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Abstract

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(Short communication)

Chromosome stability in lymphocytes of rabbit (*Oryctolagus cuniculus)* feed in presence of Verbascoside and/or Lycopene

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Abstract

Phenylpropanoid glycosides (PPG), like other phenolic compounds, are powerful antioxidants with the Verbascoside (VB) showing the highest scavenger activity in the PPG. Previous cytogenetic studies 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 Lycopene (LP) by feeding the animals without VB and LP (control), in presence of VB or/and LP for 80 days. Peripheral blood cultures were performed in three different times: 0, 40 and 80 days of the experiment and the two cytogenetic test we used (CA-test, chromatid and chromosome breaks and SCE-test) revealed no mutagenic effects on chromosome blood lymphocytes. Indeed mean values of CA at 0, 40 and 80 days were lower or not statistically different in cells exposed to VB alone or with LP, compared to unexposed cells (without VB and/or LP). SCE-test revealed no statistical differences between the SCE-mean values achieved in all animal groups at 0, 40 and 80 days, thus confirming that VB (alone or with LP) does not originate any chromosome instability being not cytotoxic for the animals.

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

1. Introduction

Phenylpropanoid glycosides (PPG), like other phenolic compounds, are powerful antioxidants and Verbascoside (VB) is that showing the highest scavenger activity in comparison with other phenolic compounds. Indeed, the presence of VB in the diet reduces cardiovascular risk factors (Campo et al., 2015) and a 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 a., 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 (together to α-carotene, β-carotene, β-kryptoxanthin and lutein) present in the human carotenoid 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 (16), 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).

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). Since in other *in vitro* studies VB was found to be not genotoxic (Anter et a., 2014; Santos-Cruz et al., 2012), in the present study we applied two cytogenetic tests to evaluate the effects of the *in vivo* exposure to VB (and LP) of lymphocytes cell cultures from four homogeneous groups of rabbit feed in presence or absence (control) of VB (and LP). Our data reveal no cytotoxic effects in lymphocytes of rabbits feed with VB alone or together to LP. In our knowledge, this is the first time that cytogenetic 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) 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 cages in a kind 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 with VB contained a water-soluble extract of Verbenaceae leaves (*Lippia* spp.) prepared on an industrial scale with a standardized procedure that includes extraction by ultrasound with a 60% aqueous solution of ethyl-alcohol from drying, followed with malt dextrin as an excipient. The quantitative analysis of phenolic compounds was performed by HPLC-UV-DAD, in accordance with Piccinelli et al. (2004). To prevent oxidation of the feed, the nutritional supplement has been micro-encapsulated with a protective matrix of hydrogenated vegetable fat with the spray-cooling technology (Sintal Husbandry, 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).

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 the CA-test (chromosome and chromatid breaks, duration time 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 with acridine orange (0.01%) for 10 min, washed with tap and distilled water, mounted in P-buffer (pH=7.0) and the coverslip sealed with 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 and stained with acridine orange as reported above. 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.

2.3. Statistical analysis

Mean values and standard deviations of both CA- and SCE-data were calculated for both single animals and animal groups. Statistical analyses were performed between the four groups of rabbits by using a t-student test. Bonferroni correction was applied as default restriction and differences were considered significant if $P \leq 0.05$.

3. Results

3.1. CA-test

Figure 1 shows a rabbit male metaphase plate with a chromatid break (CA-test) while Table 1 shows all data achieved at 0, 40 and 80 days. CA-mean values at zero day were relatively high in both group A (0.55±081) and all animals (0.40±0.71). Since at both 40 and 80 days CA-mean values of all groups were all significantly lower ($P<0.01$) than those achieved at day 0, we made the comparison between exposed animal groups with the control group A at 40 and 80 days only.

At 40 days CA-mean values were 0.25±0.51 in group B (LP), 0.30±0.63 in group C (VB) and 0.28±0.63 in group D (LP±VB). These values were all lower than those achieved in the control at 40 days (0.34±0.63) but the differences were not statistical significant (Table 1).

At the 80 days, CA-mean values were 0.25±0.53 in group B (LP), 0.17±0.43 in group C (VB) and 0.13±0.38 in group D (LP+VB), being significantly lower ($P<0.05$) than those achieved in the control (0.23±42) only for group D (LP+VB) (Table 1).

3.2. SCE-test

Mean values of SCEs (Figure 2) at zero day were 8.82±3.81 in all animals, 8.40±3.60 in group A (control), 9.30±3.94 in group B (LP), 8.75±3.95 in group C (VB) and 8.84±3.66 in group D (LP+VB) (Table 2). At 40 days, SCE-mean values were 7.08±3.20 in group B (LP), 8.03±3.50 in group C (VB) and 7.78±3.62 in group D (LP+VB) and no statistical differences were found compared to the control group A at 40 days (7.60±2.92) (Table 2). At 80 days, SCE-mean values were 7.93±3.62 in group B (LP), 8.36±5.68 in group C (VB) and 8.50±3.48 in group D (LP+VB) and no statistical differences were observed compared to the control group at 80 days (8.20±3.48) (Table 2).

4. Discussion

Cytogenetic test 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) (Santoro et al., 2008, Iannuzzi et al., 2004; Perucatti et al., 2006; Di Meo et al., 2011; Genualdo et al., 2012; Genualdo et al., 2015; Perucatti et al., 2015; Iannuzzi et al., 2016).

In the present study, data shown at 0, 40 and 80 days of the experiment reveal, in general, that chromosome fragility decreases starting from 0 to 40 and 80 days in both control and exposed groups to VB, LP and to both LP+VB considering only the CA-test, while SCE-mean values are essentially stable in all groups at 0, 40 and 80 days. Considering the higher values of CA-test at zero day, we can hypothesize that since rabbits were 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 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. For this reason, we performed the comparison among groups at 40 and 80 days to test the mutagenic activity of VB and LP alone or together, using the control group A at 40 and 80 days only and not at zero day.

CA-test at 40 days of feeding in presence of VB, LP and VB+LP reveals CA-mean values lower than that achieved in the control, but the differences were statistically not significant. At the 80 days CA-mean values were lower in groups C (VB,) and D (VB+LP) than those achieved in the control group A, but the differences were significant (P>0.05) only for group D (VB+LP), further supporting that VB (especially in combination with LP) is not cytotoxic (Table 1).

The absence of negative cytotoxic effects on rabbit cells was also confirmed by SCE-test. Indeed, SCE-mean values at both 40 and 80 days do not reveal statistical differences between the four groups (Table 2).

Our data differ from those reported earlier where a statistical higher chromosome fragility was reported (with both CA- and SCE-test) in human lymphocytes exposed *in vitro* to VB (18). 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 most 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 (19). Also *in vitro* studies 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).

5. Conclusions

VB diet supplements alone or with LP were not cytotoxic on rabbit lymphocytes based on the two cytogenetic test we applied in four homogeneous groups of animals. This could suggest the use of VB in both animal and human diets considering the various benefits it can do, as demonstrated by several studies.

Acknowledgements. The study was supported by the project "Premiale 2013" National Research Council (CNR) of Italy

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 acridine orange and showing a chromatid break (arrow).

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

Table

Table 1. Mean values per cell of CA (chromosome + chromatid breaks) 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
All animals	0.42 ± 0.71	0.29 ± 0.58	0.20 ± 0.45
A (control)	0.55 ± 0.81	0.34 ± 0.63	0.22 ± 045*
B (LP)	0.39 ± 0.71	0.25 ± 0.51	0.25 ± 0.53
C (VB)	0.35 ± 0.64	0.30 ± 0.63	0.17 ± 0.43
D (LP+VB)	0.40 ± 0.68	0.28 ± 0.54	0.13 ± 0.38*

*The result is significant at P< 0.05

Table

Table 2. 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
All animals	8.82 ± 3.81	7.61 ± 3.31	8.23 ± 4.20
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

Figure

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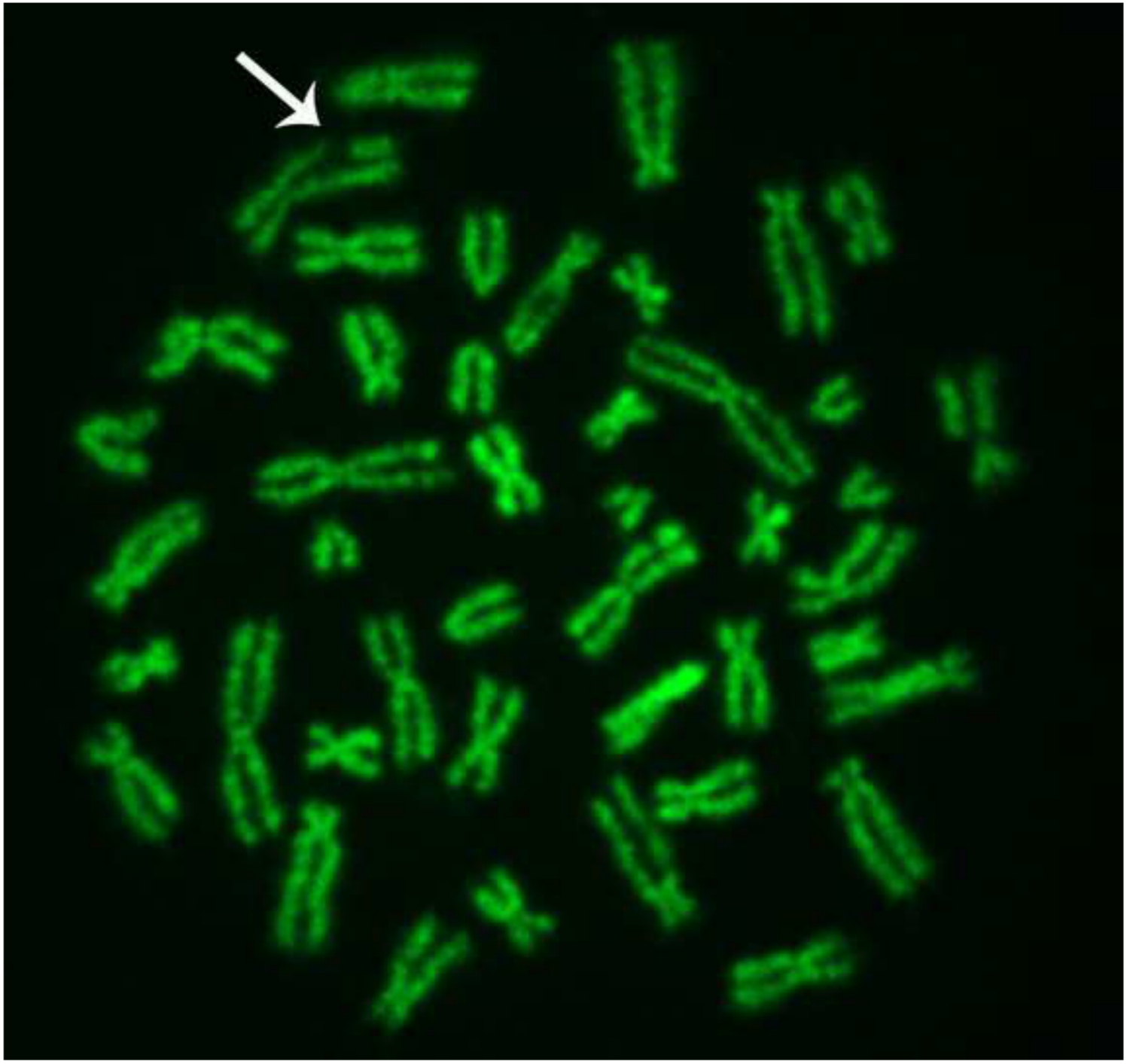

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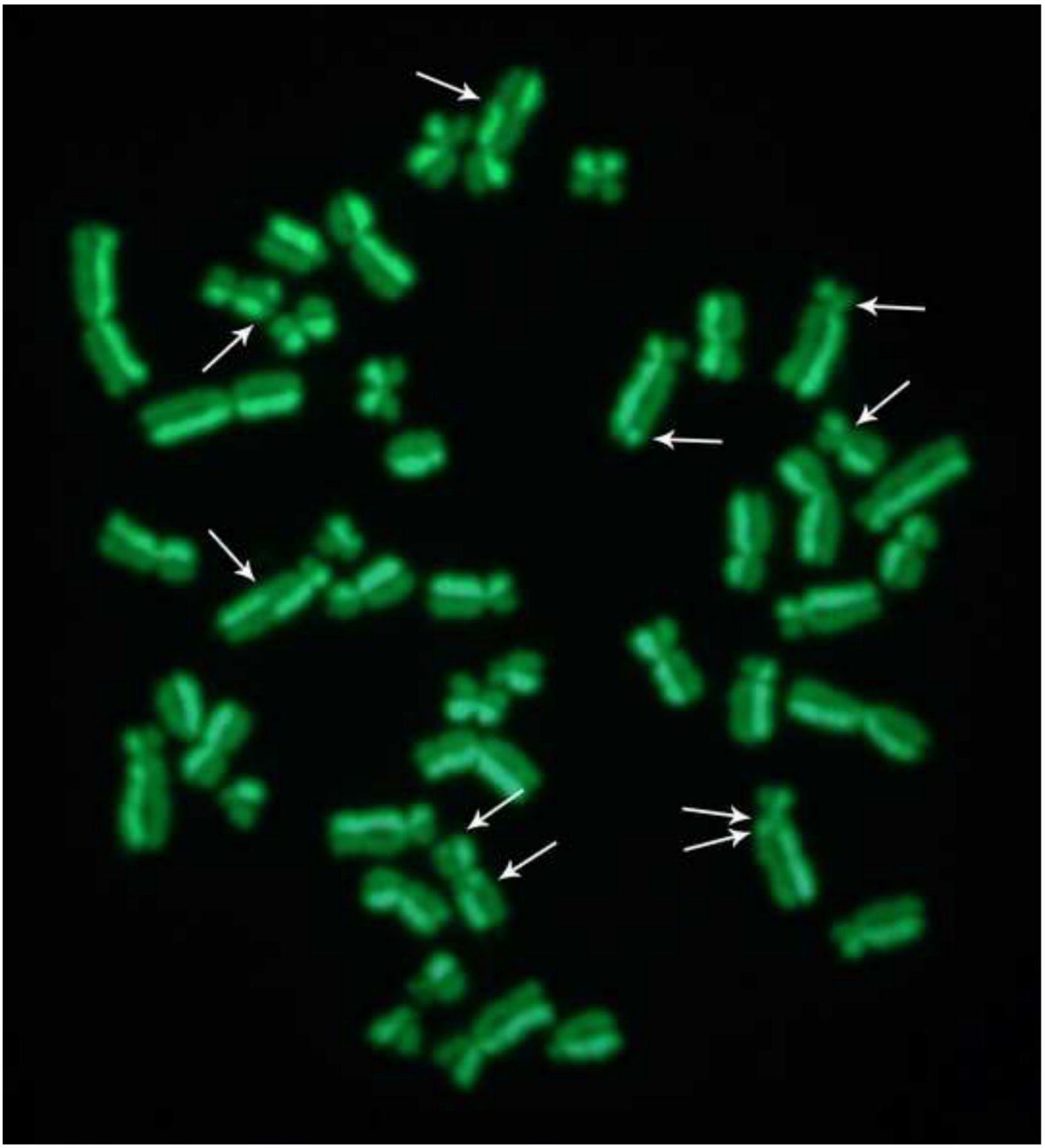

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