Increased oxidative stress in the placenta tissue and cell culture of tumour-bearing pregnant rats

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ABSTRACT
Placental dysfunction leads to foetal damage, which jeopardises the exchange between the maternal and foetal systems. We evaluated the effects of tumour growth on the activity of antioxidant enzymes and oxidative stress in placental tissue and cell culture from tumour-bearing pregnant rats compared to non-tumour-bearing pregnant rats that were ascitic fluid injected. Ascitic fluid is obtained from Walker tumour-bearing rats and contains a cytokine called Walker factor (WF), which is a molecule similar to proteolysis-inducing factor (PIF), and induces changes in protein metabolism and oxidative stress. Pregnant Wistar rats were distributed into control (C), tumour-bearing (W) and ascitic fluid injected (A) groups and were sacrificed on days 16, 19 and 21 of pregnancy to analyse the profile of enzyme activities (glutathione-S-transferase (GST), catalase (CAT), alkaline phosphatase (AP)) and malondialdehyde (MDA) content in placental tissue. Meanwhile, placenta samples from all groups were obtained on day 21, placed in primary culture and treated with WF for 72 h. The presence of tumour or ascitic fluid reduced the protein content of the placental tissue. On day 16 there was a significant reduction in AP activity in W rats, and on day 19, CAT activity and MDA content significantly increased. These results indicate that the presence of cancer decreased antioxidant enzyme capacity in the placenta, increasing the amount of oxidation in these cells, which may contribute to irreversible placental damage and compromise foetal development. WF treatment induces similar changes in placental cells in primary culture, resulting in less cell viability and increased oxidative stress. These results indicate that WF, provided by the tumour or inoculation of ascitic fluid, has negative effects on placental homeostasis, which impairs foetal health.

1. Introduction

The placenta is a remarkable organ that enables the exchange of nutrients and metabolic products between the mother and fetus to ensure proper foetal development [1]. The placenta contains highly specialised trophoblast cells that form a barrier between the maternal uterus and the fetus [1–3]. The growth of this organ is maintained by the maternal blood supply [4], which provides an adequate balance of nutrients, growth factors and hormones [5]. Alterations in this balance may result in several disorders characterised by increased oxidative stress in the placenta, such as pre-eclampsia, which is believed to result from maternal endothelial dysfunction [6,7].

Some pathological conditions (e.g., diabetes and pre-eclampsia) or conditions of malnourishment (e.g., protein–calorie malnutrition and adolescent pregnancy) are correlated with a reduced placental adaptation to oxidative stress [8–11]. An imbalance between the production of reactive oxygen species (ROS) and the antioxidants capacity to inhibit oxidative damage could result in damage to macromolecules [12,13], which can result in the impairment of placental cell function and nutrient exchange between the mother and fetus. Antioxidant enzymes include Mn or Cu superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and thioredoxin peroxidase (TPx) [14], which can counteract the deleterious actions of ROS and protect against cell damage [15].

Cancer during pregnancy is uncommon but does occur with an average frequency of 1 in 1000 births. Therefore, in the case of cancer during pregnancy, the course of treatment must take into account the unborn infant. The mechanisms that insure the survival of fetus during pregnancy presumably also favour the progression of neoplasia [16]. In previous work, we showed that pregnant rats with Walker 256 tumours had impaired foetal growth that resulted in significant changes in placental weight and protein content. In addition, increased haemorrhage and oedema were associated with high foetal resorption [17].

Tumours can increase the amount of pro-oxidant compounds and reduce the activity of antioxidant enzymes in extratumoural
tissues [18]. Because tumours can markedly alter the oxidative metabolism of distant tumour-free tissues and host organs in general, we examined the activity of key antioxidant enzymes and the level of oxidative stress in both placental tissue and placental cells in culture that were obtained from tumour-bearing and non-tumour-bearing, pregnant rats.

2. Materials and methods

2.1. Animal

Adult (90-day-old), female Wistar rats (n=92), obtained from the State University of Campinas (UNICAMP), were maintained in the Nutrition and Cancer Research Laboratory at 22 ± 2°C on a 12 h light/dark cycle with free access to a semi-synthetic control diet [AIN-93] [19] and water. All of the experimental protocols were approved by the Institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP, number # 034-5) and followed the general UKCCR guidelines for animal welfare.

Pregnant rats were confirmed by analysing vaginal smears after mating for 12 h. The pregnant rats were distributed into three groups: control (C), tumour-bearing (W), and ascitic fluid injected (A). In order to evaluate the response profile of the placenta to oxidative stress, rats were sacrificed on days 16, 19 and 21 after mating.

2.2. Tumour implantation and collection of ascitic fluid and walker factor

A suspension of Walker 256 carcinoma cells (approximately 2.5 × 10^6 viable cells in 0.5 mL) was subcutaneously injected on the 2nd day of pregnancy; control rats were injected with 0.5 mL of 0.9% NaCl (w/v) solution [20].

Ascitic fluid was obtained from the peritoneal cavity of Walker 256 tumour-bearing rats and centrifuged at 500 × g for 10 min to remove neoplastic cells. The ascitic fluid (2 mL) was administered daily by i.p. injection beginning on the 9th day of gestation. Repeated injections of NaCl, used as a control, and administered similarly to ascitic fluid, had no effect on placental development and growth [20]. All experiments were conducted on days 16, 19 and 21 after tumour transplantation or ascitic fluid treatment.

Walker factor (WF), a proteolysis-inducing factor (PIF)-like molecule, was purified from ascitic fluid of Walker tumour-bearing animals. Ascitic fluid was centrifuged and processed by precipitating the large proteins and the 24 KDa WF protein was purified and concentrated by filtering in a MILLIPORE membrane system 5 KDa [21]. The presence of WF was confirmed by western blot analysis according to Yano et al. [21].

2.3. Placental cell culture

Four separate placental tissues were collected from each rat in the C and W groups on the 21st day of pregnancy (minimum of 5 dams per group). After removing the residual foetal and maternal tissues, small pieces of placental tissue were dissected and washed twice in sterile PBS with 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% Fungisone. After trypsin digestion (0.25%), 2 × 10^6 viable cells were cultured in DMEM culture medium containing 10% FBS and the above supplements in a humidified atmosphere of 5% CO2 at 37°C. All experiments were initiated using cells grown to 90–100% confluence after the 10th day of culture. For all assays, the confluent placental cells were distributed into three experimental groups: control (C), tumour-bearing (W, placental tissue from pregnant, tumour-bearing rats) and control treated with 1.0, 3.0, 5.0 or 10.0 μg/mL WF protein in DMEM (WF, placental tissue from pregnant, control rats). After 24, 48 or 72 h of treatment with or without WF, the cells were compared under a microscope (50x) to assess the cell viability using trypan blue.

After 72 h, cells with and without treatment were washed in cold PBS to arrest cellular metabolism and were then collected in homogenisation buffer (HB, 20 mM Tris, 1 mM DTT, 2 mM ATP and 5 mM MgCl2, pH 7.2). The homogenates were centrifuged for 10 min at 500x g, and the supernatant was collected for biochemical analysis (protein concentration; GST, catalase and alkaline phosphatase activities; lipid peroxidation and DNA fragmentation), as described below.

2.4. Analytical methods

2.4.1. Biochemical analysis

Tissue samples from the placenta were weighed, homogenised in homogenisation buffer and centrifuged for 30 min at 4000 rpm; the supernatant was collected for biochemical analysis. The protein concentrations of tissue and cell homogenates were determined using the Bradford method [22] using bovine serum albumin (BSA) as a standard. All samples were assayed in triplicate using appropriate dilutions of the placental extracts.

Aliquots of supernatants of the homogenate (tissue or cells) were assayed for glutathione-S-transferase (GST) following the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione. The activity of GST was expressed in nmol/min/μg protein [23]. Catalase activity was measured as described by Cohen et al. [24] and the results were expressed in nmol/min/μg protein. Alkaline phosphatase activity was measured using 37 mM 4-nitrophenyl disodium phosphate (p-NPP) and expressed in nmol/min/μg protein [25]. The malondialdehyde (MDA) content was determined using n-methyl-2-phenylindole (MPO) as the substrate and expressed in nmol/μg protein [26]. To determine the DNA content of the placenta, the placenta was incubated with 0.5 N sodium hydroxide for 1 h and absorbance was measured at 280 and 290 nm. To assess DNA fragmentation, the DNA was isolated from cultured placental cells and separated into fragment and integral subunits using a Trizol/Triton-X method. The amount of DNA from both samples was determined using SYBR-Green and expressed in ng/mL. Data are expressed as fragment/total DNA [27].

2.5. Statistical analysis

The results were expressed as the mean ± SEM. One-way ANOVA [28] followed by Bonferroni’s test was used to compare the different experimental groups. A value of p < 0.05 indicates significance.

3. Results

3.1. Morphological parameters — cell culture

The cell viability curve (Fig. 1) shows that placental cells from control (C) and Walker tumour (W) cultures maintained confluence after 72 h. However, 21% of the cells in the W group were dead compared to the C group (Fig. 1). Cell death was observed in placental cells 48 h after treatment with 1 μg/mL Walker factor...
and increased by 19% 72 h after treatment. Treatment with 3, 5 or 10 µg/mL of WF reduces the number of viable cells by 50, 74 and 84%, respectively (Fig. 1).

3.2. Placental tissue analysis

**Protein Content**: The protein content in placenta tissue decreased 64.4% and 25.4%, in the tumour-bearing groups (W16 and W19, respectively) and 67.6% and 52.4% in the ascitic fluid groups (A16 and A19, respectively) (Fig. 2A). On day 21, there was a 28.2% reduction in the protein content of the placenta in the ascitic group (A21), when compared to C21 (Fig. 2A).

**Alkaline Phosphatase**: On day 16, alkaline phosphatase activity decreased by 51.3% in the tumour-bearing group (W16). Although not statistically significant, due to high variability among samples, mean alkaline phosphatase activity tended to average 24% lower in the tumour-bearing group at day 19 and by 41% and 29% in the ascitic groups at day 16 and 19, respectively, when compared to the control groups (C16 and C19) (Fig. 2B).

**Glutathione-S-transferase (GST) and Catalase (CAT)**: GST activity in placental tissue was similar in both control and tumour-bearing rats (Fig. 2C). There was a significant reduction (up to 50%) in the GST activity in the placenta from the ascitic fluid group (A16) (Fig. 2C). The CAT activity was increased by 3.6 fold in tumour-bearing dams (W19) and was reduced in the ascitic fluid group (A16) by 44% compared with the C16 group (Fig. 2D).

**Placental Malondialdehyde content (MDA)**: The MDA levels in the placenta tissue increased approximately 71% and 125.9% in tumour-bearing pregnant rats after 16 and 19 days of pregnancy (W16 and W19, respectively) (Fig. 2E).

**DNA Content**: There was no difference in the placental DNA content among all groups after 16 days of pregnancy. However, the presence of tumour decreased the DNA content after 21 days of pregnancy (Fig. 2F).

3.3. Placental cell culture analysis

**Protein content**: The protein content was similar in all placental cell cultures independent of the treatment (Fig. 3A).

**Alkaline phosphatase**: The alkaline phosphatase activity increased in the placental cells obtained from tumour-bearing rats (W). Walker factor treatment, regardless of concentration, did not alter the alkaline phosphatase activity (Fig. 2B).

**Glutathione-S-transferase and Catalase**: There was no statistical difference in the GST activity of placental cells treated with WF (Fig. 3C). In contrast, the CAT activity was reduced in the WF10 treatment group (62.7% lower) (Fig. 3D) compared with the other treatments.

**MDA content**: There was no statistical difference in the MDA levels of cultured cells treated with Walker factor (WF) (Fig. 3E).

**Fragmented DNA**: The placental cell cultures from the Walker tumour group (W) and the group treated with 5 mg/mL of Walker

![Fig. 2.](image-url) Protein content (A), alkaline phosphatase activity (B), glutathione-S-transferase activity (C), catalase activity (D), MDA content (E) and DNA content (F) of placenta tissue from dams sacrificed on days 16, 19 or 21 of pregnancy. Legend: C- control, W- tumour-bearing, A- injected with ascitic fluid. The results are expressed as mean ± SEM. Number of animals is 10 rats/group for each day of sacrifice. *p < 0.05, compared to the corresponding control groups.
4. Discussion

Few studies have examined the effects of cancer on pregnancy. Although uncommon, there are many tumours, such as breast cancer, one of the most common cancers that can occur concomitantly with pregnancy [29–31]. Tumour growth can be detrimental to the mother, fetus and maternal-foetal unit. The rapid growth of Walker 256 tumours during pregnancy adversely affects foetal development [32] and placental homeostasis, as previously described [17,33–35]. Because the growth and metabolic characteristics of the fetuses are very similar to those of rapidly growing tumours, there is competition for the nutritional supply between the fetus and the tumour in a pregnant mother with cancer. In addition, factors produced by the tumour or by the host in trying to fight the tumour, such as cytokines and humoral factors, can jeopardise the foetal development. Our previous studies have demonstrated that tumour-produced factors have a greater effect on foetal development than inadequate nutrition. For example, ascitic fluid injected into pregnant, non-tumour-bearing rats results in a similar level of foetal resorptions and deaths compared to tumour-bearing pregnant rats, which is not observed in rats with low foetal weight [17,20,36]. The fetuses of malnourished, pregnant rats have a lower weight rather than undergoing foetal resorption and death [37,38]. The present work demonstrates an increase in oxidative stress in both placental tissue and placental cells in culture in response to the tumour. These effects can be seen throughout the growth of the tumour and also by the Walker factor resulting in changes in antioxidant enzyme activity, oxidative stress biomarkers (MDA content) and DNA fragmentation.

As shown in our previous studies, tumour growth significantly reduced foetal and placental weight indicating impairment in foetal development [33,36]. In association with advanced gestational age, Walker tumour leads to a high number of foetal resorption sites [20,33]. In addition, haemorrhage and oedema were seen in placental tissue from tumour-bearing and ascitic fluid injected rats, suggesting that tumour-produced factors are responsible for placental damage [17,20]. In the present study, the results suggest that placental tissues or placental cells in culture obtained from tumour inoculated and ascitic fluid injected rats were negatively affected by tumour factors throughout the duration of the pregnancy.

The normal development of a placenta is essential for the growth of a healthy fetus, and placenta function is critical at all stages of pregnancy. Protein malnutrition and a calorie deficit can restrict intrauterine growth [37,39]. On the other hand, food restriction during pregnancy does not compromise placental growth [40]. Maternal malnutrition resulting from cancer does not interfere with normal placental growth. However, as seen here, direct and/or indirect tumour effects can lead to reduced weight and protein content of the placenta [20], possibly a consequence of
increased protein degradation and/or reduction in placental protein synthesis. Studies have reported that the maximum rate of protein synthesis in rat placenta is achieved on day 19 of pregnancy, as we have confirmed in our study. Placental protein synthesis can also be regulated through the Akt/mTOR pathway [34]. Investigations by Yang et al. [41] demonstrated that genetic knockout of Akt1 in the mouse resulted in placental and foetal growth retardation. Yung et al. [35] demonstrated that a decrease in 4E-BP1 phosphorylation in human placentas derived from fetuses with intrauterine growth restriction (IUGR) could result in a reduction in placental protein synthesis. We have previously demonstrated that Walker tumour and Walker factor (PIF-like) can change muscle protein synthesis by reducing eukaryotic initiation factors (eIF4E, eIF4G and p70S6 kinase). Presumably this can also occur in placental cells [21].

Reduction in placental protein content appears to be indicative of tissue damage mediated by tumour factors [42,43]. For example, Walker Factor treatment damaged placenta tissue and cultured cells, resulting in reduced cell viability and increased DNA fragmentation.

We have found that tumour growth damages placental tissue by altering the levels of apoptotic precursors [36]. There is some evidence for the occurrence of distinct apoptotic pathways in the placenta of tumour-bearing and ascitic fluid injected rats. Activated caspase-3 cleaves PARP, one of many caspase substrates, to prevent this enzyme from requiring damaged DNA, which results in the initiation of apoptosis. In the present study, we have shown that placental tissue or placental cells in culture are affected by the tumour during the course of pregnancy resulting in decreased DNA content or increased DNA fragmentation.

The formation of ROS during apoptosis could activate other apoptotic pathways. Various studies have shown an increase in apoptosis following B19 infection of villous trophoblast cells [44], an elevation in caspase-3 activity and cytochrome c release in placentas of pregnant animals [45] and higher rates of apoptosis in placenta from pregnancies complicated with intrauterine growth restriction [46]. Since trophoblast cells are responsible for maintaining normal placenta function, the uncontrolled death of these cells could have a negative impact on foetal development. As our viable cell assays confirm, WF treatment which mimics tumour derived factors, decreases placental cell viability suggesting a possible direct link between tumour factors and damage to the placenta.

An imbalance between lipid peroxides and the antioxidant system may result in placental dysfunction and subsequent inadequate nutrient transfer to the fetus. The production of lipid peroxides and free radicals by the placenta affects its integrity leading to placental damage as seen in pre-eclampsia [47] or pregnancy complications. The elevated level of MDA in pregnant, tumour-bearing rats by day 19 of pregnancy (W19) could be an indicator of lipid peroxidation in placental tissue. In contrast, catalase enzyme activity in this same group is increased, perhaps due to the ability of this antioxidant enzyme to eliminate free radical. The presence of antioxidant enzymes in the placenta increases with normal gestational progression to protect the own cell activities and also the fetus against oxidative damage [48,49]. In contrast, cancer causes oxidative stress, which results in an increased production of free radicals and a decreased activity of antioxidant enzymes. To confirm this, Sinha et al. [50] demonstrated an increase in blood levels of MDA and a reduction in catalase and SOD levels in breast cancer patients. In the present study, the antioxidant enzyme system in the tumour-bearing groups was not effective at neutralising the oxidants because the activity of catalase was increased in tumour-bearing groups and GST activity was similar to control groups. Other factors released by the tumour, such as TNF-α, have been implicated in placenta and foetal weight loss and a decrease in placental protein content in pregnant rats [17,20,33]. In many cell types, including human trophoblasts, oxidative stress serves as a signal to directly or indirectly activate apoptosis. Although apoptosis occurs as part of the normal cell turnover in the placenta, an excessive amount of apoptosis and oxidative stress may compromise the functions of the trophoblast and could be a primary pathological event or a contributing factor in complications experienced during pregnancy.

In this experimental model, we observed the direct and indirect effects of Walker Factor (PIF-like) treatment on placental cells. Since Walker Factor has immunological and molecular weight characteristics identical to PIF, it also acts by changing host protein turnover [21] altering cell activity. The cultured cells from tumour-bearing, pregnant rats (W) showed an increase in alkaline phosphatase activity, suggesting alterations in cellular activity [36]. The increase in alkaline phosphatase activity may influence apoptosis in these cells. Cells cultured from tumour-bearing rats (W) may be correlated with the W21 group (in vivo experiment) that tends to have enhanced alkaline phosphatase activity. We observed a correlation between in vivo and in vitro antioxidant enzyme activities (Cat and GST) and lipid peroxidation (MDA) whereby the C and W groups demonstrated similar results observed in the C21 and W21 groups. These data indicate that Walker Factor may exacerbate placental dysfunction, which is characterised by changes in placenta architecture and loss of cell adhesion. Treatment of placental cells in culture with WF resulted in a dose- and time-dependent decrease in the number of cells, as well as modified cell morphology, which is important in placenta physiology [50]. Walker factor treatment may interfere in reduction—oxidation reactions resulting in an increase in oxidative stress. While cellular mechanisms (slight increase in GST and CAT activities) attempt to neutralise lipid peroxidation, these mechanisms may be insufficient to compensate for the increased oxidative stress produced by the tumour. This results in Walker factor (PIF-like) interfering in placental cellular activity, reduced cell viability by altering enzyme activities and caused DNA fragmentation. Many malignancies could alter the normal course of pregnancy and impair the maternal/foetal unit by restricting foetal health and placenta homeostasis. Further studies are now underway in our laboratory to determine the cell signalling events in placenta that occur during pregnancy as a result of tumour development.

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