Estudi de la regeneració miocàrdica en la miocardiopatia alcohòlica i la seva relació amb el dany funcional i estructural miocàrdic, activació d'apoptosi i activitat miostatina

Meritxell Lluís Padierna

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Corresponding Author: JOAQUIM FERNANDEZ-SOLA, MD, Ph.D.

Corresponding Author's Institution:

First Author: JOAQUIM FERNANDEZ-SOLA, MD, Ph.D.

Order of Authors: JOAQUIM FERNANDEZ-SOLA, MD, Ph.D.;MERITXELL LLUIS, M.D.;EMILIO SACANELLA, M.D., Ph.D.;RAMON ESTRUCH, M.D., Ph.D.;EMILIA ANTUNEZ, M.D., Ph.D.;ALVARO URBANO-MARQUEZ, Prof.

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INCREASED MYOSTATIN ACTIVITY AND DECREASED MYOCYTE PROLIFERATION IN CHRONIC ALCOHOLIC CARDIOMYOPATHY.

Joaquim Fernández-Solà[1,2] M.D., Ph.D.; Meritxell Lluis M.D, Ph.D[1].; Emilio Sacanella[1,2] M.D., Ph.D.; Ramón Estruch[1,2] M.D., Ph.D.; Emilia Antúnez[1,3] M.D, Ph.D,; Alvaro Urbano-Márquez[1] M.D, Ph.D.

[1]Alcohol Research Unit,. Hospital Clinic. Institut d'Investigació August Pi i Sunyer (IDIBAPS).Department of Medicine University of Barcelona. España

[2]CIBEROBN Fisiopatologia de la Obesidad y la Nutrición. Instituto de Salud Carlos III, España

[3]Department of Medicine.University of Barcelona.Catalunya.España

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Corresponding author:

Joaquim Fernandez-Solà M.D.

Department of Internal Medicine. Alcohol Unit

Villarroel 170. 08036. BARCELONA. ESPAÑA

Phone and FAX: 00.34.93. 2279365

E-mail: jfernand@clinic.ub.es

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KEY WORDS: Alcohol, Myocardium, Apoptosis, Myostatin, Myocyte proliferation.

INTRODUCTION

Alcoholic cardiomyopathy (CMP) is a recognised major complication of chronic alcoholism, (Moushmoush and Ali-Mansour, 1991; Urbano-Márquez and Fernández-Solà, 2004). The disorder is consequence of a common, dose-related, toxic effect of alcohol, affecting one third of long-term alcohol abusers (Urbano-Marquez et al, 1989). This distinct form of congestive heart failure is responsible for 21–36% of all cases of non-ischaemic dilated CMP in Western society (Spies et al, 2001). The end-stage of this CMP is largely irreversible and is characterized by a significant loss of myocytes, hypertrophy of the remaining muscle cells and also development of progressive interstitial fibrosis (Fernández-Solà et al, 1994). Without complete abstinence, the 4-year mortality for alcoholic CMP is close to 50% (Laonigro et al, 2009). On the other hand, complete abstinence from alcohol may allow partial clinical and functional reversibility of alcoholic CMP (Guillo et al, 1997). In addition, Nicolás et al (2002) showed that controlled drinking (<60g/day) without complete abstinence was as effective as complete abstinence.

Mechanisms of alcohol-induced myocardial damage are multifactorial, with disruption of membrane composition, disturbances in calcium transients, excitation-contraction coupling mechanisms, energy and protein turnover, and changes in cell mechanisms of genetic control. All these factors are involved in the pathogenesis of this disease, often synchronously (Preedy et al, 1996; Molina et al, 2002; Urbano-Márquez and Fernández.Solà, 2004; Ren and World, 2008). As an end consequence of this process, ethanol induces myocyte damage that progress to myocyte death and cell loss, with replacement by interstitial fibrosis. This myocyte loss has long been attributed to non-inflammatory myocyte necrosis. However, as described in other CMP (Beltrami et al, 2001, 2003; Gürtl et al, 2009), myocyte apoptosis may play a role in the development of structural heart damage in this setting (Hajnóczky et al, 2005; Fernández-Solà et al, 2006).

Apoptosis has been clearly implicated in the pathogenesis of alcohol-induced organ damage (Saraste et al, 1999; Neuss et al, 2001), as evidenced in several tissues (Altura and Gebrewold, 1998; Casey et al, 2001; Hoeck and Pasimoro, 2002). In alcoholic patients without heart damage both pro-(BAX) and anti-(BCL-2) apoptotic mechanisms

are activated. When structural heart damage appears, the BCL2/BAX ratio decreases, and apoptosis is present in the heart muscle of high-dose alcohol consumers as occurs to a similar degree in other causes of structural heart damage such as longstanding hypertension (Fernández-Solà et al, 2006) or end-stage heart failure (Saraste et al, 1999; Williams, 1999).

Whereas cardiac myocytes have traditionally been considered to be terminally differentiated cells, the last few years have witnessed exciting new challenges to this dogma, including the discovery of a natural rate of apoptosis and renewal of cardiac myocytes via circulating bone marrow stem cells and resident cardiac progenitor cells (Anversa and Nadal-Ginard 2002; Bergmann et al 2009). The emerging concept of myocyte homeostasis supposes an equilibrium that is maintained with a balance between myocyte death and myocyte renewal (Hill and Olson, 2008; Buja and Vela, 2008). It is important to consider that myocyte death itself activates mechanisms of proliferation of the remaining cells and renewal from pluripotential local or systemic stem cells (Regula et al, 2004). In this scenario of heart remodelling and plasticity, there is place for physiological growth, for pathological growth, and also for antigrowth heart mechanisms (Hill and Olson, 2008). One of these antigrowth factors that control myocyte hypertrophy and proliferation is myostatin.

Myostatin is the growth differentiation factor-8 (GF-1), a potent inhibitor of skeletal muscle and heart growth (Joulia- Ekaza and Cabello, 2006). Myostatin controls cell cycle progression and inhibits skeletal myoblast proliferation and terminal differentiation. An increase in myostatin activity protects the cell from apoptosis. Its disruption causes increased skeletal mass with hypertrophy and hyperplasia of myocytes and increased myocyte proliferation (Joulia- Ekaza and Cabello, 2006; McKoy et al, 2007). Experimental studies suggest that myocyte myostatin increases with ethanol exposure (Lang et al, 2004). This effect of myostatin on skeletal myoblasts is not necessarily related to the same effects on the mature heart

We therefore tested the hypothesis whether the chronic effect of alcohol on the myocardium may induce an increase in apoptosis as well as an inhibition of myocyte proliferation through increased myostatin activity. For this purpose we used myocardial

tissue from human donors in which we can observe the effect of previous chronic alcohol consumption on the regulation of cardiac myostatin expression and also evaluate the induction of apoptosis and the degree of myocyte proliferation. We compared this effect of alcohol with that produced by longstanding hypertension and other causes of CMP, as well as in non-alcoholic, non-hypertensive controls.

MATERIAL AND METHODS

Patient and Control Selection

Over a four-year period (January 2003 to December 2006), we consecutively studied hearts from subjects who had brain death either of traumatic or cerebrovascular origin, and had been considered suitable as organ donors by the transplant team of the Hospital Clínic of Barcelona. Of 127 cadaveric donors younger than 70 years of age, 67 hearts were not suitable for transplantation. Of these latter organs we selected: 1) donors with chronic hypertension, 2) donors with a history of ethanol intake ($\geq$ 60 g/day, longer than 10 years), 3) donors with other causes of heart disease, and 4) control hearts from healthy people who were not eligible for implantation because of a lack of matched receptor or size inadequacy.

All patients were white Caucasians of Spanish descent, who lived with their families in or around Barcelona and none was indigent. The study protocol was approved by the Ethics Committee of the Hospital Clínic and included informed consent from the families of the donors concerning the use of myocardium tissue for this research protocol study. One third of these subjects had been included in previous studies on heart antioxidant status (Fatjó et al, 2005) and cardiac apoptosis (Fernández-Solà et al, 2006).

All cases had been admitted to the Intensive Care Unit and ventilatory and haemodynamic parameters had been appropriately maintained at normal values throughout hospitalisation: P_aO_2 greater than 60 mmHg, systolic blood pressure greater than100 mmHg, and arterial pH within the normal range. None of the patients required in-hospital cardiopulmonary resuscitation maneuvers.

Clinical and laboratory evaluation

A detailed history of ethanol intake was retrospectively obtained by consultation with family members using a structured questionnaire ("time-line follow-back method") (Sobel et al, 1979), as previously reported (Urbano-Márquez et al, 1989; Fernández-Solà et al, 1994). Duration of ethanol intake was calculated in each group as the total cumulated period of alcohol consumption in years, either recent or previous. The body mass index was determined as the actual body weight relative to the square of the body height (BMI, Kg/m^2). Patients were considered to have caloric malnutrition if the BMI was less than 17 Kg/m^2. Protein malnutrition was assessed by the following parameters obtained at hospital admission: haemoglobin, lymphocyte count, total protein, retinol-binding protein, pre-albumin and albumin.

Cardiac Studies

Past and present signs and symptoms of heart failure were evaluated in consultation with medical records and family members of the donors, and the New York Heart Association (NYHA) functional class was determined according to the Goldman activity scale (Goldman et al, 1981). Chest X-ray with measurement of cardiothoracic index and conventional electrocardiography were performed in all cases. Moreover, a bidimensional echocardiography was performed (Hewlet Packard Sonos 2500, USA) in 24 patients compared to none of the controls with a cardiothoracic index greater than or equal to 0.48 compared to none of the controls. End-diastolic and end-systolic diameters, the shortening fraction, left ventricular (LV) mass, and the ejection fraction (EF) were measured according to the standards of the American Society of Echocardiography (Gottdiener et al, 2004). Cardiomyopathy was defined in the presence of LVEF < 50% and LV enlargement. We observed a good correlation between the cardiothoracic index and the left-ventricle end-diastolic diameter ($r = 0.68$, $p < 0.01$). The personnel who performed and evaluated these tests had no knowledge of the alcoholic history of the patients.

<u>Myocardium histological studies</u>

A distal 3 cm sample of the left-ventricle apex was surgically excised (total weight of 4-5 g) at the time the donor was under cold perfusion. The specimen was cut into fragments, and one of these was processed for further histological analysis. The remaining fragments were immediately frozen under liquid nitrogen, until apoptosis,

myostatin and Ki-67 studies were performed. For histological studies, specimens were stained with haematoxylin-eosin and toluidine-blue in semi-thin sections. Two independent observers (JF-S and AU-M) morphologically differentiated myocytes from other cells and evaluated the degree of myocardial cell and nuclear hypertrophy, myocytolysis (defined as the presence of myofiber disarray, or cell vaquolization) and interstitial fibrosis. In case of discordance a consensus agreement was established. The amounts of interstitial fibrosis (volume fraction of fibrosis) and cardiac muscle cells (volume fraction of the myocytes) were assessed as previously reported (Fernández-Solà et al, 1994). The degree of global histology involvement was graded as normal, mild, moderate, or severe according to previous histologically defined criteria (Fernández-Solà et al, 1994; Nicolás et al, 2002).

Evaluation of myocardial apoptosis.

Myocardial apoptosis was evaluated using immunohistochemical studies. BAX and BCL-2 activities were considered as indicators of pro and anti-apoptotic status, respectively. Semi-thin cryostat sections of 6μ were obtained with ultra-microtome and fixed by paraformaldehyde 4% PBS solution at pH 7.40. After PBS washing, sections were permeabilized with 0.1% Triton X-100 sodium citrate solution for 2 minutes at 4ºC. Incubation with TUNEL *(deoxyribonucleotidyl-transferase-mediated-dUTP-biotin nick-end labelling)* was performed using the *In Situ* cell Death Detection Kit® (Boehringer, Manheim, Germany) for 60 min at 37ºC inside a wet chamber, as described by the manufacturer. After PBS washing, a second incubation with Converter-Alkaline phosphatase was performed during 30 minutes under the same conditions. The reading process was performed by adding Fast Red chromogen for 10 min, under microscopic control. Incubation with BAX and BCL-2 reagents were performed in semi-thin cryostat sections of 6μ fixed by ketone at -4ºC. After primary antibody exposure (Biogenex, San Ramon CA, USA AR 347-5R for BAX, and AM 287-5R for BCL-2), link and labelling reactants were sequentially exposed for 20 min each (Kit Detection Supersensitive, Biogenex, San Ramon CA, USA, QA000-5L), with a final Fast Red chromogen incubation of 10 minutes. Negative control slides were made in all the procedures using the same process without reagent incubation. Positive controls were performed using human palatinal amygdalar tissue, in which lymphoid cells take high TUNEL, BAX and BCL-2 activities.

The apoptotic index (Ai) was determined as the percentage of apoptotic cells, and calculated by dividing the total number of positive staining heart myocyte nuclei in the TUNEL assay by the total number of the myocyte nuclei evaluated and multiplying this value by 100 as previously reported (Narula et al,1996; Paul et al, 2001). Cell nuclei at the edges of the tissues or in the interstitium were not counted. The count was made on at least 5 different fields (magnification 250x) of each sample, with at least 500 nuclei per field, according to the method described by Sandri et al (1995). Positive cells for BAX and BCL-2 staining were considered in the presence of diffuse cytoplasmic hyperenhancement, subsarcolemmal or perinuclear deposition of the reagent, and expressed as a percentage of positive to total evaluated cells. Interstitial deposition of these reagents and peripheral zones of the samples were not considered in the evaluation. We carefully performed a morphological identification of different cell types to exclude fibroblasts, adipocytes and endothelial cells in these counts.

Evaluation of myocardic repair mechanisms: Myostatin and Ki-67 immunohistochemical studies.

The evaluation of myocardium repair mechanisms was performed by means of myostatin and Ki-67 inmunohistochemmical assays on frozen tissue.

Myostatin (GF-β1) myocardium assay was performed using a commercial kit with the monoclonal antibody (GDF8 -ab996- datasheer, Abcam, USA) with human specificity for myostatin. This antibody has an immunogen sequence common to myostatin and its precursor AA 348-364 (NMLYFNGKEQIIIGKI) that detects all the forms of myostatin: precursor, dimmers and monomers. Dilution was performed at 1/1,000 in a buffer-citrate solution at pH 6.0. Lecture was performed by the compatible secondary antibody ab6722 linked to peroxidase This antibody has nuclear and cytoplasmic reactivity. To proof the anti-myostatin antibody specificity of this antibody we used muscle specimen from myostatin knockout mouse (Se-Jin Lee, Molecular Biology & Genetics, Johns Hopkins University School of Medicine. Baltimore, USA) and compared the reactivity to muscle of wild-type mouse. Evaluation of myostatin activity was performed by means of a semiquantitative study evaluating the percentage of positive cells with respect to total evaluated myocardial cells. In each case a minimum of 3,000 myocytes were evaluated. We compared results from cases (alcoholics) with

healthy donors and also pathological controls either with hypertension or other causes of CMP.

Ki-67 myocardium activity was evaluated by immunohistochemical assay on frozen myocardium tissue. Myocardium samples were fixed by ketone at-4ºC during 10 minutes, followed by PBS washing and serum blockade during 30 minutes. Primary antibody incubation was performed with the nuclear Ki-67 marker in a wet chamber, followed by washing and incubation with the secondary antibody linked to alkaline phosphatase during 30 minutes in a wet chamber. Lastly, the sample was submitted to gentle washing, revealed with substrate and counterstain with haematoxylin.

Statistical Analysis

The data were analysed using SPSS-PC 14.0 statistical software (SPSS, Chicago, IL). Differences between groups were analysed using a chi-square test, analysis of variance (ANOVA), and a two-tailed unpaired Student *t*-test. Pearson correlation coefficients between the variables were calculated. As all variables followed a normal distribution, variables were expressed as mean ± standard deviation. A two-tailed P value <0.05 was considered statistically significant.

RESULTS:

Clinical data

From the 67 donors selected, three heart specimens from subjects with concomitant hypertension and alcoholism were not included in order to maintain the homogeneity of the study groups. Two samples which were not adequately cryopreserved were also excluded. Therefore, a total of 62 organ donors fulfilled the selection criteria and were finally included in the study. Twenty-two were high-dose chronic alcohol consumers, 22 non-alcoholic individuals had long-standing essential hypertension, 8 individuals had other causes of CMP (3 coronary disease, 3 idiopathic CMP and 2 valve heart disease). Ten previously healthy donors without regular alcohol consumption did not report any significant disease and were included as control group.

Table 1 shows the epidemiological and clinical data of the patients and controls. The groups of controls, chronic alcoholics, those with hypertension and donors with CMP of

other origin were similar in age and gender. Cerebrovascular disease was the main cause of death in the alcoholics and hypertensive patients, whereas cranial trauma was the main cause in the controls group and in those with other causes of CMP. The time from hospital admission to organ extraction was similar in the four groups. According to family members chronic alcoholics had a mean daily ethanol consumption of 155.1 ± 50.4 g during a period of 22.8 ± 5.1 years, with a lifetime dose of ethanol of 16.9 ± 5.8 Kg ethanol/Kg body weight (Table 1). Alcoholic patients reported a greater active smoking habit, with a mean of 35 ± 12 packs-year, compared to the other groups of donors ($P < 0.01$). Hypertensive patients reported a mean duration of high blood pressure of 16.4 ± 5.6 years.

Evaluation of the medical record revealed that all control subjects had NYHA I functional class. Of the 22 chronic alcoholics, 13 (59%) had NYHA I class, seven (32%) NHYA II class and two (9%) NYHA III class. In the group of 22 subjects with hypertension, six (27%) had NYHA II class and two (9%) NYHA III class. In the group of 8 donors with other causes of CMP, two subjects (25%) had NYHA II class and one (12%) NYHA III.

Neither controls nor alcoholics had received any regular medication over the three months prior to admission. By contrast, most of hypertensive patients were under maintenance treatment with angiotensin-converting enzyme inhibitors at the usual doses (n = 18) and/or diuretics (n = 4). Alcoholic, chronic hypertensive and patients with other cardiac diseases presented a greater cardiothoracic index than controls. The electrocardiogram was normal in all controls but one who showed signs of left ventricular hypertrophy, being abnormal in 10 alcoholics (45%), and in 15 (68%) chronic hypertensive patients who showed changes in ST wave, conduction defects and signs of left ventricular hypertrophy. All subjects with other causes of CMP showed electrocardiogram abnormalities with signs of ischaemia (n=3) and left ventricular hypertrophy (n=5). The left ventricular ejection fraction was similar in alcoholics, hypertensive and donors with other causes of CMP, being significantly lower in all these groups than in controls ($p<.01$) (Table 1).

Biochemical and nutritional parameters.

With respect to nutritional parameters the BMI was comparable in all the groups studied. Only two subjects with chronic alcoholism, one subject with hypertension and

other with ischaemic CMP exhibited borderline signs of caloric malnutrition. Regarding protein nutritional parameters, alcoholics showed lower mean total serum protein and albumin concentrations compared to the other groups ($p < 0.05$, all). In addition, alcoholics exhibited higher levels of aspartate aminotransferase and gammaglutamyl transpeptidase compared to the other groups ($p < 0.05$, all). Leukocyte, lymphocyte, and platelet counts as well as haemoglobin concentrations and serum muscle enzymes were similar in all groups.

Cardiac evaluation

Cardiomyopathy was defined according to the criteria described in the Methods section in 12 chronic alcoholics (55%), 13 individuals with essential hypertension (59%) and in all 8 cases of CMP of other origins. Table 3 discloses the cardiac functional data of the subgroups of patients according to the presence or absence of CMP. All alcoholics, hypertensive patients and subjects with other causes of CMP with NYHA class II-III had enlarged hearts. No difference in nutritional data was observed among the subgroups of patients. Alcoholic patients with CMP consumed a significantly greater daily and lifetime ethanol intake compared to those without CMP.

Myocardial histological analysis

Except for slight signs of nuclear and myocyte hypertrophy in two specimens, histological evaluation of myocardial samples from control donors was normal. Thirty out of 33 patients with CMP (91%) had myocyte cell and/or nuclear hypertrophy, and more than two-thirds demonstrated interstitial fibrosis. By contrast, only slight myocyte abnormalities were detected in one-third of the 19 patients without CMP. The histological degree of myocardial lesion was low in 64% of cases and moderate in 36%. Considering the origin of CMP, no differences were observed in the analysis of the histological parameters and degree of histological involvement on comparing the three different groups of donors.

Apoptosis immunohistochemical studies

Table 2 reflects the evaluation of apoptosis immunohistochemical studies in the different group of donors. Figure 1 shows BAX activitiy in a control donor compared to a subject with alcoholic CMP. Considering the presence of apoptosis in cardiac myocytes observed with the TUNEL immunohistochemical assay the apoptotic index did not differ among the groups studied (2.8 $\pm$ 1.6 % ; 3.7 $\pm$ 3.4 % ; 4.0 $\pm$ 3..2 % and 4.1 $\pm$ 5.0 % for controls, alcoholics, hypertensives and donors with other causes of CMP, respectively, p= NS). With respect to pro-apoptotic BAX and anti-apoptotic BCL-2 immunohistochemical assays, both BAX and BCL-2 reactivity was significantly higher in chronic alcoholics, hypertensives and donors with other causes of CMP compared to control donors ($p< 0.05$, all). Alcoholics showed the lowest BCL2/BAX ratio, although the differences did not achieve significance (p= 0.238, F 1.426, ANOVA).

Table 3 shows the evaluation of these immunohistochemical apoptosis parameters in the groups of donors with CMP. The results of the TUNEL assay measurements were significantly higher in alcoholics with CMP compared to those with normal heart function (5.2 $\pm$ 3.9 % vs 2.8 $\pm$ 1.6%, $p< .05$). BAX and BCL-2 activities were significantly higher in alcoholic and hypertensive donors with CMP compared to their partners without CMP ($p<0.05$, both). Alcoholics with CMP showed the lowest BCL2/BAX ratio.

Evaluation of myocardial repair mechanisms: **Myostatin and Ki-67 immunohistochemical studies.**

Figure 2 shows absence of myostatin immunohistochemical reactivity in skeletal muscle of myostatin GDF8 knockout mouse in comparison to focal perinuclear and cytoplasmic myostatin reactivity in wild-type mouse. Myostatin immunohistochemical reactivity was detected in the perinuclear area of 5.7 ± 1.4 % of control donors. The groups of donors with hypertension and the group with other causes of CMP presented a non-significant increase of myostatin values compared to controls (9.8 ± 5.3 and 9.4± 6.9%,

respectively, p= n.s.).In contrast, the group of alcoholic donors presented a significant increase in nuclear myocyte myostatin activity (12.4± 5,8%) compared to controls (P <0.01) (Table 2). Figure 3 shows a comparison between a low-reactivity myostatin assay in a control donor compared to a high-positive reactivity in an alcoholic with CMP.

Positive immunohistochemical reactivity for Ki-67, as a reflection of the myocyte proliferative process, was detected in 3.8 ± 0.9 % nuclei of control donors. As expected, the groups of donors with hypertension or other causes of CMP showed a significant increase in Ki-67 activity values compared to controls (11.1 ± 3.8 and 12.3 ± 2.5 %, p<0.01, respectively). Similarly, the group of alcoholic donors showed a significant increase in Ki-67 reactivity compared to controls (7.5 ± 3.9 % vs 3.8 ± 0.9%, p<0.05) (Table 2). Figure 4 shows a case of increased perinuclear Ki-67 reactivity in a donor with hypertensive CMP compared to low Ki-67 reactivity in a control subject.

When data analysis was performed separating the groups of donors according to the presence of CMP, we observed that alcoholic donors with CMP showed a significant increase in myocardium myostatin activity compared to alcoholic donors without CMP (15.7 ± 2.8.% vs 8.5 ± 2.3%, p<0.05). Inversely, myocardium Ki-67 activity was significantly lower in alcoholics with CMP compared with those without CMP (8.9 ± 1.6 vs 2.1 ± 1.8, p<0.05). Hypertensive donors with CMP showed a non-significant increase in myostatin and Ki-67 expression compared to hypertensive donors without CMP (13.5 ± 2.2 vs 9.2 ± 1.9, p=n.s.) (Table 3)

Table 4 shows the comparison of myostatin and Ki-67 myocardial immunohistochemical reactivity between control donors and the different groups of heart donors with hypertension or other causes of CMP. Compared to controls, the relative increase in myocardium myostatin activity in alcoholics with CMP was higher

that that observed in hypertensives and donors with other causes of CMP (2.7 fold vs 1.5 and 1.6 fold, respectively). In the ANOVA analysis, myostatin activity was significantly higher in the groups of donors with any cause of CMP compared to subjects without of CMP ($F = 2.449$, $p=.047$). The expected increase in Ki-67 myocardial proliferative activity in alcoholic donors with CMP was lower that that observed in the other group of donors with hypertension and other causes of CMP (3.3, 3.5 and 3.3 fold, respectively).

The ANOVA analysis demonstrated significant differences in the inter donor groups analysis with respect to myocardial myostatin activity, being significantly higher in alcoholics compared to the other groups of donors ($F= 2.45$, $P= 0.047$). In addition, alcoholic donors with CMP presented a significantly lower increase in Ki-67 expression compared to the other groups of donors with CMP of hypertensive or other origin (ANOVA, $F= 24.8$, $p <.001$).

DISCUSSION

In the present study, performed by immunohistochemical methods in myocardium tissue proceeding from heart donor subjects, we found an increase in myocardial myostatin expression in subjects with different causes of CMP either of alcoholic, hypertensive, coronary, valvular or idiopathic origin. These results may be expected since ventricular myostatin expression is reportedly up-regulated in the presence of diverse causes of myocyte damage (Sharma et al, 1999; Shyu et al, 2006). It is of interest to remark that in the setting of alcoholic CMP, myocardium myostatin expression is highly up-regulated in comparison to other groups of CMP. This fact corroborates the previously suggested hypothesis of alcohol up-regulation of myocyte myostatin and other growth factor activities (Lang et al, 2004).

With respect to the parameters of myocyte proliferation, evaluated in this study by myocardium Ki-67 expression, we also observed an increase in their expression in all the causes of CMP compared to healthy control donors. This is also an expected result as a reflection of the capacity of the myocardium cells to proliferate against a persistent lesion either of toxic (alcohol), hypertensive, coronary or valvular origin (Anversa and Nadal-Ginard, 2002; Beltrami et al, 2003).

However, in the case of donors with alcoholic CMP, this myocyte proliferative response was significantly lower compared to donors with hypertension or other causes of CMP, reflecting a relative inhibition of the normal proliferative response of the myocardium against persistent structural damage such as that induced by excessive alcohol consumption (Urbano-Márquez and Fernández-Solà, 2004). Thus, we observed that the relative increase in Ki-67 expression in ACM in comparison to control donors was 67% lower than the increase present in CMP of hypertensive or other origin. The relative increase in myocyte myostatin activity and the decrease in Ki-67 proliferative response in alcoholic subjects are in concordance with previous reports in experimental studies (Lang et al, 2004). In fact, alcohol consumption has been suggested to up-regulate myostatin activity in other tissues such as skeletal muscle (Lang et al, 2004). Due to the clear clinical, functional and structural relationship evidenced in the deleterious effects of chronic alcohol consumption on skeletal and cardiac muscle (Fernández-Solà et al,1994), a similar effect of alcohol was expected on myostatin activity in these two tissues.

Similar to what has already been previously reported (Fernández-Solà et al, 2006), in the present study we observed an increase in myocardium apoptotic activity in subjects with alcoholic-dilated CMP measured by detection of DNA fragmentation by the immunohistochemical TUNEL assay. In addition, we detected activation of pro- and

anti- apoptotic-regulating mechanisms (BAX and BCL-2, respectively) in organ donors with CMP either of alcoholic, hypertensive or other origin. The degree of apoptosis in alcoholics was of a similar magnitude to that observed in hypertension and other causes of CMP. Although the differences were not significant, alcoholic donors showed a lower BCL2/BAX ratio that the other groups of donors, reflecting a relative increase in pro-apoptotic mechanisms. However, apoptosis was significantly higher in donors with CMP compared to their partners without CMP. This increase in myocardium apoptosis is a reflection of a common pathogenic mechanism inducing progressive heart damage, independently of the origin of the damaging agent (Nadal-Ginard et al, 2003). In alcoholics, myocardium apoptosis does not only depend on ethanol consumption but also to the presence of structural heart damage (Jänkälä et al, 2002; Fernandez-Solà et al, 2006), demonstrating that myocardium apoptosis is a complex mechanism related not only to the original trigger agent but also to intermediate causes of myocardial damage that activate mechanisms of cell lesion such as mitochondrial caspases, disturbances in intracellular cell transients (Nicolás et al, 1998) protein synthesis (Fernández-Solà et al, 2007) or oxidative damage (Fatjó et al, 2005).

Myostatin activity has been reported to protect myocardium cells from apoptosis (Joulia Ecaza and Cabello, 2006). Therefore, the increase in myostatin activity could be a protective mechanism developed by cardiac myocytes against the toxic aggression of alcohol. However, a negative consequence of this myocardial myostatin up-regulation induced by alcohol is the inhibition of myocardium Ki-67 proliferation activity because of the anti-growth effect of myostatin (Shyu et al, 2005; McKoy et al, 2007). In the present study the negative effect of alcohol is more evident than the protective effect of myostatin in the induction of myocardial apoptosis. In addition, up-regulation of myostatin is not a single mechanism influencing the decrease in the myocardial

proliferative response. Other factors such as IGF-1 activity may also influence this process (Lang et al, 2004; Shyu et al, 2005). The existence of these multiple effects may explain the diversity of factors influencing changes in the cardiac homeostasis induced by ethanol misuse.

Thus, the chronic alcohol effect on the myocardium has a multi-factorial noxious effect, not only inducing a clear cell loss because of the increase in myocyte apoptosis but also inhibiting the relative capacity that conserves heart myocytes to proliferate though up-regulation of myostatin or other myocyte anti-growth factor activity. The final result of this process is a clear imbalance in myocyte homeostasis status, inducing a net loss in total ventricular myocyte mass, with progression to subsequent ventricular dysfunction as reflected in the natural history of alcoholic-dilated CMP (Fernández-Solà et al, 2008). In alcoholic CMP, this global effect implies a lower capacity of myocardial repair in comparison to hypertensive or other causes of CMP.

Due to the difficulty in obtaining human myocardium samples, the present study is restricted to a relatively small number of middle-age chronic alcoholic subjects of Caucasian origin, individuals with essential hypertension, other causes of CMP and controls. Due to the abstinence from alcohol established in the period comprised from hospital admission to heart donation, the acute effects of alcohol could not be considered. With the design of this study, only a retrospective approach for the drinking habits of all donors by the family members was possible. Since alcoholic donors had significantly higher active smoking consumption compared to the other groups, we cannot exclude an additional noxious effect of nicotine in the myocardium as reported in experimental (Rajiyah et al, 1996) and clinical studies (Zakhari, 1991), although nicotine heart effect mainly induces coronary artery lesions. Evaluation of myocardial apoptosis is limited to TUNEL, BAX and BCL-2 activities. With TUNEL

assay some degree of apoptosis over-detection is possible, since some DNA fragmentation is still reversible (Kanoh et al, 1999). Evaluation of myocardial repair mechanisms was limited to myostatin and Ki-67 activities. We corroborated the anti-myosin antibody specificity comparing the negative reactivity of this antibody in GDF8 knockout to the positive reactivity in wild type mouse. Since myocardial biopsy was taken only in the left-ventricle apex, this study did not consider architectural or functional changes within different sites of the myocardium considered in this study. With respect to the relatively high percentage of Ki-67 positive nuclei found in controls, we should consider that some studies have identified Ki-67 positivity in myocardiocytes as an expression of kariorrhexis but not proliferation (Kajstura et al, 1998; Beltrami et al, 2003).

Despite the reported limitations, the homogeneity of the sample and the significance of the results allow corroboration of the role of myostatin activity in alcoholic CMP in human samples as suggested in previous experimental animal studies (Lang et al, 2004).

In conclusion, we have demonstrated the existence of diverse toxic effects of alcohol on the myocardium. Myocye loss was partially due to an increase in apoptosis as well as a decrease in myocyte proliferation through an up-regulation in myostatin expression.

In this regard, treatments able to inhibit myocardial myostatin activity (Wagner et al, 2005; Yang et al, 2005), as well as to reduce myocyte apoptosis (Webster and Bishopric, 2003; Von Harsdorf , 2004) may be useful to avoid the negative influence of this mechanism on the myocyte proliferative response and decrease the progressive myocyte damage in alcoholic cardiomyopathy.

Authors contribution:

J F-S, ML and RE were responsible for the study concept and design.
ML and EA contributed to the acquisition of clinical and heart function data
ML and JF-S performed the cardiac histology and immunohistochemistry studies
ES assisted in the data analysis and interpretation of findings
AUM and RS provided critical revision of the manuscript for important intellectual contents.

All authors critically reviewed the content and approved the final version for publication

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FIGURE LEGENDS:

Figure 1: Myocardium BAX immunohistochemical activity in a control donor compared to a subject with alcoholic cardiomyopathy.

1A. Non-alcoholic heart donor (control). Low reactivity in BAX assay is present in all myocytes. (Magnification x 250)

1B.- Alcoholic donor with dilated cardiomyopathy. Increased reactivity in perinuclear and subsarclolemmic areas may be detected in TUNEL, BAX and BCL 2 assays (arrows) (Magnification x 400).

Figure 2. Skeletal muscle myostatin immunohistochemical assay.

2 A. Myostatin knockout mouse with absolute absence of myostatin activity (Magnfication x250)

2 B. Wild-type mouse with focal areas of perinuclear and cytoplasmic myostatin reactivity (arrows) (magnification x400).

Figure 3. Myocardium myostatin immunohistochemical assay in a control donor compared to a subject with alcoholic cardiomyopathy. Low myostatin reactivity is evident in the control donor (3A) compared to a high-positive reactivity in the perinuclear area in the subject with alcoholic cardiomyopathy (arrow) (3B). (Magnification x 250, both).

Figure 4. Myocardium Ki-67 immunohistochemical assay in a control donor compared to a subject with alcoholic cardiomyopathy. Absent Ki-67 reactivity is evident in the control donor (3A, magnification x 250) compared to a positive reactivity in the perinuclear area of some myocytes in the subject with alcoholic cardiomyopathy (arrow) (3B, magnification x 400).

*Marked-up Manuscript (tracking changes made)

INCREASED MYOSTATIN ACTIVITY AND DECREASED MYOCYTE PROLIFERATION IN CHRONIC ALCOHOLIC CARDIOMYOPATHY.

Joaquim Fernández-Solà[1,2] M.D., Ph.D.; Meritxell Lluis M.D, Ph.D[1].; Emilio Sacanella[1,2] M.D., Ph.D.; Ramón Estruch[1,2] M.D., Ph.D.; Emilia Antúnez[1,3] M.D, Ph.D,; Alvaro Urbano-Márquez[1] M.D, Ph.D.

[1]Alcohol Research Unit,. Hospital Clinic. Institut d'Investigació August Pi i Sunyer (IDIBAPS).Department of Medicine University of Barcelona. España

[2]CIBEROBN Fisiopatologia de la Obesidad y la Nutrición. Instituto de Salud Carlos III, España

[3]Department of Medicine.University of Barcelona.Catalunya.España

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Corresponding author:

Joaquim Fernandez-Solà M.D.

Department of Internal Medicine. Alcohol Unit

Villarroel 170. 08036. BARCELONA. ESPAÑA

Phone and FAX: 00.34.93. 2279365

E-mail: jfernand@clinic.ub.es

ABSTRACT

Background: Apoptosis mediates in alcohol-induced heart damage leading to cardiomyopathy (CMP). Myocyte proliferation may compensate for myocyte loss. Myostatin is up-regulated after cardiac damage and by alcohol consumption thereby decreasing myocyte renewal. We assess the potential role of alcohol in inducing myocyte apoptosis as well as in inhibiting myocyte proliferation.

Methods: Heart samples were obtained from organ donors, including 22 high alcohol consumers, 22 with hypertension, 8 with other causes of CMP, and 10 healthy donors. Evaluation included medical record with data on daily, recent and lifetime ethanol consumption, chest X ray, left ventricular (LV) function assessed by 2-D echocardiography and LV histology and immunohistochemistry. Apoptosis was evaluated by TUNEL, BAX and BCL-2 assays. Myocyte proliferation was evaluated with Ki67 assay. Myostatin activity was measured with a specific immunohistochemical assay. CMP was assessed by functional and histological criteria.

Results: Alcoholic and hypertensive donors with CMP showed higher apoptotic indices than did their partners without CMP. Myostatin activity was higher in alcoholics than in controls, mainly in those with CMP. The increase in myostatin expression in alcoholic CMP was higher than in other groups. The Ki-67 proliferation index increased in all groups with CMP compared to those without CMP, with alcoholics showing a lower increase in this proliferation response.

Conclusions: Alcohol produces cardiac myocyte loss through apoptosis but also partially inhibits myocyte proliferation through myostatin up-regulation. The final result may suppose an imbalance in myocyte homeostasis, with a net loss in total ventricular myocyte mass and progressive ventricular dysfunction.

KEY WORDS: Alcohol, Myocardium, Apoptosis, Myostatin, Myocyte proliferation.

INTRODUCTION

Alcoholic cardiomyopathy (CMP) is a recognised major complication of chronic alcoholism, (Moushmoush and Ali-Mansour, 1991; Urbano-Márquez and Fernández-Solà, 2004). The disorder is consequence of a common, dose-related, toxic effect of alcohol, affecting one third of long-term alcohol abusers (Urbano-Marquez et al, 1989). This distinct form of congestive heart failure is responsible for 21–36% of all cases of non-ischaemic dilated CMP in Western society (Spies et al, 2001). The end-stage of this CMP is largely irreversible and is characterized by a significant loss of myocytes, hypertrophy of the remaining muscle cells and also development of progressive interstitial fibrosis (Fernández-Solà et al, 1994). Without complete abstinence, the 4-year mortality for alcoholic CMP is close to 50% (Laonigro et al, 2009). On the other hand, complete abstinence from alcohol may allow partial clinical and functional reversibility of alcoholic CMP (Guillo et al, 1997). In addition, Nicolás et al (2002) showed that controlled drinking (<60g/day) without complete abstinence was as effective as complete abstinence.

Mechanisms of alcohol-induced myocardial damage are multifactorial, with disruption of membrane composition, disturbances in calcium transients, excitation-contraction coupling mechanisms, energy and protein turnover, and changes in cell mechanisms of genetic control. All these factors are involved in the pathogenesis of this disease, often synchronously (Preedy et al, 1996; Molina et al, 2002; Urbano-Márquez and Fernández.Solà, 2004; Ren and World, 2008). As an end consequence of this process, ethanol induces myocyte damage that progress to myocyte death and cell loss, with replacement by interstitial fibrosis. This myocyte loss has long been attributed to non-inflammatory myocyte necrosis. However, as described in other CMP (Beltrami et al, 2001, 2003; Gürtl et al, 2009), myocyte apoptosis may play a role in the development of structural heart damage in this setting (Hajnóczky et al, 2005; Fernández-Solà et al, 2006).

Apoptosis has been clearly implicated in the pathogenesis of alcohol-induced organ damage (Saraste et al, 1999; Neuss et al, 2001), as evidenced in several tissues (Altura and Gebrewold, 1998; Casey et al, 2001; Hoeck and Pasimoro, 2002). In alcoholic patients without heart damage both pro-(BAX) and anti-(BCL-2) apoptotic mechanisms

are activated. When structural heart damage appears, the BCL2/BAX ratio decreases, and apoptosis is present in the heart muscle of high-dose alcohol consumers as occurs to a similar degree in other causes of structural heart damage such as longstanding hypertension (Fernández-Solà et al, 2006) or end-stage heart failure (Saraste et al, 1999; Williams, 1999).

Whereas cardiac myocytes have traditionally been considered to be terminally differentiated cells, the last few years have witnessed exciting new challenges to this dogma, including the discovery of a natural rate of apoptosis and renewal of cardiac myocytes via circulating bone marrow stem cells and resident cardiac progenitor cells (Anversa and Nadal-Ginard 2002; Bergmann et al 2009). The emerging concept of myocyte homeostasis supposes an equilibrium that is maintained with a balance between myocyte death and myocyte renewal (Hill and Olson, 2008; Buja and Vela, 2008). It is important to consider that myocyte death itself activates mechanisms of proliferation of the remaining cells and renewal from pluripotential local or systemic stem cells (Regula et al, 2004). In this scenario of heart remodelling and plasticity, there is place for physiological growth, for pathological growth, and also for antigrowth heart mechanisms (Hill and Olson, 2008). One of these antigrowth factors that control myocyte hypertrophy and proliferation is myostatin.

Myostatin is the growth differentiation factor-8 (GF-1), a potent inhibitor of skeletal muscle and heart growth (Joulia- Ekaza and Cabello, 2006). Myostatin controls cell cycle progression and inhibits skeletal myoblast proliferation and terminal differentiation. An increase in myostatin activity protects the cell from apoptosis. Its disruption causes increased skeletal mass with hypertrophy and hyperplasia of myocytes and increased myocyte proliferation (Joulia- Ekaza and Cabello, 2006; McKoy et al, 2007). Experimental studies suggest that myocyte myostatin increases with ethanol exposure (Lang et al, 2004). This effect of myostatin on skeletal myoblasts is not necessarily related to the same effects on the mature heart

We therefore tested the hypothesis whether the chronic effect of alcohol on the myocardium may induce an increase in apoptosis as well as an inhibition of myocyte proliferation through increased myostatin activity. For this purpose we used myocardial

tissue from human donors in which we can observe the effect of previous chronic alcohol consumption on the regulation of cardiac myostatin expression and also evaluate the induction of apoptosis and the degree of myocyte proliferation. We compared this effect of alcohol with that produced by longstanding hypertension and other causes of CMP, as well as in non-alcoholic, non-hypertensive controls.

MATERIAL AND METHODS

Patient and Control Selection

Over a four-year period (January 2003 to December 2006), we consecutively studied hearts from subjects who had brain death either of traumatic or cerebrovascular origin, and had been considered suitable as organ donors by the transplant team of the Hospital Clínic of Barcelona. Of 127 cadaveric donors younger than 70 years of age, 67 hearts were not suitable for transplantation. Of these latter organs we selected: 1) donors with chronic hypertension, 2) donors with a history of ethanol intake ($\geq$ 60 g/day, longer than 10 years), 3) donors with other causes of heart disease, and 4) control hearts from healthy people who were not eligible for implantation because of a lack of matched receptor or size inadequacy.

All patients were white Caucasians of Spanish descent, who lived with their families in or around Barcelona and none was indigent. The study protocol was approved by the Ethics Committee of the Hospital Clínic and included informed consent from the families of the donors concerning the use of myocardium tissue for this research protocol study. One third of these subjects had been included in previous studies on heart antioxidant status (Fatjó et al, 2005) and cardiac apoptosis (Fernández-Solà et al, 2006).

All cases had been admitted to the Intensive Care Unit and ventilatory and haemodynamic parameters had been appropriately maintained at normal values throughout hospitalisation: P_aO_2 greater than 60 mmHg, systolic blood pressure greater than100 mmHg, and arterial pH within the normal range. None of the patients required in-hospital cardiopulmonary resuscitation maneuvers.

Clinical and laboratory evaluation

A detailed history of ethanol intake was retrospectively obtained by consultation with family members using a structured questionnaire ("time-line follow-back method") (Sobel et al, 1979), as previously reported (Urbano-Márquez et al, 1989; Fernández-Solà et al, 1994). Duration of ethanol intake was calculated in each group as the total cumulated period of alcohol consumption in years, either recent or previous. The body mass index was determined as the actual body weight relative to the square of the body height (BMI, Kg/m^2). Patients were considered to have caloric malnutrition if the BMI was less than 17 Kg/m^2. Protein malnutrition was assessed by the following parameters obtained at hospital admission: haemoglobin, lymphocyte count, total protein, retinol-binding protein, pre-albumin and albumin.

Cardiac Studies

Past and present signs and symptoms of heart failure were evaluated in consultation with medical records and family members of the donors, and the New York Heart Association (NYHA) functional class was determined according to the Goldman activity scale (Goldman et al, 1981). Chest X-ray with measurement of cardiothoracic index and conventional electrocardiography were performed in all cases. Moreover, a bidimensional echocardiography was performed (Hewlet Packard Sonos 2500, USA) in 24 patients compared to none of the controls with a cardiothoracic index greater than or equal to 0.48 compared to none of the controls. End-diastolic and end-systolic diameters, the shortening fraction, left ventricular (LV) mass, and the ejection fraction (EF) were measured according to the standards of the American Society of Echocardiography (Gottdiener et al, 2004). Cardiomyopathy was defined in the presence of LVEF < 50% and LV enlargement. We observed a good correlation between the cardiothoracic index and the left-ventricle end-diastolic diameter ($r = 0.68$, $p < 0.01$). The personnel who performed and evaluated these tests had no knowledge of the alcoholic history of the patients.

<u>Myocardium histological studies</u>

A distal 3 cm sample of the left-ventricle apex was surgically excised (total weight of 4-5 g) at the time the donor was under cold perfusion. The specimen was cut into fragments, and one of these was processed for further histological analysis. The remaining fragments were immediately frozen under liquid nitrogen, until apoptosis,

myostatin and Ki-67 studies were performed. For histological studies, specimens were stained with haematoxylin-eosin and toluidine-blue in semi-thin sections. Two independent observers (JF-S and AU-M) morphologically differentiated myocytes from other cells and evaluated the degree of myocardial cell and nuclear hypertrophy, myocytolysis (defined as the presence of myofiber disarray, or cell vaquolization) and interstitial fibrosis. In case of discordance a consensus agreement was established. The amounts of interstitial fibrosis (volume fraction of fibrosis) and cardiac muscle cells (volume fraction of the myocytes) were assessed as previously reported (Fernández-Solà et al, 1994). The degree of global histology involvement was graded as normal, mild, moderate, or severe according to previous histologically defined criteria (Fernández-Solà et al, 1994; Nicolás et al, 2002).

Evaluation of myocardial apoptosis.

Myocardial apoptosis was evaluated using immunohistochemical studies. BAX and BCL-2 activities were considered as indicators of pro and anti-apoptotic status, respectively. Semi-thin cryostat sections of 6μ were obtained with ultra-microtome and fixed by paraformaldehyde 4% PBS solution at pH 7.40. After PBS washing, sections were permeabilized with 0.1% Triton X-100 sodium citrate solution for 2 minutes at 4ºC. Incubation with TUNEL *(deoxyribonucleotidyl-transferase-mediated-dUTP-biotin nick-end labelling)* was performed using the *In Situ* cell Death Detection Kit® (Boehringer, Manheim, Germany) for 60 min at 37ºC inside a wet chamber, as described by the manufacturer. After PBS washing, a second incubation with Converter-Alkaline phosphatase was performed during 30 minutes under the same conditions. The reading process was performed by adding Fast Red chromogen for 10 min, under microscopic control. Incubation with BAX and BCL-2 reagents were performed in semi-thin cryostat sections of 6μ fixed by ketone at -4ºC. After primary antibody exposure (Biogenex, San Ramon CA, USA AR 347-5R for BAX, and AM 287-5R for BCL-2), link and labelling reactants were sequentially exposed for 20 min each (Kit Detection Supersensitive, Biogenex, San Ramon CA, USA, QA000-5L), with a final Fast Red chromogen incubation of 10 minutes. Negative control slides were made in all the procedures using the same process without reagent incubation. Positive controls were performed using human palatinal amygdalar tissue, in which lymphoid cells take high TUNEL, BAX and BCL-2 activities.

The apoptotic index (Ai) was determined as the percentage of apoptotic cells, and calculated by dividing the total number of positive staining heart myocyte nuclei in the TUNEL assay by the total number of the myocyte nuclei evaluated and multiplying this value by 100 as previously reported (Narula et al,1996; Paul et al, 2001). Cell nuclei at the edges of the tissues or in the interstitium were not counted. The count was made on at least 5 different fields (magnification 250x) of each sample, with at least 500 nuclei per field, according to the method described by Sandri et al (1995). Positive cells for BAX and BCL-2 staining were considered in the presence of diffuse cytoplasmic hyperenhancement, subsarcolemmal or perinuclear deposition of the reagent, and expressed as a percentage of positive to total evaluated cells. Interstitial deposition of these reagents and peripheral zones of the samples were not considered in the evaluation. We carefully performed a morphological identification of different cell types to exclude fibroblasts, adipocytes and endothelial cells in these counts.

Evaluation of myocardic repair mechanisms: Myostatin and Ki-67 immunohistochemical studies.

The evaluation of myocardium repair mechanisms was performed by means of myostatin and Ki-67 inmunohistochemmical assays on frozen tissue.

Myostatin (GF-β1) myocardium assay was performed using a commercial kit with the monoclonal antibody (GDF8 -ab996- datasheer, Abcam, USA) with human specificity for myostatin. This antibody has an immunogen sequence common to myostatin and its precursor AA 348-364 (NMLYFNGKEQIIIGKI) that detects all the forms of myostatin: precursor, dimmers and monomers. Dilution was performed at 1/1,000 in a buffer-citrate solution at pH 6.0. Lecture was performed by the compatible secondary antibody ab6722 linked to peroxidase This antibody has nuclear and cytoplasmic reactivity. To proof the anti-myostatin antibody specificity of this antibody we used muscle specimen from myostatin knockout mouse (Se-Jin Lee, Molecular Biology & Genetics, Johns Hopkins University School of Medicine. Baltimore, USA) and compared the reactivity to muscle of wild-type mouse. Evaluation of myostatin activity was performed by means of a semiquantitative study evaluating the percentage of positive cells with respect to total evaluated myocardial cells. In each case a minimum of 3,000 myocytes were evaluated. We compared results from cases (alcoholics) with

healthy donors and also pathological controls either with hypertension or other causes of CMP.

Ki-67 myocardium activity was evaluated by immunohistochemical assay on frozen myocardium tissue. Myocardium samples were fixed by ketone at-4ºC during 10 minutes, followed by PBS washing and serum blockade during 30 minutes. Primary antibody incubation was performed with the nuclear Ki-67 marker in a wet chamber, followed by washing and incubation with the secondary antibody linked to alkaline phosphatase during 30 minutes in a wet chamber. Lastly, the sample was submitted to gentle washing, revealed with substrate and counterstain with haematoxylin.

Statistical Analysis

The data were analysed using SPSS-PC 14.0 statistical software (SPSS, Chicago, IL). Differences between groups were analysed using a chi-square test, analysis of variance (ANOVA), and a two-tailed unpaired Student *t*-test. Pearson correlation coefficients between the variables were calculated. As all variables followed a normal distribution, variables were expressed as mean ± standard deviation. A two-tailed P value <0.05 was considered statistically significant.

RESULTS:

Clinical data

From the 67 donors selected, three heart specimens from subjects with concomitant hypertension and alcoholism were not included in order to maintain the homogeneity of the study groups. Two samples which were not adequately cryopreserved were also excluded. Therefore, a total of 62 organ donors fulfilled the selection criteria and were finally included in the study. Twenty-two were high-dose chronic alcohol consumers, 22 non-alcoholic individuals had long-standing essential hypertension, 8 individuals had other causes of CMP (3 coronary disease, 3 idiopathic CMP and 2 valve heart disease). Ten previously healthy donors without regular alcohol consumption did not report any significant disease and were included as control group.

Table 1 shows the epidemiological and clinical data of the patients and controls. The groups of controls, chronic alcoholics, those with hypertension and donors with CMP of

other origin were similar in age and gender. Cerebrovascular disease was the main cause of death in the alcoholics and hypertensive patients, whereas cranial trauma was the main cause in the controls group and in those with other causes of CMP. The time from hospital admission to organ extraction was similar in the four groups. According to family members chronic alcoholics had a mean daily ethanol consumption of 155.1 ± 50.4 g during a period of 22.8 ± 5.1 years, with a lifetime dose of ethanol of 16.9 ± 5.8 Kg ethanol/Kg body weight (Table 1). Alcoholic patients reported a greater active smoking habit, with a mean of 35 ± 12 packs-year, compared to the other groups of donors ($P < 0.01$). Hypertensive patients reported a mean duration of high blood pressure of 16.4 ± 5.6 years.

Evaluation of the medical record revealed that all control subjects had NYHA I functional class. Of the 22 chronic alcoholics, 13 (59%) had NYHA I class, seven (32%) NHYA II class and two (9%) NYHA III class. In the group of 22 subjects with hypertension, six (27%) had NYHA II class and two (9%) NYHA III class. In the group of 8 donors with other causes of CMP, two subjects (25%) had NYHA II class and one (12%) NYHA III.

Neither controls nor alcoholics had received any regular medication over the three months prior to admission. By contrast, most of hypertensive patients were under maintenance treatment with angiotensin-converting enzyme inhibitors at the usual doses (n = 18) and/or diuretics (n = 4). Alcoholic, chronic hypertensive and patients with other cardiac diseases presented a greater cardiothoracic index than controls. The electrocardiogram was normal in all controls but one who showed signs of left ventricular hypertrophy, being abnormal in 10 alcoholics (45%), and in 15 (68%) chronic hypertensive patients who showed changes in ST wave, conduction defects and signs of left ventricular hypertrophy. All subjects with other causes of CMP showed electrocardiogram abnormalities with signs of ischaemia (n=3) and left ventricular hypertrophy (n=5). The left ventricular ejection fraction was similar in alcoholics, hypertensive and donors with other causes of CMP, being significantly lower in all these groups than in controls ($p<.01$) (Table 1).

Biochemical and nutritional parameters.

With respect to nutritional parameters the BMI was comparable in all the groups studied. Only two subjects with chronic alcoholism, one subject with hypertension and

other with ischaemic CMP exhibited borderline signs of caloric malnutrition. Regarding protein nutritional parameters, alcoholics showed lower mean total serum protein and albumin concentrations compared to the other groups ($p < 0.05$, all). In addition, alcoholics exhibited higher levels of aspartate aminotransferase and gammaglutamyl transpeptidase compared to the other groups ($p < 0.05$, all). Leukocyte, lymphocyte, and platelet counts as well as haemoglobin concentrations and serum muscle enzymes were similar in all groups.

Cardiac evaluation

Cardiomyopathy was defined according to the criteria described in the Methods section in 12 chronic alcoholics (55%), 13 individuals with essential hypertension (59%) and in all 8 cases of CMP of other origins. Table 3 discloses the cardiac functional data of the subgroups of patients according to the presence or absence of CMP. All alcoholics, hypertensive patients and subjects with other causes of CMP with NYHA class II-III had enlarged hearts. No difference in nutritional data was observed among the subgroups of patients. Alcoholic patients with CMP consumed a significantly greater daily and lifetime ethanol intake compared to those without CMP.

Myocardial histological analysis

Except for slight signs of nuclear and myocyte hypertrophy in two specimens, histological evaluation of myocardial samples from control donors was normal. Thirty out of 33 patients with CMP (91%) had myocyte cell and/or nuclear hypertrophy, and more than two-thirds demonstrated interstitial fibrosis. By contrast, only slight myocyte abnormalities were detected in one-third of the 19 patients without CMP. The histological degree of myocardial lesion was low in 64% of cases and moderate in 36%. Considering the origin of CMP, no differences were observed in the analysis of the histological parameters and degree of histological involvement on comparing the three different groups of donors.

Apoptosis immunohistochemical studies

Table 2 reflects the evaluation of apoptosis immunohistochemical studies in the different group of donors. Figure 1 shows BAX activitiy in a control donor compared to a subject with alcoholic CMP. Considering the presence of apoptosis in cardiac myocytes observed with the TUNEL immunohistochemical assay the apoptotic index did not differ among the groups studied (2.8 $\pm$ 1.6 % ; 3.7 $\pm$ 3.4 % ; 4.0 $\pm$ 3..2 % and 4.1 $\pm$ 5.0 % for controls, alcoholics, hypertensives and donors with other causes of CMP, respectively, p= NS). With respect to pro-apoptotic BAX and anti-apoptotic BCL-2 immunohistochemical assays, both BAX and BCL-2 reactivity was significantly higher in chronic alcoholics, hypertensives and donors with other causes of CMP compared to control donors ($p< 0.05$, all). Alcoholics showed the lowest BCL2/BAX ratio, although the differences did not achieve significance (p= 0.238, F 1.426, ANOVA).

Table 3 shows the evaluation of these immunohistochemical apoptosis parameters in the groups of donors with CMP. The results of the TUNEL assay measurements were significantly higher in alcoholics with CMP compared to those with normal heart function (5.2 $\pm$ 3.9 % vs 2.8 $\pm$ 1.6%, $p< .05$). BAX and BCL-2 activities were significantly higher in alcoholic and hypertensive donors with CMP compared to their partners without CMP ($p<0.05$, both). Alcoholics with CMP showed the lowest BCL2/BAX ratio.

Evaluation of myocardial repair mechanisms: **Myostatin and Ki-67 immunohistochemical studies.**

Figure 2 shows absence of myostatin immunohistochemical reactivity in skeletal muscle of myostatin GDF8 knockout mouse in comparison to focal perinuclear and cytoplasmic myostatin reactivity in wild-type mouse. Myostatin immunohistochemical reactivity was detected in the perinuclear area of 5.7 ± 1.4 % of control donors. The groups of donors with hypertension and the group with other causes of CMP presented a non-significant increase of myostatin values compared to controls (9.8 ± 5.3 and 9.4± 6.9%,

respectively, p= n.s.).In contrast, the group of alcoholic donors presented a significant increase in nuclear myocyte myostatin activity (12.4± 5,8%) compared to controls (P <0.01) (Table 2). Figure 3 shows a comparison between a low-reactivity myostatin assay in a control donor compared to a high-positive reactivity in an alcoholic with CMP.

Positive immunohistochemical reactivity for Ki-67, as a reflection of the myocyte proliferative process, was detected in 3.8 ± 0.9 % nuclei of control donors. As expected, the groups of donors with hypertension or other causes of CMP showed a significant increase in Ki-67 activity values compared to controls (11.1 ± 3.8 and 12.3 ± 2.5 %, p<0.01, respectively). Similarly, the group of alcoholic donors showed a significant increase in Ki-67 reactivity compared to controls (7.5 ± 3.9 % vs 3.8 ± 0.9%, p<0.05) (Table 2). Figure 4 shows a case of increased perinuclear Ki-67 reactivity in a donor with hypertensive CMP compared to low Ki-67 reactivity in a control subject.

When data analysis was performed separating the groups of donors according to the presence of CMP, we observed that alcoholic donors with CMP showed a significant increase in myocardium myostatin activity compared to alcoholic donors without CMP (15.7 ± 2.8.% vs 8.5 ± 2.3%, p<0.05). Inversely, myocardium Ki-67 activity was significantly lower in alcoholics with CMP compared with those without CMP (8.9 ± 1.6 vs 2.1 ± 1.8, p<0.05). Hypertensive donors with CMP showed a non-significant increase in myostatin and Ki-67 expression compared to hypertensive donors without CMP (13.5 ± 2.2 vs 9.2 ± 1.9, p=n.s.) (Table 3)

Table 4 shows the comparison of myostatin and Ki-67 myocardial immunohistochemical reactivity between control donors and the different groups of heart donors with hypertension or other causes of CMP. Compared to controls, the relative increase in myocardium myostatin activity in alcoholics with CMP was higher

that that observed in hypertensives and donors with other causes of CMP (2.7 fold vs 1.5 and 1.6 fold, respectively). In the ANOVA analysis, myostatin activity was significantly higher in the groups of donors with any cause of CMP compared to subjects without of CMP ($F = 2.449$, $p=.047$). The expected increase in Ki-67 myocardial proliferative activity in alcoholic donors with CMP was lower that that observed in the other group of donors with hypertension and other causes of CMP (3.3, 3.5 and 3.3 fold, respectively).

The ANOVA analysis demonstrated significant differences in the inter donor groups analysis with respect to myocardial myostatin activity, being significantly higher in alcoholics compared to the other groups of donors ($F= 2.45$, $P= 0.047$). In addition, alcoholic donors with CMP presented a significantly lower increase in Ki-67 expression compared to the other groups of donors with CMP of hypertensive or other origin (ANOVA, $F= 24.8$, $p <.001$).

DISCUSSION

In the present study, performed by immunohistochemical methods in myocardium tissue proceeding from heart donor subjects, we found an increase in myocardial myostatin expression in subjects with different causes of CMP either of alcoholic, hypertensive, coronary, valvular or idiopathic origin. These results may be expected since ventricular myostatin expression is reportedly up-regulated in the presence of diverse causes of myocyte damage (Sharma et al, 1999; Shyu et al, 2006). It is of interest to remark that in the setting of alcoholic CMP, myocardium myostatin expression is highly up-regulated in comparison to other groups of CMP. This fact corroborates the previously suggested hypothesis of alcohol up-regulation of myocyte myostatin and other growth factor activities (Lang et al, 2004).

With respect to the parameters of myocyte proliferation, evaluated in this study by myocardium Ki-67 expression, we also observed an increase in their expression in all the causes of CMP compared to healthy control donors. This is also an expected result as a reflection of the capacity of the myocardium cells to proliferate against a persistent lesion either of toxic (alcohol), hypertensive, coronary or valvular origin (Anversa and Nadal-Ginard, 2002; Beltrami et al, 2003).

However, in the case of donors with alcoholic CMP, this myocyte proliferative response was significantly lower compared to donors with hypertension or other causes of CMP, reflecting a relative inhibition of the normal proliferative response of the myocardium against persistent structural damage such as that induced by excessive alcohol consumption (Urbano-Márquez and Fernández-Solà, 2004). Thus, we observed that the relative increase in Ki-67 expression in ACM in comparison to control donors was 67% lower than the increase present in CMP of hypertensive or other origin. The relative increase in myocyte myostatin activity and the decrease in Ki-67 proliferative response in alcoholic subjects are in concordance with previous reports in experimental studies (Lang et al, 2004). In fact, alcohol consumption has been suggested to up-regulate myostatin activity in other tissues such as skeletal muscle (Lang et al, 2004). Due to the clear clinical, functional and structural relationship evidenced in the deleterious effects of chronic alcohol consumption on skeletal and cardiac muscle (Fernández-Solà et al,1994), a similar effect of alcohol was expected on myostatin activity in these two tissues.

Similar to what has already been previously reported (Fernández-Solà et al, 2006), in the present study we observed an increase in myocardium apoptotic activity in subjects with alcoholic-dilated CMP measured by detection of DNA fragmentation by the immunohistochemical TUNEL assay. In addition, we detected activation of pro- and

anti- apoptotic-regulating mechanisms (BAX and BCL-2, respectively) in organ donors with CMP either of alcoholic, hypertensive or other origin. The degree of apoptosis in alcoholics was of a similar magnitude to that observed in hypertension and other causes of CMP. Although the differences were not significant, alcoholic donors showed a lower BCL2/BAX ratio that the other groups of donors, reflecting a relative increase in pro-apoptotic mechanisms. However, apoptosis was significantly higher in donors with CMP compared to their partners without CMP. This increase in myocardium apoptosis is a reflection of a common pathogenic mechanism inducing progressive heart damage, independently of the origin of the damaging agent (Nadal-Ginard et al, 2003). In alcoholics, myocardium apoptosis does not only depend on ethanol consumption but also to the presence of structural heart damage (Jänkälä et al, 2002; Fernandez-Solà et al, 2006), demonstrating that myocardium apoptosis is a complex mechanism related not only to the original trigger agent but also to intermediate causes of myocardial damage that activate mechanisms of cell lesion such as mitochondrial caspases, disturbances in intracellular cell transients (Nicolás et al, 1998) protein synthesis (Fernández-Solà et al, 2007) or oxidative damage (Fatjó et al, 2005).

Myostatin activity has been reported to protect myocardium cells from apoptosis (Joulia Ecaza and Cabello, 2006). Therefore, the increase in myostatin activity could be a protective mechanism developed by cardiac myocytes against the toxic aggression of alcohol. However, a negative consequence of this myocardial myostatin up-regulation induced by alcohol is the inhibition of myocardium Ki-67 proliferation activity because of the anti-growth effect of myostatin (Shyu et al, 2005; McKoy et al, 2007). In the present study the negative effect of alcohol is more evident than the protective effect of myostatin in the induction of myocardial apoptosis. In addition, up-regulation of myostatin is not a single mechanism influencing the decrease in the myocardial

proliferative response. Other factors such as IGF-1 activity may also influence this process (Lang et al, 2004; Shyu et al, 2005). The existence of these multiple effects may explain the diversity of factors influencing changes in the cardiac homeostasis induced by ethanol misuse.

Thus, the chronic alcohol effect on the myocardium has a multi-factorial noxious effect, not only inducing a clear cell loss because of the increase in myocyte apoptosis but also inhibiting the relative capacity that conserves heart myocytes to proliferate though up-regulation of myostatin or other myocyte anti-growth factor activity. The final result of this process is a clear imbalance in myocyte homeostasis status, inducing a net loss in total ventricular myocyte mass, with progression to subsequent ventricular dysfunction as reflected in the natural history of alcoholic-dilated CMP (Fernández-Solà et al, 2008). In alcoholic CMP, this global effect implies a lower capacity of myocardial repair in comparison to hypertensive or other causes of CMP.

Due to the difficulty in obtaining human myocardium samples, the present study is restricted to a relatively small number of middle-age chronic alcoholic subjects of Caucasian origin, individuals with essential hypertension, other causes of CMP and controls. Due to the abstinence from alcohol established in the period comprised from hospital admission to heart donation, the acute effects of alcohol could not be considered. With the design of this study, only a retrospective approach for the drinking habits of all donors by the family members was possible. Since alcoholic donors had significantly higher active smoking consumption compared to the other groups, we cannot exclude an additional noxious effect of nicotine in the myocardium as reported in experimental (Rajiyah et al, 1996) and clinical studies (Zakhari, 1991), although nicotine heart effect mainly induces coronary artery lesions. Evaluation of myocardial apoptosis is limited to TUNEL, BAX and BCL-2 activities. With TUNEL

assay some degree of apoptosis over-detection is possible, since some DNA fragmentation is still reversible (Kanoh et al, 1999). Evaluation of myocardial repair mechanisms was limited to myostatin and Ki-67 activities. We corroborated the anti-myosin antibody specificity comparing the negative reactivity of this antibody in GDF8 knockout to the positive reactivity in wild type mouse. Since myocardial biopsy was taken only in the left-ventricle apex, this study did not consider architectural or functional changes within different sites of the myocardium considered in this study. With respect to the relatively high percentage of Ki-67 positive nuclei found in controls, we should consider that some studies have identified Ki-67 positivity in myocardiocytes as an expression of kariorrhexis but not proliferation (Kajstura et al, 1998; Beltrami et al, 2003).

Despite the reported limitations, the homogeneity of the sample and the significance of the results allow corroboration of the role of myostatin activity in alcoholic CMP in human samples as suggested in previous experimental animal studies (Lang et al, 2004).

In conclusion, we have demonstrated the existence of diverse toxic effects of alcohol on the myocardium. Myocye loss was partially due to an increase in apoptosis as well as a decrease in myocyte proliferation through an up-regulation in myostatin expression.

In this regard, treatments able to inhibit myocardial myostatin activity (Wagner et al, 2005; Yang et al, 2005), as well as to reduce myocyte apoptosis (Webster and Bishopric, 2003; Von Harsdorf , 2004) may be useful to avoid the negative influence of this mechanism on the myocyte proliferative response and decrease the progressive myocyte damage in alcoholic cardiomyopathy.

Authors contribution:

J F-S, ML and RE were responsible for the study concept and design.
ML and EA contributed to the acquisition of clinical and heart function data
ML and JF-S performed the cardiac histology and immunohistochemistry studies
ES assisted in the data analysis and interpretation of findings
AUM and RS provided critical revision of the manuscript for important intellectual contents.

All authors critically reviewed the content and approved the final version for publication

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FIGURE LEGENDS:

Figure 1: Myocardium BAX immunohistochemical activity in a control donor compared to a subject with alcoholic cardiomyopathy.

1A. Non-alcoholic heart donor (control). Low reactivity in BAX assay is present in all myocytes. (Magnification x 250)

1B.- Alcoholic donor with dilated cardiomyopathy. Increased reactivity in perinuclear and subsarclolemmic areas may be detected in TUNEL, BAX and BCL 2 assays (arrows) (Magnification x 400).

Figure 2. Skeletal muscle myostatin immunohistochemical assay.

2 A. Myostatin knockout mouse with absolute absence of myostatin activity (Magnfication x250)

2 B. Wild-type mouse with focal areas of perinuclear and cytoplasmic myostatin reactivity (arrows) (magnification x400).

Figure 3. Myocardium myostatin immunohistochemical assay in a control donor compared to a subject with alcoholic cardiomyopathy. Low myostatin reactivity is evident in the control donor (3A) compared to a high-positive reactivity in the perinuclear area in the subject with alcoholic cardiomyopathy (arrow) (3B). (Magnification x 250, both).

Figure 4. Myocardium Ki-67 immunohistochemical assay in a control donor compared to a subject with alcoholic cardiomyopathy. Absent Ki-67 reactivity is evident in the control donor (3A, magnification x 250) compared to a positive reactivity in the perinuclear area of some myocytes in the subject with alcoholic cardiomyopathy (arrow) (3B, magnification x 400).

Table 1

Table 1. Epidemiologic and clinical data of the different groups of heart donors.

	Control subjects (n = 10)	Alcoholic patients (n = 22)	Hypertensive patients (n = 22)	Patients with other causes of CMP (n=8)
Age (y; mean ± SD)	52.2 ± 13.2	53.3 ± 12.4	58.5 ± 9.1	57.3 ± 11.3
Male/female ratio (n)	7:3	16:6	13:9	6:2
Daily alcohol intake (g; mean ± SD)	0	155.1± 50.4***	15.3 ± 45.4	12.8 ± 43.9
Duration of ethanol intake (y; mean ± SD)	0	22.8 ± 5.1***	5.3 ± 2.1	5.0 ± 2.4
Lifetime dose of ethanol (kg ethanol/kg body weight; mean ± SD)	0	16.9 ± 5.8***	1.3 ± 0.2	1.1 ± 0.1
Active smokers [n (%)]	1 (14)	15 (79)***	4 (20)	2(24)
Cause of death [n (%)]				
Cranial trauma	6 (60)	5 (23)	2 (9)**	5 (63)
Cerebrovascular disease	4 (40)	15 (68)	19 (86)	2 (25)
Other	0	2 (9)	1 (5)	1 (12)
Time from admission to donation (h; mean ± SD)	31 ± 2	32 ± 3	29 ± 3	31 ± 2
NYHA function [n (%)]				
Class I	10 (100)	13 (59)	14 (64)	5 (63)
Class II	0	7 (32)	6 (27)	2 (25)
Classes III and IV	0	2 (9)	2 (9)	1 (12)
Cardiothoracic index (mean ± SD)	0.47 ± 0.01	0.54 ± 0.06**	0.55 ± 0.05**	0.55 ± 0.04**
Left ventricular ejection fraction (%; mean ± SD)	60 ± 3	45 ± 8**	44 ± 9**	40 ± 8**
Electrocardiogram [abnormal cases; n (%)]	1 (10)	10 (45) **	15 (68)*	8 (100)*

NOTE. Data are expressed as mean ± SD.

An abnormal electrocardiogram is characterised by the presence of rhythm disturbances, conduction defects, signs of left ventricular hypertrophy, or abnormal repolarization.

CMP: cardiomyopathy

* P< 0.05 compared to the other groups.
** P <0 .01 compared with control subjects.
***P< 0.01 compared to the other groups

Table 2

Table 2. Myocardial immunohistochemical apoptosis, myostatin and Ki-67 studies in the different groups of heart donors.

	Control subjects (n =10)	Alcoholic patients (n = 22)	Hypertensive patients (n = 22)	Patients with other causes of CMP (n=8)
TUNEL (% of myocardial cells)	2.8 ± 1.6	3.7 ± 3.4	4.0 ± 3.2	4.1 ± 5.0
BAX (% of myocardial cells)	3.8 ± 4.4	215 ± 10.*	14.9 ± 13.5*	13.3 ± 12.1*
BCL-2 (% of myocardial cells)	2.6 ± 2.1	11.9 ± 8.0*	11.5 ± 11.6*	13.2 ± 10.8*
BCL2/BAX (ratio)	0.68 ± 0.6	0.55 ± 0.3	0.77 ± 0.8	0.99 ± 0.9
MYOSTATIN (% of myocardial cells)	5.7± 1.4	12.4 ± 5.8**	9.8 ± 5.3	9.4 ± 6.9
Ki-67 (% of myocardial cells)	3.8 ± 0.9	7.5 ± 3.9 *	11.1 ± 3.8**	12.3 ± 2.5**

NOTE. Data are expressed as mean ± SD.
* P< 0.05 compared with control subjects .
** P < 0.01 compared with control subjects
CMP: cardiomyopathy

Table 3

Table 3. Functional and immunohistochemical myocardium studies in apoptosis, myostatin and Ki-67 activities in the different group of heart donors according to the presence of cardiomyopathy (CMP)

	Alcoholic patients without CMP (n=10)	Alcoholic patients with CMP (n = 12)	Hypertensive patients without CMP (n = 9)	Hypertensive patients with CMP (n = 13)	Patients with other causes of CMP (n= 8)
Left ventricular ejection fraction (%; mean ±SD)	59 ± 4	38 ± 6*	60 + 3	37 + 4 **	36 + 5
Cardiothoracic index (mean ± SD)	0.48 + 0.02	0.59 + 0.04*	0.49 + 0.01	0.57 + 0.01**	0.47 + 0.02
TUNEL (% of myocardial cells)	2.8 ± 1.6	5.2 ± 3.9*	3.3 ± 3.0	5.3 ± 4.7	4.1 ± 4.5
BAX (% of myocardial cells)	12.1± 3.2	31.7 ± 13.2*	11.3 ± 3.1	20.4 ± 4.9**	13.3± 12.1
BCL-2 (% of myocardial cells)	8.3 ± 5.1	17.7 ± 8.1*	7.5 ± 5.3	15.5 ± 9.3**	13.2 ± 10.8
BCL2/BAX (ratio)	0.68 ± 0.5	0.56 ± 0.4	0.66 ± 0.9	0.75 ± 0.6	0.99 ± 0.9
MYOSTATIN (% of myocardial cells)	8.5 ± 2.3	15.7 ± 2.8*	8.3 ± 3.3	12.2 ± 3.0	9.4 ± 6.9
Ki-67 (% of myocardial cells)	2.1 ± 1.8	8.9 ± 1.6*	9.2 ± 1.9	13.5 ± 2.2	12.3 ± 2.5

NOTE. Data are expressed as mean ± SD.
* P < 0.05 compared with alcoholics without cardiomyopathy.
** P < 0.05 compared with hypertensive patients without cardiomyopathy

Table 4

Table 4. Comparison of myocardial immunohistochemmical studies in myostatin and Ki-67 activities in controls versus different groups of heart donors with hypertensive or other causes of cardiomyopathy (CMP)

	Controls (n = 10)	Alcoholic patients with CMP (n=12)	Hypertensive patients with CMP (n = 13)	Patients with other causes of CMP (n = 8)
MYOSTATIN (% of myocardial cells)	5.7 ± 1.4	15.7 ± 2.8*	8.3 ± 3.3	9.4 ± 6.9
% increase with respect to the controls		x 2.7	x 1,5	x 1.6
Ki-67 (% of myocardial cells)	3.8 ± 0.9	8.9 ± 1.6**	13.5 ± 2.2	12.3 ± 2.5
% increase with respect to the controls		x 2.3	x 3.5	x 3.3

NOTE. Data are expressed as mean ± SD.
* .P < 0.01 compared with control subjects.
** .P = 0.07 compared with control subjects.

Figure 1 A

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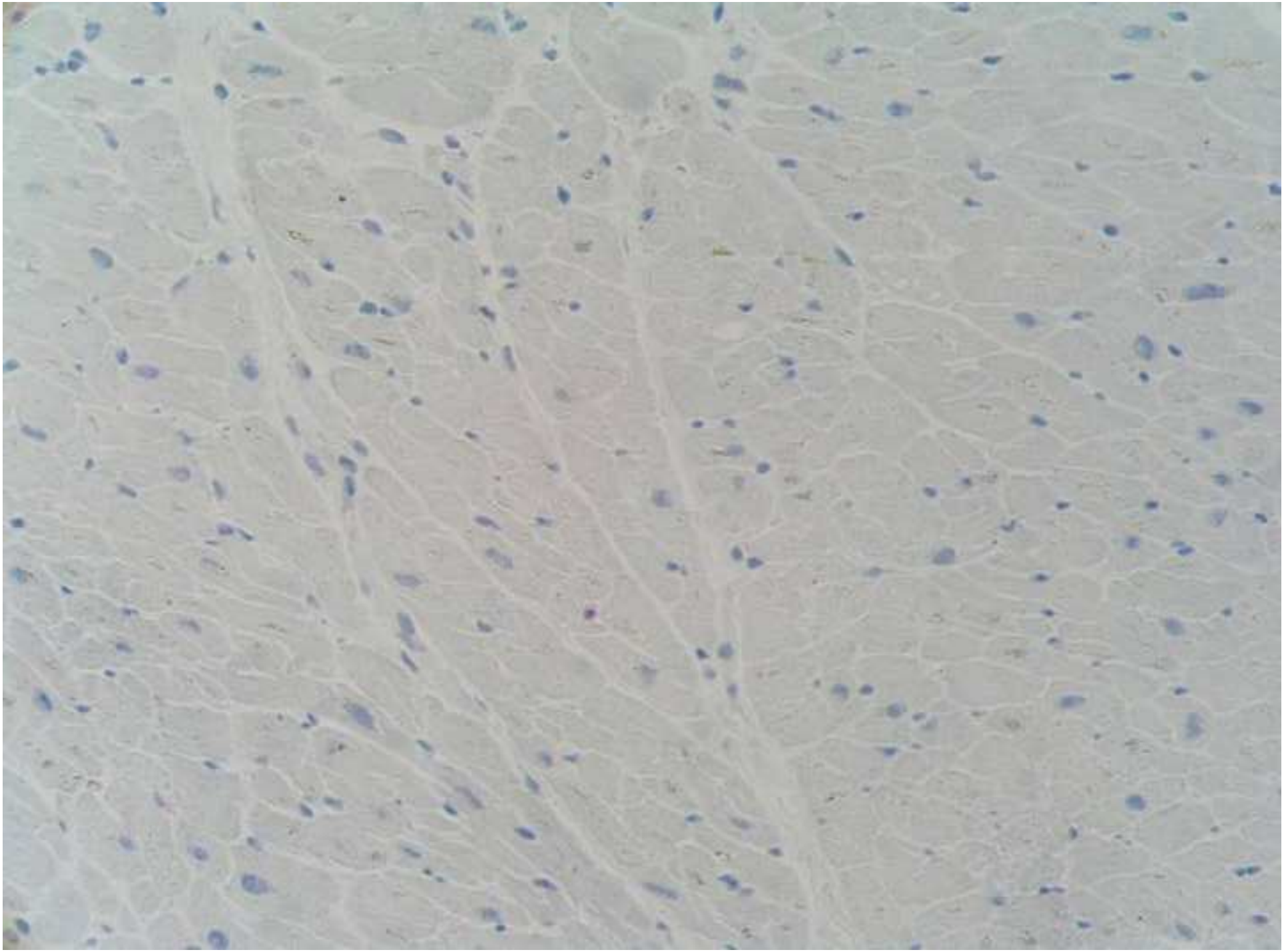

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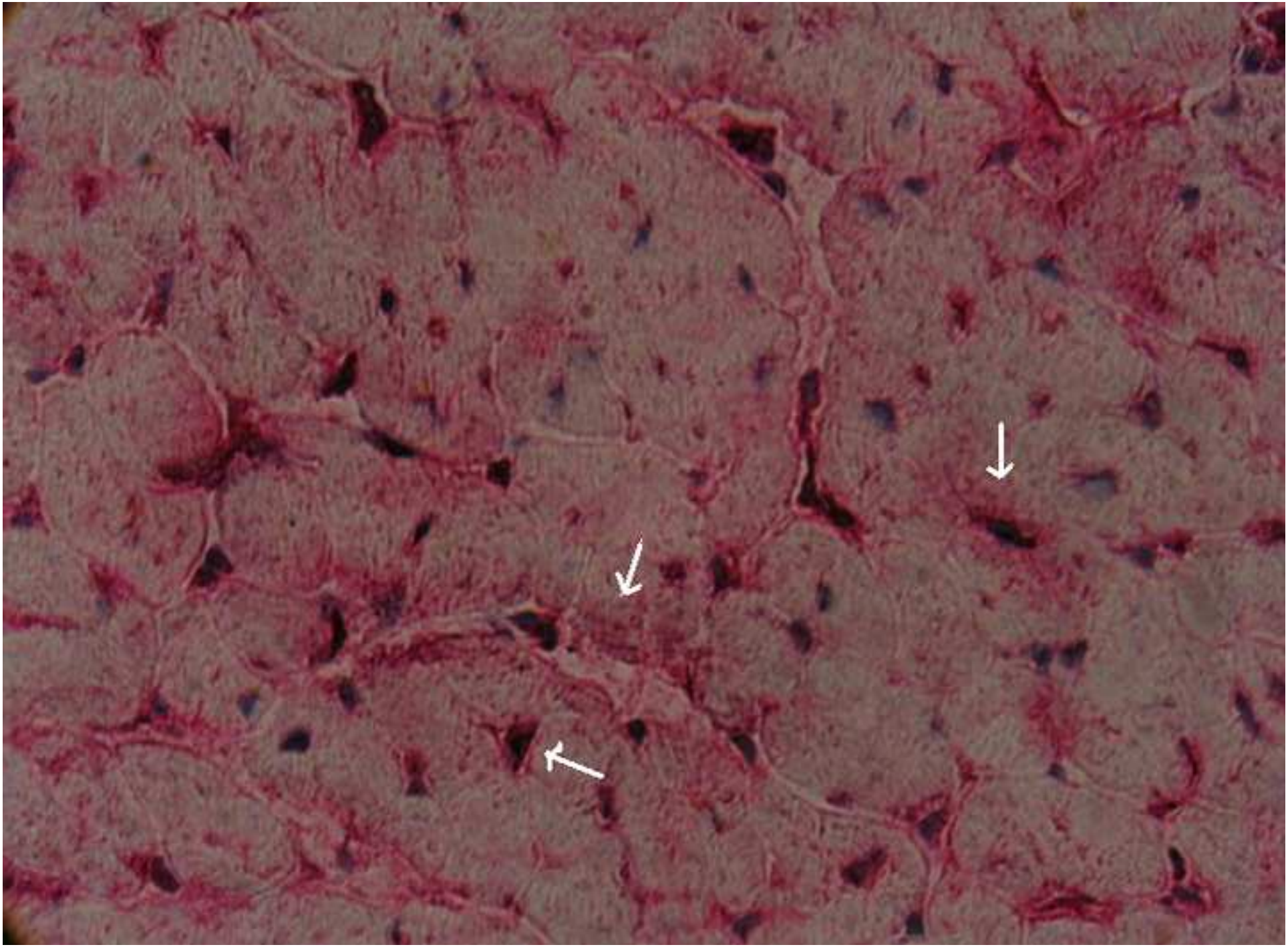

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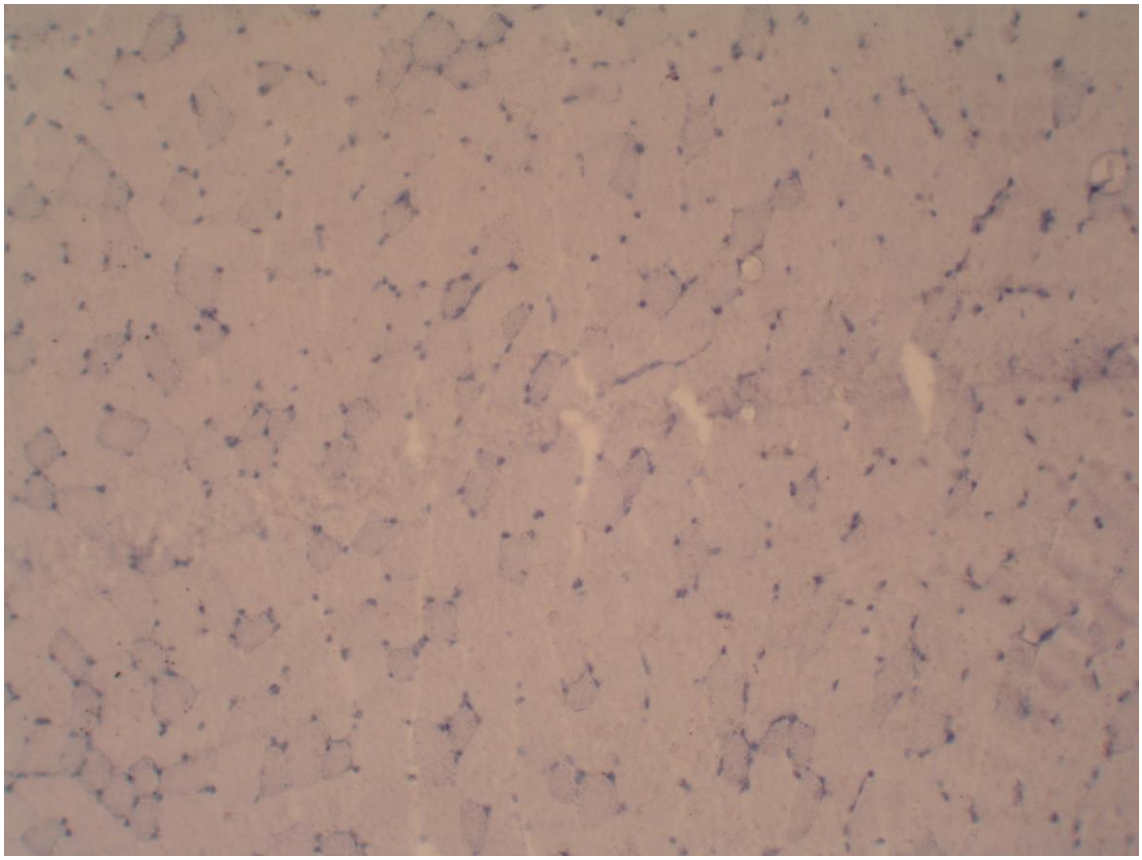

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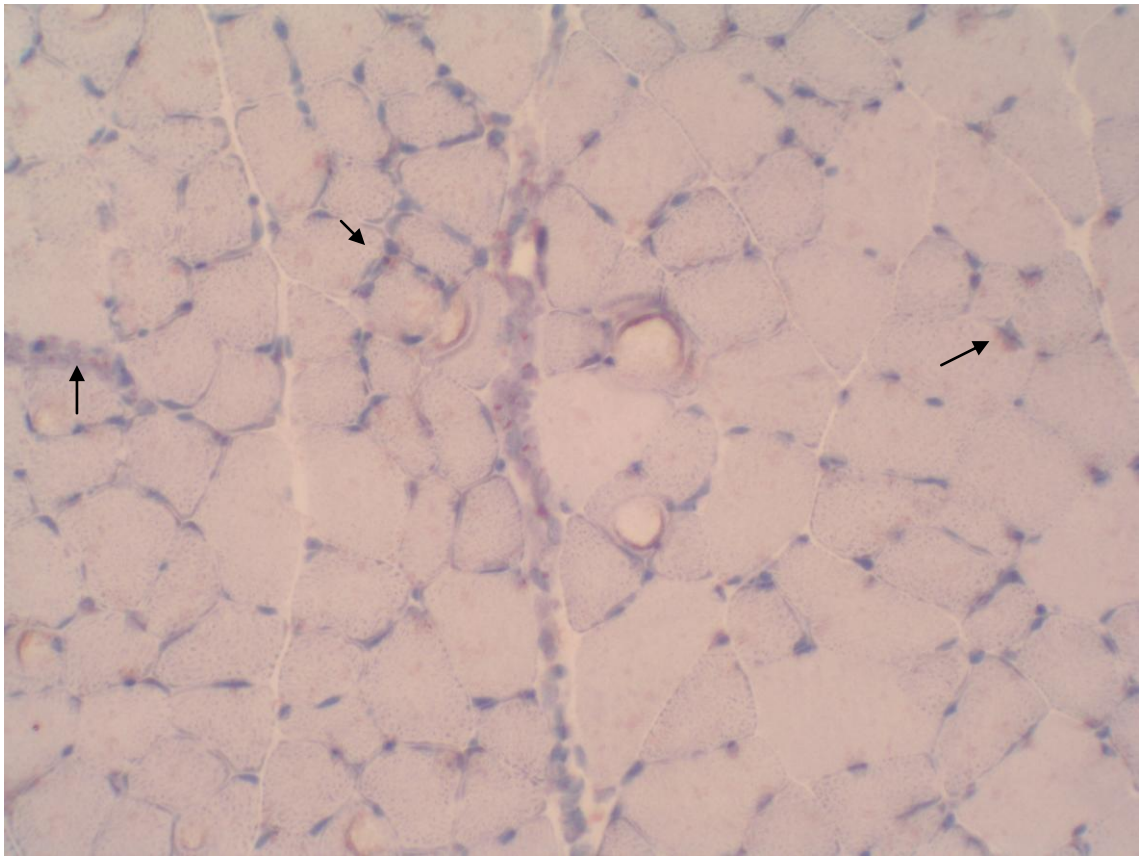

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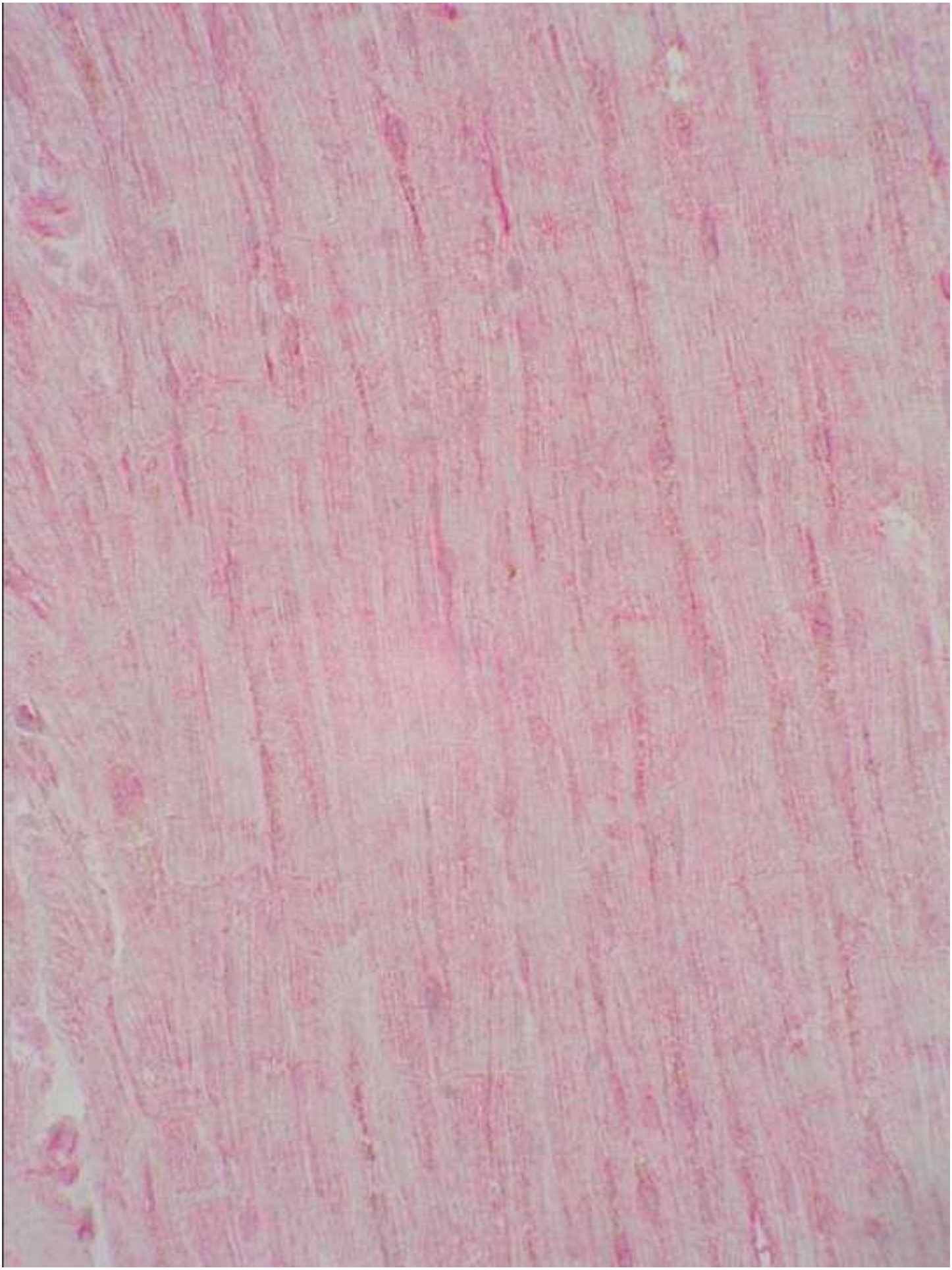

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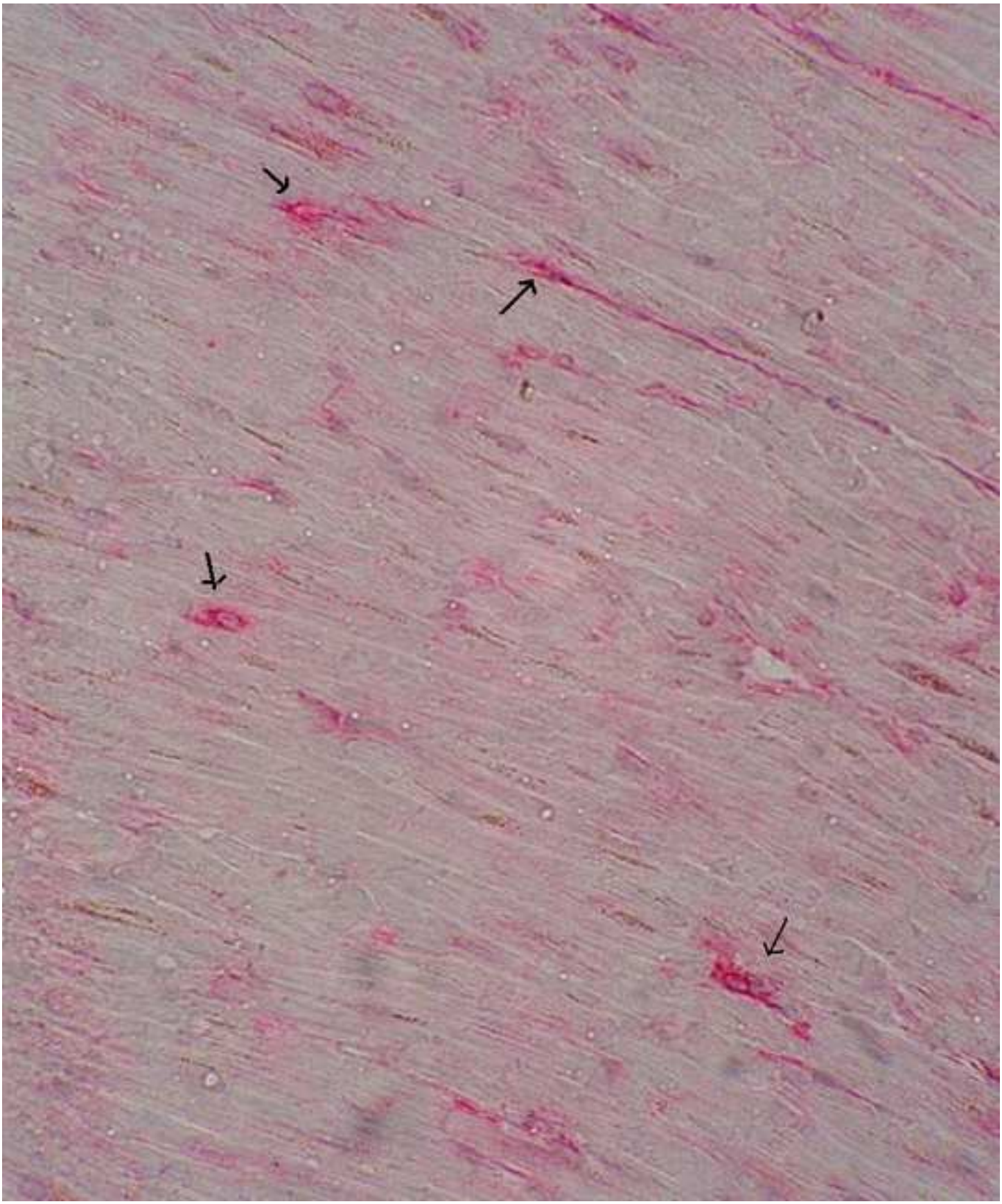

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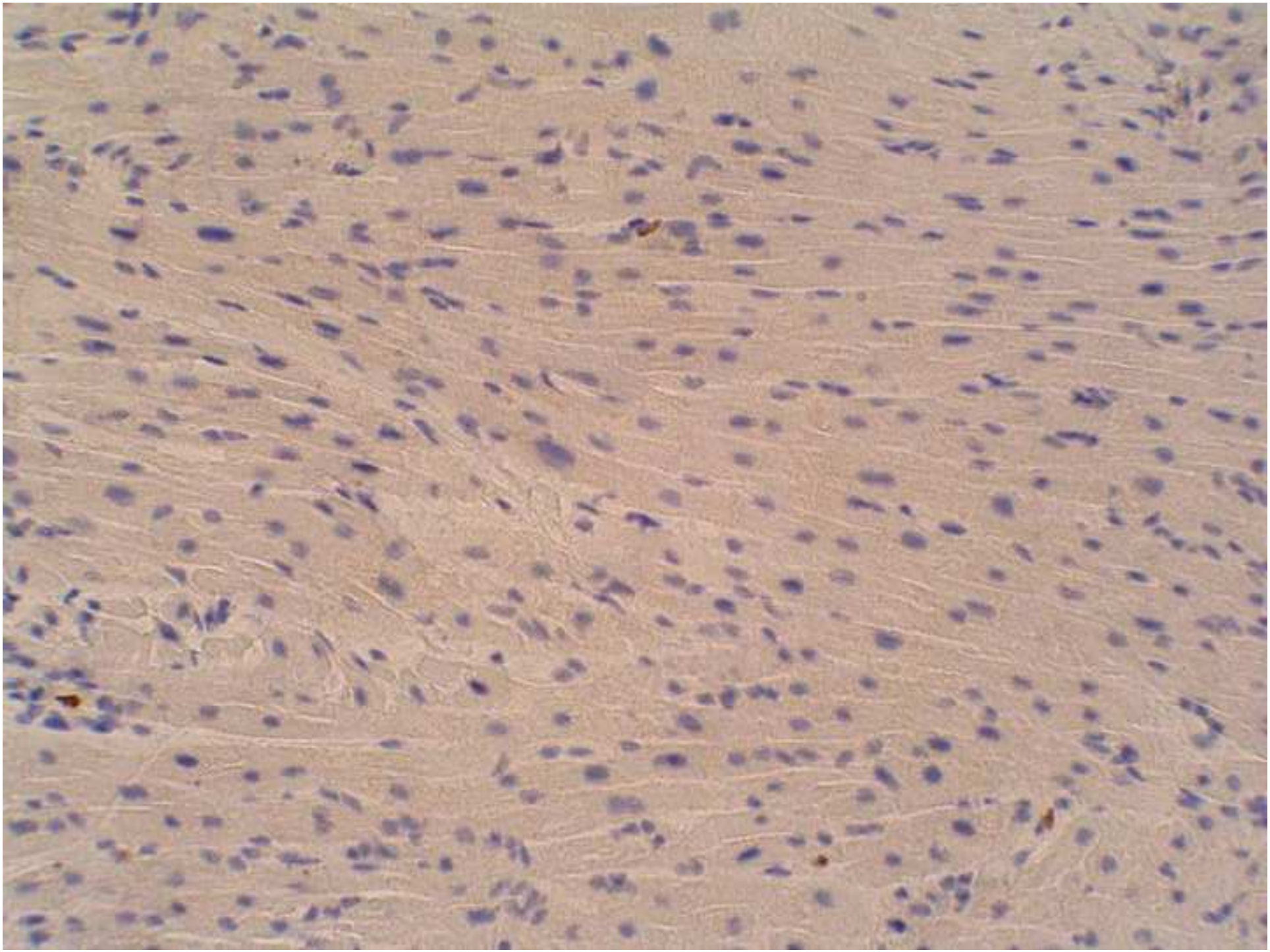

Figure 4B

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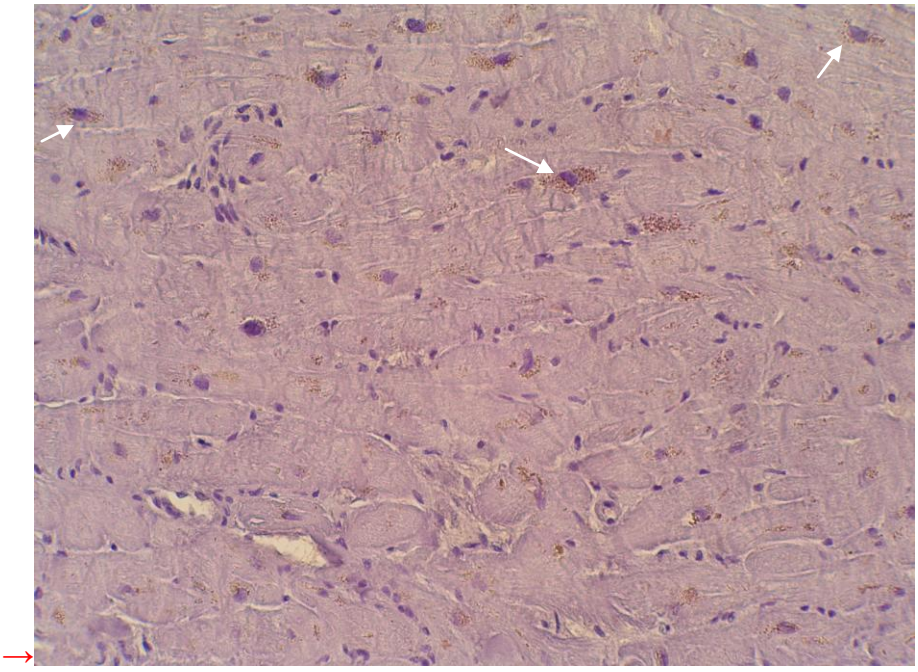

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To the Editor
Alcoholism Clinical & Experimental Research

Barcelona (Spain) April 20th,2010

Ref: *1st manuscript submission as original article*

Please find attached the original article entitled "**INCREASED MYOSTATIN ACTIVITY AND DECREASED MYOCYTE PROLIFERATION IN CHRONIC ALCOHOLIC CARDIOMYOPATHY**" to be considered for publication as a ORIGINAL ARTICLE in *Alcoholism Clinical & Experimental Research.*

This is an original study in which we evaluated the role of myostatin in alcoholics versus patients with other causes of cardiomyopathy, as well as the influence that alcohol may have in decreasing myocyte proliferation using a model of human myocardium proceeding from organ donors. To our knowledge no papers in this field have been previously published elsewhere.

This study was performed by the Alcohol Research Group of Hospital Clinic, University of Barcelona, which has been studying the pathogenic aspects of alcohol cardiomyopathy for more than 25 years. We have carefully followed the instructions for authors in the preparation of the manuscript. All authors qualify for authorship and have significantly contributed to the paper and have approved this submitted version. Conflict of interest: none declared for all authors.

The submitted manuscript has been read and approved by all signatories, All authors acknowledge that they have exercised due care in ensuring the integrity of the work, and none of the original material contained in the manuscript has been submitted for consideration nor will any of it be published elsewhere except in abstract form in connection with Meeting of the European Society for Biochemical Research on Alcoholism (ESBRA) June 2009, Helsinki, Finland.

We hope that the manuscript will found suitable for publication in your journal.
Looking forward to hearing from you.
Yours sincerely,

Joaquim Fernández-Sola M.D, .Ph.D.

Meritxell Lluis M.D, Ph.D[1].

Emilio Sacanella M.D., Ph.D.

Ramón Estruch M.D., Ph.D

Emilia Antúnez M.D, Ph.D

Alvaro Urbano-Márquez[1] M.D, Ph.D.

HOSPITAL CLÍNIC I PROVINCIAL DE BARCELONA
Villarroel, 170 - 08036 Barcelona (España)
Tel. 93 227 54 00 Fax 93 227 54 54

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Names of all authors in the order in which they appear in the Article: ..... Joaquim Fernández-Solà[1,2] M.D., Ph.D.; Meritxell Lluis M.D, Ph.D[1].; Emilio Sacanella[1,2] M.D., Ph.D.; Ramón Estruch[1,2] M.D., Ph.D.; Emilia Antúnez[1,3] M.D, Ph.D,; Alvaro Urbano-Márquez[1] M.D, Ph.D. .....

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INCREASED MYOSTATIN ACTIVITY AND DECREASED MYOCYTE PROLIFERATION IN CHRONIC ALCOHOLIC CARDIOMYOPATHY
Alcoholism: Clinical and Experimental Research

Dear Dr. Harper,

Please find the second reviewed version of this original article.

Sorry for delay in answering, but the requested modifications were difficult, and take us some time to accomplish.

We appreciate all the comments by the reviewers and the editor which have contributed to improving the article.

We have submitted a revised version of the manuscript in the online ACER system.

In concrete, as requested by the reviewer, we have performed a proof of the anti-myostatin antibody GDF8 ABCAM specificity. Prof See-Jin Lee provided us muscle form GDF8 knockout and also wild-type mouse. We performed immunohistochemmical myostatin assays in those specimens. As expected, myostatin KO mouse do not show any reactivity. In wild-type mouse, myostatin reactivity was evident in focal myocyte cytoplasma and peri-nuclear areas of myocytes. We consider that this assay proofs a good myosatin specificity of the used antibody. We have included a new Figure 2 showing this concrete myostatin KO vs wild type assay. We have modified accordingly the manuscript and re-ordered the Figure 3-4 numbers.

All changes in the reviewed version-2 are highlighted in yellow.

Below please find point by point answers to the reviewers.

We hope that the manuscript is now found suitable for publication in Alcoholism Clinical and Experimental Research.

Looking forward to hearing from you.

Yours sincerely,

Joaquim Fernandez-Sola M.D., Ph.D.
Alcohol Unit, Hospital Clinic. Barcelona SPAIN

ANSWERS TO REVIEWERS' COMMENTS:

REVIEWER # 1:

We appreciate the evaluation performed and the considerations of this reviewer to impove this paper.

As requested by the reviewer, we have performed a proof of the anti-myostatin antibody specificity.
Prof See-Jin Lee provided us muscle from myostatin GDF8 knockout and also wild-type mouse. We performed immunohistochemmical myostatin assays in those specimens.
As expected, myostatin KO mouse did not show any reactivity.
In wild-type mouse, myostatin reactivity was evident in focal myocyte cytoplasma and perinuclear areas of myocytes.

We have included a new Figure 2 showing this concrete myostatin KO vs wild type assay.

We have modified accordingly the manuscript by including a paragraph:

1.- In the methods section (page 8, 2nd parg, lines 10-14):

"To proof the anti-myostatin antibody specificity of this antibody we used muscle specimen from myostatin knockout mouse (Se-Jin Lee, Molecular Biology & Genetics, Johns Hopkins University School of Medicine. Baltimore, USA) and compared the reactivity to muscle of wild-type mouse".

2.- In the result section (page 12, 2nd parg, lines 1-3):

"Figure 2 shows absence of myostatin immunohistochemical reactivity in skeletal muscle of myostatin GDF8 knockout mouse in comparison to focal perinuclear and cytoplasmic myostatin reactivity in wild-type mouse".

3.- In the discussion (page 18, lines 3-5):

"We corroborated the anti-myosin antibody specificity comparing the negative reactivity of this antibody in GDF8 knockout to the positive reactivity in wild type mouse".

All changes are highlighted in the new reviewed version of the manuscript