ABSTRACT

Breast cancer not only causes morbidity but is also responsible for reducing the quality of life among younger premenopausal survivors due to chemotherapy-induced premature ovarian failure and infertility. This doctoral thesis examines the toxic effects of commonly used breast cancer chemotherapeutics (doxorubicin and cyclophosphamide) on ovarian carcinoma (KGN) cells and murine ovarian follicles *in vitro*.

The breast cancer chemotherapy regimen has adverse side-effects caused by the generation of reactive oxygen species (ROS), as well as the specific mechanisms of action targeting proliferating cells. Tocopherols (alpha, beta, gamma and delta) have proven antioxidant activity. Moreover, previous studies demonstrated cytotoxic effects of gamma tocopherol (gToc) against breast cancer cells *in vitro* and *in vivo.* Hence, it was hypothesised that the addition of gToc to doxorubicin and cyclophosphamide would neutralize ROS generated during chemotherapy and increase ovarian follicle cell viability and hormone synthesis.

Clinically relevant doses of the activated form of cyclophosphamide, 4-hydroxycyclophosphamide (4OHCYC), were not toxic to ovarian carcinoma cells *in vitro*. Therefore, the dose of doxorubicin (5µM) at which 50% of KGN cells were killed (EC50) was combined with 4OHCYC at a dose (0.5µM) that maintains the 10:1 ratio found in patient serum. This combination significantly reduced KGN cell viability after 72h continuous exposure but the addition of gToc neither inhibited nor stimulated KGN cell viability or estrogen production. This study showed that doxorubicin but not 4OHCYC was cytotoxic to ovarian carcinoma cells at clinically relevant doses *in vitro*. Therefore, the inhibitory concentrations (IC values) of doxorubicin, 4OHCYC and gamma tocopherol against the MCF-7 breast cancer cell line were determined.

To study the direct effects of chemotherapeutics and antioxidants on follicles, it was necessary to isolate them from ovarian tissue in a way that avoided structural damage and maintained the physiological functions and viability of the follicles. Therefore, an easy, economical and robust method for isolating follicles from ovarian tissue and assessing the viability of the follicular cells were developed and characterised. The latter involved disaggregating follicles to generate a single cell suspension that could be assessed by trypan blue assay. There was a linear relationship between granulosa cells (GC) per follicle and follicle diameter (R^2: 0.9). From this study it was determined that morphologically intact, freshly isolated murine secondary follicles (125±6µm to 185±6µm) have 287

to 488 GCs of which only 61% to 72% are viable. This study also showed that follicle cell viability was not affected by the follicle isolation technique. However, the numbers of GCs derived from image analysis of intact, whole fluorescent-stained follicles were approximately 50% lower than from disaggregated follicles combined with trypan blue assessment. This difference was attributed to the 3-dimensional structure of the intact follicles because this may have obscured the fluorescent signal. The granulosa cell numbers derived from this study were higher than values estimated using fixed ovarian sections. It was concluded that the direct quantification of viable GC isolated from disaggregated follicles is the most accurate and reproducible method for assessing GC number and viability.

Follicles cultured in a 2D *in vitro* system did not grow during a 72h culture period. Their spherical structure was disrupted but the cell viability was similar to that found in freshly isolated follicles. Follicles produced 0.84±0.07pg/mL estrogen and 5.66±6.82pg/mL progesterone after 24h which was lower than that produced in a 3D culture system. Since the 2D culture of follicles led to the disruption of follicle morphology and dispersion of GCs across the surface of the culture plate, it was decided to examine and develop 3D follicle culture systems. Two 3D matrices were examined in a 48h study. Matrigel was performed because it supported the maintenance of spherical follicle morphology (47% with high M1 or M2 grade), granulosa cell viability (64% to 69%) and synthesis of both estrogen and progesterone. None of the 2D or 3D culture conditions examined supported AMH synthesis. Therefore, Matrigel was selected for further studies in which murine secondary follicles were exposed to commonly used breast cancer chemotherapeutics with or without antioxidants.

Doxorubicin and 4OHCYC alone and in combination were more toxic to primary derived follicular GCs within an intact follicle *in vitro*, than to the breast cancer cell line MCF-7, because the IC25 values of doxorubicin and 4OHCYC obtained from 24h MCF-7 exposures killed higher proportions (74% and 63% respectively) of cells inside intact murine secondary follicles *in vitro*. The combination of doxorubicin and 4OHCYC was additive; instead of killing 50% of the cells as exposure to the combined IC25 values of the two chemotherapeutics would predict, the combination killed 84% of the follicular cells. Exposure to the IC25 value of gamma tocopherol (gToc) obtained from the MCF-7 cells however was not at all cytotoxic to follicle cells and the addition of gToc to the doxorubicin and 4OHCYC combination also had no additional cytotoxicity. However, in this study the vehicle control for gToc, 0.3% DMSO, also maintained the morphological integrity and viability of follicles. Leading to the conclusion that the study should be repeated with a higher concentration of gToc to be able to give an unequivocal description of its separate effects on follicles.