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A Timeline Morphological Study of TNF-α Induced Changes in Mice Oocytes

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Abstract

Inflammatory cytokines induce cellular degenerative and apoptotic changes. Studies have shown increase in cytokine levels in patients with premature ovarian aging or failure (POF) where excessive oocyte atresia is the major pathogenic feature. Enhanced expression of tumour necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine, and its receptors have been found in the oocytes of immunized mice with experimental immune ovarian failure. The aim of the present work was to study the direct effects of TNF-α on naked oocytes. Oocytes were isolated from superovulated Swiss Albino female mice and treated with different concentrations of TNF-α (0.1, 1, 10 and 100ng/ml). Morphological scoring was assessed based on shape, cytoplasmic and extra-cytoplasmic features of the oocytes. It was found that TNF-α could induce morphological degenerative changes in mice oocytes at all the concentrations, maximum being at 10 ng/ml, suggesting a direct effect of this inflammatory cytokine on mammalian oocytes. Present study was a small piece of work that could highlight the possible direct effects of TNF-α on mice oocytes. Similar larger studies need to be planned in near future for validation. Further studies should also be planned in animal models of immune POF or autoimmune ovarian disorders to see the effect of anti-TNF-α treatment on ovarian physiology. Our lab is planning to continue the study further to confirm the morphological features as apoptotic by TUNEL or caspase staining and to find out the possible preventive or therapeutic options.

Keywords: Cytokines, Tumor necrosis factor-alpha, Oocytes

Introduction

The pool of primordial follicles that a woman born with undergoes continuous atresia during reproductive years. It is a well-known fact that the underlying mechanism of ovarian atresia is apoptosis but the mode of death of atretic/degenerated oocytes is yet to be confirmed (1). Apoptosis or programmed cell death is an energy dependent biochemical event that is involved in regulating the immune reactions happening in response to a foreign agent. Apoptosis is a controlled process and is necessary for the survival of an organism but when it becomes uncontrollable, it can lead to various pathologies (2). Role of cytokines have been very well studied in mediating apoptosis; one such cytokine is TNF-α.

TNF-α is a 17.3kDa protein product that induces pleotropic cellular responses and is chiefly produced by activated macrophages. Numerous cell types such as osteoblasts, hepatocytes, spleen cells contain TNF-α and have also been identified in the ovaries of humans and mice. TNF-α can exist as an insoluble trans-membrane protein and in a soluble form which requires a proteolytic cleavage by a matrix metalloproteinase known as TACE (TNF-α converting enzyme) (3). To exert its biological activities, TNF-α has two distinct Type1 trans-membrane receptors, $TNFR_1$ and $TNFR_2$ (4). Both receptors have a similar cysteine rich extracellular domain and a unique intracellular domain indicating different intracellular signalling pathway. $TNFR_1$ receptor has primarily pro-inflammatory function such as apoptosis (5). It has been reported that $TNFR_1$ is necessary for cytotoxicity as it consists of an intracellular site, Death Domain which is absent in $TNFR_2$ but can stimulate NF-κB signalling and activation of various kinases (6-8). Enhanced expression of TNF-α and its receptors are found in the oocytes of immunized mice model of immune ovarian failure (9). Since it has been proved that oocytes have membrane receptors for TNF-α, we hypothesised that TNF-α can directly induce degenerative changes in the oocytes. Therefore, the aim of the present work was to study the timeline of morphological changes induced by TNF-α on mice oocytes in vitro.

Methods

All animal experiments were approved by Central Animal Ethics Committee of All India Institute of Medical Sciences, New Delhi (53/IAEC-1/April 2018) and methods were carried out in accordance with approved guidelines. 10 Swiss Albino female mice, aged 6 to 8 weeks were issued from Central Animal Facility, All India Institute of Medical Sciences, New Delhi. Animals were housed in 12-hour light dark cycle provided ad libitum food and water throughout the study. Chemicals were obtained from Merck, India unless otherwise indicated. On Day 1, mice were given an intra-peritoneal injection of 10IU PMSG (Prospec, India). After 48 hours, 10 IU of hCG was injected intra-peritoneally. After 12-14 hours of hCG injection, the oviducts were dissected out. Oviducts were collected in a dish containing 2 ml of M2 Media. Using a needle, the cumulus was flushed out of the oviduct. To remove cumulus cells from the oocyte, 5µL of hyaluronidase was added to 500µL of M2 media in mineral oil and incubated at 37˚C for 5 minutes. After the cumulus cells got separated, oocytes were then transferred to the third dish with 100µL drop of M2 media in mineral oil.

For further experiments different concentrations of TNF-α were used (0.1, 1, 10, 100 ng/ml) along with M2 media. The experiments were conducted initially for standardization and later the experiments were replicated for morphological observations. The morphological changes were observed at different hrs (1, 2, 4, 8, 12, 24, 48) and scoring was done based on the degenerative features described earlier (10).

An arbitrary morphological score of the degenerative

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changes was assessed in 5 oocytes in each concentration under inverted microscope (light cytoplasmic granulations: 1; shrinkage of cytoplasm/ distorted shape/cytoplasmic oozing: 1; increased perivitelline space: 1; dark/densely granulated cytoplasm: 2).

Results

The morphological changes (cytoplasmic granulations, increased perivitelline space, cytoplasmic shrinkage, cytoplasmic oozing, distorted shape etc.) were observed with all the concentrations of TNF-α. By 8 hrs majority of oocytes in TNF-α showed degenerative changes. At 8 hrs, the morphological scores of 5 oocytes each at 0.1, 1, 10, 100 ng/ml concentrations of TNF-α were 13, 19, 20, 16 respectively (Table 1). The score was maximum at 10 ng/ml concentration of TNF-α and lesser at extreme lower and higher concentrations. Due to technical issues, the morphological observations were further continued with two concentrations only (1 and 100 ng/ml). Majority of oocytes in M2 media were normal till 8 hrs. Under TNF-α supplementation to media, majority of the oocytes showed all the degenerative changes by 8 hrs. Within 1st hour, mild granulations were visible and by 12 hrs, all oocytes were completely atretic (Figure 1 and 2).

Discussion

TNF-α induced morphological features have been studied on ovarian granulosa and theca cells thereby affecting follicle number and oocyte status (11). Oocyte needs surrounding cumulus cells for its development. The oocyte atresia in the previous study has been interpreted indirectly via granulosa cells or follicular cell death. Results of our study showed the degenerative effects of TNF-α on mice oocyte morphology, maximum at 10 ng/ml as compared to lower and higher concentrations. This suggests that effects are produced only at optimum concentration. Similar result was shown in the previous study as well. The concentrations used in our study were based on that work with minor variations (11). Few studies have shown that apoptosis is the process of cell death in degenerative changes of unfertilized oocytes (12). This study showed that majority of unfertilized ova did not show the classical features of apoptosis (cytoplasmic shrinkage and fragmentation) until 24 hrs. of incubation. The apoptotic features appeared in the cells incubated beyond 40 hrs. One study showed that all the apoptotic cells as proved by TUNEL assay did not show cytoplasmic fragmentation (13). Another controversial result was given by a study that suggested a doubt on apoptosis occurring in atretic oocytes (14). Cells undergo continuous turnover by the process of apoptosis or programmed cell death (15). An apoptotic cell shows nuclear and cytoplasmic condensation and fragmentation along with formation of membrane bound apoptotic bodies. TNF-α is the mediator of extrinsic apoptotic pathway (15,16). Cytoplasmic fragmentation is the end point of apoptosis and the features observed in our study might be the initial changes produced by TNF-α. There have been various markers of apoptotic changes such as TUNEL, M30 immunostaining, annexin v staining etc. but the gold standard method remains the morphological criteria (17, 18). The clinical implications of excessive rate of cellular apoptosis and proliferation have been discussed in many studies (2, 19). The effect of excessive rate of oocyte apoptosis has been linked with ovarian disorders such as POF. The effect of cytokines has also been studied in recurrent pregnancy loss apart from POF and other autoimmune ovarian disorders (20).

Conclusion

To the best of our knowledge TNF-α induced timeline morphological changes on mice oocytes have not been studied so far. Present study was done in smaller number of oocytes but could highlight the possible direct effect of TNF-α on mice oocytes. Results showed the degenerative effects of TNF-α on mice oocytes. Presence of cytoplasmic shrinkage and oozing of cytoplasm are the initial features of oocyte apoptosis. Still our study needs further confirmation of whether these changes are attributed to apoptosis or not. Similar larger studies should be planned for validation. Animal models of immune POF should be designed to see the effect of anti-TNF-α on oocyte growth. Our lab is planning to continue the study to confirm TNF-α induced morphological features as apoptotic by TUNEL or caspase staining and to find out the possible preventive or therapeutic options.

Table 1: Morphological score of oocytes with different concentrations of TNF-α at 8 hrs

Concentration of TNF-α (ng/ml)	Morphological features at each concentration in 5 oocytes	Total apoptotic score
0.1	Dark granulated cytoplasm (2) Dark granulated cytoplasm (2) Dark Granulated Cytoplasm + Increased Perivitelline space (2+1) Dark Granulated Cytoplasm + Increased Perivitelline space (2+1) Dark Granulated Cytoplasm + Increased Perivitelline space (2+1)	13
1	Dark Granulated Cytoplasm + Increased Perivitelline space + Shrinkage of cytoplasm (2+1+1) Dark Granulated Cytoplasm + Increased Perivitelline space + Shrinkage of cytoplasm (2+1+1) Dark Granulated Cytoplasm + Increased Perivitelline space + Shrinkage of cytoplasm (2+1+1) Dark Granulated Cytoplasm + Increased Perivitelline space (2+1) Dark Granulated Cytoplasm + Increased Perivitelline space + Shrinkage of cytoplasm (2+1+1)	19
10	Dark Granulated Cytoplasm + Increase Perivitelline space + Shrinkage of cytoplasm (2+1+1) Dark Granulated Cytoplasm + Increased Perivitelline space + Shrinkage of cytoplasm (2+1+1) Dark Granulated Cytoplasm + Increased Perivitelline space + Shrinkage of cytoplasm (2+1+1) Dark Granulated Cytoplasm + Increased Perivitelline space + Shrinkage of cytoplasm (2+1+1) Dark Granulated Cytoplasm + Increased Perivitelline space + Shrinkage of cytoplasm (2+1+1)	20
100	Dark Granulated Cytoplasm + Increase Perivitelline space (2+1) Dark Granulated Cytoplasm + Increase Perivitelline space (2+1) Dark Granulated Cytoplasm + Increase Perivitelline space (2+1) Dark Granulated Cytoplasm + Increase Perivitelline space (2+1) Dark Granulated Cytoplasm + Increased Perivitelline space + Shrinkage of cytoplasm (2+1+1)	16

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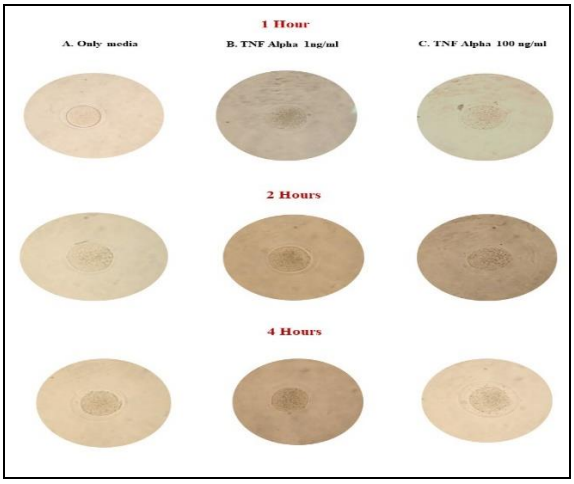

Figure 1. **Oocyte in media** at 1 hr: Normal; 2 hrs: Normal; 4 hrs: Only 1 oocyte showed slight increase in perivitelline space, rest was normal. **Oocyte in media+ TNF-α 1 ng/ml** at 1 hr: light granulations; 2 hrs: light granulations, slight increase in perivitelline space; 4 hrs: dark granulations, perivitelline space larger. **Oocyte in media+ TNF-α 100 ng/ml** at 1 hr: light granulations, distorted shape; 2 hrs: dark granulations, distorted shape; 4 hrs: dark granulations, perivitelline space larger

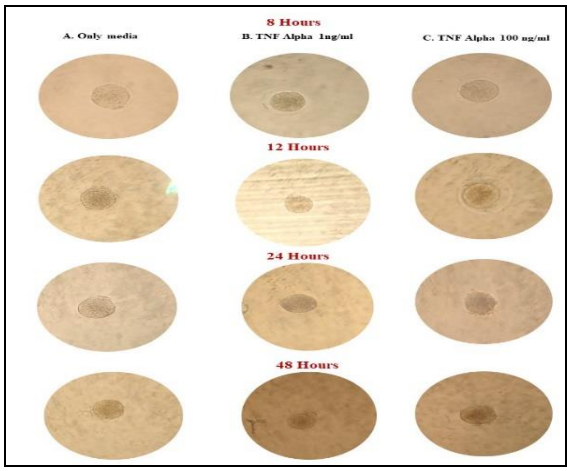

Figure 2. **Oocyte in media** at 8 hrs: Only 1 oocyte showed slight increase in perivitelline space, rest were normal; 12 hrs: diffuse granulations; 24 hrs: light granulations, distorted shape; 48 hrs: dark granulations, distorted shape, cytoplasmic shrinkage, cytoplasmic oozing; **Oocyte in media+ TNF-α 1 ng/ml** at 8 hrs: dark granulations, perivitelline space larger; 12 hrs: dark granulations, cytoplasmic shrinkage and oozing; 24 hrs: dark granulations, distorted shape, cytoplasmic shrinkage; 48 hrs: dark granulations, distorted shape, cytoplasmic shrinkage and oozing; **Oocyte in media+ TNF-α 100 ng/ml** at 8 hrs: dark granulations, perivitelline space larger; 12 hrs: dark granulations, perivitelline space larger, distorted shape; 24 hrs: dark granulations, distorted shape, cytoplasmic shrinkage and oozing; 48 hrs: dark granulations, distorted shape, cytoplasmic shrinkage and oozing

Ethical issue

Authors are aware of, and comply with, best practice in publication ethics specifically with regard to authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests and compliance with policies on research ethics. Authors adhere to publication requirements that submitted work is original and has not been published elsewhere in any language.

Competing interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

Authors' contribution

All authors of this study have a complete contribution for data collection, data analyses and manuscript writing.

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