Life Sciences 229 (2019) 93–97

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Review article

Pharmacological modulation of autophagy as a novel potential target in the successful implementation of *in vitro* fertilization

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ARTICLE INFO

Keywords:
Apoptosis
Autophagy
Endoplasmic reticulum stress
in vitro fertilization
Obesity
Oxidative stress

ABSTRACT

Autophagy is an important intracellular process to maintain homeostasis and studies have shown the key role of autophagy in modulating the functions of reproductive system. Alongside with it, the activation of autophagy has also been found to regulate a number of important processes involved in *in vitro* fertilization including degeneration of granulosa cells and oocyte defects in obese and aging women; apoptosis of oocytes during vitrification-warming; quality and viability of embryo; developmental competence and pre-implantation development of *in vitro* produced blastocysts; placental vascularization and fetal growth. The different mechanisms that may contribute in autophagy-mediated increase in developmental competence and pre-implantation development include decrease in endoplasmic reticulum (ER) stress, activation of poly(ADP-ribosyl)ation (PARP) and reduction in free radical production. The present review discusses the role of autophagy activation in increasing the efficiency of *in vitro* fertilization by modulating different aspects related to fertilization.

1. Introduction

Since the birth of a child from *in vitro* fertilization in 1978, it has been extensively used clinically and *in vitro* fertilization-conceived children account for approximately 0.2%–4.2% of all births worldwide [1]. The demand of *in vitro* fertilization is continuously increasing [2] owing to increase in the number of cases of infertility in women including polycystic ovarian syndrome (PCOS) [3]. *In vitro* fertilization along with embryo transfer involves extraction of oocytes from an infertile woman followed by *in vitro* fertilization with sperm. Thereafter, these fertilized eggs are transferred to the uterine cavity of woman for the development of fetus [4]. There is a need of persistent innovations to increase the overall efficiency of *in vitro* fertilization and scientists have unfolded the usefulness of autophagy activation in increasing the efficiency of *in vitro* fertilization [5–7].

The term autophagy was coined by C de Duve for the process in which cytoplasmic contents are degraded in the lysosomes [8]. Since the discovery of existence of autophagy in the biological system, there have been a large number of studies showing the important role of autophagy in regulating physiological as well as pathophysiological states including polycystic kidney disease [9], inflammatory bowel disease [10], neurodegenerative diseases [11], cancer and cardiovascular diseases [12]. Apart from these, scientists have found that there is an important role of autophagy in reproductive system [13], including apoptosis of oocytes [14], embryonic development [15], placental and fetal growth [16]. Moreover, autophagy has also been shown to modulate different steps involved in *in vitro* fertilization including degeneration of granulosa cells [17], oocyte defects [18], apoptosis of oocytes during vitrification-warming [19], quality and viability of embryo [5], developmental competence and pre-implantation development of blastocysts [6,7], placental vascularization and fetal growth [20]. The present review discusses the role of autophagy activation in increasing the efficiency of *in vitro* fertilization by modulating different aspects related to fertilization.

2. Induction of autophagy overcomes obesity-induced degeneration of human granulosa cells, oocyte dysfunction and endometrial stromal cell decidualization

It has been well documented that in obese women or polycystic ovarian syndrome patients, there is an increased degeneration of human granulosa cells due to increased circulating levels of oxidized low-density lipoprotein (oxLDL). It leads to reduction of overall efficiency of *in vitro* fertilization therapy. It is shown that treatment with antioxidant such as resveratrol or desferoxamine protects the granulosa cells from oxLDL-induced damage by virtue of induction of protective autophagy and reduction of oxidative stress markers. It suggests that induction of autophagy may be protective in preventing the

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https://doi.org/10.1016/j.lfs.2019.05.032
Received 22 January 2019; Received in revised form 1 May 2019; Accepted 11 May 2019
Available online 13 May 2019

degeneration of granulosa cells, which in turn may be useful for obese women or patients of PCOS [17]. The previous study from the same group of scientists exhibited that the activation of autophagy in the granulosa cells during high circulating levels of oxLDL are dependent on the activation of oxidized lipoprotein receptor 1 and toll-like 4 receptors [21]. Other studies also confirmed that there is greater degree of death in the isolated granulosa cells (about 50%) harvested from aged, obese women in comparison to younger, normal-weight females (20%) mainly due to decrease in reparative autophagy and decline in antioxidant mechanism [22]. It suggests that apart from obesity, there is also a negative influence of aging on the survival of granulosa cells, which is again related to decrease in autophagy activation. A very recent study aiming to explore the relationship between parental age and gene expression profiles in human blastocysts reported that there is a decrease in the expression of autophagy-related proteins in the blastocysts with advancing age [23].

Moreover, it is also proposed that obesity decreases the performance of oocytes by promoting accumulation of damaged mitochondria, which may be possibly attributed to the decrease in clearance of damaged mitochondria in a process. In other words, a decrease in mitophagy (autophagy of mitochondria) may be responsible for accumulation of damaged mitochondria in the oocytes of obese women. Moreover, these oocytes carry forward these damaged mitochondria to blastocysts on fertilization. Indeed, it has been depicted that *in vitro* fertilization of high fat, high sugar-exposed oocytes led to development of blastocysts with damaged mitochondria and lower ATP levels. More significantly, these deleterious effects in the blastocysts were correlated to decrease in autophagy process [18]. Moreover, negative influence of obesity on endometrial stromal cell decidualization has been reported, which is attributed to obesity-induced impairment in autophagy [24]. Therefore, it may be proposed that the activation of autophagy may overcome deleterious effects of obesity on the various aspects associated with *in vitro* fertilization including degeneration of granulosa cells, oocyte dysfunction and endometrial stromal cell decidualization (Fig. 1).

3. Induction of autophagy during vitrification-warming of oocytes and induction of cell death on inhibition of autophagy

Vitrification and warming is critical step in *in vitro* fertilization process, in which eggs are vitrified (stored at very low temperature) using cryoprotectants and liquid nitrogen. Thereafter, warming of these vitrified oocytes is done at the time of fertilization. It has been shown that autophagy is activated during vitrification-warming process in mouse oocytes, which tends to protect the oocytes from cold stressor. Indeed, upregulation of autophagy-related markers including Atg genes (Atg5, Atg7, Atg12), LC3 and Beclin1 was documented in the metaphase II (MII) of oocytes that were vitrified and stored in liquid nitrogen for 2 weeks [25]. Furthermore, inhibition of autophagy using 3-methyladenine was shown to induce the mRNA and protein expression of apoptotic enzymes including caspase-3, −9 and −12 suggesting that cryopreservation activates protective autophagy, whose inhibition induces apoptosis of oocytes during vitrification and warming [19]. Interestingly, addition of rapamycin (activator of autophagy) to activate autophagy in MII oocytes during vitrification, before subjecting to *in vitro* fertilization decreased the fertilization and developmental rate. It suggests the negative influence of rapamycin on the efficiency of *in vitro* fertilization on its administration during the time of vitrification [26]. It might be possible that excessive activation of autophagy during vitrification may be deleterious. Nevertheless, more studies are needed to explore the role of pharmacological modulators of autophagy on fertilization and development rate, during their administration at the time of vitrification.

4. Induction of autophagy improves quality of embryo and employment of autophagy as a marker to select embryo

Studies have described that induction of autophagy improves the quality as well as the viability of embryo and pharmacological activation of autophagy has been suggested as the potential mechanism to increase the quality of embryo during *in vitro* fertilization procedure. Normally, autophagy is not triggered during first 6 h of somatic cell nuclear transfer (SCNT), probably due to depolymerization of actin filaments. However, pharmacological activation of autophagy (by rapamycin or pp242) has been shown to trigger the activation of autophagy during SCNT and significantly increase the percentage of SCNT embryos progressing to the stage of blastocyst (69% *vs.* 42%). It suggests that induction of autophagy increases the survival rate and viability of embryo. In other words, due to activation of autophagy, more number of embryos may reach to the stage of blastocysts [5]. The selection of

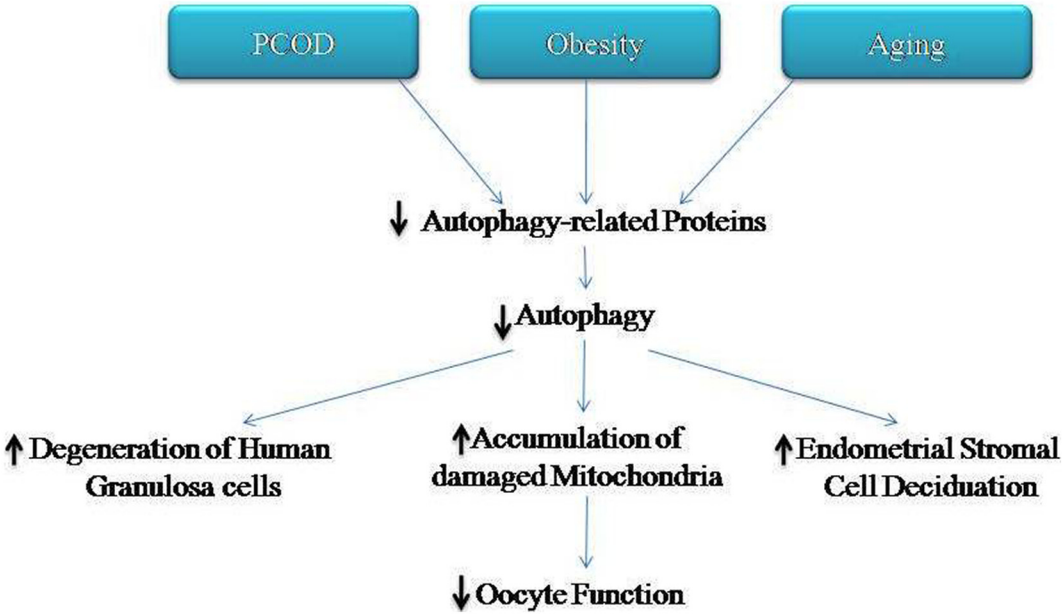

Fig. 1. Decrease in autophagic functions during obesity, PCOD and aging may decrease contribute in decreasing oocyte functioning, increasing degeneration of human granulosa cells and endometrial stromal cell deciduation.

high-quality embryos is of paramount importance in *in vitro* fertilization procedure for more efficient infertility treatments. Mostly, the identification of embryo is done on the basis of morphological basis. However, scientists have advocated the use of autophagy as a parameter to assess the quality of embryo in assisted reproduction studies [28]. Indeed, Tsukamoto et al. have developed a simple fluorescence-based technique to visualize autophagic activity in the live mouse embryos. These scientists employed green fluorescent protein (GFP)-linked LC3 to assess the activation of autophagy and demonstrated that embryonic autophagic activity is directly associated with the developmental viability of the embryo [29].

5. Induction of autophagy increases developmental competence and pre-implantation development of *in vitro* produced blastocysts

The importance of autophagy in other steps of *in vitro* fertilization including increase in developmental competence and pre-implantation development has also been very well described. It has been reported that fertilization triggers autophagy, which is maintained in the embryos. Indeed, the activation of autophagy is necessary for degrading maternal proteins in the oocytes. Along with it, autophagy-induced degradation of proteins supplies nutrients and amino acids to the developing embryo. The importance of autophagy in blastocyst development was delineated by the finding that autophagy-defective oocytes, obtained from oocyte-specific Atg5 knockout mice, fail to develop into mature embryo on fertilization with Atg5-null sperm. It emphasizes the critical role of autophagy in pre-implantation development in mammals [7]. The study of Song et al. described the importance of induction of autophagy in the developmental competence of *in vitro* produced bovine embryos. The authors reported the abundance of autophagy-associated gene transcripts, including LC3, Atg5, and Atg7 along with extensive autophagosome formation in fertilized embryos in the early stages of pre-attachment. Moreover, using selective pharmacological activators and inhibitors of autophagy, it was shown that transient elevation of autophagic activity during early pre-attachment phase led to increase in the blastocyst development rate, trophectoderm cell numbers, along with increased survival rate of blastomeres. Interestingly, inhibition of autophagy as well as prolonged induction of autophagy led to decreased blastocyst development rate and had negative effect on embryo development. It suggests that balanced (short duration) activation of autophagy may be a good strategy to induce high developmental competence in *in vitro* produced bovine blastocysts [6]. Based on the study conducted on women undergoing *in vitro* fertilization, it was deduced that decreased activation of autophagy is associated with defective implantation. However, excessive activation of autophagy is associated with ectopic pregnancy, thereby suggesting the need of optimal activation of autophagy *in vitro* fertilization procedure [30].

It has been emphasized that induction of autophagy improves nuclear and cytoplasmic maturation of porcine oocytes during *in vitro* fertilization. The authors revealed that treatment with 1 nM rapamycin activated autophagy as evidenced by increase in the expression of LC3-II, an autophagy marker. Moreover, rapamycin treatment also led to improvement in nuclear maturation, cytoplasmic maturation (increase in level of p34(cdc2), a cytoplasmic maturation marker), monospermic fertilization rate, increase in blastocyst formation rate, total number cells along with increased cell survival. Concomitantly, there was decrease in the levels of pro-apoptotic (Bax) transcript levels and increase in the anti-apoptotic (Bcl-xL) transcript levels again suggesting the beneficial role of low dose rapamycin treatment (transient induction of autophagy) in enhancing developmental process in *in vitro* fertilized porcine oocytes [31]. The study of Lee et al. also describes the important role of autophagy activation in pre-implantation blastocyst development. The authors reported that the exposure of embryos to 3-methyladenine, an autophagy inhibitor, increases the rate of apoptosis, suppresses the development of porcine blastocysts and decreases the expression of autophagy-related genes (ATG5, BECLIN1, and LC3) [26]. A recent study has also supported the above results showing that treatment with rapamycin during post-activation or *in vitro* culture facilitated higher blastocyst formation [32].

5.1. Autophagy leads to decrease in endoplasmic reticulum (ER) stress

It has been shown that there is a negative correlation between induction of autophagy and development of endoplasmic stress. In the study of Song et al., it was shown that transient increase in autophagy led to decrease in ER stress, which in turn exerted a positive effect on the developmental competence of *in vitro* produced bovine blastocyst. Moreover, inhibition of autophagy led to increase in ER stress, which led to developmental defects. The treatment with ER stress inhibitor overcame the developmental defects arising due to inhibition of autophagy suggesting that autophagy is a negative regulator of ER stress and the balanced autophagy/ER stress pathway is key for the optimal early embryogenesis [6]. Another study has shown that treatment of obese female mice with ER stress inhibitor *i.e.* salubrinal led to significant improvement in the developmental potential of blastocysts following *in vitro* fertilization. Indeed, it was shown that oocytes obtained from obese mice have high ER stress and demonstrate reduced developmental growth along with mitochondrial dysfunction. Moreover, the blastocysts transferred to normal weight surrogates gave rise to heavier fetuses that also exhibited lower mitochondrial DNA content per cell. However, these abnormalities observed during *in vitro* fertilization were significantly attenuated with administration of salubrinal in obese mice before performing *in vitro* fertilization [33]. Therefore, it may be proposed that induction of autophagy improves developmental competence of blastocysts by decreasing ER stress (Fig. 2).

5.2. Activation of poly(ADP-ribosyl)ation (PARP) induces autophagy

It has been shown that the positive effects of autophagy activation on the blastocyst development are associated with PARP activation. During pre-implantation development, there is a parallel increase in the levels of LC3 (marker of apoptosis) and activation of PARP. Exposure of embryos to 3-aminobenzamide (PARP inhibitor) suppressed the development of blastocysts, increased the rate of apoptosis and decreased the expression of autophagy-related genes. It suggests that activation of PARP is necessary for the induction of autophagy and selective autophagic degradation of proteins during pre-implantation development [27] (Fig. 2).

5.3. Induction of autophagy decreases production of oxygen free radicals

It has been suggested that activation of autophagy may decrease the production of oxygen free radicals to promote the blastocyst development. Indeed, treatment with rapamycin facilitates the blastocyst formation in *in vitro* culture by increasing the glutathione content and decreasing the formation of reactive oxygen species. It suggests that rapamycin-mediated activation of autophagy positively regulates pre-implantation development of pig embryos by decreasing cellular redox state [32] (Fig. 2).

6. Autophagy promotes placental vascularization and fetal growth

Studies have shown that there is a limited vascularization in placenta and hence, there is limited fetal growth in pregnancies obtained by assisted reproductive technologies. However, it has been documented that activation of autophagy may help in early placental development after transfer of *in vitro* produced sheep embryos. Therefore, it has been proposed that activation of autophagic activity using pharmacological agents may overcome the retarded vasculogenesis and reduced fetal growth observed in pregnancies after transfer of *in vitro* embryos [20].

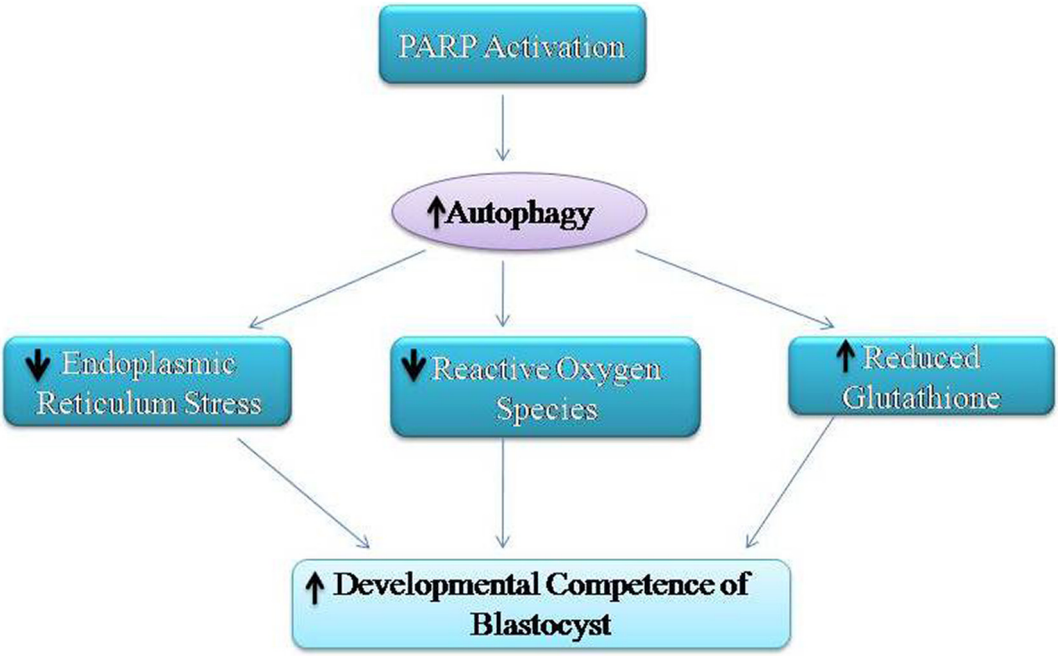

Fig. 2. Autophagy-linked mechanisms involved in increasing developmental competence of blastocysts.

7. Conclusion

Autophagy is found to modulate different steps involved in *in vitro* fertilization and pharmacological agents-induced increase in autophagy decreases degeneration of granulosa cells and oocyte defects in obese and aging women; decreases apoptosis of oocytes during vitrification-warming; improves quality and viability of embryo; increases developmental competence and pre-implantation development of *in vitro* produced blastocysts; promotes placental vascularization and fetal growth. The mechanisms involved in autophagy-mediated increase in developmental competence and pre-implantation development involves decrease in ER stress, activation of PARP and reduction in free radical production. Therefore, it may be concluded that autophagy may be potentially employed as an important target to increase the efficiency of *in vitro* fertilization.

Funding source

There is no funding source to declare.

Declaration of Competing Interest

There is no conflict of interest.

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