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A stereological study of the volume-weighted volume and of the relative volume of the nucleus of normal and preneoplastic hepatocytes in a trout model of hepatocarcinogenesis

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ABSTRACT

The fish liver has been the main subject of the biomonitoring and laboratory studies dealing with environmental carcinogenesis. The foci of cellular alterations are accepted pre-neoplastic hepatic lesions, and histopathology is the primary tool for their characterization. Despite its potential, using stereology to study quantitatively nuclear features of those lesions has not been evaluated. Herein, we estimated the volume density and the volume-weighted volume of the nucleus of normal and preneoplastic hepatocytes, using stereology and the brown trout (*Salmo trutta* f. *fario*) as model. In the hepatocarcinogenesis protocol the *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was used as initiator, and the 17-β estradiol (E2) as promoter. Three groups of 30 animals were considered: negative controls (non-exposed), initiator exposed and initiator plus promoter exposed. Estimates of both stereological parameters were significantly higher in preneoplastic hepatocytes, also showing an excellent discriminatory power when used to differentiate those hepatocytes from the normal ones. Besides, in the normal parenchyma the two parameters also differed among the three tested groups. The exposure to MNNG and/or to E2 leaded to modifications in the hepatocyte nuclei that could be unbiasedly quantified with two stereological parameters. We showed that quantitative nuclear morphology represents a valuable auxiliary tool in assessing hepatocarcinogenesis in fishes.

1. Introduction

Liver cancer is a serious condition present worldwide and across species. The lesions and patterns of neoplastic development are interestingly similar in mammals and fish. In fact, fishes have been used for long, not only as alternative models to rodents in hepatocarcinogenesis studies, but also as important environmental sentinels, testifying the presence and effects of xenobiotics in aquatic systems. In fishes, the lesions with hepatocellular origin are represented by the foci of cellular alteration (FCA), benign and malignant tumors. FCA have been described in various feral fish and in experimental fish models of hepatocarcinogenesis (Bannasch, 1986); these lesions consist of a group of cells presenting alterations in glycogen storage or in distribution and/or number of organelles, like ribosomes (Bannasch, 1986). FCA are classified according to the distinctive cytoplasmatic staining features, in comparison to the normal hepatic parenchyma (Feist et al., 2004; Blazer et al., 2006). Clear cell, vacuolated cell, eosinophilic, basophilic, amphophilic and mixed FCA have been described in fish species, and all share the following diagnostic features: normal tubular architecture, absence or slight compression of the surrounding tissue and exclusion of other hepatic elements, namely bile ducts (Boorman et al., 1997; Feist et al., 2004). The amphophilic FCA, composed by hepatocytes with increased granular acidophilia and randomly cytoplasmic basophilia, is a controversial subtype, since it has been argued that its distinction from the basophilic subtype is unclear (Boorman et al., 1997).

Like in rodents, FCA in fishes are considered preneoplastic lesions with full capacity to progress to neoplastic lesions (Hendricks et al., 1984; Brown-Peterson et al., 1999; Blazer et al., 2006). This has been substantiated, from early studies, by the concomitant presence of FCA and hepatic neoplasms in fishes from polluted waters and different hepatocarcinogenesis models (Myers et al., 1991; Brown-Peterson et al., 1999; Blazer et al., 2006). However, the nuclear and proliferative characteristics of FCA in fishes are still largely unknown.

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Conventional histopathology is still nowadays the gold standard for evaluating the presence, type and extension of preneoplastic and neoplastic lesions in sentinel and laboratory fishes (Feist et al., 2004). Besides histopathology, auxiliary tools (like morphometry and stereology) have been used to discriminate hepatocellular lesions in rodents (Broxup et al., 1988; Jack et al., 1990; Xu and Pitot, 2006), but these have never been evaluated in fishes. As far as we know, only one study focused the attention on the use of quantitative methods in hepatic neoplasms and FCA in fishes (Stine et al., 2004). These authors used stereology to evaluate the volume, distribution and shape of the lesions, and to determine the optimal sampling strategy for diagnosis, using the mummichog fish (*Fundulus heteroclitus*) exposed to environmental contaminants (Stine et al., 2004). Considering that quantitative nuclear changes in hepatocytes of the hepatocellular nodules could be markers of alterations in the cellular machinery and genetic instability, which characterizes pre-cancerous and cancerous lesions (Köhler and Ellesat, 2008), we performed a histopathological and stereological study of FCA in a fish model of hepatocarcinogenesis. Our main goal was to investigate if quantitative nuclear parameters of hepatocytes from FCA are significantly different from non-focal hepatocytes, and if the cytoplasmic characteristics of FCA are associated with nuclear quantitative variations. For these aims, we estimated the volume density (also called relative or fractional volume) of the hepatocyte nucleus (V_V) and of its volume-weighted nuclear volume ($\bar{v}_v$), using stereological tools and the brown trout (*Salmo trutta* f. *fario*) as model. We also established cut-off values to differentiate normal and preneoplastic hepatocytes using the data of both parameters.

2. Materials and methods

2.1. Experimental induction of preneoplastic hepatocytes

Hepatocarcinogenesis induction was made in embryos, and used *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) as the initiator, and 17-β estradiol (E2) as a promoter. Brown trouts were exposed in the eyed-eggs phase to an aerated water bath of 50 ppm of MNNG for 1 h. Eight-weeks after an experimental (group A) and a control group (group B) were created by exposing the animals for 8 months to 50 μg/L of 17-β estradiol (E2) as a promoter and to vehicle only (ethanol saline at 0.001%), respectively. A third group (group C) of non-initiated/non-promoted negative controls, *i.e.*, fish without exposition to MNNG or to E2 but maintained in the same conditions, was also included. Animals were kept in well-aerated closed circuits, with dechlorinated freshwater being totally renewed every 48 h, with a reposition of the nominal concentrations. Water quality parameters were checked two times a day to confirm that they were within optimal ranges for brown trout maintenance. At the end of the experimental protocol, every fish was euthanized with an overdose of anesthetics (ethylene glycol monophenyl ether), weighted, measured, and the liver was then excised. It is opportune to mention that the animals presented a mean weight of 19 g (CV = 0.60), which did not vary among groups. After excision, the livers were sliced into 3 mm thick slabs, fixed for 24 h in buffered formaldehyde at 4%. Then, the fragments were routinely processed for paraffin embedding, sectioned (at 4–5 μm in thickness) and stained with hematoxylin–eosin (the time of staining was strictly the same for all the slides). For this study, we randomly selected livers of 30 animals per group. The experiment and fish handling were done in accordance with the Guidelines of the European Union Council (86/609/EU) and national law (Decreto-Lei no. 129/92).

2.2. Histopathological criteria

The histopathological classification of a focal lesion as a FCA was done according to the following criteria: cells blended into surrounding parenchyma without compressing it, normal or exacerbated (by sinusoidal dilation) tubular architecture without noticeable increased cellularity, absence of bile ducts and macrophages aggregates, nuclear morphology similar to the parenchyma, rare or absent mitotic figures (Boorman et al., 1997; Feist et al., 2004). Only FCA with more than 12 cells were considered for this study (as in Boorman et al., 1997). The FCA were subclassified in either eosinophilic, basophilic or amphophilic, according to the fine staining qualities of the involved hepatocytes. In order to obviate the mild differences in the intensity of the stain among slides, the subclassification of the FCA took the normal adjoining parenchyma as a reference for staining. Using a criterion from human and rodent analyses, the amphophilic subtype was considered whenever there were concomitantly areas of eosinophilia and basophilia within the cytoplasm of the hepatocytes (Su et al., 1997; Mayer et al., 2003).

2.3. Stereological analysis

The diagnosed FCA were studied with stereological tools. In each case (*i.e.*, fish) we also sampled a morphologically normal (non-nodular) area of liver parenchyma in the section, which was considered as an internal control. The extension of this normal parenchyma depended on the number and relative size of the focal lesions appearing in each section. This methodology was also applied for animals of the groups B and C. In all cases, we used a systematic sampling approach in the selection of the fields for the stereological analysis, which was performed with a workstation that comprised a microscope (Olympus, BX-50) equipped with a 100× oil immersion objective (Olympus Uplan), a CCD video camera (Sony) connected to a PC monitor and a motorized stage (Prior) for stepwise displacements in *x*–*y* directions; the workstation was controlled by the software CAST-Grid (Version 1.5, Olympus, Denmark).

Two stereological parameters were estimated: (1) V_V (hepatocyte nucleus, parenchyma) and (2) nuclear $\bar{v}_v$. The former was determined by the point counting method (Freere and Weibel, 1966) (Fig. 1A). This is based in a direct counting of points hitting the object of interest (herein nuclear profiles) and points hitting the reference space. For analytical purposes, this space was the hepatic parenchyma, excluding the vessels that were larger than a sinusoid. This is an operational definition adopted in stereological studies in liver (Rocha et al., 1997). The V_V was calculated with the following formula:

$$V_v(\text{nucleus},\ \ \text{reference space}) = \frac{\sum P(\text{nucleus})}{\kappa \cdot \sum P(\text{reference space})}$$

in which κ is the ratio between the number of grid points used for targeting the nuclei and those used for the reference space (that could be the parenchyma within a FCA or the one within a analyzed normal zone). We planned a test grid in which 81 points were used for targeting the nuclei and 9 points for hitting the parenchyma, so that $\kappa = 9$.

As to the nuclear $\bar{v}_v$ we used the point-sampled intercepts method (PSI) (Gundersen and Jensen, 1985). This parameter quantifies the nuclear size and pleomorphism, being estimated with a test grid made of parallel lines bearing a systematic pattern of points (Fig. 1B). Only the nuclear profiles randomly hit by one of these points were sampled. On these profiles, the line segments crossing the point-sampled nucleus were measured from boundary to