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Assessment of lipid peroxidation by TBARS assay in spent media of oocytes obtained by trans vaginal ovum pickup subjected to *in vitro* maturation

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Abstract

Oxidative stress during *in vitro* maturation (IVM) of oocytes compromise oocyte quality and developmental competence. The current study was undertaken to understand the lipid peroxidation levels in spent media supplemented with natural antioxidants during oocyte maturation. A total of 338 oocytes were recovered through transvaginal ovum pick-up (OPU) and randomly allocated to four treatment groups viz., Vitrogen maturation medium (Group 1), Media-T (TCM- 199 based; Group 2), Media-T supplemented with silymarin (40 µg/ml; Group 3), and Media-T supplemented with Quercetin (10 µg/ml; Group 4). Oocytes were cultured in 100 µl respective media droplets for 24 hours. Following maturation, spent media was collected and assessed for lipid peroxidation by using TBARS assay. About 288 oocytes were successfully matured, yielding maturation rates of 88.89%, 87.18%, 88.41%, and 82.61%, respectively. The mean MDA concentration (n mol/ml) detected in spent media was 31.14, 25.16, 11.64 and 13.96 in Group 1 to 4, respectively indicating numerically lower lipid peroxidation among all groups compared to control media (Group 1). However, the overall lipid peroxidation detected in spent media suggests that antioxidant supplementation during oocyte maturation would help to maintain redox balance.

Keywords: *In vitro* maturation, lipid peroxidation, oxidative stress, quercetin, silymarin, TBARS assay

Introduction

Reactive oxygen species (ROS) are produced as by-products of cellular during oocyte maturation. At physiological levels, ROS play a Janus-like role by supporting signalling pathways required for meiotic resumption; however, excessive ROS causes oxidative stress that damages cellular lipids, proteins, and nucleic acids [1, 2]. Lipid peroxidation generates toxic end-products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which disrupt membrane integrity, impair mitochondrial bioenergetics, and alter cellular metabolism [3, 4]. Consequently, lipid peroxidation leads to reduced oocyte quality, spindle abnormalities, mitochondrial dysfunction, and decreased developmental competence [5].

During *in vitro* embryo production (IVEP), particularly in IVM, ROS generation is higher than *in vivo* due to artificial culture conditions [6]. Therefore, control of ROS and lipid peroxidation will improve IVEP outcomes by maintaining oocyte integrity. Oxidative damage during oocyte maturation is commonly assessed by measuring lipid oxidation metabolites formed during ROS-induced membrane degradation [3]. However, direct intracellular assessment is difficult because of the limited number and small size of oocytes and the need to preserve them for embryo production as per the technical programme of the project. Hence, analysis of spent culture media provides a non-invasive alternative, with extracellular oxidative stress markers serving as indirect indicators [1, 2]. Although lipid peroxidation assessment in IVEP using spent media has gained interest, available literature is limited and often based on modified serum or tissue assays [3, 5]. Therefore, refinement and validation are needed to establish reliable protocols for detecting oxidative stress markers in oocyte culture systems.

The present study was aimed to detect lipid peroxidation in IVM media of oocytes and to evaluate the effect of antioxidant supplementation on lipid peroxidation levels.

Materials and Methods

A total of 338 oocytes were obtained from Sahiwal donor cow through OPU method and were randomly allocated to four treatment groups viz., Vitrogen maturation medium (Group 1), Media-T (TCM- 199 based; Group 2), Media-T supplemented with silymarin (40 µg/ml; Group 3), and Media-T supplemented with Quercetin (10 µg/ml; Group 4). Oocytes (10-15 no.) were subjected to maturation in 100µl respective maturation media droplets overlaid with mineral oil for a period of 24 hours under standard maturational settings of 38.8°C, 5%CO₂, 95% RH. Each droplet was allocated 15-20 oocytes.

Following maturation, the spent media was carefully collected and was subjected to lipid peroxidation by TBARS assay.

Principle of TBARS Assay: Breakdown product of polyunsaturated fatty acids is malondialdehyde which produces pink colour chromogen upon reaction with thiobarbituric acid which was measured at 540nm^[7] with little modification that suit to minute volumes of spent media. Following reagents were prepared by using TMP standard - 10 mM in double distilled water, TBARS Reagent- 1:1:1 ratio of 0.25N HCl, 10% TCA and 0.67%TBA (equal volumes), 10% TCA (Trichloro acetic acid) prepared in 0.25N HCl and

0.67% TBA (Thiobarbuteric Acid).

Procedure

- **Preparation of standards:** Standard curve was prepared by using 500 µl of TMP standard with varying concentrations of 2, 4, 8, 12, 16, 20, 40, 80, 120, 160 and 200 nano moles diluted with distilled water.
- **Preparation of blank:** 50 µl of distilled water was added to 200 µl of TBA-TCA-HCl reagent and was used as blank.
- **Preparation of test:** 200 µl of TBA-TCA-HCl reagent was added to each test tube, 20 µl of sample and 30 µl of distilled water was added mixed thoroughly.
- All the tubes were boiled at 95°C for 15 minutes and then cooled.
- Later the tubes were centrifuged at 2500 rpm for 5 min and then the supernatant was collected.
- The absorbance of supernatant was measured at 540 nm against the blank.
- A standard curve plotted with standard TMP solution in corresponding concentration range of samples was used for calculation of MDA concentration in test samples of spent medium (Fig. 1)

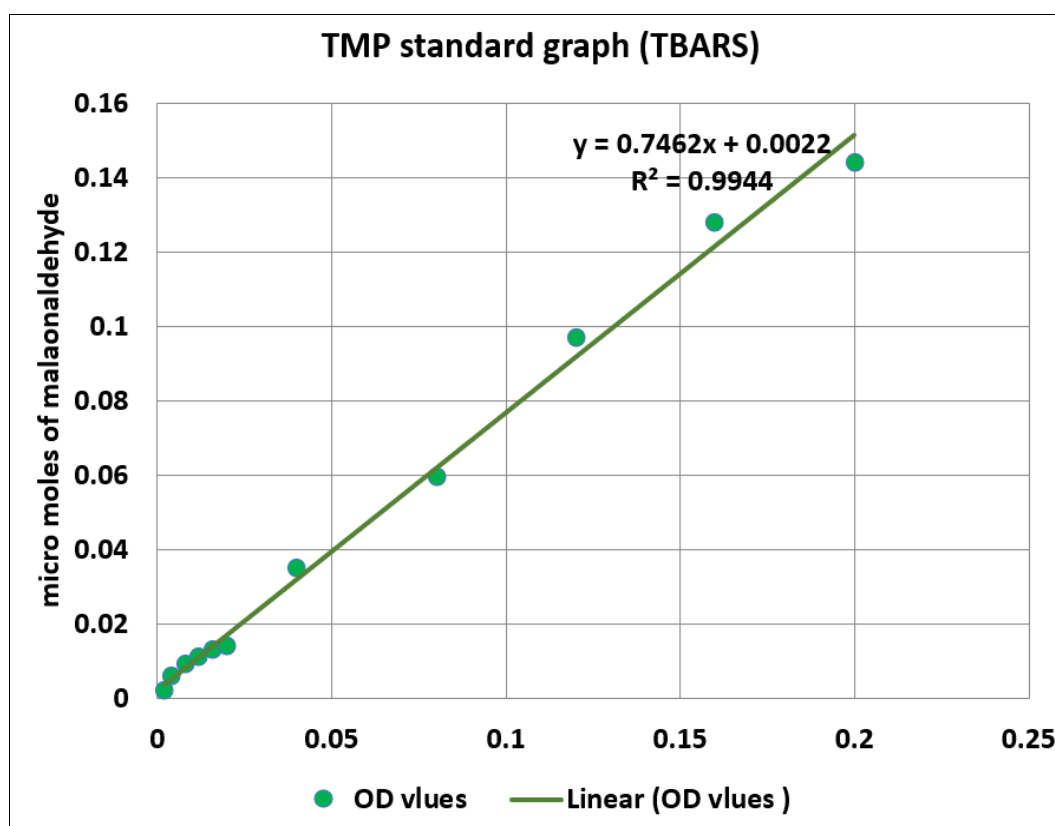


Fig 1: The lipid peroxidation level in spent media was measured using TBARS assay using various concentrations of TMP standard

Results and Discussion

Out of 338 oocytes subjected to maturation, 288 oocytes successfully matured. The maturation outcomes in different groups were as follows: Group-1 - 83 out of 99 (88.89%),

Group-2 - 68 out of 78 (87.18%), Group-3 - 61 out of 69 (88.41%) and Group-4 - 76 out of 92 (82.61%) indicating that oocyte maturation rates were relatively comparable across all treatment groups (Fig. 2).

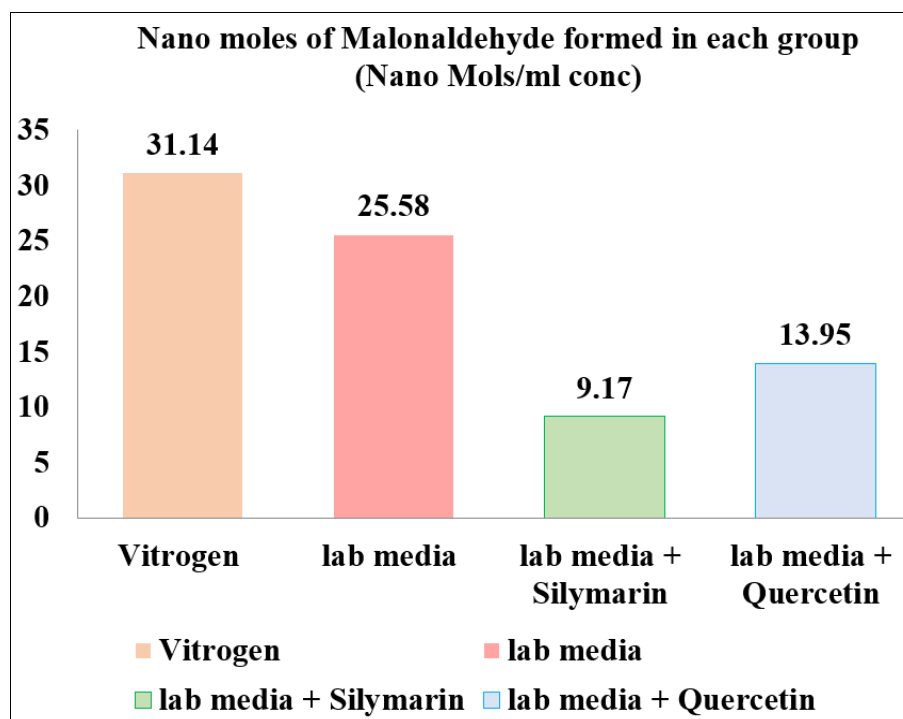


Fig 2: Concentration of Malondialdehyde formed in each group in Nano Mols/ ml concentration in spent media

Lipid peroxidation in the spent media was assessed using TBARS assay by quantifying malondialdehyde (MDA) an end-product of oxidative degradation of membrane lipids. The mean MDA concentrations (nmol/ml) recorded were Vitrogen - 31.14, Lab media - 25.16, Silymarin - 11.64, and Quercetin - 13.96. The comparatively lower MDA concentrations observed in the antioxidant-supplemented groups, particularly silymarin and quercetin, suggest reduced lipid peroxidation and improved control of oxidative stress during *in vitro* maturation [8, 9].

However, the overall levels of lipid peroxidation detected in the present study were relatively low. This may be attributed to controlled *in vitro* maturation conditions such as regulated temperature, humidity and gaseous environment, which helps to maintain free radical levels within physiological limits and thereby restrict excessive lipid peroxidation [1, 2]. Another factor that may have influenced the findings of present study is the inherent variability in oocyte quality. Oocytes obtained through transvaginal ovum pick-up (OPU) differ in morphological grade and the extent of cumulus cell layers. The cumulus-oocyte complex (COC) plays an important protective role by regulating intra-oocytic redox balance and scavenging free radicals generated during maturation [10, 11]. Variations in the proportion of high-quality and low-quality oocytes across treatment groups could therefore influence ROS generation and subsequent lipid peroxidation levels.

It is also important to note that lipid peroxidation measured in spent media reflects extracellular metabolites of oxidative stress rather than direct intracellular oxidative damage. Before oxidative by-products are released into the surrounding medium, they might have been neutralized by intracellular antioxidant defence mechanisms. Consequently, mitochondrial and cytoplasmic oxidative stress occurring within the oocytes might not be fully reflected in extracellular MDA concentrations [5]. Previous reports have indicated that although extracellular oxidative markers remain modest, intracellular oxidative stress can still be alleviated through antioxidant supplementation during *in vitro* maturation [6].

Natural antioxidants such as silymarin and quercetin possess

strong free radical scavenging and membrane-stabilizing properties, which can mitigate lipid peroxidation and enhance cellular antioxidant status in reproductive tissues [8, 12]. This mechanism likely explains the reduced MDA levels observed in the respective treatment groups. Overall, these findings suggest that supplementation of natural antioxidants during *in vitro* maturation may contribute to maintaining redox homeostasis by limiting lipid damage, thereby potentially supporting improved oocyte developmental competence.

Conclusion

In the present study, assessment of lipid peroxidation in spent media did not reveal any significant differences in the MDA concentrations among treatment groups but the antioxidant-supplemented groups showed numerically lower values than vitrogen or control media suggesting a possible reduction in oxidative stress. Variations in oocyte quality and grading might have influenced oxidative damage. Therefore, it is concluded that overall antioxidant supplementation would maintain redox homeostasis during oocyte maturation. In future larger studies incorporating both intracellular and extracellular oxidative stress markers and grade-wise oocyte analysis are required to better understand their protective role.

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Conflict of interest

None

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