Strontium ranelate induces genotoxicity in bone marrow and peripheral blood upon acute and chronic treatment

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Abstract

Strontium is a naturally occurring element that exists in the environment mainly as a free metal or in the (II) oxidation state. In this study, rats were treated by gavage with 500 mg/kg of strontium ranelate dissolved in saline three times per week for 12 weeks (chronic treatment) and 24 hours (acute treatment). The genotoxic potential of strontium ranelate was investigated in Wistar rat peripheral blood, using the micronucleus (MN) test systems. In addition to this test system, we also investigated the ratio of polychromatic erythrocytes (PCEs) to normochromatic erythrocytes (NCEs) as a cytotoxicity marker. Strontium ranelate induced micronucleus formation in peripheral blood and bone marrow of rats. It is determined that strontium ranelate has cytotoxic effect on peripheral blood cell population upon both acute and chronic treatment (p<0.001).

Keywords: Strontium ranelate, polychromatic erythrocytes, genotoxicity, micronucleus, cytotoxicity

Özet

Stronsiyum doğal bir element olup çevrede serbest metal ya da oksidasyon (II) halinde var olur. Bu çalışmada, sıçanlar stronsiyumun tuzlu suda çözünmüş 500 mg/kg’lık dozu ile haftada 3 kez olmak üzere 12 hafta için (kronik muamele) ve 24 saat için (akut muamele) gavaj yöntemi ile muamele edildiler. Stronsiyum ranelatın genetoksisik potansiyeli mikronükleus test sistemi kullanılarak Wistar sıçanlarının periferik kanında ve kemik iliğinde araştırıldı. Bu test sistemine ilaveten sitotoksiske belirteci olarak polikromatik eritrositlerin normokromatik eritrositlere oranı da araştırıldı. Stronsiyum ranelat periferik kanda ve kemik iliğinde mikronükleus oluşumunu indüklemişdir. Stronsiyum ranelatın periferik kan hücre popülasyonu üzerine hem akut uygulamada hem de kronik uygulamada sitotoksik etkiye sahip olduğu belirlenmiştir (p<0.001).

Anahtar Sütçüklər: Stronsiyum ranelat, polikromatik eritrosit, genetoksisite, mikronükleus, sitotoksiske
**Introduction**

Strontium is a naturally occurring element that exists in the environment mainly as a free metal or in the (II) oxidation state. Cyto-genotoxicity of metals is important because some metals are potential mutagens, which are able to induce tumors in humans and experimental animals. Strontium is fairly reactive and therefore is rarely found in its pure form in the earth’s crust. Examples of common strontium compounds include strontium carbonate, strontium chloride, strontium hydroxide, strontium nitrate, strontium oxide, and strontium titanate. The most toxic strontium compound is strontium chromate, which is used in the production of pigments and can cause cancer via inhalation route (Toxicological Profile for Strontium U.S. Department of Health and Human Services Health Service Agency for Toxic Substances and Disease Registry, 2004).

The terminal elimination half-life for strontium in humans has been estimated to be approximately 25 years. Estimates of the terminal elimination half-lives of strontium reflect primarily the storage and release of strontium from bone. Over shorter time periods after exposure, faster elimination rates are observed, which reflect soft-tissue elimination as well as elimination from a more rapidly exchangeable pool of strontium in bone. Strontium ranelate (SR), newly developed drug, was first listed on the Pharmaceutical Benefits Scheme (PBS) on April 1st, 2007 for the treatment of established osteoporosis in postmenopausal women. On November 1st, 2007 the listing of SR was extended to the treatment of osteoporosis in some postmenopausal women without fracture. Cellular and subcellular functions of strontium metal are not described in any detail (Meunier *et al.* 2004; Reginster *et al.* 2005). There is little evidence for genotoxicity of stable strontium. However, radioactive strontium isotopes release ionizing radiation that, within an effective radius, is known to damage DNA. No studies were located regarding genotoxic effects in humans following exposure to stable strontium. The only in vivo genotoxicity study for stable strontium in animals involved acute oral exposure (U.S. Department of Health and Human Services, 2004). Genotoxicity testing of pharmaceuticals prior to commercialization is mandated by regulatory agencies worldwide. For the most part, a three or four-test battery including bacterial mutagenesis, in vitro mammalian mutagenesis, in vitro chromosome aberration analysis and an in vivo chromosome stability assay are required. These assays have not been modified substantially since the initiation of their use and they remain the best approach to genotoxicity hazard identification (Snyder and Green, 2001).

In recent years, the in vivo micronucleus assay has become increasingly accepted as the model of choice for evaluation of chemically induced cytogenetic damage in animals. The earliest applications of this model focused on the frequency of micronuclei in polychromatic (immature) erythrocytes (MN-PCE) in rodent bone marrow (Heddle, 1973). Reports were eventually developed indicating that the peripheral blood of treated rodent is an acceptable cell population for this kind of study as long as sampling schedule was modified to account for the release of newly formed micronucleated erythrocytes from bone marrow to the blood (MacGregor *et al.* 1980; Schlegel and MacGregor 1983 ).

This approach opened the way for incorporation of micronucleus assessments into on-going repeat dose conventional toxicology studies in mice (MacGregor *et al.* 1980; Ammann *et al.* 2007; Jauhar *et al.*, 1988). However, rat is the most frequently used rodent species in repeat dose toxicology studies. Several recent studies have demonstrated the feasibility of measuring MN-PCE in bone marrow at the termination of repeat dose rat toxicology studies (MacGregor *et al.*, 1995; Albanese and Middleton 1987; Garriot *et al.*, 1995; Çelik *et al.*, 2003; Çelik *et al.*, 2005) thus taking advantage of the opportunity to correlate genetic with conventional toxicity data in this species. The circulating blood of the mouse has been accepted as an appropriate target for micronucleus assessment for both acute and cumulative damage. Very recently, studies conducted in Japan have addressed the issue of the suitability of rat blood for micronucleus assessment. These studies support the use of rat peripheral blood for evaluation of micronucleus induction in PCE.
No studies on the genotoxic effect of SR on any cell type could be found in the literature in vivo and/or in vitro test systems. The aim of present study is to provide new data on genotoxic potential risks of strontium ranelate on the rat peripheral blood using acridine orange staining- micronucleus test in acute and chronic treatment.

**Materials and methods**

**Animal treatment**

The Institutional Animal Care and Use Committee at Mersin University Medical Faculty approved the experiments described in this study. Thirty, twelve-week-old Sprague-Dawley female rats each weighing 200–250 g were used. The animals were acclimatized for 1 week to our laboratory conditions before experimental manipulation. They had free access to standard laboratory chow and water ad libitum was maintained on 12 h/12 h light dark cycle throughout the experiment. This study utilizes two treatments, acute and chronic. Rats were assigned randomly to a negative control group (n=5), a positive control group (n= 5) and chronic strontium group (n = 5). The rats were treated by gavage with 500 mg/kg of SR (Figure 1) dissolved in saline three times per week for 12 weeks for chronic treatment and once for 24 hours. Each treatment includes negative and positive control groups. Since positive controls can be administered by a different route and treatment schedule than the test agent, a single dose of MMC (2 mg/kg, i.p.) was administered at the 12th week dosing time.

**Dose selection**

Strontium ranelate [PROTOS® (strontium ranelate 2g)] was obtained as a characterized drug from Servier Pharmaceuticals.

**Description**

Description of substance and solubility: Strontium ranelate (SR) is a yellowish-white non-hygroscopic powder. It crystallises as a nonahydrate form but one water molecule is particularly labile and this leads to a compound containing either 8 or 9 water molecules per strontium ranelate molecule. Strontium ranelate is slightly soluble in purified water (3.7 mg/mL at saturation point) and practically insoluble in organic solvents (eg, methanol).

**Excipients**

Aspartame (E951, a source of phenylalanine), maltodextrin, mannitol. Chemical name: Strontium ranelate. CAS Registry Number: 135459-90-4 Molecular formula: C12H6N2O8S, Sr2 (Figure 1). The chemical name applied to SR is 5-[bis (carboxymethyl)amino]-2-carboxy4-cyano-3- thiophenacetic acid distronium salt. The Sr content of SR is 34.1% for a relative molecular weight (anhydrorous) of 513.49.

**Figure 1. Chemical structure of strontium ranelate**

**Presentation**

Granules for oral suspension. PROTOS 2g sachets contain 2g strontium ranelate as a yellow powder. The dose selection of SR was based on human exposures. The 500 mg/kg dose was an approximate environmental daily level. In literature, there are toxicity studies conducted on adult rats with 225–900 mg/kg per day dose (Marie 2005; Ammann et al. 2007).

MMC (2 mg/kg) was used as a positive control. The positive control and the untreated control rats were identically treated with equal volumes of normal saline only via intraperitoneal (i.p.) injection. It is acceptable that a positive control is administered by a different route from or the same as the test agent and that it is given only a single time (Hayashi et al. 1994).

MMC was given as a single dose.

**Tissue preparation**

All the animals used for experiments were
anesthetized by ketamine hydrochloride (Ketalar, Eczacibasi ilac Sanayi ve Ticaret A.S., Istanbul, Turkey). Blood samples were taken from their hearts into tubes. Then the both femora bone were removed by dissection.

**MN assay in peripheral blood and bone marrow smears**

Whole blood smears were collected on the day following the last strontium administration or 1st day after chronic and MMC treatment. Whole blood smears were prepared on clean microscope slides, air dried, fixed in methanol and stained with acridine orange (125 mg/ml in pH 6.8 phosphate buffer) for 1 min just before the evaluation with a fluorescence microscope using a 40X objective (Hayashi et al., 1994). The frequency of PCEs per total erythrocytes was determined using a sample size of 2000 erythrocytes per animal. The number of MN-PCEs was determined using 2000 PCE per animal.

The frequency of micronucleated erythrocytes in femoral bone marrow was evaluated according to the procedure of Schmid (1976), as performed in femoral bone marrow, with slight modifications. The bone marrow was flushed out from both femora using 1 mL fetal bovine serum and centrifuged at 2000 rpm for 10 min. The supernatant was discarded. Bone marrow smears were prepared on clean microscope slides, air-dried, fixed in methanol, and stained with acridine orange (125 mg/ml in pH 6.8 phosphate buffer) for 1 min just before the evaluation with a fluorescence microscope. In order to determine the frequency of micronucleus, 2000 PCEs per animals were scored to calculate the MN frequencies, and 200 erythrocytes (immature and mature cells) were examined to determine the ratio of PCE to normochromatic erythrocytes (NCEs) for bone marrow analysis.

Briefly, immature erythrocytes, i.e. PCEs, were identified by their orange–red color, mature erythrocytes by their green color and micronuclei by their yellowish color.

**Statistical analysis**

Data were compared by one-way variance analysis. Statistical analysis was performed using the SPSS for Windows 9.05 package program. Multiple comparisons were carried out by least significant difference (LSD) test. P ≤ 0.05 was considered as the level of significance.

![Figure 2. Arrow indicates acridin-orange stained micronucleus in immature (polychromatic) erythrocyte of rat treated with SR (500 mg/kg).](image)

**Results**

A representative fluorescence photomicrograph of MN-PCE from a SR-treated rat is shown in Figure 2. SR (500 mg/kg b.w) treatment induced the frequency of MN in both rat bone marrow and peripheral blood. There is a significant difference between SR-treated rats and negative control rats for micronucleus induction. In peripheral blood and bone marrow tissue, although the MN-PCE frequencies (4.80±0.48 and 5.00±0.31, respectively) in rats treated with SR were significantly higher than the frequency in negative control (1.60±0.24 and 2.20±0.20, respectively), they were much less than the MN-PCE frequency induced by the positive control, 2 mg/kg MMC (41.0 ±0.44, 42.4±0.92, respectively). Table 1 represents micronucleus induction and the PCEs/NCEs ratios in bone marrow and peripheral blood. SR treatment significantly decreased the PCE number when compared to controls in both bone marrow and peripheral blood. SR treatment significantly decreased the PCE number when compared to controls in both bone marrow and peripheral blood (p < 0.001). SR is a toxic substance in both bone marrow at acute treatment and peripheral blood at chronic treatment. While PCE number was 2.60±0.25 in the control group of chronic treatment, this value reached 1.2±0.20 at chronic treatment of SR. While PCE number was 103±1.40 in the control
group of acute treatment, this value reached 76.8±1.82 at acute treatment of SR.

**Discussion**

From a drug development standpoint, it is important to have a thorough understanding of the mechanism of any positive genetic toxicology findings, so that informed decisions can be made with respect to risk. This is particularly important because of an increasing experience suggesting that many “positive” gene-tox results may arise artifactually as a consequence of cytotoxicity rather than from true drug/DNA interactions. For example, cytotoxicity may be due to lysosomal damage and release of DNA endonucleases, ATP depletion or impairment of mitochondrial function (Galloway, 2000). The field of toxicology, especially toxicology practices for regulatory purposes, has not changed in several decades. Preclinical safety testing is centered on in vivo laboratory animal studies. These in vivo studies have been valuable in the prevention of some toxic drug candidates from further development, as they are effective in the detection of toxicity that are common to both humans and non-human animals.

Table 1. Micronucleus induction and the PCEs/NCEs ratios in bone marrow and peripheral blood of female Wistar rat induced by SR (500 mg/kg) treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Chronic treatment (peripheral blood)</th>
<th>Acute treatment (Bone marrow)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic saline</td>
<td>1 1 2.1  2 108</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 2 2.2  2 105</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 2 3.2  3 100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 2 3.4  2 102</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 1 2.1  2 102</td>
<td></td>
</tr>
<tr>
<td>Mean ±SE</td>
<td>1.60±0.24  2.60±0.25</td>
<td>2.20±0.20  103±1.40</td>
</tr>
<tr>
<td>SR(500mg/kg b.w.)</td>
<td>1 6 1  4 75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 4 1  5 75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 6 1  5 82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 4 2  5 80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 4 1  6 72</td>
<td></td>
</tr>
<tr>
<td>Mean ±SE</td>
<td>4.80±0.48***</td>
<td>1.2±0.20**</td>
</tr>
<tr>
<td>MMC(2 g/kg b.w.)</td>
<td>1 41 1.2  20 45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 42 1.3  22 44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 40 1  21 42</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 42 1  20 41</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 40 1  21 40</td>
<td></td>
</tr>
<tr>
<td>Mean±SE</td>
<td>41.0±0.44***</td>
<td>1.10±0.06***</td>
</tr>
</tbody>
</table>

***p<0.001, **p<0.01 MMC: Mitomycin C; SR: Strontium Ranelate; MN: Micronucleus; n: rat number in study group. PCE: Polychromatic erythrocytes; SE: Standard error.
An advantage of animal studies is that they provide a complete biological system, which can evaluate the overall effect of subtle changes observed in cell systems. Carefully controlled animal studies are essential steps in the extrapolation of biological effects to human health safety. The fundamental similarities in cell structure and biochemistry between animals and humans provide a general valid basis for prediction of likely effects of chemicals on human populations (Garriot et al., 1995; Çelik et al., 2003). In this study, SR induced micronucleus formation in both peripheral blood and bone marrow and lead to decreasing of the PCE number at chronic and acute treatment in rats.

Important contribution to the knowledge of strontium was obtained in the 1950s and 1960s. A comprehensive review on strontium was published in 1964. Strontium in human biology and pathology has attracted less attention than the other divalent metals such as magnesium and calcium and over the years been an object of academic rather than clinical interest. Strontium is not metabolized in the body. However, strontium does bind with proteins and, based on its similarity to calcium, probably forms complex formation with various inorganic anions such as carbonate and phosphate, and carboxylic acids such as citrate and lactate. Strontium is also found in the soft tissues, although at much lower concentrations than in bone. Strontium toxicity was studied by many investigators. Intravenous administration of high doses of strontium induces hypocalcaemia due to increased renal excretion of calcium. Stable strontium containing chemicals is considered as harmful to humans (Meunier et al. 2004, U.S. Department of Health and Human Services, 2004). In this study, SR (new pharmaceutical) induced the micronucleus frequency and decreased the PCE ratio in peripheral blood and bone marrow chronic and acute treatment, respectively.

Genotoxicity activity is normally indicated by a statistically significant increase in the incidence of micronucleated immature erythrocytes for the treatment groups compared with the control group; historical vehicle/negative control results are also taken into account. Bone marrow cell toxicity (or depression) is normally indicated by a substantial and statistically significant decrease in the proportion of immature erythrocytes; a very large decrease in the proportion would be indicative of a cytostatic or cytotoxic effect. Pollution by heavy metals is an important problem due to their stable and persistent existence in the environment. The in vivo micronucleus test used in this study was a very sensitive method to evaluate the chromosomal damage in mammalian cells exposed to chemical substances. Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments or intact whole chromosomes lagging behind in the anaphase stage of cell division. Their presence in cells is a reflection of structural and/or numerical chromosomal aberrations arising during mitosis (Holden et al., 1997, Heddle et al., 1991). In general, genome damage caused by accidental over exposure may result from interactions such as the formation of DNA damage directly or via free radicals, but also from damage to the nuclear membrane, lipid peroxidation, methylation disturbances, activation of a chain of signal molecules influencing the expression of apoptosis, and other mechanisms including hormonal, age related bioaccumulation of pollutants, metabolism and clearance (Giles, 2005).

Recently, strontium has been studied for bone tissue engineering in osteoblastic ROS17/2.8 cell culture. Osteoblastic cells were seeded on strontium-doped calcium polyphosphate scaffolds. This novel strontium-releasing scaffold system was found to be a promising material for bone tissue engineering (Qiu et al., 2006). Senkoylu et al. (2008) evaluated the effect of SR on H2O2-induced apoptosis of CRL–11372 cells. They assessed quantitatively with a fluorescent dye and qualitatively with agarose gel electrophoresis the apoptotic index and viability of cells. Concentrations of 1–100 µM of SR with a 6 h treatment and only 1 µM concentration with a 12-h treatment inhibited the apoptotic effect of H2O2 on cultured osteoblasts significantly (P<0.05). SR was shown to inhibit H2O2-induced apoptosis of CRL–11372 cells in a dose-dependent manner. Enhancement of osteoblastic cell replication and activity by SR, a stable salt of strontium, has been
indicated in in vitro studies. Furthermore, SR decreases preosteoclast differentiation and osteoclastic activity dose dependently (Canalis et al., 1996; Baron and Tsouderos, 2002).

The absorption of strontium and calcium from the gastrointestinal tract is carried out by the same mechanisms. It has long been suggested that excessive doses of strontium could disturb the calcium metabolism (Takahashi et al., 2003). In the study performed to assess the toxic dose levels by Morohashi et al. (1994), rats received daily strontium doses ranging from 77–770 mg/kg per day for 1 month. Net intestinal calcium absorption, fractional calcium absorption (relative to intake) and calcium retention in the body were all markedly reduced in the group that received 770 mg/kg per day, but none of these parameters were significantly affected in the groups receiving less than 153 mg/kg per day. Morohashi et al. (1994) determined that the toxic effect of strontium is dependent on doses. Some drugs such as alendronate and tibolone, is advised in order to therapy the osteoporosis. In another study performed in postmenopausal women with osteoporosis, Bayram et al. (2006) investigated the genotoxic effects of the alendronate treatment with or without tibolone using comet assay. They reported that the Comet assay revealed that tibolone did not cause any DNA damage, but alendronate did at the end of the 1-year administration of these drugs. In other studies performed in relation to drugs used in osteoporosis treatment, conclusive results were obtained for genotoxic damage. Şahin et al. (2000) reported that alendronate did not show any signs of genotoxic effects according to the sister chromatid exchange (SCE) assay. However, some of the bisphosphonates like pamidronate and zoledronate have been reported to cause DNA fragmentation (Şahin et al., 2000). Taking into consideration the long years of accumulation of these drugs in the bone, DNA damage may be important. Considering that there is still a lack of information regarding the essentiality and toxicity of SR, plasma data showed large individual variation, resulting in uncertain pharmacokinetic profiles. No studies on the genotoxic effect of SR on cells could be found in the literature. Oral administration of 130 mg strontium/kg body weight as strontium chloride to Swiss albino female mice increased the incidence of chromosomal aberrations (gaps, breaks, nondisjunction, polyploidy) in bone marrow cells 5-fold after 6 hours (Ghosh et al., 1990). Genotoxicity in male mice administered a similar dose (140 mg/kg) was only doubled, and therefore, less severe than in females. At higher dose (1,400 mg/kg), the incidence of chromosomal aberrations was similar in both sexes after 6, 12, or 24 hours. In study performed by Berköz et al. (2008) it is shown that SR decreased the paraoxonase level in rats receiving SR only one time, underwent ovariectomy operation and did not receive any drug and treated with strontium ranelate for three months after three months from the ovariectomy operation. Paraoxonase protects from oxidation the lipoproteins. Therefore in our opinion, this issue is very important in explaining for its use in treatment of established osteoporosis in postmenopausal women.

In conclusion, although the studies regarding the geno-cytotoxic effects of drugs used in osteoporosis therapy are contradictory, our results clearly demonstrated that chronically and acutely administration of SR (500 mg/kg) significantly increased the frequency of MNPCEs and decreased the % PCEs in peripheral blood of rats. Evaluation of the role of drug metabolism and toxicity is arguably a necessary activity for the evaluation of human drug toxicity. It allows a rationale design of a safer molecule (e.g. by blocking sites critical for toxic metabolite formation), assessment of sensitive human population (e.g. populations with high level of the drug metabolizing enzyme pathway for the formation of toxic metabolites; populations with low detoxifying activities; environmental factors leading to high levels of “activating” activities or low levels of “detoxifying” activities). Future studies will be necessary on experimental animal models using different doses-period and test methods.

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References


