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Report

Chemosensitivity of normal human trophoblasts evaluated by a newly developed ATP-based luminescence assay

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Trophoblast injury may be one of the possible causes of fetal distress associated with chemotherapy administered during pregnancy. The purpose of this study was to investigate the *ex vivo* chemosensitivity of normal trophoblasts (NTB) against commonly used antineoplastic agents. Using the newly developed *ex vivo* ATP-based trophoblast assay (ATP-TBA), 31 NTB freshly sampled from human placentas (gestational week 7–42) were tested against dactinomycin (Act-D), 5-fluorouracil (5-FU), 4-OOH-cyclophosphamide (4-HC), vincristine (VCR) and methotrexate (MTX) alone or in combination with calcium folate (LV). All agents were studied at concentrations relevant to clinical dosages normally used for chemotherapy of solid neoplasms. Of 31 samples studied with the ATP-TBA, 20 (65%) were evaluable. VCR, Act-D and 4-HC were the most active drugs with 55, 45 and 45% of samples responding *ex vivo*. Antimetabolites were less active, producing *ex vivo* response rates of 25 (MTX) and 20% (5-FU), respectively. MTX activity was largely neutralized by adding LV. The chemosensitivity of NTB showed considerable inter-individual variations and did not decrease with increasing gestational age. We therefore conclude that NTB of any gestational age exhibit considerable *ex vivo* sensitivity against common anticancer agents which is comparable to that observed for various solid tumors. The ATP-TBA may be helpful in planning future trials with both single agents and drug combinations in order to standardize and optimize chemotherapy during pregnancy. [© 2002 Lippincott Williams & Wilkins.]

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Introduction

Approximately 0.1% of pregnancies are complicated by the primary diagnosis of malignant disease.[1] However, cancer is a leading cause of death in women at childbearing age.[1,2] In industrialized countries, changes in the generative behavior largely attributable to socioeconomic improvements have increased the median age of pregnant women. As a result, cancer during pregnancy may become a more frequent problem in the near future. Carcinomas of the cervix uteri and other pelvic locations, breast, thyroid gland, and colorectum, malignant melanomas, lymphomas and leukemias are the most common neoplasms encountered during pregnancy.[1,3,4] Among these, breast and ovarian cancers as well as hematological malignancies frequently require chemotherapy. When administered during the first trimester of pregnancy, chemotherapy often results in both miscarriages and a high incidence of fetal malformations, particularly if antifolates or procarbazine are used.[1] Chemotherapy beyond week 16 of gestation is not associated with an increased risk of teratogenicity,[1] but a considerable number of these pregnancies will be compromised by intrauterine growth restriction (IUGR), premature delivery and low birth weight of the neonate.[1,4]

The reasons for these events are poorly understood. Most of the common antineoplastics penetrate the placenta at effective concentrations, and may disturb both metabolic and bone marrow functions of the unborn.[1,5,6] Before reaching the fetus, however, drugs firstly must penetrate the placenta. Both the well known methotrexate (MTX) sensitivity of ectopic pregnancies[7,8] and the high chemosensitivity of gestational trophoblastic neoplasia (GTN)[4,9,10] suggest that the placenta may be the first target of cytostatics delivered to the mother before affecting the unborn. Impairment of the trophoblast function may ultimately cause relative placental insufficiency and, consecutively, IUGR. Whereas the effects of MTX on normal trophoblasts (NTB) derived from first trimester placentas have been investigated in depth,[11,12] studies focusing on both NTB of later gestational age and other antineoplastics are still lacking. This trial was thus initiated to investigate the effect of a variety of commonly used anticancer drugs on NTB derived from both early and late gestational week placentas.

Material and methods

Cell culture system and trophoblast cell preparation

A microplate format adenosine triphosphate (ATP) luminescence assay was adapted for this study from a similar method used for *ex vivo* pretherapeutic chemosensitivity testing of native human malignancies.[13] The ATP tumor chemosensitivity assay (ATP-TCA)[14,15] utilizes commercially available test kits (TCA-100; DCS Innovative Diagnostik Systeme, Hamburg, Germany) which provide all of the reagents needed for the newly developed ATP trophoblast assay (ATP-TBA), unless otherwise specified.

Collection of tissue was performed in accordance to the institutional ethical guidelines including informed written consent in all cases. NTB derived from 31 normal placentas between week 7 and 42 of gestation were investigated: placentas up to week 24 of gestations were derived from normal spontaneous abortuses; material of later gestational age was collected from placentas after vaginal or cesarean delivery. All tissues were obtained under sterile conditions.

Isolation of NTB was performed using a simplification of the approach described by Sand *et al.*[11] Villi were sharply dissected from adjacent decidua and connective tissue. Some milliliters of Hank's balanced salt solution (HBSS; Gibco Life Sciences, Paisley, UK) containing 100 U/ml penicillin and 100 μg/ml streptomycin were added, and tissue was gently moved to remove blood and debris. Villi were minced into pieces of approximately 1 mm^3 and then poured into a 50-ml conical tube containing 15 ml Tumor Dissociation Enzyme Reagent (TDE; DCS Innovative Diagnostik Systeme). The tube was placed into a gently moving shaker bath at 37°C for 2 h. After centrifugation at 200 *g* for 10 min, residual TDE was discarded. Then 20 ml of HBSS enriched with 10% fetal bovine serum (FBS; ICN Flow, Meckenheim, Germany) was added and tissue was dispersed by vigorous pipetting for 15 min. The suspension was filtered through a 60-μm mesh gauze (Schweizerische Seidengazefabrik, Thal, Switzerland) after adding another 10–20 ml of HBSS/10% FBS. The filtrate was further processed by Ficoll-Hypaque density centrifugation (Lymphoprep; ICN, Meckenheim, Germany) for 10 min at 200 *g*. Cells at the interface were placed into a 50 ml conical centrifuge tube, diluted to 40 ml with HBSS and washed by centrifugation at 200 *g* for 10 min. The pellet was resuspended in 15 ml of Chang medium (Irvine Scientific, Santa Ana, CA). Viability and quality of resultant single suspensions were determined by exclusion of 0.2% Trypan blue dye (Merck, Darmstadt, Germany), and subsequent cytological and immunocytochemical stains for human chorionic gonadotropin (hCG).

Antineoplastic agents

For this study, commercial formulations of dactinomycin (Act-D; Lyovac-Cosmegen; MSD Scharp & Dohme, Munich, Germany), 5-fluorouracil (5-FU; Fluorouracil RP; Rhône-Poulenc Rohrer, Cologne, Germany), methotrexate (MTX; Methotrexat 'Lederle'; Wyeth-Lederle, Münster, Germany), vincristine (VCR; Vincristin Liquid; Elly Lilly, Bad Homburg, Germany) and calcium folate (LV; Leucovorin; Wyeth-Lederle, Münster, Germany) were utilized. Instead of cyclophosphamide (CPA) which needs *in vivo* activation, 4-OOH-cyclophosphamide (4-HC; Asta Medica, Frankfurt, Germany) was used. Preparation and storage of stock solutions was performed according to the manufacturers' advice and previous investigations.[13,16] 4-HC was freshly dissolved in sterile saline for every assay. Agents were tested at six test drug concentrations (TDC), ranging from 6.25 to 200% TDC. Values for 100% TDC are generally similar to the clinically achievable plasma peak levels after administration of an i.v. standard dose used for chemotherapy of human solid

neoplasms: Act-D, 0.1 μg/ml; 5-FU, 22.5 μg/ml; 4-HC, 3.0 μg/ml; LV 1.2 μg/ml; MTX, 2.8 μg/ml; and VCR, 0.4 μg/ml.[13,17] MTX was tested alone and in combination with LV.

Chemosensitivity testing

TDC of the different antineoplastic agents were prepared directly in 96-well polypropylene microplates by serial 1:2 dilutions with Chang medium. Each drug concentration was set up in triplicate. The MTX–LV combination was prepared by mixing the individual stocks prior to plating. A no inhibition control (M0) with blank medium and a maximum inhibition control (MI) with Maximum ATP Inhibitor were added to either six wells of each microplate. The enriched NTB suspension was seeded into all the wells of a microplate at 20 000 viable NTB cells/well.

After incubation at 37°C for 6 days in a humidified 95% air, 5% CO_2 atmosphere, cellular ATP was extracted and stabilized by adding 50 μl of Tumor Cell Extraction Reagent (TCER) to each well of every culture plate. A 50-μl aliquot of each lysate was then transferred to a LB-953 luminometer (Berthold, Wildbad, Germany) for measurement of luminescence after automated injection of 50 μl of luciferin–luciferase light reagent. Luminescence was expressed as relative light units (RLU=photons/10). Assays with RLU values (M0) below 20 000, a MI/M0 ratio above 0.01 or evidence of microbiological contamination were considered as non-evaluable.[13,15] For evaluable assays, individual percent trophoblast growth inhibition (TGI) for a particular drug concentration (x) was determined from the mean of triplicate well results as: $TGI\ (\%) = [1-(RLU_x - RLU_{MI})/(RLU_{M0} - RLU_{MI})] \times 100$.

TGI values were graphed resulting in dose response plots for each sample and drug, respectively. Both 90 and 50% inhibitory concentrations (IC_{90} and IC_{50}) were determined by linear interpolation. Additionally, a dimensionless sensitivity index ($Index_{AUC}$) represented by the area under the dose–response curve was calculated by a trapezoidal rule.[13,15] By adapting and modifying previously described criteria for human breast and ovarian cancers,[13,15,18] four categories of *ex vivo* chemosensitivity were defined as:

High sensitivity (HS):	$Index_{AUC} \geqslant 12\,500$
	$IC_{90} \leqslant 100\%$ TDC
	$IC_{50} \leqslant 25\%$ TDC
Partial sensitivity (PS):	$Index_{AUC} \geqslant 12\,500$
	$IC_{90} > 100\%$ TDC
	$IC_{50} \leqslant 25\%$ TDC
Weak sensitivity (WS):	any other with $Index_{AUC} \geqslant 12\,500$
Resistance (R):	any other with $Index_{AUC} < 12\,500$

The *ex vivo* response rate (EVRR) was given by the following equation:

$$EVRR\ (\%) = [(HS + PS + WS)/(HS + PS + WS + R)] \times 100.$$

Immunocytochemical stains

Air-dried cytocentrifuge slides were prepared from both the enriched NTB suspension before performing the ATP-TBA as well as from non-lysed M0 controls after the 6-day incubation period. Cytospin preparations were processed both according to the Papanicolau method and immunocytochemically in order to detect hCG expression. For hCG staining, a polyclonal anti-human hCG rabbit IgG antibody (Dako, Hamburg, Germany) in conjunction with the alkaline phosphatase–anti-alkaline phosphatase (APAAP) method and a 3-hydroxy-2-napthoic acid-2,4-dimethylanilide/Fast Red TR stain (Dako) was utilized. Nuclei were counterstained with Meyer's hematoxylin.

Statistics

All statistical calculations were performed using GraphPAD software (San Diego, CA). The increase of untreated positively hCG-stained cells from day 0 to 6 of incubation was analyzed by Student's t-test for matched pairs. EVRR in early ($\leqslant 14$ weeks) and late (> 14 weeks) NTB were compared by Fisher's exact test. Individual $Index_{(AUC)}$ values were correlated with week of gestation using linear regression analyses. For all statistical operations, a $p < 0.05$ was considered to indicate significance.

Results

Growth characteristics of untreated trophoblast cells *ex vivo*

Of 31 ATP-TBAs set up, 20 were found to be evaluable, resulting in an evaluability rate of 65%. Reasons for non-evaluability were microbiological contamination in two and a lack of clear dose responsibility and/or insufficient M0 values in the remaining nine cases (probably the result of low NTB cell numbers). Before incubation, 36±22% (range 10–96%) of cells were positively stained for hCG.

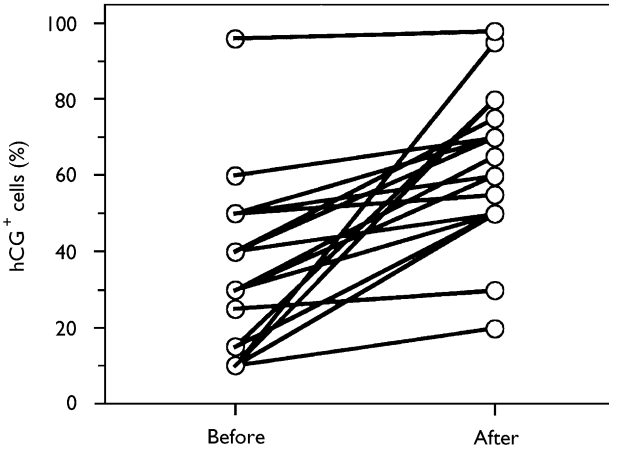

Figure 1. Percentage of hCG-expressing trophoblast cells before and after 6 days of incubation using the ATP-TBA.

After 6 days of culture, 63 ± 19% (range 20–98%) of cells isolated from M0 controls showed cellular hCG expression, representing an average 28% increase of positively stained cells within the culture period ($p < 0.0001$). In none of the 20 evaluable assays was a decrease of hCG-expressing cells noted (Figure 1).

Patterns of *ex vivo* chemosensitivity are summarized in Table 1. VCR, Act-D and 4-HC were found to be the most active agents, producing EVRR of 55, 45 and 45%, respectively. In particular, Act-D and 4-HC produced HS in a considerable number of samples. In contrast, VCR usually produced PS or WS. The EVRR for MTX (25%) and 5-FU (20%) was low. In all but one case, addition of LV completely neutralized the antineoplastic activity of MTX.

There was no significant difference in sensitivity between early (≤14 weeks) and mature NTB (>14 weeks). It should be noted that only 4-HC and Act-D produced slightly higher EVRR in early NTB (56%) compared to late ones (36%). Nevertheless, large interindividual differences with regard to *ex vivo* chemosensitivity were found independently of the gestational age (Figure 2). Figure 3 demonstrates correlations between NTB age and $Index_{AUC}$ values produced by the five single agents tested. Mostly, no correlation was found between gestational age and single agent activity. Only 4-HC tended to be more active in early NTB, although the observed inverse correlation between gestational age and $Index_{AUC}$ failed to show statistical significance ($r = -0.487$; $p = 0.067$).

Discussion

In this report, we describe the chemosensitivity of NTB tested *ex vivo* against five standard antineoplastic agents using an ATP-based luminescence assay. Of these, Act-D, MTX, CPA (4-HC) and VCR are widely used for chemotherapy of both human solid and hematologic malignancies.[19,20] These drugs also display marked activity in gestational trophoblastic disease.[4,9,10,20] In addition, 5-FU is a standard agent for the treatment of various solid neoplasms including colorectal, gastric, pancreatic, breast, bladder, cervical, vulvar, and head and neck cancers.[19,20]

Table 1. Patterns of *ex vivo* chemosensitivity of 20 normal trophoblast specimens evaluated with the ATP-TBA

Drug	Gestational age (weeks)	Pattern of *ex vivo* chemosensitivity [*n* (%)]				Response rate (%)
		High	Partial	Weak	Resistance	
MTX	≤14	–	–	3 (33)	6 (67)	33
	>14	–	–	2 (18)	9 (82)	18
	total	–	–	5 (25)	15 (75)	25
MTX–LV	≤14	–	–	–	9 (100)	0
	>14	–	1 (9)	–	10 (91)	9
	total	–	1 (5)	–	19 (95)	5
Act-D	≤14	1 (11)	1 (11)	3 (33)	4 (44)	56
	>14	1 (9)	3 (27)	–	7 (64)	36
	total	2 (10)	4 (20)	3 (15)	11 (55)	45
VCR	≤14	–	5 (56)	–	4 (44)	56
	>14	1 (9)	–	5 (45)	5 (45)	55
	total	1 (5)	5 (25)	5 (25)	9 (45)	55
4-HC	≤14	3 (33)	1 (11)	1 (11)	4 (44)	56
	>14	1 (9)	1 (9)	2 (18)	7 (64)	36
	total	4 (20)	2 (10)	3 (15)	11 (55)	45
5-FU	≤14	1 (11)	1 (11)	–	7 (78)	22
	>14	–	2 (18)	–	9 (82)	18
	total	1 (5)	3 (15)	–	16 (80)	20

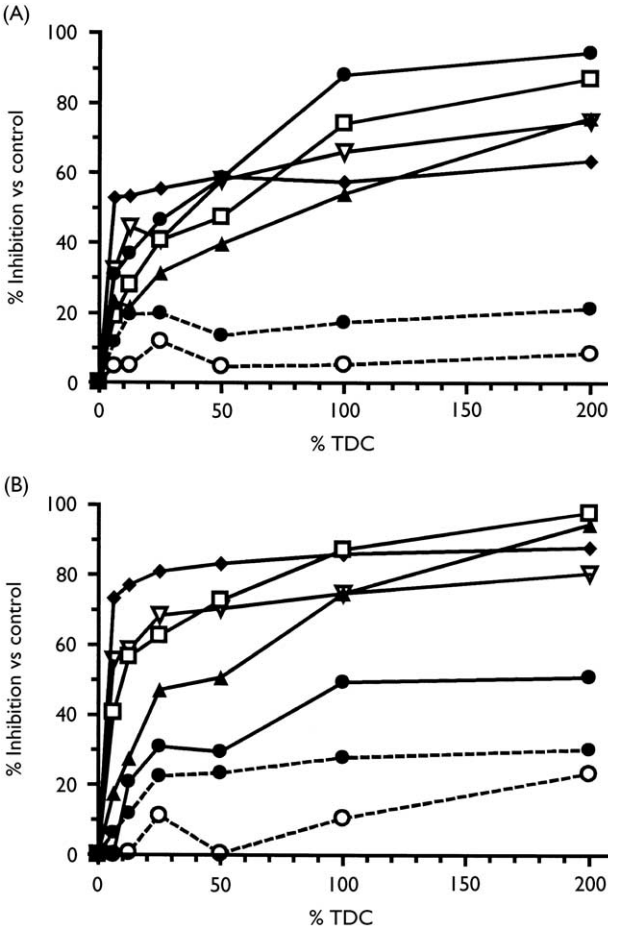

Figure 2. Typical *ex vivo* sensitivity profiles of two trophoblast samples tested with the ATP-TBA. (A) Moderately sensitive trophoblast cells derived from a week 11 abortus. (B) Highly sensitive trophoblast cells derived from a week 29 placenta. (▲) 4-HC, (□) Act-D, (◆) VCL, (△) 5-FU, (●, solid line) MTX, (○) LV and (●, dashed line) MTX+LV.

The newly developed ATP-TBA system provides evaluability rates that are clearly below those achieved with malignant tumors using the original ATP-TCA technique. In evaluable cases, however, NTB enrichment observed during the 6-day culture period was close to that reported for tumor cells,[13,15] suggesting that the ATP-TBA promotes selective growth of NTB. As a major technical improvement, the ATP-TBA avoids intensive and both time-consuming and cell-damaging NTB enrichment procedures prior to incubation, which was previously required.[11,12] Therefore, studies of the reactions of entire NTB populations against toxins or cytotoxic drugs should be facilitated by the use of this new methodology. As demonstrated by the results of pre- and post-culture immunocytochemistry, a number of stromal cells that are likely to influence the chemosensitivity of NTB might be present during the first days of incubation. As a contrast to approaches using NTB isolates, the preservation of these feeder cells which potentially exhibit chemomodulating activities may provide a more accurate estimate of the real cytotoxicity of drugs displayed in the entire specimen studied.

The chemosensitivity of NTB *ex vivo* is clearly below that found with trophoblast tumors in several experimental studies.[21–23] In particular, the relatively low anti-trophoblastic activity of MTX observed here appears to contradict clinical results with this drug in both GTN[4,9] and extrauterine pregnancy.[7,8] It should be considered, however, that the *ex vivo* concentrations of MTX utilized in this study approximate a clinical dosage of 40 mg/m^2 given at a 3-week interval.[14,17,18,24] This MTX dosage is normally used for combination chemotherapy of human solid tumors.[20] The minimum doses suggested for the treatment of both ectopic pregnancies and GTN generally are much higher (i.e. 1 mg/kg every second day).[7,8] Even then, ectopic pregnancies with serum β-hCG levels beyond 5000 U/ml often fail to respond as do trophoblastic tumors with serum β-hCG levels exceeding 100 000 U/ml.[8,10] This suggests that small volume trophoblasts or those with reduced viability are the ones which most likely respond to MTX therapy. As expected, LV completely neutralized the MTX action on NTB in all but one of the cases. Therefore, the low antifolate activity observed in this study does not appear to be an artifact and may give a realistic estimate of the NTB sensitivity against MTX concentrations routinely used for chemotherapy of common solid neoplasms. Generally, our findings agree well with those of Sand *et al.*[11] who found that NTB derived from week 8–11 abortuses were 3 logs less sensitive to MTX, as compared to previously reported results in choriocarcinoma cells.[21]

On the other hand, the chemosensitivity of NTB observed here is similar to non-trophoblastic solid tumors showing monotherapeutic EVRR between 0 and 57% in the ATP-TCA.[13–15,18,22–27] In particular, VCR, Act-D and 4-HC produced considerable cytotoxic effects on NTB. These drugs are widely used in solid and hematologic neoplasms which frequently occur in women at childbearing age such as leukemia, lymphoma, melanoma, breast cancer or GTN.[1,3,4] With the exception of the observed trend suggesting 4-HC to be somewhat more active in early NTB, the activity of the cytostatics studied did not decrease with increasing gestational age. Therefore, the trophoblast of any gestational age principally has to be considered a chemosensitive organ. Trophoblast damage may well be the primary mechanism

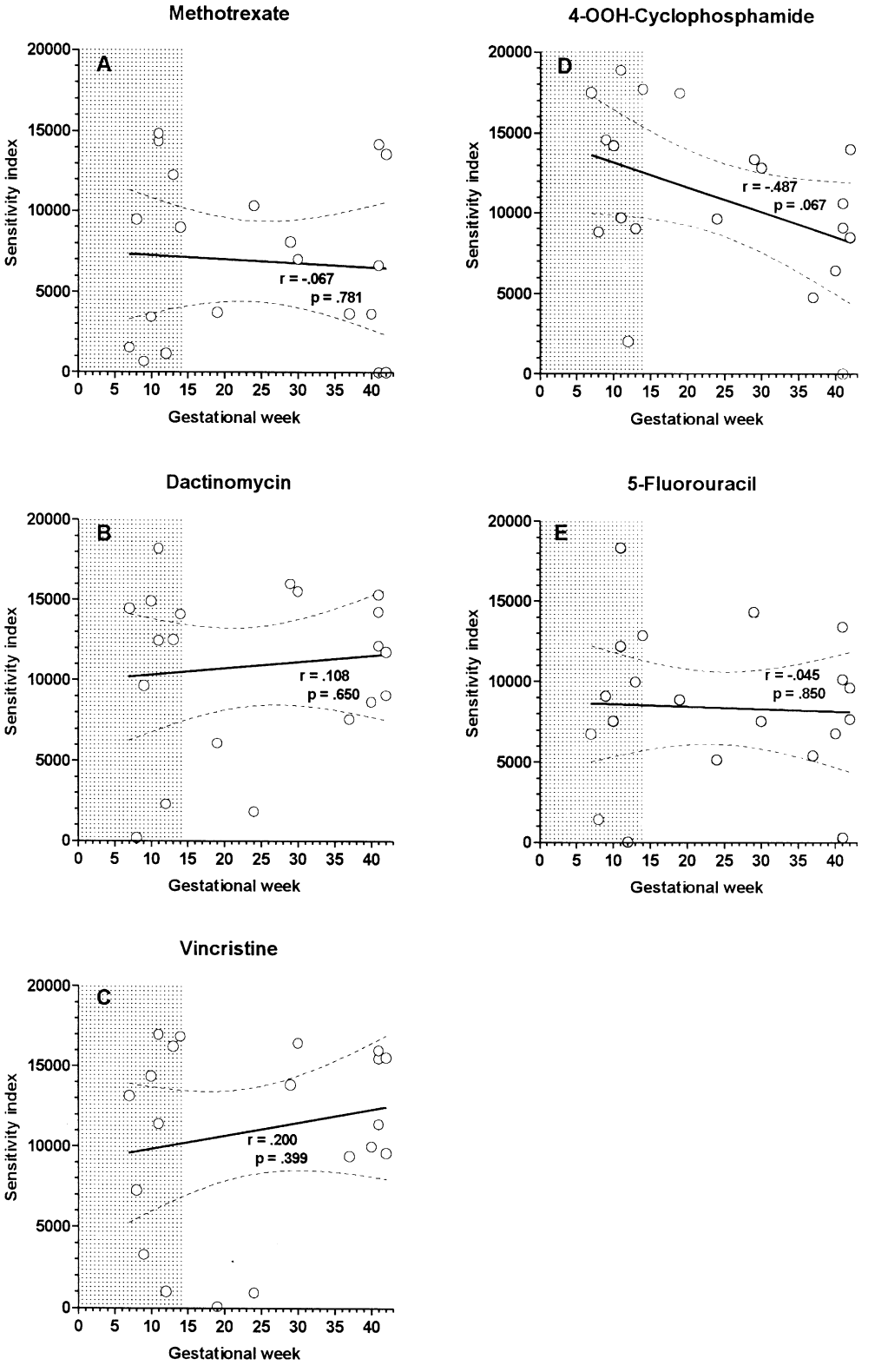

Figure 3. Correlations of gestational age of 20 evaluable trophoblasts tested with the ATP-TBA and sensitivity indices produced by MTX (A), Act-D (B), VCR (C), 4-CH (D) and 5-FU (E). The shaded areas represent weeks 1–14 of gestation.

leading to intrauterine growth restriction when antineoplastic chemotherapy is given during the second or third trimester of pregnancy. Although treatment delay mostly may be unjustified in regard to the underlying maternal disease, both physicians and patients should be aware that chemotherapy administered during pregnancy is likely to produce adverse cytotoxic effects in the placenta (and not only in the fetus) that are of the same extent as the intended antiproliferative activity to be displayed in the tumor.

Our trial represents the first systematic evaluation of NTB chemosensitivity against a panel of commonly used cytostatics. Further studies including both other drugs like anthracyclines, platinum compounds or taxanes and drug combinations are now warranted in order to optimize chemotherapy administered during the second or third trimester of pregnancy.

Conclusion

The newly developed ATP-TBA is an effective and easy-to-use methodology to selectively culture NTB freshly derived from human placentas. As a major advantage over previous approaches, the ATP-TBA respects the interaction between NTB and the surrounding stromal cells. Using this test system, we were able to show that NTB of any gestational age display remarkable chemosensitivity to different antineoplastic agents which is comparable to that of various human neoplasms studied with ATP-based luminescence assays. Therefore, the trophoblast must be considered a chemosensitive organ even at late gestational stage. Fetal growth restriction frequently associated with chemotherapy during pregnancy appears to be an indirect effect which is most likely due to relative placental insufficiency produced by the anti-trophoblastic action of the antineoplastic agents administered. The ATP-TBA is now ready to use for additional investigations increasing the efficacy and safety of therapy when cytotoxic drugs must be administered during pregnancy.

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