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# **Cardiac impairment induced by oxidative stress in rabbits fed a high-fat diet is counteracted by dehydroepiandrosterone supplementation**

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**Running head** Cardiomyopathy in rabbit fed a high-fat diet

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## Abstract

2 The role of oxidative stress in the activating of downstream signalling leading to structural and  
3 functional changes in the left ventricle of rabbits fed a high-fat diet for 12 wks and the effect of  
4 dietary dehydroepiandrosterone supplementation, were analyzed. The animals showed  
5 hyperglycemia, insulin-resistance, dyslipidemia and early features of cardiomyopathy, i.e. altered  
6 myosin heavy-chain isoforms, tissue degeneration and reduced contractility of the papillary muscle.  
7 Dietary supplementation with dehydroepiandrosterone (0.02%,wt/wt) in these animals reduced  
8 oxidative stress evaluated as levels of reactive oxygen species and hydroxynonenal levels, without  
9 affecting high plasma glucose level or insulin resistance. Dehydroepiandrosterone supplementation  
10 also counteracted both the activation of redox-sensitive transcription factor and of nuclear  
11 transcription factor-  $\kappa$ B, and the expression of tumor necrosis factor- $\alpha$  and of C-reactive protein-  
12 mRNA. Improvement of the oxidative balance counteracted the shift to myosin heavy chain  
13 isoforms, ameliorating the heart contractility. A high-fat diet induced oxidative stress and metabolic  
14 syndrome in this rabbit model, and that dehydroepiandrosterone, by restoring oxidative balance,  
15 lowering lipid levels and down-regulating inflammation, prevented molecular and functional  
16 alterations of the cardiac muscle, thus exerting a beneficial effect and delaying the onset of diabetic  
17 cardiomyopathy.

18

18 **Introduction.**

19 Diabetic cardiomyopathy is characterized by systolic and diastolic dysfunction and has been reported  
20 in diabetic patients with no ischemic, valvular or hypertensive heart disease. Its development  
21 includes metabolic disturbances, small-vessel diseases, autonomic dysfunction, insulin-resistance and  
22 myocardial fibrosis (1,2). Recently, an important role in its pathophysiology has been attributed to  
23 the generation of reactive oxygen species, which activates a number of secondary-messenger  
24 pathways, finally leading to cardiac dysfunction (3-5). We recently showed recently that free radical  
25 overproduction appears early in human type 2 diabetes (6) and that, in a rat model of type 1 diabetes,  
26 oxidative damage plays a key role in the early development of cardiomyopathy (7). Antioxidants  
27 might counteract insulin resistance associated with type 2 diabetes and cardiovascular diseases (8-  
28 10). We reported elsewhere that in streptozotocin rats, the treatment with dehydroepiandrosterone  
29 (DHEA), a compound that possesses multi-targeted antioxidant properties (11-15), prevents  
30 myocardial damage induced by oxidative stress, while avoiding impairment of cardiac myogenic  
31 factors and the switch to myosin heavy chain expression (16). DHEA also counteracts oxidative  
32 imbalance and advanced glycation end-product formation in type II diabetic patients (7).  
33 The study targets diabetic cardiomyopathy, in a model of type 2 diabetes induced by a high-fat (HF)  
34 diet in rabbits, an animal species that is highly susceptible to cardiovascular damage (17). Data show  
35 that DHEA treatment counteracted biochemical changes in the left ventricle induced by oxidative  
36 stress, improving myocardial function.

37 **Materials and methods**

38 **Experimental procedure.**

39 Male New Zealand White rabbits, 15 weeks old (Harlan-Italy, Udine, Italy) weighing 3.0 -3.5 Kg were  
40 cared for in compliance with the Declaration of Helsinki as revised in 1983, the Italian Ministry of  
41 Health Guidelines (no. 86/609/EEC) and with the *Principles of Laboratory Animal Care* (NIH no.  
42 85-23, revised 1985). The rabbits were acclimatized for 2 weeks prior to the experiment in a room with  
43 12h light dark cycle, individually housed in stainless steel cages in a temperature- and humidity-

44 controlled room ( $23 \pm 3^{\circ}\text{C}$ ,  $50 \pm 5\%$ ) and fed 100 g per day of standard rabbit non-purified diet,  
45 composition of 100 g: 16.2 g crude protein, 3.0 g crude fat, 7.3 g crude ash, 14.8 g fiber, 12 g  
46 moisture, added mineral 2.1, 40U /kg vitamin E) with appropriate certificate from the Association of  
47 Official Agricultural Chemists, 1975, and relative procedure analyses (Laboratorio Dottori Piccioni,  
48 Gessate Milanese, Italy). Rabbits were randomly distributed into four groups. The control group (C)  
49 ( $n=4$ ) received standard rabbit diet; the DHEA-alone treated group (C-D) ( $n=4$ ) received the same  
50 diet supplemented with 0.02% DHEA (Sigma Aldrich, Milan, Italy); the high-fat group (HF) ( $n=4$ )  
51 received a high-fat diet, consisting of standard rabbit non purified diet, composition of 100g: 16.2 g  
52 crude protein, 7.3 g crude ash, 14.8 g fiber, 12 g moisture, added mineral 2.1, 40U/kg vitamin E)  
53 plus 10 g added fat (6.7g com oil and 3.3 g lard) (18); the HF plus DHEA group (HF-D) ( $n=4$ )  
54 received the high-fat diet supplemented with 0.02% DHEA. Two days before being killed, the  
55 rabbits were fasted overnight and the glucose tolerance test was performed. The rabbits were killed at  
56 3 mo from the start of the experiment, by aortic exsanguination after anesthetization with Zoletil100  
57 (Tiletamine-Zolazepam, Virbac, Carros, France). Blood was collected and plasma and serum were  
58 isolated. The heart was rapidly excised and weighed, and portions of left ventricle were taken to  
59 obtain nuclear and total extracts. Other heart portions were utilized for histological microscopy. The  
60 papillary muscles were immediately removed for functional parameter detection.

#### 61 **General parameters.**

62 Body weight and length of rabbits were measured at time zero and prior to death. The body mass  
63 index (BMI) was calculated as body weight in kilograms divided by body length in meters, squared  
64 ( $\text{kgm}^2$ ).

#### 65 **Oral glucose tolerance test**

66 After a fasting period of 12h, a 50% glucose solution was orally administered to the rabbits at 1.5  
67 g/Kg. Blood samples were collected via the auricular artery before 15, 30, 45, 60, 90, 120 and 240  
68 min after glucose loading. Glucose levels were tested using an Accu-Check Compact kit (Roche  
69 Diagnostics GmbH, Mannheim, Germany)

70 **Parameters in plasma.**

71 Triglyceride (TG), total cholesterol (Te), low density-lipoprotein (LDL)- and high-density-  
72 lipoprotein (HDL)-cholesterol were determined by standard enzymatic procedures using reagents kits  
73 (Hospitex Diagnostics, Florence, Italy). Aspartate aminotransferase (AST) and lactate dehydrogenase  
74 (LDH) were determined using an enzymatic kit (DiaSys Diagnostic Systems GmbH, Holzheim,  
75 Germany). Plasma insulin was measured with an ultrasensitive insulin enzyme-linked  
76 immunosorbent assay kit from DRG Diagnostics (Marburg, Germany). Insulin sensitivity was  
77 calculated using the homeostasis model assessment (HOMA): fasting glucose (mmol/L) x fasting  
78 insulin (U/L)/22.5.

79 **Tissue extracts (cytosolic, nuclear and total extracts).**

80 Cytosolic and nuclear fractions from rabbit left ventricle were prepared by the Meldrum *et al.*  
81 modified method (19). Briefly, left ventricle (100 mg) was homogenized at 10% (w/v) in a Potter  
82 Elvehjem homogenizer (Wheaton Science Products, Millville, NJ, USA) using a homogenization  
83 buffer. Homogenates were centrifuged at 1,000 g for 5 min at 4°C. Supernatants were removed and  
84 centrifuged at 15,000 g at 4°C for 40 min to obtain cytosolic fraction. The pellets were resuspended  
85 in extraction buffer and incubated on ice for 30 min for high-salt extraction, followed by  
86 centrifugation at 15,000 g for 20 min at 4°C. The resulting supernatants containing nuclear proteins  
87 were carefully removed and samples were stored at -80°C until use.

88 Total extract was obtained by homogenizing at 10% (w/v) directly with extraction buffer and were  
89 centrifuged at 1,000 g for 5 min at 4°C. Supernatants (total extract) were stored at -80°C until use.

90 Protein content was determined using the Bradford assay (20).

91 **Oxidative biochemical parameters.**

92 Reactive oxygen species (ROS) were measured in total extracts using 2',7'-dichlorofluorescein  
93 diacetate (DCFH-DA) as a probe (21). Reduced and oxidized glutathione content was measured in  
94 cytosolic fractions by Owens's method (22). The difference between total glutathione and GSH  
95 content represents the oxidized glutathione (GSSG) content (expressed as *fmol/mg* proteins.); the ratio

96 between GSSG content and GSH is considered a good parameter of antioxidant status. 4-  
97 hydroxynonenal (HNE) was detected by an HPLC procedure. The extract sample was directly  
98 injected for HPLC (Waters Assoc., Milford, MA, USA) using an RP-18 column (Merck, Darmstadt,  
99 Germany). The mobile phase used was 42% acetonitrile/bidistilled water (v/v). Serial concentrations  
100 of HNE (0.5-10  $\mu\text{mol/L}$ ) were used to prepare a standard curve (23). Catalase activity was evaluated  
101 in the cytosolic fraction following Aebi's method (24). Total superoxide dismutase (SOD) activity  
102 was assayed by the method described by Flohè and Otting (25). Since the activity of xanthine-  
103 oxidase may vary, sufficient enzyme was used to produce a rate of cytochrome c reduction of at least  
104 0.025 absorbance units/min in the assay without SOD.

#### 105 **Advanced glycation end-products (AGE) detection with HPLC-MS.**

106 Cytosol fractions were treated with 6 mol/L hydrochloric acid for 2 h at 40°C and then centrifuged  
107 (1860 g) (26); only the supernatant was utilized. A Thermo-Finnigan Surveyor instrument (Thermo  
108 Electron, Rodano, Milan, Italy) equipped with autosampler and PDA-UV 6000 LP detector was used.  
109 Mass spectrometry analyses were performed using an LCQ Deca XP plus spectrometer, with  
110 electrospray interface and ion trap as mass analyzer. The chromatographic separations were run on a  
111 Varian Polaris C18-A column (150 x 2 mm, 3  $\mu\text{m}$  particle size) (Varian, Leinì, Turin, Italy). Flow  
112 rate 200  $\mu\text{L min}^{-1}$ . Gradient mobile phase composition was adopted: 95/5 to 0/100 v/v 5  $\text{mmol/L}$   
113 heptafluorobutanoic acid in water/methanol in 13 min. The LC column effluent was delivered to a  
114 UV detector (200-400 nm) and then to the ion source, using nitrogen as sheath and auxiliary gas  
115 (Claind Nitrogen Generator apparatus, Lenno, Como, Italy). The source voltage was set to 4.5 kV in  
116 the positive mode. The heated capillary was maintained at 200°C. The acquisition method used had  
117 previously been optimized in the tuning sections for pentosidine quasi-molecular ion (capillary,  
118 magnetic lenses and collimating octapole voltages) to maximize sensitivity. Collision energy (CE)  
119 was chosen to maintain about 10 % of the precursor ion. The tuning parameters adopted for the ESI  
120 source were: source current 80.00  $\mu\text{A}$ , capillary voltage 3.00 V, tube lens offset 15 V; for ion optics,  
121 multipole 1 offset -5.25 V, inter multipole lens voltage -16.00 V, multipole 2 offset -9.00 V. Mass



122 spectra were collected in tandem MS mode: MS<sup>2</sup> of (+) 379 *m/z* with 33 % CE in the range 100-400  
123 *m/z*.

#### 124 **Western blotting.**

125 NFκB-p65 on cytosol and nuclear extracts of left ventricle were detected by Laemmli's method (27).  
126 Anti-α-actinin antibodies served as loading control for cytosolic proteins and anti-Lamin-B1 for  
127 nuclear NFκB. Specific bands were quantified by densitometry using analytic software (Bio-Rad,  
128 Multi-Analyst, Munchen, Germany) and the net intensity of bands in each experiment was  
129 normalized for the intensity of the corresponding α-actinin or lamin-B1 bands, before comparison  
130 between control and treated samples.

#### 131 **RNA isolation and RT-PCR .**

132 Total RNA was isolated using the RNA fast kit (Molecular Systems, San Diego, CA, USA). Total  
133 DNA was amplified using sense and antisense primers specific for the C-reactive protein (CRP) gene  
134 (sense 5'-AGGATCAGGATTCGTTTG-3' and antisense 5'-CACCACGTACTTGATATGTC-3'),  
135 the tumor necrosis factor alpha (TNFα) gene (sense 5'-AGGAAGAGTCCCCAAACAACCT-3' and  
136 antisense 5'-GGCCCGAGAAGCTGAT CTG), the myosin heavy-chain α (MHCα) gene (sense 5'-  
137 GCCAAGGTGAAGGAGA TGAA-3' and antisense 5'-CTCTCCTGGGTCAGCTTCAG-3'), the  
138 myosin heavy-chain ~ (MHC~) gene (sense 5'-GGTCGAATACGTTACCATCTG-3' and antisense .  
139 5' -AA TCGCTGTCCACAGTGGTCG-3') or for the glyceraldehyde-3-phosphate dehydrogenase  
140 (GAPDH) gene (sense 5'-CGCCTGGAGAA AGCTGCTA-3' and antisense 5'-  
141 CCCCAGCATCGAAGG TAGA-3').

142 The PCR reaction system contained 1 μL of RT product, 200 μmol/L dATP, dGTP, dCTP and dTTP  
143 (Finnzymes, Espoo, Finland), 1.25 units of *Taq* DNA polymerase (Finnzymes, Espoo, Finland) and  
144 50 pmol of sense and antisense primers in a total volume of 50 μL. All experiments were performed  
145 on at least three independent cDNA preparations.

146 PCR products were electrophoresed on 2% agarose gels and amplification products were stained with  
147 GelStar nucleic acid gel stain (FMC BioProducts, Rockland, ME, USA). Gels were photographed  
148 and analyzed with Kodak 1D Image Analysis software. The net intensity of bands in each experiment

149 was normalized for the intensity of the corresponding GAPDHband before comparison between  
150 control and treated samples.

#### 151 **Histological staining.**

152 For standard histology, portions of left ventric1e were fixed in 4% neutralized formalin. Fixed  
153 material was processed for hematoxylin and eosin staining. Six-micron paraffin-wax sections of left  
154 ventric1e were used.

#### 155 **Isolated papillary muscle and contractility determination.**

156 Papillary muscles were driven at constant frequency (120 beats/min) with a pair of electrodes  
157 connected to a 302 T Anapulse Stimulator via a 305-R Stimulus Isolator (W.P. Instruments, New  
158 Haven, CT, USA) operating in constant current mode. Isometric twitches were evaluated by a  
159 Harvard transducer (60-2997), visualized on a Tektronix 2211 digital storage oscilloscope and  
160 continuously acquired and recorded in a Power Mac computer, using Labview Software (Nationai  
161 Instruments Corp., Austin, Texas, USA). The same software was used to measure developed peak  
162 mechanical tension ( $T_{max}$ ), maximum rate of rise and fall of developed mechanical tension ( $+dT/dt$   
163  $_{max}$  and  $-dT/dt_{max}$ ), time-to-peak mechanical tension (ttp) and duration of contraction.

#### 164 **Statistical analysis.**

165 Results are expressed as means  $\pm$  SD. ANOVA and post-hoc analyses included Bonferroni's test  
166 were used (28). The SPSS 14.0 package for Windows (SPSS Inc., Chicago, IL) was used for the  
167 statistical analysis. A value of  $P < 0.05$  was viewed as statistically significant.

### 168 **Results.**

169 **General features.** Body weight, abdomen length, glucose and insulin were significantly increased in  
170 rabbits fed a HP diet versus control values ( $P < 0.05$ ) after three months' treatment. DHEA  
171 supplementation did not modify these values (TABLE 1). Body mass index (BMI) and HOMA were

] 72 also significantly higher in HP rabbits than in controls ( $P<0.05$ ) but not different from those of HF  
] 73 plus DHEA rabbits. The ratio heart/body weight ratio was the same in all groups, whereas the heart  
] 74 weight of rabbit fed the HF increased vs controls; DHEA supplementation reduced this increase.  
175 The results of the glucose tolerance test are in **FIG. 1**. After oral loading, glucose levels remained a  
176 high level for up to 240 min, in the HP and DHEA treated HF groups. TG, TC and plasma LDL-  
] 77 cholesterol concentrations were significantly higher in HF rabbits versus controls ( $P<0.01$ ) and were  
] 78 lower in HF plus DHEA rabbits than in the HP alone group. ( $P<0.05$ ). HDL-cholesterol was also  
] 79 significantly lower ( $P<0.01$ ) in the HF group versus controls; in the HF plus DHEA group its value  
] 80 was similar to that of control animals ( $P<0.05$ ).

#### ] 81 **Oxidative parameters in the left ventricle.**

] 82 Rabbits fed a HP diet for 3 mo showed a significant increase in ROS levels in total extract of left  
183 ventricle vs the control group ( $P<0.01$ ). 4-hydroxynonenal, an end-product of lipid peroxidation, also  
184 significantly increased in HP rabbits ( $P<0.01$ ) (**FIGURE 2**). In rabbits fed with HF plus DHEA, the  
185 ROS and HNE levels were significantly lower than in the HP rabbits ( $P<0.05$ ). Moreover, total SOD  
186 and catalase activities were also increased in the HP rabbits, and DHEA supplementation partially  
187 restored these activities to control levels. No significant difference in the GSSG/GSH ratio was  
188 observed among groups (data not shown). The level of pentosidine was significantly lower in the HF  
189 plus DHEA than in the HF alone group ( $P<0.05$ ) (**TABLE 1**). In samples from both control and  
190 DHEA groups, gas-mass-HPLC analysis failed to detect any peak for pentosidine, apparently  
191 indicating its absence in these animals.

#### 192 **NFκB-p65.**

193 Western Blot analysis detected NFκB-p65 protein in the nuclear and the cytosolic fractions of left  
194 ventricle (**FIGURE 3, panel A and panel B**). Nuclear p65-NFκB of HF rabbits was increased  
195 versus the control groups ( $P<0.05$ ) (**panel A**) while p65-NFκB protein was reduced in the cytosolic  
196 fraction of HF rabbits ( $P<0.05$ ) (**panel B**). In the rabbits fed the HF plus DHEA diet, cytosolic p65  
197 protein content was less markedly reduced than it was in the HF group, corresponding to an increase

198 of the nuclear level of p65, and thus indicating lower activation of the NFκB transcription factor  
199 ( $P<0.05$ ).

200 Inflammatory parameters.

201 Both indices of the proinflammatory state, TNF-α and CRP, were significantly increased in the heart  
202 of rabbits fed the HF diet ( $P<0.01$ ) (TNF-α: FIGURE 3, panel C and CRP: FIGURE 3, panel D).

203 The HF plus DHEA diet significantly decreased expression of both TNF-α and CRP, vs the HF  
204 group ( $P<0.05$ ).

205 Myosin expression.

206 PCR analysis was used to evaluate expression of two isoforms (α and β) of the myosin heavy-chain  
207 (MHC) protein (FIGURE 4) in the left ventricle of control, DHEA, HF and HF plus DHEA rabbits.

208 The HF diet determined a significantly ( $P<0.01$ ) decreased expression of α-MHC and an increased  
209 expression of β-MHC. When DHEA was added to the HF diet, α-MHC was brought closer to the  
210 control value, and β-MHC was reduced versus the control value ( $P<0.05$ ).

211 Necrosis markers

212 LDH and AST release were evaluated in the plasma (FIGURE 5). Both LDH (panel A) and AST  
213 (panel B) significantly increased in HF rabbits ( $P<0.01$ ). In the HF plus DHEA rabbits, the levels of  
214 LDH and AST were significantly lower than in the HF alone rabbits ( $P<0.05$ ).

215 Histological analysis.

216 In the left ventricle of the HF rabbits, histological preparations clearly showed extensive and diffuse  
217 lipid deposition (FIGURE 6, panel C). This lipid infiltration was not observable in either the control  
218 or the DHEA alone group (panel A and panel B). Tissues obtained from HF plus DHEA rabbits  
219 (panel D) showed rare areas of slight lipid deposition.

220 Cardiac function.

221 The contractile force developed by electrically-driven papillary muscles was evaluated in basal  
222 conditions (TABLE 2). Basal contractility was weaker in papillary muscles from HF rabbits versus

223 controls; this was evident not only for maximal developed mechanical tension ( $T_{max}$ :  $P < 0.01$ ) by  
224 ANOVA), but also for maximum rate of rise ( $+dT/dt_{max}$ ;  $P < 0.01$ ) and maximum rate of fall of  
225 developed mechanical tension ( $-dT/dt_{max}$ ;  $P < 0.01$ ). In contrast, no significant difference was found  
226 between papillary muscles from control and HF rabbits in regard to time to peak mechanical tension  
227 (TPT) or duration of contraction. Treatment with DHEA significantly reduced the effects of the HF  
228 diet ( $P < 0.05$ ). However, DHEA did not per se affect contractile properties of the papillary muscles.

## 229 **Discussion**

230 Male New Zealand rabbits fed a HF diet for 3 mo developed abdominal obesity, hyperglycemia,  
231 insulin-resistance, dyslipidemia, and cardiac dysfunction. DHEA supplementation did not affect the  
232 high plasma glucose levels induced by the HF diet, nor did it protect against hyperinsulinemia or the  
233 HOMA index. There is conflicting evidence about the effect of supplemental DHEA on glucose  
234 metabolism and insulin-sensitivity in healthy human subjects (29,30): our results are in agreement  
235 with several human studies showing only slight or no effect of DHEA on glucose homeostasis (31-  
236 33). As far as the plasma lipid profile is concerned, when DHEA was added to the HF diet, HDL  
237 increased in the plasma while triglycerides, total cholesterol and LDL were reduced. These results  
238 are in agreement with studies reporting that DHEA decreases serum triglycerides and the body  
239 weight in hyperlipidemic rats and diabetic mice (34,35). The mechanisms whereby DHEA exerts its  
240 anti-lipid effects are incompletely understood. However, it has been reported that the anti-obesity  
241 effect of DHEA may in part be related to changes in lipase activity and in beta-adrenergic receptor  
242 density (36,37). Moreover, DHEA accelerates lipid catabolism by direct regulation of hepatic lipid  
243 metabolism (38) and also has antiglucocorticoid activity, caused by direct modulation of the hepatic  
244 glucocorticoid receptor (39).

245 However, alongside its effects on lipid metabolism and on insulin axis, we believe that DHEA's key  
246 action is against oxidative imbalance (6,7). Indeed, DHEA counteracted the increase in glycoxidative  
247 products observed in the plasma of rabbits fed a HF diet, in agreement with a previous rat study (13-  
248 16). Oxidative stress was also observed in the cardiac tissue of HF rabbits, in which there were

249 significant increases in ROS, end-products of lipid peroxidation (HNE) and activation of  
250 transcription factor NFkB. Activation of NFkB, which triggers in the inflammatory cascade, was  
251 demonstrated by the increased expression of TNF -alpha and CRP in cardiac tissue. Here we show  
252 that DHEA, by decreasing oxidative stress and reducing activation of NFkB transcription factor; may  
253 could determine a reduced expression of TNF and of CRP. Moreover, the anti-inflammatory effects  
254 of DHEA, due to cytokine reduction caused by decreased NFkB activation, might be amplified by  
255 the reduction of cholesterol levels, which in turn directly decreases CRP secretion from the tissues  
256 (40). CRP has been shown to predict morbidity and mortality from coronary heart disease (41).  
257 Moreover, it has been shown that high dietary cholesterol intake can increase the production of  
258 atherogenic inflammatory cytokines, such as IL-6 and TNF-a, and that reducing dietary cholesterol  
259 concentration leads to a reduction in CRP production (42).

260 We suggest that the pro-oxidant and inflammatory states, observed in HF rabbits, cause activation of  
261 intracellular signaling responsible for impaired myocardial function, as been reported in cases of  
262 heart failure (43). *HP* rabbits showed a switch of cardiac heavy-chain myosin from the alpha to the  
263 beta isoform: this event comprises the heart's "molecular motor" because contractile properties  
264 depend to a great extent on the isoform composition of MHC proteins. A switch in MHC isoform  
265 composition has been reported to cause reduced contractile velocity and energy expenditure (44). In  
266 man as in animals, a reduced content of  $\alpha$ -MHC, which is expressed exclusively in the myocardium,  
267 has been reported to be responsible for the reduced myocardial contractility during heart failure (45)  
268 and in diabetes (46). In a previous model of type I diabetes cardiomyopathy (7), we reported that  
269 DHEA treatment, by avoiding impairment of cardiac myogenic factors and producing a switch in  
270 MHC gene expression, protected against tissue damage, an early event in diabetic cardiomyopathy.  
271 DHEA's modulatory effects on MHC expression were also observed in *HP* rabbits: expression of  
272 two isoforms, alpha and beta, in DHEA treated rats were not significantly different from those of the  
273 control group.

274 Rabbits on a HF diet clearly showed alterations in papillary muscle contractility. This was shown by  
275 altered basal contractility, including reduced maximal developed tension, maximum rate of rise, and  
276 maximum rate of fall of developed tension, which are signs of diastolic dysfunction. As in the case of  
277 rat cardiac muscle (7), these alterations of the mechanisms controlling intracellular calcium handling  
278 within cardiac myocytes are probably related to the structural damage caused by the HF diet.

279 Histological analysis of tissue from HF rabbits showed extensive and diffuse lipid deposition, as  
280 reported in other animal models (47). The increased release of LDH and AST in the plasma of HF  
281 rabbits confirmed myocardial damage. Treatment with DHEA protects the cardiac tissue from this  
282 altered basal contractility, as well as minimizing histological changes and reducing cell damage  
283 caused by the HF diet.

284 The role of DHEA in the cardiovascular system has been highlighted by the recent finding, in the  
285 human heart, of DHEA production and CYP-17 gene expression, a key factor in DHEA synthesis  
286 (48,49) and it has recently been proposed that the vascular protective effect of DHEA might be  
287 dependent on G-protein-mediated activation of the phosphatidylinositol 3-kinase/ Akt  
288 signaling pathway (50). Several explanations have been put forth for the multi-targeted antioxidant  
289 effects of DHEA, including its effect on catalase expression (51), and its up-regulation of the  
290 thioredoxin system (52), of the fatty-acid composition of cellular membranes and of cytokine  
291 production. However, the precise mechanisms remain to be clarified, and additional non-antioxidant  
292 effects cannot yet be ruled out. Whether the effect of DHEA is due to DHEA itself, to its metabolites,  
293 or to a combination of both remains unclear. DHEA is also considered to be a pre-hormone, and it  
294 has been speculated that, through hormonal effects it might modulate several metabolic pathways  
295 which have nothing to do with antioxidant function. However, we found negligible variations of  
296 either 17 $\beta$ -estradiol or testosterone concentrations in rats treated with 4 mg DHEA. Nevertheless,  
297 we report elsewhere that DHEA, but not a variety of other steroids including 17 $\beta$ -estradiol, androstadiol and  
298 dihydrotestosterone, protects bovine retinal capillary pericytes against glucose-induced lipid  
299 peroxidation (53).

300 In conclusion, we show that DHEA supplementation can prevent molecular and functional alterations  
301 of the cardiac muscle, restoring oxidative balance and lowering lipid levels, in rabbit fed a high-fat  
302 diet. Since similar results have been reported in a model of diabetes type I diabetes, we suggest that  
303 heart damage is chiefly dependent on impaired glucose metabolism, and not on insulin-resistance.  
304 These data, together with our recent observations on type II diabetes patients (6), suggest that DHEA  
305 treatment might prevent many events that lead to the cellular damage induced by hyperglycemia,  
306 thus delaying the onset or progression of cardiac complications in type II diabetes.