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Evolution of DNA strand-breaks in cultured spermatocytes: The Comet Assay reveals differences in normal and γ-irradiated germ cells

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Abstract

In reproductive toxicity assessment, *in vitro* systems can be used to determine mechanisms of action of toxicants. However, they generally investigate the immediate effects of toxicants, on isolated germ cells or spermatozoa. We report here the usefulness of *in vitro* cultures of rat spermatocytes and Sertoli cells, in conjunction with the Comet Assay to analyze the evolution of DNA strand-breaks and thus to determine DNA damage in germ cells. We compared cultures of normal and γ-irradiated germ cells. In non-irradiated spermatocytes, the Comet Assay revealed the presence of DNA strand-breaks, which numbers decreased with the duration of the culture, suggesting the involvement of DNA repair mechanisms related to the meiotic recombination. In irradiated cells, the evolution of DNA strand-breaks was strongly modified. Thus our model is able to detect genotoxic lesions and/or DNA repair impairment in cultured spermatocytes. We propose this model as an *in vitro* tool for the study of genotoxic injuries on spermatocytes.

Keywords: *In vitro* model systems; Reproductive toxicology; DNA damage; Spermatocytes; Sertoli cell; Ionizing radiation; Comet Assay; Caspase

1. Introduction

Despite a protective effect of the blood-testis barrier, germ cells remain highly sensitive to numerous genotoxic agents. Among the established or potential germ cells genotoxic agents, one of the most documented is ionizing radiation (reviewed in Shelby, 1994; Sankaranarayanan and Wassom, 2005), which is responsible for different levels of cellular injury. Therapeutic or accidental exposure to γ-radiation causes cytotoxic effects on germ cells and subsequent decreases in sperm counts, which could lead to complete azoospermia (Meistrich, 1986). These lesions result from direct or indirect effects, involving the participation of endogenous reactive oxygen species (Cadet et al., 2004).

In reproductive toxicology studies, the use of *in vivo* models has led to the assessment of the dose-effect and the stage-dependent effect of ionizing radiations. It has been demonstrated that the intensity and nature of biological effects due to γ-irradiation are linked to dose exposure and the stage at which germ cells are exposed. Consequently, the

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biological impact of irradiation is linked to the period between exposure and the end of spermatogenesis. Several reports have indicated that the vulnerability of male germ cells is related to the differentiation stage, and thus to chromatin condensation, changes in nucleoproteins and variations in repair capacity (Mayer et al., 1981; Meistrich et al., 1981). In adult mouse testis, mitotically active spermatogonia are the most radiosensitive cells, whereas spermatocytes, which undergo meiotic cell divisions, and spermatids, which develop into spermatozoa, are more resistant to ionizing radiation (Oakberg and Diminno, 1960; Joshi et al., 1990; Van der Meer et al., 1992a,b; Beumer et al., 1997; Somers, 2006). Germ cell death resulting from irradiation occurs via apoptosis (Hasegawa et al., 1997). In addition to cell death, it has also been demonstrated that exposure of germ cells to radiation may lead to chromosomal abnormalities in spermatozoa (Alvarez et al., 1997) and may have mutagenic and developpemental consequences (Ellis et al., 1970; Oakberg, 1974; Hoyes et al., 1994; Brinkworth, 2000; Dubrova et al., 2000; Dobrzynska and Czajka, 2005). In germ cells, such lesions could be inheritable. Recent evidence has shown that in mouse, γ-irradiated spermatogonia develop a capacity to transmit a type of heritable genomic instability to four generations of offspring (Baulch and Raabe, 2005; Barber et al., 2006).

During spermatogenesis, a close interaction exists between germ cells and the somatic Sertoli cells. *In vivo* irradiation of the testis exposes both somatic and germ cells, thus it is not possible to study the impact of irradiation on a single cell type. The evaluation of irradiation impact in a single cell population needs to set up *in vitro* models in which cells can be exposed individually to irradiation. Research on reproductive toxicology has been advanced significantly by the introduction of *in vitro* testing systems (Lamb and Chapin, 1993; Mi et al., 2005; reviewed in Hareng et al., 2005). This approach is especially needed in genetic toxicology on germ cells. The effect of ionizing radiations on freshly isolated spermatocytes or on mature spermatozoa has been reported following *in vitro* studies. It has been shown that γ-irradiation causes significant damage to DNA during *in vitro* acute culture of rat spermatocytes and round spermatids (Joshi et al., 1990; Atorino et al., 2001). Major differences have been pointed out between primary spermatocytes and round spermatids in their response to genotoxic stress. However, the possible evolution of DNA damage during spermatogenesis or epididymal transit of spermatozoa could not be taken into consideration using these approaches.

Recently, several studies have reported the achievement of *in vitro* models of Sertoli-germ cell co-cultures. These models allow the development of meiosis in mammalian spermatogenic cells and thus permit the study of almost all spermatogenesis stages. Germ cells cultivated *in vitro* were monitored using several criteria such as ultramicroscopic examination of the different types of germ cells throughout the culture period, determination of the changes in DNA contents per germ cell nucleus with time in culture, examination of the steady-state level of germ cell specific genes in culture, and monitoring the fate of BrdU-labeled leptotene spermatocytes in culture (Parvinen et al., 1983; Toppari and Parvinen, 1985; Le Magueresse-Battistoni et al., 1991; Weiss et al., 1997; Hue et al., 1998; Staub et al., 2000; Lee et al., 2001; Sousa et al., 2002; Perrard et al., 2003). Such models could be used to study the impact of genotoxic agents by taking into consideration the evolution and repair of genotoxic-induced lesions. The main interest of these models is that they allow the specific study of lesions in a single cell type, and not lesions resulting from complex interactions between different cell types.

Spontaneous DNA strand-breaks occur during normal spermatogenesis. The meiotic recombination is initiated by DNA double strand-breaks (DSBs) that are generated by a topo-isomerase II-like protein called spo 11 (Keeney et al., 1997). DSB formation also requires the products of several other genes that are involved in DNA stabilization or recruitment mechanisms (Richardson et al., 2004). In mammals, DSB repair probably proceeds by homologous recombination, according to the model of Szostak et al. (Szostak et al., 1983). The strand invasion results in Holliday junction formation and to the displacement of single-strand DNA breaks. This process has already been analyzed by the detection γ-H2AX, a highly specific marker of the presence of DSB and by the detection of several DNA repair proteins (Anderson et al., 1997; Plug et al., 1997; Eijpe et al., 2000; Talasz et al., 2002).

Single-cell gel electrophoresis (SCGE), also called Comet Assay, is a reliable and rapid method for DNA double- and single-strand-breaks in eukaryotic individual cells. This assay resolves breaks frequencies up to a few hundred per cell (McKelvey-Martin et al., 1993; Collins et al., 1997). In comparison with other sensitive methods, the Comet Assay is relatively robust and economical. This assay is now widely used for the estimation of potential genotoxic insults *in vivo* and *in vitro* (reviewed in Rojas et al., 1999; Tice et al., 2000). In early spermatocytes, DNA strand-breaks related to recombination processes and to repair mechanisms should be efficiently detected by the Comet Assay. If DNA repair mechanisms take place in cultured spermatocytes, the number of DNA strand-breaks revealed by the Comet Assay should decrease during the culture.

In the present study, we demonstrated that the use of *in vitro* models of rat spermatocytes, co-cultured with Sertoli cells, could be useful in the study of DNA strand-breaks in germ cells. We showed that the Comet Assay provided the detection of DNA strand-breaks in normal germ cells, but also in irradiated germ cells, in which the kinetics of DNA strand-breaks evolution were strongly modified.

2. Materials and methods

2.1. Animals

Male Wistar rats were provided by Charles River Laboratories (L'Arbresle, France) and housed in the Experimen-