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Decreased numbers of dense granules in fetal and neonatal platelets

Some mucocutaneous bleeding disorders are associated with platelet granule deficiencies that result in platelet function defects. Several studies have found that platelets from neonates have reduced activation responses compared to platelets from adults, but this relative platelet hyporeactivity is not associated with excessive bleeding in healthy infants. Little information is available concerning the ultrastructure of neonatal or fetal human platelets. Using whole mount electron microscopy to count calcium-rich platelet dense granules, we observed that neonatal and fetal platelets obtained from umbilical cord blood contain significantly fewer dense granules compared to cells from children and adults obtained from peripheral blood.

It is well established that most plasma coagulation factor concentrations and/or activities are decreased in neonates compared to children and adults. The notable exceptions are clotting factor VIII and von Willebrand factor, which are unchanged and increased, respectively.[1] Less is known about the function of neonatal platelets, partly due to the lack of standardized methods, until recently, for assessing platelet function[2] and also due to the large blood volumes required for assays of platelet function. In a study of 47,000 patients, Wiedmeier *et al.*[3] reported that platelet volumes remain relatively constant throughout gestation, and show a transient increase (along with platelet number) in neonates a few days following birth. A recent study[4] also confirmed earlier reports that neonatal platelets have reduced activation responses compared to adults, which contrasts with the apparent hyperactivity of the neonatal hemostatic system when assessed by bleeding time and *in vitro* assays.[5,6]

Electron microscopy (EM) is a well-established tool in the diagnosis of inherited platelet abnormalities.[7] It can be performed using small blood volumes, making it ideal for studying neonatal platelets. Thin section transmission EM is used to examine platelet ultrastructure and quantify the relatively abundant α-granules, which are decreased/absent in conditions such as gray platelet syndrome. Whole mount transmission EM can provide accurate counts of the less numerous (3-8/platelet) dense granules (δ-granules), which are electron-opaque due to their high calcium and polyphosphate content,[7] allowing them to be visualized as dark spots (Figure 1). Dense granules also contain adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin and other small molecules and ions.

Few studies have investigated the ultrastructure and granule content of neonatal platelets. One study using thin section EM reported no morphologic differences between neonatal and adult platelets.[8] Another reported that neonatal platelets have fewer pseudopods, smaller glycogen stores, a less developed microtubule assembly and more α-granules.[9] The same study reported no differences in dense granule counts among preterm, term neonate and adult platelets, but the accuracy of this result is questionable since there is a low probability of detecting platelet dense granules in thin sections. With regards to dense granule contents, neonatal platelet serotonin concentrations have been reported to be significantly

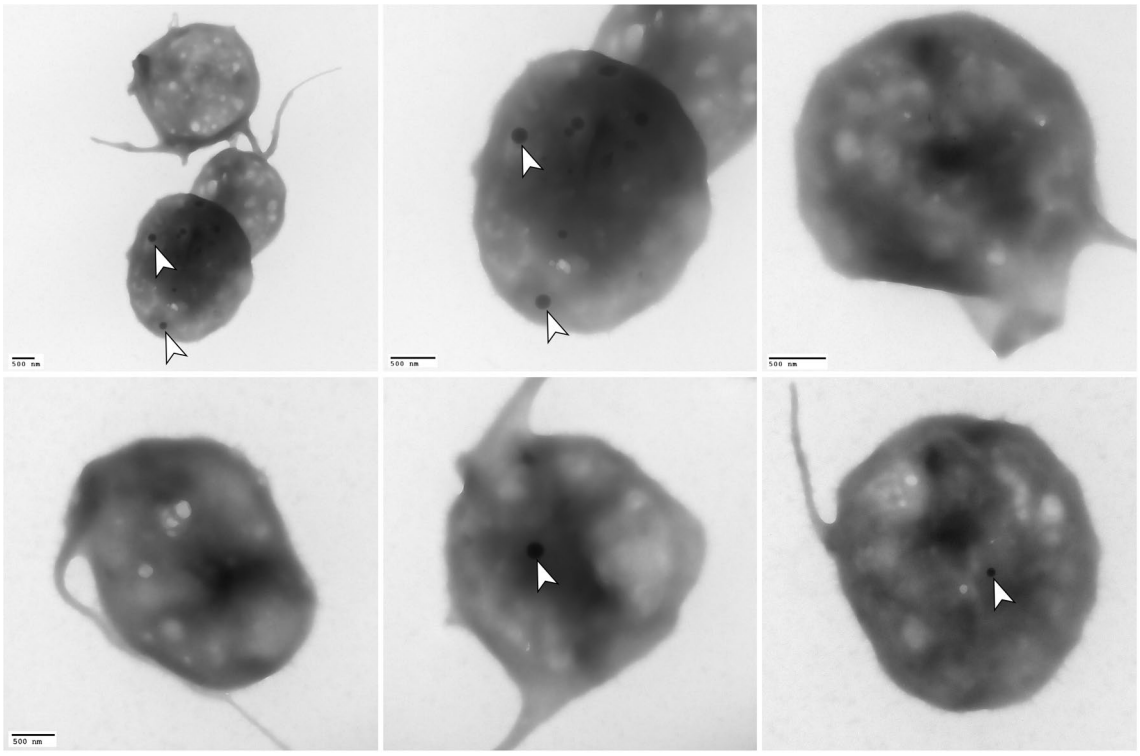

Figure 1. Imaging of cord blood platelets by whole mount electron microscopy. Representative resting platelets from two full-term neonate samples are shown in separate rows. Dense granules are visible as dark spots (white arrowheads). Top row: Left and center the same platelets imaged at 15000X and 30000X; rightmost panel shows another platelet imaged at 40000X. Bottom row: 3 platelets imaged at 30000X. Scale bars = 500 nm.

lower compared to platelets from older children and adults,[10] and to increase to near adult levels during the first month.[11] In order to clarify potential developmental differences in platelet dense granules, we employed whole mount EM to accurately count them in platelets from fetuses, neonates, children and adults.

This study was approved by the Research Ethics Board at The Hospital for Sick Children and Mount Sinai Hospital. Informed consent was provided according to the Declaration of Helsinki. Samples of umbilical cord blood were obtained from 3 first trimester fetuses after the termination of normal pregnancies, and 19 healthy full-term neonates delivered by cesarean section (C-section). Peripheral blood samples were obtained from 10 healthy children (aged 2-8 years) and 20 healthy adults (>18 years). Blood was collected in tubes containing 3.2% sodium citrate, and platelet whole mount and thin section EM was performed as previously described.[12] Briefly, 2-3 drops of platelet rich plasma were placed onto Formvar-coated nickel grids (Electron Microscopy Sciences, Fort Washington, PA, USA) for 5 minutes. Excess liquid was removed with a filter paper prior to fixation with 2.5% glutaraldehyde in phosphate buffered saline (PBS) pH 7.4 for 5 min. After rinsing with distilled water, the grids were placed into a JEOL JEM-1230 electron microscope with a 300/20 μm condenser/objective aperture (Mississauga, ON, Canada) and examined at 15000-100000X magnification. Dense granules were counted in 50 platelets for each sample, except for one fetal sample where only 25 platelets were scored. Platelets prepared from cord blood did not show abnormal levels of activation relative to cells in peripheral blood samples (Figure 1), and their resting morphology and α-granules were also comparable (*Online Supplementary Figure S1*).

The dense granule counts expressed as scatter plots (*Online Supplementary Figure S2*) show consistent results among individuals within each age group. A comparison of pooled results for all platelets scored within each group (Figure 2), shows that the mean dense granule count was significantly lower ($P<0.001$) in fetal platelets (mean 0.83 dense granules/platelet; ± SD 1.62) compared to the other age groups, while neonatal platelets (mean 2.28 ± SD 2.15) had significantly lower counts than platelets from children (mean 4.35 ± SD 2.72) or adults (mean 4.80 ± SD 3.68). Dense granule counts in platelets from children and adults did not differ significantly. Age-specific differences in dense granule counts can also be visualized *via* frequency distributions (Figure 3), which show a striking contrast between fetal platelets, where most platelets had no dense granules, and platelets from children and adults, where only a small proportion lacked granules. The total range in dense granule numbers among platelets also appeared to increase with age.

Neonatal platelets have been reported to have low levels of serotonin[10,11] relative to those from older children/adults, and to show decreased re-release of serotonin[13] and mepacrine,[14] despite comparable levels of uptake. The mepacrine observation was interpreted as indicating that neonatal platelets do not have decreased dense granules relative to adults, but rather a defect in an unspecified release mechanism.[14] It could also be argued, however, that the lower serotonin content observed in neonatal platelets is consistent with our observation that neonatal and fetal cord blood platelets have fewer mature dense granules. Since dense granules are detected *via* EM owing to their high calcium content, it is possible that these platelets also contain granular structures that lack sufficient calcium to be detected. If these immature granules are capable of taking up serotonin/mepacrine *ex vivo*, but incapable of releasing these molecules like mature granules, this would account for the normal uptake and decreased release observed in neonatal platelets.

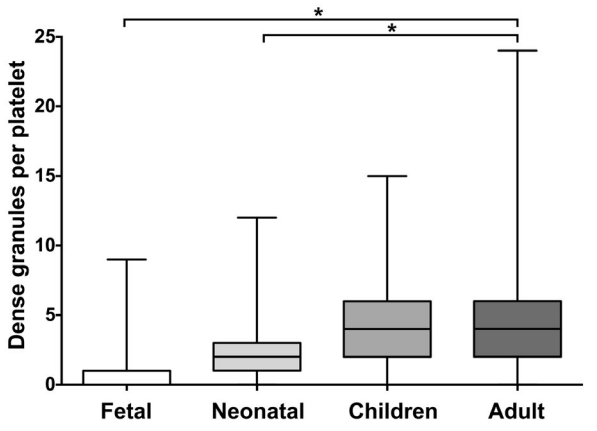

Figure 2. Dense granule counts in fetal, neonatal, child and adult platelets assessed by whole mount electron microscopy. Box plots for Fetal (N=3), Neonatal (N=19), Children (N=10), and Adults (N=20) showing midspreads and total ranges. Brackets show significant differences in mean counts for fetal platelets compared to others (top), and neonatal platelets compared to children and adult platelets (*$P<0.001$; one-way ANOVA Kruskal-Wallis/Dunn's multiple comparison test). All distributions were non-Gaussian (D'Agostino & Pearson omnibus normality test).

To our knowledge this study presents the first attempt to accurately evaluate dense granule counts in fetal and neonatal platelets using whole mount EM. As was discussed in a recent review,[15] studies of neonatal platelets have been complicated by difficulties in obtaining samples of suitable quality and volume to perform functional assays. While this challenge can be partially addressed by using cord blood as a source of neonatal platelets, they may be functionally different from circulating neonatal platelets.[16] Thus, while there is room for further investigation, we believe that our results indicate a progressive increase in EM-detectable platelet dense granules during early human development. This finding highlights the importance of taking developmental factors into consideration when diagnosing dense granule deficiencies in neonates.

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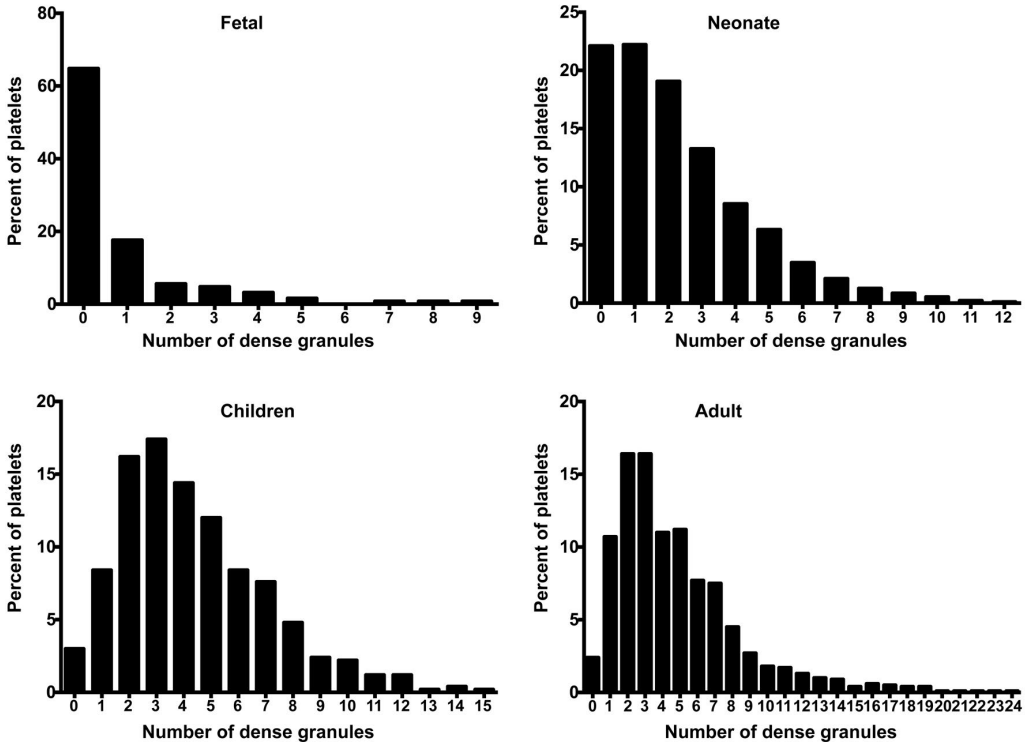

Figure 3. Frequency distributions of dense granule counts in fetal, neonatal, child and adult platelets. Dense granules were not observed in a majority of fetal platelets, while in neonates 45% had one or no granules. This contrasts with platelets from children and adults, where fewer than 4% had no dense granules and wider distributions of counts were observed.

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