

# Effects of Asphyxia on Colony-forming Ability of Hematopoietic Stem Cell of Cord Blood

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## ABSTRACT

**Background:** Asphyxia is a medical situation resulting from the deprivation of oxygen to a newborn lasting long enough during the birth process to cause physical harm, especially to the brain. Human umbilical cord blood (UCB) is a well-established source of hematopoietic stem/progenitor cells (HSPCs) for allogeneic stem cell transplantation. A low level of O<sub>2</sub> in neonates with asphyxia during labor can affect proliferation and differentiation of stem cells in cord blood.

**Methods:** The quality and colony-forming ability of hematopoietic stem cells in the cord blood of neonates with severe asphyxia with Apgar score 3-5 or need to cardiac pulmonary resuscitation 5 min after delivery were compared with the group with normal Apgar score. Thereafter, hematopoietic stem cells were isolated, and cells were cultured in an enriched media (MethoCult H4435) special for HSPCs for 7 days to assess the growth and colony formation.

**Results:** Based on the results, there was a significant difference in the number of colonies of RBC (P=0.0016) and WBC precursor (P=0.006), in a plate with 10<sup>4</sup> cord blood hematopoietic stem cells in newborns exposed to hypoxemia during labor.

**Conclusion:** Umbilical cord blood is valuable for its content of stem cells. Severe hypoxia in the perinatal period does not negatively affect the viability of UCB-derived HSPCs to grow and form colonies. Furthermore, it was found that transient severe asphyxia does not exert negative effects on the banking quality of HSPCs for likely problems in the future.

**Keywords:** Asphyxia, Cord blood, Hematopoietic stem cell, Hypoxemia

## Introduction

Asphyxia which is a major cause of neonatal mortality and neurological problems (1) is defined as a mixture of hypoxemia (oxygen deficiency in arterial blood), hypercapnia (a condition of abnormally elevated carbon dioxide (CO<sub>2</sub>) levels in the blood), and metabolic acidemia. Apgar score is one of the main criteria suggesting severe asphyxia. Although the majority of these disorders are transient, severe cases of asphyxia lead to hypoxic-ischemic encephalopathy (HIE) which occasionally results in permanent neurological damage, which can finally lead to cerebral palsy, epilepsy, and learning disabilities (2, 3).

Umbilical cord blood (UCB) stem cells were

historically considered an unused product of the birthing process but are now known to have more stem cells than adult bone marrow. When UCB is selected for therapeutic use, acute reactions in the host are really low, in comparison with other sources of stem cells, such as Bone Marrow Stem Cells (BMSCs) (4, 5). Autologous intravenous UCB transplantation is safe and possible in young children with *acquired* neurological disorders, and UCB transplantation recovers sensorimotor deficiencies after HIE (6).

Stem cells, such as hematopoietic *stem* and progenitor *cells* (HSPCs) are really sensitive to oxygen pressure levels and hypoxia. In other

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words, the low level of oxygen plays a key role in the regulation of stem cells and their fate (7). Expansion and function of the hematopoietic system, self-renewal and maintenance of embryonic stem cells, and differentiation capacity of mesenchymal stem cells (MSCs) are under the influence of the Hypoxia-induced factor (HIF) mechanism (8-12). Scientists have demonstrated that when HSPCs are cultured at reduced oxygen pressure, they enhance the potential of self-renewal and in vitro expansion (13-16). Moreover, hypoxia can affect the expansion of MSCs derived from bone marrow, UCB, and amniotic fluid (17). The MSCs which are derived from UC regulate energy consumption and metabolism during hypoxia, and hypoxia leads to an increase in the growth of UC-derived MSCs. The incubation of UC-derived MSCs at various concentrations of oxygen led to a rise in cell proliferation at hypoxia conditions (18).

UCB is a potentially vast precursor of HSPCs for hematopoietic reconstitution which can be clinically used as a reliable alternative for bone marrow transplantation in adults and pediatrics. Serious hematological malignancies, such as sickle cell disorders, leukemia, thalassemia immunological defects, bone marrow failure, as well as genetic diseases, have been considerably treated with cord blood (19-27). Since the first cord blood transplantation in 1988(28), more than 25,000 allogeneic cord blood transplantations (ACBT) have been globally performed, and their results can be compared with the bone marrow as the donor source for transplantations(29). These achievements demonstrate the role of UCB cells in the treatment of human diseases(30).

Today, more than 20% of states in the USA are involved in educating residents about the importance of UCB banking and its clinical benefits. Therefore, UCB banking for autologous and/or allogeneic transplantation and other ongoing purposes seems necessary. To demonstrate the quality of HSPCs exposed to transient severe hypoxia condition during labor by evaluating the HSPCs colony-forming efficiency, the present study aimed to assess whether primary hypoxia can affect the properties and abilities of HSPCs for making colonies even after culture in a normoxia environment and can save in public banks for therapeutic purposes.

## Methods

### Subjects

The current study was conducted on 11

neonates as the asphyxia group (umbilical blood gases measured, Apgar score 3-5) and 10 normal newborns with more than 8 Apgar score as a normal group. The exclusion criteria for normal neonates were any diseases or anomalies, and the exclusion criteria for the asphyxia group was Apgar score more than 5 without severe hypoxia condition. The written informed consent was obtained from neonates' parents before participating in the research project. It is noteworthy that this study was confirmed by the Ethical Committee of Mashhad University of Medical Sciences.

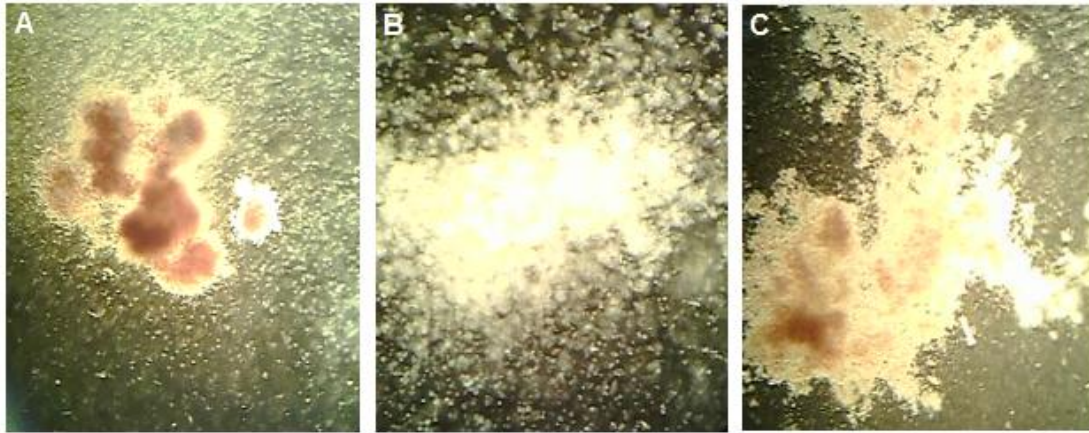
### Blood samples

Umbilical cord blood cells were collected from the Maternity ward of Imam Reza Hospital, Mashhad, Iran, within November 2014-May 2015. The umbilical cord with 6-8 cm extent of the umbilicus and other side was tied and transected between both sides 5 min after delivery. Blood was collected from the placenta end of the cord or by needle aspiration of the removed placenta. 15-25cc cord blood was collected and mixed with 5-6cc citrate anticoagulant (Citrate-dextrose solution- C3821-Sigma-Aldrich). The maximum elapsed time for transporting the samples to the clean room of the stem cell laboratory was 30 min, and they were transferred to sterile conditions.

### Methods

Depending on the sample size, 5-7cc Hydroxyethyl starch 10% (HES 200/0.5) (Fresenius Kabi, 14CA7310) was added to each sample and shaken vigorously. For efficient cell separation and red blood cell (RBC) precipitation by HES, the immobilized samples were placed in a sterile laminar class II cabinet. After 1 h, the supernatant was removed by sterile syringe and centrifuged at 600 g at 25°C for 20 min. The progenitor cells were placed on a buffy coat layer which was precisely separated by a needle immediately after centrifugation. To achieve the required concentrations of progenitor cells, they were counted by trypan blue Neubauer slide (31). Moreover, samples were evaluated by a cell counter (Sysmex XP300, USA) for further investigation.

In 12-well plates, 1ml of medium special for HSPCs (MethoCult H 4435 enriched, 04445, stem cell technology) (32), along with  $10^4$  progenitor cells obtained from buffy coat layer, was plated and 2 wells were cultured with double progenitor cell concentration. The cultured samples were incubated at 37°C, 85% humidity, and 5% CO<sub>2</sub> for



**Figure 1.** A. Red blood cell (erythrocyte) progenitor colonies ( $\times 100$ ). B: Separated white blood cell (leukocyte) progenitor colonies ( $\times 400$ ). C: white blood cell progenitor colonies ( $\times 100$ )

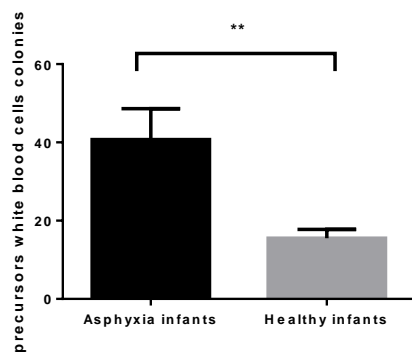
7 days based on our instruction (Colony Identification Guide) (33) and previous results. After incubation time, the colonies were counted by an inverted microscope. All the calculated cells were alive. Red colonies were regarded as red blood cell precursors, and white colonies were considered white blood cell precursors (Figure 1).

### Statistics

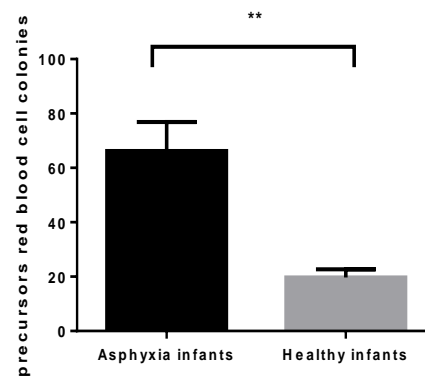
The collected data were analyzed in GraphPad Prism software (version 3) using the unpaired t-test. The significance level was considered less than 0.05 with a confidence interval of 95 %.

### Results

The mean number of colonies of white blood cell precursors in cord blood of neonates with asphyxia exposed to hypoxia was obtained at  $40.72 \pm 7.9$  in culture media for 7 days, while it was reported as  $15.5 \pm 2.3$  in newborns without asphyxia; therefore the difference was significant ( $P=0.006$ ; Graph 1). Moreover, the results of



**Graph 1.** Number of colonies of WBC precursors in cord blood after the culture cells for 7 days in H4435 culture media was significant in neonates exposed to hypoxia following asphyxia, compared to healthy ones ( $P$ -value=0.006)



**Graph 2.** Significant difference of colonies of RBC precursors in cord blood in two groups of neonates after culture cells in H4435 culture media for 7 days ( $P$ -value=0.0019)

the independent T-Test showed a significant difference in the mean number of colonies of red blood cell precursors (red colonies) in cultured media for the newborns who were exposed to hypoxemia ( $66.2 \pm 10.65$ ), compared to healthy neonates ( $16.34 \pm 5.1$ ;  $P=0.001$ ; Graph 2).

### Discussion

As evidenced by the results of the current study, hypoxia which results from low oxygen pressure in newborns with asphyxia increased the colony-forming efficiency of HSPCs in *in vitro* study. Hypoxia is one of the most important environmental factors affecting cells in various ways. Hypoxia plays a significant role in different features of cell biogenesis, such as metabolism, migration, proliferation, and differentiation. In the current research, the obtained results demonstrated that hypoxia in neonates with asphyxia increased the ability of HSPCs to form colonies and did not exert negative effects on the

viability and quality of HSPCs in culture media. Therefore, to the best of our knowledge, banking of cord blood of neonates with asphyxia and severe hypoxia poses no problem for therapeutic purposes in the future.

The obtained data demonstrated that the colony-forming efficiency of RBC and WBC precursors significantly increased in the UCB of neonates born with asphyxia, in comparison to the normal group. Based on the study conducted by Sushmita Roy et al., higher oxygen pressure has potentially been destructive on UCB-derived HSPCs, whereas the proliferation of UCB-derived HSPCs enhanced significantly under hypoxic conditions (15). We observed a 2.62-fold increase in the colony-forming efficiency of WBC progenitors, while that of RBC precursors was elevated 4 folds in UCB-HSPCs of neonates with Apgar 5 and less, while Roy et al. showed a 27-fold expansion in HSCs under hypoxic condition.

Grayson et al. indicated that MSCs and HSPCs have progressive growth under 5% O<sub>2</sub> during culture, and total cell numbers were induced in serial passages (34). Different studies showed that using low oxygen levels has significant effects on cell proliferation (35, 36). Embryonic stem cells have better growth and more proliferation in low levels of oxygen, compared to room air. Numerous investigations have demonstrated that in asphyxia conditions, the nucleated red blood cells (NRBC) were significantly more than the control group (37-39).

The present study demonstrated that 7-day incubation in 80% humidity, 37°C, and 5% carbon dioxide provides the most appropriate condition for UCB-derived HSPCs. Based on our observation, after 16 days of incubation, more than half of the expanded colonies showed signs of cell degeneration, and almost all colonies were completely destroyed when the incubation continued beyond 20 days. Our represented observations were in agreement with the study performed by Nakahata and Ogawa. They showed that when culture condition is sustained more than 16 days, some colonies were totally disintegrated, and on day 25 after seeding, just four types of colonies were recognizable (33).

The current study aimed to show that asphyxia has no negative effects on UCB banking; nonetheless, it was observed that the hypoxia caused by asphyxia significantly improved the colony-forming efficiency of UCB-derived HSPCs. On the other hand, oxygen levels are really important in the proliferation and differentiation of stem cell populations, especially HSPCs (40-43).

Low oxygen level has been reported to stimulate quiescence and adjust their differentiation, thereby maintaining stem cell phenotype. Even very low oxygen concentrations are permissive for slow self-renewing divisions of hematopoietic stem cells (44-47)

The current pilot study was conducted on limited samples obtained from one hospital; therefore, it is suggested that future studies be performed on a complicated population. In fact, the use of transplants of UCB over BMSCs has some advantages, such as ease of collection, no risk for mother and neonate, less time needed for processing, less risk for infection transmission, less need for stringent antigen typing, and less rejection risk.

## Conclusion

As evidenced by the obtained results, UCB is valuable for its content of stem cells. There is growing interest in the use of cord blood for novel indications in regenerative therapy and repair of neurological conditions, including cerebral palsy. Preserving UCB in public and/or private banks is beneficial for every family. The findings of the present study can raise public awareness about the positive outcomes of using cord blood banking. In brief, the obtained results indicated that transient severe asphyxia did not negatively affect the banking ability of HSPCs for likely problems in the future.

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## Conflicts of interest

The authors declare that they have no conflict of interest regarding the publication of the current study.

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