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Evaluation of micronucleus and erythrocytic nuclear abnormalities in Balkan whip snake *Hierophis gemonensis*

Ivancica Strunjak-Perovic · Duje Lisicic · Rozelindra Coz-Rakovac · Natalija Topic Popovic · Margita Jadan · Vesna Benkovic · Zoran Tadic

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Abstract Over recent years, changes of erythrocytic nuclei have been increasingly used to evaluate genotoxic effects of different compounds such as polycyclic aromatic hydrocarbons (benzo-*a*-pyrene, naphthalene, *β*-naphthoflavone), heavy metals (cadmium, mercury), textile mill effluent especially in aquatic ecosystem. However, in fish, both micronuclei and erythrocytic nuclear abnormalities also appear spontaneously and their frequency can be seasonally dependent. The aim of the study was to evaluate the frequency of micronuclei (MN), nuclear abnormalities (NA) including vacuolated nuclei (VN) and cytoplasmic vacuoles (CV) in erythrocytes of Balkan whip snake *Hierophis gemonensis* and establish the level of spontaneous appearance during the annual cycle. Average frequency of NA was $10.89 \pm 4.72\%$ while the MN ($0.03 \pm 0.03\%$) and VN ($0.04 \pm 0.08\%$) were seldom detected. NA significantly positively correlated with MN ($r = 0.319$; $P < 0.05$) and VN ($r = 0.363$; $P < 0.05$). Appearance of CV did not correlate with other measured parameters and average frequency was $11.06 \pm 8.33\%$. Significant seasonal variation was found in NA appearance with the lowest value in spring and the highest in winter. VN increase was observed in autumn. MN and CV levels varied between seasons but not significantly. Considering the biological cycle, frequency of NA, VN, MN and CV recorded in pre-hibernation/hibernation increased compared to the active phase, but only NA elevation was significant. Although the obtained results showed differences according to sex, statistical analysis of measured parameters showed the same pattern of seasonal variation in both sexes.

Keywords Erythrocyte nuclei · Seasonality · Genotoxicity · Fish · Reptiles

I. Strunjak-Perovic (✉) · R. Coz-Rakovac · N. Topic Popovic · M. Jadan
Division of Materials Chemistry, Rudjer Boskovic Institute, P.O. Box 180, 10002 Zagreb, Croatia
e-mail: strunjak@irb.hr

D. Lisicic · V. Benkovic · Z. Tadic
Division of Biology, Department of Animal Physiology
Faculty of Science, University of Zagreb, Zagreb, Croatia

Introduction

Interpretation of the blood cells morphology, erythrocytes particularly, became an important bioindicator of pollution. Over recent years, changes of erythrocytic nuclei have been increasingly used to evaluate genotoxic effects of different compounds such as polycyclic aromatic hydrocarbons (benzo-*a*-pyrene, naphthalene, *β*-naphthoflavone; Gravato and Santos 2002), heavy metals (cadmium, mercury; Ayllon and Garcia–Vazquez 2000), and textile mill effluent (Çavaş and Ergene-Gözükara 2003). Investigations are mainly conducted on fish that have nucleated erythrocytes and are suitable for testing the pollution of aquatic ecosystems. Carrasco et al. (1990) categorised these nuclear abnormalities into four groups (blebbed, lobed, notched and vacuolated nuclei). Although they did not find a consistent correlation between any variation of nuclear morphology and measured level of chemical contamination, many researches later included these abnormalities in the assessment of genotoxicity as a complementary test to micronucleus assay (Ayllon and Garcia–Vazquez 2000; Baršienė et al. 2006; Bolognesi et al. 2006; Çavaş and Ergene-Gözükara 2005; Fenech 2000; Gravato and Santos 2002; Pacheco and Santos 2002). In fish, both micronuclei and erythrocytic nuclear abnormalities also appear

spontaneously and their frequency can be seasonally dependent (Bolognesi et al. 2006; Jiraungkoorskul et al. 2008; Strunjak-Perovic et al. 2009). In other non-mammalian species the occurrence of nuclear abnormalities were recorded in birds (Gómez-Meda et al. 2006), amphibian (Barni et al. 2007; Marques et al. 2009) and some reptilian species (Zúñiga-González et al. 2000). Moreover, similar anomalies were observed in fibroblasts, osteosarcoma cell line exposed to irradiation, and different tumor types (mesenchymal as well as epithelial) regardless the grade of malignancy (Gisselsson et al. 2001), folic acid deficient human lymphocytes (Fenech and Crott 2002) and laminopathies (Jacob and Garg 2006; Mattout et al. 2006; Vigouroux et al. 2001).

Since their occurrence was studied more frequently in fish than in other poikilotherms such as amphibians and reptiles, the aim of this study was to establish the level of spontaneous appearance of nuclear abnormalities, micronuclei including cytoplasmic vacuoles in erythrocytes of Balkan whip snake *Hierophis gemonensis* (Colubridae) and evaluate their frequency during the annual cycle. Balkan whip snake is one of the most common snakes of costal Croatia and islands and can be found up to 800 m above sea level, but is most abundant near the sea level. Adults are usually under 100 cm of length, but can reach 130 cm, even more. They feed on lizards and large insects (e.g. grasshoppers), small mammals, nestling birds. Its natural habitats are Mediterranean-type shrubby vegetation, pastureland, plantations, and rural gardens and can be found on dry rocky places, bushy terrain, vineyards, overgrown ruins, open woods and low macchia. It is also spread in Italy, Greece, Slovenia, Albania, Bosnia and Herzegovina, Serbia and Montenegro.

Methods and materials

The Balkan whip snakes (*Hierophis gemonensis*; five males and five females) 35–148 g of weight were collected on the island Vis (43°3′32″N – 16°11′41″E), Croatia. This island is situated in the mid-eastern Adriatic, some 44 km off the coast and has the area of 91 km^2. Most of the island is covered with typical Mediterranean vegetation, consisting of pine forests, low scrubs and scattered bushes on the stony terrain. It has about 3,700 habitants, mostly situated in two main villages (Vis and Komiza).The main source of income is tourism which is run on relatively small scale. There were never any industrial facilities on the island. Snakes were caught on the hills, as far as possible from human settlements, to minimize the potential influence of pollutants. They were transferred to the laboratory where they were marked and kept in plastic cages. They had ad libitum access to water and were fed with prekilled mice on a weekly basis. Daily temperature for active period ranged from 28 to 30°C, and from 25 to 27°C during the night. During entry to the pre hibernation and arousing period animals were exposed to temperature between 13 and 21°C depending of day–night cycle for 3 weeks in order to physiologically adapted to hibernation and activity, respectively. Hibernating animals were kept in small plastic terrariums at constant temperature from 6 to 8°C in darkness.

Peripheral blood samples from every snake were drawn from the ventral caudal vein every two months, in March (arousing animals/end of hibernation), May, July, September (normal activity), November (pre-hibernation) and January (hibernation). Blood was smeared immediately on the clean microscope slides, air-dried, fixed in 95% methanol for 3 min and stained with May-Grünwald/Giemsa (MGG) stain. Three slides were prepared per animal and total of 1,000 erythrocytes per slide were examined by light microscopy (Olympus BX51) at 1,600× magnification. Slides were coded and scored by the same observer.

Statistical analyses

All data were analyzed using SPSS Statistics (*Sigma Stat for Windows ver. 1.0* and *Sigma Plot Scientific Graphing Software ver. 2.01*). For statistical analysis, blebbed, lobed and notched nuclei were interpreted together as nuclear abnormalities (NA). Micronuclei (MN), vacuolated nuclei (VN) and cytoplasmic vacuoles (CV) were analyzed separately. The results were expressed as the mean value (%) and standard deviation (SD) for each obtained parameter. Correlations between measured parameters and the strength of association were measured by Spearman's rank order correlation test. Since samplings were performed on the same animals in different months, one-way repeated measures analysis of variance and Friedman repeated measures analysis of variance (RM ANOVA on Ranks) were conducted. For seasonal comparison, data which represent spring included sampling in March and May, summer July and September, autumn November and winter January.

Results

Although most erythrocytes observed presented normal, oval nuclei of erythrocyte just like those of other vertebrates such as amphibians, fish and birds, some of them clearly deviated from their normal shape. Analyses of blood smears revealed presence of all types of irregular nuclei which were described by Carrasco et al. (1990) including micronuclei and cytoplasmic vacuoles in all

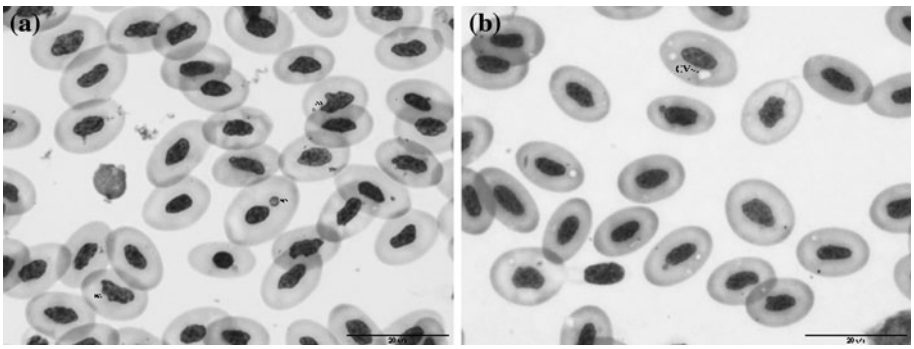

Fig. 1 **a** Photomicrographs of erythrocytic nuclear abnormalities (*NA*) and micronuclei (*MN*); **b** Cytoplasmic vacuoles (*CV*) of *Hierophis gemonensis* (×2,000)

examined individuals (Fig. 1). The data indicates that the baseline mean frequency of CV and NA were 11.06 ± 8.33% and 10.89 ± 4.72%, respectively, while VN (0.04 ± 0.08%) and MN (0.03 ± 0.03%) were seldom detected. Statistical analysis revealed significant positive correlation of NA with MN ($r = 0.319$; $P < 0.05$) and VN ($r = 0.363$; $P < 0.05$). There was no significant relationship between other measured parameters (i.e. CV vs. MN vs. VN). Analyzing data for each animal, significant inter-individual difference was recorded. Sex comparison revealed significant difference in NA and CV levels. In males, mean MN was slightly higher and VN frequency was lower than in females, but these differences were not significant (Table 1).

Concerning the sampling time and seasonal variation, the NA frequency demonstrated gradual but significant elevation in September, November and January (late summer, autumn and winter) when compared with March, May (spring) and July (summer). Significant ($P < 0.05$) elevation of CV was detected in March, September and November but grouping the data according to the season, this significance was not found (Table 2, Fig. 2a). The most frequent appearance of VN was detected in November (autumn) and significantly differs from other months (seasons) while MN increase was perceptible in January (winter), though not statistically significant (Table 2, Fig. 2b). In both males and females, the same seasonal pattern was seen.

Considering the biological cycle, NA recorded in snakes in pre-hibernated/hibernated phases (November/January: 13.20 ± 4.27%) was significantly ($P < 0.005$) higher than in active phase (March/May/July/September: 9.73 ± 4.55%). Frequency of VN, MN and CV increase was detected in pre-hibernation/hibernation comparing to the active phase, but differences were not significant ($P > 0.05$).

Discussion

Presence of erythrocyte nuclei with irregular margins in reptiles was pointed out by different authors as physiologic condition (Arikan et al. 2009; Claver and Quaglia 2009; Mayer et al. 2005) or consequence of malnutrition, anorexia and chronic diseases (Bessis 1977; Pendl 2006; Reavill 2005). However, there is no data about assessment of this phenomenon as pollution indicator in snakes. Analysis of micronuclei and other nuclear abnormalities is a common protocol to evaluate genotoxic effects especially in aquatic organisms such as fish. Since reptilian erythrocytes are nucleated it also could be suitable for implementation of this test in assessment of exposure to environmental contaminants. However, prior to involving snakes as convenient organism for testing genotoxicity, it is necessary to evaluate erythrocyte nuclear morphology in healthy, non-exposed animals.

Comparing the erythrocytic nuclear morphology detected in Balkan whip snakes with those previously recorded in gilthead sea bream, *Sparus aurata* L. (Strunjak-Perovic et al. 2009), we have found the same type of nuclear changes and depending on seasonal variation. Interspecies comparisons revealed that the lowest NA values were recorded in the spring in both species while the highest

Table 1 Mean frequencies (%) and ranges of nuclear abnormalities (*NA*), micronuclei (*MN*), vacuolated nuclei (*VN*) and cytoplasmic vacuoles (*CV*) in erythrocytes in males and females of Balkan whip snake *Hierophis gemonensis* (mean ± S.D.)

Measured parameters	Males ($n = 5$)	Range	Females ($n = 5$)	Range	Mann–Whitney rank sum test (P)
NA	12.94 ± 3.85	6.39–23.47	8.83 ± 4.66	1.44–18.72	<0.001
MN	0.03 ± 0.03	0.00–0.10	0.02 ± 0.03	0.00–0.15	>0.05
VN	0.02 ± 0.03	0.00–0.12	0.05 ± 0.11	0.00–0.47	>0.05
CV	7.99 ± 5.22	0.67–19.71	14.12 ± 9.72	0.62–38.71	<0.05

Table 2 Frequency (%) of nuclear abnormalities (*NA*), micronuclei (*MN*), vacuolated nuclei (*VN*) and cytoplasmic vacuoles (*CV*) expressed as mean ± S.D. in peripheral blood erytrhocytes of *Hierophis gemmonensis* recorded in different sampling times

Sampling time	*n*	NA	MN	VN	CV
March[a]	10	$9.85 \pm 5.77^{b,e,f}$	0.02 ± 0.02	0.02 ± 0.03^{e}	$14.78 \pm 5.77^{b,c,f}$
May[b]	10	$8.68 \pm 4.08^{a,c,d,e,f}$	0.02 ± 0.03	0.02 ± 0.02^{e}	$7.10 \pm 3.06^{a,d,e}$
July[c]	10	$9.46 \pm 4.35^{b,e,f}$	0.03 ± 0.05	0.03 ± 0.06^{e}	$3.30 \pm 2.54^{a,d,e,f}$
September[d]	10	$10.93 \pm 4.23^{b,e,f}$	0.02 ± 0.02	0.05 ± 0.11^{e}	$16.83 \pm 10.30^{b,c,f}$
November[e]	10	$12.28 \pm 4.26^{a,b,c,d,f}$	0.02 ± 0.03	$0.10 \pm 0.14^{a,b,c,d,f}$	$15.21 \pm 10.02^{b,c,f}$
January[f]	10	$14.11 \pm 4.29^{a,b,c,d,e}$	0.04 ± 0.04	0.02 ± 0.03^{e}	$9.14 \pm 6.29^{a,c,d,e}$

Letter superscripts indicate which months are significantly different from other months ($P < 0.05$)

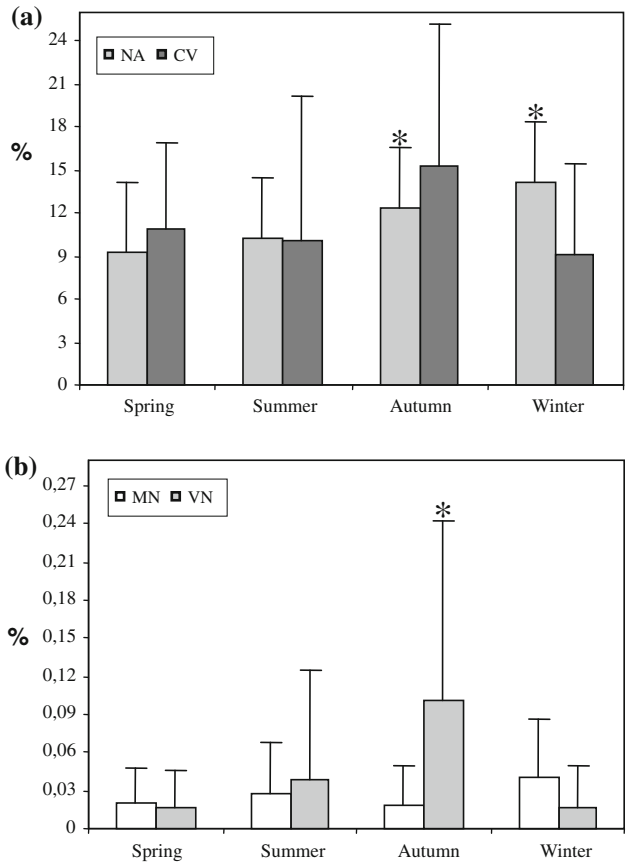

Fig. 2 The seasonal variations: **a** Nuclear abnormalities (*NA*) and cytoplasmic vacuoles (*CV*); **b** Micronuclei (*MN*) and vacuolated nuclei (*VN*) frequency in peripheral blood erytrhocytes of *Hierophis gemmonensis* (*$P < 0.05$). Error bars represent the standard deviation. Spring $n = 20$; Summer $n = 20$; Autumn $n = 10$; Winter $n = 10$

level was reached during the winter (cold period) in snakes and summer (warmer period) in fish. This seasonality correlated with biological cycle of snakes and NA more frequently appeared in pre-hibernated/hibernated than in active phase. Gender also affects the frequency of detected changes, NA particularly. Nevertheless, the same seasonal pattern was observed in both males and females. Although the number of micronucleated erythrocytes observed in peripheral blood samples can differ among species, the appearance of spontaneous MN is generally low. In our previous investigation of gilthead sea bream, mean frequency ranged from 0 to 0.04% (Strunjak-Perovic et al. 2009). Zúñiga-González et al. (2000, 2001) recorded similar values in different species of snake such as coachwhip, *Mastigophis flagellum* (0.03%), bullsnake, *Pituophis depei* and boa, *Boa constrictor* (0.01%). The mean frequencies of MN in Balkan whip snakes also were within this range (0.03 ± 0.03%). Increased VN frequency was detected in autumn, but in gilthead sea bream (Strunjak-Perovic et al. 2009) the highest values were detected in spring. Whereas snakes are ectothermic organisms and their physiological reactions are dependent on environmental temperature, seasonal variability of erythrocyte nuclear morphology could be influenced by process such as erythropoesis, lifespan of the circulating erythrocytes, eliminating old erythrocytes or those containing micronuclei (Udroiu 2006) and irregular shaped nuclei, respectively.

In snakes, cytoplasmic vacuoles can occur in most cell types spontaneously as an adaptive physiological response (Canfield 1998), as a common feature of apoptosis caused by ischemic stress, osmolar stress and growing factor deprivation (Araki et al. 2006; Morris et al. 1984; Mower et al. 1994) or in pathologic condition in other reptiles such as *Herpesvirus* infection in *Iguana* spp. Cytoplasmic vacuoles probably remain fully reversible, but can also become irreversible and lead to cell death (Henics and Wheatley 1999). In mammals, birds and lizards, CV may occur in chronic disease and malnutrition (Pendl 2006). Sakamoto et al. (1997, 2004) reported that occasionally observed vacuole-like structures in the cytoplasm of frog erythrocytes under light microscopy corresponded to swollen mitochondria under electron microscopy and degenerate mitochondria (Reavill 2005). Apart from natural toxins, many drugs and other chemicals can induce vacuolation in cells. Thus cytoplasmic vacuoles were detected in fish erythrocytes after exposure to mercury (Panigrahi and Misra 1979) and malathion (Sawhney and Johal 2000). In

fish, spontaneous appearance was detected in our previous investigation of meagre *Argyrosomus regius* with increase in spring and decrease during summer (not published). Salakij et al. (2002) reported cytoplasmic vacuoles in less than 1% erythrocytes of puff-faced watersnake (*Homalopsis buccata)* which were much lower than the levels observed in the current study. Cytoplasmic vacuoles did not correlate with any other measured parameters (NA, MN, and VN).

In conclusion, the current study provides baseline data regarding micronuclei and nuclear abnormalities in Balkan whip snakes and suggests that these assays may be useful for evaluating exposure of reptiles to genotoxic agents. However, prior to including them in the regular assessment, it will be necessary to determine confounding factor which may influence the level of spontaneous appearance of MN, and NA and to determine to what extent the presence of erytrhocytic nuclear anomalies can be inherent to certain species of snakes. The current study provides additional information which gives rise to new evidence about the impact of seasonal variation and sex dependences of erythrocyte morphology in Balkan whip snake.

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