Dietary stress triggers autophagy and apoptotic signaling pathways in the rat ovary

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Short title: Food deprivation-mediated autophagy and apoptosis in the rat ovary

Abbreviations

Follicular stimulating hormone (FSH); Luteinising hormone (LH); Autophagy-related genes (ATG); Phosphate buffer saline (PBS); Optical density (OD); Tris-buffered saline-tween 20 (TBS-T); Tris-buffered saline (TBS); Bovine-serum albumin (BSA); Standard error of the mean (SEM); One-way analysis of variance (ANOVA); Mammalian target of rapamycin (mTOR); UNC51-like kinase-1 (ULK1); Microtubule-associated protein light chain 3 (LC3); Lysosomal-associated membrane protein 1 (Lamp1); Mitochondrial outer membrane permeability (MOMP); Hypothalamo-pituitary-gonadal (HPG)

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ABSTRACT

Dieting is a global emerging trend in recent years as more people strive to adhere to food restriction plans for weight management in obese people and to achieve desired slim body. This strategy may have unforeseen repercussions in females that may affect reproductive potential. Therefore, we aimed to investigate the impact of dietary stress on reproductive hormone levels, histoarchitecture of the ovary, autophagy and apoptosis markers in the rat ovary. Data suggest that dietary stress caused due to food deprivation decreased body weight and relative ovary weight, luteinising hormone (LH), follicular stimulating hormone (FSH) and estradiol-17β levels. The dietary stress reduced number of primary follicles, altered the histoarchitecture of the ovary, increased number of fragmented and irregular shape oocytes. Dietary stress induced autophagy signaling by inhibiting mTOR and increasing Lamp-1, LC-3 and Beclin-1 in the ovarian follicles. In addition, dietary stress induced proapoptotic signaling pathway by decreasing Bcl-2 and increasing Bax as well as Cytochrome-c expressions in the ovary. Taken together, these finding suggest that dietary stress caused due to food deprivation reduced reproductive hormones level, induced autophagy and apoptotic signaling pathways that affected histoarchitecture of the ovary, ovarian function, oocyte quality and thereby reproductive potential.

Keywords: Food deprivation; Autophagy; Apoptosis; Rat ovary

1. INTRODUCTION

Dieting is a global emerging trend in recent years as more people strive to adhere to food restriction plans in order to achieve desired slim body or body weight and form. Dieting is a first choice for weight management in obese people to prevent the onset of serious co-morbidities such as diabetes and hypertension. A recent trend among the youth is the maintenance of a perfect and healthy figure through dieting. Unfortunately, this strategy may have unforeseen repercussions that may results in the onset of various disorders including malnutrition, weakened immune system and reproductive potential such as hormonal and menstrual disorder, sub-fertility and even infertility[(1)]. In addition, the intermittent fasting (complete food deprivation for a day or two days) is very common among young girls and women in several countries that may generate potential risk on their reproductive health[(2)].

Mammalian females are born with a fixed number of germ cells housed in functional units known as follicles. Each follicle contains an oocyte as well as specialized somatic granulosa cells required for the growth, development, maturation and survival of follicular oocytes. Pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) surge at the time of puberty trigger follicular growth and development. The selective recruitment occurs in the ovary that enables only dominant follicles are allowed to progress further, while others are eliminated from the cohort of the ovary through the process called follicular atresia[(3)].

The massive depletion of a majority of germ cells (>99%) from the cohort of fetal ovary prior to birth has been reported in most of the mammalian species including human females[(4–8)]. Players and pathways that trigger massive depletion of germ cells via follicular atresia that may lead to premature ovarian failure (POI) and thereby infertility remains poorly understood. Therefore, players and pathways that drive rapid germ cell/oogonia/oocyte depletion have gained global attention in the recent past.

Growing evidences suggest that a variety of apoptotic and non-apoptotic cell death pathways are involved during germ cell depletion from the cohort of the ovary[(9–13)]. The apoptosis, autophagy, necroptosis and necrosis pathways have been reported during germ cell depletion in mammalian ovaries[(8,11,14,15)]. Although in order to maintain the highest-quality oocytes capable of developing into the appropriate female gametes, elimination of poor-

quality germ cells may be part of the selection process[16], the rapid depletion of germ cells and oocytes may exhaust ovarian reserve and trigger POI that may cause infertility[9–11].

Apoptosis and autophagy have been observed in oocytes at all stages of oogenesis and in the ovum after ovulation under *in vitro* culture conditions[11,14,15,17,18]. Some pathways work together or in parallel to destroy germ cells from the ovary under various inducing factors, pathological conditions and aging[11,14,19]. Emerging evidences suggest that along with the apoptosis, autophagy plays an essential role during germ cell depletion from the ovary in mammals[20,21].

Autophagy is a regulated cell death pathway mediated by autophagy-related genes (ATG) that regulate autophagosome formation[22]. The autophagosomes then fuse with liposomes to degrade the internal components. Studies suggest that impaired autophagy or excessive autophagy may lead to cell death[21] so called autophagy-mediated cell death[23]. The involvement of impaired autophagy have recently been proposed in follicular somatic as well as germ cells in mammalian ovary[12,13,24]. Emerging evidences suggest that both apoptosis and impaired autophagy could be actively involved in germ cell depletion from mammalian ovary and may be interconnected during developmental-regulated cell death[8,12,24,24].

Mammalian ovary is a dynamic organ that performs several functions including folliculogenesis, oogenesis, steroidogenesis and generation of competent oocytes required for successful fertilization and early embryonic development[12,13,24–26]. Stress affects the ovarian functions and oocyte quality in several mammalian species including human[24,26]. Although the negative impact of dietary stress on various reproductive health issues have already been documented[27–29], the molecular mechanism by which dietary stress induce cell death in the ovary remains poorly understood. Therefore, the present study was designed to investigate whether dietary stress generated due to complete food deprivation (fasting) or partial food deprivation (half diet food restriction for one or two estrous cycle) could induce autophagy and apoptosis signaling in ovary of female rats, *Rattus norvegicus*.

2. MATERIAL AND METHODS

2.1. Chemicals and experimental animals

Chemicals used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Adult female rats of 1 year old (body weight 220±10 grams) of Charles-foster strain were separated from existing colony of departmental animal facility and subjected to the determination of their estrous cycle following standard procedure. In brief, a small amount of fluid collected from the vagina using normal saline was used to prepare vaginal smear on the cleaned slide and then observed under a microscope. A vaginal cytology with abundance of non-nucleated cornified epithelial cells was considered as estrus stage and 24 female rats having almost 5 days of estrous cycle with estrus stage were subjected to the determination of half diet under a 12 hours light/dark cycle with food and water *ad libitum* in the departmental animal facility. All procedures were approved by the Institutional Animal Ethical Committee (Wide letter no. BHU/DoZ/IAEC/2018-2019/015) and performed in accordance with the Institutional guidelines.

2.2. Determination of half diet for experimental female rats

The half diet for experimental female rats were determined by analysing the daily food intake following the methods published earlier[30,31]. For the purpose, 1 year aged 24 adult female rats in the estrus stage were subjected to routine diet observation for one week and the daily food intake was monitored for all individual rats under controlled light (12 hours of light/dark cycle), temperature (24±2^{o}C), food and water *ad libitum.* The daily food intake was determined as 5-6 grams and expressed per 100 gm body weight for experimental female rats. Accordingly, the 50% diet (half diet) was calculated from the total food intake of each female rat every after 24 hours based on the total body weight.

2.3. Generation of dietary stress

Studies suggest that aged ovaries exhibit increased sensitivity to stress and dysregulated cell death and survival pathways[32,33]. Therefore, the dietary stress was induced in 1-year-old adult female rats in the present study that roughly correspond to advance reproductive aging and considered the equivalent of perimenopausal stage in humans. These reproductive aged female rats represent a biologically relevant model to study the decline in

ovarian function, altered hormonal milieu and increased susceptibility to cellular stress responses such as autophagy and apoptosis. A short-term dietary stress was induced in experimental female rats either by subjecting them to complete food deprivation for 48 hrs or partial food deprivation for 5 (one estrous cycle) and 10 days (two estrous cycle) in order to find out short term dietary stress response of ovary at the cellular and molecular levels during reproductive aging. In brief, after determination of half diet, 24 experimental female rats were randomly divided into 4 groups and each group has 6 experimental rats (n = 6/group). The first group of experimental female rats were maintained as control and provided food and water *ad libitum.* The second group was completely deprived of food for 48 hours and then immediately subjected to sample collection and further analysis of various parameters. The third group of experimental rats was supplied with the 50% of normal diet (half diet) for 5 days, while the fourth group was supplied with the 50% of the normal diet (half diet) for 10 days. At the end of the experimental period, control and experimental groups of rats were subjected to ovary isolation and blood serum collection.

2.4. Blood serum collection and ovary isolation

The blood serum samples were collected from all 24 experimental female rats from caudal vein after mild ether anaesthesia. Immediately thereafter, animals were sacrificed by cervical dislocation and their ovaries were isolated in ice-cold phosphate buffer saline (PBS). After 15 minutes at room temperature blood samples were centrifuged for 15 minutes at 2400xg in order to separate and collect the serum. The blood serum samples were stored at -20^{0}C and used for the analysis of FSH, LH and estradiol-17β using commercially available ELISA kits purchased from G-Biosciences, MO, USA.

2.5. Analysis of serum levels of FSH, LH and estradiol-17β using ELISA

The serum levels of FSH, LH and estradiol-17β were analysed using commercially available ELISA kits (Rat FSH ELISA Kit Cat No. ITLK01315; Rat LH ELISA Kit Cat No. ITLK02367; Rat Estradiol ELISA Kit Cat No. ITLK08714; G-Bioscience, MO, USA) following company manual protocol. In brief, kits as well as serum samples were brought to the laboratory temperature (22±2^{0}C). Subsequently, a 50 µL solution containing either a working standard or a serum sample was introduced into each well and then 50 µL of biotinylated conjugate was added. After gentle shaking in order to mix the sample loaded in each well, the plate was sealed and incubated at a temperature of 37°C for 1 hour. At the end

of incubation period, the plate was washed three times with wash buffer 200 µL. The wash buffer residual in the plate was eliminated by inverting the plate and pressing it against a clean blotting towel. The 100 µL Streptavidin-HRP conjugate was added in each well and further incubated for 1 hour at a temperature of 37°C. At the end of incubation period, the plate was washed five times with wash buffer and then 90 µL of substrate solution was added to each well and the plate was placed in dark room at 37°C for 20 minutes. Thereafter, 50 µL of stop solution was added in each well that resulted in the development of colors. The optical density (OD) of the microplate was measured at 450nm using an ELISA reader (Thermo Fisher Scientific, MA, USA). The concentration of hormones in the sample was determined by constructing a standard curve, which involved plotting the optical density (OD) values against the known concentrations of the hormone in the samples. Each sample was run in duplicate and minimum 6 serum samples obtained from each group of experimental animals were subjected to analyze FSH, LH and estradiol-17β levels.

2.6. Histology of the ovary

For histology, one ovary from all 24 experimental female rats were fixed in 4% paraformaldehyde in PBS for 24 hours and then washed in PBS then underwent a series of dehydrations in various grades of ethanol. The tissue was then cleared using the xylene (Qualigen, India) three times each lasting 30 minutes and then dipped in a mixture of prewarmed paraffin wax and xylene (Merck, Darmstadt, Germany) and held in a hot air oven for 45 minutes. The procedure was repeated and then the tissue was submerged in molten wax for 30 minutes three times to completely permeate it with wax. Additionally, using steel moulds and cassettes the paraffin blocks were prepared. The semiautomatic Leica ultramicrotome was used to sectioning the paraffin block containing the tissue into thin sections, each measuring 6 µm in thickness. The microsections floating in hot water bath were then transferred to Poly-L-Lysin (Sigma; St. Louis, MO, USA) coated slides where they were allowed to dry for the next day before being stored at 4°C until further analysis.

The slides were removed and kept in a hot air oven for 30 minutes at 55°C to allow the wax to soften. The slides were subsequently immersed in xylene three times in order to remove the wax. Thereafter, slides with tissue sections were exposed to serial rehydration by being dipped twice for 5 minutes each in a graduated ethanol solution starting at 100% ethanol and ending with 30% ethanol and then distilled water. The sections were then stained with hematoxylin for 20 minutes, rinsed with running water and exposed to acid alcohol

before being maintained in distilled water for 15 minutes to allow for colour differentiation. Thereafter, slides were dehydrated by submerging two times for 5 minutes each in ethanol that was gradually increased in strength ranging from 70% ethanol to absolute ethanol. The cytoplasmic stain, eosin was used to counterstained the slides after which they were washed twice for five minutes each in absolute ethanol and xylene. The slides were then used for imaging after being mounted with cover slips using DPX (Merck, Darmstadt, Germany) mounting medium. Minimum of 6 sections from each experimental group (n=6) were selected, photographed and analysed for cellular changes in the histoarchitecture of the ovary using a Brightfield Trinocular Research Microscope (Model: BX63, Olympus Tokyo, Japan) outfitted with cellSens Imaging software at the Central Discovery Center, Banaras Hindu University and a representative photograph from each experimental group is shown in the result section.

2.7. Analysis of autophagy and proapoptotic proteins expression using Western Blotting

Another ovary from each experimental female rat of all groups was made free of fat and other tissues using forceps. Thereafter, ovary weight was taken quickly and then lysed for an overnight period at 4^0C in RIPA buffer (ThermoFischer, MA, USA) mixed with a protease inhibitor cocktail (Sigma; St. Louis, MO, USA) and phosphatase inhibitor cocktail (Sigma; St. Louis, MO, USA). The total protein was analysed using Bradford assay in various lysates. The total protein content was equated and then subjected to various autophagy marker proteins analysis using Western Blotting.

In brief, equated protein samples were denatured by boiling at 95°C for five minutes in 2x lamellae buffer (Sigma; St. Louis, MO, USA) in a 1:1 v/v ratio. Samples were loaded onto 8% or 12% SDS-PAGE (Bio-Rad, CA, USA) until it was completely resolved. The resolved proteins were then electroblotted on a PVDF membrane (Millipore, MA, USA) with a pore size of 0.45 µm for 3 hours at 350 mA. Thereafter, blotted membrane was exposed to 3% Bovine-serum albumin (BSA) in Tris-buffered saline-tween 20 (TBS-T) for overnight at 4°C to block the non-specific binding sites. The blocked membranes were then incubated either with the p-mTOR antibody (rabbit monoclonal, Cat No. 5536T, Cell Signaling Technology, MA, USA), mTOR (rabbit monoclonal mTOR antibody, Cat No. 2983S; Cell Signaling Technology, MA, USA), p-ULK-1 antibody (rabbit polyclonal, Cat No. ITA-0629, G-Bioscience, MO, USA), ULK-1 antibody (rabbit monoclonal, Cat No. 8054S, Cell Signaling Technology, MA, USA), p-Beclin-1 antibody (rabbit polyclonal, Cat No. PA5-

104576, Invitrogen, CA, USA), Beclin-1 antibody (rabbit monoclonal, Cat No. 3495T, Cell Signaling Technology, MA, USA), or LC-3 antibody (rabbit monoclonal, Cat No. L8918, Sigma; St. Louis, MO, USA) at 1:1500 dilution.

The blocked membranes were also exposed to Lamp-1 antibody (mouse monoclonal, Cat No. sc-20011, Santa Cruz Biotechnology, CA,USA), SQSTM-1 or p62 antibody (mouse monoclonal, Cat No. sc-48402, Santa Cruz Biotechnology, CA,USA), Bax antibody (mouse monoclonal, Cat No. sc-7480, Santa Cruz Biotechnology, CA,USA), Bcl-2 antibody (mouse monoclonal, Cat No. sc7382, Santa Cruz Biotechnology, CA,USA), Cytochrome-c antibody (mouse monoclonal, Cat No. sc-13156, Santa Cruz Biotechnology, CA,USA), β-Actin antibody (mouse monoclonal, Cat No. sc-47778, Santa Cruz Biotechnology, CA,USA) at 1:300 dilution in 1% BSA (SRL) in TBS-T for overnight at 4°C.

The membrane was then washed three times in TBS-T buffer for five minutes each while being continuously shaken at room temperature. The membrane was then incubated either with goat anti-mouse HRP secondary antibody (Cat No. ab6789, Abcam, MA, USA) or with goat anti-rabbit HRP secondary antibody (Cat No. ab6721, Abcam, MA,USA) at 1:10000 dilution in 1% BSA for 90 min at room temperature while being continuously shaken. The membrane was then washed three more times for five minutes each in TBS-T buffer before receiving a single wash in Tris-buffered saline (TBS). By employing a femtolucent chemiluminescence detection kit (G-Biosciences, MO,USA), specific autophagy and apoptotic protein markers band were developed and visualized using the high-resolution Charge Coupled Device (CCD) camera on the Azure 600 Western Blot Imager (Azure Biosystems, CA, USA). AzureSpot Pro image analysis software (Azure Biosystems, CA, USA). The images and the intensity of the protein of interest were normalized using the intensity of housekeeping proteins such as β-actin.

2.8. Statistical analyses

The data are the mean ± standard error of the mean (SEM) of six replicates. Data are analyzed by one-way analysis of variance (ANOVA) followed by post-hoc Tukey analysis using SPSS software version 16.0 (IBM, NY, USA). The *$p<0.05$ was considered statistically significant.

3. RESULTS

3.1. Dietary stress reduced body weight and relative ovary weight

It is generally understood that the dietary stress or partial food deprivation may lead to reduced body weight and a lower relative ovary weight in mammals. This notion is further confirmed by the data of the present study that the complete food deprivation for 48 hours reduced body weight by 3%. The dietary stress caused due to 5-days partial food deprivation (half diet) reduced body weight by 7%. The body weight loss was further increased by almost 12%, if the experimental female rats were subjected to 10-day partial food deprivation as compared to control group. Although complete food deprivation for 48 hrs did not reduce the relative ovary weight, a significant decrease in the relative weight of the ovary was observed if the experimental female rats were subjected to partial food deprivations for more than 5 and 10 days (Fig. 1).

3.2. Dietary stress reduced serum level of reproductive hormones

The impact of food deprivation on the serum levels of gonadotropic and steroid hormones are shown in Fig. 2. A significant decrease in the levels of FSH and LH were observed if the female rats were completely deprived of food for 48 hrs. The decreased of serum levels of both the gonadotrophin were maintained even after 5 or 10 days of partial food deprivation (Fig. 2 A&B). Similarly, food deprivation also declined the serum level of estradiol-17 β in female rats that were subjected to complete food deprivation for 48 hours and partial food (half diet) food deprivation for 5 and 10 days (Fig. 2C).

3.3. Dietary stress affects histoarchitecture of the ovary

As shown in Fig. 3A, a normal histoarchitecture that possess primordial germ cells, primary follicles and secondary follicles are seen with normal morphology of epithelial cell lining (black arrows). The cumulus oocyte complex encircling with several layers of granulosa cells with outer layer of theca cells are evident (black arrows). The oocyte with germinal vesicle and nucleolus are visible (blue arrows) in the control ovary (Fig. 3A). Complete food deprivation for 48 hrs did not significantly alter the histoarchitecture of the epithelial cells, theca cells as well as granulosa cells with the follicles (black arrows) of the ovary (Fig. 3B) and was almost similar to control (Fig. 3A). However, increased numbers of

fragmented, irregular shaped oocytes were evident (blue arrows) in the antral follicles (Fig. 3B).

The supplementation of half diet for 5 days significantly altered the histoarchitecture of the ovary as evident by the reduction in the number of primary follicles (Fig. 3 C). The detachment of epithelial lining of the wall of the follicles and derailment of follicular somatic cells such as theca externa, theca interna, mural granulosa as well as cumulus granulosa cells are observed (black arrows), while oocytes are showing normal morphology (black arrows) with germinal vesicle in the centre (Fig. 3C). However, the severity of histoarchitectural changes with reduction in the number of primary follicles and formation of inner lining of steroidogenic cells within the follicle are evident in the histoarchitecture of the ovary, if the female rats were subjected to 10 days on half diet (blue arrow; Fig. 3 D). The disruption of epithelial cells, dissociation and damage of various cells including theca as well as granulosa cells are seen (black arrows) in the ovary (Fig. 3 D).

3.4. Dietary stress inhibits p-mTOR and induces autophagy signaling in the ovary

The mammalian target of rapamycin (mTOR) functions as an ATP as well as amino acid sensor and considered as inhibitor of autophagy. In the presence of sufficient nutrients, mTOR is phosphorylated at Ser2448 via the PI3 kinase/Akt signaling pathway and auto phosphorylated at Ser2481 that plays a key role in cell growth and homeostasis by preventing autophagy. On the other hand, food deprivation may decrease the p-mTOR/m-TOR ratio in order to initiate autophagy signaling. Data of the present study show that the food deprivation significantly ($p<0.05$) inhibited p-mTOR/mTOR ratio in the ovary of female rats in a time-dependent manner (one-way ANOVA; $p<0.05$; Fig. 4A). One-way ANOVA followed by post-hoc Tukey analysis further strengthened our observations that protein p-mTOR/mTOR ratio was significantly decreased in the ovary of female rats after 48 hrs of complete food deprivation or subjected to half diet for 5 and 10 days (Fig. 4A).

The UNC51-like kinase-1 (ULK1) is the catalytic component of the autophagy preinitiation complex that plays a central role in the initiation stage of autophagy via phosphorylation of ATG14, Beclin1 and other autophagy proteins. Inhibition of p-mTOR/mTOR ratio in the ovary could increase p-ULK-1/ULK-1 phosphorylation ratio in order to induce autophagy signaling under dietary stress conditions. This is further strengthened by the observations that the food deprivation significantly increased p-ULK-

1/ULK-1 phosphorylation ratio in the ovary of female rats in a time-dependent manner (one-way ANOVA; $p<0.05$; Fig. 4B). One-way ANOVA followed by post-hoc Tukey analysis further supported our observations that the expression pattern of p-ULK-1/ULK-1 was significantly increased if the female rats were subjected to partial food deprivation (half diet) for 5 and 10 days as compared to control (Fig. 4B).

The AMPK-mediated phosphorylation of Beclin1 is required for autophagy induction during starvation. Therefore, p-Beclin-1/Beclin-1 phosphorylation ratio was analyzed in the present study. Data revealed that food deprivation elevated the expression pattern of autophagy marker such as p-Beclin-1/Beclin-1 in a time-dependent manner (one-way ANOVA; $p<0.05$; Fig. 4C). One-way ANOVA followed by post-hoc Tukey analysis further strengthened our observations that the expression pattern of p-Beclin-1/Beclin-1 phosphorylation ratio was significantly increased if the female rats were subjected to partial food deprivation (half diet) for 5 and 10 days as compared to control (Fig. 4C).

Microtubule-associated protein light chain 3 (LC3), is considered one of the definitive markers of autophagy. Initiation of autophagy causes the conversion of LC3-I to LC3-II that can be determined by increase of LC3-II/LC3-I ratio through western blotting. Therefore, the LC3-II/LC3-I ratio was analyzed in the present study.Data indicate that food deprivation elevated the expression pattern of autophagy marker such as LC3-I to LC3-II in a time-dependent manner (one-way ANOVA; $p<0.05$; Fig. 4D). One-way ANOVA followed by post-hoc Tukey analysis further strengthened our observations that the expression pattern of LC3-I to LC3-II ratio was significantly increased if the female rats were subjected to either complete food deprivation or partial food deprivation (half diet) for 5 and 10 days as compared to control (Fig. 4D).

The lysosomal-associated membrane protein 1 (Lamp1) is a heavily glycosylated transmembrane protein. The newly synthesized Lamp1 enter the plasma membrane where they are in dynamic equilibrium between endosomes, lysosomes, amphisomes, and autolysosomes to form degradative lysosomes that serves as hubs for autophagy. Therefore, the Lamp-1 was analyzed in the present study. Data indicate that food deprivation elevated the expression pattern of autophagy marker such as Lamp-1 in a time-dependent manner (one-way ANOVA; $p<0.05$; Fig. 4E). One-way ANOVA followed by post-hoc Tukey analysis further strengthened our observations that the expression pattern of Lamp-1 was significantly

increased if the female rats were subjected to either complete food deprivation or partial food deprivation (half diet) for 5 and 10 days as compared to control (Fig. 4E).

3.5. Dietary stress induces proapoptotic signaling in the ovary

Dietary stress may induce proapoptotic signaling in the ovary. To confirm this possibility the proapoptotic markers were analyzed in the ovary of female rats. Data of the present study suggest that dietary stress caused overexpression of Bax protein in a time-dependent manner (Fig. 5A). One-way ANOVA followed by Tukey analysis further strengthened our observations that the expression pattern of Bax was significantly increased if the female rats were subjected to either complete food deprivation for 48 hrs. or partial food deprivation (half diet) for 5 and 10 days as compared to control (Fig. 5A). On the other hand, Bcl-2 expression was decreased in a time-dependent manner (Fig. 5B). One-way ANOVA followed by Tukey analysis further strengthened our observations that the expression pattern of Bcl-2 was significantly reduced if the female rats were subjected to either complete food deprivation for 48 hrs or partial food deprivation (half diet) for 5 and 10 days as compared to control (Fig. 5B).

The overexpression of Bax and under expression of Bcl-2 might have altered Bax/Bcl-2 ratio and thereby mitochondrial outer membrane permeability (MOMP). The altered MOMP allows the release of cytochrome-c from the mitochondrial intermembrane space to the cell cytoplasm that induce downstream apoptotic pathway. Therefore, cytochrome-c expression level was analyzed in the present study. Results of the present study revealed that the dietary stress increased cytochrome c expression level in a time-dependent manner (Fig. 5C). One-way ANOVA followed by Tukey analysis further confirm our observations that the expression pattern of Cytochrome c was significantly increased if the female rats were subjected to partial food deprivation (half diet) for 5 and 10 days as compared to control (Fig. 5C).

4. DISCUSSION

Nutrient metabolism homeostasis is critical for maintenance of the integral health of mammals including human. Under nutrition, partial or complete starvation limit the daily supply of energy and nutrients required for the maintenance of overall health thus reduces body as well as various organs weight. Ovary is a dynamic organ that performs several functions and very sensitive to dietary stress especially during reproductive aging. This

notion is supported by the observations that aged ovary exhibit increased sensitivity to stress and dysregulated cell death as well as survival pathways[32,33]. The 1-year-old female rats are normally considered towards advance reproductive aging that correspond to perimenopausal stage in humans and represent biologically relevant model to study the decline in ovarian function, altered hormonal milieu, and increased susceptibility to cellular stress responses such as autophagy and apoptosis. Therefore, 1-year-old female rats were subjected to short dietary restriction in order to understand how aging ovaries respond to nutritional stress at the cellular and molecular levels, thereby broadening our understanding of fertility decline and ovarian pathophysiology during reproductive aging.

Data of the present study further confirms that the short-term dietary stress generated due to complete food deprivation for 48 hrs and partial food deprivation (half diet for 5 days or 10 days that correspond to one or two estrous cycle) significantly decreased body weight by 3%, 7% and 12%, respectively. Further, the dietary stress reduced relative ovary weight in a time-dependent manner (Fig. 1). Dietary stress may induce a cascade of metabolic and hormonal changes that may be associated with the reduced body weight and relative ovary weight in the present study. Since the body weight is one of the most noticeable and direct effect of dietary stress especially in female, a drop in the body weight is mainly due to utilization of fat and glycogen stores[34,35]. Similarly, dietary stress deprives daily requirement of various nutrients to the body that negatively affects the growth, development and performance of various organs, reduces body weight and increases susceptibility towards various diseases in mammals including humans[1,36].

The nutritional investment in reproduction is much greater in female compared to male in mammals including human[37]. Therefore, the females are more sensitive to dietary stress as compared to males and may cause reduction in gonadotrophin secretion as well as ovarian functions. This is supported by the data of the present study that dietary stress generated due to complete food deprivation or partial food deprivation not only significantly ($p<0.05$) reduced serum levels of FSH (Fig. 2A) as well as LH (Fig. 2B) secretion but also the relative ovary weight (Fig. 1) as well as estradiol-17 β in circulation in a time-dependent manner (Fig. 2C). These data corroborates with the previous findings that the dietary stress caused due to food deprivation and food restriction significantly reduced gonadotrophin secretion, ovarian weight and thereby reproductive potential in mammals[38–40]. The decrease of estradiol-17β level has been reported in experimental rats under starvation conditions[41].

These data together with previous findings suggest that dietary stress not only reduce body weight but also gonadotrophins levels lading to reduced relative ovary weight as well as estradiol-17β level.

Dietary stress-mediated decline in gonadotropic hormones, relative ovary weight as well as estradiol-17β level may adversely affect the histoarchitecture of the ovary. This possibility is further strengthened by the data of the present study that dietary stress caused due to complete food deprivation for 48 hrs increased number of fragmented and irregular shape oocytes (Fig. 3B) as compared to control (Fig. 3A). Further, the dietary stress generated due to partial food deprivation (half diet) for 5 days significantly reduced number of primary follicles and altered the histoarchitecture of the ovary (Fig. 3 C). The severity of damage in the ovary was increased and the reduced number of primary follicles, if the female rats were subjected to 10 days on half diet (Fig. 3 D). These data led us to hypothesize that the dietary stress generated in experimental female rats might have affected hypothalamo-pituitary-gonadal (HPG) axis[42,43], FSH and LH secretions as well as folliculogenesis in the ovary[44–46]. Similarly, the dietary stress-induced structural alterations in the ovary have been reported in adult female rats and women[47,48]. Although the partial starvation is less severe, it can nevertheless decrease the size of the ovarian reserve and impede the growth of follicles[49].

It is established that nutritional stress induce autophagy signaling through the major metabolic kinases such as AMP-PK and mTOR that are responsible for monitoring cellular energy and the concentration of amino acids in a cell[50]. Since mTOR is a prominent autophagy inhibitor, dietary stress generated in the present study may decrease the p-mTOR/m-TOR ratio in order to initiate autophagy signaling. This possibility was further strengthened by the data of the present study that food deprivation inhibited p-mTOR/mTOR protein expression ratio in the ovary of female rats in a time-dependent manner (Fig. 4A) suggesting the initiation of autophagy signaling in rat ovary. Similarly, the inhibition of mTOR was associated with the dietary stress-mediated initiation of autophagy signaling in mammalian ovary[51].

Downstream to mTOR, ULK1 is essential for initiation of autophagy and the inhibition of p-mTOR/mTOR protein expression ratio might have allowed the initiation of autophagy signaling by inducing the p-ULK-1/ULK-1 phosphorylation ratio that provide valuable information regarding the activation state of autophagy[52]. Our results suggest that

dietary stress increased p-ULK-1/ULK-1 phosphorylation ratio in the ovary of female rats in a time-dependent manner (Fig. 4B). These data are in agreement with the previous observations that food deprivation induced autophagy in lung cancer cell line[53]. The AMPK is an energy sensor kinase and initiates autophagy by acting on the ULK complex and mTORC1[54–56]. The AMPK binding phosphorylates ULK1/2 complex that induce phosphorylation of other units of the same complex to activate the whole complex. The activated ULK1/2 complex recruits endoplasmic reticulum, mitochondria and plasma membrane for the formation of phagophore[56–58].

For phagophore formation, Class III phosphatidylinositol 3-kinase (Class III PI3K) complex is required that consist of factors like Beclin1[59,60]. Beclin1 is one of the important units of class III PI3K that undergo AMPK-mediated phosphorylation during starvation to induce autophagy[59]. The Beclin-1 plays a crucial role in the autophagic process by acting as a pivotal regulator of the production of autophagosomes. The evaluation of Beclin-1 phosphorylation through the p-Beclin-1/Beclin-1 ratio provides insight into the regulatory action of Beclin-1 in autophagy[61]. Therefore, p-Beclin-1/Beclin-1 phosphorylation ratio was analyzed in the present study. Data revealed that food deprivation elevated the expression pattern of autophagy marker such as p-Beclin-1/Beclin-1 in a time-dependent manner (Fig. 4C). These results are in consistent with the previous observations that the p-Beclin-1/Beclin-1 ration increased as an early autophagic response to dietary stress[62].

The microtubule-associated protein 1 light chain 3 (LC3) in conjugation with Atg12-Atg5 is important for elongation and maturation of phagophore. The autophagy induction leads to an elevation LC3-II/LC3-I ratio and commonly employed to determine autophagy induction[63] as well as autophagic activity[51]. Therefore, the LC3-II/LC3-I ratio was analyzed in the present study and results indicate that food deprivation elevated the expression pattern of LC3-I to LC3-II in a time-dependent manner (Fig. 4D) suggesting the involvement of LC3-II in phagophore formation during autophagy. Similarly, studies suggest the involvement of LC3-II to form autophagosome during induction of autophagy[55,56,64].

The lysosomal-associated membrane protein 1 (LAMP1) is a heavily glycosylated transmembrane protein. The examination of Lamp-1 expression under dietary stress conditions could offer the dynamics of lysosomes in the regulation of autophagy. Therefore, the Lamp-1 was analyzed in the present study and our results suggest that the dietary stress elevated the expression pattern of Lamp-1 in a time-dependent manner (Fig. 4E). The newly

synthesized LAMP1 enter in the plasma membrane where they are in dynamic equilibrium between endosomes, lysosomes, amphisomes, and autolysosomes to form degradative lysosomes that serves as hubs for autophagy[64]. A positive association between increased Lamp-1 expression and lysosomal activity have already been documented[55]. Cells increase their autophagic capability, potentially as a means to recover vital metabolites and maintain cellular energy balance in situations where external nutrition availability is restricted[65]. Our results together with previous observations suggest that the dietary stress might have increased Lamp-1 expression in the ovary to elevate lysosomal activity and thereby autophagy[66].

The dietary stress not only induce autophagy signaling, growing evidences suggest that it may also induce stress-mediated intrinsic apoptotic signaling pathway[8,12,13,67]. For instance, the nutritional stress triggers overexpression of proapoptotic proteins such as Bax in the ovary[68]. The primary role of Bax is to facilitate the release of Cyt-c from the mitochondria that activates downstream caspases and thereby apoptotic cell death[69,70]. Hence, proapoptotic markers such as Bax, Bcl-2 and Cyt-c levels were analysed in order to determine the onset of apoptotic markers in the ovary. Data of the present study suggest that dietary stress caused overexpression of Bax protein in a time-dependent manner (Fig. 5A). On the other hand, Bcl-2 expression was decreased in a time-dependent manner (Fig. 5B). The overexpression of Bax and under expression of Bcl-2 might have altered Bax/Bcl-2 ratio and thereby mitochondrial outer membrane permeability (MOMP). The altered MOMP allows the release of Cytochrome-c from the mitochondrial intermembrane space to the cell cytoplasm. This possibility was further strengthened by our results that the dietary stress increased Cytochrome-c expression level in a time-dependent manner (Fig. 5C).These findings are in agreement with the previous observations that under stress conditions, under expression of Bcl-2 and over expressions of Bax as well as Cytochrome-c initiate intrinsic apoptotic pathways in the ovary of female rats[11,71–74]. Thus, the dietary stress generated due to complete food deprivation for 48 hrs and partial food deprivation for 5 and 10 days caused cellular stress sufficient enough to induce both autophagy as well as apoptotic signaling in parallel. The stress-mediated induction of both autophagy and apoptotic signaling has been reported in rat ovary[75–77].

5. CONCLUSION

The dietary stress caused due to complete food deprivation for 48 hrs and partial food deprivation (half diet) for 5 as well as 10 days decreased body weight, relative ovary weight, serum levels of FSH, LH and estradiol-17β. The decreased gonadotropins and steroid levels affected histoarchitecture of the ovary, reduced number of follicles, deteriorated follicular somatic cells and oocyte architecture, initiated autophagy and apoptotic signalling in the ovary (Fig. 6). Indeed, the dietary stress may initiate reproductive health disorders by altering the reproductive hormones level, structure and functions of the ovary. These findings could be useful in addressing the necessity for adequate precision lifestyle habits for women's reproductive health.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authorship

Author's credit role: PKY: Writing-original draft, Writing-review & editing, Methodology, Investigation, Formal analysis, Investigation, Data curation, Visualization,

Validation. **SNP:** Writing-review & editing. **SKC**: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Conceptualization.

Ethical approval

All procedures were performed in accordance with the guidelines of the Institutional Animal Ethical Committee of Institute of Science, Banaras Hindu University, Varanasi-221005, India (Approval number: BHU/DoZ/IAEC/2018-2019/015).

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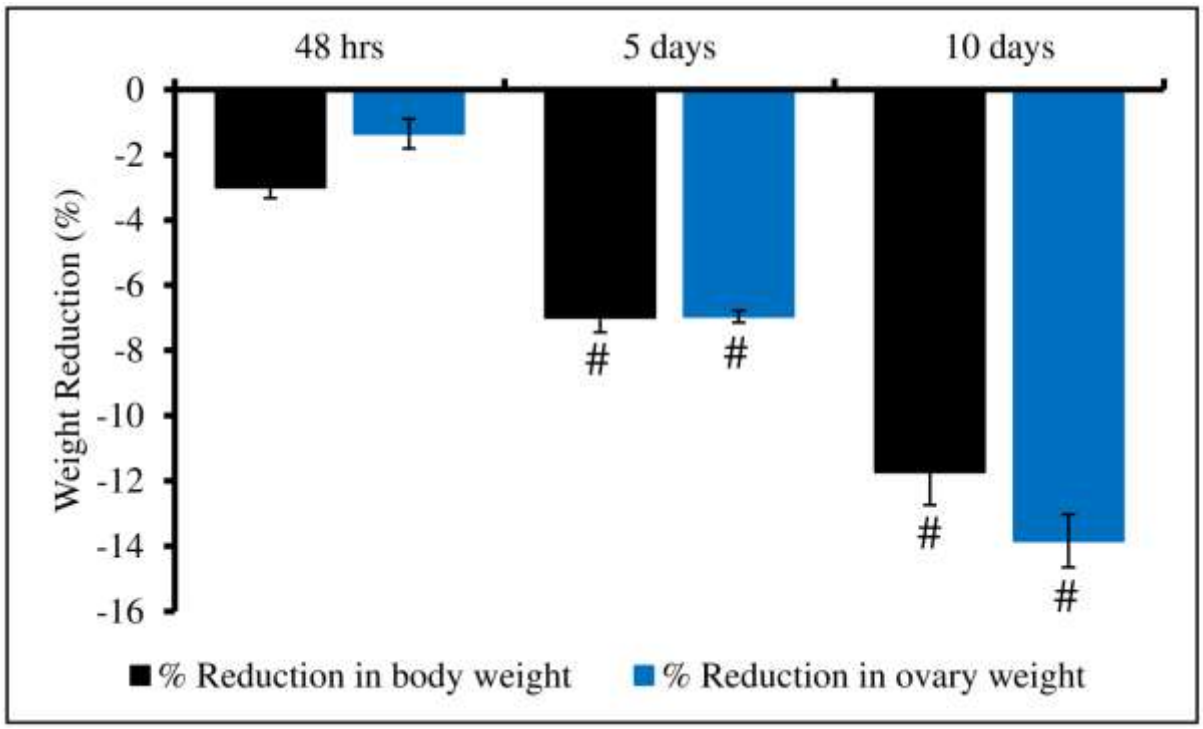

Fig. 1: Impact of dietary stress generated due to food deprivation or restriction on the relative ovary weight of the female rats. Data are mean ± SEM of six replicates and analysed by one-way ANOVA followed by a post hoc Tukey test. "#" denotes a significant decrease ($p<0.05$) as compared to control.

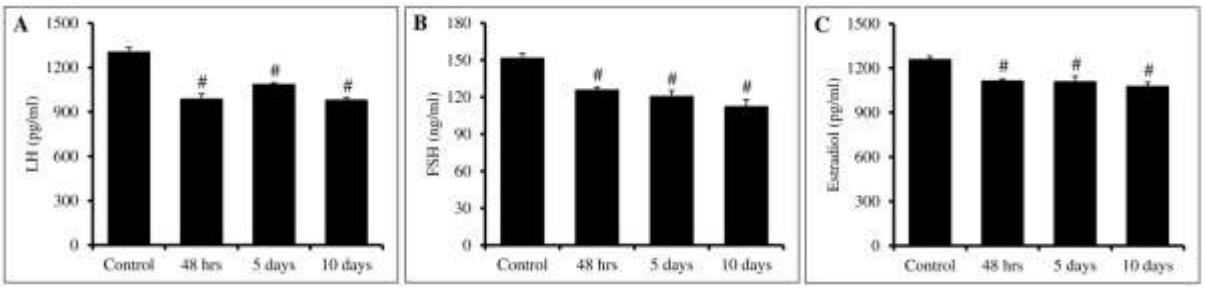

Fig. 2: Impact of dietary stress generated due to food deprivation or restriction on the serum level of (A) FSH, (B) LH and (C) Estradiol-17β in female rats. Data are mean ± SEM of six replicates and analysed by one-way ANOVA followed by a post hoc Tukey test. "#" denotes a significant decrease ($p<0.05$) as compared to control.

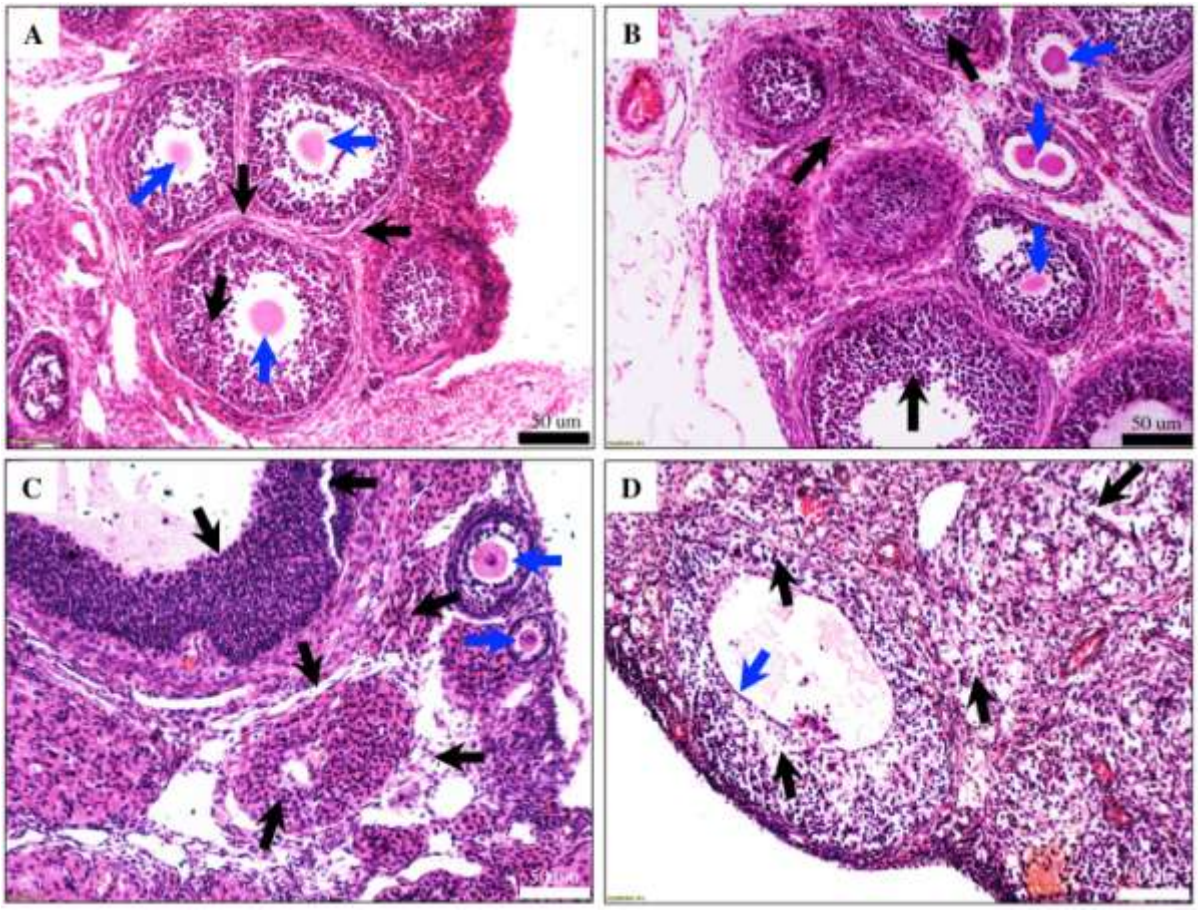

Fig. 3: Representative photographs showing the impact of dietary stress generated due to food deprivation or restriction on the histoarchitecture of ovary. (A) Control group, (B) 48 hours group,(C) 5 days group (D) 10 days group. The 50µm scale shows the 20x magnification image of the phase contrast microscope.

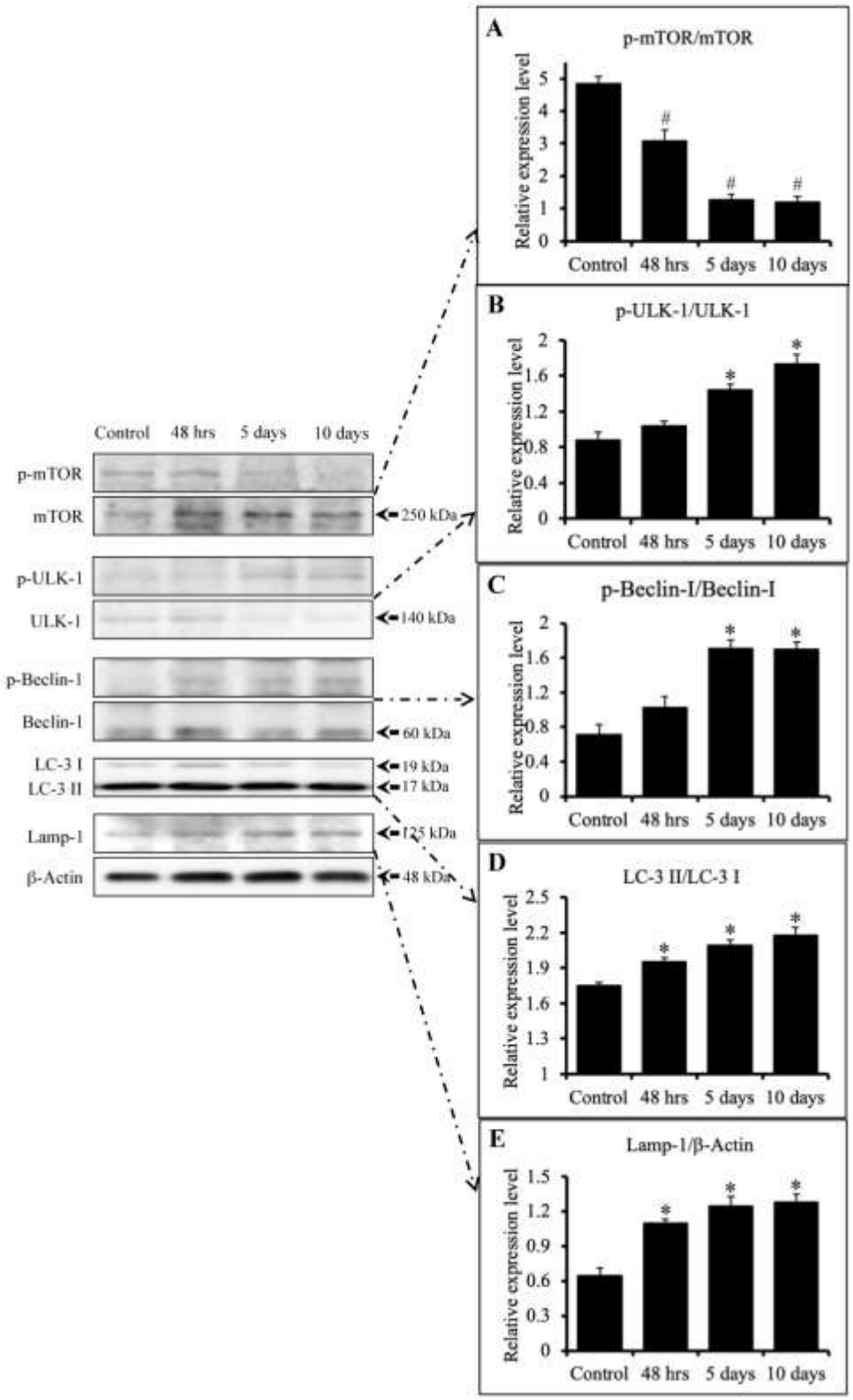

Fig. 4: Impact of dietary stress generated due to food deprivation or restriction on the autophagy marker proteins expression in the ovary. (A) p-mTOR/mTOR,(B) p-ULK-1/ULK-1,(C) p-Beclin-1/Beclin-1, (D) LC-3 II/LC-3 I and (E) Lamp-1. Data are mean ± SEM of six replicates and analysed by one-way ANOVA followed by a post hoc Tukey test. "*" denotes a significant increase and "#" denotes a significant decrease as compared to control.

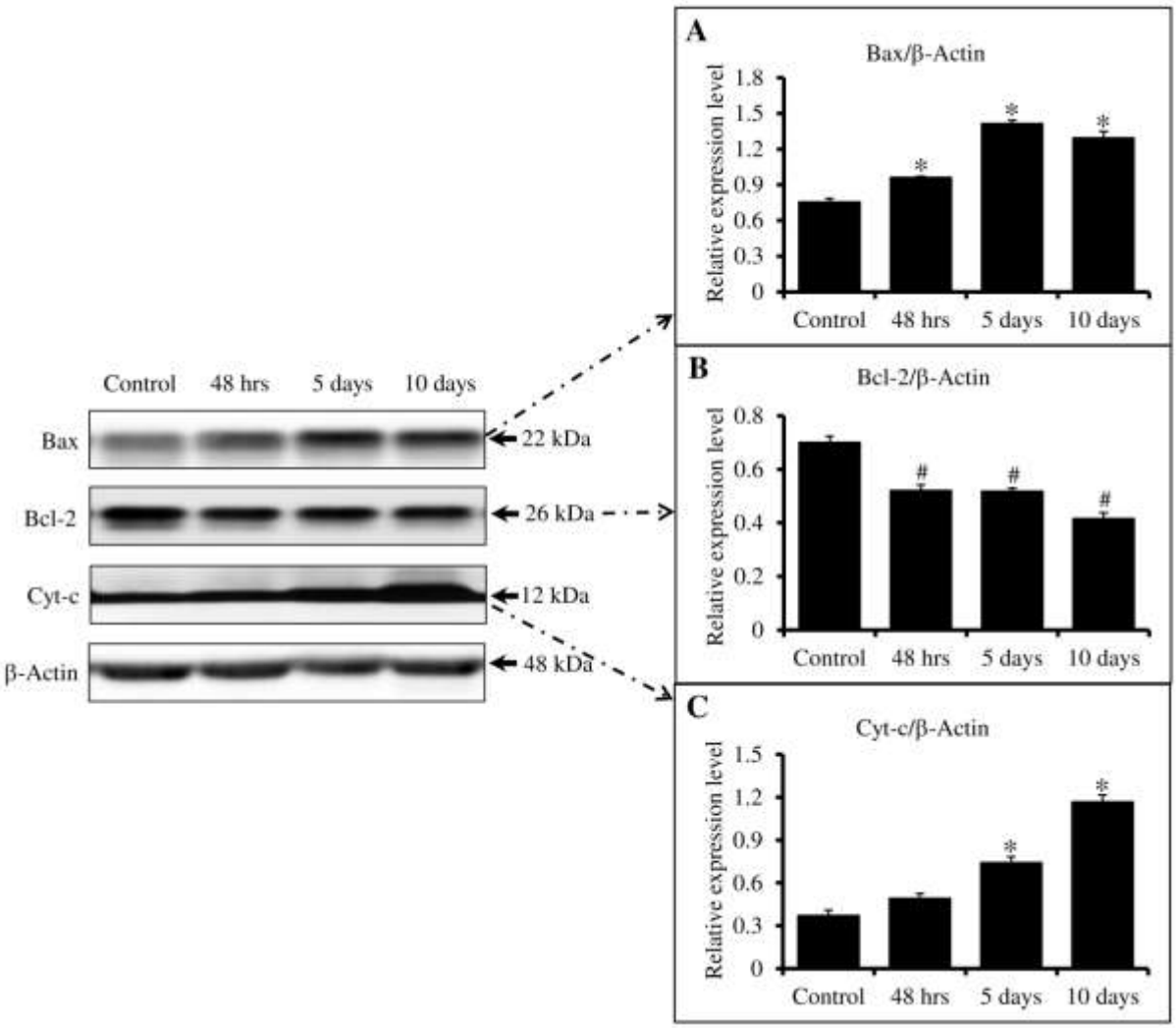

Fig. 5: Impact of dietary stress generated due to food deprivation or restriction on the apoptotic marker proteins expressions in the ovary. (A) Bax (B) Bcl-2 and (C) Cyt-c expression. Data are mean ± SEM of six replicates and analysed by one-way ANOVA followed by a post hoc Tukey test. "*" denotes a significant increase and "#" denotes a significant decrease as compared to control.

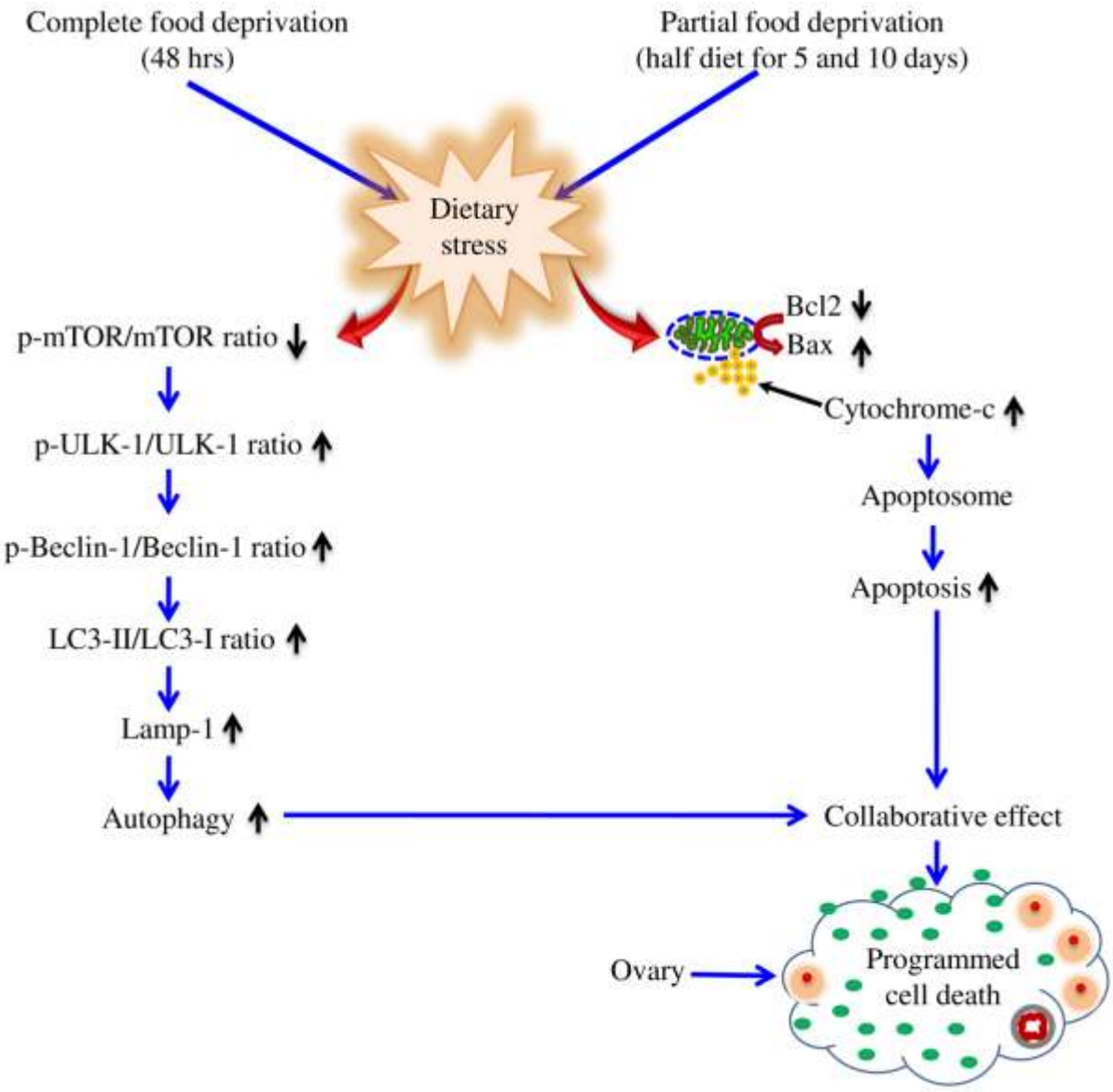

Fig. 6: Schematic diagram showing the effects of dietary stress on initiating autophagy and apoptotic signaling in the ovary. Dietary stress inhibited p-mTOR/mTOR ratio and elevated p-ULK-1/ULK-1 ratio. The phosphorylated ULK-1 activated Beclin-1/ LC3-II/ Lamp-1-mediated pathway to induce autophagy. The dietary stress increased Bax/Bcl2 ratio and cytochrome-c release that induce apoptosis. The collaborative effects of autophagy and apoptosis result in programmed cell death in the ovary.