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Nuclear phenotypes and DNA fragmentation in tendon fibroblasts of NOD mice

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Abstract — Changes in DNA/chromatin structure, ploidy degrees and cell death possibly caused by oxidative stress during the insulin-dependent diabetes have been reported for different cell types. However, all these studies have been carried in streptozotocin-induced diabetic rats or mice and showed contradictory results. In this work, nuclear phenotypes and DNA fragmentation were investigated in fibroblasts from mice spontaneously developing insulin-dependent diabetes (NOD) and compared with healthy (BALB/C) mice. Geometric, densitometric and textural parameters obtained for Feulgen-stained nuclei by image analysis were used to define nuclear phenotypes. Significant differences were observed for nuclear sizes and for densitometric and textural parameters of the tendon nuclei. Optical density, Feulgen-DNA values, transmittance variability per nucleus and nuclear entropy values were significantly higher in the NOD mice. The Feulgen-DNA amounts for the NOD and BALB/C mice were found to be distributed into several doubling Feulgen-DNA classes. The frequency of nuclei with the smallest Feulgen-DNA amounts, which may represent DNA fragmentation and loss, was lower in fibroblasts of the NOD mice (2.3%) in comparison to the BALB/C mice (38%). In contrast, the frequency of polyploid nuclei in NOD mice was higher (24.5%) than that in BALB/C mice (1.9%). Based on optical density, transmittance variability per nucleus, and nuclear entropy data, a larger contrast between highly and less densely packed states, was demonstrated for the fibroblasts of the NOD mice. Maybe the deeper condensation of the highly packed chromatin evident in NOD fibroblasts is related to silencing of some genes involved with changes in tendon supraorganization with the diabetes.

Key words: cell death; diabetes; DNA content; fibroblasts; NOD mice; nuclear phenotype.

INTRODUCTION

Image analysis of Feulgen-stained nuclei using geometric, densitometric and textural parameters can be considered a sensitive probe for the evaluation of changes in chromatin supraorganization in different cell types under various physiological and pathological conditions (Mello 1989, 1999; Mello and Russo 1990; Mello *et al.* 1995; Vidal *et al.* 1998; Maria *et al.* 2000; Moraes *et al.* 2005). Alterations in nuclear phenotype characteristics including patterns of chromatin supraorganization, induced by stressful conditions such as thermal shocks, exposure to heavy metals, gamma radiation, viral infection, tumorigenesis, starvation and refeeding have been detected by several authors, using the Feulgen reaction. Ploidy degrees, apoptotic indices, relationship between cell cycle and programmed cell death (PCD), and DNA content during apoptosis/PCD can also be assessed by image analysis of Feulgen-stained preparations (Camby *et al.* 1995; Wallet *et al.* 1996; Vidal *et al.* 1998; Maria *et al.* 2000; Mello 2005). Image analysis of Feulgen-stained preparations, in comparison with results obtained by the TUNEL assay, can detect early PCD and/or apoptosis stages by associating slight changes in nuclear phenotypes with a decrease in DNA content due to chromatin fragmentation or loss (Maria *et al.* 2000).

Changes in DNA/chromatin structure and cell death caused by oxidative stress during the insulin-dependent diabetes have been reported for different cell types (Mooradin 1992; Herrman *et al.* 1999; Frustaci *et al.* 2000). Insulin-dependent diabetes is an endocrine disorder characterized by hyperglycemia due to an absolute or relative deficiency in insulin (Ahmed 2005). This diabetes

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form results from a genetically and immunologically complex autoimmune process that is specifically directed against the pancreatic beta cells (AHMED 2005). Actually, the diabetes affects 1-2% of the population worldwide (AHMED 2005).

Recent studies have reported that insulin-dependent diabetes affects the cell metabolism, causing alterations in various aspects of the cell cycle, including hypertrophy, hyperplasia and apoptosis, three basic aspects of tissue growth (HERRMAN *et al.* 1999; AHMED 2005). Diabetes also affects apoptotic ratios and ploidy degrees of several cell types like hepatocytes, pericytes, myocytes and endothelial cells (HERRMAN *et al.* 1999; FRUSTACI *et al*. 2000). Polyploidization has been detected in diabetic vascular smooth muscle cells from mesenteric arteries (VRANES *et al.* 1999). In diabetic livers, there is morphological evidence that cell death can be decreased by 23% to 76%, while in myocardial cells the diabetes activated cell death. Additionally, flow cytometry has indicated a 613% increase in diploid hepatocytes in the livers of streptozotocin-induced diabetic rats compared to control (DOI *et al.* 1997; HERRMAN *et al*. 1999).

In face of these reports, it is clear that many studies involving diabetic animals are still required. In this context, non-obese diabetic (NOD) mice spontaneously developing a form of autoimmune diabetes, closely resembling the disease in humans (YANG and SANTAMARIA 2003), should be a useful model. Like human diabetic patients, NOD mice have an unfortunate combination of apparently normal alleles at numerous recessive *loci* associated with insulin-dependent diabetes (COLUCCI *et al.* 1997; YANG and SANTAMARIA 2003). Each of these allelic variants shows a small degree of susceptibility to the mentioned disease (YANG and SANTAMARIA 2003). Additionally, alterations in the PCD mechanisms in the immune system may contribute to the development of diabetes (COLUCCI *et al.* 1997). The percentage of apoptotic cells in the immune system of NOD mice is 4.5 – 18%, compared to 28 – 61.7% in healthy mice (COLUCCI *et al.* 1997). NOD mice are resistant to various agents, including cyclophosphamide, that are capable of inducing apoptosis in other mouse strains (COLUCCI *et al.* 1997). However, it is not known whether the high resistance to apoptosis observed in the immune cells of NOD mice is restricted to these cells or instead is a general feature for cells and tissues of mice affected by the mentioned type of diabetes.

In this work we investigated whether the insulin-dependent diabetes affects the nuclear phenotype characteristics of fibroblasts of tail tendons in NOD mice as assessed by image analysis, in comparison to results for fibroblasts from non-diabetic mice. DNA fragmentation, which is typical for apoptosis, was studied by image analysis and the TUNEL assay.

MATERIALS AND METHODS

Animals - Ten female NOD^+ mice (16 weeks old and 28 days of diabetes expression) and ten female BALB/C mice (controls) of the same age were obtained from the Multidisciplinary Center of Biological Investigation (CEMIB) of the State University of Campinas, SP, Brazil. The mice were killed by decapitation and their tails were removed. All the protocols involving animal care and use were approved by the Committee for Ethics in Animal Experimentation of the State University of Campinas and met the Guidelines of the Canadian Council on Animal Care.

Tails of five animals of each mouse strain were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.2, under vacuum, for 2 h. Then, the skin was removed and the tendons were detached from muscles and bone. The tendons were fixed for more 24 h, rinsed in water, processed for routine embedding in histosec® (Merck, Darmstadt, Germany), and sectioned at 8 μm thickness. These preparations were used for the study of the Feulgen staining and responses to the TUNEL assay. Tendons of other five animals of each mouse strain were fixed in absolute ethanol – acetic acid (3:1, v/v), rinsed in 80% ethanol, and subjected to the Feulgen reaction *en bloc*.

Feulgen reaction - Sections subjected to the Feulgen reaction were previously treated with 0.5% sodium borohydrate for 7 min for removal of aldehyde groups thus created during fixation with paraformaldehyde.

Histological sections and tendons *en bloc* were hydrolyzed in 4 M HCl for 90 min at 25°C and then rapidly rinsed with cold 0.1 M HCl to stop the hydrolysis. The tendons *en bloc* were subsequently dissociated and squashed between a slide and a coverslip and then frozen in liquid nitrogen for removal of the coverslip.

The hydrolyzed material was treated with Schiff reagent for 40 min in the dark, rinsed three times (5 min each) in sulfurous water and once in

distilled water, air dried, and mounted in natural Canada balsam (n_D = 1.54).

The histological sections were used to define nuclear phenotypes, including those assumed to represent stages of apoptosis (MARIA *et al.,* 2000), by image analysis. The tendon squashes were used for general morphological observations at the light microscope; these were not used for image analysis since many nuclei appeared piled up.

Video image analysis - The Feulgen-stained nuclei were analyzed using Carl Zeiss Kontron equipment and Kontron KS 400 software (Oberkochen/München, Germany).

The microscope images were obtained with a Zeiss Axiophot 2 microscope equipped with a Neofluar 40/0.75 objective, 0.90 condenser and λ = 546 nm. The images were captured with a Sony CCD-IRIS/RGB Hyper HAD color video camera and transmitted to a Pentium computer. Under these conditions, 1 μm corresponded to 7.23 pixels. The minimum area possible to be measured in this apparatus corresponded to four pixels. The software provided quantitative information on nuclear area (μm^2), nuclear perimeter (μm), feret ratio (minimum feret/maximum feret as an indication of the degree of ellipticity of the nuclei), optical density (OD, absorbance), integrated optical density (IOD or, in this case, Feulgen-DNA values), and textural parameters like SDtd (standard deviation of the grey average, in pixels, per nucleus or transmittance variability per nucleus) and entropy (E = k. $\log_2$n, or number of bytes needed to save the densitometric values per nucleus, in pixels). The number of nuclei analyzed was 310 for BALB/C and 129 for NOD mice.

TUNEL assay - The fibroblasts were examined for DNA fragmentation using the TUNEL POD assay, as described by the manufacturer (Roche/Amersham). Since UTP was labeled with fluorescein, anti-fluorescein antibodies coupled to peroxidase were incubated with 3-3'-diaminobenzidine (Fluka) for 10 min at room temperature (25°C). Counter-staining was done with methyl green (Merck), followed by rinsing in distilled water. The preparations were then air dried and mounted in natural Canada balsam. Endogenous peroxidase was blocked with 3% H_2O_2 in absolute methanol for 30 min at room temperature. A negative control was done by excluding the TdT enzyme from the reaction. As positive controls, preparations incubated with DNase I (3000 U/mL in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ 1 mg/mL BSA, for 10 min at 25°C), HL60 promyelocytic leukemia cells incubated with actinomycin D for 19 h, for induction of apoptosis, and Vero cells cultured for 72 h were used. At the mentioned growth time many Vero cells become confluent, activating their PCD mechanisms (DINI *et al.,* 1996). The TUNEL assay was not studied in tendon squashes since rupture of collagen bundles, consequently promoting a positive peroxidase response is elicited in this case.

Statistical analysis - All calculations and statistical analyses were done using the Minitab 12^{TM} software (State College, PA, USA) and involved analysis of variance (ANOVA), regression plots and the Mann-Whitney non-parametric test. Differences were considered as statistically significant for P-values

RESULTS

No general morphological differences were apparently observed for the Feulgen-stained tendon fibroblast nuclei of NOD mice compared to the BALB/C ones. The fibroblast nuclei were very elongated and thin, and distributed all along the tendons (Fig.1 a-b). Morphological differences were neither found for nuclei of histological sections compared to those in squash preparations (Fig. 1 c-e). However, higher values for nuclear geometric parameters were found in the NOD mice (Table 1). The nuclear areas and perimeters of NOD and BALB/C mice were found to differ significantly ($P_{0.000}$ level). No significant difference was found for the feret ratio of the mouse strains compared here (Table 1; $P_{0.05}$ level).

Significant differences were also observed for the densitometric and textural parameters of the tendon nuclei after NOD vs. BALB/C comparisons. OD (for nuclear populations), IOD, SDtd (for individual nuclei) and entropy values for the NOD mice were significantly higher than those for the BALB/C mice (Table 2; $P_{0.000}$ level). When the correlation between SDtd and entropy was expressed as a quadratic regression plot (Minitab 12^{TM} software), it was smaller for NOD mice (R^2 = 85.5%) than for control mice (R^2 = 97.6%). Since the regression analysis is used to investigate the relationship between the response variable (SDtd) and the predictor (entropy), the quadratic regression plot appears to provide a good fit to these data, and their visual inspection reveals that the data are evenly spread on the regression line, implying in a non-systematic lack of fit. The NOD mice showed textural parameters

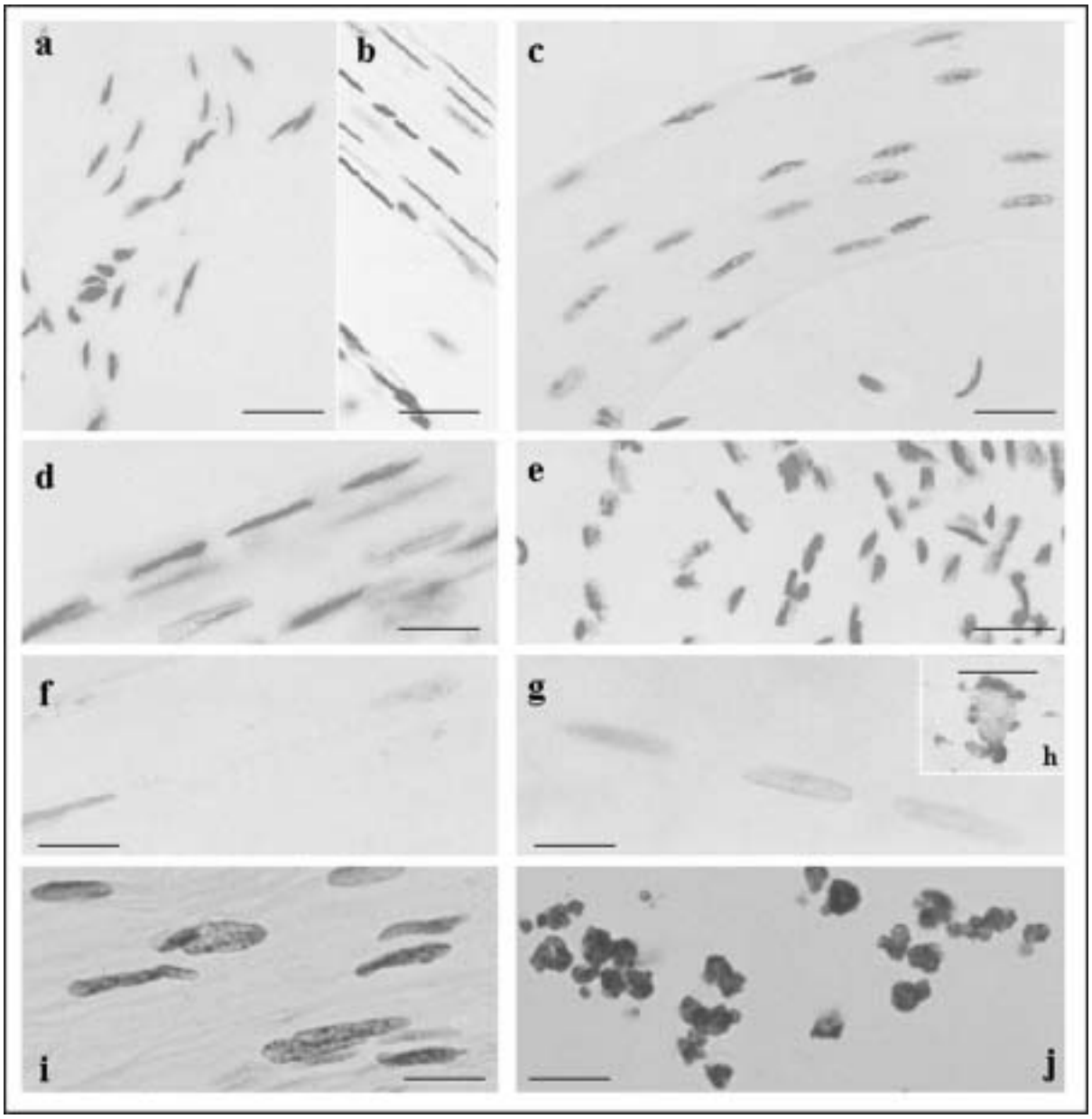

Fig. 1 — Feulgen-stained fibroblast nuclei (a, c, e: BALB/C mice; b, d: NOD mice) and fibroblasts (f: BALB/C mouse; g: NOD mouse) and Vero cell (h) subjected to the TUNEL assay. Mouse fibroblasts (i) subjected to treatment with DNase, and actinomycin D-treated HL60 cells (j) were also positive controls for the TUNEL assay. a, b f, g, i histological sections; c-e: tendon squashes. Bar: a,c,e = 25 μm; b = 100 μm; d, f, g, h, I, j: 10 μm.

Table 1 — Analysis of nuclear geometric parameters of the Feulgen-stained mouse tendon fibroblasts.

Mice	N	Area (μm2)			Perimeter (μm)			Feret ratio		
		$\overline{X}$	SD	η	$\overline{X}$	SD	η	$\overline{X}$	SD	η
BALB/C	310	22.75*	12.76	23.04	31.38*	12.51	29.79	0.19	0.07	0.18
NOD	129	28.55*	10.80	26.40	35.50*	10.99	33.46	0.22	0.11	0.18

* Statistically significant differences ($P_{0.01}$)
N = number of nuclei measured; SD = standard deviation; $\overline{X}$ = *mean;* η = median

Table 2 — Analysis of nuclear densitometric and textural parameters of the Feulgen-stained mouse tendon fibroblasts.

Mice	N	OD			IOD			SDtd			Entropy		
		$\overline{X}$	SD	η	$\overline{X}$	SD	η	$\overline{X}$	SD	η	$\overline{X}$	SD	η
BALB/C	310	0.32*	0.05	0.32	7.48*	4.56	7.10	15.56*	5.51	14.72	5.64*	0.47	5.66
NOD	129	0.49*	0.14	0.46	13.68*	4.72	12.96	22.38*	7.92	22.14	6.10*	0.50	6.17

* Statistically significant differences ($P_{0.01}$)
IOD = integrated optical density (Feulgen-DNA values); N = number of nuclei measured; OD = optical density; SD = standard deviation; SDtd = standard deviation grey averages values per nucleus; $\overline{X}$ = mean; η = median

more spread on the regression line when compared to the control mice (Fig. 2).

When nuclear areas were plotted against Feulgen-DNA values (IOD) (Marginal Plot, Minitab 12™ software), four subgroups of nuclei for BALB/C and NOD mice as regards doubling IOD values were distinguished: 1-5, 5.1-10, 10.1-20 and >20 IOD values (Fig. 3). As the number of nuclei measured by image analysis were not the same for the samples studied, the total number of nuclei for each Feulgen-DNA discriminated subgroup was expressed as percentages, in order for the data to become more consistent. The percentages of nuclei with 1-5 (2.3%) and 5.1-10 (17.8%) IOD values in NOD mice were significantly lower than in BALB/C mice (38% and 35.5%, respectively). In addition, the percentages of nuclei with 10.1-20 (69.8%) and >20 (10%) IOD values in NOD mice were much higher than in control mice (24.5% and 1.9%, respectively). In NOD mice a significant positive correlation between the nuclear area and IOD (R^2 = 53.6%) was smaller than that for control mice (R^2 = 89.2%). This correlation was expressed as a regression plot. Statistical analysis of the IOD values, after sorting data manipulation (Minitab 12™ software), revealed that the percentages of nuclei with 1-2.5 IOD values no significantly differ for the mouse strains compared here (0.77% for NOD mice and 0.80% for BALB mice).

DNA fragmentation typical of PCD and/or apoptosis was not detected by the TUNEL assay in NOD and BALB/C mice (Fig. 1 f-g); a positive response was obtained in Vero cells cultured by 72 h (Fig. 1 h), in positive control fibroblasts (Fig. 1 i), and in the apoptotic HL60 cells (Fig. 1 j).

DISCUSSION

Present results revealed differences in nuclear phenotypic characteristics of fibroblasts in tails of NOD mice in comparison to BALB/C mice, as assessed by image analysis, and considering geometric, densitometric and textural parameters.

The analysis of the geometric parameters lead us to conclude that nuclei of the fibroblasts of the NOD mice were larger than those of the BALB/C control, although not differing in their ellipsoidal shape (feret ratio not significantly different at the $P_{0.05}$ level).

When considering the distribution of IOD values (Feulgen-DNA amounts) in terms of doubling classes, four classes of values could be discriminated for the fibroblasts of NOD and BALB/C mice. As regards the class which contained the smallest Feulgen-DNA values (1-5 IOD values), a very low frequency of fibroblast nuclei was contributed by the NOD mice (2.3%) in comparison with the frequency detected for the BALB/C mice (38%). This class is assumed to contain nuclei with a certain DNA fragmentation and subsequent loss of fragmented material, as induced by apoptosis (Vidal *et al.,* 1998; Maria *et al.,* 2000), or because the apurinic acid produced is more susceptible to be released from the cell nuclei during hydrolysis pertinent to the Feulgen reaction, due to their less packed chromatin states (Mello and Vidal 1980; Mello 1997; Miyamoto *et al.*, 2005).

Whatever explanation for the DNA/apurinic acid fragmentation will be more adequate in the present case, it should not overlook the fact that this fragmentation is less frequent in the nuclei of fibroblasts of the NOD mice. Apoptosis is a phenomenon of cell death regularly occurring at a low level in the healthy tissues of different animal species (Kerr *et al*., 1972; Hockenberry 1995). Thus, it is not expected that a large frequency of the tail fibroblasts such as that included in the lowest above-mentioned Feulgen-DNA class represents in their totality apoptotic cells. Possibly, only that no positive response to the TUNEL assay has been revealed in the tail fibroblasts studied here does not exclude the possibility of apoptosis being occurring (Mello *et al.,* 2001).

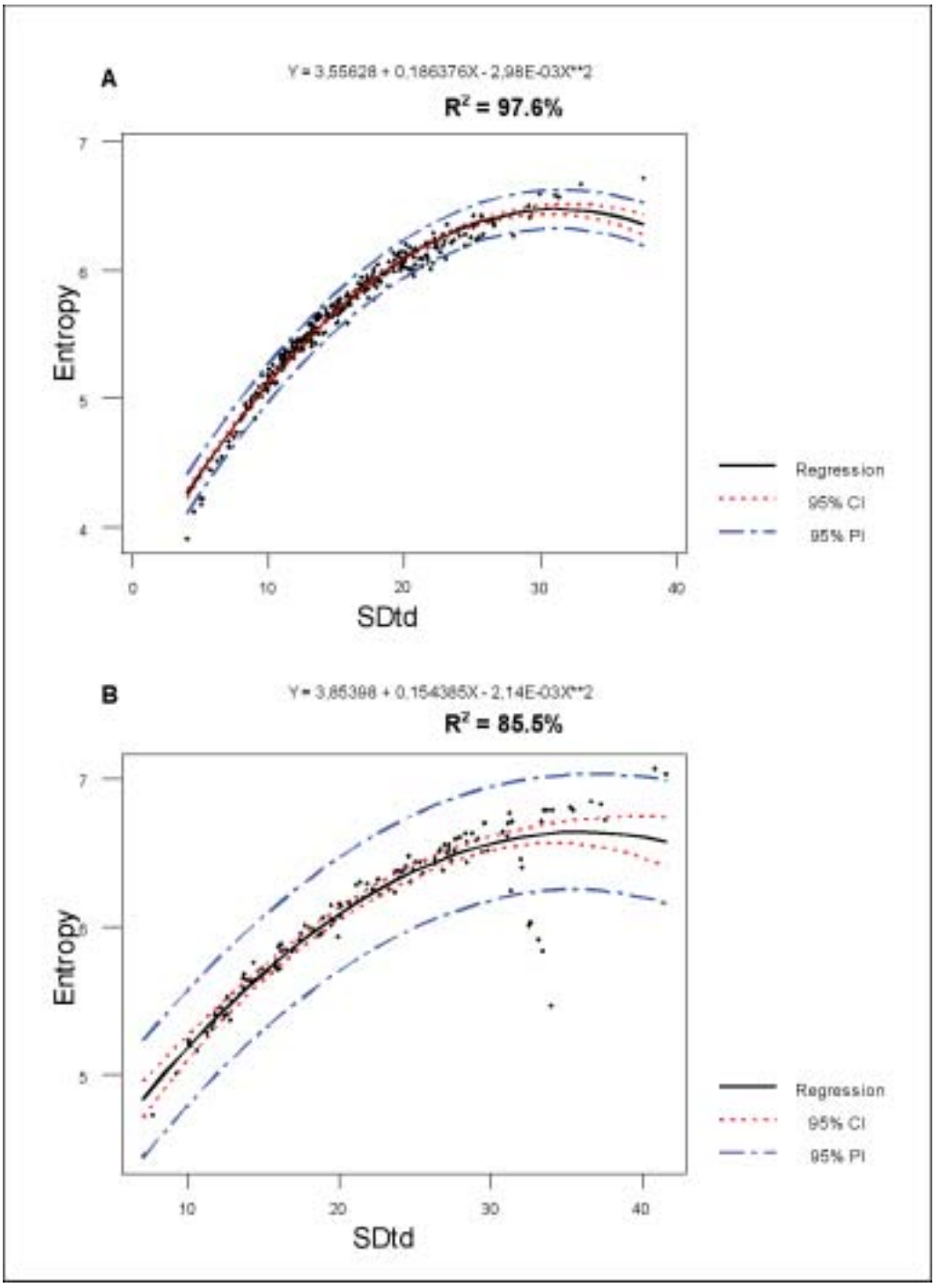

Fig. 2 — Correlation, expressed as a quadratic regression plot, between entropy and SDtd for Feulgen-stained nuclei in tendon fibroblasts of BALB/C (A) and NOD (B) mice.

In favor of the chromatin packing states being responsible for the lowest Feulgen-DNA values in the fibroblasts, more representative in the BALB/C mice, is the observation that chromatin condensation was highlighted in the fibroblasts of the NOD mice, as deduced from their higher OD values in comparison with BALB/C mice. On the other hand, since diabetes has been described as an "accelerated aging" due to similarities between those processes in terms of intra/extracellular alterations (Galeski *et al.* 1977; Yue *et al.* 1983), and no DNA loss associated with PCD has been verified at a detectable frequency during the senescence/aging process involved in dermal fi-

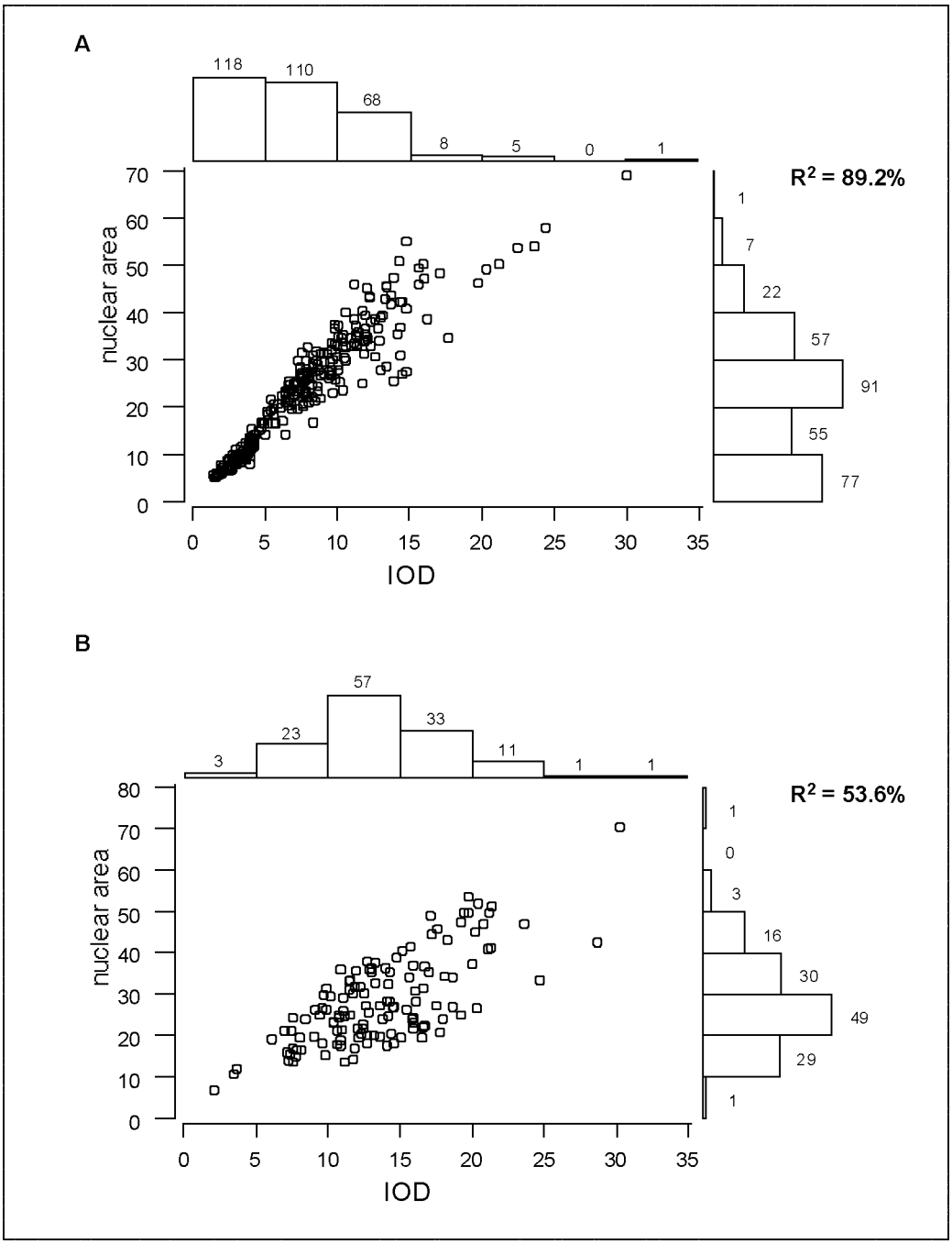

Fig. 3 — Relationship between nuclear areas and integrated optical density (IOD) values for Feulgen-stained nuclei in tendon fibroblasts of BALB/C (A) and NOD (B) mice. Four subgroups of nuclei were discriminated as regards to IOD values: 1-5, 5.1-10, 10.1-20 and >20 IOD values. The correlations of the nuclear areas on IOD were expressed as a regression plot (R^2).

broblasts (WANG 1995), it is expected that no apoptosis is occurring in the tail fibroblasts of the NOD mice.

The Feulgen-DNA classes with IOD values in the range of 5.1-10 and 10.1-20 are assumed to contain diploid fibroblasts in the G_1 and S phases of the cell cycle, respectively. The Feulgen-DNA values class with IOD values > 20 contain polyploid nuclei. In terms of relative frequencies, polyploidy would be more frequent in fibroblasts of the NOD mice. Polyploidy results from incomplete mitotic cycles generally related to increased physiological demands and aging (BRODSKY and URYVAEVA 1977; NICOLINI 1980). Various exam-

ples of biological species and physiological conditions associated with high degrees of ploidy have been described in the literature. In murine fibroblasts, polyploidy was associated with high levels of $p16^{INK4a}$ gene expression, whereas diploidy was associated with deletions of both $p16I^{NK4a}$ alleles (Zindy *et al.* 1997). Polyploidization has also been observed in smooth muscle cells from diabetic animals, however, its function during the diabetes, is far from being clear (Vranes *et al.* 1999).

Higher values for the textural parameters, SDtd and entropy, and smaller correlation expressed as quadratic regression plot between these parameters were revealed in the Feulgen-DNA stained tail fibroblasts of the NOD mice, indicating a larger contrast between highly and less densely packed states of chromatin in the cells of the diabetic mice. SDtd and entropy are correlated by the fact that the image is saved in bytes containing the total values of grey level (values). SDtd represents the variation (diffusiveness) of these values, i.e., chromatin transmittance. Entropy, on the other hand, is calculated by $E = k. \log_2 n$, where n represents the number of pixels/bytes per nucleus, i.e., amount of information.

Since OD values were simultaneously larger in the NOD fibroblasts, a deeper condensation of the highly packed chromatin is evident in these cells. This condensation, possibly associated to silencing of certain genes, may be related to changes in the extracellular matrix of the tendons with insulin-dependent diabetes. The signaling pathway from extracellular matrix to fibroblasts nuclei involves interaction among extracellular macromolecules, tenascin C, cell surface receptors and cytoskeletal mechanotransduction processes that trigger gene activation or silencing (Chiquet *et al.,* 2003), and is dependent on the tail tendon supraorganization (Vidal 1969, 1995). Since diabetes affects the tail tendon supraorganization in NOD mice through the glucose non-enzymatic incorporation to collagen bundles (Aldrovani *et al.,* 2005), it is possible that the signal transduction from the extracellular matrix to fibroblast nuclei have been altered by the disease, thus causing modifications in gene transcription (Bishara *et al.,* 2002) and chromatin texture. Recent studies have demonstrated that several cell types, including fibroblasts, possess cell surface receptors for glycosylated extracellular proteins; the interaction of these receptors with glycosylated collagen causes intracellular osmotic stress and modifies the intracellular concentration of Ca^{++} involved in the gene transcription and in the activities of endonucleases associated with DNA fragmentation (Bishara *et al.,* 2002).

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