# Immunological Study of CD34 Positive Stem Cells in Cord Blood

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Abstract: Problem statement: The Cluster of differentiation CD34 molecule is present on certain cells within the human body .It mediate attachment of stem cells to bone marrow extra cellular matrix or directly to stromal cells. Approach: In this study we studied the amount of expression of CD34 on cord blood stem cells in babies of normal healthy pregnant females and in unhealthy conditions as diabetes. Materials and methods; the count of $CD34^+$ cells was assessed by flow cytometric analysis. Results: There was statistically significant difference between the count of $CD34^+$ cellsin the cord blood. The count was higher with normal vaginal delivery, heavier infants and higher leukocytic count, a significant reduction in the count of $CD34^+$ cells in the diabetic group.
[Omar M. Eldaly; Raghda A. Hafez and Lobna A. Elqurashi. **Immunological Study of CD34 Positive Stem Cells in Cord Blood**. *J Am Sci* 2013;9(7):254-260]. (ISSN: 1545-1003). http://www.jofamericanscience.org. 31

**Key words:** Significant reduction, diabetic group, human embryonic stem, mediate attachment, embryonic membranes, developed organism

**Abbreviation**:(CD) cluster of differentiation.

## 1. Introduction

Stem cells are defined as undifferentiated or 'blank' cells found in the human body that are capable of producing exact duplicates, capable of dividing indefinitely and capable of differentiating into multiple cell line ages (De Filippis L, Binda E.,2012).giving different cell types that carry out different functions (Little *et al*., 2006).

Stem cells can be classified by different means:

- According to their potency
- According to developmental origin
- According to their potency
- Totipotent: can differentiate into embryonic and extraembryonic cell types (Sell, 2004)
- Pleuripotent: can differentiate into nearly all cells except cells of the embryonic membranes (Trounson, 2002)
- Multipotent stem cells can differentiate into a number of cells, but only those of a closely related family of cells (Sell, 2004)

Unipotent cells can produce only one cell type of their own but have the property of self-renewal which distinguishes them from non-stem cells (Sell, 2004).

According to developmental origin:

- Embryonic stem cells: The embryos from which human embryonic stem cells are delivered are typically four or five days old and are hollow microscopic ball of cells called the blastocyst (Ulloa *et al*., 2005)
- Fetal stem cells: derived from the fetus itself (Karahuseyinoglu*et al*.,2007) and blood obtained from placenta and cord blood (Guillot *et al*., 2006)
- Adult stem cells: Refers to any cell found in a developed organism that has two properties: the ability to divide and create another cell like itself and also divide and create a cell more differentiated than it. Also, it is classified as somatic stem cells and germline (giving rise to gametes) stem cells (Jiang *et al*., 2002)

The CD34 family of cell surface transmembrane proteins consist of the antigen CD34, podocalyxin and endoglycan. CD34 protein has serine, thereonine and proline rich extracellular part that is extensively N- and O-glycosylation sites, followed by a cysteine bonded globular part and a trans-membrane stalk.

In addition, each protein contains a single transmembrane helix as well as a cytoplasmic tail that contains phosphrylation sites and carboxy terminal end.It is expressed on hematopoeitic stem cell, mesencymal stem cell, vascular endothelial cells, eosinophils, hair follicle stem cells and fibrocytes (Nielsen and McNagny, 2008).

Expression of CD34 on the cell surface of HSCs and its progressive down-regulation on more mature cells suggest that it may prevent HSCs differentiation and maintain them in an undifferentiated state.

Also, CD34 enhance migration of HSC between endothelial cells and into HSC niches within the BM by binding to L-selectin (Gangenahalli *et al*., 2006).

It has been discovered that cord blood contains stem cells which have the capacity to develop into neuronal, muscle and bone forming cells (Kogler *et al*., 2004) and into endothelial cells (Bompais *et al*., 2004).

Other studies suggest that UCB is a source of non-hematopoietic stem or progenitor cells, such as mesenchymal and endothelial precursors (Tondreau *et al*., 2005). But the frequency of UCB-derived MSC was extremely low compared with BM-derived MSC (Bieback *et al*., 2004).

Diabetes is the most common medical complication of pregnancy. The incidence of pregestational diabetes is 35% had type I and 65% had Type II diabetes (Correa *et al*., 2008).

Diabetes mellitus has one of the most important effects on the fetus. Its ill-effect on the outcome of pregnancy in terms of high rates of foetal loss, neonatal deaths and congenital malformations has been known for many years (Street *et al*., 2004).

The traditional approach to treat this disease is injection of exogenous insulin and subsequent follow up of blood glucose level. The other promising approach is transplantation of pancreas and islet cells for beta-cell replacement therapy. There was considerable success to treat diabetic patient from this approach. The follow up studies after transplantation of beta-cells from 2-5 years in different studies show great achievement for insulin independency (Street *et al*., 2004).

Several sources of stem cells have been suggested that can repopulate the damaged beta-cells and that include ES cells, HSCs and MSCs (Mishra *et al*., 2010). Also a number of studies have suggested the existence of stem cells within the pancreas that can give rise to insulin-producing cells (Zulewski *et al*., 2001;Whalley NM*et al*., 2011).

## 2. Materialsand Methods

This study was conducted in Medical Microbiology and Immunology Department, Faculty of Medicine, Zagazig University, during the period from 2010 and through 2011.

The study included 90 mothers divided into two groups:

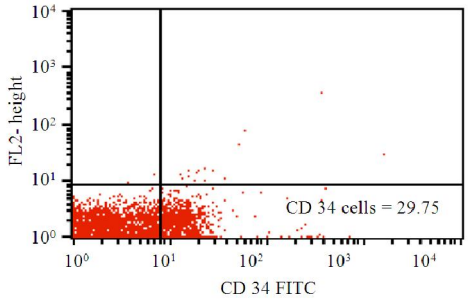


Fig. 1: Flow cytometric enumeration of $CD34^+$ cells in UCB of diabetic mother (type II) by dot blot

Table 1: Relation between Mothers' age, Mode of delivery, Neonate sex, birth weight and the median of cord blood CD34+ cells of the studied groups

| | | No. | Range CD34+ Cells/µL | Median CD34+ Cells/µL | Kruskal-Wallis test | P-value |
|---|---|---|---|---|---|---|
| Mothers' age | | | | | | |
| | 20-25 | 32 | (25-2032) | 419 | 3.77 | 0.15 |
| | 25-30 | 25 | (20-3591) | 167 | | |
| | 30-35 | 33 | (60-1247) | 215 | | |
| Mode of delivery | | | | | | |
| NVD | 33 | | (211-3591) | 677 | 280.6 | 0.00* |
| | CS | 57 | (25-1239) | 159 | | |
| Neonate sex | | | | | | |
| | Male | 32 | (25-3591) | 198 | 920.0 | 9.46 |
| | Female | 58 | (26-2032) | 291 | | |
| Birth weight | | | | | | |
| Normal | 46 | | (110-3591) | 131 | 343.0 | 0.00* |
| | birth weight | | | | | |
| | Large for gestational age | 44 | (25-2032) | 426 | | |
| Group | Control | 30 | (120-2032) | 519 | 514.0 | 0.001* |
| | Diabetic | 60 | (20-3591) | 191 | | |

**Case group:**

Sample populations include 60 diabetic pregnant mothers (20 type I, 20 type II and 20 gestational) that was diagnosed by history-taking and 2 hour OGTT according to American Diabetes Association (2011), Their range of age was from 20-32 yeaars (29±6 SD).

**Control group:**

It includes 30 healthy mothers, with ages ranging from 20 to 35 years (28±5SD).

The neonates were classified into two subgroups: neonates with birth weight ≥3000 g but ≤3600 g (normal birth weight) and neonates with birth weight >3600 g (large for gestational age).

All cases and controls were delivered at the delivery theater of obstetric ward at Zagazig University

Hospital; there was an informed verbal consent from the pregnant women prior to delivery. Confidentiality was respected.

All steps of the study were obtained after ethical approval by the medical microbiology and immunology department and CLINICAL pathology department and the obstetric Department at Zagazig university hospital.

**Collection of cord blood samples:**

Immediately after delivery, the umbilical cord was double-clamped and dissected. Then after the removal of the baby and before the separation of the placenta, the umbilical cord blood (about10 mL) is drained by gravity in sterile Falcon tubes 14 cc which contained 2 mL EDTA as an anticoagulant followed by inversion several times and labeling of the sample

**Manual white blood cell count of cord blood:**

By adding 1µL $^{1}$ of EDTA-anticoagulated blood to 19 µL of WBC Diluting Fluids as 2% Acetic Acid, then they were mixed well and left for 10 m before charging the hemacytometer.

WBC/ µL = average of cells × dilutional factor (20) ×10. Average of cells = the average of the total number of cells counted in the four large squares on both sides of the hemacytometer

**Low-density mononuclear cells layer separation:**

3mL of phosphate buffered saline solution was added to an equal volume of the blood and mixed gently by inversion.

The diluted blood was layered onto lymphoflot solution (Ficoll 1.077 g mL $^{1}$) in a ratio of 2:1.

The tubes were transferred to the centrifuge without disturbing the interface, spun at 400xg for exactly 30 m at room temperature.

Mononuclear cells are less dense than the separation medium and form a band at the interface (buffy coat). The mononuclear cells band was very carefully aspirated and transferred to a fresh Falcon tube, then washing was done by filling the tube with PBS followed by gentle mixing by inversion then centrifugation at 400× g for 10 m. A visible pellet of mononuclear cells was formed in the bottom of each tube. The supernatant was discarded by inversion; the cells were re-suspended using a pipette. A second wash was done as previously mentioned. The supernatant was discarded by inversion.

**Mononuclear cells counting:**

After the last wash, the pellet was re-suspended in 1ml buffer and counted by haemocytometer. The cell count in 1 ml was calculated by multiplying the average of cells by $10^4$ and the cell concentration was adjusted to 2 million mL $^{1}$.

**Flowcytometric assessment of stem cells in the separated mononuclear layer:**

By having 100 µL of re-suspended pellet, then addition of 10 µL of the CD34 antibody. They were mixed well and kept at room temperature for 10 m in the dark.

Washing of cells was done by adding 1 mL $^{1}$ of PBS and centrifugation at 300×g for 10 m, then aspiration of the supernatant completely. The cell pellet was re-suspended in 0.5 mL of PBS for analysis by flow cytometry.(figure 1)

**Statistical analysis:**

Data were collected, entered and checked to an SPSS version 15.

**3. Results**

The median of $CD34^+$ cells in the younger group (20-25) was more than other groups. But, there was no statistically significant difference between them ($p$ = 0.15).

There was higher count of $CD34^+$ cells in the UCB of neonates delivered by NVD than the UCB of neonates delivered by CS and the difference was statistically significant ($p$ = 0.00) .

The median of $CD34^+$ cells in the female neonates $p$ = 9.46)

There was higher count of $CD34^+$ cells in the large for gestational age group of neonates than the normal birth weight group of neonates and the difference was statistically significant ($p$ = 0.00).

There was higher count of $CD34^+$ cells in the control group than the diabetic group and the difference was statistically significant ($p$ = 0.001).

There was no statistically significant difference between the median of $CD34^+$ cells among the three types of diabetic UCB ($p$ = 0.05) but the $CD34^+$ cells count was higher in the gestational group than the other groups.

There was no statistically significant correlation between gestational age of all neonates included in the study (90 cases) and the $CD34^+$ cells in UCB (r = 0.203; $p$ = 0.05).

There was statistically significant correlation between leucocytic count of all neonates included in the study (90 cases) and the $CD34^+$ cells in UCB (r = 0.26; $p$ = 0.01). The gestational diabetic group (n = 20) shows statistically significant difference between the median of $CD34^+$ cells in UCB and the neonate's birth weight ($p$ =0.001), also between the controlled and Uncontrolled diabetic mothers ($p$ = 0.001) and the controlled diabetic had higher $CD34^+$ cells. But, there was no statistically significant difference between the median of $CD34^+$ cells in UCB and the neonate sex ($p$ = 0.38), mode of delivery ($p$ = 0.135) and the mothers' age ($p$ = 0.13).

The type I diabetic group (n = 20) shows statistically significant difference between the median

of $CD34^+$ cells in UCB and the neonate's birth weight ($p$ = 0.001), also between the controlled and Uncontrolled diabetic mothers ($p$ = 0.001) and between neonates delivered by NVD and CS ($p$ = 0.001) But, there was no statistically significant difference between the median of $CD34^+$ cells in UCB and the neonate sex (p = 0.38) and the mothers' age ($p$ = 0.17).

The type II diabetic group (n = 20) shows statistically significant difference between the median of $CD34^+$ cells in UCB and type of delivery ($p$ = 0.02), also between the controlled and Uncontrolled diabetic mothers ($p$ = 0.03). But, there was no statistically significant difference between the median of $CD34^+$ cells in UCB and the neonate sex ($p$ = 0.06), the neonate's birth weight ($p$ = 0.540) and the mothers' age ($p$ = 0.45)(.Table 1)

## 4. Discussion

In the present study, there was higher count of $CD34^+$ cells in the younger age group (20-25years) (n = 32; median = 419 μL $^{1}$) but there was no statistically significant difference between the mean of $CD34^+$ cells in UCB and mothers' age ($p$ = 0.15) .This was similar to what Jan *et al*. (2008) had found. They have reported that there was no statistically significant difference between the count of $CD34^+$ cells in UCB and mothers' age ($p$ = 0.801).

Omori *et al*. (2008) had found a relationship between $CD34^+$ cells count and the gravid status of the mother. They found that the total $CD34^+$ cells from 1-gravidae were significantly higher than those of 2- gravidae (medians, $120.0\times10^4$/mL and $83.0\times10^4$ mL $^{1}$, respectively; $p$<0.05). However, there was no statistically significant difference between the mean of $CD34^+$ cells and the maternal age. These results are consistent with several previous studies showing that maternal age does not affect $CD34^+$ cells count (Nakagawa *et al*., 2004; George *et al*., 2006; Mancinelli *et al*., 2006).

In the present study; there was higher count of $CD34^+$ cells in NVD (n = 33; median = 677 μL $^{1}$) than CS (n = 57; median = 159 μL $^{1}$). The difference between the median of $CD34^+$ cells in NVD and CS delivered neonates was statistically significant ($p$ = 0.00). This was against to what Sparrow and his group of researchers have found in (Sparrow *et al*., 2002). They have reported that a significantly higher CB volume was seen following CS (n = 61) than following vaginal delivery (n = 157) where the median volume was 76 mL $^{1}$ Vs. 63 mL $^{1}$, respectively ($p$<0.0001) and in contrast that CB from vaginal delivery had a significantly higher WBC concentration compared with CB from CS (medians, $17.1\times10^9$ and $13.6\times10^9$ WBCs/L, respectively; $p$<0.0001), but the mode of birth did not influence the $CD34^+$ cells count ($p$>0.05).

Mitchell *et al*. (2005) demonstrated that the mode of birth did not influence the $CD34^+$ subset ($CD34^+/CD61^+$,$CD34^+/CD38^-$,$CD34^+/CD90^+$), respectively ($p$ = 0.035, 0.120 and 0.069).

As confirmed by Sparrow *et al*. (2002) and considering that cesarean section results in volume increase while spontaneous delivery results in WBC increase, we can conclude that type of delivery does not influence the number of TNC and the $CD34^+$ cells in UCB.

There was statistically significant correlation between Leucocytic count of infants included in our study and the $CD34^+$ cells in UCB (r = 0.26; $p$ = 0.01). This was similar to what Sparrow and his group of researchers have found in (Sparrow *et al*., 2002) (r = 0.58; $p$ = 0.001).

In the present study, there was higher count of $CD34^+$ cells in female neonates (n = 58; median = 291 μL $^{1}$) than male neonates (n = 32; median = 198 μL $^{1}$). There was no statistically significant difference between the count of $CD34^+$ cells in UCB of male and female neonates ($p$ = 9.46).This was similar to the results reported by Ballen *et al*. (2001) that the $CD34^+$ cell counts of male neonates (n = 644, mean = $3.2\pm2.7\text{x}10^6$ mL $^{1}$) and female neonates (n = 596, mean = $2.9\pm2.6\times10^6$ mL $^{1}$) showed no statistically significant difference ($p$ = 0.06).

But, Jan *et al*. (2008) reported that The $CD34^+$ cell counts of male neonates (n = 660) was significantly higher than that of female neonates (n = 652) ($p$ = 0,007). Mitchell *et al*. (2005) demonstrated that male sex was associated significantly with an increase in the CD34+/CD61+ subset ($p$= 0.001) while other subsets ($CD34^+/CD38^-$ , $CD34^+/CD90^+$) did not have this significance($p$ = 0.111 and 0.195).

Mancinelli *et al*. (2006) noticed that female neonates had amplified WBC ($p$ = 0.013) and $CD34^+$ cells ($p$ =0.019) more than male neonates. They reported the fact there was greater number of WBC that in female neonates other than $CD34^+$ total as a very new datum and the interpretation of which is unknown.

In the present study, the neonates were classified into two subgroups: normal birth weight (n = 46, median = 131 μL $^{1}$) and large for gestational age (n = 44, median = 426 μL $^{1}$). The count was higher in the large for gestational age group .There was statistically significant relationship between the count of $CD34^+$ cells and the birth weight ($p$ = 0. 00).

Ballen *et al*. (2001) reported that a significant relationship was found between the mean of $CD34^+$ cells and the birth weight (r = 0.22, $p$ = 0.001). The birth weight of the babies (n = 1240) analyzed in this study ranged from 2155 to 5925 gram. Mitchell *et al*. (2005) demonstrated that $CD34^+/CD61^+$subset had a statistically significant relationship with the birth weight (r = 0.03, $p$ = 0.020) while other subsets ($CD34^+/CD38^-CD34^+/CD90^+$) did not have this significance(r = 0.01, p = 0.566; r = 0.02, $p$ = 0.154).

Mancinelli *et al*. (2000) reported that a significant relationship was found between the mean of $CD34^+$ cells and the birth weight ($p = 0.003$).

In the present study, on assessing the relationship between gestational age and $CD34^+$ cells in UCB there was no a statistically significant correlation between gestational age of neonates included in the study and the $CD34^+$ cells in UCB (r = 0.203, $p = 0.05$). This was in line with Surbek *et al*. (2010) who reported that the $CD34^+$ cell count per cord blood sample was independent of gestational age (r = -0.13, $p = 0.870$) .On the other hand, Omori *et al*. (2007) had found that total number of $CD34^+$ cell had a reverse correlation with the gestational age (r = -0.30, $p = 0.008$) . Similarly, Mitchell *et al*. (2005) had found a reverse correlation between the gestational age and $CD34^+$ subsets ($CD34^+/CD61^+$, $CD34^+/CD38^-$ ,$CD34^+/CD90^+$) (r = 0.09, $p<0.001$ ; r = 0.06, $p<0.001$ ; r = 0.05, $p = 0.001$).

Ballen *et al*. (2001) reported that even within the close range of term neonates (37-40 weeks), the effect of a longer gestational age was found to be associated with a higher nucleated cell count ($p<0.001$), but it has a slightly negative effect on CFU-GM ($p = 0.01$)..

Mancinelli *et al*. (2006) reported that gestational age > 39 week increase CD34% significantly ($p = 0.016$). This was explained by the fact that with increased gestational age there is placental aging and the fetus encounters a progressive hypoxia resulting in defense mechanisms that tend to increase hematopoietic cells and circulating blood volume.

Mihaela *et al*. (2007) had demonstrated that $CD34^+$ cells percentage appear to be higher on units yielded at lower gestational age (35-37 weeks) regarding those collected from 37-40 weeks old gestations(0.42 Vs. 0.32%) but the total number of $CD34^+$ cells remains almost constant around $3.56\times10^6$ mL $^{1}$ UCB. In our study, such analysis could not be performed due to the fact that all 90 deliveries considered had a gestational age >36 weeks and the differences had not been significant.

In the present study, we found a trend toward reduction of cord blood $CD34^+$ cell counts in diabetic (n = 60; median= 191 µL $^{1}$) versus non-diabetic pregnancies (n = 30; median = 519 µL $^{1}$) ($p= 0.001$). However, no significant differences were present between Type I, Type II and GDM ($p = 0.05$). Uncontrolled maternal diabetes is associated with a significant reduction of $CD34^+$ cell counts in Type I, Type II and GDM ($p = 0.001$- 0,03 - 0,001).

This was more or less similar to that obtained by Fadini *et al*. (2007). They enrolled 24 nondiabetic and 17 diabetic pregnant women: two had pregravidic type I diabetes and 15 had GDM that was diagnosed according to the American Diabetes Association guidelines. There were no significant differences in maternal age, gestational age, leukocyte count, neonatal weight and sex ($p>0.05$). There was significant reduction of cord blood $CD34^+$ cell count in diabetic versus nondiabetic pregnancies ($p = 0.042$). No significant differences were present in cell counts between type I diabetes and GDM ($p>0.05$). The cord blood $CD34^+$ cell count is not significantly reduced in the presence of well-controlled maternal diabetes ($p>0.05$).

Also, Acosta *et al*. (2011) found a significant reduction of $CD34^+$ cell counts in GDM group (n = 13) when compared with the control group (n = 10) ($p>0.04$). On the other hand, Benian *et al*. (2001) studied $CD34^+$cells in pregnant women with pre-eclampsia as another example of immunological disorder with pregnancy. They had decidual samples from the central part of the placenta to quantify $CD34^+$ cells by flow cytometry. There were no significant differences in age, parity, weight and gestational age at birth between the groups ($p>0.05$). The mean placental levels of CD34 (6.55±2.48% vs. 3.16±1.23%) were significantly higher in pre-eclamptic group (n = 21) compared with the control group (n = 20) ($p>0.05$).

Surbek *et al*. (2001) reported that There was a lower relative percentage of CD34+ cells in cord blood from pre-eclamptic patients (n = 11) versus the control group (n = 22) (0.22 Vs. 0.35%), but this difference did not reach statistical significance ($p= 0.229$). There were no significant differences in age, parity, weight, neonates sex and gestational age at birth between the groups ($p>0.05$).

One possible explanation might be the influence of cytokine and growth factor patterns in fetuses affected by preeclampsia. (Acosta*et al*., 2011) But, the explanation of higher count of $CD34^+$cells in the pre-eclamptic placentas compared with normal placentas is unknown (Surbek *et al*. 2001).

## Conclusion

The results of the present study show that the count of stem cells in UCB of controlled diabetic pregnancies is nearly the same as normal pregnancies .Therefore, UCB from controlled diabetic pregnancies should not be excluded from UCB banking for possible future clinical utility. But the uncontrolled diabetic pregnancies have lower count of these cells, so it is better to control their blood glucose levels.

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5/22/2013