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Glucose-induced increase in circulating progenitor cells is blunted in polycystic amenorrhoeic subjects

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BACKGROUND: Glucose-induced kinetics of bone marrow-derived stem cells in healthy females is presently unknown. The objectives of this study were to determine whether circulating levels of $CD133^+$, $CD34^+$ and $CD133^+CD34^+$ cells increase in response to glucose load in healthy females and whether the kinetics is altered in amenorrhoeic women. The other objective of the work was to compare the endothelial differentiation potential of peripheral blood-derived endothelial progenitor cells (EPCs) from healthy versus amenorrhoeic women.

METHODS: In this case–control study, 44 amenorrhoeic subjects and 36 age-matched females with no menstrual disturbance were recruited at Apollo Hospitals, a Tertiary health care center in Chennai, India. Circulating bone marrow-derived stem cells were measured by two color direct flow cytometry. Cultured progenitor cells were characterized at Day 7 and 14 for expression of endothelial markers and production of nitric oxide (NO) via immunofluoroscence.

RESULTS: The amenorrhoeic subjects were insulin resistant with homeostatic model of assessment of insulin resistance values of 3.33 ± 0.3 versus 1.75 ± 0.148 observed for controls ($P < 0.0001$). Among the amenorrhoeic subjects, 38 subjects had polycystic ovaries with no signs of hyperandrogenism. Fasting levels of $CD133^+$, $CD34^+$ and $CD133^+CD34^+$ cells were reduced in amenorrhoeic subjects ($P < 0.001$). There was a 1.5 to 2-fold increase in the circulating levels of these cells in response to 75 g oral glucose challenge at 1 and 2 h post-load conditions in controls, which was significantly blunted for $CD133^+$ ($P < 0.001$) and $CD133^+CD34^+$ ($P < 0.001$) cells in amenorrhoeic subjects. A positive correlation was observed between estrogen and fasting $CD133^+$ ($r = 0.205$, $P = 0.070$), $CD34^+$ ($r = 0.249$, $P = 0.027$) and $CD133^+CD34^+$ ($r = 0.217$, $P = 0.055$) cell counts. Additionally, fasting counts for $CD34^+$ and $CD133^+CD34^+$ cells positively correlated with FSH and inversely correlated with LH and C-peptide in the polycystic group. Cultured cells from polycystic subjects exhibited reduced adherence to fibronectin and expressed lower levels of endothelial nitric-oxide synthase and NO.

CONCLUSIONS: Oral glucose-induced increase in circulating numbers of $CD133^+$ and $CD133^+CD34^+$ cells and endothelial differentiation potential of peripheral blood-derived EPCs is attenuated in insulin resistant amenorrhoeic subjects.

Key words: amenorrhea / polycystic ovaries / bone marrow-derived stem cells / insulin resistance / OGTT

Introduction

The mammalian endometrium is composed of glandular epithelial, stromal and vascular cells that are recycled monthly from endometrial stem cells residing in the basalis layer of the tissue (Du and Taylor, 2007; Gargett *et al.*, 2007) and from bone marrow-derived endothelial progenitor cells (EPCs) which contribute to cyclic regeneration of the endometrial vasculature (2007) (Masuda *et al.*, 2007). Additionally, transplantation studies have detected donor-derived bone marrow cells in epithelial and stromal fraction of recipient's endometrium (Taylor, 2004; Du and Taylor, 2007; Ikoma *et al.*, 2009). Others have reported that stem cells present in the basalis layer of the endometrium are enriched in the endometrial side population which preferentially expresses the hematopoietic marker CD34 and endothelial markers VEGF receptor 2 (KDR) and CD31 (Tsuji *et al.*, 2008). Hematopoietic markers c-Kit and CD133 are also expressed in endometrial tissue (Lynch *et al.*, 2007; Chan *et al.*, 2011). Thus, bone marrow-derived stem cells and cells expressing hematopoietic markers are reported in human endometrium.

Circulating $CD34^+$ and $CD133^+$ cells are bone marrow-derived stem cells exhibiting extreme plasticity (Krause *et al.*, 2001). They

give rise to progenitors of varying lineages such as vascular, neuronal, lymphoid or skeletal (Handgretinger *et al.*, 2003; Yeh *et al.*, 2003; Urbich and Dimmeler, 2004; Matsumoto *et al.*, 2006). EPCs are a subset of this fraction and are mobilized into circulation from bone marrow upon receipt of an appropriate physiological cue (Urbich and Dimmeler, 2004). Controversy still exists on the exact definition of EPCs with some classifying them as $CD34^+KDR^+$ or $CD133^{+}$-$CD34^+KDR^+$ cells (Fadini *et al.*, 2008, 2010), while others consider $CD133^+KDR^+$ to be a more suitable phenotype (Urbich and Dimmeler, 2004). Nonetheless, studies have demonstrated rhythmic oscillations in $CD34^+KDR^+$ (Fadini *et al.*, 2008) and $CD133^+CD34^{+}$-KDR^+ cells during the menstrual cycle (Robb *et al.*, 2009). These cells express estrogen receptors and are responsible for estrogen-regulated cyclic neo-vascularization in the uterus (Masuda *et al.*, 2007; Foresta *et al.*, 2010). Intriguingly, it has been observed that the progressive decline in glucose metabolism during the progression of diabetes is associated with a concomitant decrease in fasting levels of $CD34^+KDR^+$ EPCs in diabetics (Fadini *et al.*, 2010). Even their putative precursors ($CD34^+$ cells) are less abundant both in insulin resistant pre-diabetic and diabetic subjects (Fadini *et al.*, 2007) and their numbers are negatively correlated with increasing risk factors of the metabolic syndrome (Fadini *et al.*, 2006). Surprisingly, treatment with the insulin sensitizer metformin not only improves the circulating numbers but also enhances the endothelial differentiation potential of these $CD45^{low}CD34^+KDR^+$ cells in type 2 diabetes patients (Chen *et al.*, 2010). However, it is not known whether the endothelial differentiation potential of circulating EPCs is compromised in insulin resistant amenorrhoeic women.

Insulin resistance (IR) is an underlying feature of anovulation and/or amenorrhea observed in polycystic ovary syndrome (PCOS) patients (Goodarzi *et al.*, 2011). Intriguingly the pattern of IR varies with different stages of the menstrual cycle with homeostatic model of assessment (HOMA)-IR values being maximal during the luteal phase (Yeung *et al.*, 2010). Adequate glucose uptake and its appropriate metabolism are necessary for uterine differentiation and embryo implantation (Fornes *et al.*, 2010; Frolova and Moley, 2011). However, the machinery required for insulin-dependent glucose transporter 4 translocation is attenuated in endometrial cells of PCOS subjects (Fornes *et al.*, 2010). Surprisingly, in these patients, insulin sensitizers improve menstrual cyclicity, ovulation, fertility and endometrial receptivity (Kocak *et al.*, 2002; Genazzani *et al.*, 2004; Krstevska *et al.*, 2006) thereby demonstrating a strong link between IR and female reproduction. We have recently shown that oral glucose challenge increases circulating levels of $CD34^+$ and $CD133^+CD34^+$ cells in young Asian Indian males, which is attenuated in insulin resistant individuals (Nathan *et al.*, 2011). However, it is not known whether a similar increase in response to glucose is seen for bone marrow-derived stem cells in healthy females. Given that $CD34^+$ cells have been detected in uterine endometrium right from the fetal stage (Cho *et al.*, 2004) and that bone marrow-derived stem cells home into the endometrium (Taylor, 2004; Du and Taylor, 2007; Ikoma *et al.*, 2009) an active glucose utilizing tissue, the objectives of the present study were to determine: (i) whether circulating levels of $CD34^+$, $CD133^+$ and $CD133^+CD34^+$ stem cells increase in response to glucose load in healthy females? (ii) If so, is the surge compromised in insulin resistant amenorrhoeic subjects and (iii) does the endothelial differentiation potential of peripheral blood-derived EPCs differ between healthy versus amenorrhoeic polycystic ovary (PCO) subjects.

Materials and Methods

Study design

In this case–control study, 80 subjects were recruited at Apollo Hospitals, Chennai, India. Following an over-night fast, blood was drawn for quantification of circulating progenitor cells and for determination of biochemical and hormonal profiles. This was followed by a 75 g oral glucose tolerance test and blood was redrawn at 1 and 2 h post-load for measurement of bone marrow-derived stem cells, glucose and insulin levels. We chose to enumerate $CD34^+$, $CD133^+$ and $CD133^+CD34^+$ cells for this study as they are capable of differentiating into progenitors of varying lineages in addition to expressing angiogenic markers (Pomyje *et al.*, 2003). For a subset of these subjects, the endothelial differentiation potential of circulating EPCs isolated from fasting blood was determined. Circulating EPCs are fibronectin adherent mononuclear cells which take up acetylated low-density lipoprotein (LDL) and bind to lectin upon endothelial differentiation. Isolated mononuclear cells were cultured on fibronectin-coated dishes in endothelial differentiation medium for up to 2 weeks prior to assessment of endothelial differentiation as described below.

Subjects

Forty-four amenorrhoeic subjects with self-reported menstrual irregularities were recruited following informed consent. The study was approved by Institutional Ethics Committee in accordance with Declaration of Helsinki as revised in 2000. Thirty-six age-matched subjects with regular menses were recruited as controls. All measurements (biochemical assays and enumeration of stem cells) were done at cycle day 3 for controls and anytime for amenorrhoeic subjects. Hypothalamic amenorrhea, primary ovarian failure, Cushing syndrome, hyper-prolactinemia, uncontrolled hypothyroidism, recent surgery and ongoing pregnancy were excluded. There was no history of alcohol consumption or smoking among any of the subjects. PCOs were defined as the presence of 12 or more cysts in each ovary with stromal volume of at least 10 ml. The morphometric, clinical and metabolic features of study subjects are summarized in Table I. The clinical and biochemical profiles of single cyst versus polycystic subjects within the amenorrhoeic group are shown in Supplementary data, Table SI.

Biochemical parameters

Free testosterone was measured by radioimmunoassay (Roche, USA). Serum glucose, high-density lipoprotein (HDL) cholesterol, LDL cholesterol, triglycerides, insulin, C-reactive protein (CRP), C-peptide, estrogen, FSH, LH and prolactin measurements were done with respective Siemens Adrian Kits from USA. Serum glucose levels were measured by glucose oxidase–peroxidase method (Washko and Rice, 1961). HDL cholesterol was measured by polyethylene glycol-pretreated enzymes and LDL cholesterol was measured by the direct LDL method (Izzo *et al.*, 1981; Lopez-Suarez *et al.*, 2008). Triglyceride was measured by the glycerol phosphate oxidase–peroxidase-4-aminophenazone method (Lopez-Suarez *et al.*, 2008). Total protein was measured by Biuret method and serum albumin and globulin were measured by the bromcresol green method (Gustafsson, 1976; Pelley *et al.*, 1978). Insulin levels, CRP, C-peptide, progesterone, estrogen, FSH, LH and prolactin levels were measured by chemiluminescence-based immunoassay methods. The percentage intra- and inter-assay coefficient of variations (CV%) for these assays were in the range of 3–8%. IR was estimated using HOMA-IR calculated as fasting insulin (μU/ml) × fasting glucose (mmol/l) divided by 22.5.

Table I Anthropometric, clinical and biochemical characteristics of study subjects.

Parameters	Controls ($n = 36$)	Amenorrheic subjects ($n = 44$)	*P*-value
Anthropometric measures			
Age (years)	22.3 ± 0.59	22.9 ± 0.71	0.530
Height (cm)	156 ± 0. 9	158 ± 0.8	0.183
Weight (kg)	62.2 ± 1.99	71.3 ± 1.93	0.0017**
BMI (kg/m^2)	25.5 ± 0.76	28.7 ± 0.76	0.0047**
Waist (cm)	87.2 ± 2.13	97.7 ± 1.78	0.0003***
Hip (cm)	87.5 ± 2.74	96.4 ± 1.74	0.0058**
WHR	1.01 ± 0.020	1.02 ± 0.019	0.802
Systolic pressure (mmHg)	113 ± 2.1	104 ± 1.0	0.120
Diastolic pressure (mmHg)	70 ± 1.2	742 ± 1.2	0.171
Oral glucose tolerance test			
Fasting glucose (mmol/l)	4.7 ± 0.15	5.1 ± 0.09	0.017*
1 h post-load glucose (mmol/l)	6.6 ± 0.36	6.9 ± 0.33	0.101
2 h post-load glucose (mmol/l)	5.4 ± 0.23	5.9 ± 0.26	0.204
Fasting insulin (pmol/l)	58. ± 4.5	98 ± 8.1	<0.0001***
2 h post-load insulin (pmol/l)	600 ± 98.5	644 ± 89.5	0.745
HOMA-IR	1.75 ± 0.148	3.33 ± 0.300	<0.0001***
Hormonal measures			
Testosterone (nmol/l)	5.4 ± 3.10	6.5 ± 2.12	0.210
Estradiol (pmol/l)	808 ± 151.5	315 ± 34.3	0.0010**
Progesterone (nmol/l)	0.02 ± 0.0047	0.008 ± 0.0036	0.0406*
LH (mIU/ml)	8.6 ± 1.57	13.5 ± 1.69	0.0378*
FSH (mIU/ml)	5.1 ± 0.55	6.2 ± 0.45	0.1197
LH/FSH	1.9 ± 0.99	2.2 ± 0.15	0.2822
Prolactin (pmol/l)	491 ± 46.9	532 ± 45.7	0.5338
Lipid profile			
Total cholesterol (mmol/l)	4.0 ± 0.15	4.3 ± 0.11	0.0451*
Triglyceride (mmol/l)	1.0 ± 0.07	1.2 ± 0.07	0.062
HDL cholesterol (mmol/l)	1.2 ± 0.04	1.1 ± 0.035	0.619
LDL cholesterol (mmol/l)	2.4 ± 0.13	2.6 ± 0.09	0.069
Total cholesterol/HDL	3.5 ± 0.13	4.0 ± 0.13	0.0361*
Others			
Total protein (g/l)	72 ± 2.2	75 ± 0.7	0.284
Serum albumin (g/l)	44 ± 1.3	45 ± 0.4	0.343
Serum globulin (g/l)	28 ± 1.0	30 ± 0.6	0.309
CRP (nmol/l)	44 ± 4.4	68 ± 10.0	0.0445*
C-peptide (pmol/l)	9 ± 5.0	15 ± 6.0	0.0132*

WHR, waist-to-hip ratio; HOMA-IR, homeostatic model of assessment–insulin resistance; TSH, thyroid-stimulating hormone; CRP, C-reactive protein.
Values are expressed as mean ± SEM.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$ versus controls.

Enumeration of circulating progenitor cells via flow cytometry

Blood was drawn in EDTA vacutainers (Becton Dickinson, USA). Following erythrocyte lysis [fluorescence-activated cell sorter (FACS) lysing solution, Becton Dickinson] and fixation, mononuclear cells in the lymphomonocyte fraction were morphologically gated based on forward and side scatter (please refer to representative flow cytogram in Supplementary data, Fig. S1A). A minimum of 5×10^5 events were scored as per the EUROSTAR guidelines (Distler *et al.*, 2009). Progenitor cells were analyzed for surface expression of CD133 and CD34 using direct two color flow cytometry. Samples were blocked with 5% fetal bovine serum and

FcR reagent and were stained with CD133-phycoerythrin (PE) and CD34-FITC antibodies from Miltenyi Biotech, Germany (Supplementary data, Fig. SIC). Corresponding isotype immunoglobulin G (IgG)1-PE and IgG2a-FITC (Miltenyi Biotech) antibodies were used as controls (Supplementary data, Fig. SIB). The instrument was optimized daily by analyzing the expression of anti-CD3 and anti-CD4 antibodies in peripheral blood. Data were processed using the Flowjo software program (Version 7.6.1). The FACS operator was blinded to the clinical status of the subjects. The data are represented as number of cells per million cytometric events.

Isolation and culture of peripheral blood mononuclear cells

The functional experiments were performed with mononuclear fractions obtained from four control and nine amenorrhoeic subjects from the study population. All the amenorrhoeic subjects analyzed for functional studies had polycystic appearing ovaries. Peripheral blood mononuclear cells (PBMCs) consisting of the lymphocyte and monocyte fraction were isolated by the Histopaque gradient centrifugation method (Sigma-Aldrich, USA). CD45-PerCP (BD Bioscience) was used to verify the purity of the isolated fraction and it was found to be greater than 95% for all the isolations (data not shown). Isolated mononuclear cells were cultured in fibronectin-coated (2.5 μg/ml) (Becton Dickinson) tissue culture plates in endothelial-specific EGM-2 Bullet kit medium (Lonza, USA). The first medium change was done on the 4th day following which the medium was changed once every 2 days. The endothelial differentiation potential of the PBMCs was assessed by dual staining with Ac-dil-LDL (10 μg/ml; Invitrogen) and fluorescein-conjugated Ulex-europaeus agglutinin I (10 μg/ml; Sigma-Aldrich). The expression of endothelial markers [endothelial nitric-oxide synthase (eNOS), CD31, Von Willebrand factor (vWF) and vascular endothelial (VE)-cadherin] was confirmed by immunofluorescence. Antibodies for eNOS, CD31 and VE-cadherin were from Santa Cruz (USA) and for vWF from Dako. Following 7 and 14 day culture, the medium was removed and cells were washed with 1× phosphate-buffered saline (PBS). Cells were fixed with 4% paraformaldehyde and were permeabilized with 0.25% Triton-X. Prior to addition of respective primary antibodies as per the manufacturer's instructions, blocking was done for 1 h with 3% bovine serum albumin. Respective FITC-conjugated secondary antibodies were used to visualize positive cells. Corresponding isotype control antibodies were used as negative control for immunofluoroscence. Images were analyzed using ImageJ software from NIH. eNOS expression is represented as an average eNOS intensity per cell. Intracellular nitric oxide (NO) in endothelial cells was measured via DAF2-DA imaging. DAF2-DA (Sigma-Aldrich) is a cell permeable dye which fluoresces upon binding to NO. Briefly cultured cells were incubated with 200 μM L-arginine for 3 min prior to addition of diaminofluorescein-2-diacetate (DAF-2DA). Images were captured using blue filter. Each experiment for immunofluoroscence was done in triplicates and for each well six to eight field views were assessed with a minimum of 100 cells in each field view.

Adhesion assay

Freshly isolated PBMCs were labeled with 4 μM cell tracker dye PKH26 from Sigma for 3 min. The reaction was stopped with complete growth medium containing 10% fetal bovine serum. Cells were washed three times with PBS and were seeded at a density of 10 000 cells/well in a 24-well plate coated with fibronectin (2.5 μg/ml), gelatin (1%w/v) or collagen (0.1% w/v). After 3 h of incubation, non-adherent cells were removed by washing and images were taken using an immunofluoroscence microscope (Olympus). Each sample was assessed in triplicates and for each well four field views were assessed. Viability of cells prior to and after adhesion experiment in parallel plates were assessed with the Trypan blue exclusion assay and was found to be greater than 95% for the total number of cells counted in each group (data not shown). At the time of standardizing, the assay we used a different number of cells in the linear range (1000–100 000) for initial seeding on matrix proteins. Best results were obtained when the seeding density on 24-well plates was 10 000/well. With increased number of 50 000–100 000 cells per well, the cells tended to clump together and assessment became difficult. Adhesion is represented as percentage of adhered cells compared with control subjects.

Statistical analysis

Clinical data are expressed as mean ± SEM. Progenitor cells are expressed as mean ± SEM of the number of cells counted per 1 million cytometric events. Parametric analysis was performed with log-transformed values of cell numbers where ever required. Comparisons within and across groups were performed by paired and unpaired Student's *t*-test, respectively. Correlations of clinical parameters with progenitor cells were assessed by Pearson's (*r*) correlation analysis. To determine factors which correlate with cell counts irrespective of the morphological state of the ovary a merged group analysis was done. In order to see if there is a stronger link between progenitor mobilizations with biochemical features in polycystic ovary patients, group-wise correlation analysis was subsequently performed. No corrections were made for multiple comparisons. Statistical significance was accepted at $P < 0.05$. Statistical analyses were done by SPSS (Version 15.1).

Results

Characteristics of study population

Upon application of the 2003 consensus Rotterdam screening criteria to the 44 recruited amenorrhoeic subjects, 38 exhibited the non-classical PCOS phenotype of PCOs with no signs of clinical or biochemical hyperandrogenism while the others had normal appearing ovaries. As seen in Table I, compared with the controls, the amenorrhoeic subjects had higher body weight, BMI and waist and hip circumference. They also had higher fasting glucose and insulin levels and were insulin resistant as indicated by the HOMA-IR ($P < 0.0001$). They did not exhibit hyperandrogenism but had lower estrogen and progesterone levels and higher LH levels than controls. Total cholesterol, total cholesterol-to-HDL ratio, CRP and C-peptide levels were higher in amenorrhoeic women (Table I), while the prolactin levels and thyroid function were normal (data not shown). Amenorrhoeic subjects with single cysts were also insulin resistant and had higher fasting insulin values (Supplementary data, Table SI). Additionally, they had reduced estrogen levels as opposed to controls.

Circulating levels of progenitor cells in control versus amenorrhoeic group

At fasting the amenorrhoeic group had significantly fewer of all three $CD133^+$, $CD34^+$ and $CD133^+CD34^+$ cell types than the controls (Fig. 1). In the controls 1 h after taking 75 g of glucose, there was a 2-fold increase in $CD133^+$ cells which dropped by 40% at 2 h (Fig. 1A). Intriguingly, this post-load increase for $CD133^+$ cells was significantly attenuated in amenorrhoeic subjects. Similarly for $CD133^+CD34^+$ cells, there was a progressive increase in cell counts upon glucose challenge in controls which was absent in

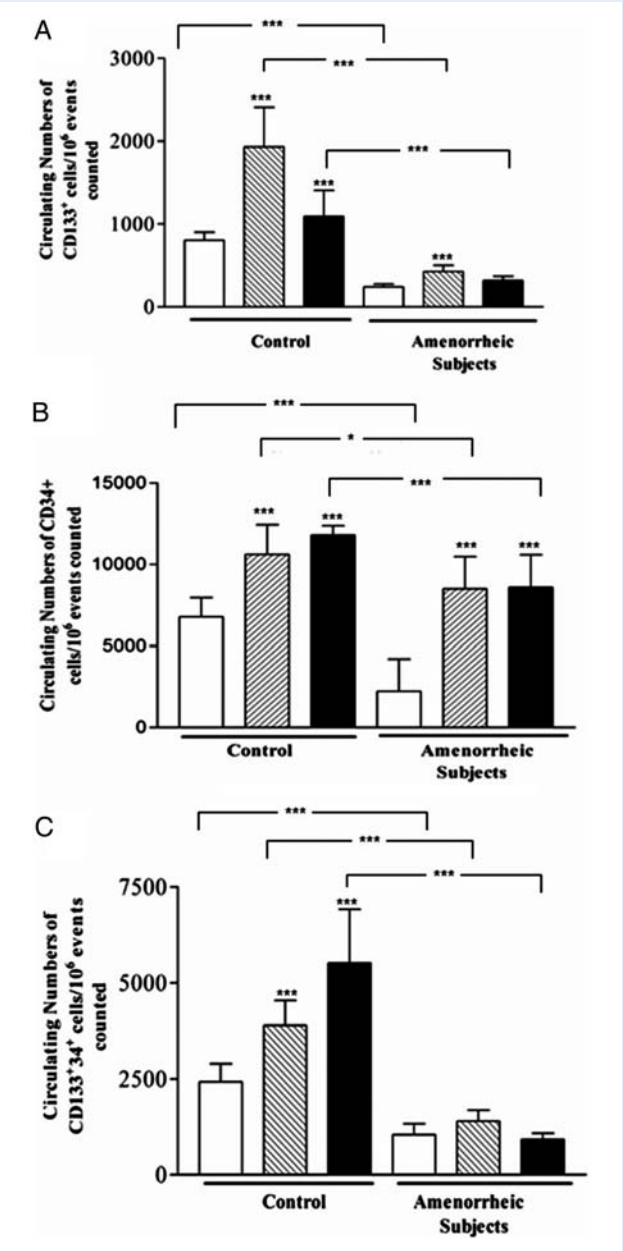

Figure 1 Circulating progenitor cells in amenorrhoeic study subjects following glucose challenge. (**A**) $CD133^+$ cells (**B**) $CD34^+$ cells and (**C**) $CD133^+CD34^+$ cells after fasting and after oral administration of 75 g glucose (1 h PL and 2 h PL). Bar graphs summarize data as mean ± SEM; $^*P < 0.05$ and $^{***}P < 0.001$ represents statistical significance in paired and unpaired Student's *t*-test. The emptied box represents fasting, box with cross line represents 1 h post-load and the filled box represents 2 h post-load.

amenorrhoeic subjects (Fig. 1C). For $CD34^+$ cells, the increase in cell numbers in response to glucose load was slightly attenuated but not blocked in amenorrhoeic women (Fig. 1B). Upon segregation of amenorrhoeic subjects based on ovarian morphology, fasting $CD133^+$ and $CD133^+CD34^+$ cell counts were much lower in subjects with PCOs than with those having single cyst (Supplementary data, Fig. SIIA and C). A partial increase in $CD133^+$ cell counts was observed in single cyst amenorrhoeic women in response to glucose load at 1 h. However even this increase was lost in polycystic women. No major differences were seen for $CD34^+$ cells in single versus polycystic subjects (Supplementary data, Fig. SIIC).

Table II Pearson's correlation analysis of progenitor cells in the merged study group.

Cell types versus parameters	Correlation coefficient (*r*)	*P*-value
Cell numbers		
FCD133 versus FCD133/34	0.263	0.018*
FCD34 versus FCD133/34	0.649	<0.0001***
1 h CD133 versus 1 h CD34	0.467	<0.0001***
1 h CD133 versus 1 h CD133/34	0.621	<0.0001***
1 h CD34 versus 1 h CD133/34	0.699	<0.0001***
2 h CD133 versus 2 h CD34	0.578	<0.0001***
2 h CD133 versus 2 h CD133/34	0.593	<0.0001***
2 h CD34 versus 2 h CD133/34	0.711	<0.0001***
Morphometric parameters		
FCD34 versus weight	−0.214	0.057
FCD34 versus BMI	−0.206	0.068
Hormones		
FCD133 versus estrogen	0.205	0.070
FCD34 versus estrogen	0.249	0.027*
FCD133/34 versus Estrogen	0.217	0.055
Proteins		
FCD34 versus CRP	−0.310	0.007*
FCD133/34 versus C-peptide	−0.306	0.008*
FCD34 versus C-peptide	−0.286	0.010*
FCD133/34 versus CRP	−0.264	0.018*

F, fasting cell count; 1 h, one hour post-load cell count and 2 h, two hour post-load cell count.
The cell numbers were log transformed for Pearson's correlation analysis.
$^*P < 0.05$.
$^{**}P < 0.01$.
$^{***}P < 0.0001$.

Correlation analysis

Upon merging the two study groups (36 controls and 44 amenorrhoeic), the log-transformed values of cell numbers were subjected to Pearson's correlation analysis. A positive correlation was observed for $CD133^+$ and $CD34^+$ cells with double positive $CD133^+CD34^+$ cells both during fasting and post-load conditions (Table II). Fasting counts of all three cell types exhibited positive correlation with estrogen (Table II). We also observed inverse correlation of fasting $CD34^+$ cells with risk factors of metabolic syndrome, i.e. weight, BMI, C-peptide and CRP. Similarly, fasting $CD133^+CD34^+$ cell counts inversely correlated with CRP and C-peptide.

We subsequently carried out group wise Pearson's correlation analysis (Supplementary data, Table SII). Since the distributions of data were parametric in each of the groups, raw cell values were correlated with biochemical parameters. Among amenorrhoeic subjects, fasting

Table III Group wise Pearson's correlation analysis of fasting cell counts with clinical and biochemical parameters.

Parameters	Control ($n = 36$)		Polycystic subjects ($n = 38$)	
	Correlation coefficient (r)	P-value	Correlation coefficient (r)	P-value
$CD34^+$ cells				
C-peptide	−0.469	0.004**	−0.202	0.225
LH	−0.031	0.861	−0.341	0.036*
FSH	0.220	0.190	0.448	0.0001***
$CD133^+$ cells				
Estradiol	0.104	0.552	0.360	0.002**
$CD133^+CD34^+$ cells				
LH	−0.092	0.172	−0.327	0.045*
FSH	0.139	0.426	0.339	0.003**
CRP	−0.316	0.064	−0.380	0.034*
C-peptide	−0.049	0.770	−0.346	0.039*

The raw cell numbers in each of the study group were correlated with biochemical parameters.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.0001$.

$CD133^+$ cells positively correlated with estrogen and FSH, and negatively correlated with HOMA-IR, C-peptide, testosterone, total cholesterol and LDL cholesterol. Upon exclusion of six single cyst subjects from analysis (Table III), fasting $CD34^+$ and $CD133^+CD34^+$ cell counts positively correlated with FSH and negatively correlated with LH and C-peptide for polycystic subjects.

Culture characteristics of PBMCs from amenorrhoeic subjects

We next sought to determine the adhesion and endothelial differentiation potential of mononuclear cells from four healthy and nine amenorrheic polycystic subjects upon culture in the presence of endothelial differentiation medium EGM-2. Adhesion to fibronectin, but not to gelatin or collagen, was significantly attenuated in study subjects, (Fig. 2). Also, the expression of eNOS was significantly lower in study subjects than controls at Day 7 ($P = 0.04$) and the NO index measured as DAF-2DA fluorescence compared with controls was significantly attenuated (Fig. 3). The expression of other markers (vWF, PECAM-1 and VE-cadherin) and Ac-dil-LDL uptake and Ulex lectin binding was similar in the amenorrheic and control groups (data not shown).

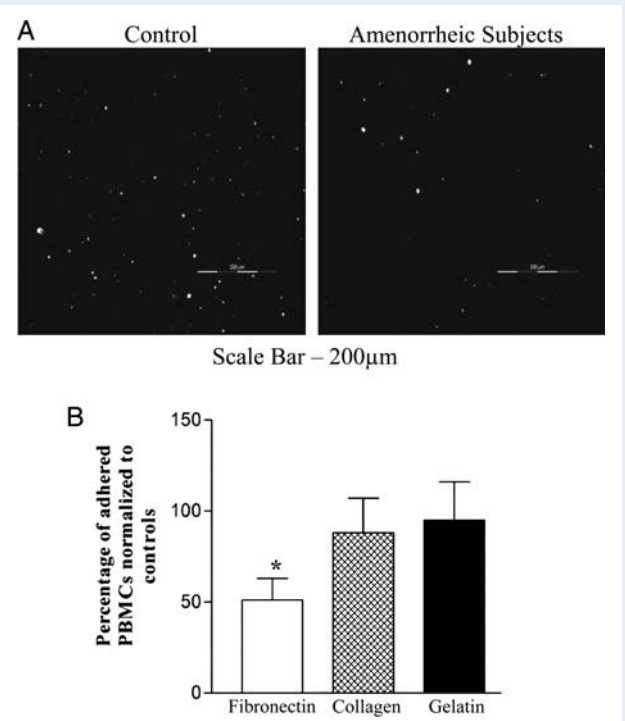

Figure 2 Adhesion potential of isolated PBMCs on extracellular matrices. (**A**) Immunofluorescence images of adhered PBMCs on fibronectin. (**B**) Bar graph summarizing percentage of PBMCs adhered per field of view normalized to control on the mentioned matrices. Results are expressed as mean ± SEM. *$P < 0.05$ versus control. The scale bar for Fig. 2A corresponds to 200 μm.

Discussion

In the current study, we report a decrease in fasting levels of circulating $CD133^+$, $CD34^+$ and $CD133^+CD34^+$ cells in insulin resistant amenorrhoeic subjects. Fasting counts for $CD133^+$ and $CD133^+CD34^+$ cells were significantly less in subjects with PCOs as opposed to single cyst amenorrhoeic or control women. Aging restricts the bone marrow reserve of progenitor cells and also compromises their mobilization (Heiss *et al.*, 2005) hence the study was performed with young age-matched individuals. Reasons for decreased fasting cell counts of bone marrow-derived stem cells can be either decreased bone marrow reserve of these cells to begin with or their defective mobilization. We also observed a surge in circulating levels of these bone marrow-derived stem cells in response to glucose load in healthy females, which was however attenuated for $CD133^+$ and

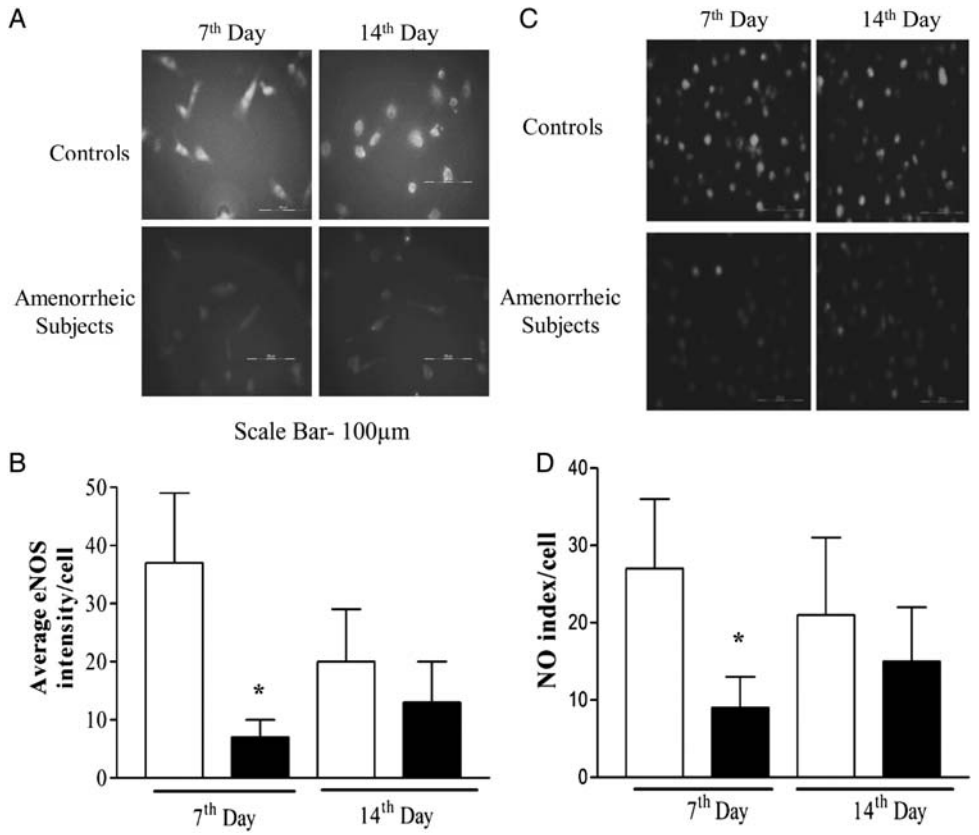

Figure 3 Comparison of eNOS expression and NO index on 7th and 14th day of culture. (**A**) Representative immunofluoroscence picture for eNOS staining in cultured cells obtained from control ($n = 4$) and amenorrheic polycystic subjects ($n = 9$). (**B**) Bar graph summarizing average fluorescence intensity per cell. (**C**) Representative picture depicting NO levels through DAF-2DA fluorescence. (**D**) Bar graphs summarizing densitometry analysis of DAF-2DA fluorescence as mean ± SEM. *$P < 0.05$ versus control. The emptied box represents controls and the filled box represents study subjects. The scale bar for Fig. 3A corresponds to 100 μm.

$CD133^+CD34^+$ cells in amenorrhoeic women. A positive correlation was observed between $CD133^+$ and $CD133^+CD34^+$ cells and between $CD34^+$ and $CD133^+CD34^+$ cells at all time points in accordance with previous studies showing that they all are a subset of a common bone-marrow pool (Fadini *et al.*, 2006). To the best of our knowledge, this is the first study to demonstrate that the glucose induced increase in bone marrow-derived stem cells in healthy women is attenuated in insulin resistant amenorrhoeic women. Based on these observations, we propose that post-prandial glucose peaks in healthy females may induce periodic mobilization of bone marrow-derived stem cells to the female reproductive tract for suitable endometrial wall buildup and IR will compromise this process in polycystic amenorrhoeic women. Mechanisms responsible for blunted glucose-induced kinetics are currently unknown. It can however be envisaged that IR alone or in combination with metabolic imbalance such as altered lipid profile alters mobilization of stem cells from bone marrow in to the circulation.

Fasting cell counts of all the three cell types positively correlated with estrogen in this study. Estrogen receptors are expressed in bone marrow-derived $CD34^+$ cells and circulating EPCs and play a major role in estrogen-mediated endometrial angiogenesis (Masuda *et al.*, 2007; Foresta *et al.*, 2010). *In vitro* culture experiments with progenitor cells from amenorrheic polycystic subjects demonstrated reduced eNOS expression and NO index. It should be noted that eNOS-derived NO plays an essential role in estrogen-mediated angiogenesis (Kim and Bender, 2009; Jesmin *et al.*, 2010). Additionally, isolated EPCs from study subjects had reduced adherence to fibronectin. In the light of these observations and the recent study demonstrating reduced tube formation by vascular progenitor cells obtained from non-obese PCOS subjects (Dessapt-Baradez *et al.*, 2011), it is tempting to speculate that compromised endothelial differentiation of circulating progenitor cells and their blunted kinetics will greatly hamper neo-vascularization and angiogenesis in the female reproductive tract. Whether IR affects the responsiveness of these progenitor cells towards estrogen in polycystic patients however remains to be determined.

We also observed a positive correlation of $CD34^+$ and $CD133^+CD34^+$ cells with FSH and a negative correlation with LH in polycystic subjects. It is hence tempting to propose that bone marrow-derived stem cells play an important role in follicle maturation and/or ovulation. Appropriate angiogenesis is also a prerequisite for follicle maturation, and ovulation in female reproduction (Masuda

et al., 2007; Demir *et al.*, 2010; Foresta *et al.*, 2010). For example maturation of oocytes in ovarian follicles in response to FSH is accompanied by increased angiogenesis (Gougeon, 2010). Similarly following ovulation in response to LH surge, the luteinizing follicle exhibits vascularization which is assessed as CD34 positive immunostaining (Suzuki *et al.*, 1998). Even the cumulus oocyte complex which surrounds the released oocyte expresses CD34 and the expression of CD34 increases right before ovulation (Hernandez-Gonzalez *et al.*, 2006). Hence it is likely that bone marrow-derived stem cells home into the maturing follicle to support angiogenesis and/or ovulation. Alternatively, it is likely that these bone marrow-derived stem cells are a subset of bone marrow-derived germ cells. This contention is based on recent studies demonstrating that in chemo-ablated female mice receiving bone marrow or peripheral blood cell transplantation, donor-derived oocytes or ovarian follicles are detected in the recipient's ovaries (Johnson *et al.*, 2005; Lee *et al.*, 2007). In contrast in chemo-ablated mice which do not undergo transplant, the ovaries consist of cystic follicles.

Obesity, IR, hyperinsulinemia and systemic inflammation are hallmark features of PCOS (Dunaif *et al.*, 1989; Castelo-Branco *et al.*, 2010; Cho *et al.*, 2011; de Groot *et al.*, 2011; Herlihy *et al.*, 2011). We observed inverse correlation of $CD34^+$ and $CD133^+CD34^+$ progenitor cells with BMI, weight, C-peptide and CRP thus establishing a link between progenitor cells and low-grade chronic inflammation and metabolic imbalance in these subjects. IR is associated with oxidative stress and pro-inflammatory cytokines which can accelerate cellular senescence (Anderson *et al.*, 2001). Unfortunately, neither the oxidative stress nor the apoptosis was scored for these cells in this study. Another limitation of this study is that the amenorrhoeic subjects conform to the non-classical phenotype of PCOS which exhibits anovulation with PCOs without signs of clinical or biochemical hyperandrogenism (Rotterdam ESHRE/ASRM sponsored PCOS consensus workshop group, 2004). This phenotype of PCOS has a lower Ferriman-Gallaway score. Whether attenuation in glucose induced progenitor kinetics is also observed in classical PCOS subjects remains to be seen. It is also not known whether these defects in progenitor kinetics occur in other ethnic groups. Hyperandrogenism has been shown to have an ethnic distribution, with classic hyperandrogenemia being less reported in Asian subjects such as Thai and Japanese women (Iwasa *et al.*, 2007; Vutyavanich *et al.*, 2007). Unfortunately, well defined studies on presentation of hyperandrogenism in the Indian subcontinent are currently missing.

In conclusion, we demonstrate that fasting counts of circulating bone marrow-derived $CD34^+$, $CD133^+$ and $CD133^+CD34^+$ stem cells are reduced in insulin resistant non-classical polycystic women exhibiting amenorrhea. Moreover, the glucose-induced increase for $CD133^+$ and $CD133^+CD34^+$ is blocked in amenorrhoeic women and cultured stem cells from these polycystic subjects have defective fibronectin adherence, reduced eNOS expression and intra-cellular NO levels. It is however imperative to determine using experimental animals, whether bone marrow-derived stem cells indeed home into the female reproductive tract following glucose challenge. Should these experimental studies demonstrate homing of bone marrow-derived stem cells to endometrium, then one could employ a simple oral glucose challenge test to assess mobilization of stem cells and their *in vitro* culture characteristics as means to score for abnormal endometrial buildup or anovulation in polycystic ovary patients.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

S.B. executed the study and analyzed the data. J.G. planned the study, screened patients and analyzed data. A.A.N. and S.S.B. analyzed data. N.P.K. acquired FACS data and M.D. planed the study, reviewed the data and wrote the manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

None declared.

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