The Effect of Bromodeoxyuridine on Spontaneous Sister Chromatid Exchange Frequency in Rabbit (Oryctolagus cuniculus) and Coypu (Myocastor coypu) Chromosomes

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The sister chromatid exchange test is regarded as a highly sensitive cytogenetic assay. It measures chromosome sensitivity to particular damage factors and provides information on control and repair mechanism performance. It is instrumental in the early identification of the effects of noxious factors present in the habitat. This investigation was aimed at identifying sister chromatid exchange sites in coypu and rabbit chromosomes, as well as determining the spontaneity of the process by applying different BrdU doses. The chromosomes were obtained from an in vitro culture of blood lymphocytes, supplemented with 4 different BrdU doses: 0.25/0.5/1.0/2.5 µg/ml in order to identify spontaneous sister chromatid exchanges in both animal species. The chromosomes were stained according to the FPG method. Spontaneous SCEs were observed in coypu at a concentration of 1.0 µg/ml, and in rabbits at 0.5 µg/ml. The mean SCE/cell incidence was 1.41±1.15 in coypu, and 2.69±2.14 in rabbits. Differences in SCE incidence were identified between the analysed animal species and the applied BrdU doses.

Key words: coypu; rabbit; mitotic chromosome; sister chromatid exchange.

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Recent decades have seen cytogenetic studies of the influence of environmental factors, xenobiotics and other pollutants entering animal organisms along with consumed food and inhaled air. Such substances negatively affect the genetic material (BILBAN & JACOBIN-BILBAN 2005; WÓJCIK & SMALEC 2013). The determiners of the consequences of organism exposure to the influence of exogenous factors include both cytogenetic and molecular biological markers. Cytogenetic markers are considered to be important tools in disease diagnostics. Biomonitoring tests can be used for screening to enable the detection of the risk of disease development prior to the appearance of typical clinical symptoms, i.e. at a stage which allows appropriate preventive action (BONASII 1999). They consist in the determination of the degree of toxicity of environmental factors by means of the above assays, based on the reactions of the living organism. The analysis involves various tissues used to assess the intensity of the exogenous factors and their role in inducing changes in DNA, chromatin and chromosome structures. Exogenous substances can exert influence on the cell either directly or indirectly, as metabolites, causing interactions between the noxious factor and the cell.

An important diagnostic tool for chromosome instability is the sister chromatid exchange test (SCE). It can help identify the earliest effects of the action of environmental genotoxic factors (WÓJCIK & SMALEC 2013). It is considered to be one of the less labour-consuming, and highly sensitive, cytogenetic assays. The degree of chromosome dam-
age in the animal organism is measured by the frequency of exchanges between sister chromatids. The frequency is a measurable biomarker of sensitivity to exogenous genotoxic, mutagenic and carcinogenic factors and of the innate or acquired capacity of the organism to respond to exposure to a specific noxious factor. The detection of the instabilities at an early stage make it possible to introduce possible changes in habitat conditions, thus enabling potential reversion of certain types of damage, since the reversal of SCE changes is much quicker than that of chromosome aberrations (Mielżyńska 2007). The mechanism of the exchanges has not been fully explored. SCE consists of mutual swapping of homologous chromatid fragments in the same chromosome, in the aftermath of single-strand or double-strand DNA breaks occurring during DNA replication (Wilson & Thompson 2007). Detected unrepaired or incorrectly repaired instances of damage can be observed as discontinuities or asymmetries in the chromatid staining pattern in the chromosome (German & Alhadeff 2001). The essence of the SCE tests consists of using the process of DNA base analogue incorporation during replication. SCEs can spontaneously occur during the cell cycle or be additionally induced by various mutagenic factors. Studies using the SCE test conducted to date in man and selected animal species have shown that SCEs are characterised by considerable species-related conservatism (Ianuzzi & Di Meo 1995; Ansari & Iannuzzi 2002). This principally refers to SCE location in the chromosomes and the concentrations of the base analogues, e.g. BrdU, applied in the SCE test (Di Berardino et al. 1996).

Coypu (Myocastor coypu) and rabbit (Oryctolagus cuniculus) are herbivorous mammals. Coypus originate from the southern part of South America (Gosling & Berker 1991). Coypu farming in European countries started only in the 1920s (Carter & Leonard 2002). Coypu breeding went into significant decline in Poland in the 1990s. Despite the diminished interest in the breeding of the species, numerous studies have been conducted, e.g. to analyse meat quality, determine the genetic diversity of populations, and investigate coypu genetics and cytogenetics (Callahan et al. 2005; Jung & Jo 2012). The coypu has been insufficiently explored in cytogenetic terms. Its chromosome number is 2n=42. All the autosomes have two arms: 32 of them metacentric and 8 submetacentric. The X sex chromosome is metacentric, and Y—the smallest of all—is acrocentric (Fredga 1966, Tsigailidou 1966; Hsu & Benirschke 1971; Ilicher et al. 2009). The banding pattern standard for coypu chromosomes has not been published, although several reports have offered preliminary distributions of C bands in the chromosomes (Piątkowska et al. 1994; Ilicher et al. 2009; Kuchta et al. 2009). Moreover cytogenetic analysis of nucleolar organizer regions (NORs) revealed the presence of 2 active NORs in the coypu karyotype (Piątkowska & Świtonski 1993). The use of the G-banding method has produced a more detailed characterization of the karyotype (Lungeanu et al. 1980).

Initially, rabbits were indigenous to the Iberian Peninsula, the south of France and North Africa. Later, they spread across Europe and Asia. In Poland, rabbits appeared in 1860. Unfortunately, there has been reduced interest in the animals since the 1980s. As a result, their population considerably diminished (Piątkowski 1994). Cytogenetically, rabbits have been more thoroughly investigated than coypu. The diploid chromosome number of this species is 2n=44. The karyotype chromosomes are divided into three morphological groups: metacentric (34 autosomes), acrocentric (8 autosomes) and submetacentric (sex chromosomes) (Robinson et al. 2002). The rabbit karyotype pattern of G, Q, R, C and NOR banded chromosomes has been determined (Hageltron & Gustavsson 1979; Poulsen et al. 1988; Martin-Deleon et al. 1978; Świtonski et al. 1982; Yerle et al. 1987; Ronne 1995; Kuchta et al. 2009) and banding patterns have also been established. The rabbit karyotype has been used for comparisons with the human karyotype (Dutrillaux 1980). Comparisons of genomes of different species are feasible due to karyotypic genetic conservatism including chromosome banding patterns, nucleotide sequences and conjugated gene groups. Genetic conservatism is often analysed in species belonging to the same family (Ianuzzi & Di Meo 1995; Ansari et al. 1999).

The present work was aimed at identifying the sites of sister chromatid exchanges in the coypu and rabbit chromosomes, as well as determining the spontaneity of the process by applying different BrdU doses.

**Material and Methods**

The research was carried out on a group of 10 coypu (Myocastor coypu) and 10 domestic rabbit (Oryctolagus cuniculus) males. All the animals were aged 7-8 months. The research material was constituted by full peripheral blood taken from the V. marginal ear (rabbits) and V. cephalica antebrachi (coypu). All the animals came from the same type of environment (rural). The cytogenetic analyses were performed on chromosomes obtained from an in vitro lymphocyte culture main-
tained for 72 hours at 37°C. At the 36th hour of the culture, BrdU was added at different concentrations: 0.25 µg/ml, 0.5 µg/ml, 1.0 µg/ml and 2.5 µg/ml according to applicable procedure (DIBERARDINO et al. 1995, 1996). Only a set index of BrdU was applied in the case of the other animals.

A harlequin structure of chromosomes was revealed on the basis of the fluorescence plus Giemsa (FPG) technique according to KIHLMAN and KRON-BORG (1975). The procedure of FPG staining consisted of two stages: preliminary digestion and Giemsa staining. In the first stage, the chromosomes were digested with a RNAse solution (10 µg/ml) for 1h; incubated in Hoechst’s solution 33258 (0.5 µg/ml) for 1h; and exposed to UV radiation for 1h. The next incubation was carried out at 4°C in darkness for 24 h. On the following day, the procedure of exposure to UV radiation for 0.5 h was repeated; the preparations were incubated in 0.5xSSC (0.75M Sodium Chloride + 0.075 Sodium Citrate, pH=7.0) for 2h, at 58°C degrees; and, finally, the preparations were stained with a 3% Giemsa solution (pH=6.8) for 45 min.

The preparations were analysed using a NIKON Eclipse 80i microscope, and the estimation of SCEs was conducted using the NIS Elements F2.30 program for computer-based analysis of images. 50 metaphases were analysed for each animal. SCEs were identified in all the chromosomes in the karyotype. The total SCE number was determined as the sum of three different forms of SCEs (interstitial, terminal and centromeric). 500 metaphases were analysed for each species. The SCEs identified in the chromosomes of the animals were profiled by means of the arithmetic mean and standard deviation. The statistical assessment was based on the chi-square test.

Results

The mean SCE/cell incidence was 1.41±1.15 in the coypu, and 2.69±2.14 in the rabbits. The four different BrdU doses applied in the coypu and rabbit chromosome cultures permitted the determination of the BrdU portion at which spontaneous sister chromatid exchange occurs in the cell. The lowest BrdU concentration (0.25 µg/ml) applied in the chromosome cultures, in the case of both the coypu and rabbits, was too small to obtain a stained chromosome structure showing sister chromatid exchanges. The concentration of 0.5 µg/ml in the coypu revealed a very poorly stained chromosome structure with sister chromatid exchanges. SCE identification was highly impeded. The concentration of 0.5 µg/ml was found to be too low to carry out a viable test for the coypu. This concentration proved, in turn, to be sufficient to identify spontaneous SCEs in the rabbit cells. The BrdU concentration of 1 µg/ml was assumed as the dose at which spontaneous SCEs are observed in coypu chromosomes. On the other hand, the BrdU concentration of 1µg/ml turned out to induce SCEs in the rabbits. The highest BrdU concentration of 2.5 µg/ml applied in the analyses was determined to induce the exchanges (Fig. 1 and Fig. 2). The different exchange frequencies within a species observed with the different BrdU doses are presented in Table 1. The differences were confirmed statistically (P=0.00000). We also identified differences in SCE frequency and between the applied BrdU concentrations in these species (apart from the concentration of 0.25 µg/ml) (P=0.000000).

A detailed chromosome analysis revealed which part of the chromosome underwent the most numerous exchanges. This was the distal chromosome region in the coypu, at all three applied BrdU concentrations. At the lowest BrdU concentration (0.5 µg/1 ml of culture), no sister chromatid exchanges were observed in the interstitial and proximal q chromosome arms. Sporadic SCEs were identified in the distal and proximal region of the p arm. The least frequent exchanges at the concentrations of 1.0 and 2.5 µg BrdU/1 ml of culture took place in the interstitial area. In the rabbits, the most frequent swaps occurred in the proximal parts of the chromosomes. The same as in the case of the coypu, more SCEs were observed in the distal regions of the rabbit chromosomes at the concentra-

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<th>Species</th>
<th>BrdU concentration (µg/ml of culture medium)</th>
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*Mean±SD
tion of 0.5 µg/ml. At the other concentrations, SCE incidence varied in the particular chromosome regions. The most numerous exchanges were proximal and distal, with the fewest in the interstitial area. Fig. 3 shows the frequency distribution of sister chromatid exchanges (%) in the particular chromosome regions in both species. Moreover, a tendency was observed in the rabbits for more numerous chromatid cracks and regroupings to appear in the p arm (54 %) than in the q arm (46 %). A similar regularity was also observed in the coypus. More sister chromatid exchanges were identified in the p arm (72 %) than in the q arm (28 %) in this species.

Discussion

The sister chromatid exchange test enables the detection of the mutual exchange of DNA replication products between chromatids within the same chromosome. The observation of differentially
stained chromatids is possible through the incorporation of bromodeoxyuridine in the DNA chain. BrdU is a thymidine analogue which helps visualise spontaneous SCEs. However, at excessive concentrations it induces additional exchanges. The BrdU levels must be high enough to enable the observation of the exchanges without causing additional ones due to dosing excess. The selection of an appropriate BrdU dose is a very important step in the standardisation of the methodology for a given species. Four different BrdU concentrations were used in the present study to observe spontaneously occurring exchanges. We also tested the size of the BrdU dose that causes SCE induction. The lowest dose that reveals spontaneous SCEs in coypu cells was found to be 1.0 µg/ml, and 0.5 µg/ml in rabbits. Higher BrdU concentrations induced additional damage. Most researchers consider the standard BrdU dose to be 10 µg/ml of culture (NICOLAE et al. 2009; WÓJCIK et al. 2011; WÓJCIK & SMALEC 2012a, b). Scientists applied various BrdU doses in this type of research from

Fig. 2. The effect of BrdU concentration on the numbers of sister chromatid exchanges in the rabbit karyotype: a) 0.25µg/ml, b) 0.5µg/ml, c) 1.0µg/ml, d) 2.5µg/ml. Arrows indicate SCEs.
0.1 µg/ml of culture to 120 µg/ml (LEIBENGUHT & THIEL 1986; VIJH et al. 1992; DI BERARDINO et al. 1996, 1997; SZELESZCZUK et al. 2014). According to WILSON and THOMPSON (2007), the level of the analogue added to chromosome culture should be very low, approaching 0, since only then is it possible to observe the spontaneous phenomenon during the cell cycle. BrdU added to chromosome culture during DNA incorporation leads to a rise in the level of single DNA strand damage. The higher the analogue dose, the higher the DNA damage level.

Apart from detectability with BrdU, sister chromatid exchanges are characterised by substantial species-related conservatism relating to the frequency of their occurrence in cells. We determined spontaneously occurring SCEs in the coypu and rabbit chromosomes at 1.39 and 1.99, respectively. Exchange incidence has different profiles in different species: 2.3–6.2 in the Chinese hamster (WÓJCIK et al. 2003), 2.48 in cattle (DI BERARDINO et al. 2012a), 3.28 in goats (DI MEO et al. 1993; DI BERARDINO et al. 1996; WÓJCIK & SMALEC 2012a), 3.6–6.6 in horses (WÓJCIK et al. 2011), 3.4 in cats (SZELESZCZUK et al. 2014), 7.8 in chickens (WÓJCIK et al. 2012) and 6.04 in quail (WÓJCIK et al. 2013). Different frequencies observed within a species can stem from the specific breeds of the animals, as well as individual differences such as age and sex. These factors significantly affect SCE incidence (CATALAN et al. 1995; IANNUZZI et al. 1991a; CIOTOLA et al. 2005; PERETTI et al. 2006).

Another factor that can induce SCEs is the habitat. Some physical, chemical and biological factors have mutagenic effects on DNA and contribute to chromatid stability disruption (WÓJCIK & SMALEC 2013; GENAUALDO et al. 2015). In order to eliminate the influence of additional factors, such as breed, age and sex, two animal species, representing the same breed within a species, of the same sex (males only) and the same age were analysed in this study. The results of instability identification in such a uniform group of animals not only provide species profiles but can also be taken advantage of to assess individual genetic resistance. Despite similar exposure of the analysed animals to environmental conditions, different SCE frequencies were obtained, which can reflect innate or acquired resistance to specific noxious effects of the environment. Varied response of the organism is a specific sensitivity biomarker, enabling the detection of changes preceding the appearance of a disease so that appropriate prophylactic action can be taken.

The sister chromatid exchange site in a chromosome is not accidental. In man and cattle, SCEs have been predominantly detected in the G bands and interstitial bands (LATT 1974; IANNUZZI et al. 1991a). In goats and horses, in turn, they have been found at the point of contact between heterochromatin and euchromatin (WÓJCIK & SMALEC 2012a, 2013). In the Chinese hamster, rats and mice, SCEs predominantly occurred in the proximal region. This area is characterised by a high level of DNA repetitions, vulnerability to recombinational chromosome cracks, and thus SCE induction (MARTIN & PRESCOTT 1964; GIBSON & PRESCOTT 1972; LIN & ALFI 1976). In chickens the exchanges mainly took place in the proximal region of the chromosomes, where constitutive heterochromatin segments are located (WÓJCIK et al. 2013). In the present work, high SCE incidence was observed in the proximal and distal regions of the coypu and rabbit chromosomes. In the coypu, these regions are largely formed by constitutive heterochromatin (ILIKER et al. 2009). Heterochromatin is principally located in the centromere region in rabbits as well. However, it has also been identified in telomeres (SWITONSKI et al. 1982; KUCHTA et al. 2009).

We observed a very interesting regularity in our study. Namely, the applied dose of 0.5 µg/ml BrdU induced SCEs chiefly in the distal chromosome area both in rabbits and coypu. Higher BrdU concentrations induced exchanges in the distal, proximal and interstitial regions. Such sensitivity of the distal chromosome area can correspond with high susceptibility of the chromosome to damage even at the lowest mutagenic concentrations. The distal region contains telomeric and subtelomeric sequences which are vulnerable to damage caused by oxidative stress exerted by free oxygen radicals (KAWANISHI & OKAWA 2004; CHEN et al. 2007; BAIRD 2008). UV radiation can also contribute to DNA damage and SCE induction (WÓJCIK et al. 2003; JIN & IKUSHIMA 2004). High SCE incidence in the distal chromosome region has been
observed by WÓJCIK and SMALEC (2013) in horses (33%), and SZELESZCZUK et al. (2014) in cats (65%). According to BLAGOEV et al. (2010), telomeres are hot spots of sister chromatid exchange. The SCE incidence result in this region can be regarded as an index of the ageing process. Accumulated and unrepaired or incorrectly repaired damage, mutations and replication errors give rise to high SCE incidence and survival of cells containing damaged genetic material which contributes to neoplastic transformations (AMORGUERET 2006).

Applied for the assessment of chromosome instability in standardised conditions, the sister chromatid exchange test can help diagnose damage resulting from mutagenic, genotoxic and carcinogenic factors. It is a measure of chromosome sensitivity to a specific impairing factor and provides information on the degree of control and repair mechanism performance. It can also serve as a reliable and highly sensitive tool for the assessment of the genetic resistance of an animal, as well as lending itself to comparisons of two different animal species.

References


