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The influence of tissue antioxidant enzymes and inflammatory cascade in pathology of cystitis: An experimental rat model

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ABSTRACT

We evaluate the role of antioxidant enzyme status and inflammatory cascade in disease progression of cystitis in a rat model. The animals were injected with clinically isolated Uropathogenic *Escherichia coli* (UPEC) and study the effect of various antioxidant enzymes and inflammatory markers in disease pathology on the 0th day, 12 h and 7th day of infection. The antioxidant status of bladder tissue was decreased during the 7th day of infection. Lipid peroxidation marker MDA was increased on the 7th day of infection in rats. The histopathology of bladder tissue shows severe inflammation and edema. This study reveals the role of decreased antioxidant status during infection play a vital role in upregulation of inflammation and tissue destruction.

1. Introduction

The urinary tract is one of the most common sites of bacterial infection in humans [1]. Nearly half of all women experience at least one urinary tract infection (UTI) during their lifetime [2] and approximately a quarter of affected women will suffer recurrent UTI within 6–12 months [3]. *Escherichia coli* remain the predominant agent in 80% of acute community-acquired uncomplicated urinary tract infections. Infrequently, *Staphylococcus saprophyticus* (10%–15%), *Klebsiella*, *Enterobacter*, *Proteus* species, and *enterococci* cause uncomplicated cystitis and pyelonephritis [4]. Cystitis (lower urinary tract infection) is typically characterized by symptoms including frequency, urgency, and dysuria [5]. Host defense factors that predispose patients to UTI include urinary stasis, abnormal urinary tract anatomy, diabetes mellitus, debility, and aging. Estrogen-related issues and short urethras predispose women to UTI [6]. The bacteria that cause cystitis invade by ascending means through the urethra into the bladder. The ability of bacteria to cause UTI depends on the virulence determinants which they possess. The common virulence determinants of UPEC include adhesins, siderophore production, polysaccharide coating, hemolysin production, outer membrane proteins etc. These virulence determinants enable the organism to colonize and invade the urinary tract [7]. The intestinal *E. coli*, which are the reservoir of *E. coli* for causing UTI, lack these virulence determinants. After the invasion into the urinary tract, UPEC evokes an inflammatory response and hence bringing about pathological changes which lead to symptomatic UTI. The severity of symptomatic infections and tissue damage during the infective process depends upon the magnitude of the inflammatory response triggered by the uropathogens. This inflammatory response and disease progression in turn dependents upon the amount of extracellular release of reactive oxygen species by the phagocytic cells [7]. Reactive oxygen species generation leads to cellular and tissue damage and increases the severity of the bacterial infection. Acute inflammation was the first and early response to any noxious stimulus or injury. Leukocyte migration to the site of injury, and activation of the biochemical cascade of inflammation causing the release of mediators such as cytokines, histamines, kinins, complement factors, clotting factors, nitric oxide, and proteases [8]. The current study aims to investigate the role of antioxidant enzyme activities and MDA level in inflammatory process associated with UPEC infection. The present study, therefore, aimed to determine the effects of oxidative stress in UTI.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals used were high-quality analytical grade reagents. Phenazine methosulfate, nicotinamide adenine dinucleotide, nitroblue tetrazolium, 2, 4-dinitrophenylhydrazine (2, 4-DNPH), dithiobisnitrobenzoic acid (DTNB), glutathione and nicotinamide adenine dinucleotide reduced (NADH) were purchased from Sigma-Aldrich chemical company, USA. Tris (hydroxymethyl) amino methane, sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), 2-thiobarbituric acid, potassium ferricyanide [$K_3Fe(CN)_6$], sorbitol, trichloroacetic acid, and sucrose were procured

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from Merck, India.

2.2. Animals and microorganism

Adult female Wistar rats (weighing 150 ± 10 g) were purchased from Small Animal Breeding Station, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala used for this study. They were kept in a controlled environment for temperature (24°C–26 °C), humidity (55–60%) and photoperiod (12:12 h light–dark cycle). A commercial laboratory balanced diet (Amrut Laboratory Animal Feeds, Maharashtra, India) and tap water were provided ad libitum. The animals received humane care, in compliance with the host institutional animal ethics guidelines. Experiments were conducted as per the guidelines of Institutional Animal Ethical Committee, School of Biosciences, Mahatma Gandhi University (Reg. No. MGUSBS/IAEC/2016-B11042016/5) according to Government of India accepted principles for laboratory animals' use and care. The Uropathogenic *E-coli* were clinically isolated from a patient with severe urinary tract infection. The microorganism was collected from MOSC Medical College, Kolencherry, Kerala, India with Institutional Ethical Committee approval (Reg. No: MOSC/IEC/167/II/ext/2016).

2.3. Experimental design

Animals were categorized into three different groups with 6 rats in each group. The group-I was treated as normal control group. Group II, III were infected with clinically isolated *E-coli* for evaluating the progression of infection. Each anesthetized rat in group II, III was infected with 2.5×10^8 colony-forming units per milliliter of clinically isolated *E-coli* and 30 μL of sterile phosphate-buffered saline, transurethrally using lubricated catheters delivering about10^{-1} μL of inoculum into the bladder. After inoculation, the catheter was removed and the animal was monitored regularly for any discomfort, injury or inflammation due to the procedure [9]. The bladder was harvested from the control animals prior to the infection and the infected animals were sacrificed after 0^{th} hours 12^{th} hours, 7^{th} day. The collected tissues were processed for biochemical studies and a portion was stored in 10% neutral buffered formalin for histopathological analysis.

2.4. Measurement of microbial count

For microbiology study, the samples were homogenized in 3 mL of sterile saline at 4 °C. Appropriate dilutions of homogenized bladders were made, and 10-μl samples were placed in triplicate on MacConkey agar. The numbers of CFU of *E. coli* in the bladder were determined after an incubation of 18 h at 37° C (the CFU per milliliter of homogenate were transformed into CFU per gram of tissue). The bacterial enumeration was done at the dilution that allowed us to detect between 30 and 300 CFU/g of bladder. The limit of detection was 30 CFU/g of bladder. The bladder was considered sterile when no CFU was detected on the agar [10].

2.5. Superoxide dismutase (SOD) activity assay

SOD activity was measured by method of Kakkar et al., [11]. Assay mixture contained 0.1 mL of supernatant, 1.2 mL of sodium pyrophosphate buffer (pH 8.3; 0.052 M), 0.1 mL of phenazine methosulphate (186 μM), 0.3 mL of nitroblue tetrazolium (300 μM) and 0.2 mL of NADH (750 μM). The reaction was initiated by addition of NADH and stopped after incubation at 30 °C for 90s by the addition of 0.1 mL of glacial acetic acid. Following the addition of 4.0 mL of n-butanol and shake vigorously. The color intensity of butanol layer was measured spectrophotometrically at 560 nm. One unit of enzyme activity was defined as that amount of enzyme which caused 50% inhibition of NBT reduction/mg protein.

2.6. Catalase (CAT) activity assay

CAT activity was assayed by the method of Aebi et al., [12]. Each tissue supernatant (5 μL) was added to a cuvette containing 1.995 mL of 50 mM phosphate buffer (pH 7.0). The reaction was initiated by addition of 1.0 mL of freshly prepared 30 mM H_2O_2. The rate of H_2O_2 decomposition was measured spectrophotometrically at 240 nm.

2.7. Glutathione peroxidase (GPx) assay

GPx of bladder tissue was assayed in a 1 mL cuvette containing 0.890 mL of 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 U/mL GSH reductase and 1 mM GSH. 10 μL of each bladder tissue homogenate was added to make a total volume of 0.9 mL. The reaction was initiated by the addition of 100 μL of 2.5 mM H_2O_2, and the conversion of NADPH to $NADP^+$ was monitored spectrophotometrically at 340 nm for 3 min. GPx activity was expressed as nmol of NADPH oxidized to $NADP^+$/min/mg protein, using molar extinction coefficient of 6.22×10^6 (cm^{-1} M^{-1}) for NADPH [13].

2.8. Measurement of glutathione reductase (GR)

GR activity was measured using the method described by Racker et al., [14]. PMS was incubated with GSSG and NADPH at pH 7.5 and changes in OD were measured at 340 nm for 3 min at an interval of 30s.

2.9. Measurement of glutathione (GSH)

Glutathione content was assayed according to the method of Ellman et al., [15]. An aliquot of 1.0 mL of bladder PMS was precipitated with 1.0 mL of 10% metaphosphoric acid. The assay mixture contained 0.1 mL of aliquot, 2.7 phosphate buffer (0.1 M pH 7.4) and 0.2 mL of DTNB (1 mg/mL in phosphate buffer 0.1 M pH 7.4). The yellow color developed was read at 412 nm.

2.10. Measurement of thiobarbituric acid reactive substance (TBARS)

The double heating method was used for assaying the TBARS levels in the sample [16]. 0.5 mL of each sample was mixed with 2.5 mL of trichloroacetic acid (TCA, 10%, w/v) solution and incubated in a boiling water bath for 15 min. After cooling to room temperature followed by the centrifugation at 3000 rpm for 10 min. 2 mL of supernatant from each sample was transferred to a test tube containing 1 mL of TBA solution (0.67%, w/v). The tubes were placed in a boiling water bath for 15 min. After cooling to room temperature, the purple color generated by the reaction of thiobarbituric acid (TBA) with malondialdehyde was measured spectrophotometrically at 532 nm.

2.11. Histopathological analysis of bladder tissue

The entire bladder tissue section (5 μm) was fixed by immersion at room temperature in 10% formalin solution. For histopathological examinations, paraffin-embedded bladder tissue sections were stained with hematoxylin–eosin (H & E) followed by examination and photographed under a light microscope for observation of structural abnormality. The severity of bladder tissue inflammation was judged by two independent observers blinded to the experimental protocol [17].

2.12. Assay of protein

Protein was assayed by the method of Lowry et al., [18].

2.13. Statistical analysis

All statistical analyses were performed with the SPSS version 16.0.