that inflammation leads to hypertriglyceridaemia in both humans and animals (Phetteplace *et al.*, 2000). This may be due to an increased production of VLDL-C, diminished conversion of VLDL-C to LDL-C by the inhibition of lipoprotein lipase activity (Gouni *et al.*, 1993) or stimulation of hepatic and adipose tissue lipolysis as well as hepatic fatty acid synthesis, which serve as substrates for hepatic VLDL synthesis (Feingold *et al.*, 1992).

There are only few studies on the role of oxidative stress in bovine pneumonia. In cases of respiratory tract infections, the neutrophils are recruited to the lungs to contribute in elimination of invading pathogens by their phagocytic activity. In the present investigation there was a significant ($P \leq 0.05$) elevation in the levels of MDA with significant ($P \leq 0.05$) decrease in the levels of erythrocytic GSH, SOD and CAT in the pneumonic camel calves compared to their levels in healthy controls. The present results are in agreement with results obtained by Valko *et al.* (2007) in humans. They reported that oxidative stress may induce lipid peroxidation and severe tissue lesions which result in damaged cell organelles, nucleic acids, lipids and proteins. The present findings of oxidative stress are also in harmony with Art *et al.* (1999) and

Deaton *et al.* (2004) in horses with recurrent airway obstruction, Ledwozyw & Stolarczyk (1992) in calves with bronchopneumonia, Al-Qudah (2009) in calves with acute pneumonia and in does with gangrenous mastitis (El-Deeb, 2013).

Decreased antioxidants (SOD, GSH and CAT), as found in the current study, may be attributed to the consumption of these enzymes in the protection of cells against oxidative injury by preventing initiation of the peroxidation process and production of final products such as TBARS, which are capable in inducing severe cellular damage (Halliwell, 1996). In certain cases of bovine pneumonia, the response to infection becomes extreme and yields many tissue-damaging products. Among the various products secreted by neutrophils are reactive nitrogen intermediates and NO• which modulates both acute and chronic inflammatory reactions. NO• is a mediator of capillary dysfunction and macromolecular leakage. O_2• reacts with NO• to produce ONOO–, causing tissue damage (Lykkesfeldt & Svendsen, 2007). In bronchopneumonic calves, it was observed that isolated granulocytes produced ten times as much O_2• and had lower plasma superoxide dismutase compared with healthy calves (Ledwozyw & Stolarczyk, 1992). On the other hand, Wessely-Szponder *et al.* (2004) reported that isolated neutrophils released from infected animals produced greater amounts of NO• and myeloperoxidase, which in combination may result in protein damage. The major antioxidant enzymes responsible for the degradation of reactive oxygen species are superoxide dismutase (which converts superoxide anion radicals to hydrogen peroxide), glutathione and catalase (which reduce hydrogen peroxides to alcohols and to water, respectively) (Halliwell, 1996; Silva *et al.*,

2012). Several studies have reported the decline of these enzymes in human patients with pneumonia (Cui *et al.*, 1997). Ledwozyw *et al.* (1992) have reported a significant decrease of SOD activity in calves with bronchopneumonia correlated with high blood concentrations of superoxide radicals. Furthermore, distinct reduction of blood GSH concentrations was reported in human patients (Pacht *et al.*, 1991).

Haptoglobin is α2-globulin synthesised in the liver (Feldman *et al.*, 2000) and considered as the major APP in ruminants. Hp could be detected in infected animals before the onset of clinical signs and its concentration is used as an indicator of disease severity (Godson *et al.*, 1996). The increased levels of Hp in cattle is interpreted as the outcome of tissue damage resulting from infection or inflammation (Eckersall *et al.*, 1988). Similar results in cattle (Eckersall *et al.*, 1988; Ganheim *et al.*, 2003) reported the same significantly (P≤0.05) higher levels of Hp found in the current study (Table 4). On the contrary, other findings demonstrated either lower (Young *et al.*, 1996) or unaltered (Wittum *et al.*, 1996) Hp values of infected cows. The significant (P≤0.05) elevation of SAA of pneumonic camel-calves (Table 4) could be attributed to the physiological role of SAA in host defense during inflammation (Orro *et al.*, 2011). Recently, similar marked elevation (P≤0.05) of SAA was reported in pneumonic calves (Nikunen *et al.*, 2007; Orro *et al.*, 2011) and pneumonic buffalo calves (El-Deeb, 2011; El-Bahr & El-Deeb, 2013). Fibrinogen (Fb) is a reliable indicator of inflammation and/or bacterial infection in cattle and sheep (Nikunen *et al.*, 2007; Gonzalez *et al.*, 2008; Orro *et al.*, 2011). The substantial elevation of Fb shown in the present study may be attributed to the involvement of Fb in homeostasis, providing a substrate for fibrin formation and in tissue repair, providing a matrix for the migration of inflammatory related cells. Previous research demonstrated higher Fb levels in infected calves (Nikunen *et al.*, 2007). Since blood samples were collected only once, the time course of APP, lipids and cytokines were not estimated. However, it well known that the concentrations of acute phase proteins as SAA increased more than 1000 times within 48–72 hours following inflammation in men and rabbits (Kushner, 1993). The mechanism for stimulation of APP production is via pro-inflammatory cytokines. The cytokine groups are the primary inducers of APP gene expression and each type initiates a different pattern of APP (Baumann & Gauldie, 1994). Thus, the increase in different patterns of APP seen in the current study reflected the production of different amounts or types of cytokines (Table 4). It was reported that the resolution of pulmonary bacterial infections includes a superbly arranged balancing act of pro-inflammatory IL-1 and TNF-α, IFN-γ and anti-inflammatory cytokines IL-10 and IL-6 (Moore & Standiford, 1998) as in the present study. Similar results for elevated levels of pro-inflammatory cytokines TNF-α, IL1β and IFN-γ in inflammation and infection were observed in pigs (Reeth & Nauwynck, 2000), cattle (Gruys *et al.*, 2005) and buffalo calves (El-Deeb, 2011; El-Bahr & El-Deeb, 2013). In addition, expression of TNF and IL was significantly increased in the airways and lung lesions of infected calves (El-Deeb, 2011; El-Bahr & El-Deeb, 2013). On the early stage of bacterial infection, resident alveolar macrophages become stimulated and discharge pro-inflammatory cytokines, resulting in

the eventual creation of a pro-inflammatory amplification loop between local or recruited macrophages or neutrophils and lymphocytes. As the infection is cleared, a second wave of anti-inflammatory cytokines is produced to localise the inflammatory response within the lung microenvironment and ultimately to down-modulate this immune response (Moore & Standiford, 1998). So, the balance between pro-inflammatory and anti-inflammatory cytokines (which is disturbed in diseased calves) is a very important factor in the immnological response of calves to bacterial pneumonia.

CONCLUSIONS

The presented study shed a light on the pathophysiological role of lipid profile, oxidative stress markers, acute phase proteins and pro-inflammatory cytokines for bronchopneumonia in camel-calves. Future research efforts will be focused on the diagnostic and prognostic accuracy of such parametrs in camel calves.

ACKNOWLEDGMENTS

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