1 **Running head of the title:** Melatonin alleviates NaF-induced hepatotoxicity

2 **Title:** SIRT3-dependent mitochondrial oxidative stress in sodium fluoride-induced
3 hepatotoxicity and salvage by melatonin

4 **Authors:** Chao Song^{a, b, 1}, Jiamin Zhao^{a, b, 1}, Jingcheng Zhang^{a, b}, Tingchao Mao^{a, b},
5 Beibei Fu^{a, b}, Haibo Wu^{a, b, *}, Yong Zhang^{a, b, *}

6 **Author affiliations:** ^aCollege of Veterinary Medicine, Northwest A&F University,
7 Yangling 712100, Shaanxi, China.

8 ^bKey Laboratory of Animal Biotechnology, Ministry of Agriculture, Northwest
9 A&F University, Yangling 712100, Shaanxi, China.

10 ¹These authors contributed equally to this study.

11 **Corresponding author:** *Correspondence should be addressed to Haibo Wu or
12 Yong Zhang.

13 Tel.: +86 29 87080092

14 Fax: +86 29 87080092

15 E-mail: hbwu029@nwsuaf.edu.cn (H.W) or zhangy1956@sina.com (Y.Z)

16 This work was funded by the State Key Program (No. 31530075) of National
17 Natural Science Foundation of China and China Postdoctoral Science Foundation
18 Grant (No. 2016M590978)

19 **Keywords:** Melatonin; Sodium fluoride; Hepatotoxicity; SIRT3; PGC-1 α

20

21

22

23

24

25

26

27

28

29

Abstract: Oxidative stress induced by fluoride (F) is associated with fluorosis formation, but the underlying molecular mechanism remains unclear. In this study, Melatonin pretreatment suppressed F-induced hepatocyte injury in HepG2 cells. Melatonin increases the activity of superoxide dismutase (SOD2) by enhancing sirtuin 3 (SIRT3)-mediated deacetylation and promotes SOD2 gene expression via SIRT3-regulated DNA-binding activity of forkhead box O3 (FoxO3a), indicating that melatonin markedly enhanced mROS scavenging in F-exposed HepG2 cells. Notably, melatonin activated the peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC-1 α). PGC-1 α interacted with the estrogen-related receptor alpha (ERR α) bound to the SIRT3 promoter, where it functions as a transcription factor to regulate SIRT3 expression. Furthermore, daily injection of melatonin for 30 days inhibited F-induced oxidative stress in mice liver, leading to improvement of liver function. Mechanistic study revealed that the protective effects of melatonin were associated with down-regulation of JNK1/2 phosphorylation in vitro and in vivo. Collectively, our data suggest a novel role of melatonin in preventing F-induced oxidative stress through activation of the SIRT3 pathway.

Introduction

Environmental fluoride (F) is a toxic reagent that can affect human health in various ways (Taghipour et al., 2016). Small amounts of F can be used for strengthen bones and prevention of dentals, but excessive F exposure causes a variety of pathological changes in different cells and tissues (Fu et al., 2014). The increasing F concentration in the environment combined with the intervention of human activities is cause for great concern (Ameeramja et al., 2016). The liver is the largest internal organ and the main target of F in the body. Epidemiological and clinical data have shown that excessive sodium fluoride (NaF) exposure results in liver damage (Chattopadhyay et al., 2011). As the cellular outcome of mitochondrial dysfunction, oxidative stress is strongly implicated as one of the most important mechanisms contributing to the toxic effects of NaF (Varol and Varol, 2012). Evidence suggests that the liver is highly vulnerable to oxidative stress because of

the accumulation of mitochondrial superoxide anion ($O_2^{\bullet-}$) (Mahaboob Basha and Saumya, 2013).

As the main mitochondrial deacetylase, sirtuin-3 (SIRT3) modulates various proteins to control mitochondrial function and mitochondrial reactive oxygen species (mROS) (Pi et al., 2015; Sundaresan et al., 2009). Mitochondrial manganese superoxide dismutase (SOD2) is the main enzyme responsible for scavenging harmful $O_2^{\bullet-}$ and is a substrate of SIRT3 (Kim et al., 2016; Miar et al., 2015). The binding of SIRT3 with SOD2 causes the deacetylation and activation of SOD2 (Tao et al., 2010). Moreover, SIRT3 can also interact with forkhead box O3a (FoxO3a) to activate the FoxO3a-dependent antioxidant-encoding gene SOD2 (Padmaja Divya et al., 2015).

Melatonin and its metabolites are powerful antioxidants and free radical scavengers (Ramis et al., 2015; Siu et al., 2006). Furthermore, melatonin modulates mitochondrial function and strengthens its antioxidant defense systems (Dragicevic et al., 2011). These effects are facilitated by its amphiphilic nature, thereby enabling melatonin to penetrate all morphophysiological barriers and enter all subcellular compartments (Venegas et al., 2012). Recent studies have focused on the role of melatonin in the regulation of mROS levels in healthy and disease states (Acuna Castroviejo et al., 2002). However, the mechanism by which melatonin protects against NaF-induced hepatic oxidative injury remains obscure.

The data presented in the current report are the first to indicate that melatonin efficiently protected against NaF-induced mitochondria-derived and $O_2^{\bullet-}$ -dependent oxidative stress *in vivo* and *in vitro*. All these results contribute to the future clinical treatments of F-induced hepatotoxicity.

Results

Melatonin attenuated F-induced oxidative injury in HepG2 cells via SIRT3 pathway

Firstly, we explored the effects of melatonin on F-induced hepatotoxicity *in vitro*. As shown in Fig. 1A, F decreased cell viability in a time- and dose-dependent

88 manner in HepG2 cells. However, melatonin pretreatment significantly attenuated
 89 the adverse effect of F on cell viability (Fig. 1B). F treatment resulted in
 90 significantly elevated levels of MDA, which was attenuated by melatonin (Fig. 1C).
 91 Pre-treatment with melatonin followed by F resulted in restoration of GSH (Fig.
 92 1D). As shown in Figs 1E and F, melatonin protected the HepG2 cells against
 93 apoptosis. The expression of Bax was increased significantly in the cytosolic
 94 fraction but decreased significantly in the mitochondrial fraction in HepG2 cells
 95 when treated with F. The Bax/Bcl-2 ratio after F treatment increased in the cytosolic
 96 fraction but significantly decreased in the mitochondrial fraction of HepG2 cells.
 97 By contrast, melatonin pretreatment inhibited F-induced changes of Bax/Bcl-2 ratio
 98 in the cytosolic and mitochondrial fractions.

99 As the main mitochondrial deacetylase, sirtuin-3 (SIRT3) modulates various
 100 proteins to control oxidative stress response. Melatonin pretreatment significantly
 101 recovered the reduced SIRT3 expression (Fig. 2A) and activity (Fig. 2B), which
 102 was induced by F. Loss of SIRT3 diminished the effects of melatonin-induced the
 103 up-regulated expression and activity of SIRT3. We found that melatonin could
 104 protect against F-induced mitochondria-derived $O_2^{\bullet-}$ elevation (Fig. 2C) and cell
 105 viability reduction (Fig. 2D). As shown in Fig. 2E, melatonin significantly blocked
 106 the increase in apoptosis induced by F. Furthermore, melatonin treatment inhibited
 107 the collapse of MMP induced by F (Fig. 2F). However, these beneficial effects of
 108 melatonin were significantly attenuated by SIRT3 siRNA transfection. These data
 109 suggest a SIRT3-dependent effect of melatonin on oxidative stress response in
 110 hepatic cells exposed to F.

111 Moreover, HepG2 cells were also incubated with F in the presence of Mito-TEMPO
 112 (mitochondria-targeted SOD mimetic). Treatment with Mito-TEMPO significantly
 113 enhanced SOD2 activity but not SOD2 protein levels (Figs. S1A and B). The
 114 F-induced increase in oxidative injury was significantly attenuated in the presence
 115 of Mito-TEMPO (Figs. S1c–e).

116 **.Melatonin inhibits mitochondria-derived $O_2^{\bullet-}$ accumulation via SOD2**

117 **upregulation in HepG2 cells**

118 SOD2 is a mitochondrial antioxidant that aids in the elimination of $O_2^{\bullet-}$. As shown
 119 in Figs. 3A and B, loss of SIRT3 diminished the effects of melatonin-induced the
 120 up-regulated expression and activity of SOD2. SIRT3-mediated deacetylation of
 121 SOD2 and the subsequent regulates its antioxidant activity, we further studied the
 122 relationship between the influence of melatonin on SOD2 activity and SIRT3.
 123 Coimmunoprecipitation pull-down (Co-IP) assay results indicated that melatonin
 124 promoted the binding of SOD2 and SIRT3 in mitochondria under F exposure (Fig.
 125 3C), and caused the decreased acetylation of SOD2 (Fig. 3D). SIRT3 knockdown
 126 diminished the effects of melatonin on the acetylation levels of SOD2 (Fig. 3E).
 127 Overexpression of SIRT3 significantly rescued F-induced suppression of SIRT3
 128 expression (Fig. 4A) and activity (Fig. 4B). Moreover, overexpression of SIRT3,
 129 but not SIRT3^{H248Y} (a catalytic mutant of SIRT3 lacking deacetylase activity),
 130 decreased the expression of acetylated-SOD2 and increased SOD2 activity in
 131 HepG2 cells exposed to NaF (Figs. 4C and D). The deacetylase-deficient Sirt3
 132 mutant (H248Y) completely eliminated the protective effects of SIRT3 (Figs. 4E
 133 and F). These results indicate that the deacetylation of SOD2 induced by melatonin
 134 is mediated by SIRT3 and melatonin enhances SOD2 activity through the
 135 deacetylation of SIRT3.

136 **Melatonin increased SOD2 expression via the interaction of SIRT3 with** 137 **FoxO3a.**

138 As shown in Fig. 5A, melatonin pretreatment had little influence on the total protein
 139 level of FoxO3a in F-treated HepG2 cells. Treatment of HepG2 cells with F caused
 140 increased the phosphorylation of FoxO3a at serine 253 (Fig. 5B) and the acetylation
 141 of FoxO3a at the K-100 lysine (Fig. 5C). Both events prevented nuclear import,
 142 thereby leading to its inactivation. Melatonin pretreatment decreased the
 143 phosphorylation at Ser253 and comparably increased FoxO3a deacetylation. Loss
 144 of SIRT3 diminished the effects of melatonin on the deacetylation and
 145 phosphorylation of FoxO3a. SIRT3 and FoxO3a functionally and physically interact

146 to form a complex that regulates the activity and acetylation status of FoxO3a. Our
147 results showed that melatonin promoted the interaction of FoxO3a with SIRT3 in
148 mitochondria (Fig. 5D). Moreover, the FoxO3a-luciferase reporter gene assay
149 indicated that melatonin increased the transcriptional activity of FoxO3a. When the
150 cells were co-transfected with the pGL-FHRE-luc plasmid and SIRT3 siRNA, the
151 regulative activities of melatonin were abolished (Fig. 5E). The ChIP assay was
152 then performed to investigate the role of FoxO3a in the melatonin-induced SOD2
153 expression of FoxO3a. In the ChIP assay, melatonin promoted the binding of
154 FoxO3a to the promoter of SOD2 (Fig. 5F) under F exposure.

155 The manipulation of SIRT3 expression, but not that of SIRT3^{H248Y}, restored
156 activation of FoxO3a by decreasing phosphorylation at Ser253 (Figs. 6A and B)
157 and comparably increasing FoxO3a deacetylation (Fig. 6C). Overexpression of
158 SIRT3 increased the transcriptional activity of FoxO3a (Fig. 6D) and restored the
159 F-induced reduction FoxO3a binding to the gene promoter of SOD2 (Fig. 6E).

160 **Melatonin activated the PGC-1 α /ERR α -SIRT3 signaling pathway in HepG2** 161 **cells**

162 Next, we sought to determine which factor mediates the expression of SIRT3. As
163 shown in Figure. 7A, SIRT3 expression was regulated at the transcription level.
164 SIRT3 was previously reported to be regulated by PGC-1 α . So we examined
165 PGC-1 α expression with western blotting and found that PGC-1 α was changed with
166 a similar change pattern of SIRT3 expression (Fig.2B). PGC-1 α siRNA transfection
167 prevented the induction of SIRT3 expression in HepG2 cells, thereby indicating that
168 PGC-1 α was required for the activation of melatonin during SIRT3 expression.
169 Since ERR α acts as the downstream target of PGC-1 α and is co-activated by this
170 transcriptional coactivator. Here we found that PGC-1 α interacted with ERR α in
171 HepG2 cells (Fig. S2A). Knockdown of ERR α or PGC-1 α decreased SIRT3
172 expression and cotransfection of ERR α siRNA and PGC-1 α siRNA could decrease
173 more expression of SIRT3 (Fig. S2B), while overexpression of PGC-1 α or ERR α
174 increased SIRT3 expression and cotransfection of PGC-1 α and ERR α could

175 increase more expression of SIRT3 (Fig. S2C). These results indicate that PGC-1 α
176 and ERR α coordinately regulate SIRT3 expression in HepG2 cells. Furthermore,
177 the luciferase assay was used to determine if SIRT3 mRNA activation by melatonin
178 occurred via PGC-1 α -dependent ERR α binding to the SIRT3 promoter. As shown in
179 Fig. 7C, F exposure caused a significant decrease in the luciferase activity as
180 compared with the control group. By contrast, melatonin pretreatment significantly
181 increased the ERRE-mediated SIRT3 transcriptional activity, whereas this effect
182 was diminished by the addition of PGC-1 α siRNA.

183 The EMSA assay was further performed to test the *in vitro* binding of ERR α and
184 SIRT3 fragments. A preliminary experiment was performed to test the binding of
185 probe (Fig. 7D-1). As shown in Fig. 7D-2, a band in the lane loaded with WT probe
186 and lysates was shifted as compared with lysates alone. The protein–probe binding
187 was regulated by F and melatonin. Moreover, a specific super-shift band was
188 detected with the ERR α antibody, thereby indicating that ERR α was bound to the
189 probe (Fig. 7D-3). The results of EMSA also showed that melatonin increased the
190 binding of the exogenous consensus DNA oligonucleotide of SIRT3 with ERR α . To
191 further confirm the EMSA results and verify that ERR α physically occupies the
192 SIRT3 promoter, we performed the ChIP assay. As shown in Fig. 7E, a 3.6-fold
193 enrichment of ERR α was observed.

194 **Melatonin attenuated F-induced JNK1/2 activation in mice liver**

195 Furthermore, we also investigate whether melatonin could prevent F-induced
196 oxidative stress in mice liver. Significant accumulation of F was observed in
197 F-toxicated mice liver (Fig. S3A). Liver functions were also measured based on
198 changes in the hepatic markers ALT and AST. The results showed that the serum
199 activities of ALT and AST were significantly increased in the F group when
200 compared with the control group (Figs. S3B and C). This result confirmed that
201 F-toxicity model had been successfully established. Melatonin supplementation
202 caused a significant reduction in the accumulated F-content and the serum activities
203 of ALT and AST. Moreover, Melatonin treatment successfully attenuated the

204 F-induced upregulation of $O_2^{\bullet-}$ and MDA content (Figs. S3D and E). Moreover,
 205 GSH level were decreased significantly in F-toxicated mice livers, which was
 206 reversed by melatonin (Fig. S3F).
 207 Since apoptosis plays an important role in the pathogenetic mechanisms involved in
 208 fluorosis. NaF treatment increased caspase-3 activity (Fig. 8A), an indicator of
 209 apoptosis, and decreased the protein levels of Bcl-2 (Fig. 8B), an important
 210 anti-apoptotic factor in mice liver. Pretreatment with melatonin attenuated
 211 caspase-3 activity and increased Bcl-2 protein expression in NaF-treated mice liver.
 212 Activation of mitogen-activated protein kinase (MAPK) has been implicated in
 213 F-induced apoptosis and they are sensitive to oxidative stress, Western blot showed
 214 that the melatonin significantly reduced the phosphorylation of JNK1/2 in
 215 F-exposed mice liver.
 216 To further address the involvement of JNK1/2, HepG2 cells were pretreated
 217 melatonin or SP600125 (a potent JNK1/2 inhibitor). Our results showed that
 218 F-induced JNK1/2 activation in HepG2 cells, which was significantly reduced by
 219 melatonin or SP600125 (Fig. 8C). In addition, caspase-3 activity were reduced in
 220 F-treated HepG2 cells following pretreatment with melatonin or JNK inhibitor (Fig.
 221 8D).

222 **DISCUSSION**

223 Oxidative stress from excessive mROS plays an important role in the pathogenesis
 224 of F-mediated cytotoxicity (Chouhan and Flora, 2008; Gao et al., 2008). SOD2 is
 225 crucial for maintaining the mitochondria-derived $O_2^{\bullet-}$ balance (Li et al., 2004). In
 226 the present study, SOD2 expression and activity were significantly reduced in
 227 NaF-treated HepG2 cells. Pretreatment with melatonin promotes SOD2 activity and
 228 expression, thereby protecting liver cells from mROS induced oxidative stress
 229 under excessive F exposure. Moreover, treatment with the mitochondria-targeted
 230 antioxidant Mito-TEMPO alleviated cellular oxidative stress and increased cell
 231 viability. The maintenance of mitochondria-derived $O_2^{\bullet-}$ at tolerable levels may be a
 232 viable strategy to treat F-induced cellular damage.

233 Oxidative stress is the cellular outcome of mitochondrial dysfunction. The MMP is
 234 a measure of mitochondrial function (Gong et al., 2014). The diminished membrane
 235 potential in the present study is consistent with previous findings in fluorosis (Yan
 236 et al., 2015). Moreover, mitochondrial dysfunction was further aggravated by the
 237 down regulation of SOD2 which is critical for maintaining mitochondrial oxidative
 238 balance. As the primary mitochondrial deacetylase, SIRT3 regulates the biological
 239 functions directly involved in mitochondrial function and oxidative stress response
 240 (Ansari et al., 2016; Qiu et al., 2010). SIRT3 also has a positive role in maintaining
 241 the MMP (Zhou et al., 2014). Melatonin treatment ameliorates mitochondrial
 242 oxidative stress by scavenging for enhanced mROS and improving the
 243 mitochondrial function.

244 SOD2 is a substrate of SIRT3 in mitochondria; the binding of SIRT3 with SOD2
 245 causes SOD2 deacetylation, thereby enhancing the mitochondrial scavenging
 246 capacity (Chen et al., 2011; Qiu et al., 2010). Our current findings indicate that
 247 melatonin promotes SOD2 deacetylation in mitochondria, which was suppressed by
 248 SIRT3 siRNA transfection. Furthermore, a catalytic mutant of SIRT3 (SIRT3^{H248Y}),
 249 which lacks deacetylase activity, failed to reverse the F-induced increase in
 250 mitochondria-derived $O_2^{\bullet-}$. Therefore, SIRT3 activates SOD2 via its deacetylase
 251 activity to inhibit $O_2^{\bullet-}$ accumulation.

252 SIRT3 directly targets SOD2 and promotes SOD2 transcription by FoxO3a
 253 activation, thereby protecting cells from cellular oxidative stress (Du et al., 2013;
 254 Wei et al., 2015). Nuclear localization of FoxO3a is essential for its transcriptional
 255 activity and the transcription of FoxO3a-dependent genes (Calnan and Brunet, 2008;
 256 Tseng et al., 2013). In the present study, F treatment increased FoxO3a
 257 phosphorylation at Ser253, thereby inactivating FoxO3a and preventing its nuclear
 258 import to inactivate HepG2 cells. Melatonin promotes the interaction of FoxO3a
 259 with SIRT3 and enhances FoxO3a deacetylation in the mitochondria; thus the
 260 translocation of FoxO3a from mitochondria to nucleus is promoted, thereby causing
 261 its complex influence on SOD2 transcription, which controls mitochondria-derived

262 $O_2^{\bullet-}$. Collectively, our findings indicate that melatonin enhances SOD2 expression
 263 by promoting SIRT3-regulated FoxO3a transcriptional activity under excessive F
 264 exposure.

265 Melatonin-induced SIRT3 activation plays a pivotal role in protecting against
 266 F-induced oxidative stress. The transcriptional coactivator PGC-1 α is a crucial
 267 regulator in mitochondrial biogenesis, energy generation, and oxidative stress
 268 response (Houten and Auwerx, 2004; Millay and Olson, 2013). Furthermore, a
 269 current study indicated that PGC-1 α stimulated mouse SIRT3 activity in both
 270 hepatocytes and muscle cells, indicating that PGC-1 α acts as an endogenous
 271 regulator of SIRT3 (Park et al., 2012). Recent work highlights the key role of
 272 PGC-1 α in melatonin-regulated hepatic mitochondrial health. Our study is the first
 273 to reveal that melatonin activates SIRT3 mRNA transcription via a
 274 PGC-1 α -dependent signaling pathway. PGC-1 α interacts with the SIRT3
 275 transcription factor ERR α as a transcriptional coactivator for the expression of
 276 SIRT3 in F-induced liver cells injury.

277 Oxidative stress have been demonstrated to activate a variety of signaling pathways,
 278 among which ERK1/2 and JNK1/2 have been implicated in F-induced apoptosis
 279 (Geng et al., 2014). Here, melatonin pretreatment prevented JNK1/2 activation
 280 induced by F in vivo and in vitro. Inhibition of JNK1/2 prevented F-induced
 281 apoptosis. Thus, blocking JNK1/2 signaling may represent a potential mechanism
 282 underlying the protection of melatonin.

283 In summary, we obtained evidence for the first time to demonstrate mROS
 284 inhibition by melatonin reduces F-induced oxidative stress via SIRT3 upregulation
 285 in HepG2 cells. Melatonin enhances SOD2 activity by promoting SIRT3-mediated
 286 deacetylation, but also increases SOD2 expression by increasing the transcriptional
 287 activity of FoxO3a. Our results further highlight the potential importance of
 288 melatonin in SIRT3-mediated mROS homeostasis, thereby illustrating a novel
 289 molecular mechanism of melatonin to be explored for future clinical treatment of
 290 F-induced hepatotoxicity.

291 **Materials and methods**

292 **Ethics statement**

293 This study was performed in strict accordance with the guidelines for the care and
294 use of animals of Northwest A&F University. All animal experimental procedures
295 were approved by the Animal Care Commission of the College of Veterinary
296 Medicine, Northwest A&F University.

297 **Chemicals and reagents**

298 All chemicals and reagents were obtained from Sigma-Aldrich Chemical Co. (St.
299 Louis, MO, USA) unless otherwise stated. Antibodies against Bax, Bcl-2, GAPDH,
300 β -actin, COX IV, SOD2, SIRT3, acetylated-lysine, $ERR\alpha$, and c-Jun NH2-terminal
301 kinase-1/2 (JNK1/2) were purchased from Cell Signaling Technology (Beverly, MA,
302 USA). Antibodies against FoxO3a, phospho-FoxO3a (Ser 253), and PGC-1 α were
303 obtained from Abcam (Cambridge, UK).

304 **Cell culture and treatment**

305 HepG2 cells were purchased from the American Type Culture Collection (ATCC,
306 Manassas, USA). The HepG2 cells were cultured in Dulbecco's Modified Eagle's
307 Medium (DMEM, Gibco, USA), which was supplemented with 10 %
308 heat-inactivated fetal bovine serum (FBS, Gibco) in a 5 % CO₂ humidified
309 atmosphere at 37 °C. Cells were pretreated with 40 μ M melatonin for 2 h, washed,
310 and treated with or without NaF (2 mM) for an additional 12 h. Experimental
311 protocol are described in more details in Supporting information.

312 **Cell viability**

313 Cell viability was analyzed using Cell Counting Kit-8 (Beyotime, Jiangsu, China).
314 The absorbance was obtained with a microplate reader (Epoch, BioTek, Luzern,
315 Switzerland) set at a wavelength of 450 nm.

316 **Assay of biochemical makers of oxidative stress**

317 The concentrations of malondialdehyde (MDA) and glutathione (GSH), and the
318 activity of SOD2 were assayed by commercial assay kits purchased from Nanjing
319 Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

320 **Apoptosis analysis**

321 Cell apoptosis was detected with the Annexin V–fluorescein isothiocyanate (FITC)
322 Apoptosis Detection kit (Beyotime) and analyzed on the BD LSR II flow cytometry
323 system (Becton Dickinson, Franklin Lakes, NJ, USA).

324 **Isolation of cytosolic and mitochondrial fractions**

325 Mitochondrial fractions were immediately extracted with the Cytosolic and
326 Mitochondria Isolation Kit (Beyotime). Protein concentrations were determined
327 with the BCA Protein Assay Kit (Pierce Biotech, Rockford, IL, USA).

328 **Immunoblot analysis**

329 Immunoblot analysis was performed as described in the protocols provided by the
330 primary antibody suppliers.

331 **SIRT3 activity**

332 Protein was extracted with a mild lysis buffer (50 mM Tris-HCl, pH 8; 125 mM
333 NaCl; 1 mM DTT; 5 mM MgCl₂; 1 mM EDTA; 10 % glycerol; 0.1% NP-40).
334 SIRT3 activity was determined with the CycLex SIRT3 Deacetylase Fluorometric
335 Assay Kit according to the manufacturer's instructions (MBL International Corp.
336 Tokyo, Japan). The fluorescence intensity was monitored at excitation and emission
337 wavelengths of 355 and 460 nm, respectively.

338 **Mitochondrial O₂^{•-} assessment**

339 Mitochondrial O₂^{•-} generation was assessed in HepG2 cells by 10 μM MitoSOX
340 (Molecular Probes, CA, USA) for 20 min at 37 °C. The fluorescence intensity was
341 measured with an Infinite™ M200 Microplate Reader (Tecan, Mannedorf,
342 Switzerland) at excitation and emission wavelengths of 492 and 595 nm,
343 respectively.

344 **RNA interference**

345 The siRNA targeting SIRT3 and PGC-1α were purchased from Santa Cruz
346 Biotechnology (Santa Cruz, CA, USA). Cells were transfected with the
347 non-targeted control siRNA to target small interfering RNAs for 6 h according to
348 the manufacture's protocol. At 24 h after transfection, cells were harvested for

349 further experiments.

350 **Mitochondrial membrane potential (MMP)**

351 MMP was detected with the fluorescent, lipophilic dye, JC-1 (BioVision, Milpitas,
352 CA, USA) as previously described (Ye et al., 2011).

353 **Immunoprecipitation (IP)**

354 IP was conducted according to previously described methods (Lai et al., 2013) with
355 a few modifications. Lysates were clarified by centrifugation at 14,000×g for 15
356 min and adjusted to the same protein concentration with the respective lysis buffer
357 for IP. Briefly, protein extracts were incubated overnight at 4 °C with the
358 anti-SIRT3, SOD2 or FoxO3a antibody before fresh protein A/G-conjugated beads
359 (Santa Cruz) were added and rotated overnight at 4 °C. Finally, the beads were
360 washed thrice with the same lysis buffer, eluted with the sample loading buffer, and
361 subjected to immunoblot analysis.

362 **Plasmids and transfection**

363 SIRT3 cDNA was cloned from HepG2 cells and inserted into the *EcoRI/XhoI* site
364 of the vector pIRES-hrGFP-1a. The mutant of SIRT3 (H248Y) was generated with
365 the Quick Change Site-directed Mutagenesis Kit (Stratagene, Santa Clara, CA,
366 USA). Cells were washed after 24 h of transfection and processed for further
367 studies. All the primers used for plasmids construction are listed in Supporting
368 Information Table S1.

369 **Immunofluorescence staining**

370 Cells were fixed with 4% formaldehyde, permeabilized with 0.5% Triton X-100,
371 washed with PBS, blocked for 1 h with 10 % bovine serum albumin, and incubated
372 with rabbit monoclonal anti-pFoxO3a (S253) overnight at 4 °C. The cells were then
373 washed and incubated with FITC- conjugated secondary antibodies (Santa Cruz) for
374 1 h at room temperature, and nuclei were revealed with DAPI (10 mg/ml;
375 Sigma–Aldrich Co., St. Louis, MO). The stained cells were observed by
376 fluorescence microscopy (Nikon, Tokyo, Japan).

377 **Luciferase reporter assay**

Luciferase measurements were performed with the dual luciferase reporter (DLR) Assay System (Promega, Madison, WI, USA). pGL-FHRE-Luc (reporter plasmid for FoxO3a) and pGL-ERRE-Luc (reporter plasmid for ERR α) plasmids were obtained from Addgene (Cambridge, MA, USA). Briefly, cells were transfected with 2 μ g of reporter plasmid/well and 0.1 μ g of *Renilla* luciferase plasmid pRL-SV40 (Promega) was co-transfected as an internal control. Data were collected with a VICTOR X5 Multilabel Plate Reader (PerkinElmer).

Quantitative Real-time PCR (qPCR) analysis

Total RNA was isolated with the TRIzol Reagent (Invitrogen), which was reverse transcribed to cDNA with the SYBR PrimeScript RT-PCR Kit (Takara BIO Inc., Japan). The gene-specific primers used are listed in Supporting Information Table S2. Results were normalized to levels of GAPDH mRNA and expressed as the fold change ($2^{-\Delta\Delta C_t}$).

Chromatin immunoprecipitation assay (ChIP)

A ChIP assay was performed with the Pierce Agarose ChIP Kit as previously described (Wu et al., 2014). Briefly, cells were cross-linked with formaldehyde for 15 min at room temperature followed by glycine treatment to stop the cross-linking. Genomic DNA was isolated and sheared by ultrasonic waves and 10 % of the supernatant was regarded as input. Antibodies against FoxO3a or ERR α were used for IP. The ChIP enrichment was determined with an ABI StepOnePlus PCR system (Applied Biosystems). Primer sequences used for ChIP-qPCR are listed in Supporting Information Table S3.

Electrophoretic mobility shift assay (EMSA)

The EMSA assay was strictly performed with an Electrophoretic Mobility Shift Assay Kit (Molecular Probes, Invitrogen) according to the manufacturer's recommendations. Primer sequences used for EMSA are listed in Supporting Information Table S4.

Animal studies

A total of 40 two-mo-old Kunming mice were purchased from the experimental

407 animal center of the Fourth Military Medical University. The mice were kept in
 408 standard animal housing at 22 ± 2 °C with ventilation and hygienic conditions, as
 409 well as free access to food and water.
 410 Animals were randomly divided into four groups of 10 each. Group 1 (Control):
 411 Mice was provided distilled water and received daily injection vehicle for 30 days.
 412 Group 2 (Mel): Mice were received daily injection of melatonin alone (5 mg/kg/day,
 413 i.p.) for 30 days (San-Miguel et al., 2015). Group 3 (F): Mice were supplied with
 414 120 mg/L NaF in deionized water and received daily injection vehicle for 30 days
 415 according to our previous study (Fu et al., 2014). Group 4 (Mel + F): Mice were
 416 administrated with 120 mg/L NaF in drinking water and received daily injection of
 417 melatonin for 30 days. After treatment, the F concentration in the liver was
 418 estimated with an ion-sensitive electrode, as previously described (Zhou et al.,
 419 2013). Values were expressed as $\mu\text{g F per g dry tissue}$.
 420 The livers were homogenized in nine fold (w/v) cold normal saline using an
 421 automatic homogenizer, and centrifuged at $1500 \times g$ for 20 min at 4 °C. The
 422 supernatant was kept at -80 °C until further analysis.

423 **Liver function**

424 Liver function was evaluated by measuring serum alanine aminotransferase (ALT)
 425 and aspartate aminotransferase (AST) with an automated chemistry analyzer
 426 (Olympus AU1000, Olympus, Tokyo, Japan).

427 **Statistical analysis**

428 Raw data were analyzed with the SPSS 19.0 software (Chicago, IL, USA). Results
 429 are expressed as mean $\pm$ SD from triplicate parallel experiments unless otherwise
 430 indicated. Statistical analyses were performed with one-way ANOVA, followed by
 431 post hoc least significant difference tests. Values with $P < 0.05$ were considered
 432 statistically significant.

433 **Acknowledgments**

434 This work was funded by the State Key Program (No. 31530075) of National
 435 Natural Science Foundation of China and China Postdoctoral Science Foundation

436 Grant (No. 2016M590978).

437 **Competing interests**

438 The authors indicate no competing financial interest.

439 **Author contributions**

440 Chao Song: Conception and design, collection and assembly of data, data analysis
441 and interpretation, manuscript writing

442 Jiamin Zhao: Collection and assembly of data

443 Jingcheng Zhang: Collection and assembly of data

444 Tingchao Mao: Collection and assembly of data

445 Beibei Fu: Collection and assembly of data

446 Haibo Wu: Conception and design, collection and assembly of data, data analysis
447 and interpretation

448 Yong Zhang: Conception and design, data analysis and interpretation, financial
449 support, final approval of manuscript

450 **Figure Legends**

451 **FIGURE 1 Melatonin inhibited NaF-induced hepatic injury in HepG2 cells. (A)**

452 Cells were treated with NaF with different concentrations or for different time
453 intervals, respectively. Cell viability was determined using the CCK-8 assay. (B)
454 Confluent cells were pretreated for 2 h with various concentrations of melatonin.
455 After removing the supernatants, cells were incubated with fresh medium in the
456 presence or absence of NaF (2 mM) for an additional 12 h. Cell viability was
457 determined. (C) GSH level. (D) MDA content. (E) Representative images of flow
458 cytometric analysis by Annexin V-FITC/PI dual staining. (F) The ratio of
459 Bax/Bcl-2 in the cytosolic fraction and mitochondrial fraction. All results are
460 representative of three independent experiments and values are presented as means
461 $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ versus the control group, ### $p < 0.01$ vs. the F group.

462 **FIGURE 2 Melatonin protected against NaF-induced oxidative injury via**

463 **SIRT3 pathway.** Cells were transfected with SIRT3 siRNA. At 24-h

464 post-transfection, cells were pretreated with melatonin (40 μ M) for 2 h and then

465 treated with or without NaF of 2 mM for an additional 12 h. SOD2 expression (A)
466 and activity (B), the mitochondrial $O_2^{\bullet-}$ levels (C), cell viability (D), apoptosis (E),
467 and MMP (F) were determined, respectively. The scale bar is 50 μ m. All results are
468 presented as means $\pm$ SD of at least three independent experiments. * $p < 0.05$, ** $p <$
469 0.01 versus siNC + control group, $^{##}p < 0.01$ versus the siNC + F group, $^{$$}p < 0.01$
470 versus the siNC+ Mel + F group.

471 **FIGURE 3 Melatonin increased mROS scavenging by stimulating**
472 **SIRT3-mediated SOD2 deacetylation.** Cells were transfected with SIRT3 siRNA.
473 At 24-h post-transfection, cells were pretreated with melatonin (40 μ M) for 2 h and
474 then treated with or without NaF of 2 mM for an additional 12 h. (A) SOD2
475 expression. (B) SOD2 activity. (C) SOD2 was immunoprecipitated using SIRT3
476 antibody. (D) acetylated-SOD2 (Ac-SOD2) was immunoprecipitated using SOD2
477 antibody. (E) Ac-SOD2 was immunoprecipitated in SIRT3-deficient HepG2 cells.
478 All results are representative of three independent experiments. ** $p < 0.01$ versus
479 siNC + control group, $^{##}p < 0.01$ versus the siNC + F group, $^{$$}p < 0.01$ versus the
480 siNC+ Mel + F group.

481 **FIGURE 4 SIRT3 deacetylase deficiency does not affect SOD2 acetylation and**
482 **oxidative injury in F-treated HepG2 cells.** Cells were transfected with SIRT3
483 expression constructs (WT or H248Y) followed by exposure to NaF (2 mM) for 12
484 h. (A) SIRT3 expression, (B) SIRT3 activity. (C) Acetylation of SOD2 was
485 determined by immunoprecipitation. (D) SOD2 activity. (E) Mitochondrial-derived
486 $O_2^{\bullet-}$ production. (F) Cell viability. ** $p < 0.01$ versus scramble + F group, $^{##}p < 0.01$
487 versus the SIRT3 + F group.

488 **FIGURE 5 Melatonin regulated the expression of SOD2 through the**
489 **interaction of SIRT3 with FoxO3a in mitochondria.** (A) Mitochondria were
490 isolated after treatment and subjected to western blot analysis for FoxO3a. (B and C)
491 Expressions of p-FoxO3a and FoxO3a acetylation at lysine-100 residue. (D)
492 FoxO3a was immunoprecipitated using a SIRT3 antibody. (E) Cell lysates were
493 harvested for dual luciferase report assays. (F) ChIP analysis was used to examine

the binding of FoxO3a to the SOD2 promoter. Data are presented as the mean $\pm$ SD of three independent experiments. *P<0.05, **P<0.01 versus the Control group.

FIGURE 6 SIRT3 deacetylase deficiency does not affect SOD2 expression and in F-treated HepG2 cells. Cells were transfected with SIRT3 expression constructs (WT or H248Y) followed by exposure to NaF (2 mM) for 12 h. (A and B) The expression of pFoxO3a, FoxO3a, and SOD2. (C) FoxO3a acetylation at lysine-100 residue was examined. (D) Cell lysates were harvested for dual luciferase report assays. (E) Binding of FoxO3a to SOD2 was examined using ChIP assay. The scale bar is 50 μ m. Data are presented as the mean $\pm$ SD of three independent experiments. **P<0.01 versus the F + Scramble group, \$\$P<0.01 versus the F + SIRT3 group.

FIGURE 7 Melatonin activated PGC-1 α /ERR α -SIRT3 signaling pathway in HepG2 cells. Cells were transfected with PGC-1 α siRNA. At 24-h post-transfection, cells were pretreated with melatonin (40 μ M) for 2 h and then treated with or without NaF of 2 mM for an additional 12 h. (A) SIRT3 mRNA level. (B) The expression of PGC-1 α and SIRT3. (C) ERR α luciferase reporter plasmid was transfected into HepG2 cells. Luciferase activity was determined. (D) *In vitro* binding of ERR α and SIRT3 promoter was examined by EMSA assay. (E) *In vivo* binding of ERR α and SIRT3 promoter was examined by the ChIP assay. Data are presented as the mean $\pm$ SD of three independent experiments. *p < 0.05, **p < 0.01 versus the siNC + control group; #p < 0.05, ##p < 0.01 versus the siNC + F group, and \$p < 0.05 versus the siNC + Mel + F group. Abbreviation: WT, wild-type; MUT, mutation; Ab, antibody.

FIGURE 8 Melatonin inhibited JNK1/2 activation in mice liver. (A) Caspase-3 activity (H). (B) The representative western blot for Bax, Bcl-2, JNK1/2 in mice liver. (C) Immunoblot analysis in HepG2 cells. (D) Caspase-3 activity was determined in HepG2 cells. Data are mean $\pm$ SD; n = 6–8. *p < 0.05 or 3 different cultures., **p < 0.01 versus the control group, #p < 0.05, ##p < 0.01 versus the F group.

523

524 **Abbreviations:** Sodium fluoride, NaF; Melatonin, Mel; Mitochondrial reactive
525 oxygen species, mROS; Mitochondrial manganese superoxide dismutase, SOD2;
526 Peroxisome proliferator-activated receptor gamma coactivator 1 α , PGC-1 α ;
527 Estrogen-related receptor alpha, ERR α ; Malondialdehyde, MDA; Glutathione, GSH;
528 Chromatin immunoprecipitation assay, ChIP; Electrophoretic mobility shift assay,
529 EMSA. Alanine aminotransferase, ALT; Aspartate aminotransferase, AST; d c-Jun
530 NH2-terminal kinase-1/2, JNK1/2; WT, wild-type; MUT, mutation; Ab, antibody.

531

532 **References**

533 **Acuna Castroviejo, D., Escames, G., Carazo, A., Leon, J., Khaldy, H. and**
534 **Reiter, R. J.** (2002). Melatonin, mitochondrial homeostasis and
535 mitochondrial-related diseases. *Curr Top Med Chem* **2**, 133-51.

536 **Ameeramja, J., Panneerselvam, L., Govindarajan, V., Jeyachandran, S.,**
537 **Baskaralingam, V. and Perumal, E.** (2016). Tamarind seed coat ameliorates
538 fluoride induced cytotoxicity, oxidative stress, mitochondrial dysfunction and
539 apoptosis in A549 cells. *J Hazard Mater* **301**, 554-65.

540 **Ansari, A., Rahman, M. S., Saha, S. K., Saikot, F. K., Deep, A. and Kim, K.**
541 **H.** (2016). Function of the SIRT3 mitochondrial deacetylase in cellular physiology,
542 cancer, and neurodegenerative disease. *Aging Cell*.

543 **Calnan, D. R. and Brunet, A.** (2008). The FoxO code. *Oncogene* **27**, 2276-88.

544 **Chattopadhyay, A., Podder, S., Agarwal, S. and Bhattacharya, S.** (2011).
545 Fluoride-induced histopathology and synthesis of stress protein in liver and kidney
546 of mice. *Arch Toxicol* **85**, 327-35.

547 **Chen, Y., Zhang, J., Lin, Y., Lei, Q., Guan, K. L., Zhao, S. and Xiong, Y.**
548 (2011). Tumour suppressor SIRT3 deacetylates and activates manganese superoxide
549 dismutase to scavenge ROS. *EMBO Rep* **12**, 534-41.

- 550 **Chouhan, S. and Flora, S. J. S.** (2008). Effects of fluoride on the tissue
551 oxidative stress and apoptosis in rats: Biochemical assays supported by IR
552 spectroscopy data. *Toxicology* **254**, 61-67.
- 553 **Dragicevic, N., Copes, N., O'Neal-Moffitt, G., Jin, J., Buzzeo, R., Mamcarz,**
554 **M., Tan, J., Cao, C., Olcese, J. M., Arendash, G. W. et al.** (2011). Melatonin
555 treatment restores mitochondrial function in Alzheimer's mice: a mitochondrial
556 protective role of melatonin membrane receptor signaling. *J Pineal Res* **51**, 75-86.
- 557 **Du, K., Yu, Y., Zhang, D., Luo, W., Huang, H., Chen, J., Gao, J. and Huang,**
558 **C.** (2013). NFkappaB1 (p50) suppresses SOD2 expression by inhibiting FoxO3a
559 transactivation in a miR190/PHLPP1/Akt-dependent axis. *Mol Biol Cell* **24**,
560 3577-83.
- 561 **Fu, M., Wu, X., He, J., Zhang, Y. and Hua, S.** (2014). Natrium fluoride
562 influences methylation modifications and induces apoptosis in mouse early
563 embryos. *Environ Sci Technol* **48**, 10398-405.
- 564 **Gao, Q., Liu, Y. J. and Guan, Z. Z.** (2008). Oxidative stress might be a
565 mechanism connected with the decreased alpha 7 nicotinic receptor influenced by
566 high-concentration of fluoride in SH-SY5Y neuroblastoma cells. *Toxicol In Vitro* **22**,
567 837-43.
- 568 **Geng, Y., Qiu, Y., Liu, X., Chen, X., Ding, Y., Liu, S., Zhao, Y., Gao, R.,**
569 **Wang, Y. and He, J.** (2014). Sodium fluoride activates ERK and JNK via induction
570 of oxidative stress to promote apoptosis and impairs ovarian function in rats. *J*
571 *Hazard Mater* **272**, 75-82.
- 572 **Gong, S., Peng, Y., Jiang, P., Wang, M., Fan, M., Wang, X., Zhou, H., Li, H.,**
573 **Yan, Q., Huang, T. et al.** (2014). A deafness-associated tRNA^{His} mutation alters
574 the mitochondrial function, ROS production and membrane potential. *Nucleic Acids*
575 *Res* **42**, 8039-48.
- 576 **Houten, S. M. and Auwerx, J.** (2004). PGC-1alpha: turbocharging

577 mitochondria. *Cell* **119**, 5-7.

578 **Kim, H., Lee, Y. D., Kim, H. J., Lee, Z. H. and Kim, H. H.** (2016). SOD2 and
579 Sirt3 Control Osteoclastogenesis by Regulating Mitochondrial ROS. *J Bone Miner*
580 *Res.*

581 **Lai, L., Yan, L., Gao, S., Hu, C. L., Ge, H., Davidow, A., Park, M., Bravo, C.,**
582 **Iwatsubo, K., Ishikawa, Y. et al.** (2013). Type 5 adenylyl cyclase increases
583 oxidative stress by transcriptional regulation of manganese superoxide dismutase
584 via the SIRT1/FoxO3a pathway. *Circulation* **127**, 1692-701.

585 **Li, W., Jue, T., Edwards, J., Wang, X. and Hintze, T. H.** (2004). Changes in
586 NO bioavailability regulate cardiac O₂ consumption: control by intramitochondrial
587 SOD2 and intracellular myoglobin. *Am J Physiol Heart Circ Physiol* **286**, H47-54.

588 **Mahaboob Basha, P. and Saumya, S. M.** (2013). Suppression of mitochondrial
589 oxidative phosphorylation and TCA enzymes in discrete brain regions of mice
590 exposed to high fluoride: amelioration by Panax ginseng (Ginseng) and
591 Lagerstroemia speciosa (Banaba) extracts. *Cell Mol Neurobiol* **33**, 453-64.

592 **Miar, A., Hevia, D., Munoz-Cimadevilla, H., Astudillo, A., Velasco, J., Sainz,**
593 **R. M. and Mayo, J. C.** (2015). Manganese superoxide dismutase
594 (SOD2/MnSOD)/catalase and SOD2/GPx1 ratios as biomarkers for tumor
595 progression and metastasis in prostate, colon, and lung cancer. *Free Radic Biol Med*
596 **85**, 45-55.

597 **Millay, D. P. and Olson, E. N.** (2013). Making muscle or mitochondria by
598 selective splicing of PGC-1alpha. *Cell Metab* **17**, 3-4.

599 **Padmaja Divya, S., Pratheeshkumar, P., Son, Y. O., Vinod Roy, R., Andrew**
600 **Hitron, J., Kim, D., Dai, J., Wang, L., Asha, P., Huang, B. et al.** (2015). Arsenic
601 Induces Insulin Resistance in Mouse Adipocytes and Myotubes Via Oxidative
602 Stress-Regulated Mitochondrial Sirt3-FOXO3a Signaling Pathway. *Toxicol Sci* **146**,
603 290-300.

- 604 **Park, S. J., Ahmad, F., Philp, A., Baar, K., Williams, T., Luo, H., Ke, H.,**
605 **Rehmann, H., Taussig, R., Brown, A. L. et al. (2012).** Resveratrol ameliorates
606 aging-related metabolic phenotypes by inhibiting cAMP phosphodiesterases. *Cell*
607 **148**, 421-33.
- 608 **Pi, H., Xu, S., Reiter, R. J., Guo, P., Zhang, L., Li, Y., Li, M., Cao, Z., Tian,**
609 **L., Xie, J. et al. (2015).** SIRT3-SOD2-mROS-dependent autophagy in
610 cadmium-induced hepatotoxicity and salvage by melatonin. *Autophagy* **11**,
611 1037-51.
- 612 **Qiu, X., Brown, K., Hirschey, M. D., Verdin, E. and Chen, D. (2010).** Calorie
613 restriction reduces oxidative stress by SIRT3-mediated SOD2 activation. *Cell*
614 *Metab* **12**, 662-7.
- 615 **Ramis, M. R., Esteban, S., Miralles, A., Tan, D. X. and Reiter, R. J. (2015).**
616 Protective Effects of Melatonin and Mitochondria-targeted Antioxidants Against
617 Oxidative Stress: A Review. *Curr Med Chem* **22**, 2690-711.
- 618 **San-Miguel, B., Crespo, I., Sanchez, D. I., Gonzalez-Fernandez, B., Ortiz de**
619 **Urbina, J. J., Tunon, M. J. and Gonzalez-Gallego, J. (2015).** Melatonin inhibits
620 autophagy and endoplasmic reticulum stress in mice with carbon
621 tetrachloride-induced fibrosis. *J Pineal Res* **59**, 151-62.
- 622 **Siu, A. W., Maldonado, M., Sanchez-Hidalgo, M., Tan, D. X. and Reiter, R. J.**
623 **(2006).** Protective effects of melatonin in experimental free radical-related ocular
624 diseases. *J Pineal Res* **40**, 101-9.
- 625 **Sundaresan, N. R., Gupta, M., Kim, G., Rajamohan, S. B., Isbatan, A. and**
626 **Gupta, M. P. (2009).** Sirt3 blocks the cardiac hypertrophic response by augmenting
627 Foxo3a-dependent antioxidant defense mechanisms in mice. *J Clin Invest* **119**,
628 2758-71.
- 629 **Taghipour, N., Amini, H., Mosaferi, M., Yunesian, M., Pourakbar, M. and**
630 **Taghipour, H. (2016).** National and sub-national drinking water fluoride

631 concentrations and prevalence of fluorosis and of decayed, missed, and filled teeth
632 in Iran from 1990 to 2015: a systematic review. *Environ Sci Pollut Res Int* **23**,
633 5077-98.

634 **Tao, R., Coleman, M. C., Pennington, J. D., Ozden, O., Park, S. H., Jiang,**
635 **H., Kim, H. S., Flynn, C. R., Hill, S., Hayes McDonald, W. et al.** (2010).
636 Sirt3-mediated deacetylation of evolutionarily conserved lysine 122 regulates
637 MnSOD activity in response to stress. *Mol Cell* **40**, 893-904.

638 **Tseng, A. H., Shieh, S. S. and Wang, D. L.** (2013). SIRT3 deacetylates FOXO3
639 to protect mitochondria against oxidative damage. *Free Radic Biol Med* **63**, 222-34.

640 **Varol, E. and Varol, S.** (2012). Effect of fluoride toxicity on cardiovascular
641 systems: role of oxidative stress. *Arch Toxicol* **86**, 1627.

642 **Venegas, C., Garcia, J. A., Escames, G., Ortiz, F., Lopez, A., Doerrier, C.,**
643 **Garcia-Corzo, L., Lopez, L. C., Reiter, R. J. and Acuna-Castroviejo, D.** (2012).
644 Extrapineal melatonin: analysis of its subcellular distribution and daily fluctuations.
645 *J Pineal Res* **52**, 217-27.

646 **Wei, L., Zhou, Y., Qiao, C., Ni, T., Li, Z., You, Q., Guo, Q. and Lu, N.** (2015).
647 Oroxylin A inhibits glycolysis-dependent proliferation of human breast cancer via
648 promoting SIRT3-mediated SOD2 transcription and HIF1alpha destabilization. *Cell*
649 *Death Dis* **6**, e1714.

650 **Wu, H., Wu, Y., Ai, Z., Yang, L., Gao, Y., Du, J., Guo, Z. and Zhang, Y.**
651 (2014). Vitamin C enhances Nanog expression via activation of the JAK/STAT
652 signaling pathway. *Stem Cells* **32**, 166-76.

653 **Yan, X., Yang, X., Hao, X., Ren, Q., Gao, J., Wang, Y., Chang, N., Qiu, Y.**
654 **and Song, G.** (2015). Sodium Fluoride Induces Apoptosis in H9c2 Cardiomyocytes
655 by Altering Mitochondrial Membrane Potential and Intracellular ROS Level. *Biol*
656 *Trace Elem Res* **166**, 210-5.

657 **Ye, R., Kong, X., Yang, Q., Zhang, Y., Han, J. and Zhao, G.** (2011).

658 Ginsenoside Rd attenuates redox imbalance and improves stroke outcome after
659 focal cerebral ischemia in aged mice. *Neuropharmacology* **61**, 815-24.

660 **Zhou, X., Chen, M., Zeng, X., Yang, J., Deng, H., Yi, L. and Mi, M. T.** (2014).
661 Resveratrol regulates mitochondrial reactive oxygen species homeostasis through
662 Sirt3 signaling pathway in human vascular endothelial cells. *Cell Death Dis* **5**,
663 e1576.

664 **Zhou, Y., Qiu, Y., He, J., Chen, X., Ding, Y., Wang, Y. and Liu, X.** (2013).
665 The toxicity mechanism of sodium fluoride on fertility in female rats. *Food Chem*
666 *Toxicol* **62**, 566-72.

667