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Original Research Article

An in-vitro analysis of L-Carnitine mediated rescue of TNF-α induced apoptosis in mice oocytes

Ranjana Rana[1], **Ashutosh Halder**[1], **Surabhi Gupta**[1], **Mona Sharma**[1,*]

[1] *Dept. of Reproductive Biology, All India Institute of Medical Science, New Delhi, India*

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ABSTRACT

Background: Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine implicated in various physiological and pathological events. Carnitine is a quaternary amine which plays a significant role in fatty acid oxidation and is reported to produce antiapoptotic effects. Aim of this work was to study the effect of L-Carnitine (LC) on TNF-α induced apoptosis in mice oocytes.

Materials and Methods: Oocytes were isolated from super ovulated Swiss Albino mice and treated with different concentrations of TNF-α (0.1ng/ml, 1ng/ml, 10ng/ml, 100ng/ml) and LC (0.1mg/ml, 0.3mg/ml, 0.5mg/ml, 1.0 mg/ml). TUNEL Assay was done for the biochemical assessment of apoptosis.

Results: Apoptotic indices with different doses of TNF-α (0.1ng/ml, 1ng/ml, 10ng/ml, 100 ng/ml) were 28.5%, 71.4%, 100%, 42.8% respectively. The concentration of TNF-α that produced the highest apoptotic index was 10ng/ml. LC alone in different doses (0.1mg/ml, 0.3mg/ml, 0.5mg/ml, 1.0 mg/ml) did not elicit any apoptotic signal. Further LC was added in different doses with 10ng/ml TNF-α to study the rate of apoptosis in mice oocytes. Apoptotic index with 10 ng/ml TNF-α and different doses of LC (0.1mg/ml, 0.3mg/ml, 0.5mg/ml, 1 mg/ml) were 25%, 37.5%, 50%, 62.5% respectively. The concentration of LC that reduced the apoptotic index to the maximum was 0.1mg/ml.

Conclusion: Present study could demonstrate the anti-apoptotic effect of LC against apoptotic effects of TNF-α in mice oocytes. The study presents preliminary data suggesting a possible therapeutic role of LC in inflammatory etiologies such as ovarian failure.

1. Introduction

Normal oocyte physiology is integral to ovarian function. During oogensis, oocyte undergoes meiotic arrest and resumes during puberty as a part of development and differentiation process.[1] Reproductive aging in women occurs as a result of progressively declining follicles and oocyte quality that leads to loss of fertility and menopause. It has already been proposed that the inflammatory process may contribute to ovarian aging. An excessive rate of oocyte apoptosis leads to premature ovarian failure (POF). POF is a condition characterized by amenorrhea/oligomenorrhea, infertility and menopausal symptoms due to cessation of ovarian function in women before the age of 40 years. Causes of POF are multifactorial and inflammatory oocyte aging has been reported as one of them.[2] Ovarian function is affected by the secretion of pro-inflammatory cytokines such as TNF-α, IL-6 by macrophages and other cells. These cytokines exert local effects on follicular cells and oocyte.[3]

TNF-α is a pleotropic cytokine which is implicated in initiating apoptosis or necrosis.[4] These activities of TNF-α are mediated by specific cell surface receptors, TNFR-I and TNFR-II. These receptors have a similar extracellular domain but distinct cytoplasmic domain indicating different intracellular signalling pathways. TNFR-I is necessary for

* *Corresponding author*.
E-mail address: dr.mona18sharma@gmail.com (M. Sharma).

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cytotoxicity as it contains an intracellular site, Death Domain.[5] It is now known that TNF-α plays a role as an intraovarian regulator of follicular atresia.[6] Increased expression of TNF-α and its receptors was found in the oocytes of immunized mice with experimental inflammatory ovarian failure.[7] TNF-α was also studied in inducing apoptosis in hen granulosa cells and neonatal rat ovary which suggests the role of TNF-α as a follicular cell death enhancer.[8]

Carnitine is a quaternary amine, which plays an essential role in the fatty acid metabolism by facilitating the transport of activated fatty acids across the inner mitochondrial membrane by acting as a shuttle protein.[9,10] LC is a potent anti-oxidant as it is shown to neutralize the embryotoxic effects of exogenous oxidants. In various tumours and inflammatory diseases, TNF-α was significantly decreased after LC treatment.[11] LC has been shown to reduce apoptosis in cultured mice fibroblasts.[12]

One of our previous studies showed that by 8 hours of incubation, majority of the oocytes undergo morphological degenerative changes induced by TNF-α in mice oocytes.[13] The apoptotic nature of these changes was not confirmed in that study. As TNF-α has been found to induce apoptosis and LC has shown antiapoptotic effects, therefore, the present study hypothesized the ameliorating role of LC on TNF-α induced apoptosis in mice oocytes.

2. Materials and Methods

2.1. Mice and reagents

Ethical permission was obtained for doing animal experiments from Central Animal Ethical Committee of AIIMS, New Delhi (53/IAEC-1/2018). All the experiments were conducted in accordance with approved guidelines. 25 Swiss Albino female mice, aged 6 to 8 weeks were used for the study. They were housed in a 12-hour light-dark cycle in an animal facility at AIIMS, New Delhi and were provided with the required nutrition throughout the study. Chemicals used were purchased from Merck, India unless otherwise indicated.

2.2. Superovulation and oocyte isolation

Swiss Albino female mice were given an intraperitoneal injection of 10 IU PMSG (Prospec, India) on Day 1. Two days later, 10 IU of hCG injection was given intraperitoneally and 12-14 hours later oviducts were dissected out. Oviducts were collected in a dish containing 2 ml of M2 Media. The cumulus mass was separated from the oviduct. Cumulus cells were separated from the oocyte by adding 5μL of hyaluronidase to 500μL of M2 media drop covered with mineral oil. The culture dish was further incubated at 37°C for 5 minutes. After separation, oocytes were transferred to the third dish with a 100μL drop of media in mineral oil. Only good quality Metaphase II(M2) stage oocytes were processed further to study the TNF-α induced morphological changes.

2.3. TNF-α induced apoptosis

The next set of experiments included incubating oocytes in different concentrations of TNF-alpha (0.1ng/ml, 1ng/ml, 10ng/ml, 100ng/ml), deoxyribonuclesae (DNase) in 300 IU/ml was used as a positive control. After 8 hours of incubation, rate of apoptosis was assessed by TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) assay.

2.4. In situ cell detection, fluorescein (TUNEL Assay)

Oocytes were fixed using 4% paraformaldehyde for 1 hour. After fixation, oocytes were washed in PBS (pH=7.2). Permeabilization was done using 1% sodium citrate in 0.1% Triton-X for 2 minutes on ice. Oocytes were again washed twice with PBS. 50 μl of enzyme+label (TUNEL mixture) solution was made for every dish according to the protocol of In situ cell detection kit, fluorescein. After incubation for 1 hour with TUNEL mixture at 37°C, oocytes were washed thrice with PBS and examined via a fluorescence microscope. Oocytes incubated in only media were used as blank control. Negative control had no enzyme in the TUNEL mixture. The experiment was repeated twice. Apoptotic index was calculated as the percentage of apoptotic oocytes out of total number of oocytes in each culture plate.

2.5. L-Carnitine supplementation

Different concentrations of LC were used with 10ng/ml TNF-α (as apoptotic index was highest at this concentration) to study the effect on rate of apoptosis by TUNEL Assay. Oocyte culture dishes were made each having TNF-α (10ng/ml) and different concentrations of LC (0.1, 0.3, 0.5, 1mg/ml), with DNase (300IU/ml) (positive control), with only M2 media (blank control) and with no TUNEL enzyme (negative control). Oocytes were also incubated in different concentrations of LC alone. Culture dishes were incubated at 37°C for 8 hours.

3. Results

The results of present work showed that TNF-α induced oocyte apoptosis with maximum apoptotic changes (100%) observed at 10ng/ml (Table 1, Figures 1 and 2).

As the experiment was repeated twice, apoptotic index was calculated for each experiment separately. Figure 2, Table 1 represent the apoptotic index as Mean ± SEM for each concentration of TNF-α in both the replicates. The apoptotic indices after adding LC in doses of 0.1, 0.3,0.5 and 1 mg/ml to TNF-α 10 ng/ml was calculated as well. The apoptotic index at 0.1 mg/ml was lowest as compared

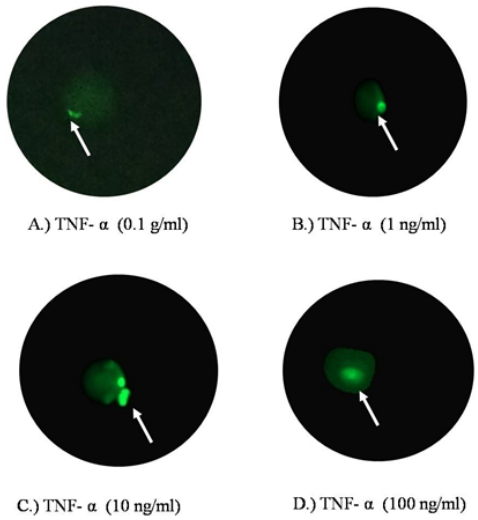

Fig. 1: Photomicrograph of TUNEL Assay with different doses of TNF-α Oocyte at 10X showing green fluorescent signal (white arrow)

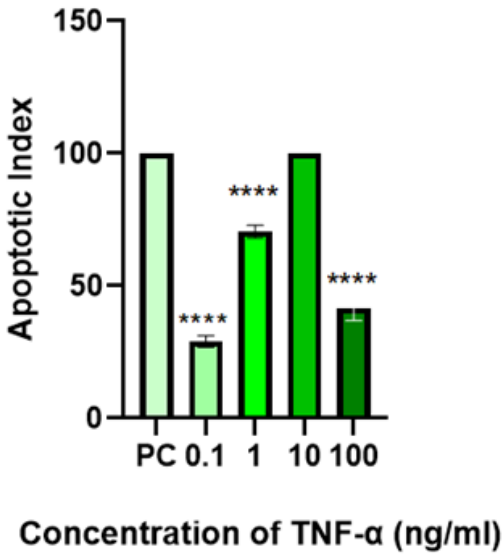

Fig. 2: Effect of TNF-α on mice oocytes. The apoptotic index is shown on the Y-axis of the graph with different concentrations of TNF-α on the X-axis. PC is positive control which is Media + DNase (300IU/ml). Values are presented in Mean ± SEM. **** is p-value <0.0001. The maximum apoptotic index was seen with 10ng/ml of TNF-α

to other concentrations of LC (Table 2, Figures 3 and 4). Table 2 and Figure 4 represent the apoptotic indices of LC in different concentrations with 10 ng/ml TNF-α as Mean ± SEM. The apoptotic index of positive control was 100% whereas negative and blank controls did not show any apoptotic signals (0%) (Figure 5). Similarly, oocytes incubated in different doses of LC alone did not elicit any apoptotic signal (Figure 6).

Data analysis was done using Student's t-test. The statistical graph was generated using GraphPad Prism Software.

4. Discussion

TNF-α is a known inducer of apoptosis in ovarian tissue. Inflammatory aging is the development of low grade inflammation that is induced with age. This process accelerates biological aging and induces several pathologies such as arthritis, osteoporosis, ovarian failure etc. Mechanisms by which inflammatory agents induce follicle depletion is not yet clear but evidences suggest apoptotic follicular death as the main mode. It has been demonstrated that TNF-α induces apoptotic DNA fragmentation in ovarian follicles and even oocyte death in vitro.[6,8]

The major pathogenic feature in POF of inflammatory etiology is excessive rate of oocyte atresia due to apoptosis. The exaggerated rate of apoptosis has found to be detrimental to the cellular functions.[14,15] Apoptosis can be induced by intrinsic or extrinsic pathway and TNF-α is one of the mediators of extrinsic pathway.[16]

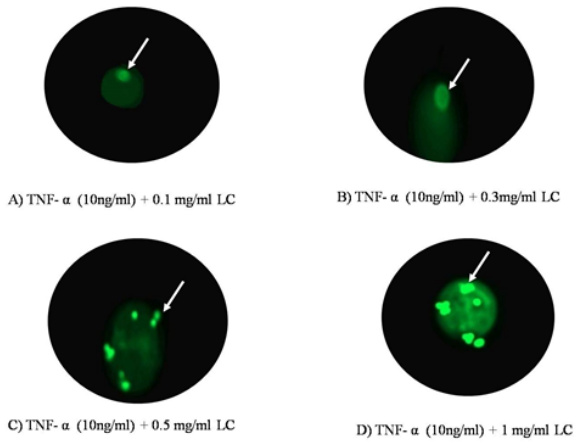

Fig. 3: Photomicrograph of TUNEL Assay with different doses of LC Oocyte at 10X: green fluorescent signals indicate apoptotic fragments (white arrow)

Table 1: Apoptotic index of oocytes incubated in different concentrations of TNF-α

S.No.	Concentration of TNF-α (ng/ml)	Total Number of oocytes in the culture plate	Mean Apoptotic index (%)	Standard Error Mean (SEM)
1.	0.1	7	29	2.13809
2.	1	7	70.5	2.405351
3.	10	7	100	0
4.	100	7	41.5	4.543441
5.	Only Media	7	0	0
6.	Media + DNase (300IU/ml)	8	100	0
7.	Media+ No TUNEL enzyme	8	0	0

Table 2: Apoptotic Index of Oocytes incubated in different concentrations of L-Carnitine and 10ng/ml TNF-α

S.No.	Concentration of L-Carnitine (mg/ml) with 10ng/ml TNF-α	Total No. of oocytes	Mean Apoptotic index (%)	Standard Error Mean (SEM)
1.	0.1	8	16.5	8.25
2.	0.3	8	25	12.5
3.	0.5	8	50	0
4.	1	8	58	4
5.	Only Media	8	0	0
6.	Media + DNase (300IU/ml)	8	100	0
7.	Media+ No TUNEL enzyme	8	0	0

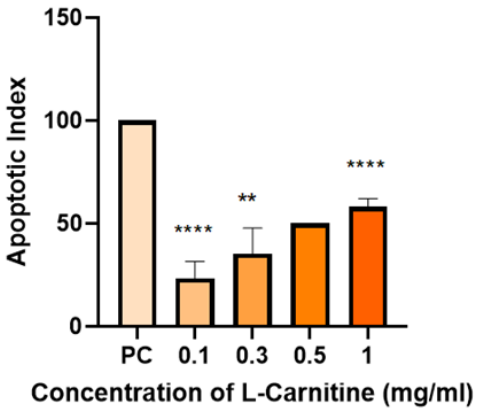

Fig. 4: Effect of LC on mice oocytes incubated with 10ng/ml TNF-α. Apoptotic index is shown on the Y-axis of the graph with different concentrations of LC on the X-axis. PC is positive control which is Media + DNase (300IU/ml). Values are presented in Mean ± SEM. **** is p-value <0.0001and **is p-value <0.001. A minimum apoptotic index was seen with 0.1mg/ml LC with 10ng/ml TNF

Results of present study showed TNF-α induced apoptotic changes in mice oocytes at each concentration used but the apoptotic index was highest at 10 ng/ml. It has already been shown that TNFRI is associated with cell apoptosis as in induced POF.[7] Earlier studies showed that granulosa cells interact with the oocyte to induce TNF-α mediated apoptosis. A study conducted in a rat model showed that TNF-α can induce DNA damage by increasing reactive oxidative species in the culture media eventually

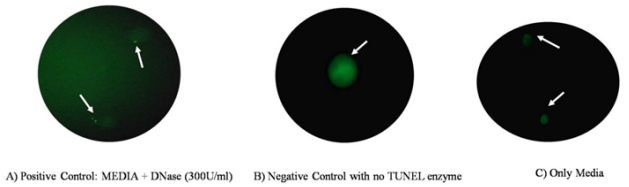

Fig. 5: Photomicrograph of TUNEL Assay Oocyte at 10X: Green fluorescent signals observed in positive control, No green fluorescent signal observed in negative and blank controls (white arrow)

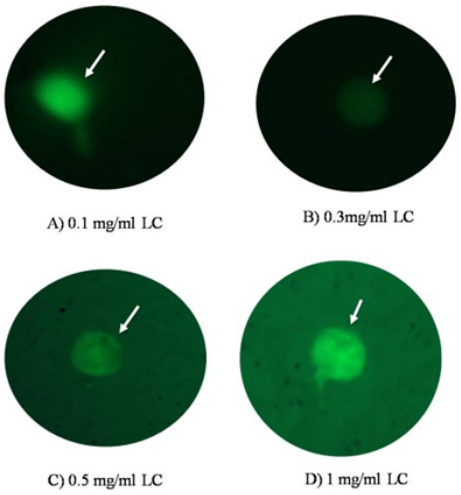

Fig. 6: Photomicrograph of TUNEL Assay with different doses of L-Carnitine Oocyte at 10X. No green fluorescent signal observed in Oocytes (White Arrow)

leading to apoptosis.[17] One of our previous studies has also shown the TNF-α induced degenerative changes in mice oocytes were maximum at 10ng/mL.[6,13] Similarly present study confirms that the degenerative effects produced were the apoptotic changes. Cellular apoptotic changes can be easily identified by various biochemical methods such as TUNEL, M30 immunostaining, caspase staining etc.[18–21] TUNEL is one of the common methods to identify DNA breaks as marker of cellular apoptotic changes.[20]

The receptor-mediated TNF-α cross-talk regulating downstream signalling has been extensively studied in cellular survival, aging, immunity and viral pneumonia.[22,23] It was shown that serum concentrations and ovarian mRNA levels of TNF-α/IL6 and inflammatory associated genes such as ASC/NLRP3 were increased with age suggesting their possible association with age-induced changes.[24]

Physiologically inflammation is an important event associated with various reproductive events such as ovulation, folliculogenesis but in a tightly regulated manner. The cytokines regulate the secretion of steroid hormones necessary for folliculogenesis. Unregulated inflammation leads to reproductive pathologies including ovarian failure.

In mice, the increasing levels of systemic and intraovarian expression levels of inflammatory proteins were associated with a decline in follicle pool with age. Mice lacking TNF-α had prolonged fertility, increased follicle count and litter size.[25]

Depending upon stages of development, TNF-α regulates granulosa cell differentiation and apoptotic demise. TNF receptors are trans-membrane proteins having a cytoplasmic domain that initiates signalling once TNF-α binds to it.[22] After binding, various other adapter proteins form complex I consisting of TNF-R1 associated death domain protein (TRADD), TNF-associated factor 2 (TRAF2), and interacting receptor protein 1. Complex II is formed by assembly of complex I with Fas associated death domain (FADD) and procaspase 8/10 that ultimately leads to apoptosis.[16,23]

One of the previous studies showed that TNF-α at 1ng/ml could produce apoptosis via TNFR1 but at higher concentrations antiapoptotic effects were observed via TNFR II.[26] Moreover, calcium dependant endonucleases taking part in apoptosis may be regulated by different doses. It was also shown that at TNF-α at 10ng/ml could mobilize calcium from intracellular stores but not at the higher doses.[27] Also, higher doses (50, 100 ng/ml TNF-α) activated antiapoptotic NF-kB by upregulating expression of apoptosis inhibitor FLIP (FLICE inhibitory peptide) in granulosa cells.[28,29]

Current study could also show that apoptotic effects of TNF-α on mice oocytes were more at lower doses (10ng/ml) as compared to higher doses. TNF-α at10ng/ml produced maximum apoptosis (100%) in mice oocytes. When LC was supplemented in different concentrations (0.1 mg/ml, 0.3 mg/ml, 0.5 mg/ml, 1 mg/ml) with 10ng/ml of TNF-α, rate of apoptosis decreased from 100% to 25%, 37.5%, 50%, 62.5% respectively. By this result LC mediated inhibition on TNF-α induced apoptosis can be suggested. Also, LC alone in different doses did not produce apoptotic changes in mice oocytes. Out of the four concentrations of LC used, 0.1 mg/ml gave the best anti-apoptotic results (lowest apoptotic index) when supplemented with 10ng/ml of TNF-α. Group of few other studies have demonstrated that LC is effective in reducing plasma as well as serum levels of TNF-α.[30,31] It can be suggested that LC produce anti-apoptotic effects at lower doses only just like TNF-α showed more apoptotic effects at lower doses. But the mechanism by which LC could inhibit TNF-α induced apoptotic effects in lower doses only is yet to be confirmed in further studies.

5. Conclusion

TNF-α is a multifunctional cytokine present in various cells of ovarian tissue. Overall, our data with previous references suggest that there exists a relationship between inflammatory agents such as TNF-α, apoptosis and ovarian aging. Our study suggests the role of LC as the antiapoptotic protein in decreasing the apoptotic effects of TNF-α in mice oocytes. As the present study was hypothesis generating so was planned in smaller sample size and therefore present study alone cannot prove the therapeutic effects of LC with confirmation until the work is extended in larger cohorts.

6. Author Contribution

Authors have contributed for conception or design of the work, planning of experiments, approval of the study design and concept, contributed essential consumables, animal procurement & animal care, analysing data, final approval of the version to be published and writing of the paper.

7. Source of Funding

None.

8. Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Author biography

Ranjana Rana, PhD Scholar https://orcid.org/0000-0001-7471-7221

Ashutosh Halder, Professor and Head https://orcid.org/0000-0002-3116-3502

Surabhi Gupta, Additional Professor https://orcid.org/0000-0001-6092-6575

Mona Sharma, Additional Professor https://orcid.org/0000-0003-0901-1905

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