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Long-term starvation effects on *Mus musculus* hepatocyte nuclear phenotypes

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Abstract — Starvation is a physiologic stress and can significantly alter the structure of hepatic cells. This work aims to detect morphological changes in mice hepatocyte under starvation physiologic stress, based on silver staining technique. Fourteen 21 day old male mice *(Mus musculus)* were used, 5 as control, 5 submitted to 72 hours of starvation, and 4 were refed during 72 hours after 72 hours of starvation. After liver imprint, 15 nuclei per mouse and their respective nucleoli were outlined in millimetric paper and their areas were obtained. The results, in mm^2, were transformed into μm^2. The number of nucleoli per nuclei were also counted. After starvation, a statistically significant rise in nuclear and nucleolar areas occurred and no significant increase in the number of nucleoli was observed. The refeeding caused a partial recovery of the nuclear area, no significant change in the nucleolar area and a statistically significant increase in the number of nucleoli. Therefore, starvation can be considered as a modifier agent of the chromatinic structure, leading to an increase of the nuclear and nucleolar areas probably due to an increment of RNA and protein synthesis. The recovery of the stress (re-feeding) did not presented a decrease of nucleolar area and evidenced a nucleoli fragmentation, probably to increase more the protein synthesis and/or due to its cycle during the interphase.

Key words: AgNOR, hepatocytes morphology, nuclear and nucleolar morphom-etry, nucleolus, Refeeding, Starvation.

INTRODUCTION

Starvation results in a total protein, total lipids, blood sugar content, body and liver weight diminution, as well as liver atrophy in rats (KRUSTEV *et al.* 1976). Hepatocyte atrophy is due to a 95% decrease in the membrane space, in which glycogen and endoplasmic reticulum membranes are contained, and to a 5 % depletion of lipid droplets, in which cholesterol and triglycerides are stored (BELLONI *et al.* 1988). Physiologically, nutrient deprivation triggers the continuous turnover of intracellular protein and other macromolecules that regulate cytoplasmic content and provide amino acids for ongoing oxidative and biosynthetic reactions. MORTIMORE *et al.* (1988) detected that the lysosomal system is the final step in the basal degradation of long-lived proteins in the hepatocyte, and a specific class of secondary lysosomes (type A) plays a direct role in its regulation dur ing caloric starvation.

Structurally, at the cellular level, the effects of physiologic stress are expressed by cytoplasmic, nuclear and chromosomal alterations (AMARAL and MELLO 1989; BRITO-GITIRANA and STORCH 1998). Puff formation on polytenic chromosomes or chromatin decompactation in *Rhinchosciara americana* (SIMOES *et al.* 1975; SIMOES and CESTARI 1982), as well as partial decompactation of heterochromatin in tumoral cells (SANDRITTER *et al.* 1974), have evidenced that stressing situations lead cells, or cellular chambers, to modify their structure in response

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to stress. Likewise, under cupreous ions action (MELLO *et al.* 1995) or starvation (MELLO and RAYMUNDO 1980), a partial heterochromatin decompactation of chromatin filaments can occur in *Triatoma infestans* and *Panstrongylus megistus*. However, some nuclei degenerate and break in both situations (ANDRADE and MELLO 1987; MELLO *et al* 1995).

The starvation stress has being studied in many organisms, from protozoa to mammals. At the cellular level it has been demonstrated that this stress causes a partial inhibition in the final ribosome formation, as well as an alteration in the content of DNA in protozoa (GALZONE *et al.* 1983; GALEGO *et al.* 1984). WINICK *et al*. (1978) verified in mouse that starvation, or poor nutrition, stops or decreases the synthesis of DNA once DNA polymerase is no longer synthesized. These alterations in the DNA total content are permanent in brain and kidneys, however they are reversible in liver. At the ultrastructural and molecular levels it was detected that bad nutrition can affect the structure of mice hepatocyte chromatin, generating modifications in nuclear proteins as well as DNA hypomethylation (CASTRO *et al.* 1986).

Therefore, considering that starvation can alter significantly the structure of hepatic cells, the purpose of this work is to detect some of these important alterations in mice under the starvation physiologic stress, based on silver staining technique.

MATERIAL AND METHODS

Fourteen 21 day old male mice *(Mus musculus* — Swiss isogenic line) were used in this work. Five as control, 5 submitted to 72 hours of starvation, and 4 were refed during 72 hours after 72 hours of starvation. Each mouse was isolated in individual cage, with water *ad libitum* and mouse food (control and refeeding treatments only).

After the treatments, the mice were dissected and the livers were removed and washed in physiologic solution. The hepatocyte nuclei were obtained through imprint in a clean slide and the materials were fixed in 3:1 ethanol-acetic acid for 2 minutes. After this period, the materials were rinsed three times in 70% ethanol and hydrated. The hepatocyte nuclei were stained by the method described by Howell and Black (1980). The nuclei were observed in microscope Zeiss-Jenaval and fields containing medium size nuclei (diploid nuclei) were photographed in Kodak-TRI-X-pan film.

Fifteen nuclei per mouse and their respective nucleoli were outlined in millimetric paper (x 3500) and their areas were obtained counting the squares inside the outlines. The results, in mm^2, were transformed into μm^2. The number of nucleoli per nuclei were also counted. An exploratory analysis using frequency histograms was employed. The histograms were constructed in the Microsoft® Excel using a geometric scale with a ratio equal to $\sqrt[3]{4}$ (MELLO and LIMA, 1978). The nuclear and nucleolar areas and also the number of nucleoli were statistically analyzed (ANOVA and Two Sample *t*-test) in the Minitab® Release version 10.1 for Microsoft® Windows.

RESULTS

The hepatocyte AgNOR-staining evidenced, in the three experimental conditions, highly individualized dots or aggregates inside the nuclei (Fig. 1). The areas obtained for the nuclei and their respective nucleoli were used to achieve an exploratory analysis of the alterations after the treatments (Figs. 2 and 3). The control animals (Fig. 2A) showed nuclei with nuclear area ranging from 105.54 μm^2 to 422.11 μm^2, with the medium size being around 175 μm^2. After starvation (Fig. 2B), a displacement of the nuclei frequency to a higher area value was observed, ranging from 152.74 μm^2 to 484.87 μm^2 with the medium size rising to around 300 μm^2. A partial recovery was detected after refeeding as a small return of the displacement to a smaller area value, ranging from 87.73 μm^2 to 462.97, with the medium size being around 250 μm^2 (Fig. 2C).

Almost the same could be observed with the nucleolar area (Fig. 3), that is, the control animals (Fig. 3 A) presented nuclei with nucleolar area ranging from 9.55 μm^2 to 55.27 μm^2, with the medium size being around 23 μm^2. After starvation (Fig. 3B), a displacement of the nuclei frequency to a higher area value was observed (medium size rising to around 44 μm^2), although almost the same range was observed (from 9.55 μm^2 to 63.49 μm^2). However, there was no return to a smaller area value after re-feeding (Fig. 3C), although a flattening of the peaks and an increasing in the area intervals were observed (from 5,74 μm^2 up to 63,49 μm^2).

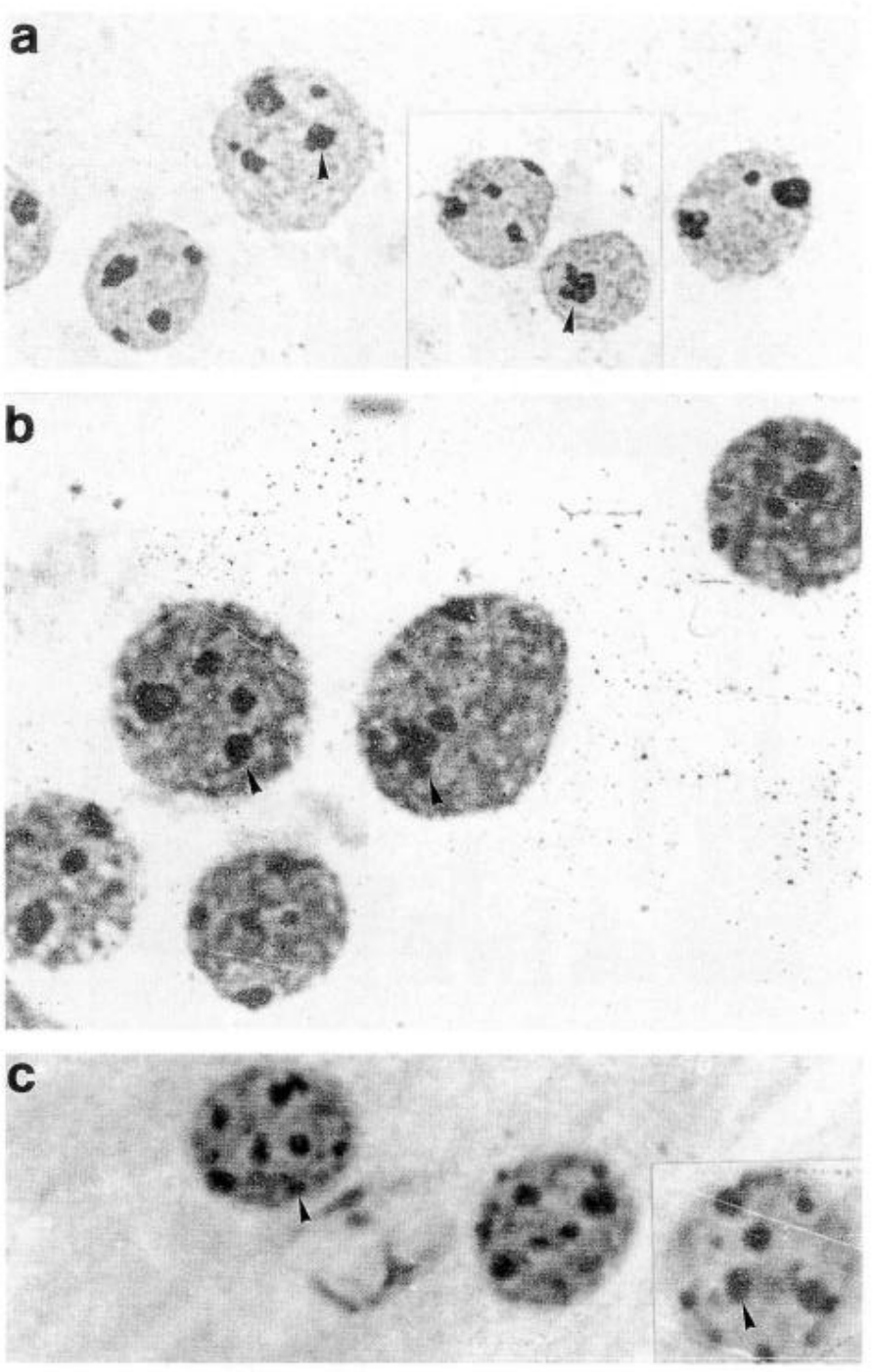

Fig. 1 — AgNOR-stainedhepatocytes nuclei. A. Control; B. Starvation; CRefeeding. AgNOR dots are evidenced by the arrows x3500.

The changes in the nuclear and nucleolar areas, and in the number of nucleoli obtained for each mouse were statistically analyzed (Tables 1 and 2). The ANOVA test analysis (Table 1) showed that the total alteration occurred in the nuclear area ($F = 15.35$, $p<0.01$), the nucleolar area ($F = 17.75$, $p<0.01$) and the number of nucleoli ($F = 8.93$, $p<0.01$) after all the treatments were statistically significant. The *t*-test (Table 2) showed that the nuclear area presented a significant change between control and starvation (t - -6.61, $p<0.01$) only. The nucleolar area+.

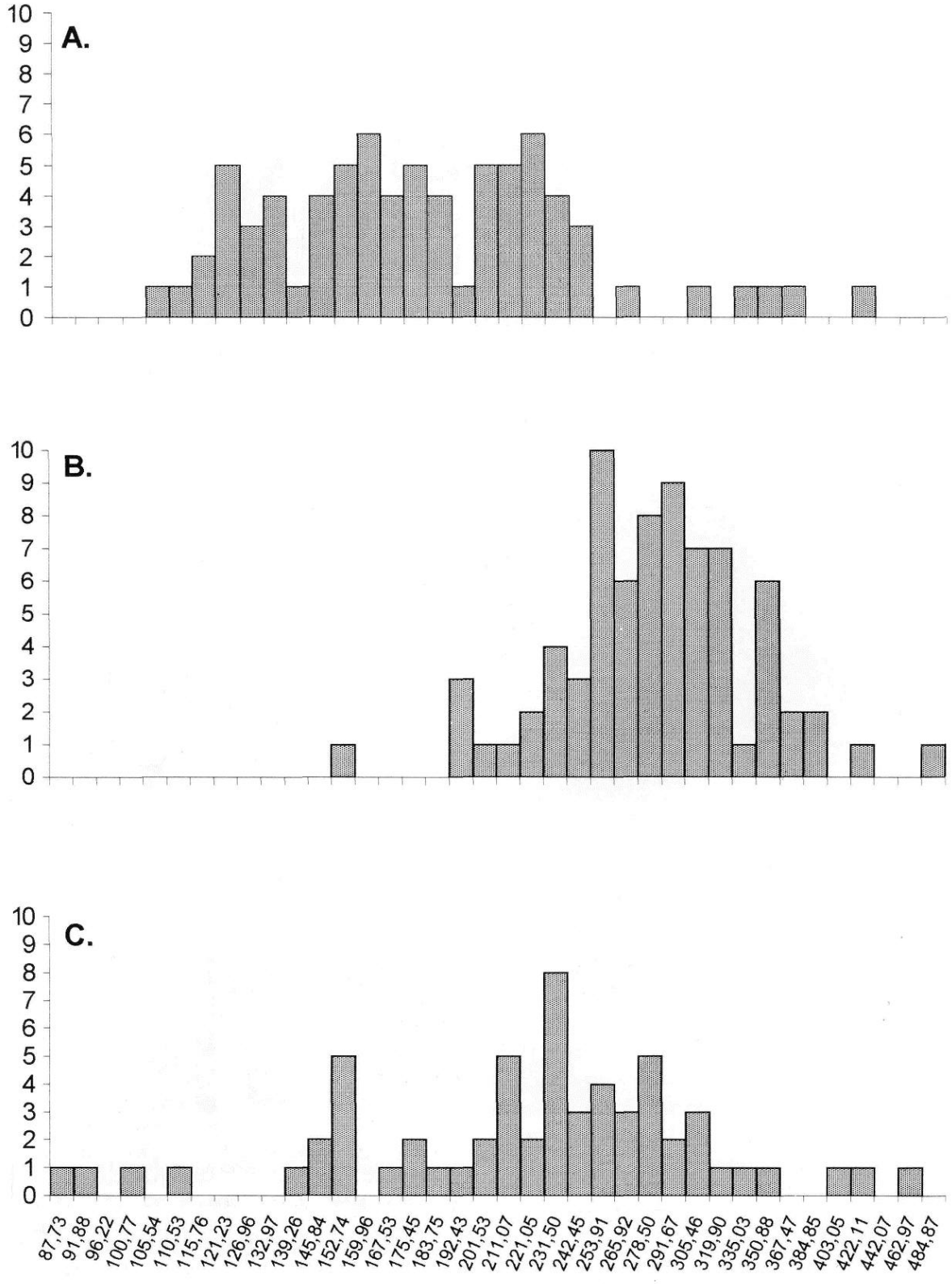

Fig. 2 — Frequency histograms of nuclear area of mice hepatocyte: A) Control; B) Starvation; and C) Refeeding. The data were distributed on a geometric scale with a ratio equal to $\sqrt[3]{4}$ (MELLO and LIMA 1978).

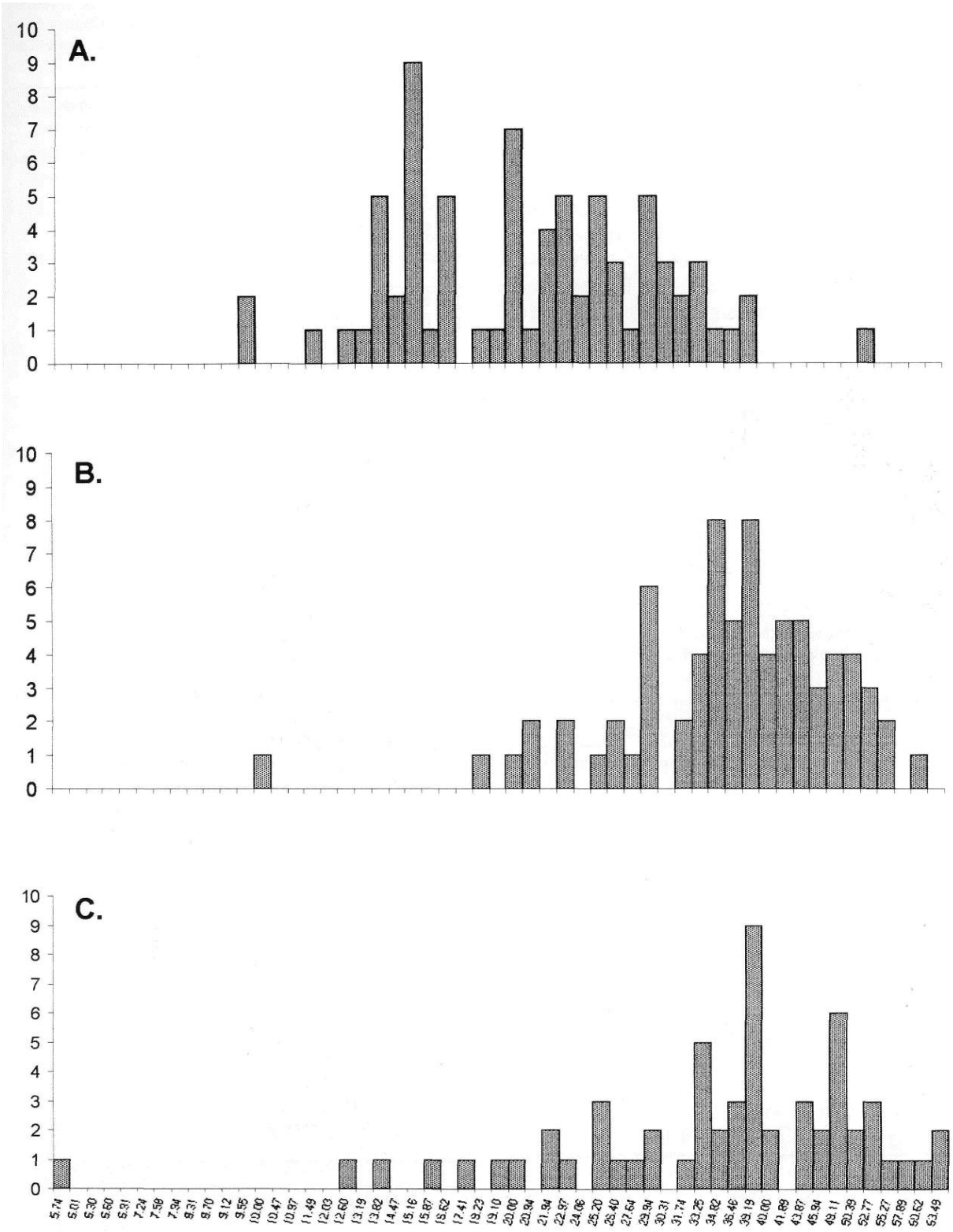

Fig. 3 — Frequency histograms of nucleolar area of mice hepatocyte: A) Control; B) Starvation; and C) Refeeding. The data were distributed on a geometric scale with a ratio equal to $\sqrt[3]{4}$ (MELLO and LIMA 1978).

Table 1A — ANOVA (Minitab) for the nuclear area (m^2) of hepatocytes of the mouse *Mus musculus* in the Control, Starvation and Refeeding conditions, x ± SD = mean ± standard deviation.

Animal	Control	Starvation	Refeeding
1	234.50 ± 15.30	284.40 ± 18.90	238.80 ± 7.20
2	176.30 ± 11.10	285.20 ± 5.30	258.50 ± 17.00
3	181.40 ± 12.60	286.50 ± 16.00	238.10 ± 24.00
4	173.40 ± 18.40	276.70 ± 15.20	171.80 ± 16.90
5	144.00 ± 8.30	271.30 ± 12.00	—
(χ ± SD)	181.92 ± 32.81	280.82 ± 6.55	226.80 ± 37.87

F = 15.35
p < 0.01

Individual 95% CIs for mean based on pooled SD

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(----*-----)
                (----*-----)
        (-----*------)
---------+---------+---------+------
       200       250       300
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Pooled SD = 28.25

Table IB — ANOVA (Minitab) for the nucleolar area (m^2) of hepatocytes of the mouse *Mus musculus* in the Control, Starvation and Refeeding conditions, x ± SD = mean ± standard deviation.

Animal	Control	Starvation	Refeeding
1	27.72 ± 1.55	36.03 ± 2.56	42.24 ± 2.11
2	19.00 ± 1.36	39.30 ± 2.80	41.89 ± 1.81
3	23.05 ± 2.21	40.50 ± 2.60	33.80 ± 4.00
4	20.90 ± 2.70	37.68 ± 1.96	29.10 ± 3.40
5	18.76 ± 1.18	31.72 ± 1.87	-
(χ ± SD)	21.89 ± 3.69	37.05 ± 3.42	36.76 ± 6.42

F = 17.75
p < 0.01

Individual 95% CIs for mean based on pooled SD

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  (-----*------)
                        (-----*-----)
                        (------*------)
--------+-----------+-----------+-----------+
     21.0        28.0        35.0        42.0
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Pooled SD = 4.523

presented significant changes between control and starvation (t = -6.74, p<0.01) and control and refeeding (t = -4.12, p<0.05). The number of nucleoli showed a significant change only between control and refeeding (t = -4.18, p<0.01).

Table 1C — ANOVA (Minitab) for the number of nucleolus of hepatocytes of the mouse *Mus musculus* in the Control, Starvation and Refeeding conditions, x ± SD = mean ± standard deviation.

Animal	Control	Starvation	Refeeding
1	4.27 ± 0.37	3.20 ± 0.28	4.40 ± 0.47
2	3.53 ± 0.31	4.53 ± 0.38	5.93 ± 0.49
3	3.40 ± 0.29	4.73 ± 0.28	5.07 ± 0.50
4	2.93 ± 0.25	4.33 ± 0.29	5.33 ± 0.55
5	3.73 ± 0.18	4.20 ± 0.20	-
(χ ± SD)	3.57 ± 0.49	4.20 ± 0.59	5.18 ± 0.63

F = 8.93
p < 0.01

Individual 95% CIs for mean based on pooled SD

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--------+-----------+-----------+-----------+-----
    (--------*--------)
          (--------*--------)
                    (----------*----------)
--------+-----------+-----------+-----------+-----
     3.20        4.00        4.80        5.60
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Pooled SD = 0.5695

DISCUSSION

Hepatocytes are cells with a highly uniform morphology and can exhibit one or two nuclei per cell. Entire nuclei with variable size can be obtained from liver imprint technique. These nuclei are from hepatocytes located in the portal-terminal vein axis, where they can be assigned for proliferation and/or quiescent compartment and can undergo differentiation and aging (ZAJICEK 1992).The variability is expected in normal conditions and is due to the correlation between nuclear area and ploidy (HINTZSCHE 1945).

The nucleolus is an interphasic nucleus compartment with its own cytochemical characteristic, property that allow its individualization and morphometry. Due to two acidic proteins which are highly argyrophilic, it can be clearly and rapidly individualized and evidenced by silver nitrate staining procedures (GOODPASTURE and BLOOM 1975; CROCKER *et al.* 1989). These two proteins were identified quite recently (ROUSSEL *et al.* 1992; ROUSSEL and HERNANDEZ-VERDUN 1994) and they are nucleolin, a phosphoprotein of 105 kDa that plays an important role in the transcription of rRNA molecules, and nucleophosmin (or B23 protein), a

Table 2 — Pairwise comparisons using the Two Sample Student *t*-test.

		Control	Starvation
Nuclear Area	Starvation	-6.61**	-
	Refeeding	-1.87	2.82
Nucleolar Area	Starvation	-6.74**	-
	Refeeding	-4.12*	0.08
Number of Nucleolus	Starvation	-1.82	-
	Refeeding	-4.18**	-2.38

* — $p < 0.05$; ** — $p < 0.01$.

phosphoprotein of 38-39 kDa that is engaged in the late steps of pre-ribosomal particle organization (DERENZINI 2000). Several works use Ag-NOR-staining as a tool for detection of alterations in the cell cycle caused by physiologic stress and cell proliferation in tumors (SCHWINT *et al.* 1996; KOBAYASHI *et al* 2000; TANNAPFEL *et al* 2000).

The number and size of interphase NORs are highly variable within the nuclei, according to rRNA transcriptional activity. Nuclei of a cell with a very low level of ribosome biogenesis (such as those of resting lymphocytes) are characterized by a large solitary interphase NOR, whereas nucleoli of activated cells (such as those of phytohemagglutinin-stimulated lymphocytes) exhibit a great number of small interphase NORs (DERENZINI and PLOTON 1991). The area occupied by the silver-stained interphase NORs within the nucleoli is related to the total nucleolar area and to the level of ribosome biogenesis. Therefore, the evaluation by image analysis of the area occupied by the silver-stained interphase NORs within the nucleoli permits to obtain precise information on the nucleolar size and function in routine cyto-histopathological samples (DERENZINI *et al.* 1998).

Our results evidenced that, in starvation stress, there were visual and statistical increase in the nuclear and nucleolar areas and no correspondent statistical significant increase in the number of nucleoli (Figs. 2 and 3; Tables 1 and 2), This indicates an increase in the demand of the synthesis of nuclear transcribed elements, mainly rRNA of the nucleolar regions (AMARAL and MELLO 1989). In mice, a high correlation between nuclear and AgNOR-stained area has been revealed to be much higher than the correlation between nuclear area and the number of AgNOR positive points or aggregates (VIDAL *et al.* 1994). Total nucleolar area increase in starvation could be indicative of an increase in the ribossome synthesis and consequently of proteins synthesis. These proteins might be involved in the cell protection as a response to the stress, or they could be Heat Shock Proteins (HSP) (ATKINSON and WALDEN, 1985; AMARAL and MELLO 1989).

After refeeding, the observed response of the nuclei and nucleoli was different, that is, nuclear area had a partial recovery to normal size (difference between control and refeeding areas is not statistically significant — Table 2) while nucleolar area showed no recovery (difference between starvation and refeeding is not statistically significant — Table 2). This indicates that the recovery of stress can be different in the cell compartments, comparing their areas individually. However, the nucleoli number showed a statistical significant increase in the AgNOR-stained dots and aggregates between control and refeeding (Table 2). This shows that the nucleoli suffered a fragmentation after refeeding, what could be a response to increase the nucleoli contact area with the nuclear surrounding, intensifying the protein synthesis, or due to its fragmentation in the interphase during the cell cycle (ALBERTS *et al.* 1994).

Therefore, we concluded that starvation is a modifier agent of the chromatinic structure, fact that could be observed by area measurements. Starvation leads to an increase of the nuclear and nucleolar areas, promoting an increment of RNA synthesis. The recovery of the stress (refeeding) did not demonstrate a decrease of nucleolar area and evidenced a nucleolus fragmentation and an increase in the area class intervals. This indicates another increase of the protein synthesis or could be due to its fragmentation in the cell cycle interphase.

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