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Investigation of the effect of L-Carnitine on Mouse Ovarian Transplantation

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ABSTRACT

Introduction: L-carnitine (LC) has yielded relevant results on ischaemic/ reperfusion (I/R) injuries in organs such as the intestine, liver, kidney, brain and heart, because of its antioxidant activity. We aimed to evaluate the potential of using L-carnitine to improve the quality of grafted ovarian tissues. *Materials and methods:* The ovaries from 4-6-week old mice were grafted into back muscle site. Transplanted ovaries were divided into four groups. 200 mg/kg LC was injected intraperitoneally one day before surgical operation

and repeated until one week after grafting. Follicular morphology and apoptosis were examined in all groups and compared to age-matched non grafted ovaries. Anti- active caspase-3 staining was performed to evaluate apoptosis in all groups. *Results:* group Morphological analysis revealed that the number of follicles decreased in all transplanted groups compared with non-grafted group. The rate of apoptosis in LC- treated group showed no significant differences compared with control. *Conclusion:* LC supplementation may be a suitable approach for the ovarian graft viability. Further experimental studies are required to clarify the mechanisms of action of L-carnitine, and clinical studies are required to determine optimum dose and treatment duration for decreasing I/ R injuries.

Keywords: Apoptosis, Follicle, L-carnitine, ovarian transplantation.

1. INTRODUCTION

Todays, several assisted- reproduction techniques have been explored to preserve fertility in cancer patients. One of the most recent and promising strategy of which is transplantation of ovarian tissue (Jeruss et al., 2009; Rodriguez et al., 2012a; Donnez and dolmans, 2014). Researchers have found that grafted ovaries could recover reproductive and endocrine function (Donnez et al., 2004; Eimani et al., 2009; Behbahanian et al., 2013). To date, about 60 live birth have been reported in humans after transplantation of ovarian tissue (Donnez et al., 2012; Kim, 2014).

However, grafting of ovaries is considered to be an experimental method, because there is a 50% to 90% reduction in follicle density during transplantation (Baird et al., 1999; Nissole et al., 2000). It has been shown that more follicles die from ischemia that occurs soon after transplantation (Dolmans et al., 2007; Nottola et al., 2008; Shikanov et al., 2011). Finding ways to accelerate graft revascularization is an urgent challenge in the development of reproducible and reliable procedures for implanting of ovarian tissue. Various studies have been performed to decrease the damage of ischemia to grafts such as the use of antiapoptotic factors, Drugs, antioxidants or angiogenic agents during transplantation (Sapmaz et al., 2003; Silber et al., 2008; Soleimani et al., 2008; Smitz et al., 2010; Damous et al., 2010; Lee et al., 2014).

L-carnitine (LC) is a vitamin-like compound that is structurally similar to amino acids. It has been reported to reduce programmed cell death in various cell types (Berni et al., 2008; Usta et al., 2008). Some studies showed that l-carnitine protects the myocardium (Vescovo et al., 2002), kidney and liver against ischemia (Atilla et al., 2002). The double role of LC as an antioxidant and an antiapoptotic agent makes it a candidate as a novel factor for improving the success rate of transplantation. The present study is therefore aimed to evaluate the effect of LC supplementation on transplantation of mouse ovarian tissue.

2. MATERIALS AND METHODS

Animals

Animal experiments were performed according to the Declaration Helsinki and the Guiding Principles in the care and use of Animals (DHEW publication, NIH, 80-23).

Female NMRI mice aged 4-6 weeks were used in all experiments. The animals were housed and kept at a temperature of 20-25^{O}C and 50% humidity under light controlled conditions (12h light/12h dark) and provided with sterile food and water in the central animal house of Royan institute according to national standards.

Chemicals

L-carnitine is produced by Sigma. It was dissolved in the normal saline at a concentration of 200 mg/Kg.

Experimental groups

Mice were divided into the following four groups: Group 1 (n=5) was the control group and no operation was performed. Group 2 (n=5): only underwent ovarian transplantation (T). Group 3 (n=5): was given saline instead of LC (T+saline). Group 4 (n=5): was administered LC intraperitoneally one hour before transplantation (T+LC) and repeated every day during one week after that.

Transplantation procedure

The surgery was performed under aseptic conditions. Mice were anaesthetized with intraperitoneal ketamine (50 mg/ml; Rotexmedica GMBH, Trittau, Germany) and xylazine (20mg, 2%, Alfasan, Woerden, The Netherlands). After shearing and sterilizing

the skin, the ovaries were brought out through small incision, only the left ovary was inserted into back muscle. The muscle fibers were sutured with non- absorbable 6-0 surgical thread. Peritoneum and the skin were sutured with absorbable thread. At the end, the animals were allowed to recover in the laboratory and then transferred to the animal house.

Recovery of grafts

In all experimental groups, the mice were killed after a period of three weeks. The ovaries were retrieved and fixed in 10% buffered formalin.

Histological method

The fixed ovarian specimens were dehydrated, clarified in xylene, and embedded manually in paraffin. The whole ovary was sectioned serially at 6 µm thickness. One of every five consecutive sections was put on slide and stained by H & E staining, while other representative slides were used for immunohistochemical staining. The follicles were examined and counted under a light microscope (magnification ×400). To avoid recounting of the same follicle, follicles were only counted when the dark staining nucleolus was seen within the germinal vesicle.

Morphometric analysis of grafts

Follicles based on Liu and colleague's (Liu et al., 2002) were classified into four groups: primordial (oocytes surrounded by one layer of flattened cells), primary (surrounded by one layer of cuboidal cells), preantral (with more than one layer of cuboidal cells without antrum), or antral (with an antral cavity) follicles.

Caspase-3 immunohistochemistry

The sections selected for immunohistochemistry were deparaffinized, rehydrated, and exposed to antigen unmasking in boiling sodium citrate buffer (PH=6.0). Then treated with 0.3% hydrogen peroxidase for blocking endogenous peroxidase activity. Sections were blocked with a 1:100 dilution of goat serum and incubated overnight at 4^{O} C with the anti-active caspase-3 (AB4051; Abcam) antibody (1:100 dilution). In negative control, the primary antibody was omitted. Bound antibody was detected using a 1:200 dilution of secondary antibody (AB97051; Abcam) for 1h at 37 ^{O}C. Subsequently, the sections were developed using diaminobenzidine (DAB) chromogen for 2-3 min. Next, the sections were washed in water and counterstained with hematoxylin, then; they were washed, dehydrated, cleared, and mounted.

Sections from the middle of grafts were analyzed for immunostaining. Red-brown coloring of the cytoplasm/nucleus in granulosa cells was specified as positive staining. A follicle containing over 30% cells positive staining was considered as apoptotic.

Statistical analysis

Results were expressed as Means± SEM. One-way analysis of variance (ANOVA) with Tukey, s post-hoc test (SPSS 16) was used to compare groups. P value of <0.05 were considered significant.

Ethical approval

The experimental protocol was approved by Animal Ethic Committee of Royan Institute (Code: EC/92/1043).

3. RESULTS

Graft development

Gross morphology of back muscle grafts showed the blood vessels that generated around the implants. The graft size was obviously smaller than that of beginning of grafting. There was an unclear margine between the muscular and ovarian tissue.

Histological analysis

Hematoxylin and eosin staining showed that follicles at different stages were observed at grafted ovaries (Fig.1), but total follicle count in the control groups were significantly higher than that in T and T+ saline groups ($p<0.05$) (Fig.2). Among the follicles that examined in all groups, primordial follicles had highest numbers, followed by primary follicles, then by pre-antral follicles and antral follicles. Compared with transplantation group, the numbers of follicles were not increased significantly, when ovaries treated with LC (200 mg/kg, $P>0.05$). Also, there was no significant difference between control and LC treated groups. The percentages of follicles in all grafted groups were similar to those seen in the control group Fig 3). Also, in some sections of all grafted groups, a

few follicles had no oocytes, forming cysts in the grafted ovaries. Medulla of grafted ovaries as there was in control groups was un-cleared. Establishment of well- organized blood vessels around the grafts was apparent.

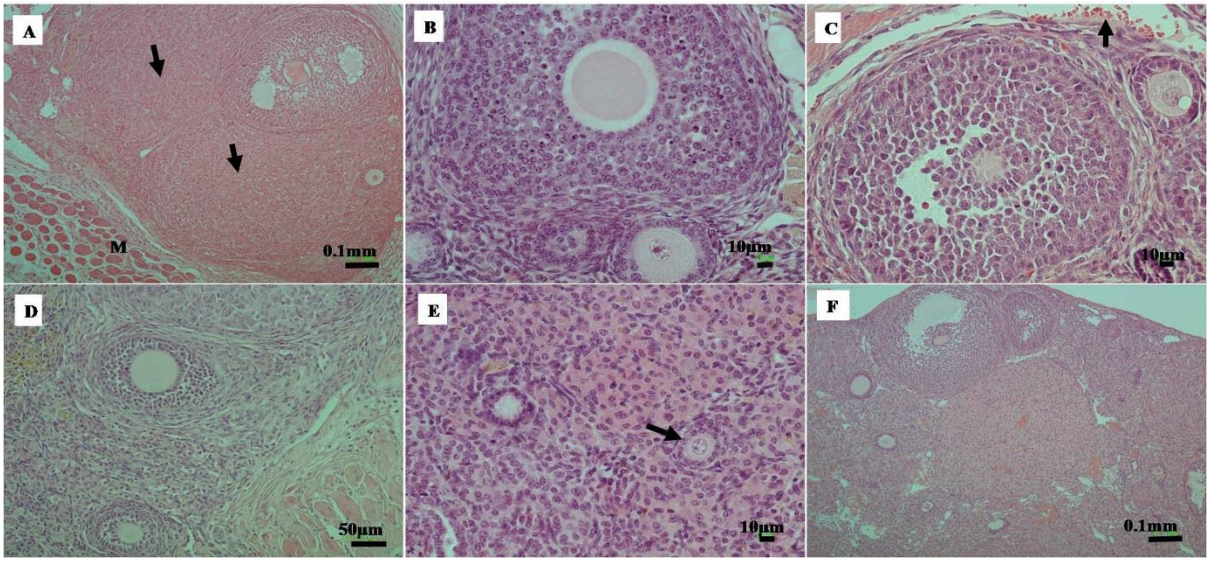

Figure 1 Ovarian tissue morphology after transplantation. H & E- stained ovarian section showing the corpora lutea in LC treated group (A). healthy follicles in T + saline group(B). Antral follicle in T group (C). Two preantral follicles (D). A healthy primary follicle (black arrow) (E). H & E- stained ovarian section of control group (F). Vascularization of the transplanted ovarian tissue (Arrow in C). M= muscle.

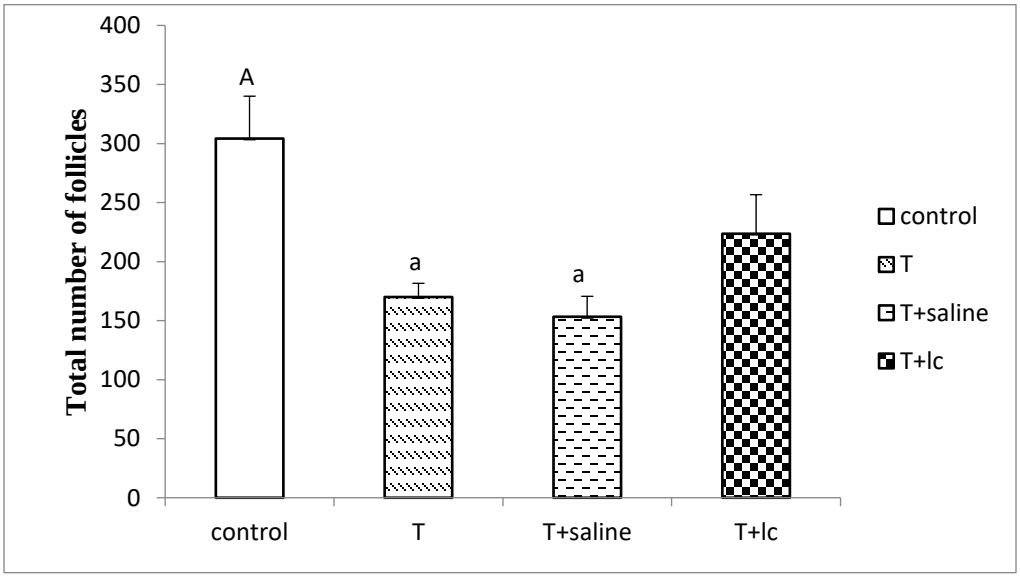

Figure 2 Total follicle number analysis of experimental groups. Capital letter vs. the same small letter shows significant differences ($p<0.05$).

Effect of ovarian transplantation on apoptosis of follicles

Caspase-3 positive apoptotic follicles were not detected in small, single-layered follicles, while they were observed in granulosa cells of pre-antral and antral follicles (Fig 4). The percentages of apoptotic follicles were significantly increased in T and T+ saline groups compared with control group ($p<0.05$), but no significant difference existed between grafted groups (Fig 5).

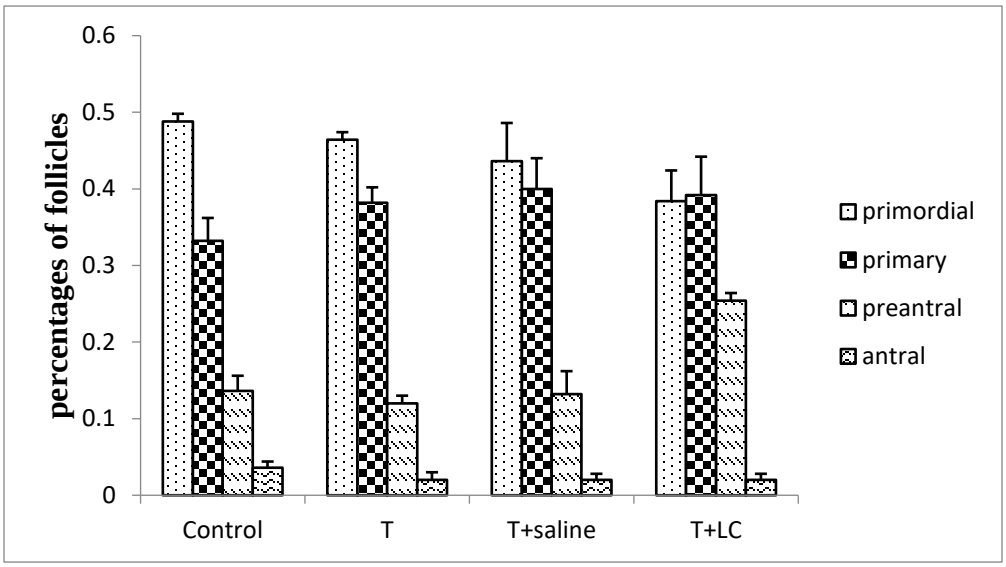

Figure 3 The percentages of follicles at different developmental stages.

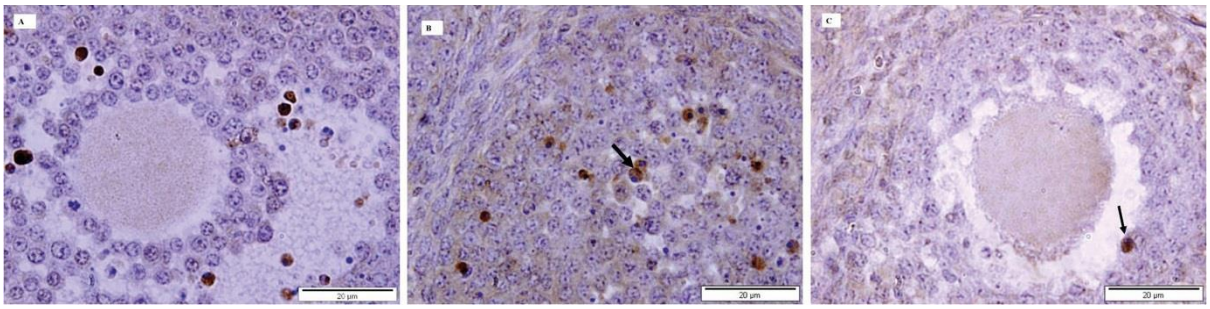

Figure 4 Representative immunohistochemistry of caspase-3 in granulosa cells of follicles in transplanted ovaries (A-C). (Shahi Sadrabadi et al., 2018)

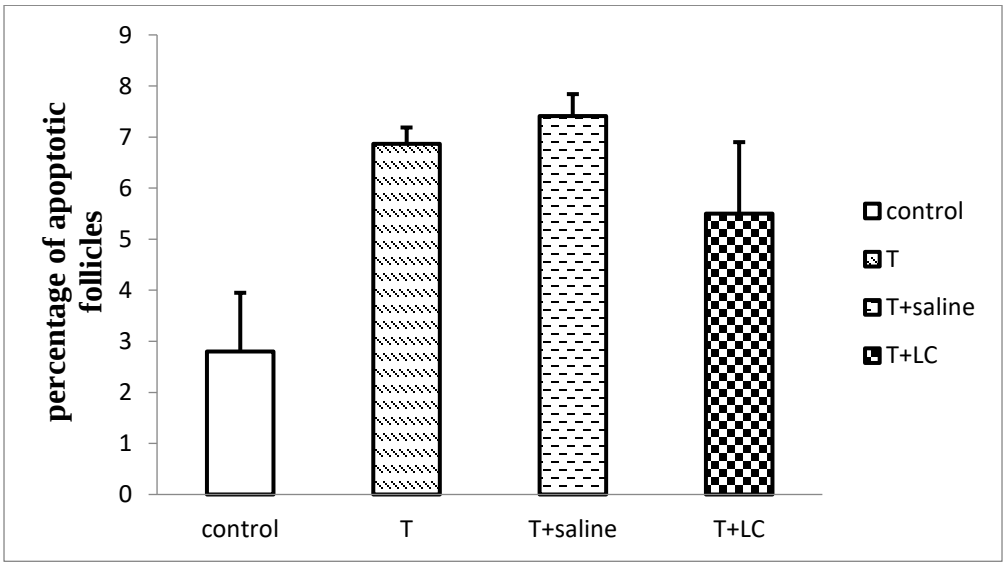

Figure 5 The percentages of immunopositive follicles in different groups. Capital letter vs. the same small letter shows significant differences ($p<0.05$). (Shahi Sadrabadi et al., 2018)

4. DISCUSSION

The major challenge of ovarian tissue transplantation is ischemia-reperfusion injury (Gosden, 2008; Rodriguez and Oktay. 2012b; Demeestre et al., 2009). There are several experimental treatments for improving graft quality and one of the proposals is addition of antioxidant agents. Local injection of Vitamin E before graft could improve follicular survival (Nugent et al., 1998). In the present study, we chose L-carnitine, because of its prevention of energy loss and its antioxidant activity to test its effect on ovarian transplantation.

Carnitine treatment has yielded relevant results on I/R injury in other organs such as the intestine, liver, kidney, brain and heart (Gulcin, 2006). In cell culture, L-carnitine had a protective effect against mouse fibroblast cell apoptosis via stabilization of mitochondrial membrane (Pillich et al., 2005). LC had a significant decrease in the level of apoptosis in mouse embryos treated with actinomycin-D (Abdelrazic et al., 2009). However, our results showed a trend of increase in total follicle numbers in LC-treated group, but it was not significantly different. Previous studies mentioned that LC did not alter survival, growth or differentiation of follicles (Dunning et al., 2011). Moreover, treatment of the grafts with LC had no effect on percentages of apoptotic follicles. A possible explanation for this finding is that the given dose and duration of LC administration did not compensate for the reducing I/R damages. Several experimental antioxidant models for testing different therapeutic strategies are described in the literature, both in vitro (mouse embryo culture) and in vivo (liver). Oyanagi et al's study has demonstrated that LC can maintain mitochondrial function and suppress membrane permeability transition (MPT) of mitochondria through acceleration of β- oxidation. The opening of MPT has been proves as a key event in cell apoptosis after I/R (Oyanagi et al., 2008). Cekin *et al*. assessed the effect of LC on oxidative damage in hepatic I/ R injury in rats. The authors observed a protective effect of LC and improvement of antioxidant defense system and lipid peroxidation levels (Cekin et al., 2013).

Kim and colleague's demonstrated that incubation of fresh ovarian cortical specimen with antioxidant ,ascorbic acid, for 48h had no effect on apoptotic primordial follicles (Kim et al., 2004).

5. CONCLUSION

Our data showed that LC supplementation may be helpful for graft viability and increased total follicle number of transplanted ovaries. However, further studies are required to elucidate the optimal concentration and application days of LC treatment.

Conflict of interest

The authors declare that there is no conflict of interest.

Finding sources

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