Association of Neonatal Asphyxia With Serum Levels of Heat Shock Protein 27 in a Small Sample of Newborns

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Abstract- Neonatal asphyxia is a state of hypoxia and hypercapnia caused by failure to breathe spontaneously and regularly soon after birth. Heat shock proteins (HSPs) are a ubiquitous and diverse group of highly conserved proteins which are rapidly up-regulated following periods of cellular stress including exposure to heat, ultraviolet irradiation, or chemical toxicity. The aim of the current study was to explore whether there is a relation between serum levels of HSP27 and neonatal asphyxia in a small sample of newborns. A total of 25 healthy newborns and 25 newborns diagnosed with neonatal asphyxia were recruited form Imam Reza Hospital, Mashhad, Iran. The Apgar score was recorded at one minute after delivery by trained nurses and newborns with the Apgar score of less than 7 were considered to be asphyctic. The mean birth weight of newborns in the case and control groups were 3110.47 ± 613.5 g and 3230.4 ± 584.83 g, respectively ($P=0.4$). Moreover, the mean maternal age of infants in the case group was higher than the mean maternal age of infants in the control group (31.1 ± 6.1 vs. 30.1 ± 5.0). Although it was marginally significant, the level of HSP27 was higher in the case group than the control group (0.23 ± 0.08 vs. 0.19 ± 0.09; $P=0.07$). Levels of HSP27 were found to be higher in newborns with neonatal asphyxia compared with healthy controls.

Introduction

Perinatal asphyxia, neonatal asphyxia or birth asphyxia is a state of hypoxia (lack of oxygen) and hypercapnia (accumulation of carbon dioxide) caused by failure to breathe spontaneously and regularly soon after birth (1,2). Approximately one million deaths each year are attributed to neonatal asphyxia worldwide, and about 0.08% of full-term neonates require cardiopulmonary resuscitation (CPR) (3). In asphyxia state, fetal oxygen supply and fetal heart rate are decreased, and the outcome can vary from non-pathological asphyxia to permanent brain death (4-6). The Apgar score, developed as an objective tool for measuring five signs of physiologic adaptation (7), is commonly used as a predictor of neonatal asphyxia (8). According to the international classification of diseases, mild birth asphyxia is defined as a 1-minute Apgar score of less than 7, and severe asphyxia is defined as a 1-minute Apgar score of 3 or less (9).

Heat shock proteins (HSPs) are a ubiquitous and diverse group of highly conserved proteins found in both eukaryotic and prokaryotic cells which play a key role in the maintenance of normal cell metabolism and function (10). HSPs are involved in a variety of cellular processes which include protein folding and degradation of misfolded proteins, membrane transport, and stability, modulating signaling pathways, nascent protein transport, and regulating immune responses (11-15). The expression of HSPs is rapidly up-regulated following periods of cellular stress, including exposure to heat, ultraviolet irradiation, or chemical toxicity (16-18). In mammalian cells, HSPs are classified into seven families according to their size and molecular structure: HSP10, HSP40, HSP60, HSP70, HSP90, HSP100, and small heat shock proteins including Hsp27 (19). The small HSPs are the most diverse family, which are composed of at least 10 small HSPs with molecular masses ranging from 12 to

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43 kDa (20). Hsp27 is a member of the small HSP family which is over-expressed following exposure of cells to oxidative stress (21).

It has been shown that various stimuli, including hyperthermia, staurosporine, and cytotoxic drugs, as well as oxidative stress, trigger the apoptotic cell death which can be protected by the overexpression of HSP27 (22,23). It has been shown that mothers whose babies were born with a birth defect to have higher levels of serum anti-HSP70 when compared with mothers who delivered healthy infants (24). Jiang and colleagues also observed increased expressions of HSP70 and HSP27 in a modified newborn rat model of hypoxic-ischemic brain damage (HIBD) (25). We previously showed that serum anti Hsp70 titers are significantly higher among asphyxiated neonates when compared with healthy neonates (26). Since there was no study investigating the relationship between neonatal asphyxia and HSP27, the present study aimed to determine whether there is an association between serum levels of HSP27 and neonatal asphyxia in a small sample of newborns.

Materials and Methods

Study population

In this case-control study, cases were 25 newborns with neonatal asphyxia diagnosed at Imam Reza Hospital, Mashhad, Iran. Controls were 25 normal newborns with no symptoms of neonatal asphyxia from the same hospital. Totally, 54% (n=27) of the study population were female and 46% (n=23) were male. Moreover, 78% of the infants were term, and 32% were preterm. The total number of infants born by vaginal delivery and cesarean section were 20 (40%) and 30 (60%), respectively. Preterm infants who were small for gestational age (SGA), suffered from congenital malformations, major birth trauma, septicemia, intrauterine growth retardation (IUGR), and those who died within 24 hours of birth were excluded from the study. All mothers gave informed, written consent to contribute to the survey, which was approved by the Ethics Committee of Mashhad University of Medical Sciences.

Apgar score

The presence of neonatal asphyxia was determined by the Apgar score invented by an anesthesiologist as a method to quickly summarize the health of newborn (7,27). Five simple criteria are used to determine the Apgar scale by evaluating the newborn at 1 and 5 minutes after birth. Each criterion has a score between 0 and 2 based on a 3-point scale. Accordingly, the final score can be a number between 0 (minimum) and 10 (maximum) (7). The five components of Apgar score are as follow: (1) Appearance (skin color); (2) Pulse (heart rate); (3) Grimace (reflex irritability); (4) Activity (muscle tone); and (5) Respiration (breathing effort) (Table 1). The Apgar score was recorded at one minute after delivery by trained nurses. The newborns with Apgar score of less than 7 were considered to be asphyctic (9).

Table 1. The five components of the Apgar score

	Score=0	Score=1	Score=2	Component of backronym
Skin color	Blue or pale	Blue at extremities (acrocyanosis)	Entire body pink	Appearance
Heart rate	No heartbeat	< 100 beats per minute	> 100 beats per minute	Pulse
Reflex irritability	No reaction	Grimace	Cry on stimulation	Grimace
Muscle tone	Flaccid	Some flexion	Active motion	Activity
Respiratory effort	No breathing	Weak, irregular	Strong, robust cry	Respiration

Determination of serum Hsp27 antigen

Venous blood samples were collected from each infant, and the prepared serum was stored at -80° C until the day of analysis. The samples were transferred to BuAli Research Institute to determine the levels of HSP27. Serum Hsp-27 antigen concentrations were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) in-house, as previously described (28). Briefly, one hundred microliters of a 2.5-mg/mL solution of monoclonal Hsp-27 antibody in phosphate-buffered saline (PBS) was used to coat a 96-well microtiter plate. After overnight incubation, the plate was washed three times with 0.05% Tween-20 in PBS and then blocked with 4% goat serum for 1.5 h. Fifty microliters of standards, 0.94, 1.875, 3.75, 7.5 and 15 ng/ml of recombinant Hsp27 (SPP-715, Stressgen Bioreagents), and 1:3 diluted samples were then added into duplicate wells. After adding 50 mL of 1:6000 dilution of rabbit anti-human Hsp-27 polyclonal antibody, 50 mL of 1:6000 dilution of goat anti-rabbit

horseradish peroxidase-conjugated antibody and a final wash of 4 cycles, 50 mL of the substrate solution tetramethylbenzidine dihydrochloride (TMB) was also added. The reaction was stopped after 20 minutes with 2 mol/L hydrochloric acid (HCl), and the absorbance was read at 450 nm. The sensitivity of the assay was 0.94 ng/mL, and the inter- and intra-assay coefficients of variation were 3.7% and 5.8%, respectively.

Statistical analysis

The data were subjected to statistical evaluation using IBM SPSS, version 19, with descriptive statistics being determined for all variables [mean±standard deviation for variables with normal distribution]. The Kolmogorov-Smirnov test was used for checking for the normality of data distribution. The Student's sample t-test was considered to compare the means of variables with a normal distribution.

Results

Out of 50 infants, 23 (46%) were male, and 27 (54%) were female. As presented in Table 2, the mean birth weight of newborns in the case and control groups were 3110.47±613.5 g and 3230.4±584.83 g, respectively (P=0.4). As expected, the mean Apgar score was significantly higher in the case group as compared to the control group (5.6±1.7 vs. 9.6±1.2; P=0.005). Although it was not statistically significant, the mean maternal age of infants in the case group was higher than the mean maternal age of infants in the control group (31.1±6.1 vs. 30.1±5.0).

Table 2. Comparison of Apgar score, birth weight, and maternal age between two groups

	Neonatal asphyxia (+) (N=25)	Neonatal asphyxia (-) (N=25)	*P*
Apgar score	5.6±1.7	9.6±1.2	0.005
Birth weight (g)	3110.47±613.5	3230.4±584.83	0.4
Maternal age (year)	31.1±6.1	30.1±5.0	0.5

Although the level of HSP27 was higher in the case group than the control group (0.23±0.08 vs. 0.19±0.09); a marginally significant difference was observed between two groups (P=0.07; Table 3).

Table 3. Comparison of HSP 27 between two groups

	Neonatal asphyxia (+) (N=25)	Neonatal asphyxia (-) (N=25)	*P*
HSP27 (ng/mL)	0.23±0.08	0.19±0.09	0.07

Discussion

In the present study, levels of HSP27 were found to be higher in newborns with perinatal asphyxia compared with control subjects.

There are several studies reporting that the serum levels of oxidative stress markers such as malondialdehyde (MDA) and protein carbonyl are significantly higher in asphyxiated babies as compared to normal healthy newborns (29-31), which in turn leads to a cascade of damaging events and neurodevelopmental sequelae (32,33). The lack of oxygen during neonatal asphyxia leads to mitochondrial dysfunction, and the subsequent failure to produce energy, accumulation of purine derivatives, and increased generation of reactive oxygen species (ROS) (34). The small HSPs such as Hsp27 can protect against protein misfolding following exposure to oxidative stress (35). Therefore, HSP27 can increase the anti-oxidant defense of cells and act as an antioxidant agent, at least in part, by holding glutathione in its reduced form and subsequently decreasing ROS cell content (36,37).

Kelly *et al.* reported that the overexpression of HSP72 might protect cornu ammonis of the hippocampus neurons from global cerebral ischemia induced by oxygen-glucose deprivation in hippocampal neuron cultures which may be mediated in part by increased Bcl-2 expression (38). In another study investigating the effect of hypoxia on some small molecular weight HSPs in the hippocampus, cortex, and cerebellum of newborn piglets, it was revealed that HSP20 is rapidly induced only in the hippocampus, while HSP27 is rapidly induced in the cortex and cerebellum (39). Jiang and colleagues also observed increased expressions of HSP70 and HSP27 from both cortical and hippocampal samples that reached a maximum at 12 or 24 h after HIBD in a

modified newborn rat model (25).

There are limited number of studies regarding the level of HSPs in human infants. We have previously shown that asphyxiated neonates have significantly higher serum anti Hsp70 titers when compared with non-asphyxiated neonates. It was concluded that serum concentrations of HSP70 antigen might be a useful marker for the early diagnosis of prenatal hypoxia (26). Moreover, it has been reported that serum anti-HSP70 level of mothers whose babies were born with a birth defect is higher than mothers who delivered healthy infants (24). The expression of HSPs was also shown to be induced in the parieto-occipital and hippocampus of subjects with a hypoxic/ischemic damage (40).

To the best of our knowledge, this is the first report to identify the association between serum levels of HSP27 and neonatal asphyxia in a small sample of newborns. One of the major limitations of our study was the small sample size, which limited our ability to draw definitive conclusions. In summary, levels of HSP27 were found to be higher in newborns with perinatal asphyxia compared with healthy controls.

Acknowledgments

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