

A KARYOMETRIC STUDY OF HEAT-SHOCKED HeLa CELLS*

Silvia R.P. Miranda¹, Maria Luiza S. Mello¹ and Aquiles E. Piedrabuena²

ABSTRACT

HeLa cells exposed to heat shock at temperatures of 40°C and 43°C for 1 hour and also cells 4h after returning to control conditions (37°C) were studied comparatively with respect to nuclear and nucleolar sizes, using karyometric procedures. Some alterations especially involving nucleolar areas were found to be promoted by the shock, though mostly detectable during the post-shock period held at control temperature. These alterations when elicited by the shock at 43°C were assumed to be associated with transcriptional activities involved in recovery of the ordinary cellular activities, whereas those related to the shock at 40°C were suggested to be due to karyoskeletal proteins appearing late with the stress and which could be mechanically restraining the nucleolar area increase. These proteins also appear in the HeLa cells exposed to the heat shock at 43°C and equally seem to affect the increase in nucleolar area in comparison to nuclear area, however they are disassembled when the cells are returned to control temperature.

INTRODUCTION

Human epithelioid carcinoma (HeLa) cells, have been reported to undergo marked morphological changes, when exposed to environmental temperatures above 37°C, which may be reversible when the heat shock is followed by a return to control temperature conditions (Heine *et al.*, 1971). Exposure of HeLa cells to a temperature of 41°C has a mild effect, while very pronounced changes are verified at 42-43°C and degenerative changes and cell death are frequent at 44-45°C (Heine *et al.*, 1971).

* Part of a thesis presented by SRPM to the Instituto de Biologia, UNICAMP, Campinas, in partial fulfillment of requirements for the Masters degree.

¹ Departamento de Biologia Celular, Instituto de Biologia, UNICAMP, 13081-970, Campinas, SP, Brasil. Send correspondence to M.L.S.M.

² Departamento de Genética e Evolução, Instituto de Biologia, UNICAMP, 13081-970, Campinas, SP, Brasil.

A new distribution and/or aggregational state of macromolecules, especially proteins, is elicited with heat shock in cell nuclei (Reiter and Penman, 1983; Welch and Suhan, 1985; McConnell *et al.*, 1987; Ornelles and Penman, 1990). The pattern of dry mass distribution, as revealed by interference microscopy, changes in HeLa cell nuclei subjected to hyperthermia (Mello and Miranda, in press).

However, data on nuclear and nucleolar sizes, which could indicate materials being produced or broken down, under the influence of heat shock conditions, is not available.

We studied morphometrically nuclei and nucleoli of HeLa cells subjected to supranormal and recovery temperatures, compared to cells under control conditions.

MATERIALS AND METHODS

Cells - HeLa cells were cultured as monolayers in Leighton tubes in RPMI/1640 medium (Flow Laboratories, England), supplemented with 10% calf serum (Microbiological, Brazil) without antibiotics. About 1×10^5 cells were resuspended in one ml of the medium and maintained for 16-21 hours in a culture incubator (New Brunswick Scientific) at 37°C, and wet atmosphere containing a 5% CO₂ concentration.

Heat shocks - The Leighton tubes were partially submerged in a water bath at 40°C and 43°C for one hour. Some cultures were then returned to control conditions (37°C) for four hours (recovery). Codes for the experiments are presented in Table I.

Table I - Experimental parameters of mild and drastic heat shock treatments of HeLa cells.

Experimental codes	Treatments			
	Initial culture at 37°C (h)	Heat shocks (h) at		Recovery at 37°C (h)
		40°C	43°C	
C1, C1*	16	-	-	-
C1-1*	17	-	-	-
C2, C2*	21	-	-	-
HS1	16	1	-	-
HS1*	16	-	1	-
HS2	16	1	-	4
HS2*	16	-	1	4

Fixation - The monolayers were fixed in an acetic ethanol mixture for one min, immersed in 70% ethanol for two min, and air dried.

Staining - The preparations were stained for 15 min with a 0.025% toluidine blue (TB) solution prepared in McIlvaine buffer at pH 4.0. After staining, the slides were rapidly rinsed in distilled water, air dried, cleared in xylene and mounted in Canada balsam ($n_D = 1.54$), according to the method of Vidal (Mello and Vidal, 1980).

Morphometry - Photographic negatives of the nuclear images were projected with a photographic enlarger and the projections used for the determination of the nuclear and nucleolar areas (Mello and Vidal, 1980).

Statistics - Since nuclear areas, similar to nuclear volumes, generally do not obey a Gaussian distribution, the nuclear area data in μm^2 were plotted as frequency histograms on a scale of values in geometric progression, starting with the lowest value for the control and using $\sqrt[3]{4}$ as the increasing ratio for intervals II with respect to I, III with respect to II and so on, up to the nuclear V interval (Mello and Vidal, 1980). This choice was based on reports that an increase of nuclear area by a factor of $\sqrt[3]{4}$ corresponds to a doubling of nuclear volume (Palkóvitz and Fischer, 1968). The comparison of the nuclear and nucleolar areas of the HeLa cells under various experimental conditions was carried out with Mann-Whitney and Kruskal-Wallis tests. A study of correlation and regression parameters was determined and their significance level established.

RESULTS

Observation of heated and unheated HeLa interphase cells after staining with toluidine blue revealed the same cell phenotypical diversity previously found by Rose (1962). Single-nucleated cells predominated in all the preparations (Table II).

Single-, bi- and multinucleolated nuclei (Figures 1A, B, C) with predominance of the former were exhibited by the experimental and control cells (Table II).

A negative response to toluidine blue staining at pH 4.0 was found around the nuclei in the heat-shocked cells (Figures 1A, B, C). Considering staining conditions, this is attributed to the aggregation of protein molecules, possibly vimentin-containing intermediate filaments, in the perinuclear region (Welch and Suhan, 1985).

Mitotic index values decreased in the heated cells, especially when the shock was at 40°C, and was not apparently recovered during the four hour post-shock period (Table II). This alteration in cell proliferation is possibly due to changes in transcriptional processes, leading to the synthesis of heat shock proteins, affecting the synthesis of proteins necessary to cell division.

Micronucleation increased in the cells subjected to heat shock at 43°C (Table III), an indication that shocks at higher temperatures can promote an increase in chromosome deletions and/or decrease in the survival ability of the HeLa cells.

Table II - Mitotic index (M.I.) and some nuclear characteristics of heat-shocked HeLa cells.

Experimental conditions ¹	Number of cells	M.I. (%)	Frequencies of cells (%)			No. of nucleoli/nucleus (%)		
			Single-nucleate	Bi-nucleate	Multi-nucleate	1	2	> 2
C1	1001	0.80	93.3	5.7	1.0	63	26	11
C1*	1001	2.10	97.2	2.2	0.6	51	28	21
C1-1*	1013	0.99	97.4	2.2	0.4	46	28	26
HS1	1017	0.39	96.5	2.7	0.8	67	26	7
HS1*	1001	1.00	97.9	1.4	0.7	51	29	20
C2	1018	0.49	95.8	3.5	0.7	67	26	7
C2*	1015	4.43	98.7	1.3	-	31	25	44
HS2	1017	0.10	96.5	2.9	0.6	68	24	8
HS2*	1007	0.38	98.9	1.1	-	61	20	19

¹Abbreviations: see Table I.

Table III - Micronucleation in heat-shocked HeLa cells.

Experimental conditions ¹	Number of cells studied	Micronuclei (%)
C1	1067	0.94
C1*	1038	2.22
C1-1*	1028	2.28
HS1	1057	1.23
HS1*	1077	3.06
C2	1016	2.56
C2*	1025	1.17
HS2	1073	2.33
HS2*	1067	4.06

¹Abbreviations: see Table I.

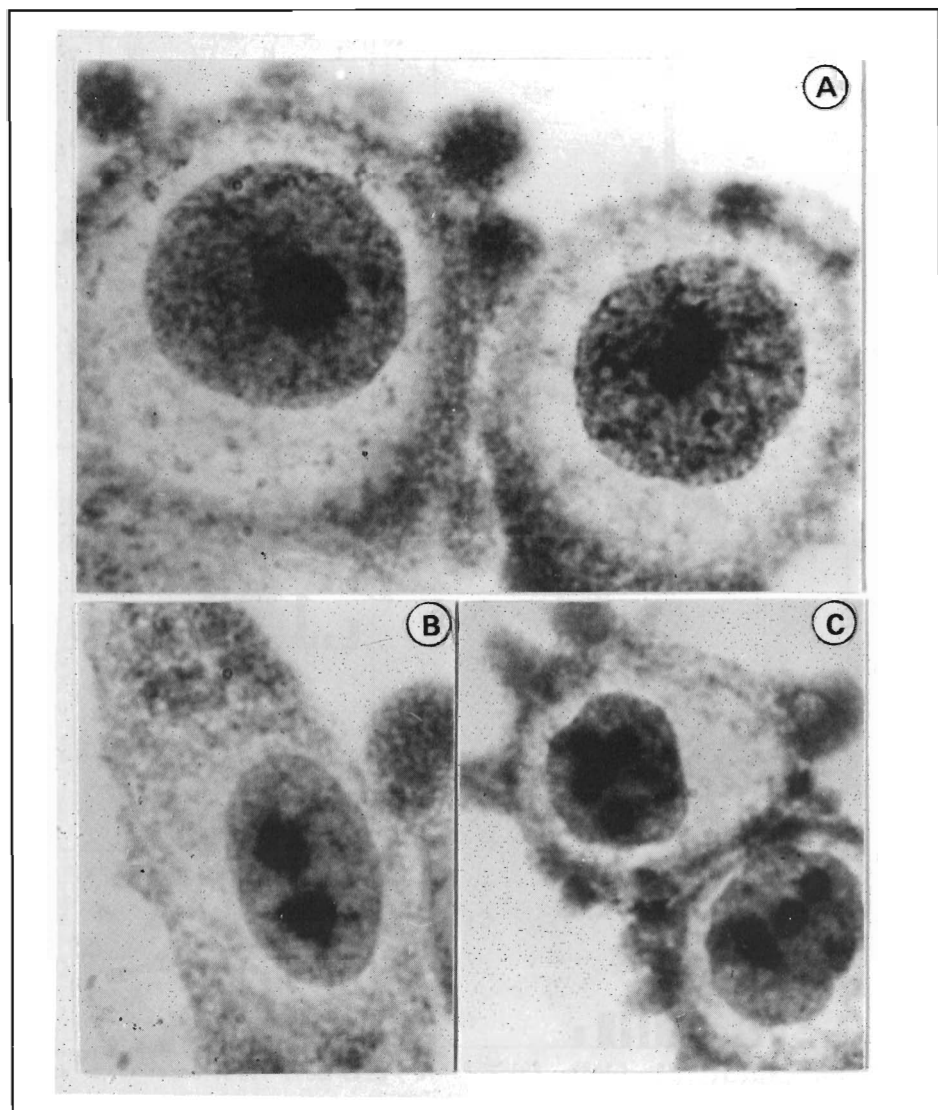


Figure 1 - Heat-shocked HeLa cells stained with a toluidine blue solution at pH 4.0. Single- (A), bi- (B) and multinucleolated (C) nuclei as well as a reactive material at cellular edges and cytoplasmic projections are observed. x2000.

The nuclear and nucleolar areas, plotted as frequency histograms for the experimental conditions described in Table I, are shown in Figures 2 and 3. Statistically significant differences were not promoted by heat shock (Table IV). However, highly

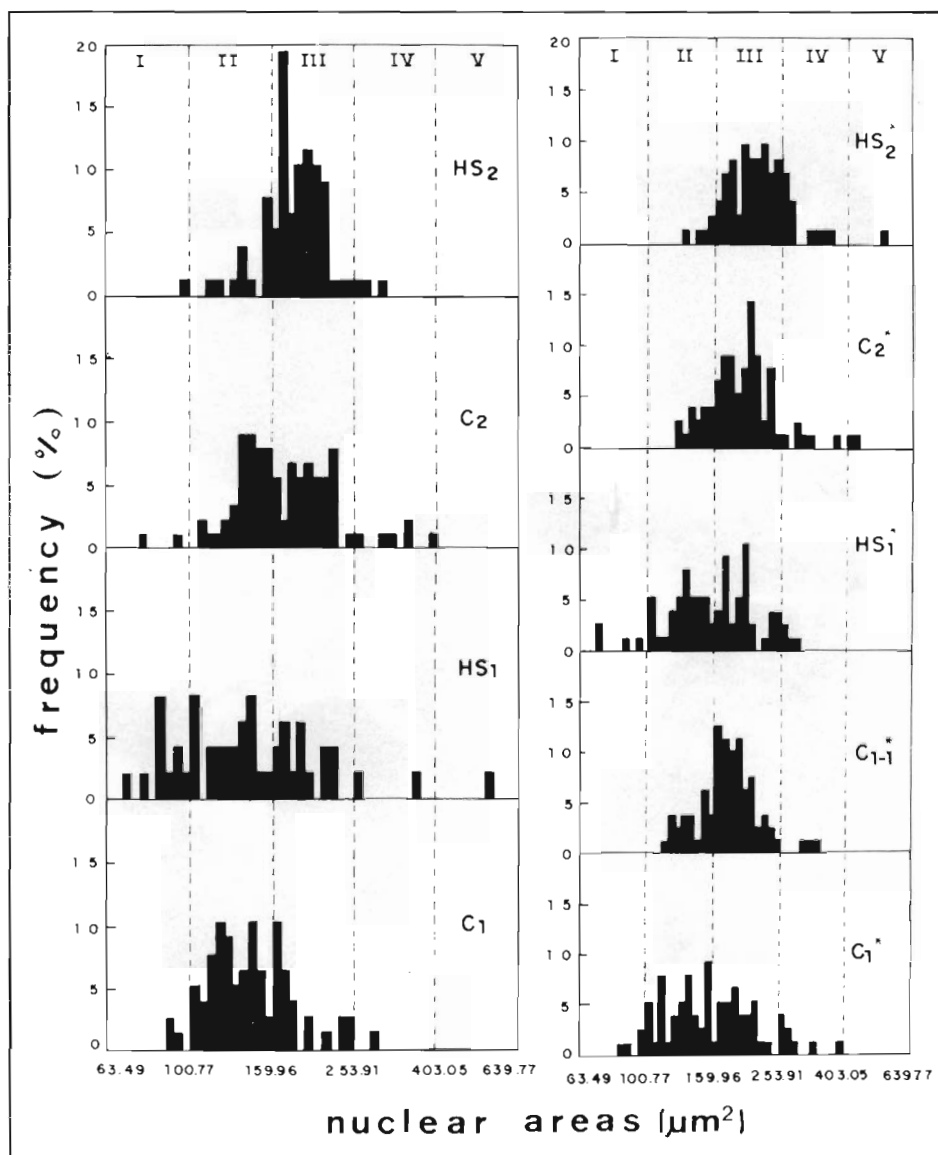


Figure 2 - Frequency histograms for nuclear areas (μm^2) of HeLa cells subjected to heat shocks. I to V: intervals with an increasing ratio of $\sqrt[3]{4}$; n: number of measurements. Abbreviations: see Table I.

significant differences were demonstrated in the shock x recovery comparison for nuclear areas, for the shock at the higher temperature for nucleolar areas (Table IV). The nucleolar

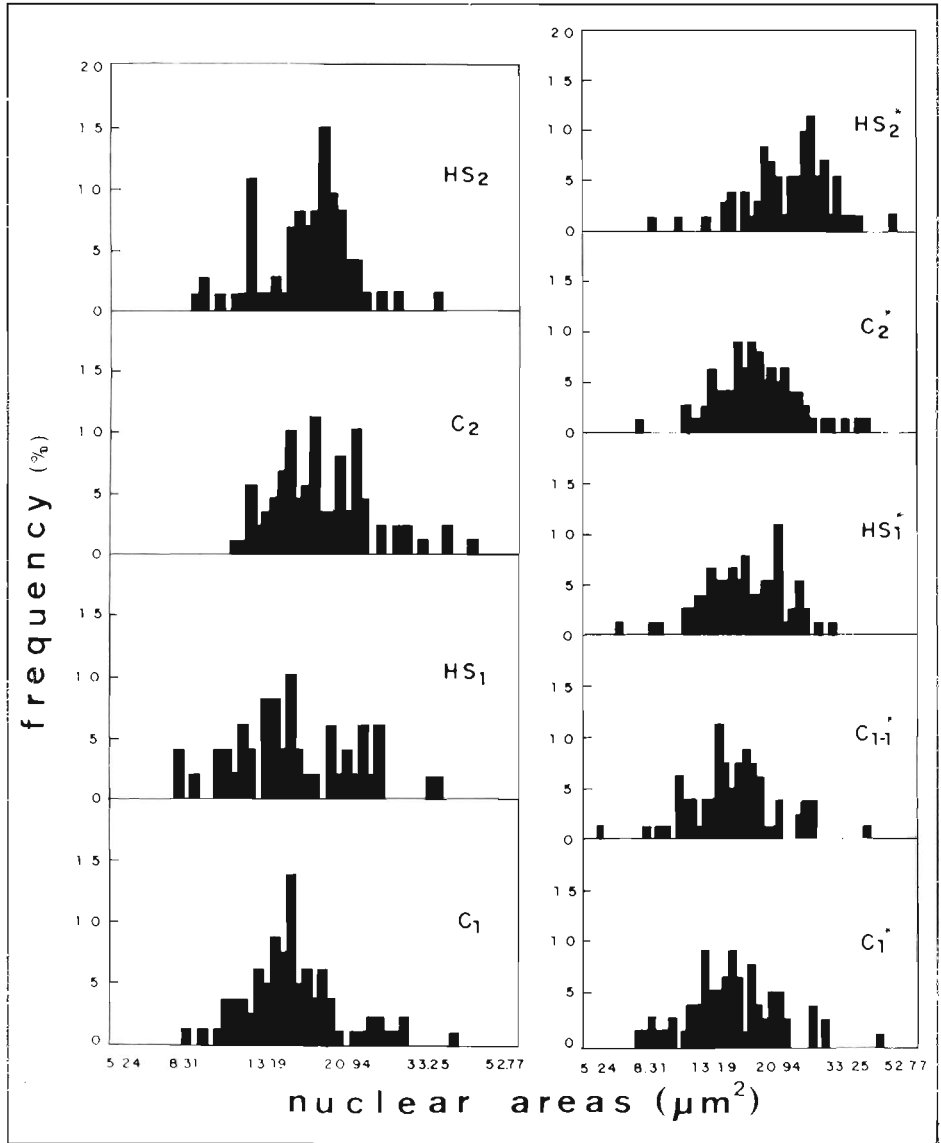


Figure 3 - Frequency histograms for nuclear areas (μm^2) of HeLa cells subjected to heat shocks. n: number of measurements. Abbreviations: see Table I.

areas from preparations recovered after the heat shock at 43°C also differed statistically from the respective control (Table IV).

Table IV - Mann-Whitney and Kruskal-Wallis tests for comparison of nuclear and nucleolar areas of HeLa cells.

Comparisons ¹	Nuclear areas		Nucleolar areas	
	40°C	43°C	40°C	43°C
C1 or C1* x shock	NS	NS	NS	NS
C2 or C2* x recovery	NS	NS	NS	S***
C1 or C1* x C2 or C2*	S***	S***	S#	S*
Shock x recovery	S***	S***	NS	S***
C1 x C1-1*	-	NS	-	NS
C1-1* x shock	-	NS	-	NS
C1-1* x C2*	-	NS	-	S*

¹Abbreviations: see Table I.

NS, not significant ($P < 0.05$).

*, significant ($0.01 < P < 0.05$).

***, significant ($P < 0.001$).

#, significant ($0.01 < P < 0.05$) for Mann-Whitney test and ($0.001 < P < 0.01$) for Kruskal-Wallis test.

Nuclear and nucleolar areas generally increased with advancing cell cultivation time (Figures 2, 3, Table IV). This could explain the above-cited differences between shock x recovery conditions for the nuclear areas, but not for the nucleolar areas (Figure 3, Table IV).

In increase in nucleolar areas during cell proliferation was mainly due to binucleolated nuclei, whereas that observed in HS2* recovered cell nuclei was not associated with any special nuclear phenotype, defined in terms of number of nucleoli.

The relationship between nuclear and nucleolar areas for the various experimental conditions is shown in Figures 4 and 5. In all cases a significantly positive correlation of nucleolar areas on nuclear areas was found.

The correlation and regression parameters of nucleolar areas on nuclear areas for heat-shocked cells were generally different from those for controls and for recovered cells (Table V). This is possibly due to the fact that the largest nucleolar areas of the heat-shocked cells were surpassed by (Figure 5) or exceeded (Figure 4) nucleolar values of control and recovered cells, considering constant nuclear sizes.

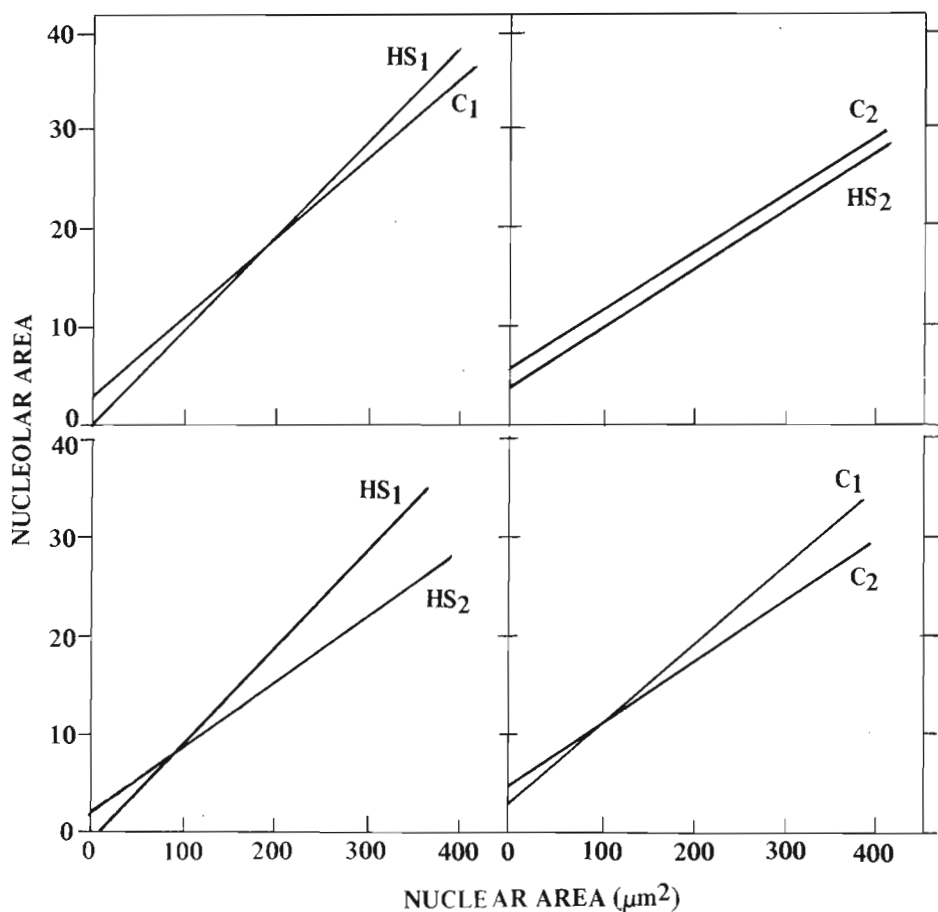


Figure 4 - Relationship between nuclear and nucleolar areas in HeLa cells treated with a heat-shock at 40°C. Abbreviations: see Table I.

DISCUSSION

Some alterations in nuclear and, especially, nucleolar sizes were promoted by heat shock conditions. This was mostly detected during a four hour post-shock period, and was more pronounced when the shock was carried out at 43°C, which is in agreement with reports on general cell ultrastructural changes under similar experimental conditions (Heine *et al.*, 1971).

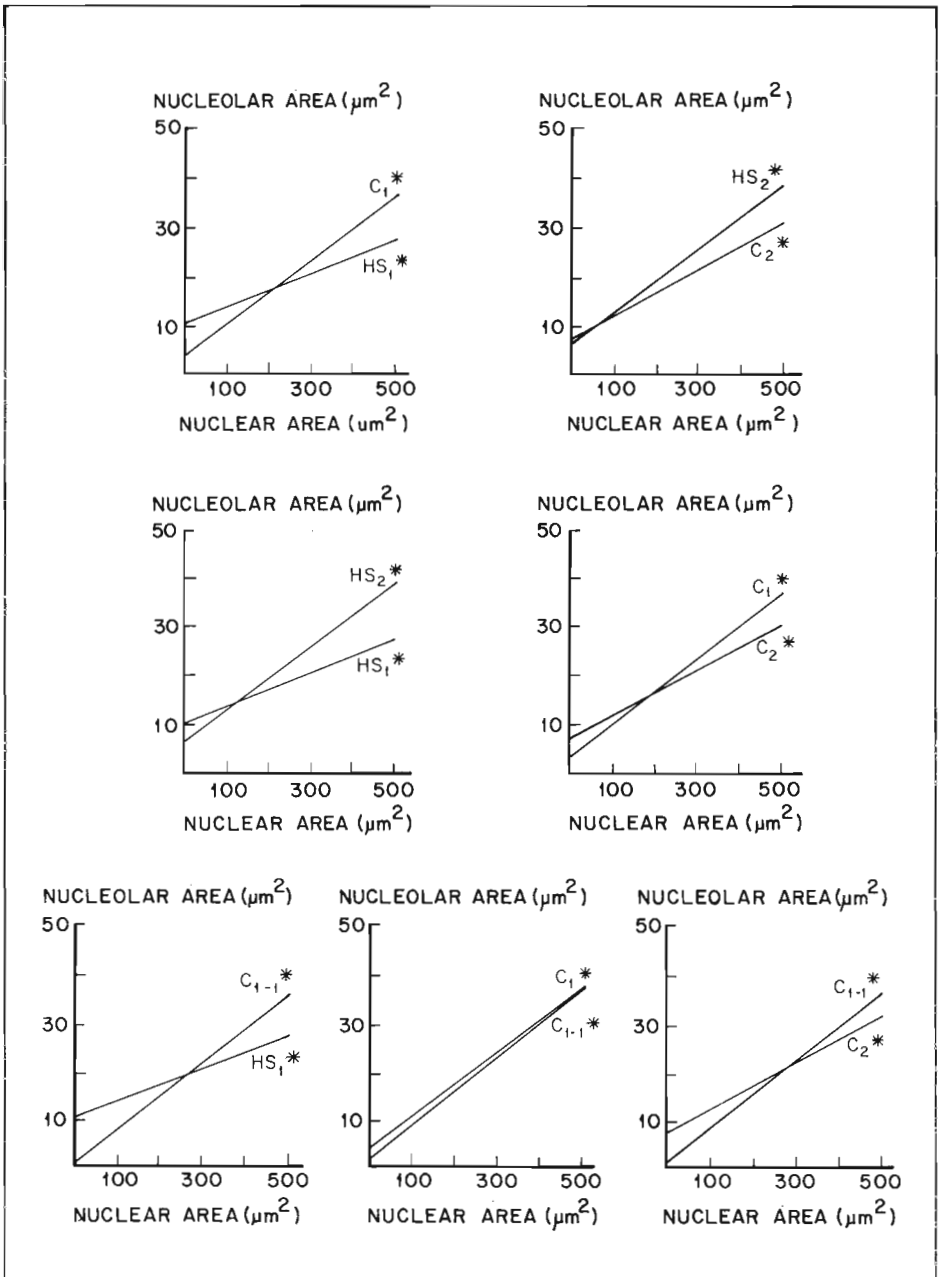


Figure 5 - Relationship between nuclear and nucleolar areas in HeLa cells treated with a heat-shock at 43°C . abbreviations: see Table I.

Table V - Correlation and regression parameters for nucleolar areas on nuclear areas in HeLa cells.

Comparisons ¹	r		Hcight		Angle	
	40°C	43°C	40°C	43°C	40°C	43°C
C1 or C1* x shock	S*	S**	S**	S**	S*	S**
C2 or C2* x recovery	NS	NS	S**	NS	NS	S**
C1 or C1* x C2 or C2*	NS	S*	S**	S**	S**	S**
Shock x recovery	S**	S*	S**	S**	S**	S**
C1 x C1-1*	-	NS	-	S*	-	NS
C1-1* x shock	-	NS	-	S**	-	S**
C1-1* x C2*	-	NS	-	S**	-	S**

¹Abbreviations: see Table I.

NS, not significant ($P < 0.05$).

*, significant ($0.01 < P < 0.05$).

** , significant ($0.001 < P < 0.01$).

The increase in nucleolar areas during the four hour period after the shock at 43°C is probably related to recovery of ordinary cellular activities (McCormick and Penman, 1969; Heine *et al.*, 1971).

Although changes in cell proliferation and cell survival ability were found and an increase in nuclear protein dry mass concentration has also been reported for HeLa cells under the same experimental conditions (Mello and Miranda, in press), no alterations in nuclear sizes sufficient to be detected statistically could be proven immediately after heat shock. Possibly nuclear areas could have been undervalued due to the increased micronucleation which is elicited with shock at 43°C.

The fact that in HeLa cells exposed to heat shock at 43°C the largest nuclear areas were not accompanied by a nucleolar area increase may be due to the presence of karyoskeletal proteins (nuclear matrix-intermediate filament cell fraction (Littlewood *et al.*, 1987)) and the tendency of these protein assemblies to stabilize or become rigid with thermal stress (McConnell *et al.*, 1987). These proteins, which in some way contribute to nuclear size, could, on the other hand, mechanically restrain an increase in size. Interference image analysis has demonstrated an increase in protein dry mass concentration around the nucleoli in HeLa cells exposed to heat shock at 43°C (Mello and Miranda, in press). Furthermore, the same experimental condition induces a partial loss of the granular component of the nucleoli (Heine *et al.*, 1971).

During the four hour post-shock period the aggregation of karyoskeletal proteins originated with the stress is broken down (Mello and Miranda, in press), and the normal characteristics of the cells are recovered by increased transcriptional activity, which was assumed from the rearrangement of the nucleolar components (Heine *et al.*, 1971), and confirmed by present data on nucleolar area increase.

The hypothesis of nucleolar area restraint due to karyoskeletal proteins would also explain the smaller nucleolar sizes than those expected from data from milder (40°C) heat shock treatments. The appearance of putative karyoskeletal proteins in response to the heat shock at 40°C, leading to an increase in nuclear dry mass concentration was found to be elicited later, extending into the four hour post-shock period (Mello and Miranda, in press). In this case, a longer period (Heine *et al.*, 1971) is probably required for full cell recovery.

As regards comparison between C2 or C2* control x recovery, the apparent discrepancy in the statistical significance decision for the parameters considered (height, angle - Table V) may be solved when analyzing Figures 4 and 5. The nucleolar areas in cells recovered from the heat shock at 40°C are smaller than those expected from nuclear areas, based on control data (Figure 4). In cells recovered from the heat shock at 43°C the larger the nuclear area the larger the nucleolar area surpassing value, as compared to control data (Figure 5).

ACKNOWLEDGMENTS

The authors are indebted to Dr. Wirla M.S.C. Tamashiro, Dr. Paulo M.F. Araújo and Miss Dirce L. Gabriel for generously providing HeLa cells.

This investigation was supported by CNPq (grants no. 304691/89-5/GE/FV and 410602/89-2/GE/FV). Miss S.R.P. Miranda was recipient of a fellowship from CAPES.

Publication supported by FAPESP.

RESUMO

As áreas nucleares e nucleolares de células HeLa, obtidas por cariométrica, foram estudadas comparativamente em condições de choque térmico a 40°C e 43°C por 1 h e também 4 h após retorno à temperatura controle (37°C). Foram encontradas algumas alterações induzidas pelo choque, porém detectáveis principalmente durante o período de 4 h pós-choque, relativas principalmente à área nucleolar. Estas alterações, quando devidas ao choque a 43°C, foram consideradas estarem associadas a atividades celulares normais, enquanto aquelas relacionadas ao choque a 40°C foram sugeridas como devidas a proteínas do cariosqueleto, que surgiram tardiamente com o estresse, e que poderiam restringir mecanicamente o aumento em área nucleolar. Estas proteínas também surgem nas células HeLa submetidas ao choque a 43°C e igualmente parecem

estarem comprometidas em uma falta de aumento em área nucleolar, comparativo à área nuclear, contudo são desagregadas no curto período de retorno das células à temperatura controle.

REFERENCES

- Heine, U., Sverak, L., Kondratick, J. and Bonar, R.A. (1971). The behavior of HeLa-S3 cells under the influence of supranormal temperatures. *J. Ultrastruct. Res.* 34: 375-396.
- Littlewood, T.D., Hancock, D.C. and Evan, G.I. (1987). Characterization of a heat shock-induced insoluble complex in the nuclei of cells. *J. Cell Sci.* 88: 65-72.
- McConnell, M., Whalen, A.M., Smith, D.E. and Fisher, P.A. (1987). Heat shock-induced changes in the structural stability of proteinaceous karyoskeletal elements *in vitro* and morphological effects *in situ*. *J. Cell Biol.* 105: 1087-1098.
- McCormick, W. and Penman, S. (1969). Regulation of protein synthesis in HeLa cells: translation at elevated temperatures. *J. Mol. Biol.* 39: 315-333.
- Mello, M.L.S. and Vidal, B.C. (1980). *Práticas de Biologia Celular*. Edgard Blucher, São Paulo.
- Mello, M.L.S. and Miranda, S.R.P. Interference image analysis of heat-shocked HeLa cells. *Acta Histochem.* (in press).
- Ormelles, D.A. and Penman, S. (1990). Prompt heat shock and heat-shifted proteins associated with the nuclear matrix-intermediate filament scaffold in *Drosophila melanogaster* cells. *J. Cell Sci.* 95: 393-404.
- Palkóvits, M. and Fischer, J. (1968). *Karyometric investigations*. Akademiai Kiadó, Budapest.
- Reiter, T. and Penman, S. (1983). "Prompt" heat shock proteins: translationally regulated synthesis of new proteins associated with the nuclear matrix-intermediate filaments as an early response to heat shock. *Proc. Natl. Acad. Sci. USA* 80: 4737-4741.
- Rose, G.G. (1962). The morphological diversity of Gey's strain HeLa after ten years in tissue culture. *Texas Rep. Biol. Med.* 20: 308-337.
- Welch, W.J. and Suhan, J.P. (1985). Morphological study of the mammalian stress response: Characterization of changes in cytoplasmic organelles, cytoskeleton, and nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. *J. Cell Biol.* 101: 1198-1211.

(Received December 12, 1991)