The Effect of Ifosfamide and Cyclophosphamide on the Pre-Pubertal Testis

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As childhood cancer survival rates improve, it is increasingly important to focus on reducing toxicities and late effects of treatment, including the adverse impact of treatment on future fertility. Alkylating chemotherapy agents are commonly used in paediatric oncology treatment regimens, and are widely accepted to be amongst the most gonadotoxic anti-cancer drugs. Much of our understanding of the detrimental effects of alkylating agents is drawn from clinical studies, and relatively less is understood about the direct effect of individual agents on gonadal tissue. Understanding this damage may enable development of protective strategies, and this is of particular relevance to pre-pubertal males, for whom there are currently no clinically available methods of fertility preservation. Here, we examine the direct effects of two commonly used alkylating agents – cyclophosphamide and ifosfamide – on the pre-pubertal testis, using an in vitro culture model. Clinical studies support that cyclophosphamide is significantly gonadotoxic, but relatively less is known about the effect of ifosfamide on male fertility.

Testes were dissected from male mice (n=6) on postnatal day 5, cut into fragments, and cultured in αMEM with 10% KSR at 34^{o} and 5% CO_2. Tissue was randomly allocated as control or to receive treatment with a metabolite of either ifosfamide (4-hydroperoxyifosfamide; 4HI) or cyclophosphamide (4-hydroperoxycyclophosphamide; 4-OOH-CP), at the following concentrations: 1 µg/ml, 10 µg/ml, 20 µg/ml, 100µg/ml. The metabolites were used because ifosfamide and cyclophosphamide are pro-drugs which require activation through hepatic metabolism; concentrations were selected based on the limited data available about patient serum levels. After six hours of exposure, tissue was moved to drug-free media. Media was then changed every 24-48 hours until day 5, at which point tissue was fixed in NBF. Immunohistochemistry was then performed to quantify germ cells (MVH), spermatogonial stem cells (SSCs; PLZF), Sertoli cells (SOX9), and peri-tubular myoid cells (SMA). ELISA was performed on culture media to assess testosterone production. Data were analysed in GraphPad Prism using one-way ANOVA with Sidak or Dunnett's post-hoc test.

Exposure to 4HI and 4-OOH-CP caused a significant loss of germ cells relative to control at all but the lowest treatment concentration (p<0.001). SSC density was significantly reduced by treatment with 20 µg/ml (p=0.049) and 100 µg/ml 4-HI (p=0.043), and by treatment with 100 µg/ml 4-OOH-CP (p=0.027). Sertoli cell density was significantly reduced only by treatment with the highest concentration of 4-OOH-CP (100 µg/ml; p=0.006), and density of peri-tubular myoid cells was not affected by any concentration of either drug. Media testosterone concentration increased in a dose-dependent manner, reaching a significant increase at 20µg/ml of either metabolite (p=0.001); however, this was followed by a significant reduction in testosterone levels relative to control following treatment with 100µg/ml of either 4HI (p=0.005) or 4-OOH-CP (p=0.002).

These findings suggest that ifosfamide and cyclophosphamide selectively damage the germ cells of the immature testis, with higher concentrations specifically targeting the subpopulation of SSCs. There is relative sparing of the somatic cells, with damage to Sertoli cells only seen at the highest concentration of the cyclophosphamide metabolite. However, testosterone production is affected in a dose-dependent manner. Given that testosterone secretion would be expected to be relatively low

before puberty, further work is required to examine whether this is a clinically relevant effect of treatment or whether it may reflect the culture model.