Descriptive Study of the Effects of Phosphoramide Mustard on the Testis

Background

The increase in survival rates of cancer patients over the last 50 years presents new challenges in improving quality of life for survivors. A major concern for these survivors is their subsequent fertility following treatment with chemotherapeutics. Fertility preservation is particularly challenging in the case of pre-pubertal boys who are not yet producing sperm which can be cryopreserved and a detailed understanding of how the drugs damage the testis is necessary for the design of protective treatments. Cyclophosphamide is one of the most widely used chemotherapy drugs. It is a pro-drug which is metabolised to give phosphoramide mustard (PM) and acrolein (Phillip et al 1999). PM is the active metabolite, exerting the anti-neoplastic effects in patients. In rats, a single injection of cyclophosphamide induces apoptosis among germ cells, particularly the differentiating spermatogonia (Drummond et al 2011). This project examined the effects of 24hr exposure to PM on mouse testis tissue in culture. The aim was to observe the concentration-dependent effects of the drug on cell death and proliferation in the different cell types of the testis.

Methods

Testes were dissected from mice at post-natal day 5 and cut into small pieces which were then cultured for 4 days. On day 2, varying concentrations of PM (0.01µM, 0.1µM, 1µM and 10µM) were added to the media, with a proportion of pieces acting as controls. Drug doses used were chosen to reflect the range of patient serum levels previously reported in the literature. Drugs were washed out on day 3 and BrdU, a thymidine analogue which becomes incorporated into replicating DNA, was added to the media to label dividing cells. The pieces were then fixed on day 4. Once fixed, the pieces were embedded in agar and processed into wax blocks. Histological sections were then cut from these blocks and floated out onto slides for immunofluorescent staining. Mvh expression was used as a marker of germ cells, Sox9 as a marker of Sertoli cells, COUPTFII as a marker of interstitial cells and cleaved caspase 3 (CC3) as a marker of apoptotic cells. Images of stained tissue were analysed using ImageJ software. Results were analysed on GraphPad Prism by one-way ANOVA, or Kruskal-Wallis test for non-parametric data.

Results

Analysis of the results indicated that PM caused a loss of germ cells. At 1µM and 10µM, there was a significant decrease ($P<0.0001$) in germ cells. Interestingly, a significant increase was seen at the lowest concentration (Fig. 1; Fig 2. A.). No significant changes were seen in BrdU (Fig 1.). Therefore, cells in the testis continued to divide even at the highest drug concentration.

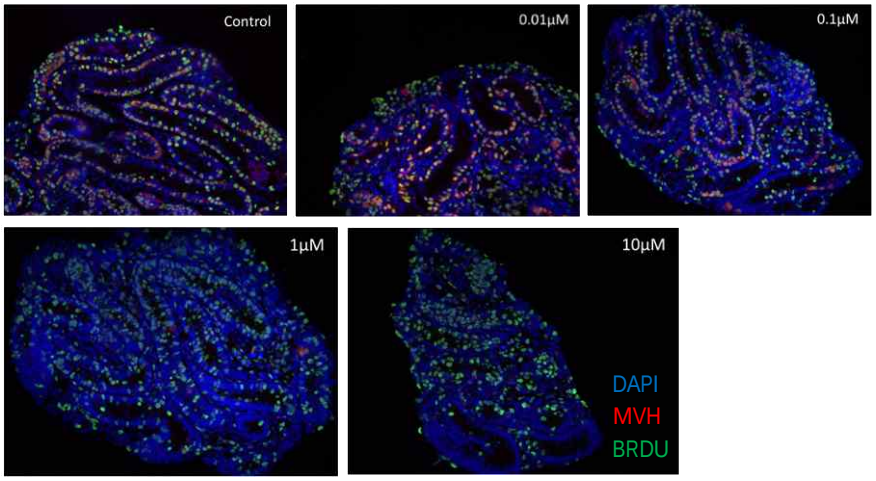

Fig. 1. Representative photos of Mvh/BrdU immunofluorescence at each concentration of PM. Germ cells significantly decrease at 1µM and 10µM concentrations.

No significant differences in Sox9 expression were observed, suggesting that Sertoli cells were not depleted by PM (Fig. 2. B.). CC3 did not differ significantly from controls at the lower drug concentrations but showed a sharp and significant increase at 10µM. At a concentration of 1µM, a significant decrease in Mvh expression was seen without a corresponding increase in CC3 expression. Perhaps the cell death caused by PM at 1µM has begun to die down by day 4 of culture, but is still ongoing at the highest concentration. A time-course analysis could provide more information on the temporal changes of CC3 expression. The unchanged expression of Sox9 at all drug concentrations suggest that PM may target the germ cells directly instead of their supporting cells. CC3 was also observed in interstitial cells. If Leydig cells were being affected by the drug, there may be hormonal changes (e.g. a drop in testosterone) which could contribute to germ cell death. In future studies, testosterone levels in the culture medium could be measured to test for any changes at each drug concentration. Although statistical analysis suggested that there was no significant change in COUPTFII with drug dose, it appears as though there may be a trend towards an increase at higher concentrations (Fig. 2. C.). It may be that tubules shrunk in response to PM, increasing the relative area of the interstitium. Detailed analysis of tubule diameter could be undertaken to test this theory. It would be interesting in the future to examine more specifically the drug's effects on the different interstitial cell types.

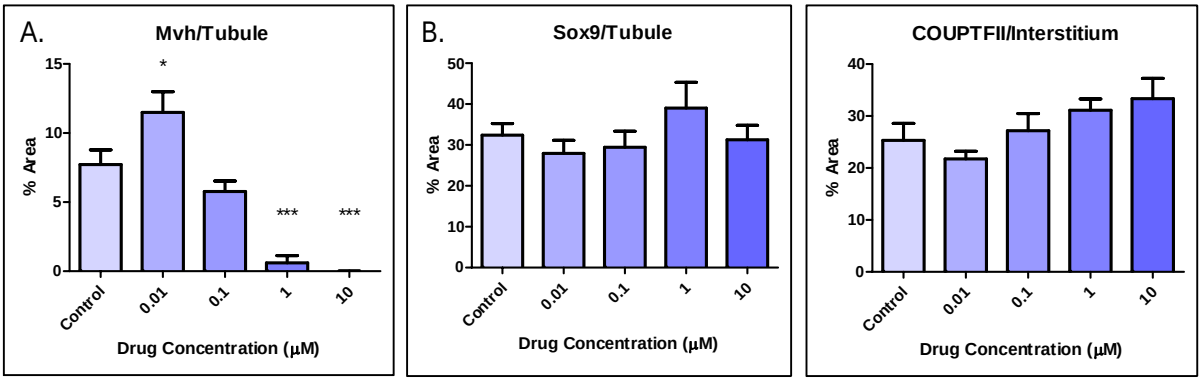

Fig. 2. A. Mvh expression at each drug concentration as a proportion of tissue section area. One-way ANOVA showed significant difference ($P<0.0001$). Significant difference from control as determined by Dunnett's Multiple Comparison test shown on graph. **B.** Sox9 expression at each drug concentration as a proportion of tissue section area. One-way ANOVA showed no significant changes. **C**. COUPTFII expression at each drug concentration as a proportion of tissue section area. One-way ANOVA suggested no significant changes ($P=0.0652$).

Conclusion

In the testis, PM significantly depletes germ cells. Sertoli cells appear to remain unaffected, at least in number, even at the highest dose. Apoptosis significantly increases at 10µM PM. It will be helpful in the future to carry out time-course analyses to better determine the effects of this drug on the testis in the time following exposure.

References

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