Toxicology Letters 265 (2017) 70–76

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Germ cell responses to doxorubicin exposure *in vitro*

Khaled Habas, Diana Anderson, Martin. H. Brinkworth*

School of Medical Sciences, Faculty of Life Sciences, University of Bradford, Bradford, Richmond Road, West Yorkshire, BD7 1DP, UK

HIGHLIGHTS

- Purified cells were treated with different concentrations of the genotoxic agent Doxorubicin (Dox) and assessed for DNA damage and apoptosis.
- Exposure of the purified germ cells to Dox yielded significant increases in DNA damage and apoptosis.
- Dox disrupts spermatogenesis by causing DNA damage and apoptosis in spermatogonia, spermatocytes and spermatids.
- The effects of Dox were cell type- and exposure-dependent with the strongest responses at the highest concentration in spermatogonia and a lack of response in spermatids at the lowest concentration.

ARTICLE INFO

Article history:
Received 12 November 2015
Received in revised form 21 October 2016
Accepted 23 November 2016
Available online 24 November 2016

Keywords:
DNA damage
Apoptosis
Doxorubicin
Comet assay
TUNEL assay
Male germ cells

ABSTRACT

Anthracyclines such as doxorubicin (Dox), widely used to treat various types of tumours, may result in induced testicular toxicity and oxidative stress. The present investigation was designed to determine whether exposure of isolated and purified mouse germ cells to Dox induces DNA damage in the form of strand breaks (presumably) resulting in apoptosis and to investigate the relative sensitivity of specific cell types. DNA damage was assessed using the Comet assay and the presence of apoptosis was determined by TUNEL assay. Isolated mouse germ cells were treated with different concentrations (0.05, 0.5 and 1 mM, respectively) of Dox, and fixed 1 h after treatment. The incidences of both DNA damage shown by single cell gel-electrophoresis and of apoptosis increased significantly in each specific cell type in a concentration-dependent manner. The DNA damage and apoptosis incidences gradually increased with concentration from 0.05 to 1 mM with Dox. Our results indicate that apoptosis plays a vital role in the induction of germ cell phase-specific toxicity caused by Dox with pre-meiotically and meiotically dividing spermatogonia and spermatocytes respectively as highly susceptible target cells.

1. Introduction

A frequently used chemotherapeutic drug is the extremely effective anthracycline doxorubicin (Dox) also known as adriamycin (Badkoobeh et al., 2013). It is the antineoplastic drug of choice in the treatment of various types of tumours such as, childhood leukemia and testicular cancer, though one of its adverse effects is male infertility (Shamberger et al., 1981; Imahie et al., 1995; Prahalathan et al., 2005; Vendramini et al., 2010).

Dox is well known to be a mutagen in both somatic cells (Smith, 2003; Pulte et al., 2008) and in early spermatogenic cells (Sjoblom et al., 1998; Zanetti et al., 2007). Even a low dose of Dox (1.0 mg/kg) damaged mouse spermatogonia, though that dose was ineffective on primary spermatocytes (Lu and Meistrich, 1979). One of the responses to DNA damage induced by Dox is apoptosis and the induction of apoptosis in the adult testis is often one of the earliest signs of genotoxic damage (Jahnukainen et al., 2000). In the neonatal rodent testis, however, spontaneous apoptosis is extremely important for the maintenance of appropriate germ-cell number relative to Sertoli cell number (Rodriguez et al., 1997). In addition, various endogenous, normal physiological processes such as insulin signalling (Dias et al., 2013) can influence levels of testicular cell apoptosis.

Dox exerts an effect on spermatogonial cells mainly because of their high division rate and slow cell cycle (Brilhante et al., 2012). Recent study has shown that the higher mitotic turnover is required for spermatogonia to generate the same number of differentiated germ cells as species with a lower turnover rate but a higher number of differentiating spermatogonial generations

* Corresponding author.
E-mail address: m.h.brinkworth@bradford.ac.uk (M. H. Brinkworth).

http://dx.doi.org/10.1016/j.toxlet.2016.11.016
0378-4274/

(Ehmcke et al., 2006; Krieger and Simons, 2015). This higher mitotic turnover could increase the risk for germ cell mutations and vulnerability to cytotoxic events (Waheeb and Hofmann, 2011).

A wide range of clinical and experimental studies have demonstrated the testicular toxicity caused by Dox (Damani et al., 2002). It has been found that even a low dose of Dox (1 mg/kgb w) given to adult mice is able to target germ cells, mainly spermatogonia, leading to seminiferous epithelium depletion (Jahnukainen et al., 2000). The intercalation of Dox into germ cell DNA during division is considered to be the principal cause of cellular death in the seminiferous epithelium and influences the number of germ cells located in the vicinity of the basement membrane of the testis (Vendramini et al., 2010). Blood-testis barrier injury from Dox exposure, mediated by the generation of free radicals, has also been reported (Jahnukainen et al., 2000). It also exerts its effects *via* a mechanism that includes intercalation with DNA and consequent inhibition of topoisomerase II (Topo II) activity, which results in replication-dependent, site-selective double-strand breaks in DNA (Myers and Chabner, 1990; Quiles et al., 2002) because anthracyclines inhibit the re-ligation of these breaks (Zunino and Capranico, 1990). Dox has also been shown to interfere transcription and the stability of chromosomes, by affecting DNA methyl transferase 1 activity, inducing apoptosis (Yokochi and Robertson, 2004), which also makes it potent in the developing germ cell line, leading to male infertility (Dacunha et al., 1983; Meistrich, 2013a). This in turn leads to up-regulation of p53, which prevents DNA replication in the presence of DNA damage and can thus lead to apoptosis (Bunz et al., 1998). Dox can also reduce the viability of cancer cells through RNA damage (Fimognari et al., 2008).

The generation of free radicals by Dox arises from its ability to bind iron and form complexes with DNA, thus resulting in DNA damage (Eliot et al., 1984; Ravi and Das, 2004) (Injac and Strukelj, 2008). This free radical generation from Dox causes genotoxicity in normal cells (Quiles et al., 2002) and in different types of cancer cells (Gouaze et al., 2001). The induction of apoptosis in the adult testis is often one of the earliest signs of genotoxic damage (Jahnukainen et al., 2000). In the neonatal rodent testis, however, spontaneous apoptosis is extremely important for the maintenance of appropriate germ-cell number relative to Sertoli cell number (Rodriguez et al., 1997). In addition, various endogenous, normal physiological processes such as insulin signalling (Dias et al., 2013) can influence levels of testicular cell apoptosis. Exposure to Dox induces intracellular oxidative stress that can be ameliorated *via* the overexpression of antioxidant enzymes that prevent apoptosis in tumour cells (Suresh et al., 2003). Blocking the activity of endogenous antioxidants, such as glutathione peroxidase-1 produced by tumour cells, or depletion of glutathione pools also enhances the sensitivity of tumour cells to Dox (Gouaze et al., 2001; Poirson-Bichat et al., 2000). It is therefore appropriate to investigate the adverse effects of Dox on susceptible normal cells such as spermatogenic cells, which are likely to be vulnerable to damage in a similar way as the tumour cells targeted during treatment.

2. Materials and methods

2.1. Animals

Male NMRI mice (National Medical Research Institute) 12 wk of age were obtained from the Institute of Cancer Therapeutics, University of Bradford, UK where they were maintained under standard conditions. All animal care procedures were carried out according to the National Research Council's Guide for the Care and Use of Laboratory Animals.

2.2. Chemicals

Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich, Poole, UK.

2.3. Preparation of cells and culture

The method for fractionation of mouse testicular germ cells was as described previously (Habas et al., 2014). Briefly, testes were collected from four male adult (10–12 week-old) NMRI mice, decapsulated, and the seminiferous tubules placed into ice cold Dulbecco's Modified Eagle's medium (DMEM), dispersed by gentle pipetting, minced and resuspended in fresh DMEM containing collagenase (5 mg/ml) and DNAse (1 μg/ml), then incubated at 32 °C for 20 min. The cells were left to stand for 5 min before being filtered through an 80 μm nylon mesh (Tetco Inc., Briarcliff Manor, NY), centrifuged at 600*g* for 10 min and bottom-loaded into the separation chamber of a Staput apparatus in a volume of 10 ml. A 2–4% w/v concentration gradient of BSA was then generated below the cells, which were allowed to sediment for a standard period of 2.5 h before 31 separate 12 ml fractions were collected at 60 s intervals. The cells in each fraction were examined under a phase contrast microscope, and consecutive fractions containing cells of similar size and morphology spun down by low-speed centrifugation and resuspended in DMEM. The identity and purity of all cell preparations used in the experiments was confirmed by Reverse Transcription PCR assay (RT-PCR) and quantitative Reverse-Transcription PCR assay (RT-qPCR) as described below in Sections 2.3 and 2.6 respectively. The viabilities of the freshly isolated spermatogonia, spermatocytes and spermatids were routinely >95%, as evidenced by trypan blue exclusion (Phillips, 1973) of these cells. The germ cells were cultured overnight at 37 °C. The following day, viability was re-checked and the cells treated with mutagen as required (Section 2.4). Viabilities were checked again and were found to be routinely >89% for cells that had been exposed to Dox. They were then used immediately for qPCR, Comet assay or TUNEL assay.

2.4. Confirmation of identity of purified, mouse spermatogonia, spermatocytes and spermatids by RT-PCR and RT-qPCR

The identity and purity of all cell preparations used in the experiments was confirmed by RT-PCR for the presence or absence of spermatogonial-, spermatocyte- and spermatid-specific mRNA. Thus, total RNA was extracted from the freshly isolated mouse spermatogonia, spermatocytes, spermatids, and mouse testis tissues, using TRIzol reagent (Invitrogen Carlsbad, CA), and total RNA quantity and quality was checked using $OD_{260/280}$ measurements. Reverse transcription (RT) was performed as described below in Section 2.5.

Portions of genes specifically expressed in each of the main categories of male germ cells were amplified from cDNA produced as described above by PCR using primers for glial cell line derived neurotrophic factor receptor (GDNFR) (spermatogonia), synaptonemal complex protein 3 (SCP3) (spermatocytes), Transition protein-1 TP1 (spermatids) and β-actin for RT-PCR. (RT-qPCR was also performed for these genes: see Section 2.6 for details.) The PCR reactions started with a single step of 94 °C for 2 min, which was followed by the following cycle pattern: denaturation at 94 °C for 30 s, annealing at 58–61 °C (depending on the primer pair) for 30 s, and elongation at 72 °C for 30 s. After 30 cycles, the samples were incubated for an additional 5 min at 72 °C. PCR products were separated by electrophoresis on 1.5% agarose gel and visualized with ethidium bromide. Images were recorded and band intensities analysed using a digital gel documentation system (UVItec, Cambridge, UK).