Sister chromatid exchange test in river buffalo lymphocytes treated in vitro with furocoumarin extracts

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Sister chromatid exchange (SCE) test in river buffalo lymphocytes treated *in vitro* with Furocoumarin extracts

Alessandra Iannuzzi[1,*], Angela Perucatti[1], Viviana Genualdo[1], Alfredo Pauciullo[1-2], Rita Melis[3], Claudio Porqueddu[3], Mauro Marchetti[4], Marianna Usai[5], Leopoldo Iannuzzi[1]

[1]*National Research Council (CNR), Institute for Animal Production Systems in Mediterranean Environments (ISPAAM), Laboratory of Animal Cytogenetics and Genetics, Naples, Italy*

[2]*Department of Agricultural, Forest and Food Sciences (DISAFA), University of Turin, Italy*

[3]*CNR-ISPAAM, UOS-Sassari, Trav. La Crucca, 3, 07100 Sassari, Italy*

[4]*CNR-IBC, UOS-Sassari, Trav. La Crucca, 3, 07100 Sassari, Italy*

[5]*Department of Science for Nature and Environmental Resources (DIPNET), University of Sassari, Via Muroni, 23A, 07100 Sassari, Italy*

*Corresponding author: alessandra.iannuzzi@cnr.it

Tel: +39 081 5964977; Fax: +39 081 5965291

Abstract

Furocoumarin extracts from *Psoralea morisiana*, the endemic Sardinian legume species, were tested for their mutagenic potential on river buffalo blood cells. The results obtained performing the sister chromatid exchange (SCE) test in blood cultures of five river buffalo calves (exposure to furocoumarins for 72 h) and five cows (exposure to furocoumarins for 3 h, in the absence and presence of S9 metabolic activator) are reported. Significant differences in mean values of SCEs were observed in cells of calves compared to control cells (unexposed), but no differences in SCE mean values were found between treated and untreated cells of cows in the presence or absence of S9. SCE mean values were much higher in cells of cows (exposed and control) than in cells of calves. Indeed, in calf cells, SCE mean values/cell (±SD) were 6.66 ± 2.45 in the control and 7.63 ± 3.01, 9.03 ± 3.90, 9.53 ± 3.60 and 9.99 ± 3.41 in treated cells at 50, 100, 200 and 400 μg/ml of furocoumarin extracts, respectively. In cow cells, grown in presence of S9, SCE mean values/cell were 11.49 ± 4.78 and 11.65 ± 5.19 in treated cells at 100 and 200 μg/ml of furocoumarins and 11.66 ± 5.45 in the control. In cow cells grown in absence of S9, SCE mean values were 11.81 ± 6.14 in the control and 12.35 ± 7.09 and 12.01 ± 5.43, respectively, in the presence of 100 and 200 μg/ml of furocoumarins. Despite their higher SCE values in the absence of S9, no statistically significant differences were found when these values were compared with those shown in presence of S9, suggesting no mutagenic action of furocoumarins in cows, at the doses used in this study.

Introduction

Furocoumarins are a class of compounds widely studied in treating many diseases, especially in photochemotherapeutic drugs applications (1). The interest for these studies originates from the effectiveness of Psoralen + Ultraviolet A –UVA (PUVA) therapy, realized by oral or topical administration of a linear furocoumarin (psoralen) followed by irradiation with UVA light, for the treatment of psoriasis and cutaneous T-cell lymphoma [reviewed in Refs. (2,3)]. Furocoumarins are strictly dependent on UVA irradiation; indeed in the presence of UVA light (365 nm, 1.2 J/cm2) all furocoumarin derivatives demonstrated the ability to inhibit cell growth [reviewed in (2)].

Psoralen, the most important furocoumarin, intercalates into dsDNA and creates covalent cross-links primarily with thymidine residues (4,5). Covalent DNA inter-strand crosslinks (ICLs) block the separation of the two DNA strands required for transcription and replication of the genetic material. ICL-inducing agents such as psoralen with ultraviolet (UV) light, mitomycin C, nitrogen mustards and cisplatin are therefore particularly toxic, especially in proliferating cells and are largely used in the treatment of cancers and skin diseases (6,7). Indeed, psoralen inhibits bone metastasis of breast cancer in mice (8). However, genotoxic effects of ionizing radiation seem to remain for decades after exposure even in subjects exposed to low-dose radiation (9). Studies on plants containing furocoumarins like bergamot, thyme and rosemary, have been demonstrated to have an antibacterial action. Indeed furocoumarins seem to prevent infections by staphylococcus species, which are one of the main causes of mastitis in dairy cattle (10). Furthermore, the use of bergamot in the lab-rat diet (i) reduced the levels of cholesterol and low density lipoproteins (LDL) and (ii) increased levels of high density lipoprotein (HDL) and the protective effects of liver parenchyma (11).

Furocoumarins are also present in the genus Psoralea belonging to the family Leguminosae. P. bituminosa L. (sin. Bituminaria bituminosa (L.) C.H. Stirt.) and P. morisiana Pignatti & Metlesics, two perennial species widespread in Mediterranean areas, are currently under study as alternative feeds for ruminants, owing to their protein content and because they are able to remain green during the summer, when other leguminous do not (12). Nonetheless, their use as feeds may be detrimental for animal health, considering the mutagenic potential of furocoumarins.

Cytogenetic tests can be very useful to detect chromosome fragility in both animal and human cells when they are exposed in vivo or in vitro to potential mutagens (13,14). The sister chromatid exchange (SCE) test has been used widely to test several chemicals in both in vitro and in vivo studies with or without use of metabolizing enzymes, such as S9 mix activation system, which has been applied in many in vitro cell cultures to test mutagens that require oxidative metabolism to reactive species before demonstrating mutagenicity.

In domestic animals, significantly increased levels of SCEs have been found in lymphocytes naturally exposed to mutagens (15‑20), or after in vitro exposure to several chemicals including chemotherapeutics for the treatment of the breast cancer (21). Previous studies using SCE test in human lymphocytes exposed to psoralen compounds have reported different results depending of type of cell cultures (dark condition, use or not of the metabolizing enzyme S9, dose of psoralen) (22‑26).

In this study, the results obtained in two different experiments performing the SCE test in river buffalo lymphocytes exposed in vitro to furocoumarin extracts from a Sardinian population of Psoralea morisana are reported. To our knowledge, these are the first observations on domestic animal cells treated in vitro with furocoumarin extracts using a cytogenetic test.

Material and methods

Extraction and determination of furocoumarins

Fresh leaves (100 g) of Psoralea morisana (accession Punta Giglio) harvested in July 2013 were macerated in cold MeOH/HCl 2M (800 ml) and kept under stirring for 20 h at room temperature. The extracts were filtered and concentrated under vacuum at a temperature below 50°C in a rotary evaporator, then dissolved in distilled H2O (250 ml) and the extraction repeated three times with 45 ml of CHCl3. The organic extract was concentrated under vacuum at 30°C and the residue used for the furocoumarin analysis by a GC (Hewlett Packard 5970) equipped with a ZB5 column (Phenomenex, length 60 m, i.d. 0.25 mm, film thickness 0.25 μm) coupled with a MS instrument (Hewlett Packard GMD) and quantified by the Standard Addition Method. The chromatographic conditions used were as follows: detector and injector 280°C; initial oven temperature 50°C, heating rate of 3°C/min until reaching 135°C, hold for 1 min, temperature increase of 5°C/min up to 225°C, hold for 5 min, temperature increase of 5°C/min up to 260°C, hold for 10 min at 260°C; carrier used, helium ppm at 1ml/min flow. All analyses were repeated three times.

Cell cultures

Peripheral blood samples from five river buffalo calves (up 3 months–2 males and 3 females) were incubated at 38°C for 72 h in RPMI medium enriched with FCS (15%), antibiotics and antimitotics (1%), concavalin A (15 μg/ml) as mitogen to reach 10 ml of final cell culture. Cells were exposed to different quantities of furocoumarin extracts: 0 (control), 50, 100, 200 and 400 μg/ml during all culture time (72 h). 5-Bromodeoxyuridine (BrdU) (10 μg/ml) and colcemid (0.5 μg/ml) were added to cell cultures 24 and 1.5 h before harvesting, respectively. Then cells were treated with hypotonic solution followed by three fixations in methanol/acetic acid. Cell suspensions were fixed on slides and air dried. A day or more later, slides were stained with Hoechst33258 (H33258) (25 μg/ml) for 10 min, then washed with distilled water and air dried. Slides were mounted in 2×SSC with coverslips and exposed for 30 min to a UV lamp (40 W, distance 7 cm), washed again with distilled water and air dried. Slides were then stained for 10 min with acridine orange (0.01 % in P-buffer pH = 7.0), washed in tap and distilled water and air dried. Slides were then mounted in P-buffer (pH = 7.0) with coverslips and sealed with rubber cement. Slides were observed a day or more later under a fluorescence microscope (Nikon E1000 and Leica RBD) connected to a CCD camera.

Additional cultures were later performed with five river buffalo cows (6 years old) from the same farm with and without S9 metabolic activator during cell cultures as follows. Six different cell cultures were performed for each of five cows using the medium described above. After 24 h of culture, three cell cultures for each cow were treated only with furocoumarins [0 (control), 100 and 200 μg/ml] and three cell cultures were treated with both furocoumarins [0 (control), 100 and 200 μg/ml] and S9 following the protocol reported by Eke and Celix (27). After 3 h of culture in the presence or absence of S9, cells were washed twice with physiological solution to eliminate both furocoumarins and S9 (in the cultures where they were present). Then cells were allocated in fresh medium and cultured for additional 72 h including the treatment with 5-bromodeoxyuridine (10 μg/ml) and colcemid (0.5 μg/ml) 24 and 1.5 h before harvesting, respectively. Cells and slides were then treated and stained as reported above for calf cells. Thirty cells for each cell culture (and furocoumarin dose) were analysed in both experiments.

Statistical analyses

Mean values and standard deviations of SCEs were calculated for both single animals and animal groups. Statistical analyses were performed between the two groups of calves and cows by using a Student's t test. Bonferroni correction was applied as default restriction and differences were considered significant if $P \leq 0.05$.

Results

Psoralea plant extracts

Table 1 reports the mean values of the components found in the extract of *Psoralea morisiana* plant from Punta Giglio (Sardinia island, Italy), while the relative chemical structures are reported in Figure 1. Among the furocumarins, angelicin and psoralen were the most abundant, reaching almost 70% of the total extract. However, other bioactive compounds, such as 'cumaric acid methyl ester' and 'plicatin B', the latter being an antimicrobial agent and a strong antioxidant,(28,29) were also found in the extract.

Experiment with calf cells

Although the cell growth (percentage of stimulated lymphocytes on total cells) appeared normal in both treated and untreated (control) cells, a significantly ($P < 0.01$) higher number of SCEs were observed in treated cells, compared to those achieved in the control (Figure 2A). Furthermore, SCE increasing mean values were strictly related to the increasing doses of furocoumarins. Indeed, SCE mean values (±SD) were 6.66 ± 2.45 in the control and 7.63 ± 3.01, 9.03 ± 3.90, 9.53 ± 3.60 and 9.99 ± 3.41 in treated cells at 50, 100, 200 and 400 µg/ml, respectively (Table 2). The comparison among SCE mean values in treated cells revealed statistical differences ($P < 0.01$) only between 50 versus 100 µg/ml of furocoumarins, suggesting that at higher doses of furocoumarins SCE mean values may reach a plateau level (see Supplementary Figure 1).

Experiments with cow cells

Cell growth appeared normal in all cell cultures. SCE mean values in cows were higher (Figure 2B) than those achieved in the calves (both in treated and untreated cells with furocoumarins) but no significant differences were observed when comparing data obtained in both treated and untreated (control) cells of cows and both in the presence or absence of S9. Indeed SCE mean values were 11.66 ± 5.45 in the control and 11.49 ± 4.78 and 11.65 ± 5.19 in treated cells at 100 and 200 µg/ ml of furocoumarins, respectively, in the presence of S9. In cells grown in the absence of S9, SCE mean values were 11.81 ± 6.14 in the control (without furocoumarins) and higher (12.35 ± 7.09 and 12.01 ± 5.43 in presence of 100 and 200 µg/ml of furocoumarins, respectively) but the differences were not statistically significant (Table 3).

Discussion

While exposure to furocoumarins gave significant increases in SCEs in calves exposed to different doses of them for all cell culture time, compared with control (Table 2), no significant increase in SCEs was found in cows exposed to furocoumarins both in presence or absence of S9 (Table 3). It is difficult to explain these different results. By examining only data from controls (without exposure to furocoumarins) in both calves and cows, it appears evident that the SCE mean value in cows (11.66 ± 5.45) is higher compared than that in calves (6.66 ± 2.45). Furthermore, the highest SCE mean value achieved in calves at the highest dose of furocoumarin used (400 µg/ml) was lower (9.99) than those achieved in the cows (exposed and unexposed cells). This reveals a higher chromosome fragility in cows than in calves. It is well known that the SCE test can be influenced by age, with higher SCE mean values in older individuals (30,31).

However, river buffalo cows generally show higher SCE rates than other domestic species (18,32). It is also possible that cow cells, starting from a higher level of SCEs, compared to those of calves, are less sensitive to furocoumarins than calf cells, at least at the doses used in this study. This could explain the different SCE values between calves and cows. Another reason could be the different cell culture time: 72 h in calves and 96 h in cows, although cow cells were all washed twice with physiological solution after treatment with furocoumarins and restarted in fresh medium. In addition, calves are fed only with milk (generally bovine in order to save that of river buffalo which is used to produce mozzarella cheese), while the cows are fed at the box with a mixture of corn silage, hay and grain (maize, soya and barley). This could be one of the causes of higher levels of SCEs in cows, compared with those in the calves, although we do not have evidence of possible contaminants of cow feed increasing SCE mean values.

Environmental agents such as ultraviolet light, several genotoxic chemicals and pollutants cause DNA damage. If not repaired, DNA damage can lead to mutations and increased risk of cancer (33). Furocoumarin derivatives demonstrated the ability to inhibit cell growth, but this propriety is strictly dependent on UVA irradiation (9). In the present study, cell growth appeared normal in cells treated with furocoumarins (with and without S9 metabolic activator). In previous studies performed in human cells, no increased frequency of SCEs was observed in lymphocytes exposed to 8-methoxyposaralen (1μM) in dark conditions (22), while Wulf (23) and Faed and Peterson (24) found that 8-methoxyposaralen alone induced SCEs at low (1 μM) and high (115 μM) concentrations, respectively. Baysal et al. (25) studied 42 psoriasis patients undergoing PUVA treatment at three different doses and a control group of 22 psoriasis patients not treated with PUVA. Mean SCE/cell values of three dosedependent patient groups were significantly higher ($P < 0.001$) than the control group (25). However, in absence of metabolizing system (S9), the differences were significant only at high doses of PUVA, varying between 155 and 1442 μM (26). By examining the data obtained in our study, it is possible to drawn the following conclusions: (i) cells exposed to furocoumarins for all period of culture (72 h) and in absence of S9 showed significant increases in SCEs compared to control, and levels of SCEs are strictly related to the quantity of furocoumarins used, although significantly increased values of SCEs were observed at lower quantities of furocoumarins; (ii) cells exposed to furocoumarins for only 3 h (SCE test in cows) did not induce significant increases of SCEs compared to the control with or without use of S9, although SCE values were much higher in cows, compared to those induced in calves, but with the limitation that two different protocols were used in this study; (iii) since cow cells did not show increasing of SCEs when using furocoumarins, especially when S9 was present, it is possible to conclude that furocoumarins have no mutagenic effects in animal cells at the doses we used in cows; (iv) this could suggest the use of plants, like those of psoralea accession varieties, for animal feeding, especially during the summer, when other leguminous plants are not green; (v) in vivo studies using cytogenetic tests on animals fed with Psoralea plants (or with a diet rich in furocoumarin extracts) should be performed to get final conclusions about their use for animal feeding.

Supplementary data

Supplementary Figure 1 is available at Mutagenesis Online.

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Conflict of interest statement: None declared.

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Table 1. Mean values of the components found in the extract of *Psoralea morisiana* plant from Punta Giglio (Sardinia Island, Italy)

Compound (r.t., min)	Maltol (15.3)	Trimethyl citrate (22.3)	Cumaric acid methyl ester (27.2)	Angelicin (28.1)	Psoralen (28.9)	Plicatin B (31.8)
%	9.0	8.5	4.0	40.5	27.5	10.5

The retention time (r.t.) of each component is reported between brackets.

Table 2. Animals, furocoumarin extract doses, examined cells, SCEs and SCE mean values in buffalo calves exposed and unexposed (control) to furocoumarin

Animal n	Furocoumarin Extract (µg/ml)	Cells (n)	SCEs (n)	Mean±SD	
5	0	150	999	6.66	2.45
5	50	150	1144	7.63*	3.01
5	100	150	1355	9.03*	3.90
5	200	150	1429	9.53*	3.60
5	400	150	1498	9.99*	3.41

* Significantly different versus controls (P<0.001)

Table 3. Animals, furocoumarin extract, examined cell and SCE mean values in river buffalo cows unexposed (control) and exposed to furocoumarin without (-) and with (+) S9.

Animal n	Furocoumarin Extract (µg/ml)	S9 (+/-)	Cells (n)	SCEs (n)	SCEs Mean±SD
5	0	+	175	2041	11.66±5.45
5	100	+	175	2010	11.49±4.78
5	200	+	175	2039	11.65±5.19
5	0	-	175	1854	11.81±6.14
5	100	-	175	2161	12.35±7.09
5	200	-	175	2102	12.01±5.43

Fig. 1. Structure of compounds found in the extract of *Psoralea morisiana* (Punta Giglio, Sardinia, Italy).

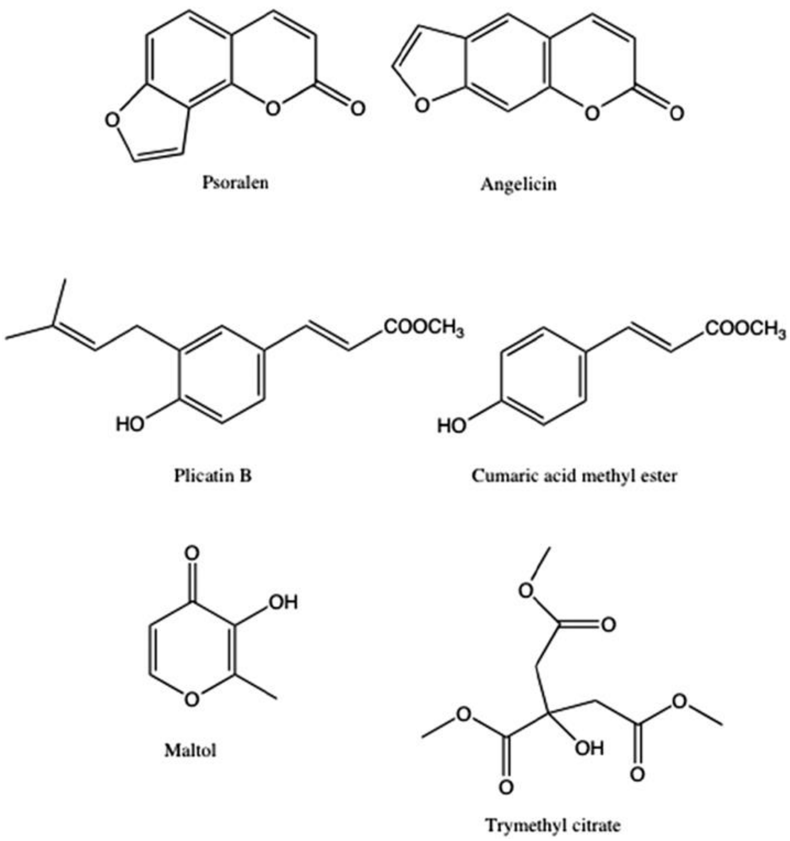

Fig. 2. River buffalo metaphase plates from calf (A) and cow (B) cells treated for SCE test. Note the low and high number of SCEs (arrows), observed on chromosomes of calf and cow cells, respectively.