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Dominant lethal effects of nocodazole in germ cells of male mice

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ABSTRACT

The ability of the anticancer drug, nocodazole, to induce dominant lethal mutations in male germ cells was investigated by the *in vivo* dominant lethal test. Mice were treated with single doses of 15, 30 and 60 mg/kg nocodazole. These males were mated at weekly intervals to virgin females for 6 weeks. Nocodazole clearly induced dominant lethal mutations in the early spermatid stage with the highest tested dose. Mice treated with 60 mg/kg nocodazole showed an additional peak of dominant lethal induction in mature spermatozoa during the first week matings after treatment. The percentage sperm count and sperm motility were significantly decreased after treatment of males with 30 and 60 mg/kg nocodazole. Moreover, the middle and highest doses of nocodazole significantly increased the percentage of abnormal sperm. Our study provides evidence that nocodazole is a germ cell mutagen. Marked alteration in the spermiogram analysis after nocodazole treatment possibly confirms that nocodazole has a significant effect on sperm maturation and development during storage and transit. The demonstrated mutagenicity profile of nocodazole may support further development of effective chemotherapy with less mutagenicity. Moreover, the cancer patients and medical personnel exposed to this drug chemotherapy may stand a higher risk for abnormal reproductive outcomes.

1. Introduction

Antimicrotubule drugs represent a critical arsenal against human cancers. Antimicrotubule drugs include nocodazole, which inhibits the addition of tubulin molecules to microtubules, leading to microtubule depolymerization (De Brabander et al., 1986). Microtubule-disrupting agents are thought to arrest cells in mitosis by triggering the mitotic checkpoint (Shah and Cleveland, 2000). When microtubules fail to attach to one or more kinetochores as a result of drug treatment, components of the checkpoint continue to generate signals that inhibit the metaphase/anaphase transition. Like most cell cycle checkpoints, the mitotic checkpoint can adapt. After prolonged treatment with microtubule-disrupting agents, cells exit mitosis without undergoing cytokinesis. These cells then enter an abnormal, tetraploid G_1-like phase (Torres and Horwitz, 1998; Woods et al., 1995) in which they are susceptible to activation of a "microtubule-sensitive" G_1 checkpoint (Notterman et al., 1998; Woods et al., 1995).

Nocodazole has been reported to induce chromosome loss and non-disjunction in human lymphocytes *in vitro* (Elhajouji et al., 1997). Micronuclei formation was also observed in cultured mouse splenocytes and human lymphocytes treated with nocodazole (Steiblen et al., 2005). *In vivo* micronucleus induction in mouse bone marrow by nocodazole was also reported (Tinwell and Ashby, 1991). Recently, the origin of nocodazole-induced micronuclei in mouse bone marrow cells was analysed by fluorescence *in situ* hybridization staining technique (Attia, 2013). The assay showed that nocodazole has high incidences of aneugenicity and low incidences of clastogenicity during mitotic phases; moreover chromosomes can be enclosed in the micronuclei before and after centromere separation. Additionally, nocodazole was also found to induce aneuploidy in mouse germ cells both *in vivo* and *in vitro* (Attia et al., 2008; Sun et al., 2005).

A dominant lethal mutation is a genetic change in a germ cell that acts early in development to cause the death of the zygote produced by that germ cell, before, at, or post-implantation. The dominant lethal assay is used to detect mutagens that produce primarily chromosomal aberrations in male germ cells over any stage of spermatogenesis that subsequently affect embryonic viability (Ashby and Clapp, 1995; Brewen et al., 1975). Embryonic death is often due to numerical chromosome aberrations that are either lost, resulting in monosomy, or that form trisomics, as a result of non-disjunction. Typically monosomics are lethal during early development, while trisomics are lethal at a later stage (Singer et al., 2006). The dominant lethal assay is the only assay commonly used for regulatory testing of chemicals suspected of being mutagenic to the germline.

So far there are no published dominant lethal mutations studies for nocodazole therefore, the aim of the present study was to investigate

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Table 1
Duration (days) of male germ cell development in mice, rats and humans.

Species	Differentiating spermatogonia (mitotic cells)	Spermatocytes (meiotic cells)	Spermatids (post-meiotic cells)	Testicular sperm	Total testicular spermatogenesis	Epididymal mature sperm
Mouse	6	14	9	6	35	4–6
Rat	10.5	19	12	8.5	50	7
Human	16	25	16	6.5	64	8–17

Timing of the stages is indicated in retrograde chronological order, representing the number of post-treatment days required before cells exposed at a particular stage can be sampled from the vas deferens as mature spermatozoa. The reader is directed to Adler (1996) for more detailed information than can be presented herein.

the ability of nocodazole to induce dominant lethal mutations in male mouse germ cells. Dominant lethal mutation assays help in the identification of agents that present a risk of transmissible genetic damage (Chamorro et al., 2003; Jha and Bharti, 2002). In this test, different stages of gametogenesis may be scored for mutations depending upon the interval between treatment and fertilization. In the present investigation, the time schedule chosen for mating represents the pre-meiotic (35–41 days), meiotic (21–35 days), and post-meiotic (1–21 days) germ cells. As shown in Table 1, weeks 1, 2, 3, 4, 5, and 6 post-treatment sperm represent the spermatozoa of epididymis, late spermatids, early spermatids, meiotic germ cells, and B-spermatogonial stages, respectively, at the time of treatment (Adler, 1996).

2. Materials and methods

2.1. Animals

Adult male and female Swiss albino mice aged 10–14 weeks and weighing 25–30 g were obtained from Experimental Animal Care Center at King Saud University. The animals were maintained in an air-conditioned animal house at a temperature of 25–28 °C, relative humidity at ~50% and photo-cycle of 12:12 h light and dark periods. The animals were provided with standard diet pellets and water *ad libitum*. This work was approved by the Ethical committee of Pharmacy College at King Saud University, Riyadh, Saudi Arabia.

2.2. Dominant lethal test

Nocodazole was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the working solution was formulated with 100% dimethyl sulfoxide and administered by intraperitoneal injection at a maximum volume of 0.1 ml per animal. Males were treated intraperitoneally with single doses of 15, 30 and 60 mg/kg nocodazole. The doses of nocodazole were selected on the basis of its effectiveness in inducing chromosome aberrations in mouse germ cells (Attia et al., 2008). A concurrent control group of males was injected intraperitoneally with equivalent volumes of DMSO as a vehicle. Each group consisted of 40 males. The dominant lethal test was performed essentially by the guidelines of Ehling et al. (1978). Males were mated 4 h after treatment at a ratio of 1:1 in the first 3 weeks or 1:2 in the rest 3 weeks to untreated virgin females. Every week, the females were replaced by fresh batch, and the system of caging was continued for 6 weeks to cover the entire spermatogenic cycle. This 42-day mating scheme provided data for the analysis of all stages of spermatogenesis except stem spermatogonia. Every morning, mating was confirmed by checking the presence of vaginal plug representing congealed contents of the seminal vesicle. At pregnancy days 14–16 the females were killed by cervical dislocation and uterus contents were inspected for number and status of all implantation sites. The total number of implants, number of live implants, number of early resorptions or moles and late deaths were recorded at the time of each dissection (Ehling et al., 1978). Dominant lethality was expressed as % dominant lethality = [1 – (live implants per female in the experimental group/live implants per female in the control group)] × 100.

2.3. Spermiogram analysis

Sperm cells from male animals were collected at the end of the experiment as previously described (Attia et al., 2005) and used for evaluation of epididymal sperm parameters as follows: sperm count and motility were determined under the light microscope using a Neubauer hemocytometer according to the World Health Organization (1992) manual for the examination of human semen and two counts per animal were averaged. For sperm-shape abnormality, aliquots of sperm suspensions were stained with 1% eosin-Y and the smears were made on clean glass slides, air-dried and made permanent. The stained slides were examined by bright field microscope and the abnormalities were categorized as close as to those described by Wyrobek and Bruce (1975). At least 500 sperms per animal were assessed for morphological abnormalities which included triangular, without hook, banana shape, amorphous, and tail abnormality.

2.4. Statistical analysis

Results were expressed as means ± SD. The data were analyzed by the non-parametric test, Mann–Whitney *U*-test using the software computer program (GraphPad InStat; DATASET1.ISD). Results will be considered significantly different if the *P*-value was <0.05.

3. Results and discussion

The importance of the dominant lethal test in the assessment of the mutagenic effects of xenobiotics is well established. Numerical and structural chromosomal abnormalities induced in parental germ cells by a mutagenic agent is believed to be the cause for dominant lethality, in which the resultant zygote dies during the process of development in heterozygous condition (Ashby and Clapp, 1995; Brewen et al., 1975). In the current study, no significant difference was found in the percentage of post-implantation loss in the 15, 30 mg/kg nocodazole-treated groups compared with untreated control animals. On the other hand, 60 mg/kg nocodazole was found to significantly induce post-implantation loss in the third week, at which time the epididymal sperm used to fertilize the females were in the early-spermatid stage at the time of exposure (Table 2). The frequency of dead implants induced by 60 mg/kg nocodazole was significantly increased by factors of 1.54 compared with the corresponding control (Fig. 1). These results are consistent with those showing that most mutagens elicit their effects in post-meiotic germ cell stages (Adler and Anderson, 1994) and in the early weeks of the dominant lethal test (Bateman, 1966).

Some increase in number of dead implants in the third week implies that chromosomal non-disjunctional and clastogenic event occurred in the second meiotic cell division when secondary spermatocytes develop into spermatids (Allen et al., 1995). The current observation is in accordance with earlier reports that nocodazole can induce aneuploidy in mammalian cells and increases the embryonic death. In

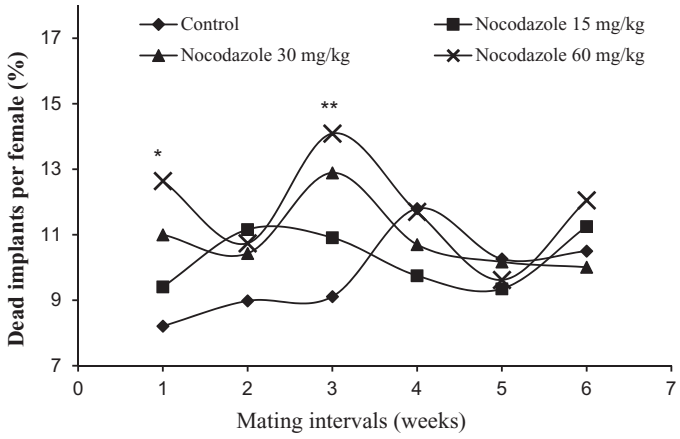

Fig. 1. Percentage of dead implants per female after nocodazole treatment at different mating intervals. *$P < 0.05$ and **$P < 0.01$ versus the corresponding control (Mann–Whitney *U*-test).