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Caspase 3 activation and PARP cleavage in lymphocytes from newborn babies of diabetic mothers with unbalanced glycaemic control

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Several epidemiological studies showed that gestational diabetes mellitus is the most frequent metabolic disorder of pregnancy, the pathogenesis of which has yet to be completely clarified.

The aim of this study was to investigate the presence and processing of caspase 3 (Casp3) and poly(ADP-ribose) polymerase 1 (PARP1) in cord blood lymphocytes as markers of apoptosis in relation to glycaemic control during intrauterine life.

Our results showed a specific positive correlation between the levels of active Casp3 (17–19 kDa) and the inactive form of PARP1 (89 kDa) in lymphocytes isolated from newborn babies of diabetic women with unbalanced glycaemic control, with a direct correlation between the activation of casp3 and the inactivation of PARP1, that makes lymphocytes unresponsive towards lipopolysaccharide stimulation, highlighting an altered functional response.

Besides more studies are required to fully correlate the activation of the apoptotic process during the intrauterine life with the foetal health later in life, our study indicates that a cord blood lymphocyte, an easily accessible source, is informative about the activation of apoptotic stimuli in circulating cells of newborn babies in relation to the glycaemic control reached by the mother during pregnancy.

KEY WORDS—caspase 3; PARP; lymphocytes; gestational diabetes mellitus

INTRODUCTION

Gestational diabetes mellitus (GDM), defined as a carbohydrate intolerance of varying severity developing or first recognized during pregnancy, is the most frequent metabolic disorder of pregnancy, occurring in 1–10% of all pregnancies.[1] GDM is considered a heterogeneous disease, and the pathogenesis of which has yet to be completely clarified.[2–4]

It is well established that an optimal regulation of maternal glucose levels continues to be a challenge in controlling perinatal morbidity. In fact, the failure in achieving early glycaemic control in maternal metabolism leads to the activation of apoptotic stimuli that could contribute to the development of birth defects.[5–8]

Although the apoptosis process is highly needed in an appropriate location and temporal pattern in the physiology of pregnancy,[7] the available data showed an higher incidence of terminal deoxyuridine triphosphate nick-end labelling-positive nuclei and lower expression of the anti-apoptotic protein Bcl-2 in placental villous trophoblasts in GDM groups compared with the normal glucose tolerant pregnant women.[8] Additionally, authors showed that hyperglycaemia up-regulates p53, triggering the mitochondrial death cascade pathway in the human placenta[9] and increasing the rate of apoptosis in cultured trophoblast cell lines.[10]

Caspase, a family of cysteinyl aspartate-specific proteases highly conserved in multicellular organisms, is the central regulator of apoptosis. A member of this family, caspase 3 (Casp3), has been identified as a key mediator of apoptosis in several cell types.[11–13] The activation of Casp3 orchestrates the demolition of the cell by cleavage of several key proteins required for cellular functioning and survival,[14] including the poly(ADP-ribose) polymerase 1 (PARP1) enzyme.[15,16] PARP1 is involved in the detection of strand

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breaks and signalling in both the base excision repair and nucleotide repair pathways. PARP1, the prototype and the most abundantly expressed member of a family of poly (ADP-ribose) polymerases,[17] is made up of three functional domains including DNA-binding domains (DBD), automodification domain (AMD) and catalytic domains (CD). The DBD contains zinc fingers that can bind to DNA breaks, and contains the nuclear localization signal, which ensures the translocation of PARP1 into the nucleus.[18] Poly(ADP-ribose) polymerase 1 enzyme (PARP) is specifically cleaved into 89 and 24-kDa fragments that contain, respectively, the active site and the DBD of the enzyme during apoptosis mediated by Casp3 in a variety of cells. This cleavage inactivates the enzyme by destroying its ability to respond to DNA strand breaks.[19,20]

The objective of our study was to investigate the expression and processing of Casp3 and PARP1 enzymes in cord blood lymphocytes from newborn babies in relation to the glycaemic control reached by the diabetic mothers.

PATIENTS AND METHODS

Patients

In the present study, eligible participants were glucose normotolerant pregnant women and pregnant women with GDM, attending to the Perinatal and Reproductive Centre, Department of Obstetrics and Gynaecology, University of Perugia (Italy).

All pregnant women were eligible to participate unless they had one or more of the following exclusion criteria: age less than 18 years and more than 45 years, conception using gonadotropin ovulation induction or by *in vitro* fertilization, smokers, hypertension or renal failure.

The GDM diagnosis was carried out following the new criteria set up by the Hyperglycaemia and Adverse Pregnancy Outcome study.[21–23] After the enrolment, all the participants were divided into two groups: (1) control group (CG), glucose normotolerant pregnant women ($n = 23$, code CG1–CG23); (2) diabetic group (DG), pregnant women with GDM ($n = 24$, Code DG1–DG24). Until delivery, pregnant women with GDM were routinely examined and subjected to the metabolic control on the basis of three critical points:

1. *Target blood glucose*. In GDM, blood sugar levels were controlled by monitoring fasting blood glucose and after 1 h the start of main meals.
2. *Treatment*. All pregnant women with GDM were treated with diet therapy: nutrient was calculated according to the international guidelines on the basis of pre-pregnancy body mass index (BMI). The intake of carbohydrates should not be less than 45–50%. Insulin was initiated if the fasting glucose level was $>5{\cdot}5\ \mathrm{mmol\,l^{-1}}$ or 1-h post-prandial glucose was $>7\ \mathrm{mmol\,l^{-1}}$.
3. *Metabolic management of delivery*. The peri-partum blood glucose was maintained between 70 and 100 mg $\mathrm{dl^{-1}}$. In the case of planned caesarean section (CS) or pharmacological induction of delivery, it was started an intravenous infusion of insulin and glucose solution (two ways) when fasting was prolonged for more than 8 h to avoid ketosis by fasting.

All pregnant women with GDM were divided into two subgroups: (a) diabetic group non-follow-up (NFU), constituted by those that did not fullfill the aforementioned three critical points of the metabolic control (DG-NFU, $n = 11$); (b) diabetic group follow-up (FU), constituted by those that reached a good glycaemic control (DG-FU, $n = 13$). The criterion for the inclusion of the mothers in FU or NFU group was the attainment of the therapeutic objective. For patients with GDM, the main goal of treatment is a fasting glucose $<95\ \mathrm{mg\,dl^{-1}}$, 1-h post-prandial glucose $<140\ \mathrm{mg\,dl^{-1}}$, and 2-h post-prandial $<120\ \mathrm{mg\,dl^{-1}}$.[24]

The CG comprised age-matched healthy pregnant women with normal glucose tolerance subject to the same exclusion criteria as those with diabetes.

Informed consent was obtained from all participants involved in this study. The study protocol has been approved by the institutional committee on human research.

Lymphocytes isolation from umbilical cord blood

An amount of 10 ml of cord blood was obtained from normotolerant pregnant women at term and from pregnant women with GDM at term, with vaginal delivery or CS; samples were collected in ethylenediaminetetraacetic acid tubes in the delivery room.

Cord blood was diluted with phosphate buffered saline (PBS), without Ca^{2+}/Mg^{2+}, containing 2 mM ethylenediaminetetraacetic acid and subjected to density gradient centrifugation (800 *g*, 20 min) using by Lympholyte® medium according to the manufacturer's protocol (Cederlane laboratories Ltd, Paletta Court, Burlington, Ontario, Canada). Peripheral blood mononuclear cells (PBMCs) were subsequently used for the negative selection of lymphocytes using a $CD14^+$ miniMACS isolation kit (Miltenyi Biotec GmbH Friedrich-Ebert-Straβe 68, Bergisch Gladbach, Germany). Lymphocytes were collected as cell population remaining from PBMCs after depletion of $CD14^+$ monocytes.

Cell extract and protein content

Lymphocytes obtained from cord blood were washed in PBS and resuspended at a concentration of 4×10^6 cells $\mathrm{ml^{-1}}$ in hypotonic sodium phosphate buffer 10 mM, pH 6·0, containing the detergent Nonidet NP-40 at final concentration of 0·1% (v/v) and sonicated using a Measuring and Scientific Equipment (MSE) ultrasonic apparatus (MSE (UK) Ltd, Worsley Bridge Road, Lower Sydenham, London) at a frequency of 10 MHz. The samples so treated were put on ice for 90 min and then used for protein analysis.

The total protein amount was determined by the Bradford's method by using the Bio-Rad assay kit and the bovine serum albumin as standard.[25]

Polyacrylamide gel electrophoresis and Western blotting

Proteins (15 μg) were separated by 12% or 8% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and

subjected to immunoblots to evaluate Casp3 and PARP, respectively, by using primary antibodies contained in the apoptosis sampler kit (#9915, Cell Signalling Technology).

Immunostaining was made using the enhanced chemiluminescence Western blotting detection reagents and analysis system (GE Healthcare, UK). The protein quantification was assessed using the β-actin (goat polyclonal antibody, Santa Cruz Biotecnology) as an internal control.[26] Densitometric analysis was performed using the Adobe Photoshop CS4™ programme.

Cell cultures and D-glucose treatments

Cord blood lymphocytes obtained from glucose normotolerant pregnant women were divided into the following groups: (1) normal glucose group incubated in Roswell Park Memorial Institute (RPMI) 1640 containing 5·6-mM glucose as controls; (2) intermittent high-glucose (IHG) group incubated in RPMI 1640 with 50 or 5·6-mM glucose, alternatively, at the interval of 12 h; (3) mannitol group incubated with 5·6-mM glucose or 44·4-mM mannitol plus 5·6-mM glucose alternatively at the interval of 12 h, as osmotic control. Cells were harvested and washed three times with PBS after 24 h of culture. At this time point, we have analysed Casp3 and PARP expression and processing by immunoblotting as described. All the glucose used in the present study was D-glucose. All experimental groups were cultured in quadruplicate.

Proliferative function of lymphocytes

Cord blood lymphocytes obtained from CG, DG that failed (DG-NF) or reached a good glycaemic control (DG-FU) and lymphocytes from CG treated by culture '*in vitro*' with intermittent high glucose (IHG) were placed in 96-well flat-bottomed microplates in triplicate at 2×10^5 cells well^{-1}, in 200 µl well^{-1} of RPMI 1640 culture medium containing 10% foetal calf serum, and then lipopolysaccharide (LPS, from Sigma, St. Louis, MO, USA) was added to the wells at a final concentration of 20 µg/l.[27,28] Serum-free RPMI 1640 medium was used as a control. For cell cycle analysis, cells were harvested, washed with PBS and fixed in 75% ethanol for 30 min at room temperature. Prior to analysis, cells were treated with RNAse for 30 min at 37 °C to remove RNA and then incubated with 20-mg l^{-1} propidium iodide for 30 min in the dark. Then the samples were analysed by a flow cytometer (EPICS XL-MCL, Beckman Coulter). Data from three independent experiments are presented as mean ±standard deviation.

Statistical analysis

Statistical analysis of Casp3 and PARP expressions, and activation in the CG and DG populations was performed using the Mann–Whitney *U*-test.

Differences in active Casp3, full-length PARP1 or inactivated PARP1 (89 kDa) between the CG and diabetic populations subgrouped in DG-FU and the DG-NF were analysed by the one-way ANOVA with Newman–Keuls test for the multiple comparison test (GraphPad Prism 5).

The correlation between the variables Casp3 and PARP 116 or 89 kDa has been investigated through the analysis of the coefficient of determination (r^2) and the Pearson index (r Pearson) (Golden Software Grapher 8™), where appropriate data are presented as mean ± standard deviation. A $p < 0{\cdot}05$ was considered to be significant.

RESULTS

Population

The study sample consisted of 47 pregnant women divided into two groups: 23 patients were the CG characterized by normal glucose tolerance, and 24 were diabetic patients. The characteristics of the two groups (mother/newborn couple) are summarized in Table 1. The CG tended to be younger ($33{\cdot}34 \pm 6{\cdot}11$ years) than the DG ($36{\cdot}08 \pm 3{\cdot}97$ years), and the gestational age at delivery was higher in normal pregnancies (CG, $38{\cdot}45 \pm 1{\cdot}37$ weeks of gestation; DG, $37{\cdot}99 \pm 1{\cdot}32$ weeks of gestation). The BMI of the CG was normal ($21{\cdot}83 \pm 2{\cdot}82$ kg m^{-2}) instead of DG's BMI that has shown an overweight condition ($27{\cdot}07 \pm 6$ kg m^{-2}).

Foetal weights resulted as follows: CG, $3{\cdot}29 \pm 0{\cdot}45$ kg; DG, $2{\cdot}95 \pm 0{\cdot}39$ kg, and a lower foetal/placenta weight ratio (CG, $5{\cdot}78 \pm 0{\cdot}43$; DG, $5{\cdot}28 \pm 0{\cdot}75$) was found in pregnancies complicated by gestational diabetes.

Babies born predominantly by CS in the DG, whereas, in the CG, half of them born by CS and half by vaginal delivery.

On the basis of the critical points for the metabolic control mentioned in the aforementioned Patients section, DG group was divided into two subgroups according to the attainment of a good glycaemic control (DG-NFU and DG-FU), and the characteristics of two diabetic subgroups are summarized in Table 2.

Increased active Casp3 in lymphocytes from newborn babies of diabetic mothers

Figure 1A shows representative Western blotting results for Casp3 on normotolerant and diabetic women. Scatter dot plots of the precursor form (Figure 1B) and of the active form (Figure 1C) of Casp3 amount found in the CG and DG groups were also reported.

Table 1. Demographic summary of control group (CG) and diabetic group (DG)

	CG, mean ± SD	DG, mean ± SD
Number	23	24
Patient age (years)	$33{\cdot}34 \pm 6{\cdot}11$	$36{\cdot}08 \pm 3{\cdot}97$
BMI (kg m^{-2})	$21{\cdot}83 \pm 2{\cdot}82$	$27{\cdot}07 \pm 6$
Gestational age (weeks)	$38{\cdot}45 \pm 1{\cdot}37$	$37{\cdot}99 \pm 1{\cdot}32$
Newborn weight (kg)	$3{\cdot}29 \pm 0{\cdot}45$	$2{\cdot}95 \pm 0{\cdot}39$
Placenta weight (g)	$579{\cdot}52 \pm 45{\cdot}87$	$564{\cdot}16 \pm 70{\cdot}8$
Fetal weight/placenta weight	$5{\cdot}78 \pm 0{\cdot}43$	$5{\cdot}28 \pm 0{\cdot}75$

Data are expressed as mean ± SD.
SD, standard deviation; BMI, body mass index.

Table 2. Demographic summary of diabetic subgroups: diabetic group follow-up (DG-FU) and diabetic group non-follow-up (DG-NF)

	DG-FU	DG-NF
Number	13	11
Patient age (years)	36·17 ± 4·39	36·0 ± 3·69
BMI (kg m^{-2})	26·3 ± 4·64	27·7 ± 7·59
Gestational age (weeks)	38·7 ± 0·57	37·46 ± 0·91
Newborn weight (kg)	3·03 ± 0·4	2·986 ± 0·38
Placenta weight (g)	546·9 ± 76·74	584 ± 60·23
Fetal weight/placenta weight	5·59 ± 0·73	4·91 ± 0·63

The subdivision criteria are described in Patients and Methods section. Data are expressed as mean ± SD.
SD, standard deviation; BMI, body mass index.

As showed in Figure 1B, we did not find statistically significant variation in the Casp3 precursor form (35 kDa) amounts between the CG and the DG.

In particular, in the CG, we found only the precursor form of Casp3, whereas the active form (17–19 kDa) was not or was barely detectable along all samples investigated (Figure 1A, B and C). Opposite results arise from lymphocytes from newborn babies of diabetic mothers. As showed in Figure 1A, we found a variable level of active Casp3 ranging from samples in which it was not or was barely detectable (i.e. DG1, patient code) to samples in which we found a high amount of the active form (i.e. DG2 and DG3).

As showed in Figure 1C, we have found a statistically significant variation in the active form amounts between the CG (0·009 ± 0·004) and the DG (0·227 ± 0·09) (**$p < 0·01$ vs that of CG). It was evident that some of the diabetic patients expressed active Casp3 amounts ranging from 0 to 0·2 with respect to CG group, whereas the other samples ($n = 6$) showed higher active Casp3 amounts, ranging from 0·5 to 1·8. As reported in Figure 1D, in the DG, the relative levels of active Casp3 were statistically significantly different between lymphocytes isolated from newborn babies of diabetic mothers with unbalanced glycaemic control during pregnancy (DG-NF, 0·40 ± 0·16; **$p < 0·01$ vs that of CG) with respect to that isolated from newborn babies of diabetic mothers that reach a good glycaemic control during pregnancy (DG-FU, 0·063 ± 0·041; **$p < 0·01$ vs that of DG-NF) that showed an active Casp3 level comparable with CG.

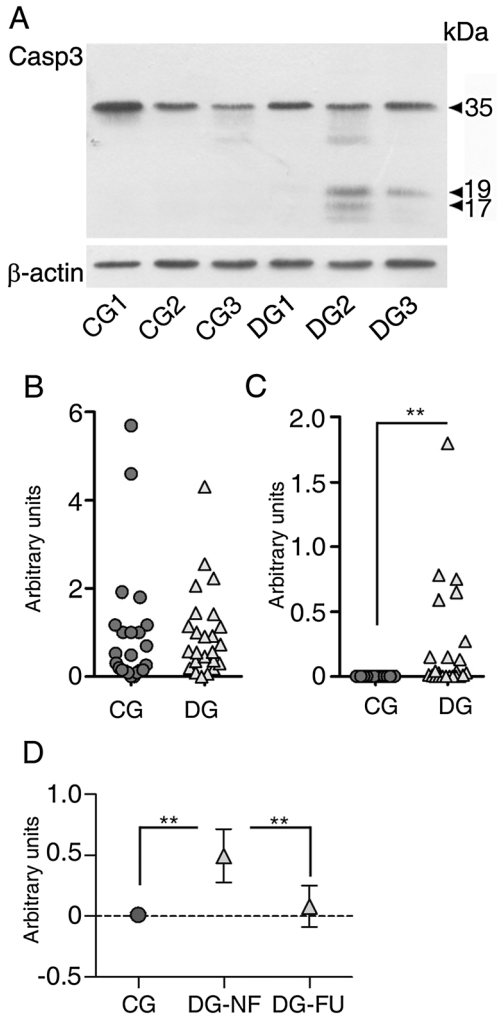

Figure 1. Analysis of caspase 3 (Casp3) levels in lymphocytes from newborn babies of diabetic mothers (DG) and from glucose normotolerant pregnant women (CG, control group). (A) Representative blots of the different situations that we have encountered during Western blotting analysis for CG and DG. CG1, CG2 and CG3 are representatives of samples from CG. DG1, DG2 and DG3 are representatives of samples from DG. Western blotting analysis was performed using β-actin as loading control. The 35-kDa band reveals the presence of full-length precursor of Casp3, whereas the 17–19-kDa band reveals the presence of active Casp3. (B) Scatter dot plot of the precursor form of Casp3 amount found in the CG (●) and DG (▲) groups. No statistically significant differences were found in inactive full-length Casp3 amount between two groups. (C) Scatter dot plot of the active form of Casp3 levels found in CG (●) and DG (▲) groups. (**$p < 0·01$ DG vs that of CG). (D) Relative levels of active Casp3 (17–19 kDa) between lymphocytes isolated from newborn babies of diabetic mothers with unbalanced glycaemic control during pregnancy (DG-NF) with respect to those of isolated from newborn babies of diabetic mothers that react a good glycaemic control during pregnancy (DG-FU). Results are expressed as means ± SD (**$p < 0·05$, DG-NF vs that of CG and DG-NF vs that of DG-FU). SD, standard deviation; PARP, poly(ADP-ribose) polymerase 1 enzyme

PARP inactivation in cord blood lymphocytes correlates with glycaemic control of mothers

To investigate the molecular mechanism by which Casp3 may exert a negative effect (or may produce a negative outcomes), we monitored the expression and processing of PARP, on the basis of the specific site of cleavage by which Casp3 mediates the inactivation of PARP,[18] catalysing the conversion of active full-length form of PARP1 (116 kDa) into the cleaved-inactive form (89 kDa).

In Figure 2A, there are summarized representative blots of PARP levels and processing; as already reported for Casp3 level, in the DG group we found heterogeneous levels of both the active and the inactive forms of PARP. In fact, the levels of the 116-kDa fragment were lower in the DG group (0·71 ± 0·059) with respect to that of the CG group (0·995 ± 0·024; Figure 2B, **$p < 0·01$), whereas no statistically significant difference between the two groups in the 89-kDa form levels was found (Figure 2C).

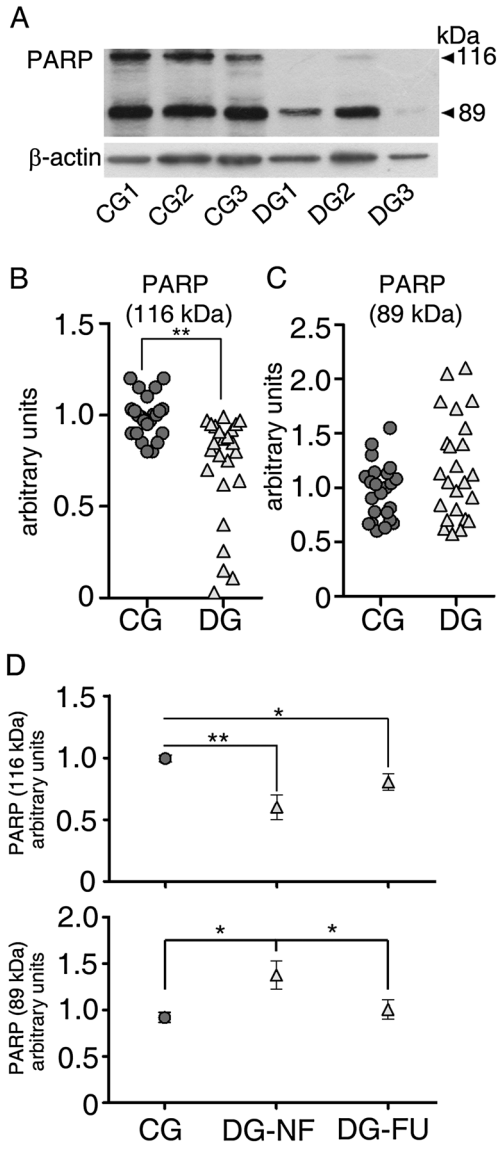

Figure 2. Analysis of poly(ADP-ribose) polymerase 1 enzyme (PARP) levels and processing in lymphocytes from newborn babies of diabetic mothers (DG) and from glucose normotolerant pregnant women (CG). (A) Representative blot of PARP levels and processing. CG1, CG2 and CG3 are representatives of samples from CG. DG1, DG2 and DG3 are representatives of samples from DG. Western blotting was performed using β-actin as loading control. (B) Scatter dot plot of the active full-length form of PARP1 (116 kDa) amount found in the CG (●) and DG (▲) groups (**$p < 0{\cdot}01$ DG vs that of CG). (C) Scatter dot plot of the cleaved-inactive form (89 kDa) amount found in the CG (●) and DG (▲) groups. No statistically significant differences were found in the levels of the 89-kDa fragment between the two groups. (D) Analysis of the active-PARP (116 kDa) levels based on the glycaemic control reached by the investigated newborn mothers. The amount of active PARP (116 kDa) was significantly lower in patients that failed the glycaemic control (DG-NF, **$p < 0{\cdot}01$ vs that of CG) than in patients reaching a good glycaemic control (DG-FU, *$p < 0{\cdot}05$ vs that of CG), (upper panel). The inactive form of PARP (89 kDa) was significantly higher in DG-NF with respect to those of the CG group (*$p < 0{\cdot}05$ vs that of CG) and DG-FU group (*$p < 0{\cdot}05$ vs that of DG-FU) (lower panel). Results are expressed as means ± SD. SD, standard deviation

The analysis of the amount of the active PARP1 (116 kDa) versus the glycaemic control reached by the mother of investigated newborn babies indicated that the level of active PARP1 was significantly lower in patients that failed the glycaemic control (DG-NF, $0{\cdot}60 \pm 0{\cdot}1$) with respect to that in CG ($0{\cdot}99 \pm 0{\cdot}12$, **$p < 0{\cdot}01$). We found an increased level of full-length PARP1 in DG-FU subgroup ($0{\cdot}80 \pm 0{\cdot}063$), with respect to that in the DG-NF($0{\cdot}60 \pm 0{\cdot}1$) although lesser than that in CG (*$p < 0{\cdot}05$ vs that of CG) (Figure 2D, upper panel).

On the contrary, the 89-kDa form of PARP arises significantly higher in DG-NF ($1{\cdot}37 \pm 0{\cdot}15$) with respect to that in the CG ($0{\cdot}92 \pm 0{\cdot}05$, *$p < 0{\cdot}05$ vs that of CG) and with respect to that in the DG-FU ($1{\cdot}01 \pm 0{\cdot}1$; *$p < 0{\cdot}05$ vs that of DG-NF; Figure 2D, lower panel).

Active Casp3 correlates with inactive PARP

To investigate the relation between the presence of active Casp3 and inactivated PARP1 in samples from DG, we employed a correlation study.

As reported in Figure 3A, a significant inverse correlation between active Casp3 level and active PARP was observed within the lymphocytes of newborn babies with diabetic mothers ($P = 0{\cdot}0001$, Pearson $r = -0{\cdot}889$ and $r^2 = 0{\cdot}791$). In fact, all samples that showed Casp3 in the active form showed also a reduction of full-length form of PARP1.

Moreover, we found a significant direct correlation between the active Casp3 level and the inactive form of PARP (89 kDa) in lymphocytes of newborn babies with diabetic mothers ($P = 0{\cdot}0048$, Pearson $r = 0{\cdot}556$ and $r^2 = 0{\cdot}31$) (Figure 3B).

High-glucose Casp3 and PARP inactivations in lymphocytes: in vitro study

To understand if PARP degradation by Casp3 arises directly from the hyperglycaemic maternal environment during pregnancy, we performed *in vitro* studies in which lymphocytes from cord blood of healthy donors were cultured in the presence of 5·6 or 50-mM glucose, alternatively, at the interval of 12 h.

By immunoblotting approach, performed after 24 h from treatment, we found that the relative levels of active Casp3 (Figure 4A) and inactive PARP (Figure 4B) were markedly increased in the IHG group compared with those in the group with normal glucose treatment ($1{\cdot}44 \pm 0{\cdot}153$-fold and $1{\cdot}67 \pm 0{\cdot}13$-fold increases, respectively, with respect to control cells; *$p < 0{\cdot}005$). There was no significant difference in levels of these proteins between the osmotic control mannitol-group and CG (Figure 4A and B).

Changes in lymphocyte proliferative function in DG-NF group

Because both Casp3 and PARP1 could influence the adaptive immune response,[29,30] we monitored the functional consequences of the altered levels of active Casp3 and inactive PARP1 in lymphocytes of newborn babies of diabetic mothers, monitoring cell proliferation after LPS

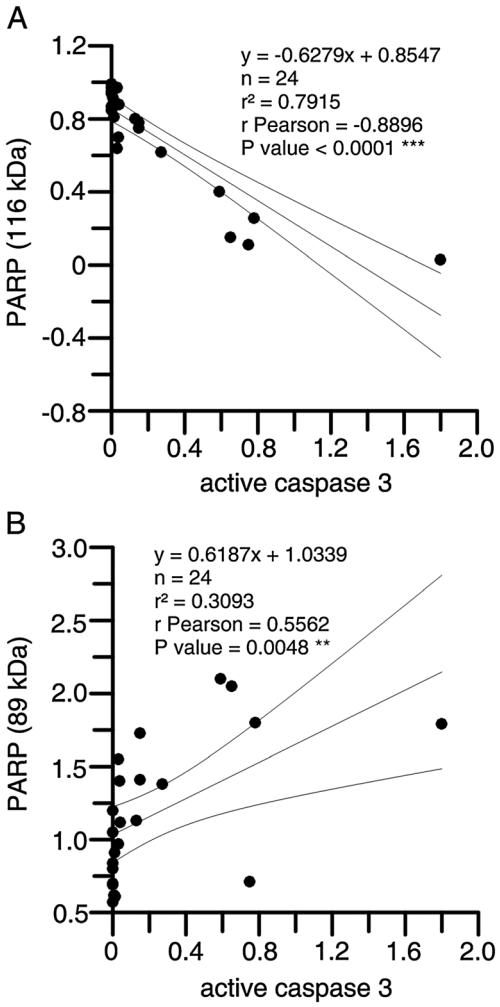

Figure 3. Correlation study between the levels of active caspase 3 (Casp3) and poly(ADP-ribose) polymerase 1 enzyme (PARP) in lymphocytes from newborn babies of diabetic mothers (DG) has been investigated through the analysis of the coefficient of determination (r^2) and the Pearson index (r Pearson). (A) Active PARP (116 kDa) inversely correlates with active Casp3 levels in cord blood lymphocytes from diabetic mothers ($n = 24$, $P = 0.0001$; Pearson $r = -0.8896$; $r^2 = 0.7915$). All samples that showed Casp3 in the active form showed also a reduction of full-length form of PARP. (B) Direct correlation between the active Casp3 level and the inactive form of PARP (89 kDa) was found in lymphocytes of newborn babies with diabetic mothers ($n = 24$, $P = 0.0048$; Pearson $r = 0.5562$; $r^2 = 0.3093$)

stimulation.[27,28] As expected, the stimulation of CG lymphocytes results in a statistically significant increased percentage of cells in the Gap2/Mitosis phase of the cell cycle (G2/M) with respect to that in the unstimulated cells ($53{\cdot}7 \pm 5{\cdot}2\%$ vs $37{\cdot}7 \pm 6{\cdot}7\%$, $p < 0{\cdot}05$; Table 3), with a corresponding decreased percentage of cells in the Gap0/Gap1 phase (G0/G1) ($28.8 \pm 4\%$ vs $46{\cdot}1 \pm 4{\cdot}5\%$, $^*p < 0{\cdot}05$, Table 3). We obtain similar results in lymphocytes from newborn babies of DG-FU group (G2/M, $49{\cdot}3 \pm 6\%$ vs $32{\cdot}1 \pm 3{\cdot}2\%$; G0/G1, $27{\cdot}6 \pm 4{\cdot}4\%$ vs $48{\cdot}7 \pm 3{\cdot}2\%$, $^*p < 0$ 05; Table 3). Interestingly, opposite results arise from newborn babies' lymphocytes from DG-NF. These cells were lesser prone to receive the proliferative stimulus by LPS. In fact, we do not observe a significant variation in both the G0/G1 or G2/M phase, although with an increased cell in the synthesis phase (S) with respect to the unstimulated lymphocytes ($32{\cdot}8 \pm 5{\cdot}1\%$ vs $19{\cdot}9 \pm 3{\cdot}1\%$, $^*p < 0{\cdot}05$; Table 3). Moreover, to monitor the relevance of the hyperglycaemic environment on the proliferative response of lymphocytes, we subjected lymphocytes from CG cultured in intermittent high-glucose condition to the LPS stimulation. Similarly to the DG-NF cells, we monitored no variation in the cell cycle distribution (Table 3).

DISCUSSION

In this work, we report, for the first time, a specific correlation between the activation of Casp3, the inactivation of PARP1 enzyme and an altered functional response of lymphocytes isolated from cord blood of newborn babies of GDM pregnant women with unbalanced glycaemic control.

We have chosen lymphocytes from cord blood to perform our investigation because these cells are informative, reproducible and sensible to the healthy status of donors as demonstrated in studies related to diabetes during pregnancy,[31–34] neurological[35,36] and metabolic disorders[37] and last but not least, they are easily accessible and can be collected without pain from newborn babies through non-invasive methods.

Our results showed a specific positive correlation between the levels of active Casp3 (17–19 kDa) and the inactive form of PARP1 (89 kDa) in lymphocytes isolated from newborn babies of diabetic women with respect to those of the controls, with a specific trend between the activation of Casp3 and the inactivation of PARP1 in relation to the glycaemic control reached by the mothers.

Growing body of literature described complex roles of caspases in influencing multiple aspects of the mammalian immune response;[29] the modulation of which is essential during implantation and throughout pregnancy.[38] To perform an investigation on functional consequences of our findings, we monitored the proliferation of lymphocytes in response to the stimulation induced by LPS. Our results showed that lymphocytes from CG and DG-FU respond to the stimulation with LPS. In fact, we monitored an increased percentage of cells in the G2/M phase of the cell cycle. Interestingly, the cells of the DG-NF group that showed a high amount of active Casp3 do not proliferate after LPS stimulation.

These results indicate that the activation of Casp3 and, in turn, the inactivation of PARP1 could alter the functional response of lymphocytes from newborn babies of DG-NF group.

Our results are in agreement with those previous reported by Woo *et al*. collaborators.[39] They demonstrate that in Casp3-deficient mice, as consequence in apoptosis defects, peripheral T cells showed an increased proliferation.[39] Moreover, they showed that Casp3 regulates cell cycle in B cells influencing the adaptive immune response by regulating B lymphocyte proliferation. In fact, loss of Casp3 in mice results in lymphadenopathy and splenomegaly because of hyperproliferation of both B and T lymphocytes.[40]

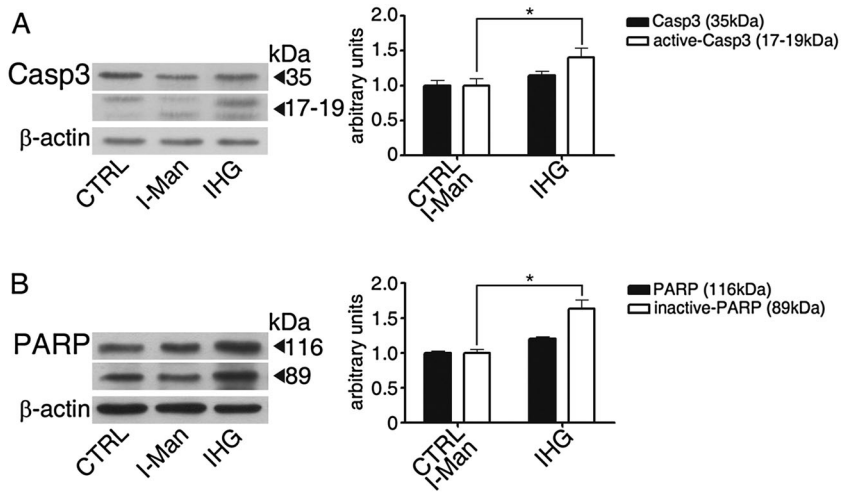

Figure 4. High-glucose treatment and caspase 3 (Casp3)/poly(ADP-ribose) polymerase 1 enzyme (PARP) processing in lymphocytes: *in vitro* study. We performed *in vitro* experiments in which lymphocytes from cord blood of healthy donors were cultured in the presence of 5·6 or 50-mM glucose, alternatively, at the interval of 12 h [intermittent high-glucose (IHG) treatment]. Mannitol group (I-Man) was incubated with 5·6-mM glucose or 44·4-mM mannitol plus 5·6-mM glucose alternatively at the interval of 12 h, as osmotic control. Cells were harvested after 24 h to analyse Casp3 and PARP levels and processing by Western blotting performed using β-actin as the loading control. Data were normalized with respect to control (CTRL)/I-Man-treated cells and are reported as fold changes with respect to untreated cells. (A) Representative blot of Casp3 levels and activation. Densitometric analysis of Casp3 (35 kDa) and active Casp3 (17–19 kDa) levels. Data from five independent experiments are presented as mean ± SD, $*p \leq 0{\cdot}05$ *versus* CTRL/I-Man. (B) Representative blot and densitometric analysis of PARP1 levels and processing after 24 h of cell culture under IHG treatment. Data from eight independent experiments are presented as mean ± SD, $*p \leq 0{\cdot}05$ *versus* CTRL/I-Man. SD, standard deviation

Table 3. Analysis of lymphocyte proliferative function: cell cycle distribution of lymphocytes from newborn babies cultured in the presence of lipopolysaccharide (LPS)

	CG		DG-NF		DG-FU		CG-IHC	
	−LPS	+LPS	−LPS	+LPS	−LPS	+LPS	−LPS	+LPS
G0/G1 (%)	46·1 ± 4·5	*28·8 ± 4	49·5 ± 6·4	41·9 ± 7·2	48·7 ± 3·2	*27·6 ± 4·4	37·8 ± 5·3	41·6 ± 5·5
S (%)	14·98 ± 3·7	18·2 ± 3·1	19·9 ± 3·1	*32·8 ± 0·5.1	18·9 ± 4·0	23·3 ± 4·9	17·65 ± 4·8	13·89 ± 5·0
G2/M (%)	37·7 ± 6·7	*53·7 ± 5·2	30·5 ± 4·2	24·9 ± 4·9	32·1 ± 3·2	*49·3 ± 6	42·5 ± 6·5	42·46 ± 6·2

CG, control group; DG-NF, newborn babies of diabetic mothers that failed a good glycaemic control; DG-FU, newborn babies of diabetic mothers that reached a good glycaemic control; CG-IHG, control group treated *in vitro* with intermittent high glucose;G0/G1: Gap0/Gap1 phase of the cell cycle; S: Synthesis phase of the cell cycle; G2/M: Gap2/Mitosis phase of the cell cycle.

Recently, several reports showed that GDM women and their newborn babies have lymphocyte subset impairments.[31–34,41,42] In particular, authors suggest that some of the changes that were observed both in neonates born to Type 1 diabetes mellitus (T1DM) and GDM mothers might have been associated with the degree of metabolic control.[43]

Constant high glucose and IHG are two general phenomena in diabetes. In particular, IHG has been suggested to be dangerous for the diabetes-related complications, and it can promote the apoptosis in several cell types.[44]

In our cellular experimental model, lymphocytes from newborn babies of mothers with normal glucose tolerance were exposed, *in vitro*, to intermittent high-glucose levels. We demonstrate that this treatment enhances the activation of Casp3 and the inactivation of PARP and makes lymphocytes unresponsive towards LPS stimulation, in a fashion similar to what we have seen in lymphocytes of newborn babies of diabetic mothers with unbalanced glycaemic control.

On the basis of our *in vivo* and *in vitro* results, we can hypothesize that, *in vivo*, the hyperglycaemic environment could lead to an enhanced activation of Casp3 with a consequent inactivation of PARP1 that generates a lymphocyte dysfunction of newborn babies with diabetic mothers, at least, in B lymphocytes, on the basis of the specific LPS-induced activation of B cells.[28,29]

This biochemical panel underlies the importance to reach an optimal regulation of maternal glucose levels. In fact, in lymphocytes from newborn babies of GDM mothers that reach a good glycaemic control during pregnancy, we found relative levels of Casp3 and PARP comparable with those of the controls.

Besides more studies are required to fully correlate the activation of the apoptotic process during the intrauterine life with the foetal health later in life, our study indicates that a cord blood lymphocyte, an easily accessible source, is informative about the activation of apoptotic stimuli in circulating cells of newborn babies in

relation to the glycaemic control reached by the mother during pregnancy.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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