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Hyperglycaemia in vitro alters the proliferation and mitochondrial activity of the choriocarcinoma cell lines BeWo, JAR and JEG-3 as models for human first-trimester trophoblast

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Abstract

Aims/hypothesis. Early intrauterine growth delay in diabetes could be caused by a reduced growth of the placenta. Our study investigates whether hyperglycaemia in vitro reduces trophoblast proliferation.
Methods. First-trimester trophoblast cell models (BeWo, JAR and JEG-3 choriocarcinoma cells) were cultured for 24 and 48 h with 5.5 mmol/l D-glucose, 25 mmol/l D-glucose (hyperglycaemia) and with an osmotic control. Cell number, total protein and nucleic acid content and mitochondrial activity (tetrazolium salt assay) were measured, the cell cycle analysed (FACS, cyclin B1 levels) and apoptosis (Annexin-V) measured.
Results. In BeWo cells hyperglycaemia reduced cell number, protein, nucleic acid and cyclin B1 levels. The reduced G_2/M and increased G_0/G_1 population after 24 h reflects growth arrest at G_0/G_1. In JAR cells after 24 h the population was arrested in G_0/G_1, whereas after 48 h the G_0/G_1 block was abrogated and the cells were arrested at G_2/M. The net effect was an unchanged cell number. In JEG-3 cells hyperglycaemia resulted in fewer cells after 24 h but not after 48 h indicating some adaptation. Mitochondrial activity was either stimulated (BeWo) or reduced (JAR, JEG-3) under hyperglycaemia. Some of these effects were also induced by hyperosmolarity alone.
Conclusion/interpretation. Hyperglycaemia has the potential to inhibit the proliferation of first-trimester trophoblast cell models. The mechanisms leading to growth arrest and to changes in mitochondrial activity are complex and depend on differentiation. We hypothesise a hyperglycaemia-induced impairment of placental growth in the first trimester of a poorly controlled diabetic pregnancy. [Diabetologia (2001) 44: 209–219]

Keywords Diabetes, pregnancy, hyperglycaemia, proliferation, placenta, growth delay, cell cycle, hyperosmolarity.

Despite considerable advances in the treatment of diabetes in the past decades, diabetic pregnancies can still be associated with fetal growth disturbances even when an optimized and intensive metabolic control has been achieved. Macrosomia of the fetus is a common and well-known consequence of maternal diabetes with an increased obstetric risk. The increase in fetal fat mass that accounts for the fetal overweight [1] is the result of fetal hyperinsulinism as a consequence of maternal and, therefore, fetal hyperglycaemia [2]. According to the dynamics of fetal growth, macrosomia is caused by metabolic alterations or hyperglycaemic insults predominantly in later stages of pregnancy [3, 4]. In contrast diabetes-associated metabolic derangements in the first trimester of pregnancy can lead to malformations [5] and to delayed growth of the developing embryo [6, 7]. Whereas the molecular mechanisms underlying embryo malformations have received enormous attention, no data are available to explain early fetal growth delay. Because of the close link between placental and fetal growth, early intrauterine growth delay could be caused by a reduced growth of the pla-

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centa. This hypothesis is supported by the reduced concentrations of the placenta-specific hormones hPl and PAPP-A in the circulation of diabetic mothers early in gestation [8]. Synthesis and secretion of these hormones is solely a function of placental or trophoblast mass.

Other abnormilities that have an increased incidence in pregnancies in which diabetes is poorly controlled or uncontrolled include spontaneous abortions [9], intra-uterine growth restriction [10], and pre-eclampsia [11]. At a cellular level the common denominator is the inhibition of proliferation of first-trimester cytotrophoblast, which could lead to reduced growth of the placenta. The lower number of trophoblast cells could also result in their impaired invasion into the decidua with subsequent inadequate widening of spiral artery lumen underlying pre-eclampsia and intra-uterine growth restriction and probably also a subset of spontaneous abortions.

During human implantation and placentation adequate proliferation and invasion of trophoblastic cells are the basic requirements for a pregnancy without complications, but the altered maternal milieu in a diabetic pregnancy can affect these processes. Disturbances of trophoblast and placental growth and differentiation in the first trimester by inadequate glycaemic control can have long-ranging consequences for placental structure and function throughout gestation [12]. Although the placenta in diabetes at term of gestation has been intensively studied [13, 14] little is known about the effects on first-trimester placentas.

The aim of our study was to investigate whether hyperglycaemia in vitro reduces trophoblast proliferation. Because of the limited availability of first-trimester placental tissue, the choriocarcinoma cell lines BeWo, JAR and JEG-3 have been used instead. These were derived from first-trimester trophoblast. The diabetic environment and hyperglycaemia could result in the generation of free oxygen radicals and these have been implicated in the generation of embryogenic dysmorphogenesis [15]. When the glutathione precursor *N*-acetylcysteine was added to rat embryo cultures malformations of the embryo were blocked. Moreover, effects of the diabetic environment on morphology [16] and function of mitochondria [17] were shown. Therefore, the mitochondrial activity was measured and in some experiments the culture medium was supplemented with *N*-acetyl-cysterne.

Material and methods

Cell culture. We obtained BeWo (CCL-98, passage number: 191), JAR (HTB-144, passage number: 722) and JEG-3 (HTB-36, passage number: 124) from the American Type Culture Collection (ATCC, Rockville, Md., USA). The JAR and JEG-3 were cultured in DMEM (Gibco, Life Technologies, Paisley, Scotland), containing 10 % FCS (HyClone Laboratories, Logan, Utah, USA) and 1 % penicillin/streptomycin (Gibco), whereas BeWo cells were cultured in F-12 medium (Gibco) supplemented with 10 % FCS, 1 % penicillin/streptomycin and 2 mmol/l L-glutamine (Gibco).

The cells were plated at a density of 4×10^4 cells/cm^2 and cultured for 24 and 48 h under a humidified 5 % CO_2/95 % air atmosphere at 37 ° in the respective medium supplemented with 5.5 mmol/l (normoglycaemic control) and 25 mmol/l D-glucose (Merck, Darmstadt, Germany) (hyperglycaemic group) as well as with 5.5 mmol/l D-glucose + 19.5 mmol/l D-mannitol (Sigma-Aldrich, Vienna, Austria) as an osmotic control. After 48 h all cultures were still subconfluent. Longer culture periods were avoided to rule out effects of cell density, i. e. contact inhibition. In several experiments the cells were also cultured under hyperglycaemic conditions with 1 mmol/l (final concentration) *N*-acetyl-cysteine (Sigma-Aldrich) added to the medium.

The mean ± SEM osmolarity was 278 ± 4 mosm in the normoglycaemic control media, 303 ± 4 mosm in the osmotic control media ($p < 001$ vs control) and 300 ± 7 mosm in the hyperglycaemic media ($p < 0.001$ vs control).

Cell viability. Viability of the cells was assessed by 2 % trypan blue (Sigma). Amount of hCG-β secreted into the culture media were measured by an enzyme-immuno assay (Hoffman-La Roche, Basel, Switzerland).

Annexin V binding assay. One $\times 10^6$ cells were cultured under the conditions mentioned above for 24 and 48 h. Thereafter Annexin V binding was measured by the ApoAlert Annexin V Apoptosis Kit (Clontech, Palo Alto, Calif., USA) following the instructions in the Kit. Jurkat cells (TIB-152, ATCC) treated with 10^{-4} M dexamethasone (Sigma) were used as a positive control.

Determination of cell number. A direct (cell counting) and an indirect (total cellular nucleic acid and protein content) method were used.

The number of cells was counted in the cell counter and analyser system CASY 1 (Schärfe System, Reutlingen, Germany) using a 150 μm capillary to detect particles between 3.4 and 30 μm in diameter. Measurements with this system are based on detection of conductivity changes along an aperture during the flow of a cell-containing liquid. The result is a size distribution curve of the cells [18], in which cell debris, dead cells and viable cells can be identified by their distinct diameters. Dead cells are characterised by the volume of their cell nucleus which alone contributes to the conductivity [19]. One $\times 10^6$ cells were cultured under conditions mentioned above for 24 and 48 h. Both adherent cells and those in the supernatant were collected for cell counting.

The total cellular nucleic acid and protein contents were determined by measuring the optical density at 260 and 280 nm, respectively. Six $\times 10^3$ cells were cultured on UV transparent culture plates (Molecular Devices, Sunnyvale, Calif., USA) for 24 and 48 h under conditions mentioned above. Medium without cells was used to determine blank values. Optical densities at 260 and 280 nm were then measured in a SpectraMAX 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, Calif., USA).

Immunoblotting of cyclin B1 and p21WAF1 protein. Proteins of adherent cells were subject to SDS-polyacrylamide gel electrophoresis and immunoblotting as described [20]. The blotting membranes were incubated (60 min, room temperature)

with anti-cyclin B1 (1:250; Pharmingen, San Diego) and anti-p21WAF1 (1:25 or 1:50; Oncogene, Cambridge, Mass., USA) antibodies. Anti-mouse IgG (H + L) horseradish peroxidase conjugate (1:250 cyclin B1; 1:200 p21WAF1; Southern Biotechnology Associates, Birmingham, Ala., USA) was used as secondary antibodies. The immunolabelling was visualised using the chemoluminescence based SuperSignal CL-HRP Substrate System (Pierce, Rockford, Ill., USA). The high performance chemiluminescence film (Hyperfilm ECL, Amersham) were developed and analysed by the ONE-Dscan program (Scanalytics, Billerica, Mass., USA).

Cell cycle analysis. The cell cycle was analysed by fluorescence-activated pulse cytophotometry (FACS) following a standard protocol [21]. One $\times 10^6$ cells were cultured in the respective media containing the corresponding glucose concentrations for 24 and 48 h. The cells were then stained with DAPI (Partec, Muenster, Germany) for FACS analysis. The percentage of cells in each phase of the cell cycle was analysed by the Multicycle program (Phoenix Flow Systems, San Diego, Calif., USA).

Mitochondrial activity. The activity of dehydrogenases as a measure of mitochondrial activity was measured using the reagent WST-1 (Boehringer Mannheim, Mannheim, Germany). This colorimetric assay is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells. Six $\times 10^3$ cells were cultured on tissue culture plates (96 wells; Falcon, Becton Dickinson, Franklin Lakes, N. Y., USA) as described above. After each culture period 20 µl of the WST-1 reagent was added to each well. In kinetic pilot experiments a 2-h incubation resulted in the best linear increase of optical density with cell number. After 2 h the optical density was measured in a microplate spectrophotometer (SpectraMAX 250) at 450 nm with 600 nm as an internal reference. Blank values of the respective media without cells were measured after incubation with WST-1 as described above and subtracted from the readings in the presence of the cells.

Statistical analysis. The data are expressed as mean values ± SEM. Data were analysed with standard methods for descriptive values and with ANOVA (analysis of variance) to compare the size of the effect. Significant overall results were further examined with Dunnett's post hoc test for comparison with the control and LSD-tests (least significant difference) for all other pairwise comparisons. A significance level of 0.05 was used for all tests. We used SPSS (SPSS, Chicago, Ill., USA) for all calculations.

Results

Cell Characterisation and Viability. The cells rapidly attached after seeding regardless of the culture condition. All cells formed aggregates but no true syncytia were observed. After harvesting the cells with trypsin-EDTA the viability of all three choriocarcinoma cells was greater than 95 % by trypan blue exclusion. None of the conditions resulted in increased Annexin V binding in any of the choriocarcinoma cells (not shown) indicating the absence of apoptosis induction. After correcting for cell numbers, the amount of hCG-β in the culture media was increased ($p < 0.05$) under hyperglycaemic conditions in all cell lines. The osmotic change alone was associated with higher amounts of hCG-β only in BeWo and JEG-3 (Table 1).

Table 1. Amount (means ± SEM) of hCG-β (mIU/1 $\times 10^6$ viable cells) secreted into culture media after 48 h culture

condition	BeWo	JAR	JEG-3
normoglycaemic	291 ± 90	504 ± 33	329 ± 80
osmotic control	416 ± 24[a]	448 ± 16	402 ± 11[a]
hyperglycaemic	495 ± 14[ab]	696 ± 60[ab]	450 ± 29[a]

[a] $p < 0.05$ vs control
[b] $p < 0.05$ vs osmotic control
$n = 3$ experiments

Table 2. Effect of 1 mmol/l *N*-acetylcysteine (NAC) on hyperglycaemia-induced changes in optical density (means ± SEM; $\times 10^{-2}$) at 260 nm, in JAR cells after 24 and 48 h

	24 h	48 h
Normoglycaemia	9.6 ± 2.3	8.6 ± 1.6
Osmotic control	4.4 ± 1.6[a]	5.3 ± 1.6
Hyperglycaemia	6.3 ± 1.5[b]	5.7 ± 1.6
Hyperglycaemia + 1 mmol/l NAC	12.2 ± 1.5[c]	13.0 ± 1.8[c]

[a] $p < 0.001$
[b] $p < 0.05$ vs normoglycaemia
[c] $p < 0.001$ vs hyperglycaemia

Cell counting. The numbers of viable and non-viable (dead) cells under the effect of the different culture media is depicted in Figure 1. In BeWo and JEG-3 growth was arrested from 24 to 48 h, but the number of JAR cells doubled within this time. The number of viable BeWo cells decreased under hyperglycaemia. Whereas after 24 h hyperglycaemia augmented, however, the hyperosmolarity-associated changes, after 48 h only hyperglycaemia alone decreased the number of viable cells. The number of dead cells was either unaffected (24 h) or reduced (48 h) by the treatments. The number of viable JAR cells was virtually unchanged but the number of dead cells dropped after 24 and 48 h. Hyperosmolarity reduced the number of JEG-3 cells after 24 h without any additional decrement by hyperglycaemia. These effects disappeared after 48 h. Notably, in JEG-3 the number of dead cells was higher after 24 and 48 h and this effect was due to the hyperglycaemia and not caused by hyperosmolarity.

Optical density at 260 and 280 nm. Measurements of absorbance at 260 nm and 280 nm reflect the total DNA/RNA and protein contents. The results at 280 nm (Fig. 2) are similar to those at 260 nm (not shown), except for a reduced optical density at 260 nm in JAR cells after 24 h under hyperosmotic and hyperglycaemic conditions (Table 2). These reduced nucleic acid concentrations were paralleled by fewer cells in the S-phase of the cell cycle (Fig. 3, Table 3). Absorbance readings of BeWo were lower

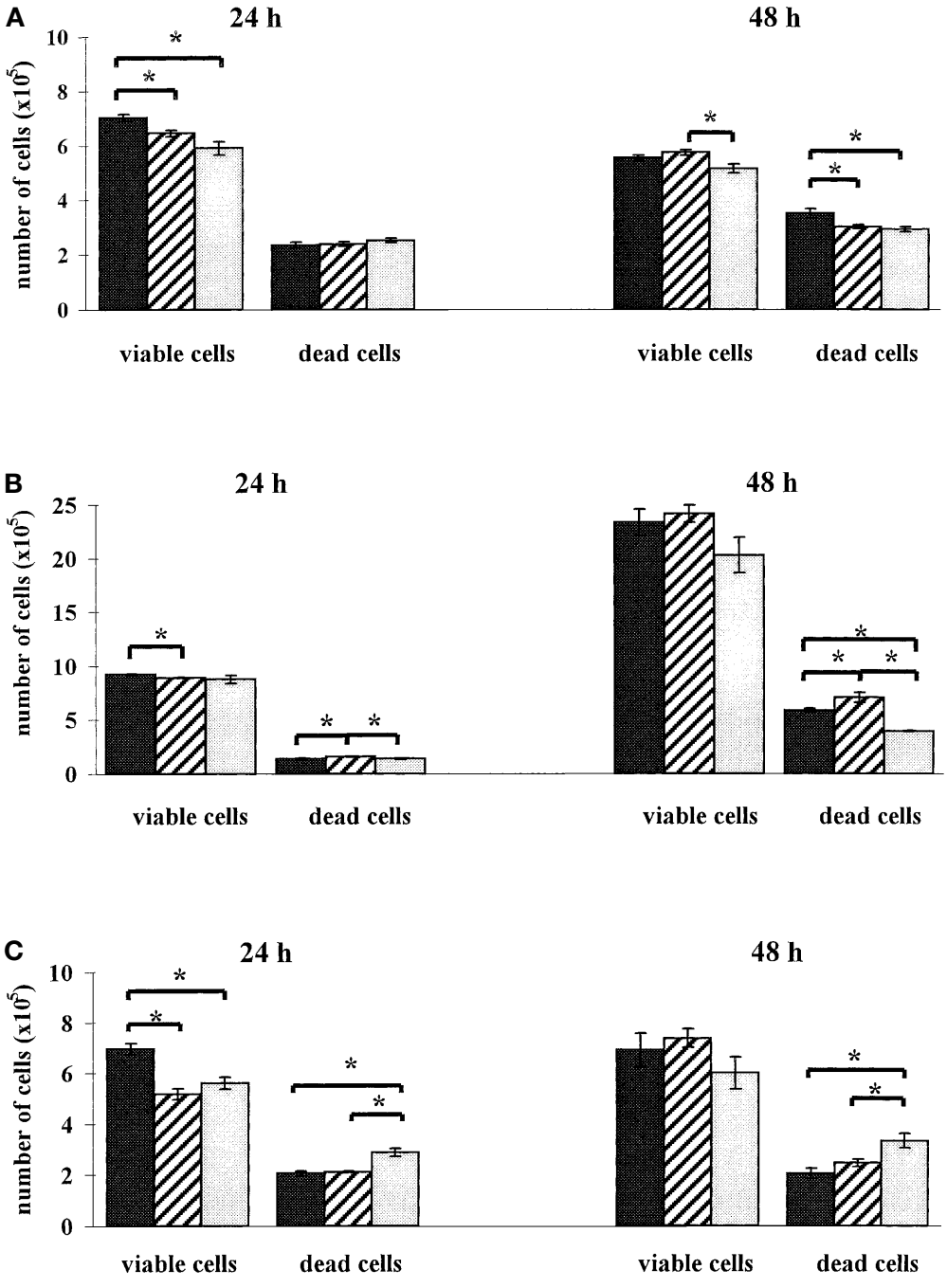


Fig. 1 A–C. Number of viable and non-viable BeWo (**A**), JAR (**B**) and JEG-3 (**C**) cells (adherent and non-adherent) after 24 h and 48 h of culture under normoglycaemic (■); osmotic control (▨) and hyperglycaemic (□) conditions. Results are presented as means ± SEM of three independent experiments. * $p < 0.05$

($p < 0.001$) after 24 and 48 h under hyperglycaemia compared with normoglycaemia and osmotic control. These results are in agreement with those of the cell cycle regulating protein cyclin B1 (Fig. 3). In JAR cells the different culture conditions did not change the absorbance readings. In JEG-3 cells the optical density was lower ($p < 0.001$) in the osmotic control. This reduction was further augmented under hyperglycaemic conditions ($p < 0.05$). The addition of 1 mmol/l *N*-acetylcysteine abolished the hyperglycaemia-induced changes only in JAR (Table 2) but not in BeWo and JEG-3 cells (not shown).

Cell cycle analysis. Immunoblotting showed the presence of cyclin B1 in all three choriocarcinoma cell lines whereas p21WAF1 was not detectable (Fig. 4). Cyclin B1 expression as well as cell cycle distribution (Fig. 3) were affected by hyperglycaemia or osmotic control depending on the cell line. In BeWo cells hy-

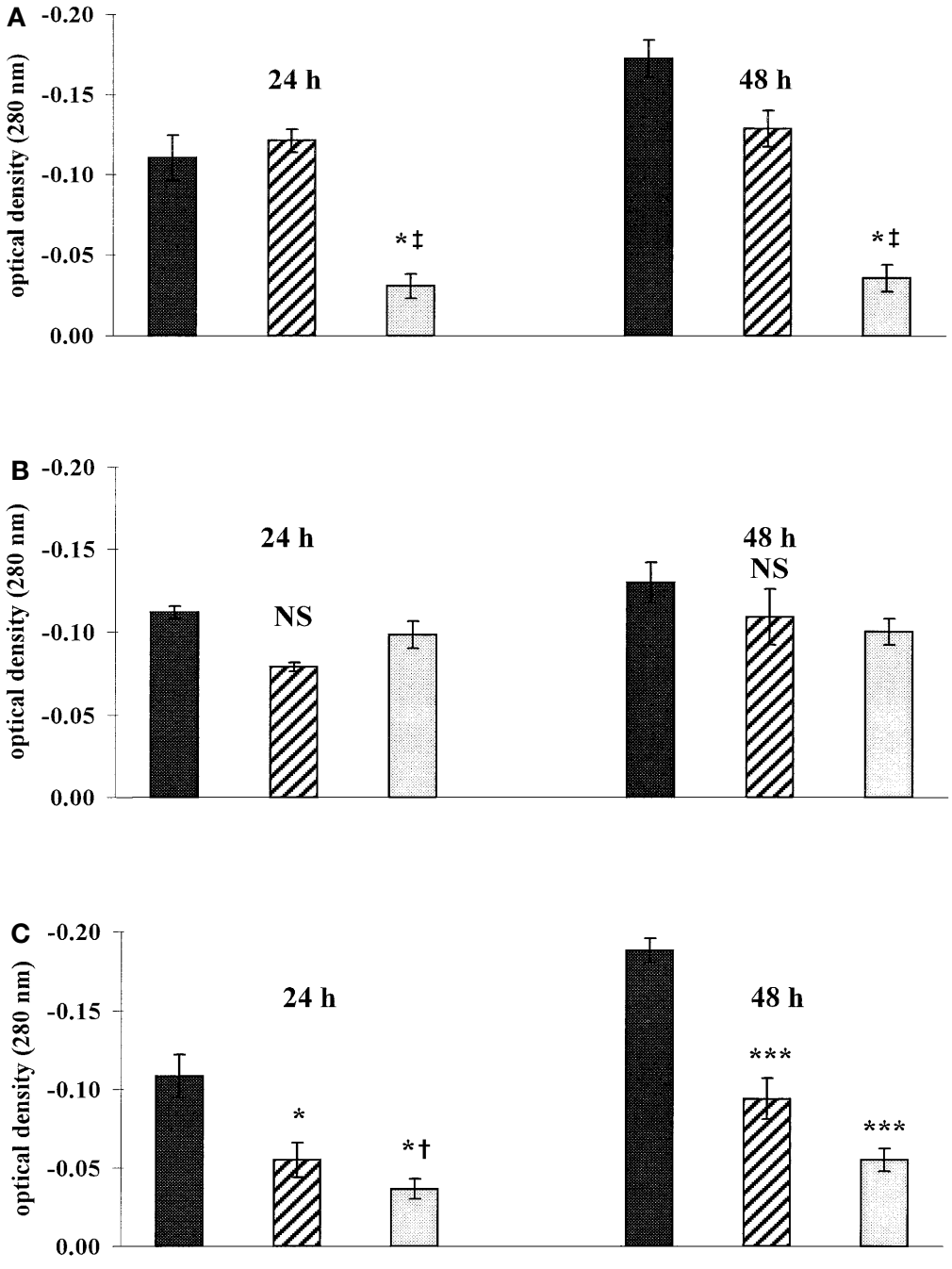
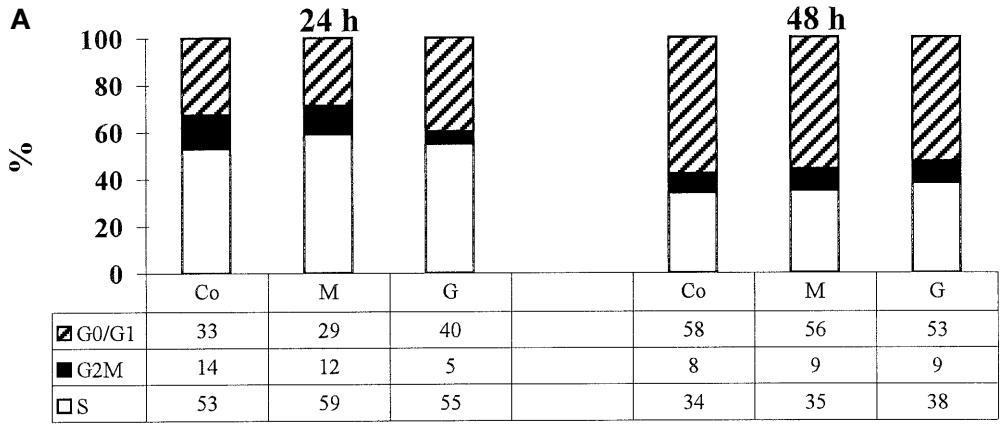
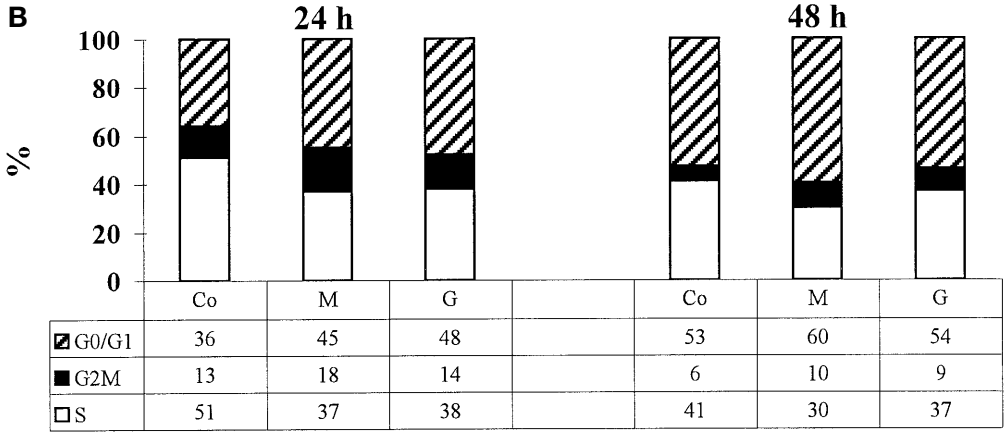


Fig. 2 A–C. Optical density at 280 nm after 24 and 48 h of culture of BeWo (**A**), JAR (**B**) and JEG-3 (**C**) cells under normoglycaemic (black bars), osmotic control (hatched bars) and hyperglycaemic (grey bars) conditions. Results are presented as means ± SEM of eight independent experiments. * $p < 0.001$ vs control; *† $p < 0.05$ vs osmotic control; *** $p < 0.001$ vs osmotic control

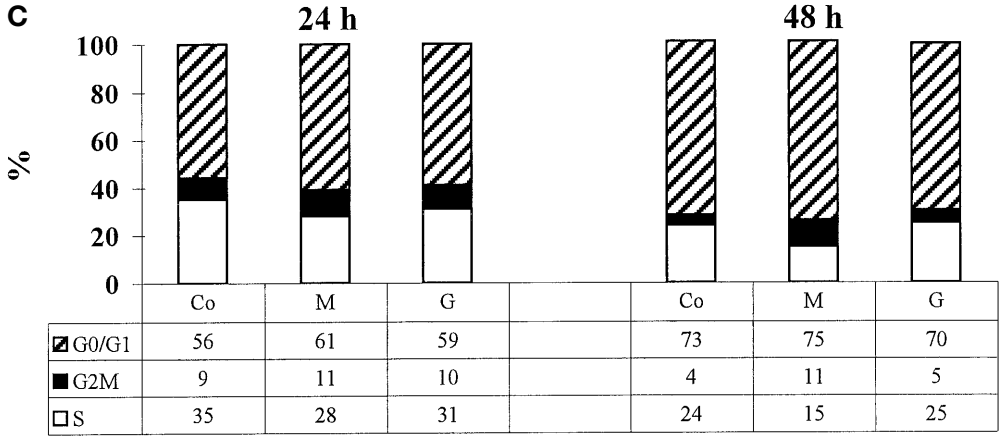
perglycaemia, but not osmotic changes, reduced cyclin B1 levels after 24 and 48 h by about 30% and 40%, respectively. This was paralleled by a smaller proportion of cells in the G_2/M-phase and more cells in G_0/G_1, indicating cell cycle arrest after 24 but not after 48 h. In JAR cells cyclin B1 levels were increased after 24 h under osmotic control conditions (+ 20%). After 48 h both hyperosmolarity and hyperglycaemia resulted in an increase by 30% and 40%, respectively. These changes were paralleled by the presence of more cells in G_2/M, whereas hyperglycaemia increased the cells in G_2/M only after 48 h. A greater proportion of cells in cell cycle arrest, i.e. in G_0/G_1, was found after 24 h of osmotic change. In JEG-3 cells cyclin B1 levels were about 30% higher in the osmotic control after 24 and 48 h than under normoglycaemia. When hyperosmolarity was superimposed by increased glucose concentrations cyclin B1 expression was reduced by about 30% compared



	24 h Co	24 h M	24 h G	48 h Co	48 h M	48 h G
G0/G1	33	29	40	58	56	53
G2M	14	12	5	8	9	9
S	53	59	55	34	35	38



	24 h Co	24 h M	24 h G	48 h Co	48 h M	48 h G
G0/G1	36	45	48	53	60	54
G2M	13	18	14	6	10	9
S	51	37	38	41	30	37



	24 h Co	24 h M	24 h G	48 h Co	48 h M	48 h G
G0/G1	56	61	59	73	75	70
G2M	9	11	10	4	11	5
S	35	28	31	24	15	25

Fig. 3. Proportion (%) of cells in the G_0/G_1-phase, S-phase and G_2/M-phase of their cell cycle after 24 and 48 h of culture under normoglycaemic (Co), osmotic control (M) and hyperglycaemic (G) conditions. Representative example of four [BeWo (**A**), JAR (**B**)] or two [JEG-3 (**C**)] independent experiments

with the osmotic control attaining levels similar to normoglycaemia. These effects induced by hyperosmolarity were paralleled by fewer cells in the S-phase of their cell cycle.

Mitochondrial activity. Mitochondrial activity differed among the cells when cultured under normoglycaemia ($p < 0.05$) with JEG-3 greater than JAR and JAR greater than BeWo after 24 h (Fig. 5). In BeWo

Table 3. Summary of changes in BeWo, JAR and JEG-3 cells induced by 24 h and 48 h culture under osmotic control (hyperosmolarity: 19.5 mmol/l D-mannitol + 5.5 mmol/l D-glucose) and hyperglycaemic (25 mmol/l D-glucose) conditions

end point	BeWo				JAR				JEG-3			
	hyperosmolarity		hyperglycaemia		hyperosmolarity		hyperglycaemia		hyperosmolarity		hyperglycaemia	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
number of viable cells	↓	—	↓	—	↓	—	—	—	↓	—	↓	—
protein content (280 nm)	—	—	↓	↓	—	—	—	—	↓	↓	↓	↓
nucleic acid content (260 nm)	—	—	↓	↓	↓	—	↓	—	↓	↓	↓	↓
cyclin B1 level	—	—	↓	↓	↑	↑	—	↑	↑	↑	—	—
G_0/G_1	—	—	↑	—	↑	↑	↑	—	↑	—	—	—
G_2/M	—	—	↓	—	↑	↑	—	↑	—	↑	—	—
S	—	—	—	—	↓	↓	↓	—	↓	↓	—	—
overall effect vs normoglycaemia	no effect	no effect	growth arrest in G_0/G_1	growth arrest site not determined	growth arrest in G_0/G_1 and G_2/M	growth arrest in G_0/G_1 and G_2/M	growth arrest in G_0/G_1	no effect	growth arrest in G_0/G_1	growth arrest in G_2/M	no effect ?	no effect ?

↑ ↓ —: increase, decrease or no change vs normoglycaemic (5.5 mmol/l D-glucose) conditions

cells the optical density increased after 24 h by the osmotic change. This increase was further augmented with superimposed hyperglycaemia. After 48 h the hyperglycaemia was still associated with a higher optical density but only compared with the osmotic control. In JAR and JEG-3 cells the hyperglycaemia resulted in a decrease in the mitochondrial activity compared with normoglycaemia and osmotic control, except for JAR after 24 h. Any hyperglycaemia-induced changes were not abolished by the addition of *N*-acetylcysteine (not shown).

Summary of results (Table 3). In BeWo cells hyperosmolarity does not seem to have pronounced effects, apart from the fewer viable cells after 24 h. Hyperglycaemia clearly results, however, in a reduced number of viable cells, lower protein and nucleic acid levels and lower cyclin B1 levels. The reduced G_2/M and increased G_0/G_1 population after 24 h is in line with a growth arrest at G_0/G_1. The absence of cell-cycle changes after 48 h could be due to the limited sensitivity of cell cycle analysis by FACS or reflect other underlying mechanisms.

In JAR cells hyperosmolarity was associated with a greater population in G_2/M, paralleled by higher cyclin B1 levels and in G_0/G_1 suggesting a block at both cell-cycle transitions. Superimposed hyperglycaemia differently affected the cell cycle: after 24 h the population was arrested in G_0/G_1 whereas after 48 h the G_0/G_1 block was abrogated and the cells were arrested at G_2/M. The net effect was no change in number of viable cells.

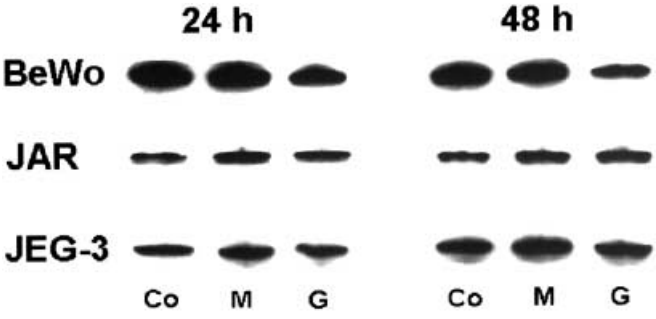


Fig. 4. Immunoblots of cyclin B1 in BeWo, JAR and JEG-3 cells after 24 and 48 h culture under normoglycaemic (Co), osmotic control (M) and hyperglycaemic (G) conditions. Representative example of two independent experiments. Note that levels cannot be compared between the different cell lines

In JEG-3 cells hyperosmolarity and hyperglycaemia were associated with a reduction in the number of viable cells after 24 but not after 48 h, which could indicate some adaptation and desensitisation. Although hyperosmolarity arrested the cells in G_0/G_1 (24 h) or G_2/M (48 h) with an associated increase in cyclin B1, these cell cycle changes were abrogated by superimposed hyperglycaemia.

Discussion

We set out to investigate the effect of hyperglycaemia in vitro on human first-trimester trophoblast proliferation to understand the consequence of poor glycae-

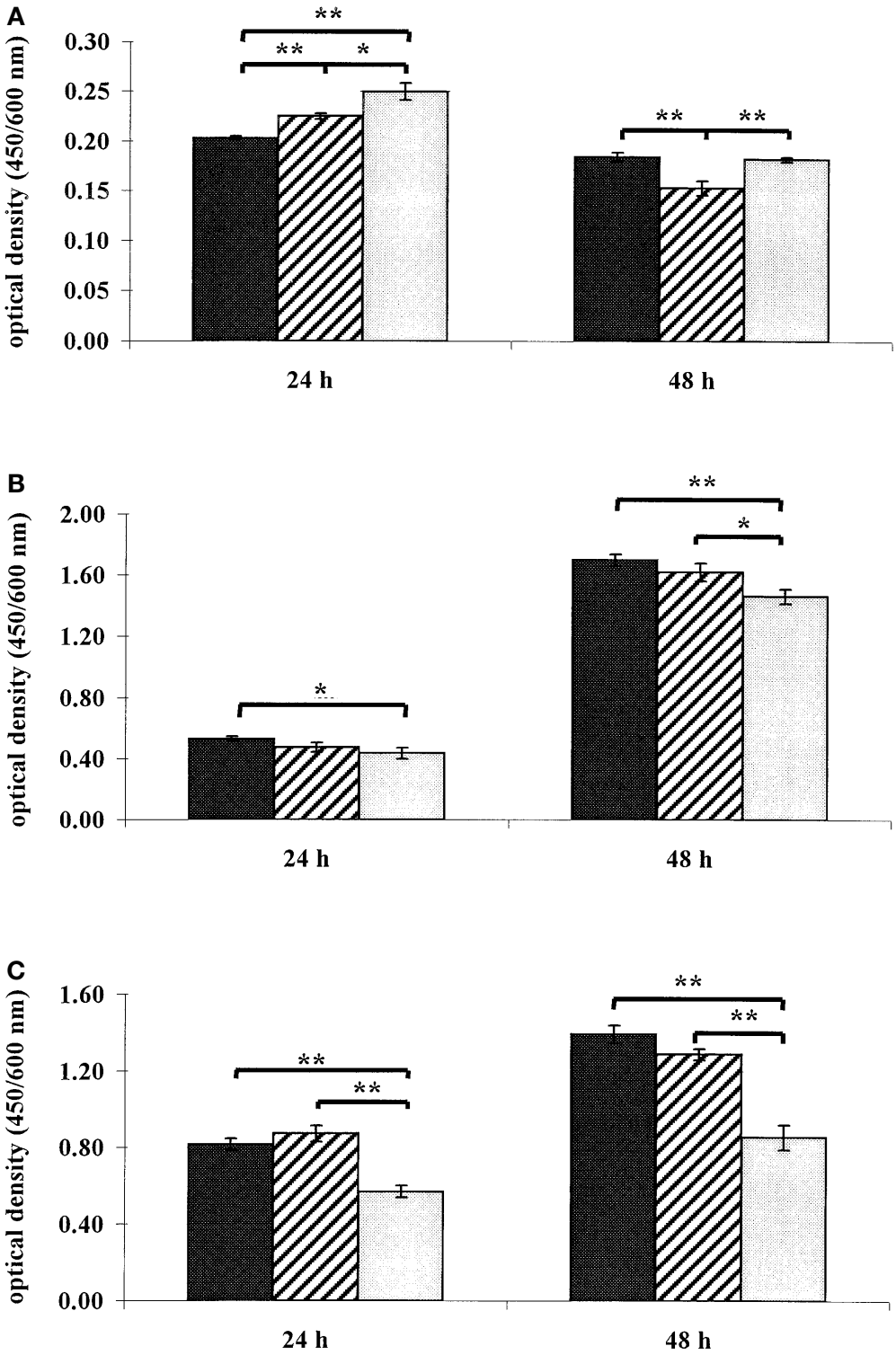


Fig. 5 A–C. Mitochondrial dehydrogenase activity measured in BeWo (**A**), JAR (**B**) and JEG-3 (**C**) cells by the WST-1 reagent after 24 and 48 h of culture under normoglycaemic (■), osmotic control (▨) and hyperglycaemic (□) conditions. Results are presented as means ± SEM of eight independent experiments. *$p < 0.05$, **$p < 0.01$. Note the different scale on the y-axis

mic control of the diabetic mother early in gestation on placental development in general and on the trophoblast in particular.

The trophoblast compartment of the human placenta comprises a variety of trophoblast types, which differ in location and degree of differentiation [22]. Despite some success in isolating trophoblasts from first-trimester pregnancies [23], the low cell yield still makes comprehensive investigations impossible.

Therefore, we used three choriocarcinoma cell lines, which differ by their proliferative activity and degree of differentiation. The hallmark of these cells is their synthesis and secretion of human chorionic gonadotropin. The JAR and BeWo choriocarcinoma are regarded as poorly differentiated cells whereas JEG-3 proliferate slowly and have a higher degree of differentiation, i.e. they contain several syncytial elements [24–26]. These cells, thus, offer the unique possibility of identifying biological responses that vary with the degree of trophoblast differentiation. This is exemplified by recent studies from our laboratory in which a hyperglycaemia-induced up-regulation of the GLUT-1 system in JAR was observed but no change in JEG-3 cells [20]. So far BeWo cells have not been investigated. The results of this study with different end-points further support the hypothesis of a differentiation-dependent susceptibility of the human first trimester trophoblast to hyperglycaemia.

Incorporation of ^{3}[H]-thymidine is widely used to measure cell proliferation. Because hyperglycaemia increases the activity of thymidine kinase, the rate-limiting enzyme for thymidine incorporation [27], this method was not a suitable option. The most accurate way to determine the cell number is by counting. The automatic counter used here detects viable and non-viable cells, which differ in their cell volume [19].

In two of three cell lines hyperglycaemia led to fewer cells supporting the hypothesis of a reduced trophoblast growth in the wake of maternal hyperglycaemia. This effect did not, however, persist until 48 h indicating some capacity of the cells to adapt their responses to the altered metabolic environment. It is not clear whether this is also an intrinsic feature of some first trimester trophoblast populations. If so, it could explain why placental growth is only retarded in poorly or uncontrolled first trimester diabetic pregnancies, implying some compensatory growth later on. Alternatively, the hyperglycaemia-induced growth arrest of trophoblasts and placenta and, hence, fetus could be released in vivo by fetally derived insulin, which can bind to the cytotrophoblast [28].

To gain potential insight into cell cycle changes cyclin B1 and p21 were measured and the cell populations in the cell-cycle phases determined. The cell cycle is regulated by cyclin-dependent kinases. Their activity is controlled positively by cyclins and negatively by inhibitor proteins such as p21, p27 and p57. Dysregulation of cyclins or cdk inhibitors or both could lead to uncontrolled proliferation, growth arrest or activation of apoptosis. Critical for the G_2/M transition is the cyclin-dependent kinase complex cdc2 bound to cyclin B1 [29]. Arrest at the G_1/S transition is in part mediated by p21 [30]. The choriocarcinoma cells either do not express p21 or the expression is below the limit of detection in our assay. In general, cells deficient in p21 lose their potential for a G_1-phase delay [31]. The hyperglycaemia-induced G_0/G_1 growth arrest in BeWo and JAR suggests the presence of other inhibitory proteins such as 14–3–3σ protein substituting for p21 [32].

In this context the absence of apoptosis under all conditions is noteworthy. Hyperglycaemia induces apoptosis in endothelial cells [33] and the trophoblast covers the placental villi in an endothelium-like position lining the maternal blood spaces. Neither the Annexin-V assay (this study) nor examination of morphology [34] indicated, however, that apoptosis occurs.

The generation of intracellular reactive oxygen species underlies several pathways of hyperglycaemia-induced intracellular damage [35]. Superoxide, one of the key reactive oxygen species, has been implicated in the teratogenicity of media containing high glucose concentrations in vitro and in vivo [17, 36, 37]. Its effects can be attenuated or even blocked by antioxidants such as vitamin E, vitamin C, superoxide dismutase or the glutathione precursor *N*-acetylcysteine [36–39]. Reactive oxygen species adversely affect the physiology and survival of cells by modulating cellular energy homeostasis and by inducing apoptosis [40]. This pathway generally links hyperglycaemia to apoptosis induction. The increased glucose flux through the glycolytic pathway could, however, also protect cells against apoptosis [41]. The absence of apoptosis in the trophoblast models could suggest the presence of some unknown pro-survival factors, a defect in the apoptosis induction machinery or the absence of increased generation of reactive oxygen species. In the term trophoblast a large proportion of the mitochondria is uncoupled [42]. Mitochondrial activity in the presence of hyperglycaemia was depressed in JAR and JEG-3 cells and this was not the result of oxidative stress because the antioxidant *N*-acetylcysteine did not lessen or abrogate the hyperglycaemia-induced alteration in mitochondrial activity. Therefore, we favour the hypothesis that the trophoblast models do not respond to hyperglycaemia by generation of reactive oxygen species above the threshold level to induce apoptosis. Experiments to test this are currently ongoing in our laboratory.

Most changes observed here were small, though significant. When blastocysts were recovered from streptozotocin-diabetic rats at day 6, the cell number of the inner cell mass was reduced by 20 % whereas that of the trophectoderm only by 9 % [43]. In vitro culture of day-5 blastocysts from the same model with 17 mmol/l D-glucose for 48 h resulted in 12 % fewer cells in the trophectoderm and in a reduction by 34 % in the inner cell mass [44]. Limited susceptibility to hyperglycaemia seems therefore to be characteristic of the trophoblast lineage throughout all stages of pregnancy.

Any study using a hyperglycaemic challenge has to control carefully for the associated increase in osmo-

larity, which alone can alter cell function [45]. Hyperglycaemia-induced changes in biological response can either be caused by osmotic changes, by modification of intracellular metabolic pathways or by generation of reactive oxygen species or by a combination thereof. The BeWo and JEG-3 cells are examples of differential cell responses to hyperosmolarity and hyperglycaemia: Hyperosmolarity alone resulted in growth arrest in JEG-3, but had to be superimposed by hyperglycaemia to produce this effect in BeWo. The partial absence of effect by the superimposed increase in external glucose concentration in JEG-3 suggests that the isolated glucose effect opposes that of the hyperosmolarity. Clearly, the mechanisms underlying any cellular change by high glucose concentrations are highly complex and cell-specific.

Collectively, the present study demonstrates the potential of hyperglycaemia to inhibit the proliferation of first trimester trophoblast models. The mechanisms leading to the growth arrest and to changes in mitochondrial activity are complex and clearly depend on the degree of differentiation of the cells [46]. A hyperglycaemia-induced impairment of placental growth in the first trimester of a poorly controlled diabetic pregnancy can be hypothesised because growth of the human placenta in the first trimester is mainly driven by trophoblast growth [22]. This is in line with the lower concentrations of circulating hormones that are derived from the trophoblast and determined by trophoblast mass [8]. It has to be kept in mind, however, that the transformed cells used here could have lost certain trophoblast-specific features relevant to the regulation of proliferation.

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