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Resveratrol supplementation at old age reverts changes associated with aging in inflammatory, oxidative and apoptotic markers in rat heart

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1   **RESVERATROL SUPPLEMENTATION AT OLD AGE REVERTS CHANGES**
2   **ASSOCIATED WITH AGING IN INFLAMMATORY, OXIDATIVE AND**
3   **APOPTOTIC MARKERS IN RAT HEART**

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18

19   **ABSTRACT**

20   Purpose

21   Aging is known to play a critical role in the etiopathogenesis of several diseases.
22   Among them, cardiovascular disorders are especially relevant since they are
23   becoming the first cause of death in western countries. Resveratrol is a
24   polyphenolic compound that has been shown to exert beneficial effects at
25   different levels, including neuronal and cardiovascular protection. Those effects
26   of resveratrol are related, at least in part, to its antioxidant and anti-inflammatory
27   properties. In the current investigation we were interested in exploring whether
28   the positive effects of resveratrol at cardiac level were taking place even when
29   the supplementation started in already old animals.

30   Methods

31   Old male rats were supplemented with resveratrol during 10 weeks. Using RT-
32   PCR, we analyzed the effects of resveratrol supplementation on the expression
33   of different genes related to inflammation, oxidative stress and apoptosis in rat
34   heart.

35   Results

36   Resveratrol reverted the age-related changes in inflammatory, oxidative and
37   apoptotic markers in the rat heart. Among others, the expression of two major
38   inflammatory markers, $\text{INF-}\gamma$ and $\text{TNF-}\alpha$ and two oxidative markers, heme
39   oxygenase and nitric oxide synthase, were increased with aging, and resveratrol
40   supplementation reduced their levels to those observed in the heart of young
41   animals. Moreover, age-related changes in apoptotic markers in rat heart were
42   also reverted by resveratrol treatment.

43   Conclusion

44 Our results suggest that resveratrol might exert beneficial effects as an anti-aging
45 compound in order to revert age-related changes in cardiac function.

46

47 **KEYWORDS**

48 Resveratrol, aging, heart, inflammation, oxidation, apoptosis

49

50 INTRODUCTION

51 Cardiovascular diseases are becoming the first cause of death in western
52 countries, and in the next two decades the proportion of total deaths worldwide
53 due to cardiovascular diseases within the elderly population (>70 yr) will be 40%
54 [1]. Although long- term exposure to risk factors, such as those related to lifestyle
55 (diet, physical inactivity...), plays a major role in the etiopathogenesis of cardiac
56 disorders, aging itself is considered to be the major determinant for developing
57 cardiac diseases [2]. Thus, the increased life expectancy has as a direct
58 consequence: a higher incidence in age-related diseases, in particular those
59 associated with the cardiovascular system.

60 During the last decades different investigations have stressed the relevance of
61 inflammation and oxidative stress both in heart aging and in the onset and
62 development of cardiac diseases, such as heart failure, cardiac hypertrophy
63 and diabetic cardiomyopathy (reviewed in [3]). Age-related increases in those
64 processes lead to reduced cellular survival and cardiac dysfunction. Moreover,
65 inflammation and oxidative stress are also believed to play an important role in
66 age- related changes in the vascular system, in particular in the reduction in the
67 endothelium-dependent relaxation and increases in endothelium-dependent
68 contraction [4]. Cytokines are major signaling proteins involved in immunity,
69 inflammation and hematopoiesis, among other processes. During aging, a shift
70 in the cytokine profile in blood occurs, with increases in inflammatory
71 substances such as tumor necrosis factor- alpha (TNF- α) and interleukins (IL)
72 1, 2 and 6, as well as decreases in anti-inflammatory substances like IL-10 [5].
73 Regarding oxidative stress, mitochondrial reactive oxygen species (ROS)
74 production is considered the most important source of cellular ROS in healthy

75 tissues since the main free radical generator, the electron transport chain, is
76 located at the inner mitochondrial membrane. Together with ROS, reactive
77 nitrogen species (RNS) also play an important role in the disruption of redox
78 signaling and in molecular damage. RNS includes the nitric oxide radical (NO),
79 which is mainly generated by NO synthases (NOS), and other compounds
80 originated by the reaction of NO with ROS, which results in peroxynitrites.
81 During aging, the age-associated organ dysfunction has been partly related with
82 the accumulation of damage induced by ROS and NOS [6].

83 Eventually, inflammatory and oxidative processes have been described to
84 contribute to mitochondrial dysfunction that may trigger apoptotic signaling [7,8].
85 According to this, an over-activation of the apoptotic pathways has been
86 observed in age-related diseases, including neurodegenerative processes and
87 cardiovascular events such as ischemia/ reperfusion [9,10].

88 Different strategies have been used trying to improve aging and postpone the
89 onset of age-related disorders. Among them, nutritional intervention is very
90 popular, including both dietary restriction and dietary supplementation. Dietary
91 restriction (DR) has been used as a model for investigating the mechanisms
92 underlying the aging process for many years and it has described to increase
93 lifespan and reduce the incidence of age-related diseases in different species
94 [11,12]. Interestingly, the positive effects of DR have been described to take
95 place even when restriction is initiated in middle-aged animals [11]. On the
96 other hand, one of the most popular dietary supplements that has been used
97 due its beneficial effects on age-related detrimental changes is resveratrol [13].
98 Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is a polyphenolic phytoalexin
99 present in some food products including grapes and berries. The relevance of

100 resveratrol exponentially rose when it was considered as a potential DR
101 mimetic, being identified as an activator of Sirt1 [14]. Nevertheless, sirt1 is not
102 the only target of resveratrol. It has been described to inhibit cyclooxygenase 1,
103 cAMP phosphodiesterases, and to interact with the estrogen receptor alpha
104 [15]. The beneficial effects of resveratrol are diverse, including suppression of
105 cancer cell growth, neuroprotection and protection against age-related
106 cardiovascular dysfunction [15]. However, most of the studies on resveratrol
107 have been performed in young/ adult animals, when the deleterious effects of
108 aging are still missing. In the current investigation we were interested in
109 exploring whether supplementation with resveratrol in old animals reverted the
110 age-related changes in different parameters covering various aspects of
111 inflammation, oxidation and apoptosis in the rat heart. We observed that when
112 old animals were supplemented with resveratrol, the expression of genes
113 related to those processes were reverted to levels observed in young animals.
114 Moreover, those effects were related, at least in part, by the changes in SIRT1
115 levels. Our results suggest that resveratrol would exert beneficial effects on
116 cardiac function even when supplementation starts at advanced age.

117 **MATERIAL AND METHODS**

118 **Animals and treatment**

119 This study was carried out in accordance with the guidelines for Ethical Care of
120 Experimental Animals of the European Union, approved by the Ethical
121 Committee for Animal Studies of the Complutense University (Madrid, Spain).
122 Young (2 month-old) and old (24 month-old) male Wistar rats were compared in
123 this study. All rats were housed in standard conditions and fed a standard

laboratory rat diet and water *ad libitum*. One group of old rats was treated with resveratrol at dosages of 10 mg/ kg per day during 10 weeks. In humans, this dosage would correspond to approximately to 100mg/ day, assuming an average human body weight of 60Kg. This would correspond to a mild dose in humans. Several studies in humans have shown that resveratrol is well tolerated with no marked toxicity [16,17], and only the daily administration at very high doses of 2.5- 5g per day for 29 days caused mild to moderate gastrointestinal symptoms [18]. Using allometric scaling [19], the human equivalent dosage to the one we used in old rats in the current investigation, based on body surface area, is 1.61 mg / kg / day. In the present study, old rats were treated with resveratrol at doses of 10 mg / kg / day for 10 weeks. The second group of rats was left untreated as a control group. Resveratrol was obtained from Actafarma Laboratories, Madrid, Spain. It was dissolved in absolute ethanol and added to the drinking water in a final ethanol concentration of 0.1%. Resveratrol solution was prepared according to water intake of the animals, in order to ensure that they received the right dosage. Water bottles were covered with aluminium foil for protection from light, and the drinking fluid was changed every day. Untreated animals received same ethanol concentration in tap water. Animals were sacrificed by decapitation and the heart (ventricles) removed.

144

145 **RNA isolation and RT-PCR**

146 RNA was isolated from heart samples using the method described by
147 Chomczynski and Sacchi [20], using the TRI Reagent Kit (Molecular Research
148 Center, Inc., Cincinnati, OH), following the manufacturer's protocol. The purity

149 of the RNA was estimated by 1.5% agarose gel electrophoresis, and
150 RNA concentration was determined by spectrophotometry (260 nm). Reverse
151 transcription of 2 µg RNA for cDNA synthesis was performed using the Reverse
152 Transcription System (Promega, Madison, WI) and a pd(N)6 random hexamer.
153 RT-PCR was performed in an Applied Biosystems 7300 apparatus using the
154 SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and 300-
155 nM concentrations of specific primers (Table 1). The thermocycling profile
156 conditions used were: 50°C for 2 m, 95°C for 10 m, 95°C for 15 s, 60°C for 1 m,
157 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. For the normalization of cDNA
158 loading in the PCR reaction, the amplification of the 18S rRNA for every sample
159 was used. Relative changes in gene expression were calculated using the 2-
160 $\Delta\Delta CT$ method [21].

161

162 **Purification of total protein extracts and western blotting**

163 In order to obtain the total protein extracts, hearts were homogenized in RIPA
164 buffer (1:10 w/v) (50 mM TRIS, 150 mM NaCl, 1 % triton 9100, 0.5 % Sodium
165 deoxycholate, 0.1 % SDS) in the presence of protease inhibitors (protease
166 inhibitor cocktail (Sigma)) and incubated 30 min on ice. After centrifugation at
167 10000g for 30 min, the supernatants were collected. Samples were aliquoted
168 and stored at -80°C until use. The protein concentration of the different
169 preparations was determined by the Lowry method (Lowry et al. 1951).
170 Total heart samples (75 µg protein) were separated on 4–15 % MiniPROTEAN
171 TGX™ gels (Bio-Rad) and transferred to PVDF membranes (Millipore). SIRT 1
172 protein levels were detected by using a monoclonal mouse antibody (1:500
173 dilution; ab110304 Abcam). β -Actin (monoclonal mouse antibody. 1:10000

174 dilution, A2228 Sigma) was used as loading control. Secondary anti-mouse
175 antibody was used at 1:5000 dilution. Chemiluminescent reaction was
176 performed using ECL plus® (GE Healthcare, Amersham) and visualized in a
177 GeneGnomeXRQ (Syngene). In order to estimate the levels of SIRT1, the
178 intensity of the bands was quantified using the specific Gene Snap software
179 (Syngene)

180

181 **Statistical analysis**

182 All data are reported as mean $\pm$ standard error of the mean (SEM) using from 4
183 (old animal group) to 6 (young group and old resveratrol treated group)
184 independent preparations, except when indicated. The results were analyzed by
185 ANOVA followed by Fisher's test. Statistical analyses were carried out using the
186 Graphpad Prism Software. The minimum level of statistical significance was set
187 at $p < 0.05$ in all analyses.

188

189 **RESULTS**

190

191 **SIRT1 levels were increased in the heart of old rats after resveratrol**

192 **treatment**

193 During aging SIRT1 levels were reduced by 20% in rat heart although this
194 reduction was not statistically significant ($p = 0.50$) (Figure 1). When old animals
195 were subjected to resveratrol treatment, SIRT1 protein levels were 20% higher
196 than old control counterparts ($p = 0.38$), reaching protein levels similar to those
197 observed in young rats.

198

199 **Resveratrol reverted the age-related changes in the transcription of**
200 **inflammation- related genes in the heart of old rats**

201 One of the main hallmarks of aging is a chronic inflammation state. In the
202 current investigation, we did observe such pro- inflammatory state in the heart
203 of old rats (Figure 2). A significant age-related increase in the transcription of
204 pro-inflammatory cytokines INF- γ and TNF- α ($p=0.04$ and $p=0.03$, respectively)
205 was observed. Such increase was especially important in the case of TNF- α ,
206 with a 2.2 –fold enhancement. We also investigated one of the main
207 transcription factors related to pro-inflammatory cytokines, NF κ B. Similarly to
208 what we observed in pro-inflammatory cytokines, the expression of NF κ B2
209 (p52) increased by 45% with age in rat heart, although it did not reach statistical
210 significance ($p=0.29$). In addition, the transcription levels of the anti-
211 inflammatory cytokine IL-10 were 25% lower in the heart of old rats than in
212 young animals, although such reduction was not statistically significant
213 ($p=0.43$). On the other hand, resveratrol treatment led to a significant reduction
214 in the levels of pro- inflammatory markers INF- γ and TNF- α ($p<0.05$ and
215 $p=0.08$), and it also reduced the age-related enhancement in p52 transcription
216 levels, yet again, not significantly ($p=0.60$). Conversely, resveratrol increased
217 the transcription of IL-10 ($p=0.39$), reaching comparable levels to those
218 observed in young animals.

219

220 **Resveratrol treatment reduced the age-related increase in the**
221 **transcription of oxidation- related genes in the heart of old rats**

222 Similarly to what we observed with inflammatory markers, the heart of aged rats
223 showed higher transcription levels of oxidative stress markers (Figure 3).

224 Expression of HO-1 was strongly induced with aging ($p < 0.001$) and although
225 resveratrol treatment significantly reduced such an increase ($p = 0.0032$), it did
226 not completely revert it. Similarly, HO-2 expression was also enhanced with
227 aging. However, this increase was not statistically significant ($p = 0.11$).
228 Nevertheless, resveratrol significantly reduced the expression levels of HO-2 in
229 the heart of old rats ($p = 0.18$). We observed an age-related increase in the
230 expression of both endothelial (eNOS; $p = 0.06$) and inducible (iNOS; $p = 0.24$)
231 NOS. Moreover, the expression of iNOS was significantly reduced in old
232 animals when they were subjected to resveratrol treatment ($p \leq 0.05$) to levels
233 similar to what we observed in young rats, whereas eNOS expression levels,
234 although diminished, did not change significantly ($p = 0.29$).

235

236 **The transcription of genes related to apoptosis was increased in the heart**
237 **of aged animals and reverted by resveratrol treatment**

238 Inflammation and oxidative stress may induce the activation of the apoptotic
239 pathways, which would eventually lead to cell death. Enhancement in apoptosis
240 would be particularly relevant in tissues containing post-mitotic cells such as the
241 heart, since the tissue function might be compromised. Therefore, we
242 investigated the expression of four different genes related to both, caspase-
243 dependent and independent apoptotic pathways (Figure 4). We observed that
244 with the exception of XIAP ($p = 0.07$), the transcription of AIF, BAD and Bcl-2
245 markers was significantly increased in the heart of old animals ($p \leq 0.05$), being
246 the increase in AIF the most important one (1.9- fold increase; $p < 0.0064$).
247 Similarly to what we observed in inflammatory and oxidative factors, resveratrol
248 treatment reduced the expression levels of all the analyzed apoptotic markers.

249 However, such reduction did not reach statistical significance for any of the
250 markers, AIF (p=0.08), BAD (p=0.20), Bcl-2 (p=0.07) and XIAP levels (p=0.35).

251

252 **DISCUSSION**

253 Nowadays, cardiovascular diseases are the first cause of morbidity and
254 mortality in western countries. Thus, searching for new strategies that may help
255 to delay the development of cardiovascular diseases is of great interest. One of
256 the main risk factors for cardiovascular dysfunction is aging [3], and different
257 approaches have been used in order to improve the age-related decline in
258 cardiovascular function and delay the onset of cardiovascular diseases. Among
259 them, supplementation with resveratrol has been described to exert beneficial
260 effects on the cardiovascular system [22,23]. However, whether resveratrol has
261 beneficial effects once the age-related deterioration has taken place, is
262 unknown. In the current investigation, we analyzed whether resveratrol
263 supplementation had beneficial effects on the cardiovascular system, even
264 when the treatment starts in animals that are already old.

265 Resveratrol acts as an activator of different signaling pathways, but the
266 activation of SIRT1 seems to be critical [24,25]. Moreover, it has been reported that
267 resveratrol induces the expression of SIRT1 as well [26,27]. SIRT1 belongs to
268 the sirtuin family of proteins and it is the most studied one in mammals. Sirtuins
269 are NAD⁺-dependent protein deacetylases that regulate key cellular processes
270 such as cell cycle, apoptosis or inflammation. Together with their role as
271 transcription regulators through histone deacetylation, sirtuins are known to
272 have different targets in cytosol and mitochondria, including, among others,
273 p53, FoxO transcription factors, PGC-1 α and NF- κ B [28]. Sirtuins also activate

274 AMP-activated protein kinase (AMPK) [29], which is linked to the regulation of
275 the mammalian target of rapamycin (mTOR) pathway with important
276 consequences at cardiac level [30-32]
277 Different studies have shown that during aging or under specific pathological
278 conditions, such as diabetes, sirtuin expression and activity are reduced
279 [26,33,34]. Thus, the beneficial effects of resveratrol in relation to aging and the
280 onset and progression of various diseases are likely to be related to its role as
281 activator of sirtuins. Accordingly and in agreement with previous investigations
282 [33,34], we observed that SIRT1 protein levels tended to be lower in aged
283 animals and, as expected, resveratrol treatment reverted SIRT1 levels to those
284 observed in young animals.

285

286 Age-related cardiac dysfunction has been associated with an increased in
287 inflammatory processes (reviewed in [3]). In agreement with that, the
288 inflammatory markers analyzed in the current study indicated a pro-
289 inflammatory state in the heart of aged rats. In cardiac tissue, chronic
290 inflammation is particularly relevant, since it has been suggested to significantly
291 contribute to heart failure [35,36]. In particular, the increase in TNF- α levels has
292 been related with cardiac hypertrophy and dilated cardiomyopathy [37]. In old
293 animals, we have observed an increased in the pro- inflammatory markers INF-
294 γ and TNF- α , together with a reduction in the levels of anti-inflammatory
295 cytokines like IL-10. The enhancement of pro- inflammatory cytokines levels
296 was related with an age-related increase tendency in the expression of p52,
297 one of the proteins constituting homodimers and heterodimers with other NF κ B
298 proteins. NF κ B is a complex transcription factor that is involved in a number of

299 physiological processes, including inflammation, oxidative stress and cell
300 survival [38]. NF κ B is chronically elevated in the aging process and in many
301 age-related disorders including cardiovascular diseases [39].
302
303 Considering the described anti-inflammatory effects of sirtuins and the effect of
304 resveratrol supplementation on SIRT1 levels in the heart of our animals, we
305 expected to observe a reversion of the inflammatory markers in old animals
306 treated with resveratrol. The expression levels of pro-inflammatory cytokines
307 were actually reduced, reaching those observed in young animals. At the same
308 time, the reduction in the anti-inflammatory IL-10 observed in old animals was
309 reverted by resveratrol treatment. However, resveratrol treatment did not
310 change significantly p52 expression levels. Resveratrol has been described to
311 inhibit NF κ B signaling by suppressing the activity of I κ B kinase (IKK β) [40].
312 Activation of IKK β is required for p52 activation, which suggests that the effect
313 of resveratrol might not be dependent on changes on p52 expression levels but
314 rather on its activity. In sum, our results support previous investigations showing
315 that resveratrol prevent inflammation in the cardiovascular system [41-43].
316 Together with inflammatory markers, we investigated the expression levels of
317 two enzymes related with oxidative stress: heme oxygenase (HO) and nitric
318 oxide synthase (NOS). The beneficial effects of resveratrol have been
319 described to be related with a lower ROS generation, which would be
320 responsible, at least in part, for its anti-oxidant effects [41,42]. Heme
321 oxygenases 1 and 2 are the inducible and constitutive isoforms respectively.
322 Both catalyze the degradation of heme to biliverdin, free iron, and carbon
323 monoxide (CO). HO-1 can be induced by several factors, including oxidative

324 stress, and HO-2, although it is constitutively expressed, may be regulated
325 differently under certain conditions [44,45]. Both HO isoforms are believed to
326 play an important role in the cellular antioxidant defense [46,47]. Moreover, the
327 HO system has been described to have an important anti-inflammatory role
328 [48]. In contrast with a recent study showing a decline in the levels of HO-1 and
329 HO-2 and in the total HO activity in female rats with aging [49], we observed an
330 age-related increase in the mRNA levels of both HO-1 and HO-2. However, it
331 has been described that estrogens play an important role in the regulation of
332 HO activity [49-51], suggesting that the pattern of age-related changes in HO
333 expression in male and females animals may differ. In fact, a previous study in
334 our laboratory showed that HO-1 levels were increased with aging in the heart
335 of male mice [52], in agreement with the current results in male rats. The
336 increase in HO levels might indicate an attempt to compensate an
337 enhancement of oxidative stress and inflammation in the heart of old animals.
338 We observed that resveratrol supplementation in old rats totally or partially
339 blocked the age-related increase in HO transcription levels. Reported effects of
340 resveratrol on HO levels are contradictory. Thus, despite it has been reported to
341 induce HO-1 expression in cell cultures [53], supplementation with resveratrol
342 has been described to revert increases in HO-1 levels observed in retina of
343 diabetic rats [54] as well as to inhibit metastasis of lung adenocarcinoma cells
344 by suppressing HO-1 activity [55]. In agreement with these studies, we
345 observed that the HO-1 and HO-2 transcription levels were lower in the heart of
346 resveratrol-supplemented rats than in aged control animals, suggesting that
347 resveratrol supplementation might reduce oxidative stress in those animals.

348 Regarding NOS, we investigated the inducible (iNOS) and the constitutive
349 (eNOS) isoforms. Nitric oxide (NO) is generated by the activity of NOS, which
350 converts L-arginine into L-citrulline. NO bioavailability is critical for vascular
351 function and hence age-related reduction on this parameter is considered as a
352 main risk factor for developing cardiovascular diseases such as hypertension,
353 which may lead to cardiac failure [56]. Different studies have described
354 decreases, increases and even no changes in eNOS expression with aging
355 [56]. In the current investigation we have observed that both eNOS and iNOS
356 expression were higher in aged animals than in young counterparts. An
357 increase in iNOS expression has been previously described in the liver, heart
358 and pancreas of the senescence-accelerated mice model SAMP8 [52,57,58] as
359 well as in pancreas and heart of aged animals [52,59]. Increased iNOS
360 expression has been suggested to play an important role in reducing eNOS
361 activity and NO bioavailability [60], and it has been related to atherosclerosis
362 development, since iNOS has been described to be the main generator of free
363 radicals in the atheromatous plaques [61,62]. Moreover, an age-related
364 uncoupling of eNOS occurs with aging, leading to higher superoxide production
365 and peroxynitrate generation [56,63]. Thus, the higher levels of both isoforms of
366 NOS in the heart of aged animals suggest that cardiac tissue is subjected to an
367 increased oxidative and nitro-oxidative stress in those animals. Similarly to what
368 we observed in HO-1 and HO-2, resveratrol treatment reverted the age-related
369 increase in NOS isoforms, supporting the antioxidant and protective effect of
370 resveratrol on the aged heart as previously reported [34,41].

371 Together with the evidences of inflammation being activated through
372 enhancements in oxidative stress, there are several reports suggesting a cross-

373 talk between oxidative stress, inflammation and initiation of apoptotic events,
374 particularly in the cardiovascular system [7,8,64,65]. Age-related enhancement
375 in apoptosis has been related to cell loss in several tissues, including the heart,
376 leading to cardiac dysfunction and cardiomyopathies [66,67]. In the current
377 investigation, we observed that one of the pro-apoptotic factors in the caspase-
378 dependent pathway, BAD, was increased during aging. BAD is one of the pro-
379 apoptotic “Bcl-2 family” proteins that promote permeabilization of the
380 mitochondrial membrane and the release of cytochrome c. We also observed a
381 very significant increase, almost 2.5 fold, in the levels of AIF expression. This
382 pro-apoptotic factor is released from mitochondria and promotes apoptosis
383 through nuclear DNA fragmentation in a caspase- independent pathway,
384 stressing an age-related pro-apoptotic state in the rat heart. However, at the
385 same time, we observed that the expression of the anti-apoptotic protein Bcl-2,
386 which binds to pro-apoptotic proteins to inhibit mitochondrial permeabilization,
387 was significantly increased in old animals, as well as the levels of the caspase
388 inhibitor XIAP. These results suggest that the age-related increase in pro-
389 apoptotic factors would be counteracted by the enhancement of anti-apoptotic
390 factors of the caspase-dependent pathway. The augmented levels of anti-
391 apoptotic factors are likely a protective mechanism that would be important in
392 tissues with primarily post-mitotic cells like the heart, where cell loss would
393 greatly compromise their functionality. We have actually observed that in the
394 liver of these animals, which is a mitotic tissue, the expression of pro-apoptotic
395 proteins was increased 20-35% while the expression of anti-apoptotic proteins
396 like XIAP experienced a 20% reduction during aging (Torregrosa-Muñumer,
397 unpublished results), suggesting a different profile of apoptosis in heart and

398 liver. These differences in apoptosis when analyzing mitotic and post-mitotic
399 tissues have been previously reported [68-70]. The effect of resveratrol
400 treatment on the expression of pro- and anti-apoptotic markers was similar to
401 the one observed in inflammatory and oxidative markers: it reverted the age-
402 related changes in all cases.

403 Since resveratrol treatment led to a general reduction in inflammatory and
404 oxidative markers in aged hearts, it was expected that its effect on pro-apoptotic
405 markers would be also similar. The apoptotic response of a cell is regulated by
406 the relative balance of pro- and anti-apoptotic proteins. Cellular control of anti-
407 apoptotic factors expression would be critical for cells' ability to modulate its
408 responses to apoptotic stimuli [71]. Thus, similarly to what we observed in pro-
409 apoptotic factors, a reduction of anti-apoptotic proteins would be expected
410 under resveratrol treatment.

411 Moreover, these results are in agreement with previous investigation showing
412 an anti-apoptotic effect of resveratrol in pancreas [34] and heart [72,73].

413 Summarizing, resveratrol had positive effects on the heart of old animals even
414 when starting supplementation at old age. Our results indicate that resveratrol
415 might be an interesting approach not only for preventing age-related increases
416 in inflammation and oxidative damage, but also for reverting those changes
417 once they take place. In the current investigation we observed that the pro-
418 inflammatory and pro-oxidative state that characterizes the aging process were
419 reverted by resveratrol. Moreover, the age-related changes in apoptotic markers
420 were also reverted by resveratrol treatment. In figure 5 we summarize some
421 aspects of the pathways that have been investigated in the current study
422 together with resveratrol effects through SIRT1. Similarly to previous studies

423 showing protective effects of resveratrol in cognitive performance [74] or
424 endothelial dysfunction [75] in aged rats, our results suggest that resveratrol
425 might be also used as anti-aging approach in order to revert the age-related
426 changes in cardiac function.

427

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430

431 **CONFLICT OF INTEREST.** The authors declare that they have no conflicts of
432 interest.

433

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691

692 **FIGURE LEGENDS**

693 **Fig. 1** Sirtuin 1 protein levels in the heart of young (C 2m), old (C 24m) and old
694 rats treated with resveratrol (RSV 24m). Results are expressed as the mean±
695 SEM relative to 2 month-old animals.

696 **Fig. 2** mRNA levels of inflammatory markers in the heart of young (C 2m), old
697 (C 24m) and old rats treated with resveratrol (RSV 24m). Results are expressed
698 as the mean± SEM relative to 2 month-old animals. n= 4- 6, except TNF- α data
699 in C 24m group (n=3). *p≤0.05

700 **Fig. 3** mRNA levels of oxidative markers in the heart of young (C 2m), old (C
701 24m) and old rats treated with resveratrol (RSV 24m). Results are expressed as
702 the mean± SEM relative to 2 month-old animals. n= 4- 6, except eNOS and
703 iNOS data in C 24m group (n=3). *p≤0.05; **p≤0.01; ***p≤0.001

704 **Fig. 4** mRNA levels of apoptotic markers in the heart of young (C 2m), old (C
705 24m) and old rats treated with resveratrol (RSV 24m). Results are expressed as
706 the mean± SEM relative to 2 month-old animals. n= 4-6, except XIAP and Bcl-2
707 data in C 24m group (n=3). *p≤0.05; **p≤0.01

708 **Figure 5.** Beneficial effects of resveratrol are partly related to sirtuin 1 actions.
709 An important positive feedback between inflammation and oxidative stress
710 takes place leading to activation of apoptotic events. Among other effects,
711 resveratrol reduces inflammation and oxidative stress. The figure depicts some
712 of the resveratrol effects through sirtuin 1. Those that have been analyzed in
713 the current investigation are highlighted with boxes. Cyt c: Cytochrome c; C3:

714 Caspase 3; C8: Caspase 8; C9: Caspase 9; Solid lines with bars indicate
715 inhibition; Arrows represent activation.

716

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718

719 **Table 1. Primers used in RT-PCR experiments**

IL10	Forward	ACTGCACCCACTTCCCAGT
	Reverse	TTGTCCAGCTGGTCCTTTGT
NFkB	Forward	TGGAACAGCCCAAACAGC
	Reverse	CACCTGGCAAACCTCCAT
INFg	Forward	TGAAAGCCTAGAAAGTCTGAAGAAC
	Reverse	CGTGTTACCGTCCTTTTGC
TNF-a	Forward	ATGAGAAGTTCCCAAATGGC
	Reverse	CTCCACTTGGTGGTTTGCTA
AIF	Forward	AGTCCTTATTGTGGGCTTATCAAC
	Reverse	TTGGTCTTCTTTAATAGTCTTGTAGGC
XIAP	Forward	GCTTGCAAGAGCTGGATTTT
	Reverse	TGGCTTCCAATCCGTGAG
BAD	Forward	GCCCTAGGCTTGAGGAAGTC
	Reverse	CAAACCTCTGGGATCTGGAAC
Bcl-2	Forward	CAGGTATGCACCCAGAGTGA
	Reverse	GTCTCTGAAGACGCTGCTCA
HO-1	Forward	GTCAAGCACAGGGTGACAGA
	Reverse	ATCACCTGCAGCTCCTCAAA
HO-2	Forward	TACGGCACCAAGAAAAGGAAA
	Reverse	GTGCTTCCTTGGTCCCTTC
eNOS	Forward	CCAGTGCCCTGCTTCATC
	Reverse	GCAGGGCAAGTTAGGATCAG
iNOS	Forward	CTTTGCCACGGACGAGAC
	Reverse	TCATTGTACTCTGAGGGCTGAC
18S	Forward	GGTGCATGGCCGTTCTTA
	Reverse	TCGTTGTTATCGGAATTAACC

720 18S was used as a housekeeping gene to compare the samples

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5