Changes in Nuclear Phenotypes Following Cold Shock in *Panstrongylus megistus* (Burmeister)

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The nuclear phenotypes of Malpighian tubule epithelial cells of 5th instar male nymphs of the blood-sucking insect Panstrongylus megistus *were studied immediately after a short (1 h) cold shock at 0ºC, and 10 and 30 days later. The objective was to compare the responses to a cold shock with those known to occur after hyperthermia in order to provide insight into the cellular effect of cold in this species. Nuclei which usually exhibited a conspicuous Y chromosome chromocenter were the most frequent phenotype in control and treated specimens. Phenotypes in which the heterochromatin was unravelled, or in which there was nuclear fusion or cell death were more abundant in the shocked specimens. Most of the changes detected have also been found in heat-shocked nymphs, except for nuclear fusion which generates giant nuclei and which appeared to be less effective or necessary than that elicited after heat shock. Since other studies showed that a short cold shock does not affect the survival of more than 14% of 5th instar nymphs of* P. megistus *with domestic habit and can induce tolerance to a prolonged cold shock, heat shock proteins proteins are probably the best candidates for effective protection of the cells and the insects from drastic damage caused by low temperature shocks.*

Key words: *Panstrongylus megistus* - cold shock - nuclear phenotypes - cell survival - cell death

Thermal shocks affect survival, molting incidence and nuclear phenotypes in blood-sucking hemipterans, including the vectors of South American trypanosomiasis. Variations in the responses are a function of the shock duration and of the insect developmental phase, sex and species (Rodrigues et al. 1991, Dantas & Mello 1992, Garcia et al. 1999b). *Panstrongylus megistus*, for instance, is less resistant than *Triatoma infestans* to heat and cold shock assays (Rodrigues et al. 1991, Garcia et al. 1999b). *P. megistus* is a very important disease vector because of its wide distribution in Brazil, its high rate of infection with *Trypanosoma cruzi* and its adaptability in invading artificial ecotopes (Forattini et al. 1978, Forattini 1980). Knowledge of how this species responds to stress is necessary not only to establish the optimal conditions for rearing specimens in the laboratory but also to evaluate the altered biological characteristics of specimens captured in natural ecotopes.

Exposure for l h at 0°C affects the survival of *P. megistus* nymphs at various developmental stages and adults, more than does a heat shock at 40°C for the same time, although this difference is relatively less pronounced in 5th instar nymphs (Garcia et al. 1999b). Cold shock also drastically affects the frequency and time of molting, to a greater extent than does heat shock (Garcia et al. 1999b). Even so, more than 50% of the *P. megistus* nymphs at different developmental phases survive for at least one month after a short cold shock (Garcia et al. 1999b). While cryoprotectants are unknown in these insects, it is hypothesized that heat shock proteins (hsp) confer a certain amount of protection to *P. megistus* against cold in a similar way to that reported for some other insect species (Chen et al. 1987, Burton et al. 1988, Komatsu et al. 1996). Indeed, cold tolerance may be induced in *P. megistus* (Garcia et al. 1999a).

Considering that changes in nuclear phenotypes indicative of cell survival and cell death are induced by heat shock in 5th instar nymphs of *P.*

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megistus (Garcia et al. 2000), we have examined the cellular effects of cold shock in specimens of the same developmental phase of this species for comparison purposes.

MATERIALS AND METHODS

Male 5th instar nymphs, descendants of a domestic population of *P. megistus* (Burmeister) (Hemiptera, Reduviidae) and reared in the laboratory were used. Control groups were maintained at 28°C and 80% relative humidity whereas the experimental specimens were subjected to cold shock at 0°C for 1 h. The shock temperature conditions were selected based on a previous report on the survival and molting incidence for this species (Garcia et al. 1999b).

The specimens underwent a 15-day moderate fasting period before the shock after which they were fed with hen blood once a week. This nutritional regime was chosen because there was no significant difference between the survival of slightly fasted and fully-nourished 5th instar nymphs of domestic *P. megistus* (Garcia et al. 1999b) while the moderate fasting facilitates the dissection procedure.

Whole Malpighian tubules were removed from the insects immediately after the shock and also 10 and 30 days later. Organs from at least three specimens under experimental and control groups were used. The control groups were the same as those used for study of the heat shock effects (Garcia et al. 2000), since heat and cold shock experiments were processed simultaneously, although cold shock results were analyzed later. The organs were mounted on histological slides, fixed in absolute ethanol: glacial acetic acid (3:1, v/v) for 1 min, rinsed in 70% ethanol for 5 min and air dried at room temperature. The tubules were then processed for the Feulgen reaction, acid hydrolysis being performed in 4 M HCl at 25°C for 65 min. The stained preparations were rinsed in three washes of sulfurous water and one wash of distilled water, air dried, cleared in xylene, and mounted in Canada balsam.

The total number of Malpighian tubule epithelial cell nuclei and the number of different nuclear phenotypes present in the organ were determined in each specimen by counting with a Zeiss light microscope. Photomicrographs were obtained using a Zeiss Axiophot II microscope. The linear relationship between the shock conditions and the various nuclear phenotypes was assessed statistically with the Minitab^{TM} software.

RESULTS

The Feulgen-stained polyploid nuclei of the Malpighian tubules of *P. megistus* males showed homogeneously distributed fine chromatin granules encircling one small heterochromatic body which contained several copies of the Y chromosome (Mello et al. 1986) (Fig. 1). This phenotype was the most frequent in both, the control and experimental specimens (Table I), although it decreased in relative frequency 30 days after the cold shock (Table II).

Other nuclear types were more prominent in the cold-shocked specimens (Table I) and were characterized by heterochromatin unravelling (Fig. 2), nuclear fusion (giants) (Fig. 3), typical and suspected apoptosis (Figs 4, 5) and necrosis (Fig. 6). Apoptosis and necrosis were defined morphologically and were also seen in some giants.

The absolute, but not relative, frequency of heterochromatin unravelling increased immediately after the shock. With increasing time after the shock, both the absolute and relative frequencies of this nuclear phenotype increased significantly (Tables I, II).

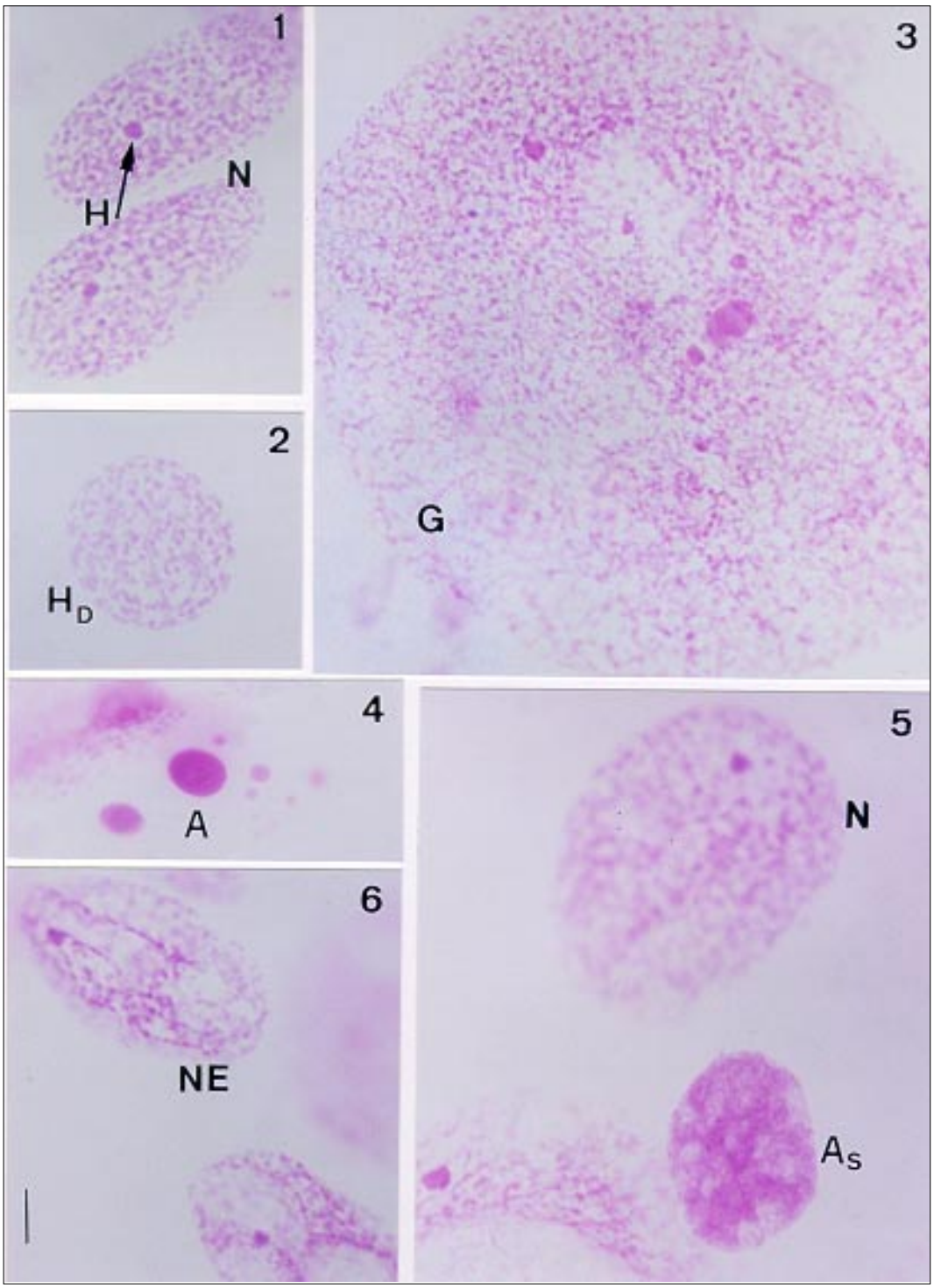
The absolute frequency of giant nuclei increased slightly just after the shock, but decreased thereafter, whereas the relative frequency of these nuclei was not significantly affected immediately after the shock but decreased moderately throughout the remainder of the experiment. Rare necrotic giant nuclei, and giant nuclei suspected of apoptosis or with heterochromatin unravelling were observed with increasing time after the shock (Tables I, II).

There was a significant increase in the frequency of apoptotic nuclei but not in that of nuclei suspected of apoptosis following the cold shock. However, the frequency of apoptotic nuclei subsequently decreased whereas that of nuclei suspected of apoptosis was not significantly correlated with increasing time after the shock (Tables I, II). The frequency of necrotic nuclei did not change immediately after the shock but increased significantly throughout the rest of the experiment (Tables I, II).

DISCUSSION

The control specimens of *P. megistus* studied here had lower nuclear frequency than that reported for fully-nourished, laboratory-reared specimens (Mello et al. 1986, Garcia et al. 2000). This may reflect nuclear fusion and cell death induced by other stressing agents (such as fasting) prior to the shock. The presence of a certain frequency of nuclear phenotypes different from that of the normal phenotype agrees with this hypothesis.

The increased frequencies of altered nuclear phenotypes following cold shock indicate mechanisms involving cell survival (heterochromatin unravelling, nuclear fusion) and cell death (apoptosis, necrosis). Most of the nuclear changes observed



Figs 1-6: nuclear phenotypes in Feulgen-stained Malpighian tubules of *Panstrongylus megistus* nymphs subjected to cold shock. Bar = 10 µm. A: apoptosis; A_S: suspected apoptosis; G: giant nucleus; H: heterochromatin; H_D: unravelled heterochromatin; N: normal; NE: necrosis

TABLE I

Absolute frequencies of nuclear phenotypes in Malpighian tubule epithelial cells of *Panstrongylus megistus* 5th instar nymphs after cold shock at 0ºC

Experimental	Nuclear phenotypes									
conditions	A	A_s	NE	G	G_{NE}	G_{HD}	G_S	HD	N	Total
[a]Control, t_0	10	1324	1255	25	4	3	0	147	5302	8070
	0	1345	192	1	1	0	0	54	8786	10378
	6	2347	1037	0	0	0	0	35	10499	13951
1 h shock: t_0	49	1982	791	15	1	1	4	80	9083	12006
	1	994	472	54	2	12	1	135	6956	8627
	51	3036	946	42	2	4	17	396	12349	16823
[a]Control, $t_{10\ days}$	7	1729	325	6	0	0	0	98	9039	11204
	9	2482	116	0	0	0	0	76	6479	9162
	16	3252	728	0	0	0	0	268	11428	15692
1 h shock: $t_{10\ days}$	52	2313	1298	7	1	0	0	110	13792	17570
	33	2004	668	11	0	0	2	75	10699	13492
	19	3587	1032	1	0	0	0	446	10495	15580
[a]Control, $t_{30\ days}$	25	2587	3	0	0	0	0	173	10242	14506
	11	1351	1882	10	0	0	8	295	8169	11726
	23	2704	1962	5	0	0	0	424	10673	15791
	8	1408	1522	5	0	1	0	164	7271	10379
1 h shock: $t_{30\ days}$	5	2529	1057	0	0	0	0	68	6653	10312
	7	2850	482	0	0	0	0	309	9830	13478
	8	2910	4905	4	0	4	3	101	8733	16668

A: apoptosis; As: suspected apoptosis; G: giant nuclei; G_{NE}: giant necrotic nuclei; G_{HD}: giant nuclei with heterochromatin decondensation; G_S: giant nuclei suspected of apoptosis; HD: heterochromatin decondensation; N: normal; NE: necrosis; *a*: Garcia et al. 2000

TABLE II

Linear correlation between cold shock and the relative frequency of nuclear phenotypes

	D	ºC	A	A_S	NE	G	G_D	G_{HD}	G_S	HD
ºC	.01									
A	**-.32**	**-.22**								
A_S	.14	-.09	-.04							
NE	**.52**	-.03	-.14	-.19						
G	**-.47**	-.01	**35**	**-.26**	-.08					
G_D	**-.35**	**.20**	**.47**	-.04	-.07	**.82**				
G_{HD}	**-.37**	.04	**30**	-.16	-.05	**.96**	**.89**			
G_S	-.16	-.14	**.47**	-.19	.10	**39**	**.29**	**.29**		
HD	**.22**	.04	.01	.07	.06	.17	.07	.09	**33**	
N	**-.55**	.08	.12	**-.40**	**-.80**	.16	.03	.08	-.05	**-.22**

D: days after shock; ºC: temperature; A: apoptosis; A_s: suspected apoptosis; N: normal nuclei with usual heterochromatin body; NE: necrosis; G: giant nuclei; G_D: necrotic giant nuclei; G_{HD}: giant nuclei with heterochromatin unravelling; G_s: giant nuclei with suspected apoptosis; HD: heterochromatin unravelling

value moderate correlation (between 20% and 50%)
value high correlation (> 50%)

here have also been reported after hyperthermia in *P. megistus* and *T. infestans* (Dantas & Mello 1992, Tavares et al. 1997, Garcia et al. 2000). Heterochromatin unravelling, with its possible role in the activation of silent genes during stress (Simões et al. 1975), is longer-lasting in *P. megistus* compared with *T. infestans*, irrespective of the eliciting temperature used (Dantas & Mello 1992).

Giant nuclei generated by fusion in the organs of several blood-sucking hemipteran species have been associated with survival mechanisms under unfavorable conditions (Wigglesworth 1967, Mello & Raymundo 1980, Mello 1989). The frequency of giant nuclei in *P. megistus* nymphs increased slightly just after the short cold shock but remained very low with increasing time after the shock. This finding contrasts with the situation found after short heat shock when there was an increase in the absolute frequency of giant nuclei 10 days after a 1 h-treatment and a decreased frequency thereafter (Garcia et al. 2000). The activation of nuclear and/or cell fusion which generates giant nuclei (Wigglesworth 1967) was found to be less effective or necessary in response to cold shock than that elicited by heat shock. In addition, some of the giant nuclei showed morphological signs of cell death.

The apoptosis and necrosis phenomena seen in the Malpighian tubules of *P. megistus* after a cold shock have also been observed after a heat shock (Garcia et al. 2000). In both cases, only the apoptosis program intensified immediately after the shocks; necrosis was a later response.

When stress is enhanced beyond a certain level and hsp are incapable of protecting the cells from the deleterious effects of stress, apoptosis is activated. If the stress damage is very severe, necrosis predominates (Lindquist & Craig 1988, Harmon et al. 1990, Sakaguchi et al. 1995, Samali & Cotter 1996). Although the effects of a short cold shock in *P. megistus* are not completely controlled by hsp or survival mechanisms involving cell/nuclear fusions and heterochromatin unravelling since cell death mechanisms are elicited, cell death was not grave enough to affect the survival of more than 14% of the 5th instar nymphs of *P. megistus* after this shock (Garcia et al. 1999b).

Prolonged cold and heat shocks are extremely deleterious to *P. megistus* nymphs (Garcia et al. 1999b). Indeed, tolerance to prolonged heat and cold shocks develops in *P. megistus*, if preceded by short heat and cold shocks, respectively (Garcia et al. 1998, 1999a). The development of tolerance after sequential shocks has been attributed to the action of hsp (Lindquist 1986, Burton et al. 1988). Thus, if hsp are activated in *P. megistus* by a short cold shock, thereby producing tolerance to a prolonged cold shock, hsp would be the best candidates for effectively protecting the cells and insect from drastic damage by low temperature.

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