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ORIGINAL ARTICLE

Autophagic deficiency is related to steroidogenic decline in aged rat Leydig cells

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Late-onset hypogonadism (LOH) is closely related to secondary androgen deficiency in aged males, but the mechanism remains unclear. In this study, we found that reduced testosterone production in aged rat Leydig cells is associated with decreased autophagic activity. Primary rat Leydig cells and the TM3 mouse Leydig cell line were used to study the effect of autophagic deficiency on Leydig cell testosterone production. In Leydig cells from young and aged rats, treatment with wortmannin, an autophagy inhibitor, inhibited luteinising hormone (LH)-stimulated steroidogenic acute regulatory (StAR) protein expression and decreased testosterone production. In contrast, treatment with rapamycin, an autophagy activator, enhanced LH-stimulated steroidogenesis in Leydig cells from aged, but not young, rats. Intracellular reactive oxygen species (ROS) levels were increased in both young and aged Leydig cells treated with wortmannin but decreased only in aged Leydig cells treated with rapamycin. Furthermore, an increased level of ROS, induced by H_2O_2, resulted in LH-stimulated steroidogenic inhibition. Finally, knockdown of Beclin 1 decreased LH-stimulated StAR expression and testosterone production in TM3 mouse Leydig cells, which were associated with increased intracellular ROS level. These results suggested that autophagic deficiency is related to steroidogenic decline in aged rat Leydig cells, which might be influenced by intracellular ROS levels.
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INTRODUCTION

Late-onset hypogonadism (LOH) is a clinical and biochemical syndrome associated with ageing and androgen deficiency, which is characterized primarily by erectile dysfunction, changes in moods, sleep disturbances, decrease in lean body mass, increase in visceral fat and decrease in bone mineral density, but the mechanism remains unclear.[1–4] Recently, a study has shown that LOH can be defined by the presence of at least three sexual symptoms associated with a total testosterone level of less than 11 nmol l^{-1} and a free testosterone level of less than 220 pmol l^{-1}.[3] And testosterone replacement therapy can alleviate the symptoms and signs with a wide range of benefits that include improvement in libido and sexual function, bone density, muscle mass, body composition, mood, erythropoiesis, cognition, quality of life and cardiovascular disease.[2,4–8]

Serum testosterone levels decrease in aged males, and dysfunction of the aged Leydig cells might plays a key role in this process.[9–11] Androgen is mainly produced by Leydig cells and many enzymes, such as steroidogenic acute regulatory (StAR) protein, cholesterol side-chain cleavage enzyme (P450scc) and 3β-hydroxysteroid dehydrogenase, are involved in regulation of testosterone production.[10–12] The functional form of StAR and P450scc are localized in the mitochondrial membrane, and their function is closely related to the mitochondria, furthermore, the StAR protein is an important and rate-limiting enzyme in the regulation of testosterone biosynthesis of Leydig cells. StAR protein expression is predominantly regulated by a cyclic adenosine monophosphate-dependent pathway.[12] It has been demonstrated that reactive oxygen species (ROS) inhibit StAR expression and testosterone production in Leydig cells through activating p38 mitogen-activated protein kinase or c-Jun.[13–16] Because mitochondria are the crucial sources of ROS in aerobic eukaryotic cells, androgen deficiency in aged males may be attributed to dysfunctional mitochondria owing to an accumulation of ROS.[17–19]

Autophagy is a cellular degradative pathway that involves the delivery of cytoplasmic cargo to lysosomes, and it is essential for cell survival, differentiation, development and homeostasis.[19–21] Autophagy can be induced by inhibiting target of rapamycin with rapamycin or blocked by inhibiting the class III PI3K with 3-methyladenine, wortmannin or bafilomycin A1 which inhibits the autophagosome fusion. Autophagy can be measured by ultrastructural observation of autophagic compartments (such as the phagophore, autophagosome and autolysosome), by detecting the expression of Atg8-PE or microtubule-associated protein light chain 3 (LC3)-II biochemically or by observing the contribution of green fluorescent protein (GFP)-tagged Atg8 or LC3 microscopically.[22,23] There are three categories of autophagy: microautophagy, macroautophagy and chaperone-mediated autophagy. Mitochondrial homeostasis is maintained by 'mitophagy',

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a particular type of autophagy in the category of macroautophagy. Clearance of dysfunctional or aged mitochondria by mitophagy plays an essential role in the anti-ageing process.[24–26] and many age-related pathologies.[27–29]

In this report, we studied the role of autophagy in regulating androgen production in Leydig cells from aged rats.

MATERIALS AND METHODS

Animals

Ten healthy male Wistar rats at 3 months old (young) and 24 months old (aged) respectively were used for research.[17,30] Animals were supplied by the Animal Center of Peking University First Hospital (Beijing, China) and were kept in a standard animal facility with a controlled temperature (21±1 °C) and photoperiod (12 h light/12 h dark), with free access to water and standard rat chow diet *ad libitum*. All rats were anaesthetized and killed by injection with sodium pentobarbitone (150 mg kg^{-1}) in sterile saline. The protocols for the use of animals were approved by the Animal Care and Use Committee of Peking University Health Center (Beijing, China) in accordance with the National Institutes of Health guidelines.

Materials

A goat anti-3β-hydroxysteroid dehydrogenase and a mouse anti-β-actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit anti-LC3 antibody was purchased from Sigma (St. Louis, MO, USA). An anti-StAR antibody was kindly provided by Dr Douglas M. Stocco (Texas Tech University Health Sciences Center, Lubbock, TX, USA). GFP-labelled LC3 plasmid was kindly provided by Dr Tamotsu Yoshimori (Osaka University, Suita, Osaka, Japan). CM-H2DCF-DA and Lipofectamine RNAiMAX Transfection Reagent were purchased from Invitrogen (Carlsbad, CA, USA). Wortmannin, rapamycin, luteinizing hormone (LH) and *N*-acetyl-*L*-cysteine (NAC) were purchased from Sigma. The TM3 mouse Leydig cell line was purchased from the American Type Culture Collection (Manassas, VA, USA).

Cell culture

Primary rat Leydig cells were prepared from rat testes by density gradient centrifugation. Briefly, decapsulated testes were incubated with type I collagenase (0.25 mg ml^{-1}) for 20 min at 34 °C. Crude interstitial cells were collected by centrifugation at 300 *g* for 10 min and then washed twice in phosphate-balanced solution containing 0.1% (w/v) bovine serum albumin. To obtain purified Leydig cells, the crude cell suspension was loaded on top of a discontinuous Percoll gradient (20%, 40%, 60% and 90% Percoll in phosphate-buffered saline) and subsequently centrifuged at 800 *g* for 20 min. The fraction enriched in Leydig cells was centrifuged in a continuous, self-generating density gradient starting with 60% Percoll at 20 000 *g* for 30 min at 4 °C. The purity of Leydig cells was determined by histochemical staining for 3β-hydroxysteroid dehydrogenase, and cell viability was assessed by Trypan blue dye exclusion. Cells (95% purity and viability) were washed twice with DMEM/F-12 medium and resuspended for use in DMEM/F-12 medium supplemented with 15 mmol l^{-1} HEPES (pH 7.4), 1 mg ml^{-1} bovine serum albumin, 365 mg l^{-1} glutamine, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. For each experiment, 1×10^6 cells ml^{-1} per well were plated into a six-well plate in a volume of 2 ml and cultured at 34 °C in a humidified atmosphere of 5% CO_2. TM3 mouse Leydig cells were cultured in DMEM/F-12 medium supplemented with 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 2.5% horse serum and 2.5% foetal calf serum at 34 °C in a humidified atmosphere of 5% CO_2.

Transmission electron microscopy

Specimens were fixed with glutaraldehyde (3% in 0.1 mol l^{-1} cacodylate buffer, pH 7.4) for 30 min at 4 °C, then post-fixed in osmium tetroxide (OsO_4) and embedded in Epon-812. Sections (0.1 mm) were stained with uranyl acetate/lead citrate and viewed with a JEM1230 transmission electron microscope (JEOL, Tokyo, Japan).

GFP-LC3 transfection and observation

Primary rat Leydig cells (8×10^5) or TM3 mouse Leydig cells with or without Beclin 1 knockdown were plated in 35-mm glass-bottomed dishes with supplemented culture medium. GFP-LC3 plasmid was transfected into the cells with the transfection reagent Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After incubation for another 48 h, the GFP-LC3 puncta in transfected cells were visualized by a confocal laser scanning microscope (OLYMPUS, Tokyo, Japan; excitation: 488 nm, emission: 507 nm). Additionally, TM3 Leydig cells were starved in Earl's balanced salt solution for 8 h before observation.

small interfering RNA (siRNA) transfection

The TM3 Leydig cells were seeded at 40% confluence per well in six-well plates overnight, and the mouse Beclin 1 siRNA (5′-GGACAGUUUGGCACAAUCA-3′)[31] or negative control siRNA (GenePharma, Shanghai, China) was transfected into TM3 Leydig cells with Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). The transfected cells were used for experiments after 72 h.

Testosterone assay by radioimmunoassay

After rats were anaesthetized, blood was collected *via* vena cava puncture. Serum was isolated by centrifugation at 1200 *g* for 20 min and stored at −80 °C until analysis. Culture media were collected and stored at −80 °C until later analysis after cells were treated. The concentration of testosterone in serum and medium samples was determined using a radioimmunoassay kit (Beijing North Institute of Biological Technology, Beijing, China) in duplicate following the manufacturer's manual. Testosterone concentration in cultured medium was normalized to cell numbers for each sample.

Western blotting

Samples were solubilized in lysis buffer (50 mmol l^{-1} Tris-HCl, 150 mmol l^{-1} NaCl, 50 mmol l^{-1} NaF, 0.5% NP-40, pH 7.5). Proteins were separated by 12% or 15% SDS-PAGE with 20 μg of total protein in each lane and then electrotransferred onto a nitrocellulose membrane. After incubation with primary antibody (1 : 1000) at 4 °C overnight, the membrane was rinsed with TBST (1% Tween-20) three times (10 min each) and then incubated with horseradish peroxidase-conjugated secondary antibody (1 : 5000) at room temperature for 1 h. After the membrane was rinsed with TBST (1% Tween-20) three times (10 min each), the signals were detected using an enhanced chemiluminescence Western blot kit from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The X-ray films were quantified by densitometry and analysed with Quantity One-4.6.2 (BIO-RAD, Hercules, CA, USA).

Measurement of intracellular ROS

CM-H_2DCF-DA (Invitrogen), an accepted cell-permeant indicator of ROS, was used to assess the ROS level in Leydig cells. Cells were

harvested by centrifugation (1000 *g* for 3 min) and washed in serum-free medium (pH 7.2, buffered with HEPES) three times. The cells were resuspended in serum-free culture medium at 10^5 cells ml^{-1}. The cells were then treated with 10 mmol l^{-1} CM-H_2DCF-DA and incubated at 34 °C for 10 min. Finally, the cells were plated into a 96-well plate and incubated with 1 mmol l^{-1} H_2O_2 for 30 min at 34 °C. The relative ROS level within cells was analysed by a fluorescence microplate fluorometer (Turner BioSystems, CA, USA) (excitation: 490 nm, emission: 530 nm), and data were expressed as relative numerical values of fluorescence.

Statistical analysis

Data are presented as mean±s.d., and significance was determined by analysis of variance followed by the Duncan's multiple comparison test. Immunoblot data were quantified by the band density relative to that of β-actin and analysed by an unpaired t-test for independent samples. $P<0.05$ was considered statistically significant.

RESULTS

Autophagic deficiency occurs in aged rat testes and is associated with inhibited StAR expression and a decline of serum testosterone

To illustrate the function of the Leydig cells, transmission electron microscope was used for ultrastructural observation, and total serum testosterone and StAR protein expression in testes were examined in young and aged male rats. Ultrastructural observation of rat Leydig cells demonstrated that swollen mitochondria with mitochondrial cristae loss were found in aged rat Leydig cells (**Figure 1d**, upper panel). The expression of StAR protein was down-regulated in the testes of aged rats (**Figure 1a**), and the total serum testosterone concentration of aged rats was lower than that in young rats ($P<0.05$) (**Figure 1b**). To assess autophagic activity, the expression of LC3, a marker for the generation of tautophagosome, was also detected by Western blot. Electron microscope (EM) was used for ultrastructural observation of autophagic compartments, including the phagophore, autophagosome and autolysosome, in Leydig cells *in vivo*. The results

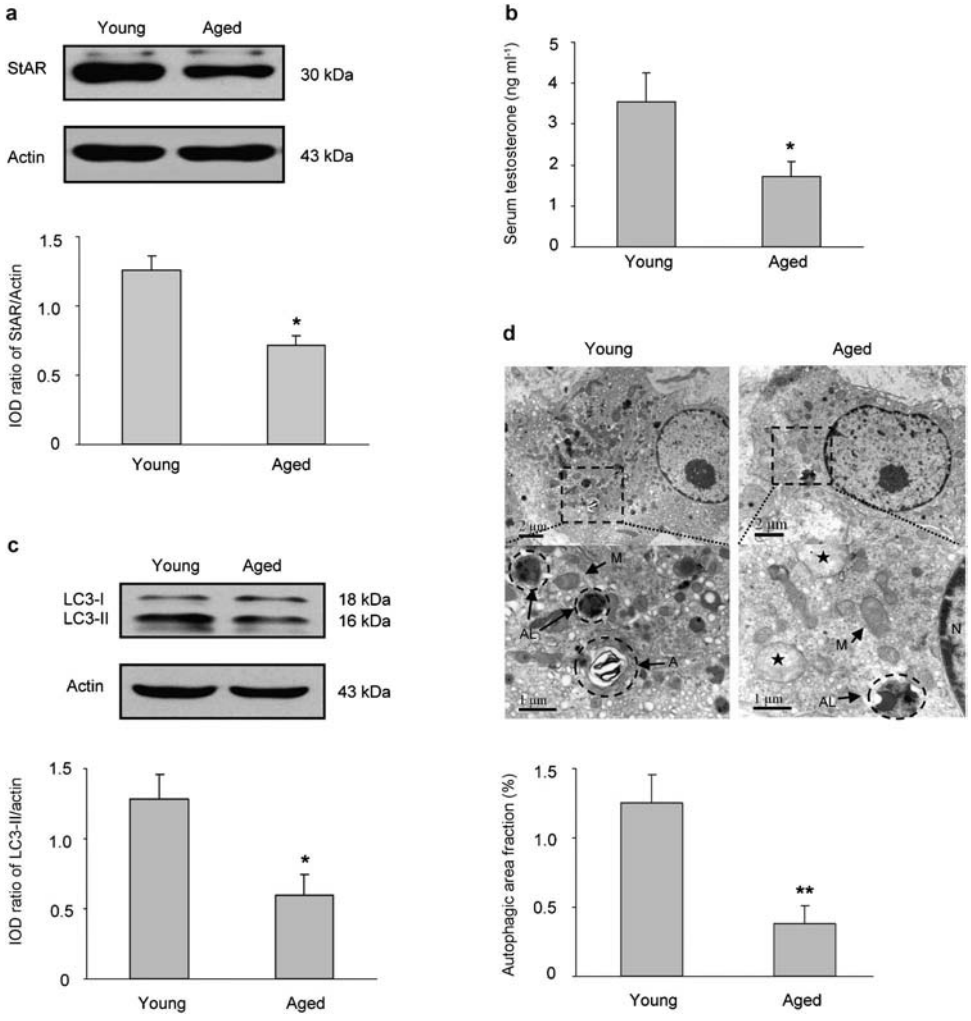

Figure 1 Autophagic deficiency occurs in aged rat testes and is associated with the inhibition of StAR expression and decline of serum testosterone. (**a**) Expression of StAR protein in testes was detected by Western blot analysis. The expression of StAR protein decreased in aged rat testes. (**b**) Total serum testosterone was lower in aged rats than in young rats. (**c**) The expression of LC3-II decreased in aged rat testes. (**d**) Fewer autophagic compartments (including phagophore, autophagosome and autolysosome) were observed in aged rat Leydig cells by EM. The data of the area ratio were not distributed normally. Data are presented as the mean of the area fraction in 50 cells randomly selected from each group. *$P<0.05$, **$P<0.01$ ($n=10$, compared with young rats). Arrow (→), Leydig cells; asterisk (★), swollen mitochondria. Abbreviations: A, autophagosome; AL, autolysosome; EM, electron microscope; IOD, integrated optical density; LC3, microtubule-associated protein light chain 3; M, mitochondria; N, nucleus; StAR, steroidogenic acute regulatory protein.

showed that LC3-II expression was decreased in the testes of aged rats compared to young rats ($P<0.05$) (**Figure 1c**). Statistical analysis consistently showed that the autophagic area fraction decreased in aged rat Leydig cells ($P<0.01$) (**Figure 1d**, lower panel).

StAR protein expression and testosterone production decrease in primary Leydig cells from aged rats, accompanied by autophagic deficiency

To compare the functional difference between young and aged rat Leydig cells *in vitro*, we isolated Leydig cells from young and aged rats and treated them for 48 h with 100 nmol l^{-1} rapamycin, 1 mmol l^{-1} NAC, 50 nmol l^{-1} wortmannin or 50 nmol l^{-1} wortmannin with a pre-treatment with 1 mmol l^{-1} NAC for 1 h. All groups, except the controls, were treated with 100 ng ml^{-1} LH for another 3 h before harvesting. To investigate the steroidogenic function of Leydig cells, StAR protein expression was determined by Western blotting, and the testosterone level was examined using the radioimmunoassay kit. StAR protein levels were increased by the stimulation of LH both in young and aged Leydig cells ($P<0.01$). Furthermore, irrespective of whether LH was present, the StAR protein expression was lower in aged Leydig cells than in young cells ($P<0.01$ and $P<0.05$, respectively) (**Figure 2a**). In parallel with StAR protein expression, the testosterone levels in the supernatant from young and aged Leydig cells were also elevated by LH stimulation ($P<0.01$). Furthermore, irrespective of whether LH was present, testosterone levels were lower in the supernatant from aged Leydig cells than in that from young cells ($P<0.01$ and $P<0.05$, respectively) (**Figure 2b**). To compare differences in autophagic activity between young and aged Leydig cells, LC3 expression was examined by Western blot analysis. LC3-II expression

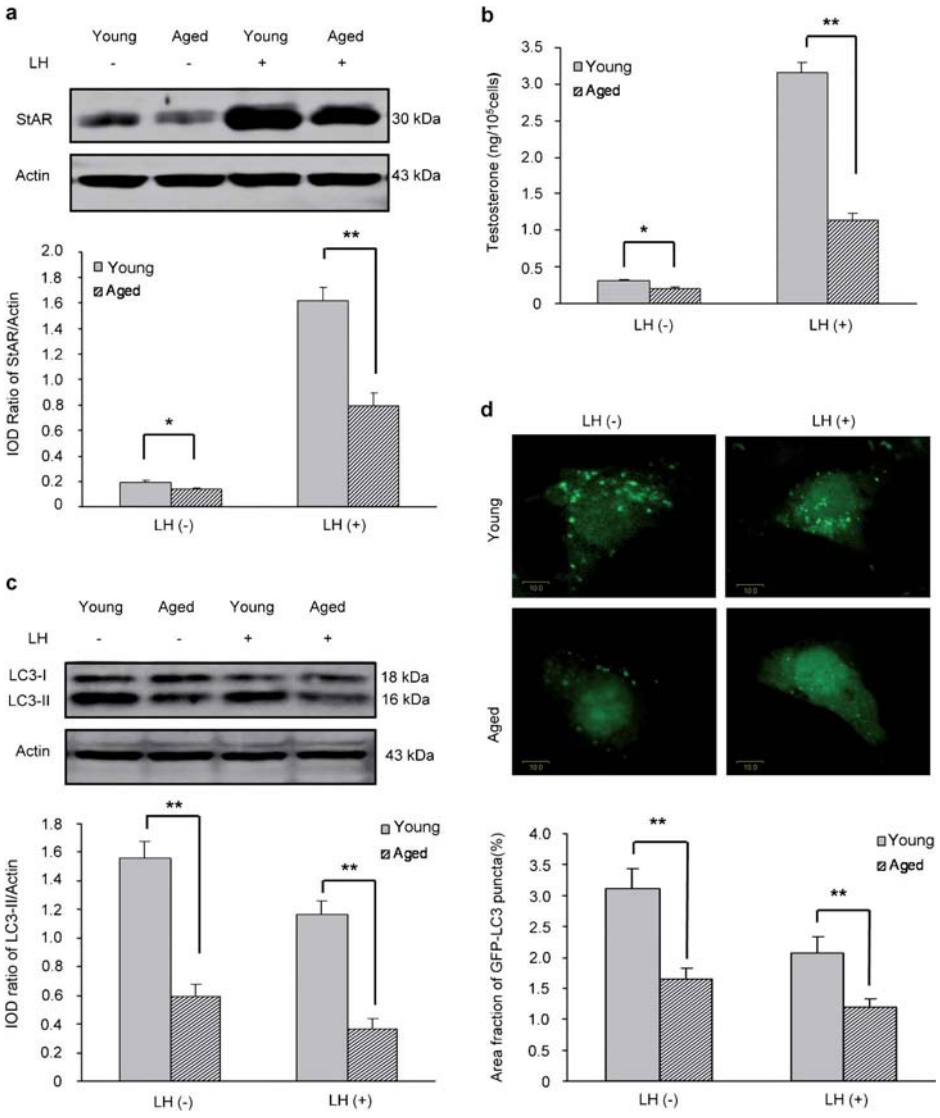

Figure 2 StAR expression and testosterone production decrease in aged primary rat Leydig cells is associated with autophagic deficiency. (**a**) The expression of StAR protein was increased by LH stimulation both in young and aged Leydig cells ($P<0.01$). StAR protein expression in aged Leydig cells was lower than that in young cells both in the presence ($P<0.01$) and absence ($P<0.05$) of LH. (**b**) The supernatant testosterone level was promoted by LH stimulation. The supernatant testosterone level of aged Leydig cells was lower than that of young cells either in the presence or absence of LH ($P<0.05$). (**c**) The expression of LC3-II in aged Leydig cells was lower than that in young cells either in the presence ($P<0.01$) and absence ($P<0.05$) of LH. (**d**) The area fraction of punctate GFP-LC3 in young Leydig cells was higher than that in aged ones. Data are presented as the mean of the area fraction in 50 cells randomly selected from each group. *$P<0.05$, **$P<0.01$ ($n=10$). Abbreviations: GFP, green fluorescent protein; IOD, integrated optical density; LC3, microtubule-associated protein light chain 3; LH, luteinizing hormone; StAR, steroidogenic acute regulatory protein.

was lower in aged Leydig cells than in young Leydig cells in the presence or absence of LH ($P<0.01$) (**Figure 2c**). Furthermore, when Leydig cells were transfected with GFP-LC3 plasmid for 48 h, the area fraction of GFP-LC3 puncta in young Leydig cells was higher than that in aged Leydig cells ($P<0.01$) (**Figure 2d**).

Alterations of autophagic activity affect LH-stimulated StAR protein expression and testosterone production in primary rat Leydig cells

To investigate the effect of autophagic alteration on steroidogenesis, primary Leydig cells were isolated from the testes of young and aged rats. The Leydig cells were treated for 48 h with 100 nmol l^{-1} rapamycin, 1 mmol l^{-1} NAC,

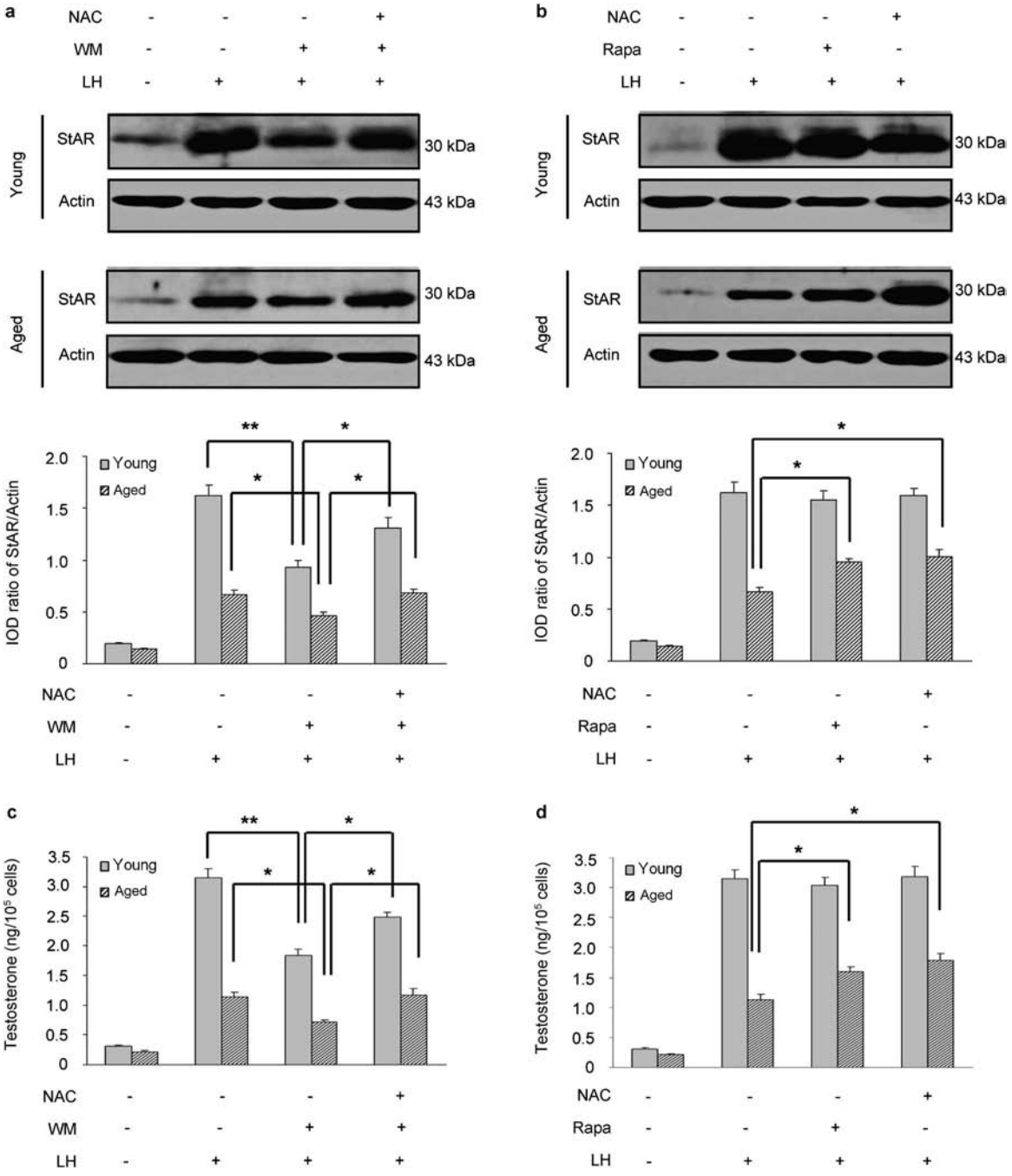

Figure 3 Alterations of autophagic activity affect LH-stimulated StAR protein expression and testosterone production in primary rat Leydig cells. (**a**) Effect of autophagic inhibition on LH-stimulated StAR protein expression. For young or aged rat primary Leydig cells, the LH-stimulated StAR protein expression was inhibited by wortmannin, which could be attenuated by NAC. (**b**) When treated with rapamycin or NAC, the LH-stimulated StAR protein expression increased in aged, but not young, Leydig cells. (**c**) The effects of autophagic alteration on LH-stimulated testosterone production paralleled that of StAR protein expression. When treated with wortmannin, LH-stimulated testosterone production decreased in both young and aged rat Leydig cells, and this decrease could be attenuated by NAC. (**d**) Treatment with rapamycin or NAC increased LH-stimulated testosterone production in aged Leydig cells, whereas there was no significant effect on young cells. $^{*}P<0.05$, $^{**}P<0.01$ ($n=4$). Abbreviations: IOD, integrated optical density; LH, luteinizing hormone; NAC, *N*-acetyl-L-cysteine; Rapa, rapamycin; StAR, steroidogenic acute regulatory protein; WM, wortmannin.

50 nmol l^{-1} wortmannin or 50 nmol l^{-1} wortmannin with a pre-treatment with 1 mmol l^{-1} NAC for 1 h. All groups, except controls, were treated with 100 ng ml^{-1} LH for another 3 h before harvest. The StAR protein was examined in the cells with different treatments. LH stimulated strong StAR protein expression in both young and aged cells ($P<0.01$) (**Figure 3a**, comparing lane 1 to 2). However, wortmannin impaired the effect of LH stimulation in both young ($P<0.01$) and aged ($P<0.05$) cells, whereas NAC negated the effect of wortmannin (**Figure 3a**, comparing lanes 3 and 4 to 2). To further confirm the role of autophagy in the regulation of StAR protein expression, we treated the Leydig cells with rapamycin. We also used NAC to induce ROS-mediated clearance of Leydig cells. In the results, the levels of StAR protein expression were increased by treating with rapacmycin or NAC ($P<0.05$) in LH-stimulated aged rat Leydig cells, however, these changes were not found in young rat Leydig cells ($P<0.05$) (**Figure 3b**). Consistent with the StAR protein level, LH-stimulated testosterone production was decreased in young and aged rats Leydig cells ($P<0.05$) by treatment with wortmannin ($P<0.01$), however, which was restored by pre-treatment with NAC (**Figure 3c**). Similarly, treatment with rapamycin or NAC were increased levels of LH-stimulated testosterone production in aged, but not young, Leydig cells ($P<0.05$) (**Figure 3d**).

ROS are involved in changes in LH-induced steroidogenesis resulting from autophagic alteration in primary rat Leydig cells

To investigate the effect of autophagic alteration on oxidative stress, the relative intracellular ROS level in primary Leydig cells was examined. ROS levels increased after H_2O_2 treatment, as shown by the positive control ($P<0.01$) (**Figure 4a**). Wortmannin enhanced the cellular ROS levels in both young and aged Leydig cells ($P<0.01$), and this enhancement could be attenuated by NAC (**Figure 4a**). Furthermore, rapamycin or NAC decreased the cellular ROS level in aged, but not in young rat Leydig cells ($P<0.01$) (**Figure 4a**). To investigate the effect on steroidogenesis, we treated primary rat Leydig cells with different concentrations of H_2O_2. Exposure of primary rat Leydig cells to H_2O_2 led to a decrease in LH-stimulated StAR protein expression (**Figure 4b**). Similarly, H_2O_2 treatments decreased the level of testosterone production (**Figure 4c**).

Knockdown of Beclin 1 leads to autophagic deficiency

To investigate the effect of autophagic deficiency on StAR protein expression and testosterone production, Beclin 1 was knocked down with siRNA in TM3 mouse Leydig cells. The protein expression of LC3-II expression as determined by Western blot (**Figure 5a**), area fraction of autophagic compartment in EM (**Figure 5b**) and GFP-LC3 puncta in fluorescence microscopy (**Figure 5c**) were all decreased in TM3 Leydig cells after knockdown of Beclin 1 ($P<0.01$).

Knockdown of Beclin 1 in TM3 mouse Leydig cells leads to a decrease in StAR protein expression and testosterone production that is associated with an increased cellular ROS level

After knockdown of Beclin 1, LH-stimulated StAR protein expression (**Figure 6a**) and testosterone production (**Figure 6b**) decreased ($P<0.01$), whereas the cellular ROS level increased in TM3 mouse Leydig cells after knockdown of Beclin 1 ($P<0.01$) (**Figure 6c**).

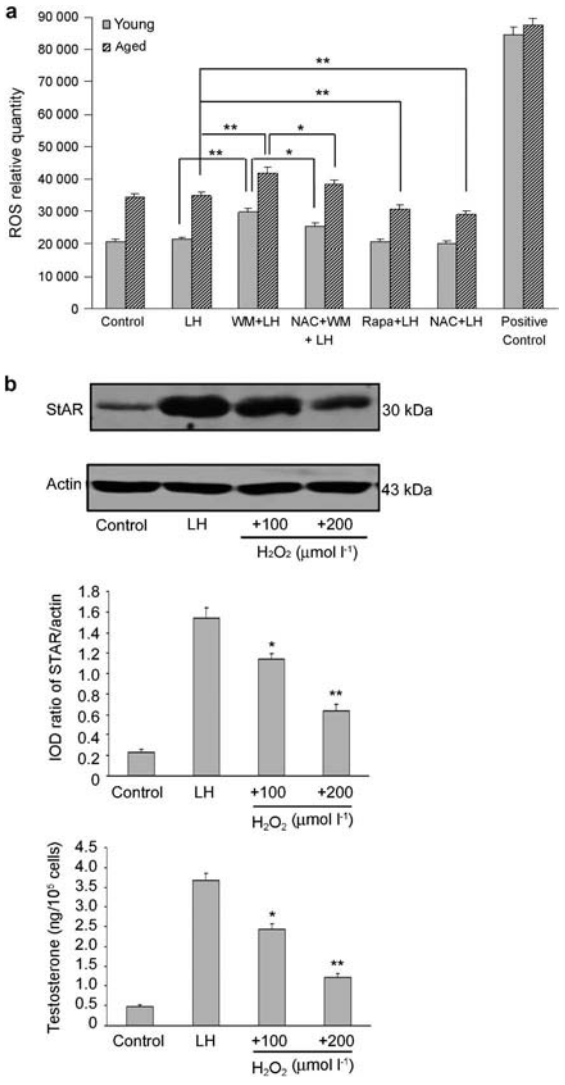

Figure 4 ROS are involved in the changes of LH-stimulated steroidogenesis resulting from autophagic alteration in primary rat Leydig cells. (**a**) The positive control was treated with 1 mmol l^{-1} H_2O_2, and the ROS level was strongly increased. Inhibition of autophagy with wortmannin led to increased ROS levels in both young and aged rat Leydig cells, which could be attenuated by NAC. Treatment with NAC or enhancement of autophagic activity with rapamycin led to a decreased ROS level in aged rat Leydig cells but not in young cells. *$P<0.05$, **$P<0.01$ ($n=4$, compared with LH group). (**b**) Treatment with H_2O_2 led to inhibition of LH-stimulated StAR protein expression and testosterone production in rat primary Leydig cells. *$P<0.05$, **$P<0.01$ ($n=4$, compared with LH group). Abbreviations: IOD, integrated optical density; LH, luteinizing hormone; NAC, *N*-acetyl-*L*-cysteine; Rapa, rapamycin; ROS, reactive oxygen species; StAR, steroidogenic acute regulatory protein; WM, wortmannin.

DISCUSSION

Androgen deficiency in elderly men may lead to LOH and decrease quality of life. Because testosterone deficiency plays a key role in LOH, testosterone replacement therapy has been used to alleviate the symptoms of LOH.[5,8,9,32] To reveal the mechanism of decreased testosterone production in aged Leydig cells and possibly identify new therapies, we studied the effect of autophagic alteration on steroidogenesis in young and aged rat Leydig cells and TM3 mouse Leydig cells. Our results indicate that decreased steroidogenesis is associated with the autophagic deficiency in aged rat Leydig cells. Previous studies have reported that StAR protein expression and testosterone production were decreased in aged Leydig cells.[9–11,33] No studies on the linkage between autophagy and testosterone production have been reported, although Taneike *et al.*[34] reported decreased autophagic activity in aged heart tissue, and Zhang *et al.*[29] reported low autophagic activity in aged smooth muscles of the corpus cavernosum. Our study showed that the autophagic activity could affect StAR protein

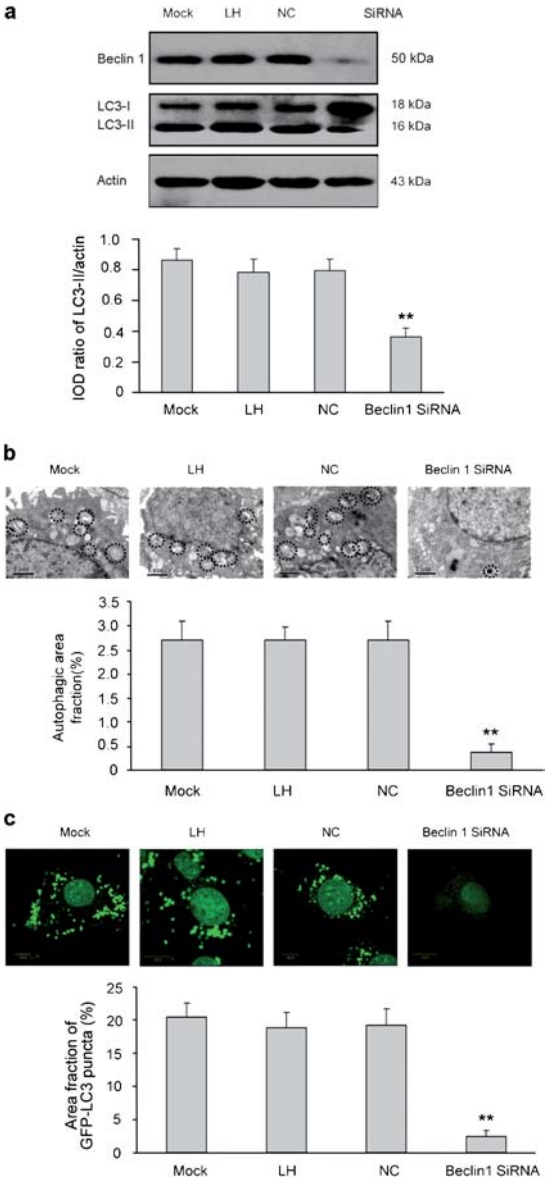

Figure 5 Knockdown of Beclin 1 leads to autophagic deficiency. (**a**) Knockdown of Beclin 1 led to decreased protein expression of LC3-II. (**b**) Knockdown of Beclin 1 led to a decreased area fraction of autophagic compartments. (**c**) Knockdown of Beclin 1 led to a decreased area fraction of GFP-LC3 puncta. $^{**}P<0.01$ ($n=4$, compared with NC group). Abbreviations: GFP, green fluorescent protein; IOD, integrated optical density; LC3, microtubule-associated protein light chain 3; LH, luteinizing hormone; NC, negative control; siRNA, small interfering RNA.

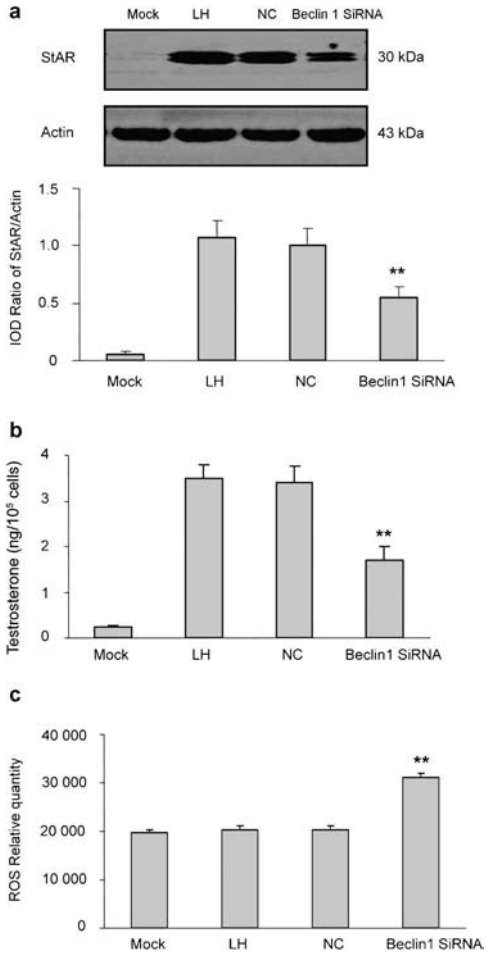

Figure 6 Knockdown of Beclin 1 leads to increased cellular ROS levels and down-regulation of LH-stimulated StAR protein expression and testosterone production. (**a**) LH-stimulated StAR protein expression decreased in TM3 Leydig cells after knockdown of Beclin 1. (**b**) The LH-stimulated testosterone level decreased in TM3 Leydig cells after knockdown of Beclin 1. (**c**) The cellular ROS level increased in TM3 Leydig cells after knockdown of Beclin 1. $^{**}P<0.01$ ($n=4$, compared with NC group). Abbreviations: LH, luteinizing hormone; NC, negative control; ROS, reactive oxygen species; StAR, steroidogenic acute regulatory protein.

expression and testosterone production in aged Leydig cells, possibly by elevating the cellular ROS level.

We attributed the linkage between lower testosterone levels and autophagic deficiency in aged Leydig cells to intracellular ROS accumulation generated from dysfunctional or aged mitochondria. Indeed, we observed that the autophagic area, identified by autophagic area fractoin of both autopagosomes in EM and puncta of GFP-LC3 in fluorescence microscope to identify the autophagic activity, was much lower in aged rat Leydig cells than in young rat Leydig cells. In addition, we observed low levels of StAR protein and testosterone production in aged Leydig cells. To confirm the role of autophagy in regulating testosterone production, the autophagy activator (rapamycin) and inhibitor (wortmannin) were used to treat primary Leydig cells. We showed that the autophagy inhibitor wortmannin decreased LH-stimulated StAR protein expression and testosterone production in both young and aged Leydig cells, whereas rapamycin had an enhancing effect in aged Leydig cells. Furthermore, we assessed the intracellular ROS levels and found that ROS levels were elevated by wortmannin, suggesting that autophagy is required for the clearance of ROS. Because ROS are harmful to steroidogenesis in Leydig cell,[13–16] accumulation of ROS during the ageing process will result in a low level of testosterone. It may be possible that mitochondrial dysfunction in ageing cells contributes to oxidative stress.[35] LH-stimulated StAR protein expression and testosterone production were reduced in primary rat Leydig cells when exposed to H_2O_2, which demonstrates the harmful effects of ROS on steroidogenesis. Taken together, our data demonstrate that aged Leydig cells have an autophagic deficiency, an increased number of dysfunctional mitochondria and an increased ROS level. We propose that the autophagic deficiency may result in ROS accumulation, leading to a decline in steroidogenesis in aged rat Leydig cells. We used the TM3 mouse Leydig cell line as the model and knocked down Beclin 1 to study the effect of autophagic

deficiency on LH-stimulated StAR expression and testosterone production. The LH-stimulated StAR expression and testosterone production decrease was associated with an increase of cellular ROS levels.

Impaired or deficient autophagy is believed to cause or contribute to ageing and a number of age-related pathologies. ROS have been linked to autophagy and ageing in several studies. Wu *et al.*[18] reported that the intracellular ROS level of Atg7$^{-/-}$ cells was increased, but it could be decreased by the antioxidant NAC. Masiero *et al.*[36] reported that deletion of Atg7 resulted in muscle atrophy and an age-dependent decrease in strength with accumulation of abnormal mitochondria and a swollen sarcoplasmic reticulum. Taneike *et al.*[34] found that Atg5-deficient mice exhibited disorders in cardiac morphology and function. The ultrastructural analysis revealed a disorganized sarcomere structure and collapsed mitochondria with decreased mitochondrial respiratory functions. Collectively, these data suggest that mitochondrial dysfunction and oxidative stress have a role in autophagic deficiency-related pathology. These studies support our hypothesis that autophagic deficiency might result in increased cellular ROS levels.

The role of autophagy on testosterone production has been implied in studies of calorie restriction (CR). The age-related changes in plasma testosterone, triglycerides, oxidative damage and glucose regulation could be attenuated by CR,[37,38] which is related to autophagic activation.[39] Sitzmann *et al.*[40] reported that moderate CR could help maintain higher levels of circulating testosterone. Kume *et al.*[41] further demonstrated that adult-onset and long-term CR in mice attenuated hypoxia-associated mitochondrial and renal damage by enhancing autophagic activity. These findings implied that autophagy is related to the testosterone production and are congruent to the findings in this study.

In conclusion, we have demonstrated that autophagic deficiency plays an important role in the decreased testosterone production of aged Leydig cells and increased intracellular ROS may be involved in this process.

AUTHOR CONTRIBUTIONS

ZCX and ZJC designed the research; WRL, LC, HX, TL, YQZ, GYL, FZ, YQG and ZZG performed the studies; WRL, ZJC and ZCX wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1 Nieschlag E, Swerdloff R, Behre HM, Gooren LJ, Kaufman JM *et al*. Investigation, treatment, and monitoring of late-onset hypogonadism in males: ISA, ISSAM, and EAU recommendations. *J Androl* 2006; **27**: 135–7.
2 Jones TH. Late onset hypogonadism. *BMJ* 2009; **338**: b352.
3 Wu FC, Tajar A, Beynon JM, Pye SR, Silman AJ *et al*. Identification of late-onset hypogonadism in middle-aged and elderly men. *N Engl J Med* 2010; **363**: 123–35.
4 Bassil N, Morley JE. Late-life onset hypogonadism: a review. *Clin Geriatr Med* 2010; **26**: 197–222.
5 Amano T, Imao T, Takemae K, Iwamoto T, Nakanome M. Testosterone replacement therapy by testosterone ointment relieves lower urinary tract symptoms in late onset hypogonadism patients. *Aging Male* 2010; **13**: 242–6.
6 Permpongkosol S, Tantirangsee N, Ratana-Olarn K. Treatment of 161 men with symptomatic late onset hypogonadism with long-acting parenteral testosterone undecanoate: effects on body composition, lipids, and psychosexual complaints. *J Sex Med* 2010; **7**: 3765–74.
7 Wang C, Nieschlag E, Swerdloff R, Behre HM, Hellstrom WJ *et al*. Investigation, treatment, and monitoring of late-onset hypogonadism in males: ISA, ISSAM, EAU, EAA, and ASA recommendations. *J Androl* 2009; **30**: 1–9.
8 Makinen JI, Huhtaniemi I. Androgen replacement therapy in late-onset hypogonadism: current concepts and controversies. *Gerontology* 2010; **57**: 193–202.
9 Arianayagam R, Arianayagam M, McGrath S, Rashid P. Androgen deficiency in the aging man. *Aust Fam Physician* 2010; **39**: 752–5.
10 Luo LD, Chen HL, Zirkin BR. Leydig cell aging: steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme. *J Androl* 2001; **22**: 149–56.
11 Leers-Sucheta S, Stocco DM, Azhar S. Down-regulation of steroidogenic acute regulatory (StAR) protein in rat Leydig cells: implications for regulation of testosterone production during aging. *Mech Ageing Dev* 1999; **107**: 197–203.
12 Manna PR, Dyson MT, Stocco DM. Regulation of the steroidogenic acute regulatory protein gene expression: present and future perspectives. *Mol Hum Reprod* 2009; **15**: 321–33.
13 Diemer T, Allen JA, Hales KH, Hales DB. Reactive oxygen disrupts mitochondria in MA-10 tumor Leydig cells and inhibits steroidogenic acute regulatory (StAR) protein and steroidogenesis. *Endocrinology* 2003; **144**: 2882–91.
14 Chen L, Xin ZC, Li X, Tian L, Yuan YM *et al*. Cox7a2 mediates steroidogenesis in TM3 mouse Leydig cells. *Asian J Androl* 2006; **8**: 589–94.
15 Abidi P, Zhang HY, Zaidi SM, Shen WJ, Leers-Sucheta S *et al*. Oxidative stress-induced inhibition of adrenal steroidogenesis requires participation of p38 mitogen-activated protein kinase signaling pathway. *J Endocrinol* 2008; **198**: 193–207.
16 Lee SY, Gong EY, Hong CY, Kim KH, Han JS *et al*. ROS inhibit the expression of testicular steroidogenic enzyme genes *via* the suppression of Nur77 transactivation. *Free Radical Bio Med* 2009; **47**: 1591–600.
17 Sahoo DK, Roy A, Chainy GB. Rat testicular mitochondrial antioxidant defence system and its modulation by aging. *Acta Biol Hung* 2008; **59**: 413–24.
18 Wu JJ, Quijano C, Chen E, Liu H, Cao L *et al*. Mitochondrial dysfunction and oxidative stress mediate the physiological impairment induced by the disruption of autophagy. *Aging (Albany NY)* 2009; **1**: 425–37.
19 Kim I, Mizushima N, Lemasters J. Selective removal of damaged mitochondria by autophagy (mitophagy). *Hepatology* 2006; **44** (Suppl 1): 241A.
20 Klionsky DJ, Emr SD. Cell biology—autophagy as a regulated pathway of cellular degradation. *Science* 2000; **290**: 1717–21.
21 Mizushima N, Levine B. Autophagy in mammalian development and differentiation. *Nat Cell Biol* 2010; **12**: 823–30.
22 Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell* 2008; **132**: 27–42.
23 Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G *et al*. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy* 2008; **4**: 151–75.
24 Mammucari C, Rizzuto R. Signaling pathways in mitochondrial dysfunction and aging. *Mech Ageing Dev* 2010; **131**: 536–43.
25 Gottlieb RA, Carreira RS. Autophagy in health and disease. 5. Mitophagy as a way of life. *Am J Physiol-Cell Ph* 2010; **299**: C203–10.
26 Weber TA, Reichert AS. Impaired quality control of mitochondria: aging from a new perspective. *Exp Gerontol* 2010; **45**: 503–11.
27 McMullen CA, Ferry AL, Gamboa JL, Andrade FH, Dupont-Versteegden EE. Age-related changes of cell death pathways in rat extraocular muscle. *Exp Gerontol* 2009; **44**: 420–5.
28 Parkington JD, LeBrasseur NK, Siebert AP, Fielding RA. Contraction-mediated mTOR, p70S6k, and ERK1/2 phosphorylation in aged skeletal muscle. *J Appl Physiol* 2004; **97**: 243–8.
29 Zhang J, Wu XJ, Zhuo DX, Liu T, Li WR *et al*. Effect of tankyrase 1 on autophagy in the corpus cavernosum smooth muscle cells from ageing rats with erectile dysfunction and its potential mechanism. *Asian J Androl* 2010; **12**: 744–52.
30 Levy S, Serre V, Hermo L, Robaire B. The effects of aging on the seminiferous epithelium and the blood–testis barrier of the Brown Norway rat. *J Androl* 1999; **20**: 356–65.
31 Fujiwara K, Daido S, Yamamoto A, Kobayashi R, Yokoyama T *et al*. Pivotal role of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 in apoptosis and autophagy. *J Biol Chem* 2008; **283**: 388–97.
32 Page ST. Reproductive endocrinology: Late-onset hypogonadism: evidence for diagnostic criteria. *Nat Rev Endocrinol* 2010; **6**: 602–3.
33 Wang X, Shen CL, Dyson MT, Eimerl S, Orly J *et al*. Cyclooxygenase-2 regulation of the age-related decline in testosterone biosynthesis. *Endocrinology* 2005; **146**: 4202–8.
34 Taneike M, Yamaguchi O, Nakai A, Hikoso S, Takeda T *et al*. Inhibition of autophagy in the heart induces age-related cardiomyopathy. *Autophagy* 2010; **6**: 600–6.
35 Seo AY, Joseph AM, Dutta D, Hwang JC, Aris JP *et al*. New insights into the role of mitochondria in aging: mitochondrial dynamics and more. *J Cell Sci* 2010; **123**: 2533–42.
36 Masiero E, Agatea L, Mammucari C, Blaauw B, Loro E *et al*. Autophagy Is required to maintain muscle mass. *Cell Metab* 2009; **10**: 507–15.
37 Roth GS, Lane MA, Ingram DK, Mattison JA, Elahi D *et al*. Biomarkers of caloric restriction may predict longevity in humans. *Science* 2002; **297**: 811.
38 Mattison JA, Lane MA, Roth GS, Ingram DK. Calorie restriction in rhesus monkeys. *Exp Gerontol* 2003; **38**: 35–46.
39 Madeo F, Tavernarakis N, Kroemer G. Can autophagy promote longevity? *Nat Cell Biol* 2010; **12**: 842–6.
40 Sitzmann BD, Leone EH, Mattison JA, Ingram DK, Roth GS *et al*. Effects of moderate calorie restriction on testosterone production and semen characteristics in young rhesus macaques (*Macaca mulatta*). *Biol Reprod* 2010; **83**: 635–40.
41 Kume S, Uzu T, Horiike K, Chin-Kanasaki M, Isshiki K, *et al*. Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney. *J Clin Invest* 2010; **120**: 1043–55.