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Expression of Nrf2 in Macrophage (RAW264.7) Induced by Serum of Burn Septic Rat

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Expression of Nrf2 in macrophage RAW264.7 induced by serum of septic rat infected with endotoxin and bacteria. Experiments were divided into animal model and cell model. For the animal model, the rats (n=45) were divided into normal control group, LPS group, Pure Burn group, *Pseudomonas* group, and *Staphylococcus* group. Rats of all groups were made 40 %total body surface area (TBSA) full thickness scald burn except normal control group. Lethal dose of *Staphylococcus aureus, Pseudomonas aeruginosa* and LPS were injected into burnt rat peritoneum according to the group respectively. Rats of all groups were killed by cardiac puncture method at 8 hours following burn and bloods collected were centrifuged to collect serum. Macrophage RAW264.7 cells were interfered with the serum separately. RT-PCR and Western blot were performed to study the expression of Nrf2. Nrf2 was prominently expressed in both experiments in all groups compared to normal control. In RT-PCR, Nrf2 mRNA expression level was the highest in the Pure Burn group ($p<0.01$) which was followed by the LPS group ($p<0.01$), *Pseudomonas* group ($p<0.01$) and Staphylococcus group ($p<0.01$) respectively. In the Western blot, Nrf2 protein expression was the highest in the LPS group ($p<0.01$) which was followed by the pure burn group ($p<0.01$), *Pseudomonas* group and staphylococcus group ($p<0.05$) respectively. The expression of Nrf2 indicates that different degree of endogenous anti oxidative system, host inflammatory response and its defense mechanism is active against oxidative stress inside the macrophage RAW264.7 interfered by different pathogens.

Key words: Nrf2; RAW264.7, Sepsis; LPS, Oxidative stress injury, Endogenous antioxidant system.

This study designed Macrophage RAW 264.7 cell as a cell model. This target cell was induced by blood serum of septic rats and undergone some experiments for Nrf2 expression. This paper describes how Nrf2 is expressed in target the cell due to oxidative stress injury and endogenous antioxidant mechanisms.

In this experiment, identification of host responses at the gene transcription levelprovides molecular profile of the events that is occurred following the infection[1]. Burn sepsis caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa* are facultative intracellular pathogensof macrophages that induce chronic infection in human anddomestic animals[1].

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Using RT-PCR and Western blot experiments, the responseof macrophages for 8 hours following the infection was analyzedin order to identify early intracellular infection events that are occurredin macrophages through the specific Nrf2 gene expression. . Nrf2 is a key transcription factor that regulates antioxidant defense in macrophages and epithelial cells, protecting against the proinflammatory and oxidizing effects of some chemicals and xenobiotics[2].

OS level plays a crucial role in the event. Itis a general term used to describe the steady state level of oxidative damage in a cell, tissue, or organ. ROS are the main cause of OS. These are free radicals and peroxides produced by the normal metabolism of oxygen. They are produced by mainly two different sources. Internal source is the normal metabolism and detoxification process in our body and external source is the excess intake of alcohol, smoking, and other poisoning including bacterial, fungal or viral infections. Here we focus on oxidative stress in burn sepsis caused by some bacteria and endotoxins.

Various pathologic processes disrupt this balance by increasing the formation of free radicals in proportion to the available antioxidants thus forming the oxidative stress[3]. Examples of increased free radical formation are immune cell activation, inflammation, ischemia, infection, cancer and so on. Free radical formation and the effect of these toxic molecules on cell function (which can result in cell death) are collectively called oxidative stress).

Burn sepsis is one of the major challenges for a surgeon in a burn unit. Infection is the most common and most serious complication of a major burn injury resulting sepsis. Sepsis accounts for 50-60% of deaths in burn patients today despite the improvements in antimicrobial therapies[5]. Burn injury leads to suppression of nearly all aspects of immune responses. Post-burn serum levels of immunoglobulin, fibronectin, and complement levels are reduced diminishing the ability for opsonization, chemotaxis, and phagocytosis, killing the function of neutrophils, monocytes, and impairing the macrophages[6]. Granulocytopenia is common following the burn injury which impairs the cellular immune response. Burn injury results in reductions of interleukin-2 (Il-2) production, T-cell and NK cell cytotoxicity, and helps suppress T-cell ratio. Furthermore, infusion of serum from burned to normal patients or animals can transmit some of these immunosuppressive effects (6).

Thus suspecting that a deregulation in the body's inflammatory response exacerbates sepsis, some researchers began looking into the genetic factors that might contribute to this syndrome. In 2002, Biswal and his colleagues discovered that Nrf2 acts as a primary regulator of most of the cellular antioxidant pathways detoxifying the enzymes that protect the body from a wide variety of environmental toxicants (6, 7). In subsequent studies, they discovered that Nrf2 is a pleiotropic protein that regulates a broad spectrum of genes used by the host to defend against a variety of stresses, including oxidative and inflammatory diseases[7].

Nrf2 is a transcription regulator factor which is important in the protection against oxidative stress through ARE-mediated transcriptional activation of several phase 2 detoxifying and antioxidant enzymes[8]. Clinical and animal studies have provided evidence that inflammation and oxidative stress from ROS are involved in the progression of burn sepsis[6-8]. In addition, recent research has demonstrated that oxidative stress can modulate inflammatory responses during tissue injury, possibly through the activation of Nrf2[8].

Nrf2 belongs to CNC-bZIP transcription factor family and its activity is pivotal for the coordinate induction of phase II detoxifying and antioxidative enzymes whose expression is under the regulatory influence of ARE[9, 10]. Thus Nrf2 contributes to cytoprotection against environmental electrophils and oxidative stress[11, 12]. Nrf2 is highly expressed in detoxication organs such as GI tract, liver, kidneys, lungs etc. for the protection from various stresses. In addition, Nrf2 is abundantly exposed in activated macrophages suggesting additional physiological roles beyond detoxication[13].

In this research we designed macrophage cell line (RAW 264.7) induced by some pathogens and endotoxins and studied its host defense activity and observed new gene (Nrf2) expression in cytoprotective mechanism from oxidative stress within it.

MATERIALS AND METHODS

Experimental animals

Wistar rats (n= 45),half male and half female, purchased fromXiangya third hospital Animal Experiment Lab with weights ranging from 250 to 300 grams were used for the experiment.

Pathogens and endotoxin

Followings are the lists of pathogens and endotoxins that were used intheexperiment: *Staphylococcus* aureus (Strain type ATCC25923) provided by Central South University, Xiangya school of medicine department of pathology, *Pseudomonas* aeruginosa (Strain type ATCC27853), provided by Central South University, Xiangya school of medicine department of pathology, Endotoxin(LPS2630), provided by Central South University, Xiangya school of medicine department of Pathophysiology, Macrophage cell line RAW 264.7(ATCC TIB71), purchased from Central South University, Xiangya school of medicine, department of Pathology.

Reagents

The follwing products were used as the experimental reagents: RT-PCR two step reagents (Promega Corporation, Foster, CA, USA); Nrf2 and β-actin Primers (Shanghai bio engineering company,Shanghai, China);Nrf2 antibody (ab92946)- Rabbit polyclonal to nrf2(Abcam company, Cambridge,UK);PCR product purification reagents,Taq DNA polymerase and dNTPs(Takara Bio Inc.Shiga,Japan);Trizol Kit (Invitrogen Biotechnology Company, Carlsbad,CA, USA); BSA (Sigma-Aldrich Corp.St. Louis, MO, USA);Tris (Serva Electrophoresis, Heidelberg Germany); 100bp DNA Ladder (*Tian Wei Shi Dai Science Technology Co.* Beijing, *China*);ECL reagent (Santa Cruz Biotechnology, Inc.Santa Cruz, CA. USA)

Design burn model

The rats were adapted and fed under strict observation for 1 week in the animal lab. Environment of the lab, equipments and the ventilation system of the lab were well maintained and kept under approved standard laboratory condition. Room temperature was maintained at 25-30 °C.The rats were kept NPO (Nil per mouth) for one day before performing the experiment. The next day hair were saved on the back of the rats and underwent 40%(TBSA) total body surface area full thickness scald burn by keeping the back of rats in 99°C boiling water continuously for 10 secondsunder general anesthesia (pentobarbital 50 mg/kg body weight) and analgesia (buprenorphin 1 mg/kg body weight)[14]. This model ensured that both groups were similar in metabolic rates. After the thermal injury, rats were immediately resuscitated by intraperitoneal injection of Ringer's Lactate (50 mL/kg body weight)[14]. The Wounds were disinfected by iodine solution and kept in metabolic wire cage for its feeding for 8 hours.

Design burn sepsis model

Burn sepsis model was designed by injecting some pathogens and endotoxins into the rat peritoneum. First all the animal models were divided into five groups; 1.Normal Control group 2.Pure Burn group 3.Staphyllococcal Burn group 4.Psedomonas Burn group and 5.LPS Burn group. Each group consisted of 10 rats except the Control Group which consisted (5 rats). Then 0.8ml (contains8×10^8cfu/ml) of *Staphylococcus aureus*, 0.5ml (contains 1×10^8cfu/ml) of *Pseudomonas aeruginosa*, and 6mgkg^{-1} of LPS were injected into the peritoneum of therats of group 3, 4 and 5 respectively. The sings of severe sepsis such as fever (One degree greater than the control group), tachycardia (Heart rate double than the control group) and tachypnoea (Respiration rate double than the control group) were observed. The metabolic conditionand the burn wound of the rats were also observed. Rats of all groups were killed at 8 hours postburn by Cardiac puncture method using 5 ml syringe and blood was collected separately in 5ml clotting tubes and centrifuged for serum. Serum was piped out and stored in below 0 °C.

Design cell model

Macrophage cell line RAW 264.7 was used as target cells for the cell model experiment. All cells were first plated into 5 different flasks and cultured in 10% FBS within CO_2 incubator at 37 0 °C for 24 hours at a concentration of 6 x106 per flask. All groups of serum stored at below 0 °C were then melted in room temperature. Cultured RAWcells were interfered by all groups of serum separately. Following infection, the cells were washed threetimes with PBS to remove extracellular bacteria. This is how the target cell model (Macrophage RAW264.7) was designed interfering by the infected serum of the burnt rats.

RT-PCR

Total RNA was extracted by using Trizol kit according to the manufacturer's protocol. After determination of its concentration (density and OD value 260/280) by using UV spectrophotometer and quality by gel electrophoresis,RNA strand is first reverse transcribed into cDNA by using reverse transcriptase (Promega Corporation, Foster, CA, USA) at 42°C in a total volume of 3 µg containing 20 µl of total RNA for one hour. According to mRNA sequence of Nrf2 and β-actin (Gene bank sequence no. NM-031789 and NM_031144), online primer design software was used to design the related primers (Table 1)The PCR amplification was performed using the primers for Nrf2 and β-actin. Primers for β-actin were used as an internal control. The upstream primer for Nrf2 (Segment length 311bp) was 5'-accggagaattcctcccaat-3' and downstream primer was 5'- agctcctgccaaacttgctc-3'.The upstream primer for β–actin (Segment length 487bp) was5'-gccgggacctgacagactac-3' and downstream primer was 5' gagccaccaatccacacaga-3'. PCRs for Nrf2 and β-actin were performed in a total reaction volume of 25 µl containing 4 µl of cDNA template, 0.5 mM primers, 2 mM MgCl2, 0.2 mMdNTP and 2.5 U of *Taq* DNA polymerase in a Perkin-Elmer thermo cycler (Norwalk, CT). Samples were heated at 95 °C for 5 min and subjected to 30 cycles of amplification (30 sec at 95°C, 30 sec at 58°C, and 60 sec at 72°C), followed by 10 min at 72°C for final extension and maintained at 4°C. PCR products were electrophoresed in 2% agarose gels. After EB staining, scanned under UV lamp to determine its OD value. Image was taken by gel imaging system and the expression was determined by the optical density ratio of target gene in reference with β–actin.

Western blotting

Serums of all groups were homogenized with lysis buffer (2xSDS sample buffer). Following centrifugation at13, 000 *g* for 20 minutes at 4^0C, the protein concentration of the supernatant was determined using Bradford's reagent. All protein samples were adjusted in same concentration and stored at –80°C. After SDS-PAGE preparation, equal amounts of protein (40 µg) were resolved on 15% SDS-PAGE and transferred onto a PVDF membrane (Millipore). The membranes were incubated with anti Nrf2 (1:1000) antibody and kept overnight in swinging bed at 4 ºC. Rabbit anti Goat IgG/HRP (1:4000) was then added and kept in room temperature for one and half hours. PVDF film was placed in HRP-ECL reagent for 1-3 minutes for reaction .Thus the blots were developed with the ECL reagent according to the manufacturer's instruction and exposed to X ray plate in dark room for obtaining and fixing image. The interest protein strap on the PVDF film was analyzed and managed by Gel Documentation System (Gel Doc 2000). The expression was determined by the optical density ratio of target gene in reference with β-actin.

Statistical analysis

Numerical data were shown as mean±standared deviation. All data were analyzed by SPSS15.0 statistical package. ANOVA and t test were applied for the comparison of each group. **Person** correlation analysis was applied for the dependability of Nrf2 mRNA and protein expression. $P<0.05$ is considered as significance difference.

RESULTS

The $OD_{(260)}/OD_{(280)}$ value of total RNAextraction

By using TRIZOL method, its value was approximately 1.7-1.9.Using 1% Agarose gel electrophoresis in randomly selected three samples showed 3 different clear straps 28s018s05s.Among them fluorescence intensity ratio of 18s and 28s strap is 16"2. It showed that RNA maintained in good molecular integrality. (Fig. 1)

Sequencing of PCR product

Designed primers were sequenced with PCR product separately and results were analyzed by using Seqman of DNASTAR software. It was verified that RT-PCR product and mRNA sequence were isogenesis. (Fig. 2)

Semi quantitative RT-PCR measurement of Nrf2 mRNA

Amplified DNA fragments of Nrf2 were seen in all gropus of serum in PCR amplification.

Heighest bands were amplified in pure burn serum group (Lane 3) and lowest bands were observed in normal serum group (Lane 1). LPS serum group (Lane 2), *Pseudomonas* serum group (Lane 4) and *Staphylococcus* serum group (Lane 5) wereat second, third and fourth place respectively. The PCR product of Nrf2 and β-actin were 311bp and 487bp respectively. (Fig. 3)

Table 1. Primer design for RT-PCR amplified genes

Target gene	Segment length	Primer sequence
Nrf2	311bp	Upstream : 5'- accggagaattcctcccaat-3' Downstream : 5'- agctcctgccaaacttgctc-3'
β-actin	487bp	Upstream : 5'- gccgggacctgacagactac-3' Downstream :5' gagccaccaatccacacaga-3'

Table 2. Nrf2 mRNA expression in RAW 264.7 cell interfered with different serum group by RT-PCR

Serum group	Normal serum group	LPS serum group	Pure Burn serum group	*Pseudomonas* serum group	*Staphylococcus* serum group
Nrf2 mRNA OD value	40.985±3.0921	71.883±2.243	81.190±3.833	65.708±2.975	60.525±3.512

All four group compared to normal control are highly significant at p<0.01. Pure burn serum group compared to LPS serum group significant at (p<0.05), Pseudomonas serum group compared to LPS serum group significant at (p>0.05), Staphylococcus serum group compared to LPS serum group significant at (p<0.01 and Pseudomonas serum group compared to staphylococcus serum group significant at (p>0.05)

Table 3. Nrf2 protein expression in RAW 264.7 cells induced with different serum group by WESTERN BLOT

Serum group	Normal serum group	LPS serum group	Pure Burn serum group	*Pseudomonas* serum group	*Staphylococcus* serum group
Nrf2 protein OD value	68.333±3.521	97.733±3.274	89.800±2.900	80.066±2.059	73.333±3.082

In the comparison with normal control; LPS serum group, Pure burn serum group & Pseudomonas serum group are highly significant at p<0.01 while Staphylococcus serum group is significant at p<0.05.Pure burn serum group compare to LPS serum group significant at (p<0.05), Pseudomonas serum group compare to LPS serum group significant at (p<0.01), Staphylococcus serum group compare to LPS serum group significant at (p<0.01), Pseudomonas serum group compared to staphylococcus serum group significant at (p>0.05)

Nrf2 mRNA expression ratio ((Nrf2/β–actin) in pure burn serum becomes 81.19±3.83247, LPS serum becomes 71.8825±2.24292, ---*Pseudomonas* serum becomes 65.70752±.97473, *Staphylococcus* serum becomes 60.525±3.512 and normal serum becomes 40.985±3.09207. Compared to normal, highest value seen in pure burn serum and lowest value seen in *Staphylococcus* serum. (Table 2 and Fig. 4)

Western Blot detection of Nrf2 protein expression level

Obvious Nrf2 bands were seen in all groups of serum in Wesrern blotting. Dark bands were seen in LPS serum group (Lane 2) and light bands were seen in normal serum group (Lane1). Pure burn serum group(Lane 3), *Pseudomonas* serum group(Lane 4) and *Staphylococcus* serum group(Lane 5) were at second ,third and fourth place respectively. (Fig. 5)

Nrf2 protein expression ratio in normal serum group becomes 68.333±3.521, LPS serum group becomes -----97.733±3.274, pure burn serum becomes 89.8±2.900, *Pseudomonas* serum group becomes 80.066±2.059, and *Staphylococcus* serum group becomes 73.333±3.082.Compare to normal, highest value seen in LPS group, and lowest value seen in *Staphylococcus* group respectively. (Table 3 and Fig. 6)

Correlation analysis indicates that there is prominent positive correlation between Nrf2 protein expression in Western blot result and Nrf2 mRNA expression in RT PCR result.

DISCUSSION

In our experiment we are going to identify the host response at the gene transcriptional level that provides a molecular profile of the events that

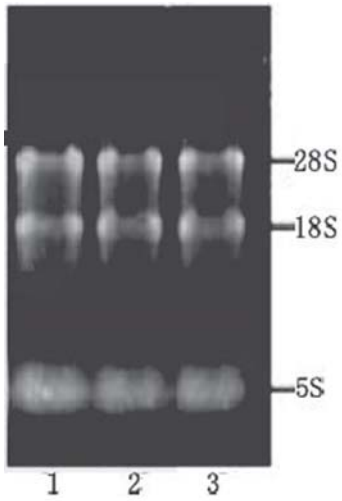
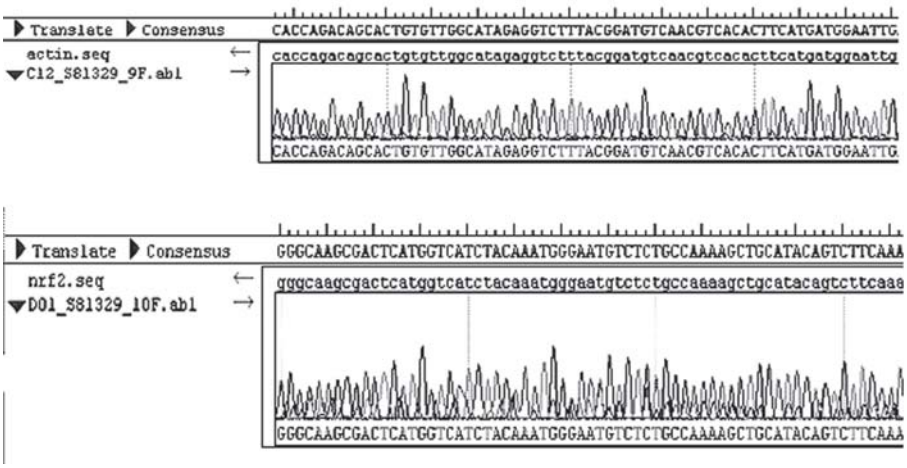


Fig. 1. Determination of total RNA quality by AGE



A:) β–actin PCR product sequencing **b)** Nrf2 PCR product sequencing

Fig. 2. PCR product sequencing

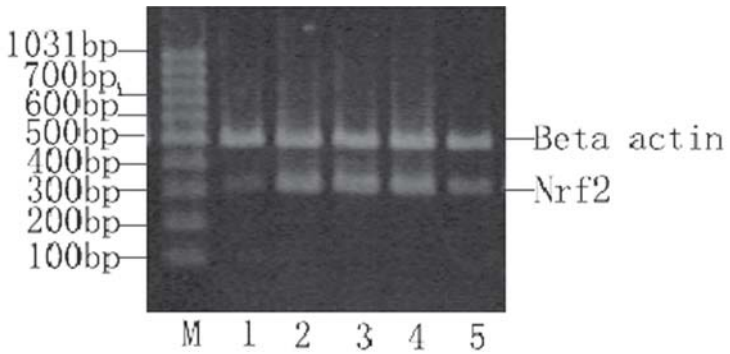
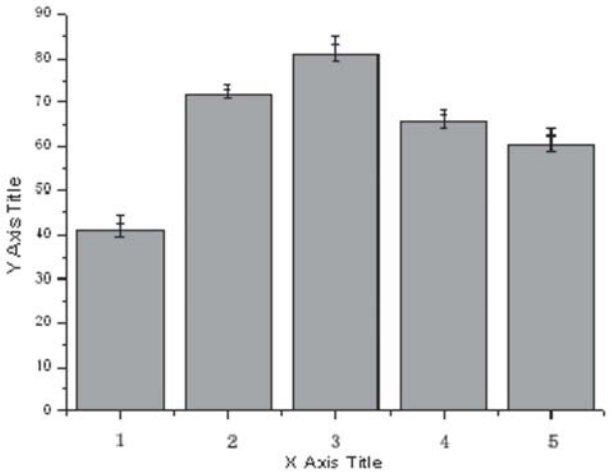
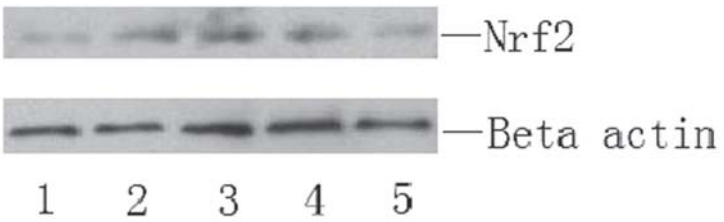


Fig. 3. Expression of Nrf2 mRNA in RAW264.7 cell in different group of 8hours Serum; Lane1: Normal control, Lane 2: LPS group, Lane 3: Pure burn group, Lane 4: *Pseudomonas* group, Lane 5: *Staphylococcus* group, M: DNA marker



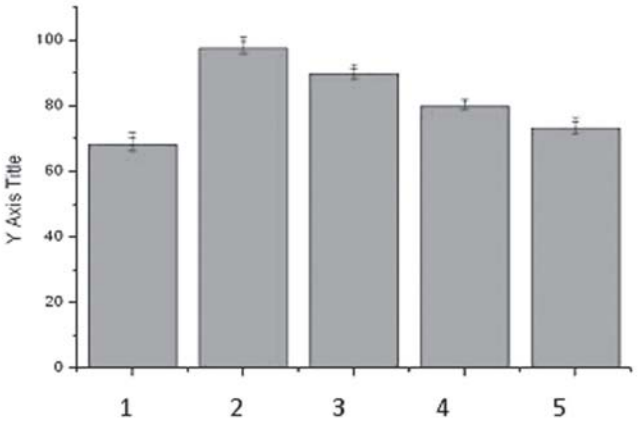
Lane1: Normal control group, Lane 2: LPS group, Lane 3:
Pure burn group, Lane 4: Pseudomonas group, Lane 5: Staphylococcus group

Fig. 4. The ratio of OD (Nrf2)/OD (β actin) value in RAW 264.7 cell interfered with different serum group by RT-PCR



Lane 1: Normal control group, Lane 2: LPS group, Lane 3: Pure burn group,
Lane4: Pseudomonas group, Lane 5: Staphylococcus group, M: DNA marker

Fig. 5. Expression of Nrf2 protein in RAW 264.7cell in all group of 8 hours serum.



Lane 1: Normal control group, Lane 2: LPS group, Lane 3: Pure burn group,
Lane 4: Pseudomonas group, Lane5: Staphylococcus group

Fig. 6. The ratio of OD (Nrf2)/OD (β actin) value in RAW 264.7 cell interfered with different serum group by Western Blot

occurs following infection. Here we used macrophage cell line RAW264.7 as host cell. Infected burn serum (causative agents are LPS, *Staphylococcus* and *Pseudomonas*) is interfered with RAW264.7 cell line and tried to identify Nrf2 gene expression in that host cell by using RT PCR and Western blot screening method using specific primers at 8 hours following infection. This study revealed that there is unregulated expression of Nrf2 genes in both RT-PCR and Western blot screening. But the expression level of target gene in that host cell following 8 hours infection differ in all pathogens compared to control.In RT-PCR**,** compared to normal serum (40.985±3.09207), expression of Nrf2 mRNA is highest in the RAW264.7 cell line interfered by pure burn serum (81.19±3.83247) and lowest in *Staphylococcus* serum (0.525±3.5116).LPS serum (71.8825±2.24292) and *Pseudomonas* serum (65.70752±.97473) are in second and third place respectively. In Western blot, compared to normal serum (68.333±3.521), expression of Nrf2 protein is highest in LPS (97.733±3.274) and lowest in *Staphylococcus* (73.333±3.082). Pure burn serum (89.8±2.9) and *Pseudomonas* (80.066±2.059) was in second and third place respectively.

In this research oxidative stress injury, host inflammatory response and its defense mechanism was proved to be important in burn sepsis. For that instant, Nrf2 gene is introduced for combating against oxidative stress during burn sepsis and studied its genetic factor for its contribution to the syndrome. Burn Sepsis is a complex disease characterized by an increased inflammatory response in body's attempt to combat bacterial, viral or fungal infection. A weak host inflammatory response leads to greater infection, whereas an excessive inflammatory response may lead to target organ damage, multiple organ failure or death[7].

Here oxidative stress injury is very important to be discussed. In critically ill burnt case, number of free oxygen radical ROS production accelerate the severity of illness leading to target organ damage or MODS. Therefore the expression level of Nrf2 in above experiment is different might be the different degree of oxidative stress caused by those pathogens. Nrf2 gene transfer to RAW264.7 macrophage produces several antioxidant enzymes such as HO-1 and with its anti inflammatory action protects tissue damage.

Many studies indicate that Nrf2 activity is expressed in genes of number of tissues and cell lines under physiological conditions through the binding of cytoskeleton-associated protein Keap1, and is activated if this interaction is interfered .Activation of Nrf2 is also dependent on mechanism that mediate its stabilization[15]. This mechanism includes two types of reactions. The Phase I reaction is mediated by cytochrome P450 mono-oxygenase systems, which promotes oxidations and reductions of compounds. Phase II enzymes promote conjugation of Phase I products with various free oxygen radical, including glutathione and glucuronic acid. Phase I enzyme genes are induced by Xenobiotics, key transcription activators while Phase II enzyme genes are induced by highly electrophilic metabolites of Phase I enzymes[16].

These Electrophiles transcriptionally activate the expression of Phase II enzyme genes, such as NQO1, GSTs, and UGTs), through the ARE or EpRE. Minimum ARE/EpRE sequence necessary for transcriptional induction by electrophiles is TGACnnnGC[16].

Nrf2 prevents septic shock by two ways. First by anti-inflammatory action prevents degranulation of host inflammatory mediators. Second by anti-oxidative property. Studies showed that Nrf2 acts as a primary transcription regulator of most of the cellular antioxidant pathways and detoxifying enzymes .Subsequent studies showed that Nrf2 is a pleiotropic protein regulating an array of genes that defend cells against variety of stresses, COPD and allergic asthma in mice models[7] and protection against cancer, neurodegenerative diseases, cardiovascular diseases, acute & chronic lung injury, autoimmune diseases in human[17].

Hence endogenous antioxidant mechanism is very crucial in this matter.Nrf2 gene transfer not only protect tissue or cells damage during sepsis, but also plays a role by inhibiting production of other inflammatory mediators.

Some studies showed that Using peritoneal macrophages from Nrf2-deficient mice, showed that Nrf2 also controls the expression of a group of electrophile- and oxidative stress-inducible proteins and activities, which includes HO-1, A170, Prx 1, and cystine membrane transport system x(c)(-) activity. The response to electrophilic

and reactive oxygen species-producing agents was profoundly impaired in Nrf2-deficient cells[18].

Using an antibody against 15d-PGJ(2), it was observed that macrophages from pleural lavage accumulate 15d-PGJ(2)(18,19).It showed that in mouse peritoneal macrophages 15d-PGJ(2) can activate Nrf2 by forming adducts with Keap1, resulting in an Nrf2-dependent induction of HO-1 and PrxI gene expression[21].

Host defense mechanism is also one of main factor for Nrf2 activation and its expression.Raw264.7 cell line is itself a scavenger for pathogens. In bacterial infection, its DNA binding capability by RAW264.7 cell is also very important. Using RAW264.7 cells as a model, Bacterial DNA can stimulate the production of cytokines and NO and IL. The immune activity of DNA is influenced by context or intracellular location and that, when transfected into cells, mammalian DNA can activate cells through signaling pathways similar to those of bacterial DNA[19].

Activated macrophages express high level of Nrf2,a transcriptional factor that positively regulates anti oxidant and detoxication enzyme[22].When animals are exposed with pathogens ,endotoxins, xenobiotics and drugs the expression of genes are essential for cellular defense mechanism. These processes of gene induction is mediated by ARE[20, 23].

Nrf2 activates cellular rescue pathway against oxidative injury, inflammation/immunity, apoptosis and carcinogenic[24]. In our experiment, RAW 264.7 cell line is interfered multiple times. First it was attacked by serum infected by *Staphylococcus*; *Pseudomonas* and LPS .Secondly interfered by Nrf2 gene. Studies have shown that pathogens and endotoxins increase the activation of macrophage RAW 264.7 cells by inflammatory response that requires a coordinated integration of various signaling pathway including COX, NO and cytokines[25, 26].

COX enzyme further catalyses the conversion of arachidonic acid to PG H2 and PGE2, an important component of inflammatory cascade that manifest cardinal sign of inflammation[20].

Our experiment shows that there is a significant expression of Nrf2 in *Pseudomonas* interfered RAW264.7 compared to *Staphylococcus*. As we discussed above, despite the oxidative stress within the target cell by two different bacteria are different, there might be other question arise that the mechanism for the counteract and subvert innate immunological responses to exacerbate pathogenesis by those two bacteria are different. *Pseudomonas* is gram negative bacteria and its early innate responses during its intervention is strong than that of *Staphylococcus* (gram positive).TLR stimulation signaling specially TLR-4 might be less strong in *Staphylococcus* than in *Pseudomonas*. Research showed that TLR signaling stimulation is also crucial for anti-inflammatory functions[21].

Activation of contact system might also be a factor for RAW264.7 activation. Different species of bacteria has different consequences of activation of the contact system. Classically it has two classical consequences; initiation intrinsic pathway of coagulation and cleavage of HK leading to the release of bradykinin, potent proinflamatory peptide[27].Inhibition of contact system promotes bacterial dissemination and growth and identify a novel and important role for the contact system in the defense against invasive bacterial infection[27] Results showed that expression of Nrf2 was high in LPS induced RAW 264.7 murine cell line in both Western blot and RT-PCR compared to other two pathogens. Because it is a strong endotoxin, a component of gram negative bacterial cell wall (30). Receptor-ligand complex is formed between the mammalian Toll-like receptor 4 (TLR4)-MD2-CD14 complex and LPS. The variability of bacterial ligands such as LPS and their innate immune receptors is an important factor in determining the outcome following infection.(28)It is released to circulation during infection and stimulate the pattern recognizing TLR4-MD2-CD14 receptor complex[29, 30].

This increases thetranscription of inflammatory and immune-response genes viamechanisms that depend on dissociation of the $I_{\kappa}B$– NF-B complex and translocation of NF-B to the nucleus,where it activates cytokine gene promoters (33). Stimulation ofthe NF-B pathway leads to increased transcription of severalproinflammatory cytokines, such as TNF- and (IL)-1, as well as prostaglandins and NO. Acting in an autocrine and paracrine manner, theseand other cytokines amplify host responses to invading pathogens[24, 32].

Although activation of the innate immune response during host-pathogeninteraction is initially adaptive, the inability to regulateimmune responses, which causes immunoparalysis, leads to sepsis[32, 33].Thus LPS challenge greatly increase the expression of Nrf2 gene in RAW264.7 murine cell line because of its strong endotoxin power.Furthemore might also caused a strong oxidative injury in target cell by releasing pro inflammatory mediators.

In RAW264.7 cell induced by pure burn serum, there is significant up regulated expression of Nrf2 in both experiment compare to other two bacteria. Burn sera markedly upregulated TNF-alpha, HMGB1 mRNA expression but downregulated IL-10 expression in normal macrophages, while these genes maintained in a very low level in normal macrophages with normal serum stimulation[34].Thus burn serum markedly increase the pro inflammatory mediators, hence expression of Nrf2 is significant.

CONCLUSION

From the study we came in conclusion that there was significant up regulated expression of Nrf2 in RAW264.7 murine cell line attacked by all group of serum. But the expression level was different in all groups. In RT-PCR high expression was seen in pure burn serum group followed by LPS serum group, *Pseudomonas* serum group respectively. Low expression was observed in *Staphylococcus* serum group. In Western blot experiment, high expression was observed in LPS serum group, then pure burn serum group, *Pseudomonas* serum group respectively. Lowest expression was seen in *Staphylococcus* serum group. There was prominent positive correlation between Nrf2 protein expression in Western blot and Nrf2 mRNA expression in RT PCR. High and low expression of Nrf2 might be the cause of different degree of oxidative stress injury in the target cell caused by those pathogens and endogenous anti oxidant system, host inflammatory response and its defense mechanism becomes different. Future studies will determine the potential role of Nrf2 for treatment of sepsis and other inflammatory diseases and can be new therapies for combating burn sepsis.

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