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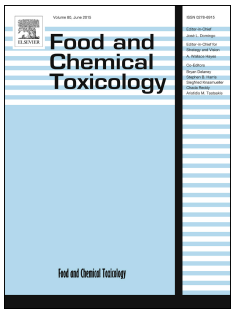

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(Short communication)

Cytogenetic tests reveal no toxicity in lymphocytes of rabbit (*Oryctolagus cuniculus, 2n=44)* feed in presence of Verbascoside and/or Lycopene

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Abstract

Phenylpropanoid glycosides (PPG), like other phenolic compounds, are a powerful antioxidants and the Verbascoside (VB) is one of the most active of them. A previous study, by using *in vitro* exposure of blood human lymphocytes to Verbascoside, reported a significant increasings of chromosome fragility compared to control. In the present study, four homogeneous groups of rabbits were used to test *in vivo* the VB and/or Lycopene (LP) by feeding the animals without VB and LP (control), in presence of VB or/and LP for 80 days. Lymphocyte cell cultures were performed in three different times: 0, 40 and 80 days of the experiment and the cytogenetic tests that we used [CA-test (Chromosome Abnormalities in terms of chromosome and chromatid breaks) and Sister Chromatid Exchange (SCE-test)] have revealed no mutagenic effects on chromosomes. Indeed, mean values/cell of CA and SCE decreased during the experiment with some difference among and within groups, with significant decreasing value only for some group. The study shows clear evidence that diets rich in Verbascoside (and/or Lycopene) do not originate any mutagenic activity, resulting no cytotoxic for the animals and, suggesting a possible their use in both animal and human diets.

Key words: Verbascoside; Lycopene; mutagenic test; chromosome stability; in vivo exposure

1. Introduction

Phenylpropanoid glycosides (PPG), like other phenolic compounds, are a powerful antioxidants and the Verbascoside (VB) is that showing the highest scavenger activity in comparison with other phenolic compounds. In fact, the presence of VB in the human diet reduces cardiovascular risk factors (Campo et al., 2015) and the memory loss reduction in humans (Peng et al., 2015). *In vitro* exposure to VB promotes apoptosis and reduces cancer cell proliferation in colon cells (Zhou et al., 2014). In addition, derivative oil products containing VB were not genotoxic in the somatic mutation and recombination test (SMART) on Drosophila melanogaster and, more importantly,

exerted anti-genotoxic activity against DNA-oxidative damage generated by hydrogen peroxide (H_2O_2) (Anter et al., 2014). An extensive review on VB has been reported by Alpieva et al. (2014).

VB has been also used in animal diet for several purposes: (**a**) to decrease stress biomarkers in swine gut by reducing levels of nitrotyrosine in enteroendocrine cell proliferation (Di Giancamillo et al., 2013); (**b**) to improve oxidative stability and color indices in *Longissimus Dorsi* in pig (Rossi et al., 2014); (**c**) to improve the homoeostatic stability in Lacaune suckling lambs by testing the plasma oxidative status (Casamassima et al., 2013a); (**d**) to influence growth performances by increasing final weight and oxidative status of piglets (Corino et al., 2007); (**e**) to positively influence the lipidic and hepatic profiles, and oxidative status of jennies as a potentially novel strategy for improving the functional properties of donkey's milk for human diet and for improving the welfare of young animals (D'Alessandro et al., 2014); (**f**) to improve the growth rate in young hares (Casamassima et al., 2013b); (**g**) to significantly improve milk yield and high density lipoprotein cholesterol and significantly decreased triglycerides and, total cholesterol in Lacaune ewes (Casamassima et al., 2012).

Lycopene (LP) is one of the most important carotenoids (in addition to α-carotene, β-carotene, β-kryptoxanthin and lutein) present in the human diet (Bolhassani, 2015), being one of the most effective oxygen radical quenching agents among the carotenoids (Sies and Stahl, 1992). LP (and α-carotene) rich diet reduces the risk of some type of cancer as that on the prostate (Wang et al., 2015). Indeed, LP has been found in relatively high concentrations in the prostate gland (Clinton et al., 1996), although large and well-designed randomized trials with clinical endpoints or lifestyle modification interventions using diets, containing also LP, are recommended (Hackshaw-McGeagh et al., 2015). High exposures to LP from dietary sources gave no indication of any significant adverse effects of LP (McClain and Bausch, 2003). More recently, a study on rats exposed to human daily intake of carotenoids revealed no genotoxicity, mutagenicity and cytotoxicity in multiple organs of rats (Larangeira et al., 2016).

In a cytogenetic study using *in vitro* exposure to VB of blood human lymphocytes from three donors, a significant increasing of chromosome fragility was found in exposed cells when compared to control, confirming the genotoxic action of VB after determination of enhanced protein expression levels of PARP-1 and p53 (Santoro et al., 2008). Considering that, in other *in vitro* studies the VB has been found to be not genotoxic (Anter et al., 2014; Santos-Cruz et al., 2012), in the present study we have applied two mutagenic tests to evaluate the effects of the *in vivo* exposure to VB (and LP) on lymphocytes cell cultures from four homogeneous groups of rabbit feed in presence or absence (control) of VB. To reduce possible cytotoxic effects of VB (on the basis of the previous study reported by Santoro et al, 2008), also LP, alone and together the VB, was added in the animal diet. Our data clearly reveal no cytotoxic effects in lymphocytes of rabbits feed with VB, alone or in addition to LP. In our knowledge, this is the first time that mutagenic test are applied on cells from animals feed in presence of VB (and LP).

2. Materials and methods

2.1. Animals and feeding

Four homogeneous groups of rabbits (six animals/group, all males, from White New Zeeland breed, randomly selected) were acquired by a private company farm at 35 days of life and used to test *in vivo* the VB and LP. Animals were kept in individual cage in an animal house respecting the animal welfare. Rabbits were fed for 80 days without supplements of VB and LP (control – group A), with LP (5mg/Kg of feeding/day, group B), with VB (5 mg/Kg of feeding/day, group C), with both VB and LP (5 mg/Kg of feeding each/day, group D).

Components of feed ration for rabbits used in both private company farm and during our experimentation phase were essentially the same (alfalfa, wheat bran, oatmeal, beet pulp, barley, protein soybean meal genet. modified, di-calcium phosphate, calcium carbonate, sodium chloride, with the addition of vitamins and minerals). The only two variables were (**a**) the classical intensive

rabbit hutch closed loop in the private company farm (against the breeding in a kind of animal house in individual cages with food in our experiment), (**b**) the practice in the private company farm to spray an antifungal (*Itraconazole as active component*) on both animals and animal hutch environment to prevent mycosis.

The dietary supplement contained a water-soluble leaf extract of *Lippia citriodora* (Verbenaceae), prepared on an industrial scale with standardized procedures including extraction by ultrasonic treatment with 60% aqueous solution of ethanol, followed by drying with malt dextrin as the excipient. To better identify natural extract polyphenols content, a quantitative analysis of phenolic compounds was performed by HPLC-UV-DAD in agreement with Piccinelli *et al.* (2004). Our duplicate-samples analyses stated that the feed additives contained verbascoside 4.47±0.08, methyl gallate 1.91±0.09, gallic acid 1.75±0.07, 3.4-dihydroxybenzoic acid 0.45±0.04 and isoverbascoside 0.43±0.04 g/kg. To prevent the oxidation of the feed, the dietary supplement was micro-encapsulated with a protective matrix of hydrogenated vegetable fat using a spray-cooling technology (Sintal Zootecnica, Isola Vicentina, Vicenza, Italy).

The dietary supplement containing lycopene (sourced on herbal) was produced by Erbamea, FR, Italy, and contained lycopene from tomato fruit 2% (Sodium alginate, pea starch, gum arabic, inulin, anti-caking agent, magnesium stearate). In our laboratory lycopene content in the commercial additive was extracted with tetrahydrofuran: dichloromethane (1:1, v/v) and analyzed on an Agilent Technology 1200 series HPLC with the visible detector set at 450 nm according to a modified method of Rautenbach et al. (2010). Twenty microliter of extracted samples was injected automatically into the column (5µm, 150x4.6mm C18 column, Phenomenex, Torrance, CA, USA) and isocratic elution performed on a mobile phase consisting of methanol : acetone (9:1, v/v) with flow rate set at 1 mL/min. Lycopene peak was identified based on the retention time of the pure standard (Sigma Aldrich St. Louis, USA).

2.2.Cell cultures and microscope observation

Peripheral blood (1 ml) cultures of rabbits were performed at three different times: 0, 40 and 80 days. For each animal two different cell cultures were performed: **culture A** (without the addition of any base analog) for CA-test (Chromosome Abnormalities in terms of chromosome and chromatid breaks), with a duration time of 48 h, and **culture B,** with the addition of 5-Bromodeoxyuridine (BrdU, 10µg/ml) for the SCE-test (duration time 72 h). Cells were grown at 38°C in RPMI1640 medium, FCS (15%), Concanavalin A (1.5%) as mitogen, antibiotics and antimycotic (1%). Colcemid (0.1 µg/ml) was added 1.5 h before harvesting for both cell cultures, while BrdU (culture B – SCE-test) was added 24 h before harvesting. Hypotonic treatment (KCl 0.5%) and three fixations in Methanol-Acetic Acid (3:1) followed. Two drops of cell suspension were spread on wet slides and air dried. A day later or more, slides from CA-test were stained for 10 min with Giemsa (8% in P-buffer pH=7.0), washed with tap and distilled water, air dried and mounted with coverslip using rubber cement.

Slides for SCE-test were first stained with Hoechst33258 (25 µg/ml in distilled water) for 10 min, then washed with distilled water, mounted in 2xSSC and exposed to UV-light for 30 min. Finally, slides were washed again with distilled water, air dried, stained with acridine orange (0.01% in P-buffer pH=7.0), washed with tap and distilled water, air dried and finally mounted with cover slip using P-buffer and sealing cover slip borders with rubber cement. For further details on protocols see in Iannuzzi and Di Berardino (2008).

At least 100 cells for CA-test and 35 cells for SCE-test for each animal were studied with three different fluorescence microscopes (Nikon E-1000, Leica DBM-RBE and Leica DM6000B) all connected with a CCD-camera and PC. For both cytogenetic tests (CA and SCE) only cells with 2n=44 were considered to avoid underestimated evaluation in cells with no complete chromosome set. Since the only chromosome abnormalities observed were chromosome and chromosome breaks and only complete diploid cells were studied for the test, no abnormal cells were reported.

2.3. Statistical analysis

The distribution of data within each group was tested for normality according to Shapiro and Wilk (1965). After the examination of data normality, they were subjected to variance analysis (ANOVA) by SAS (ver 9.2). Tukey's pairwise test was used to make all possible comparisons between the groups. Bonferroni correction was applied as default restriction and differences were considered significant if $P \leq 0.05$.

3. Results

3.1. Chromosome and chromatid breaks (CA-test)

Figure 1 shows a rabbit male metaphase plate with a chromatid break (CA-test) while table 1 shows mean value CA/cell in the four groups of animals at 0, 40 and 80 days. Comparisons within the same group show decreased CA-mean values starting from groups at the day 0, compared with those at both 40 and 80 days, including the CA-mean values of the control groups. This is also shown in the supplementary figure 1 reporting the CA/cell at 40 and 80 days in comparison to those at the 0 days within 95% standardized coefficient in the four groups of rabbits. CA-mean values at 40 and 80 days were significant lower than those at the 0 days only when data were compared with those of the control group A (table 2). As shown, diets rich in VB (and/or LP) do not originate any effect on CA/cell.

Sister Chromatid Exchange (SCE) test

A rabbit metaphase plate with several SCEs is shown in Figure 2, while table 3 shows mean values of SCE/cell in the four groups of rabbits. SCE-mean values tend to decrease within the same group starting from 0 to 80 days, but the differences were statistically significant only for LP (table 4), as also visualized in the supplementary figure 2 which reports the SCE/cell at 40 and 80 days in comparison to those at 0 days within 95% standardized coefficient in the four groups of rabbits.

While the diet rich in LP (group B) significantly decrease the effects on SCE/cell, no significant effects were observed on cells in the diet rich in VB (group C) or VB+LP (group D).

3. Discussion

Mutagenic tests have been applied to study the chromosome fragility (or stability) on cells exposed both *in vivo* and *in vitro* to mutagens or chemicals, suspected to have mutagenic activity (Mrdjanovic et al., 2014; Lovreglio et al., 2014). In particular, the cytogenetic tests we used in the present study have been applied in both human and animal lymphocytes, often revealing a higher chromosome fragility, compared to control groups (*in vivo* exposure) or control unexposed cells (*in vitro* exposure) (Rubes et al., 1997; Lioi et al., 1998; Picco et al., 2004; Iannuzzi et al., 2004; Perucatti et al., 2006; Santoro et al., 2008; Bonassi et al., 2008; Di Meo et al., 2011; Genualdo et al., 2012; Siviková and Dianovsky, 2006; Sivikova et al., 2013; Georgieva et al., 2013; Genualdo et al., 2015; Perucatti et al., 2015; Iannuzzi et al., 2016).

In the present study, data at 0, 40 and 80 days of the experiment, in general demonstrate that chromosome fragility decreases starting from 0 to 40 and 80 days in both control and exposed groups to VB, LP and to LP+VB considering both test we used although significant lower values were observed only within group A (control) with CA-test, and within group B(LP) with SCE-test (tables 1-4, supplementary figures 1 and 2).

Considering the higher values of CA at zero day, we can hypothesize that since rabbits have been acquired in a large company farm raising rabbits, it is probable that this higher chromosome fragility reflects possible stress due to a large number of animals, compared to the small groups used for this study. In addition, in the private company farm, there was the practice to spray an antifungal on both animals and animal hutch environment to prevent mycosis. However, this relative higher chromosome fragility at zero day was not confirmed by the SCE-test, indicating that CA-test may be more sensitive than SCE-test or reflecting a different mechanism-originating

chromosome and chromatid breaks related to the large and private company farm were the rabbits were acquired.

By examining all data and the statistical analysis we performed, the present study based on two mutagenic tests shows a clear evidence that diets rich in VB (and/or LP) have no cytotoxic effect on rabbit cells.

Our data differ from those reported earlier where statistical higher chromosome fragility was reported by using the same mutagenic tests (CA- and SCE-test) in human lymphocytes exposed *in vitro* to VB (Santoro et al., 2008). It's difficult to compare two experiments performed in different conditions. Our study was an *in vivo* exposure to VB, whereas the first one was carried out *in vitro* using only three donors and without the use of any metabolic activator of VB. Generally, in the majority of studies performed *in vivo* using VB in diet supplementation, the quantities of VB vary between 1 to 10 mg/Kg of feed (Di Giancamillo et al., 2013; Casamassima et al., 2013a; Pastorelli et al., 2012), although in a study on Drosophila, the *in vivo* exposure to VB reached very high values (from 27 to 173 mM) and no cytotoxic effects of VB were found (Santos-Cruz et al., 2012). Also an *in vitro* study using 0.03-0.48 mM of VB (equivalent to 0.019-0.309 mg/L, respectively) revealed no genotoxic effects on cells (Anter et al., 2014). These values were lower and higher to the lowest (0.05 mM) and highest (0.1 mM) doses of VB, respectively, used in human lymphocytes, where cytotoxic effects of VB were found (Santoro et al., 2008). Our results performed in four different groups of rabbit (6 animals per group) and by using the same mutagenic test used earlier by Santoro et al. (2008), show very clearly that VB alone or with LP is not cytotoxic for the rabbit cells.

4. Conclusions

VB diet supplement alone or with LP has been revealed no cytotoxic on rabbit lymphocytes based on the two mutagenic tests we applied in four homogeneous groups of animals. This could suggest

the use of VB (and LP) in both animal and human diets considering the various benefits it can do, as demonstrated by several studies.

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Ethical approval. The experimental project has been approved by the Ethical Commission of the National Research Council (CNR), ISPAAM of Naples, with registered number 01/2015, Jan. 20, 2015.

Conflict of interest. The authors declare to have not conflict of interest in this study.

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Figure legend

Figure 1. Rabbit metaphase plate stained with Giemsa and showing a chromatid break (arrow).

Figure 2. Rabbit metaphase plate stained with acridine orange and showing several SCEs (arrows).

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Table 1. Mean value/cell of chromosome and chromatid breaks (CA) in rabbits feed in absence (control – group A) and in presence of Lycopene (LP -group B), Verbascoside (VB – group C) and LP+VB (group D).

Animal Group	CA (chromosome + chromatid breaks)		
	Day 0 mean±sd /cell	Day 40 mean±sd /cell	Day 80 mean±sd /cell
A (control)	0.10±0.35	0.08±0.29	0.05±0.22
B (LP)	0.05±0.22	0.03±0.18	0.03±0.18
C (VB)	0.04±0.20	0.04±0.20	0.02±0.12
D (LP+VB)	0.04±0.22	0.03±0.18	0.01±0.17

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Table 2. P-values of Tukey's test for the CA count per cell. Asterisks identify significant values

		CA-test											
		Group A			Group B			Group C			Group D		
		0	40	80	0	40	80	0	40	80	0	40	80
A	0	-	0.995	0.399	0.327	0.029*	0.008*	0.146	0.090	0.000*	0.160	0.023*	0.000*
	40		-	0.977	0.959	0.449	0.224	0.824	0.712	0.052	0.843	0.399	0.023*
	80			-	1	0.997	0.966	1	1	0.737	1	0.995	0.556
B	0				-	0.999	0.982	1	1	0.803	1	0.998	0.636
	40						1.000	1	1	0.999	1	1	0.991
	80						-	0.999	0.999	1	0.998	1	0.999
C	0							-	1	0.951	1	1	0.861
	40									0.982	1	1	0.931
	80									-	0.942	0.999	1
D	0										-	1	0.843
	40												0.995
	80												-

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Table 3. Mean values of SCEs in rabbit feed in absence (control- group A) and presence of Lycopene (LP- group B) Verbascoside (VB- group C) and Lycopene+Verbascoside (LP+VB – group D)

Animal Group	SCEs		
	Day 0 mean ± sd /cell	Day 40 mean ± sd /cell	Day 80 mean ± sd /cell
A (control)	8.40 ± 3.60	7.60 ± 2.92	8.20 ± 3.48
B (LP)	9.30 ± 3.94	7.08 ± 3.20	7.93 ± 3.62
C (VB)	8.75 ± 3.95	8.03 ± 3.50	8.36 ± 5.68
D (LP+VB)	8.84 ± 3.66	7.78 ± 3.62	8.50 ± 3.48

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Table 3. P-values of Tukey's test for the SCE count per cell. Asterisks identify significant values

		SCE test											
		Group A			Group B			Group C			Group D		
		0	40	80	0	40	80	0	40	80	0	40	80
A	0	-	0.623	1.000	0.413	0.026*	0.985	0.998	0.998	1.000	0.991	0.903	1.000
	40		-	0.915	0.000*	0.969	0.999	0.093	0.993	0.694	0.049*	1.000	0.416
	80			-	0.139	0.122	0.999	0.948	1.000	1.000	0.873	0.995	0.999
B	0				-	0.000*	0.015*	0.957	0.038*	0.346	0.988	0.003	0.619
	40					-	0.530	0.000*	0.338	0.036*	0.000*	0.788	0.009*
	80						-	0.5489	1.000	0.992	0.392	1.000	0.933
C	0							-	0.744	0.996	1.000	0.295	1.000
	40								-	0.999	0.591	1.000	0.985
	80									-	0.982	0.936	1.000
D	0										-	0.183	0.999
	40											-	0.580
	80												-

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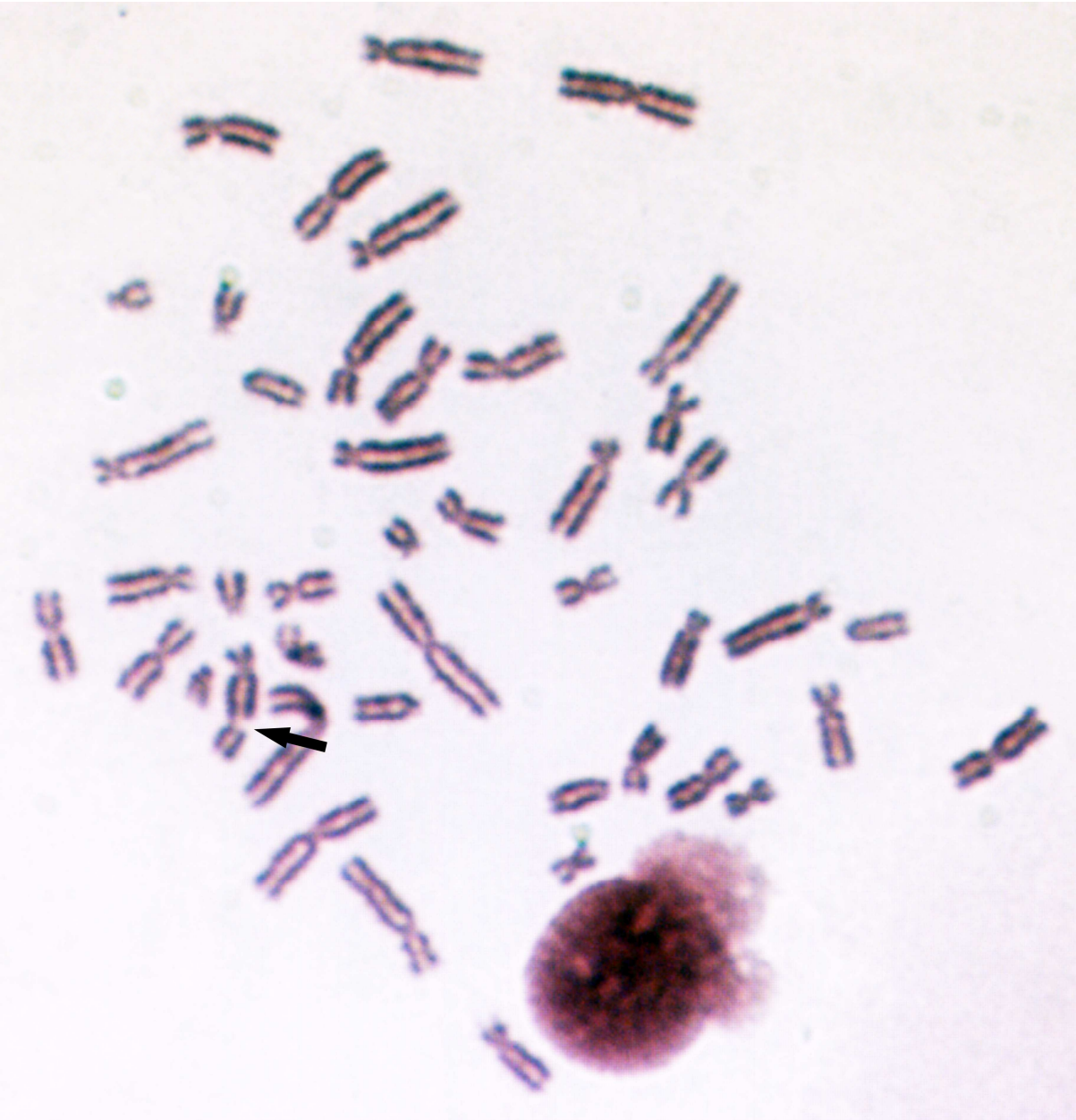

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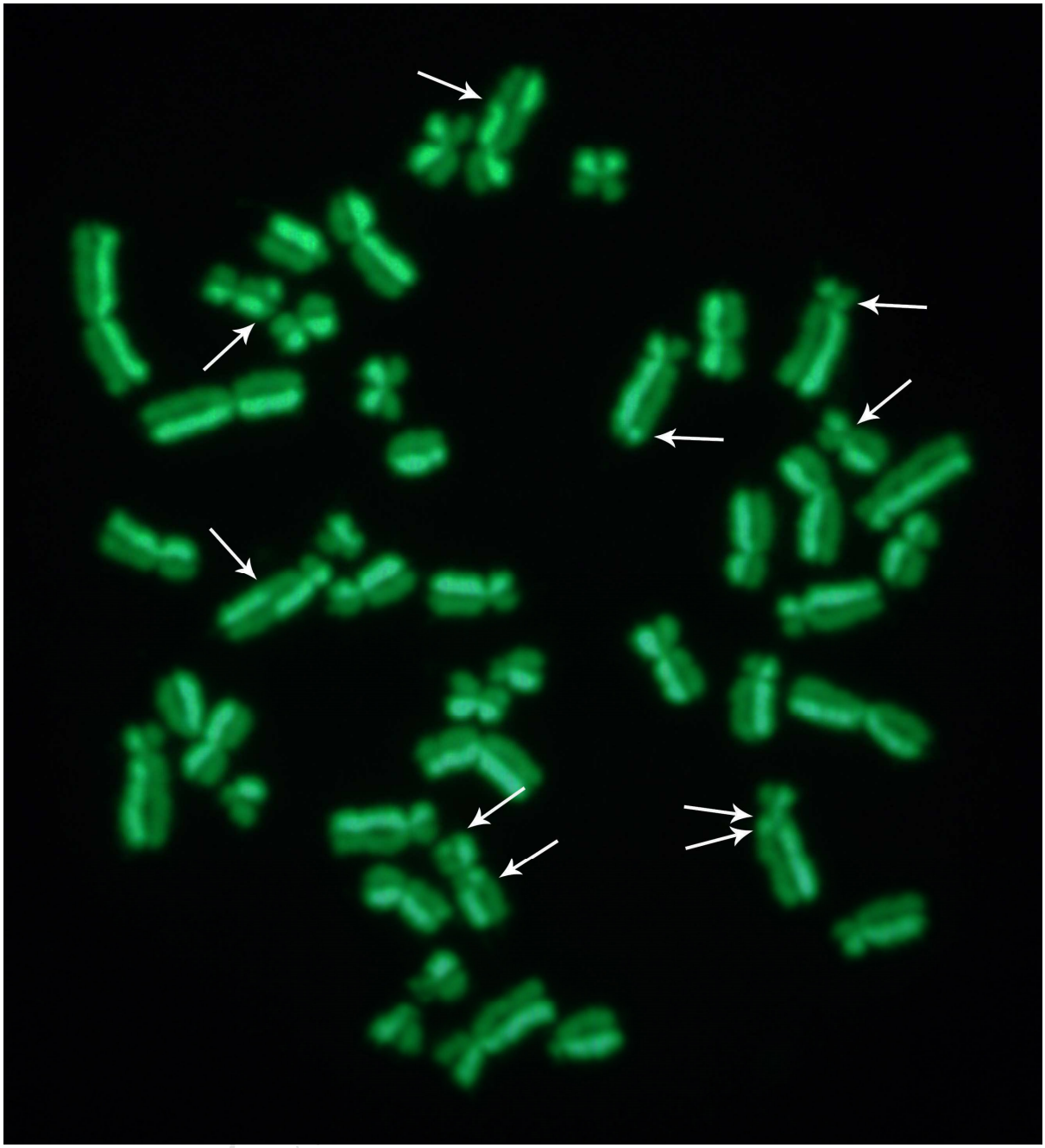

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