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TECHNOLOGICAL INNOVATIONS

Raman spectroscopy-based approach to detect aging-related oxidative damage in the mouse oocyte

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Abstract
Purpose Detection of chemical modifications induced by aging-related oxidative damage in mouse metaphase II (MII) oocytes by Raman microspectroscopy.
Methods CD-1 mice at the age of 4–8 weeks (young mice) and 48–52 weeks (old mice), were superovulated and oocytes at metaphase II stage were recovered from oviducts. MII oocytes from young animals were divided into three groups: A) young oocytes, processed immediately after collection; B) in vitro aged oocytes, cultured in vitro for 10 h before processing; C) oxidative-stressed oocytes, exposed to 10 mM hydrogen peroxide for 2 min before processing. Oocytes from reproductively old mice were referred to as old oocytes (D). All the oocytes were analyzed by confocal Raman microspectroscopy. The spectra were statistically analyzed using Principal Component Analysis (PCA).
Results PCA evidenced that spectra from young oocytes (A) were clearly distinguishable from those obtained from in vitro-aged, oxidative-damaged and old oocytes (B, C, D) and presented significant differences in the bands attributable to lipid components (C=C stretching, 1,659 cm^{-1}; CH_2 bending, 1,450 cm^{-1}; CH_3 deformation,1,345 cm^{-1}; OH bending, C-N stretching, 1,211 cm^{-1}) and protein components (amide I band,1,659 cm^{-1}; CH_2 bending modes and CH_3 deformation, 1,450 cm^{-1}; C-N and C-C stretching vibrations, 1,132 cm^{-1}; phenylalanine's vibration, 1,035 cm^{-1})
Conclusions Raman spectroscopy is a valuable non-invasive tool for the identification of biochemical markers of oxidative damage and could represent a highly informative method of investigation to evaluate the oocyte quality.

Keywords Raman spectroscopy · Aging · Oocyte · Oxidative stress

Capsule We developed a Raman spectroscopy-based approach to detect aging-related oxidative damage in oocytes derived from young and reproductively old mice. This could represent a highly informative, non-invasive tool of investigation to evaluate the oocyte quality.

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Introduction

During folliculogenesis the oocyte undergoes a remarkable array of genetic, epigenetic and cytoplasmic changes aimed to develop full competence for fertilization and production of normal offspring [35]. Development and maintenance of oocyte competence can be hampered by intrinsic and extrinsic factors targeting the oocyte during oogenesis and following ovulation. In this regard, physiological threats come from reactive oxygen species (ROS), oxygen-derived molecules formed as intermediary products of cellular metabolism [1]. These chemical species regulate cell functions through redox-dependent signalling pathways but at high concentrations they can cause a wide range of molecular damages, including lipid peroxidation, protein and DNA damage resulting in structural and functional alterations [23, 27]. To neutralize ROS, oocytes and ovary are endowed with an elaborate defence system consisting of enzymatic and non-enzymatic antioxidants [7, 8, 18, 29]. ROS scavenging ability decreases with aging [29] and can be overwhelmed by increased ROS generation related to pathophysiological conditions [22, 24] or elevated oxygen tension during in vitro manipulation [3]. It is well known that the oocyte experiences oxidative injuries during ovary aging and post-ovulatory aging [10, 22, 30]. Ovary aging is an age-dependent process related to prolonged stay of the oocyte in a

resting phase prior to follicle activation and/or its exposure to aged ovarian microenvironment during growth and maturation [31]. Post-ovulatory aging is a time-dependent process related to progressive loss of oocyte competence both in vivo and in vitro if fertilization does not occur in time [21]. Although their different aetiology, these processes of oocyte aging share common features including fertilization failure, embryo loss and development of abnormal embryos associated with faulty spindle checkpoint, predisposing to aneuploidy and decline of mitochondrial function and energy metabolism [5, 16, 32]. Based on these observations, the evaluation of oocyte molecular damage represents a crucial aspect of the overall evaluation of oocyte quality as an effort to save fertility potential and promote positive outcome of assisted fertilization procedures. This step of oocyte selection should rely on methodologies ensuring sensitiveness, reproducibility and applicability in rapid non-invasive analysis, so far poorly investigated in the field of oocyte biology.

Raman microspectroscopy (RMS) is a powerful technique for studying the composition of cells. The obtained spectra provide a unique molecular fingerprint of the macromolecular components based on their chemical composition and spatial distribution. An important advantage of RMS is that cells can be analysed under physiological conditions and do not need to be fixed or stained. This technique has been increasingly used, at the single cell level, to study the biological changes induced in various cellular components during cell cycle progression, apoptosis and response to stress [19]. However, application of RMS to the analysis of gametes is still poor. Assessment of DNA damage, mitochondrial status and differences between normal and abnormal cells were performed in human sperm [12–14, 17, 20, 26, 28] . On the other hand, studies focused on female gamete are restricted to only three works [2, 25, 34].

The aim of the present work was to evaluate the potential of Raman spectroscopy as a tool to detect biological damage in mammalian oocytes. To this end we first verified its effectiveness in the identification of molecular modifications induced by oxidative stress in mouse oocytes and then applied it in the study of oxidative damage during the aging processes employing oocytes from an animal model represented by young and reproductively old mice conveniently treated to simulate oocyte aging.

Materials and methods

Oocyte collection

CD-1 mice were obtained from Charles River Italia s.r.l. (Calco, Italy). Animal care and experiments were carried out in accordance with the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH, 80–23). At the age of 4–8 weeks (young mice) and 48–52 weeks (old mice), females were superovulated by intraperitoneal injection of 10 IU of PMSG (Folligon; Intervet-International, Boxmeer, Holland) and 10 IU of hCG (Profasi HP 2000; Serono, Roma, Italy) 48 h apart. After 15 h, mice were sacrificed by cervical dislocation and oviducts were removed. Cumulus masses were released into the M2 medium (Sigma, St. Louis, MO) and oocytes arrested at metaphase II stage (MII) were isolated after a brief exposure to 0.3 mg/mL hyaluronidase (Sigma). Oocytes were pooled and randomized before distribution into the experimental groups; degenerated or fragmented cells were discarded.

Experimental design

Oocytes from young mice were divided into three experimental groups:

A) "young oocytes" ($n=10$), processed immediately after collection;
B) "in vitro aged oocytes" ($n=10$), cultured at 37 ° C, 5 % CO_2 in M16 medium (Sigma-Aldrich) for 10 h before processing;
C) "oxidative-damaged oocytes" ($n=10$), exposed to 10 mM hydrogen peroxide (H_2O_2) for 2 min before processing in order to induce oxidative stress [6, 33].

Oocytes from reproductively old mice were referred to as:

D) "old oocytes" ($n=10$).

RMS measurements

Measurements were performed using a Bruker Senterra confocal Raman microscope; 532 nm excitation wavelength at 25 mW of power and a 60x water-immersion objective (numerical aperture: 1.1; working distance: 1.5 mm) were used. The oocytes were immersed in a 150 μl drop of PBS on quartz windows covered with mineral oil (Sigma-Aldrich).

Imaging of a single oocyte

A preliminary set of measurements was performed by recording a chemical map of the entire oocyte defining a 100 by 100 μm grid with 2.5 μm step size (Fig. 1a); each spectrum was obtained by averaging 5 acquisitions of 5 s. This acquisition required about 11 h.

Line scans

Since the recording time for generating the oocyte chemical map was too long especially for biological samples, which may be easily deteriorated, line scans were preferred as a tool to analyse all the samples [34]. Measurements were performed

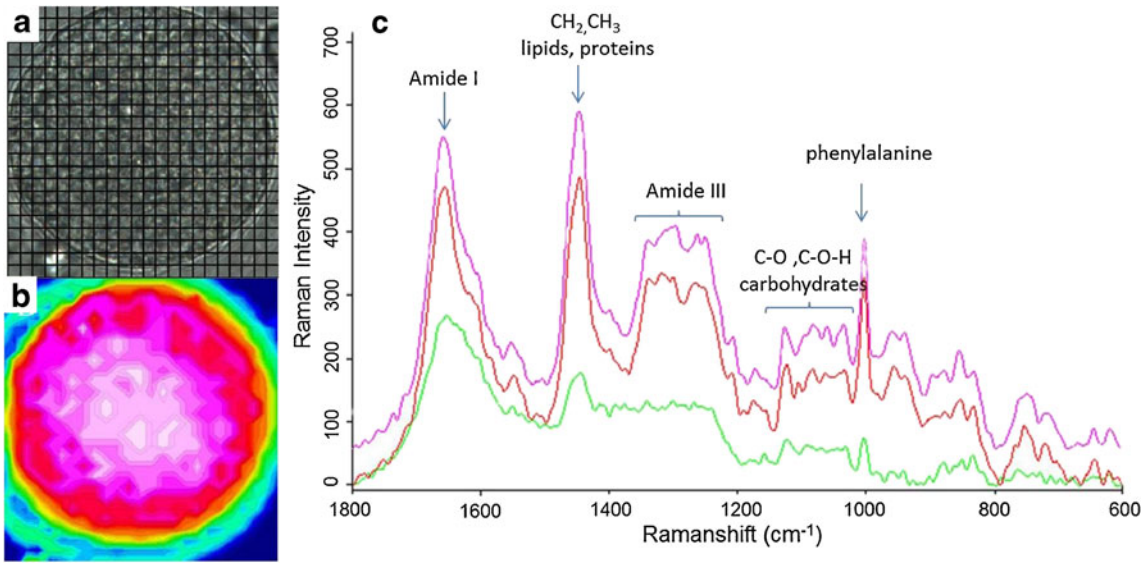

Fig. 1 Definition of measurement grid to obtain the complete mapping of an oocyte (**a**). False colour map obtained after cluster analysis (**b**). Spectra extrapolated from three different compartments of the oocytes, corresponding to zona pellucida (*green*), subcortical (*red*) and central (*pink*) zone of cytoplasm (**c**)

by recording three 60 μm-long parallel line scans across the oocyte (Fig. 2a) with 5 μm step size, totaling 32 point spectra for each oocyte (approximately 4 min. acquisition time).

RMS spectrum of each oocyte was obtained by averaging the point spectra of the three line scans. RMS spectra were cut in the 500–1,800 cm^{-1} range, vector-normalized and baseline-corrected.

Statistical analysis

Spectra obtained after recording the map of the entire oocyte were evaluated by hierarchical cluster analysis, which generates false colour maps based on spectral variation.

To investigate spectral changes between oocytes of A, B, C, D groups, principal component analysis (PCA) was applied to the line scan data in the 600–1,800 cm^{-1} spectral range. A 17-point smoothing was applied to all averaged spectra, the second derivative was calculated and PCA was applied to the second derivative spectra. Score plots was used to present the data in a form where the spectra could be represented as single points in multivariate space and loading plot is used to assign coefficient to the relevant principal components from a variable data set.

The OPUS-Quant package of the Bruker OPUS 6.5 software was used for statistical analysis.

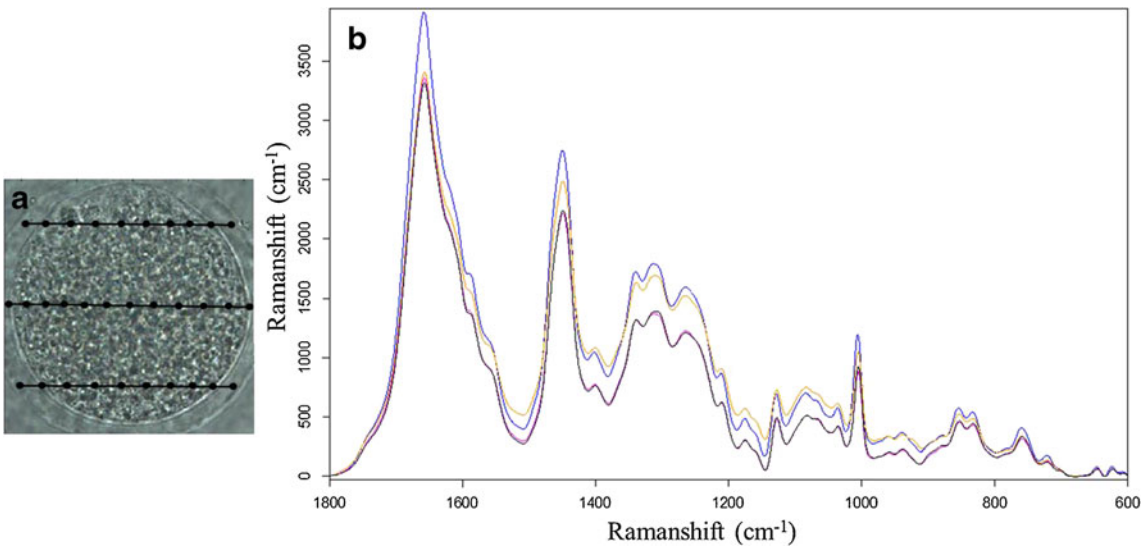

Fig. 2 Definition of measurement line scans across the oocyte (**a**). Average Raman spectra (**b**) of young (*blue*), in vitro aged (*black*), oxidative damaged (*pink*) and old (*yellow*) oocytes in the 1,800–600 cm^{-1} spectral range obtained after line scan measurements

Results

Imaging of a single oocyte

Figure 1b shows the false color chemical map that was obtained through the cluster analysis. In combination with the corresponding Raman spectra, each of the three main clusters can be, unambiguously, assigned to certain cellular compartments of the oocyte. The green cluster corresponds to the zona pellucida of the oocyte, the red cluster to the subcortical region of the oocyte and the pink cluster belongs to the central portion of the cytoplasm. Single point spectra from the zona pellucida, the subcortical and central region of the cytoplasm are showed in Fig. 1c. It is possible to identify prominent spectral peaks in characteristic spectral regions. These features arise from the molecular vibration of zona pellucida and cytoplasm components such as lipids, proteins, nucleic acids [2, 19]. The spectral regions around 1,655 cm^{-1} and in the 1,230–1,320 cm^{-1} range are from protein amide I and amide III, respectively. The amide I and amide III bands are strongly protein-conformation sensitive. The band at 1,448 cm^{-1} represents the CH_2 bending vibration and CH_3 deformation of the protein and lipids. The bands between 1,020 and 1,140 cm^{-1} result mainly from the C-O stretching and C-O-H bending modes of carbohydrates. The sharp insensitive band at 1,003 cm^{-1} and the one around 780 cm^{-1} can be assigned to ring breathing mode of phenylalanine and to nucleic acids, respectively.

All three spectra clearly differ in the spectral profile due to the chemical composition of the oocyte compartments. Spectrum of the zona pellucida shows similarities to the other regions, but with less pronounced lipids and protein bands and absence of nucleic acid band.

Line scans

Figure 2b shows the average Raman spectra of young, in vitro-aged, oxidative-damaged and old oocytes in the 1,800–600 cm^{-1} spectral range obtained after line scan measurements. Clustering of the groups in the score space diagram is observed, indicating that groups can be separated based on the spectra measurements (Fig. 3a). PCA score plots evidenced that spectra from young oocytes were clearly distinguishable and significantly different from those obtained from in vitro-aged, oxidative-damaged and old oocytes. PCA showed little variation in the measurements obtained by aged (in vitro aged, old) and oxidative damaged oocytes.

The PCA loading plot (Fig. 3b) highlights the bands (1,659 cm^{-1}, 1,587 cm^{-1}, 1,450 cm^{-1}, 1,345 cm^{-1}, 1,211 cm^{-1}, 1,132 cm^{-1}, 1,035 cm^{-1}) that are relevant in the separation, which is observed in the PC2 versus PC1 scores plot.

Compared to the fresh oocytes B, C, D groups presented significant differences in the bands attributable to lipid components; specifically, a reduction in the intensity of the peaks at 1,659 cm^{-1} (C=C stretching), 1,450 cm^{-1} (CH_2 bending), 1,345 cm^{-1} (CH_3 deformation), 1,211 cm^{-1} (OH bending, C-N stretching) was recorded.

With regard to the protein component, spectra of B, C, D groups showed modifications in the intensities of peaks 1,659 cm^{-1}, 1,450 cm^{-1}, 1,132 cm^{-1}, 1,035 cm^{-1} which refer, respectively, to amide I band (α-helix protein structure), CH_2 bending modes and CH_3 deformation of the protein, C-N and C-C stretching vibrations of proteins and phenylalanine's vibration compared to group A [19].

Discussion

This study represents the first approach to evaluate oocyte biochemical modifications caused by aging-related oxidative damage exploiting the potential of RMS as a new, non-invasive analytical tool. RMS has been applied to investigate changes in the molecular architecture of mouse oocytes at the germinal vesicle and metaphase II stages [34], for the analysis of Xenopus laevis oocytes [25] and to assess the vitrification-induced changes of the zona pellucida of ovine oocyte [2].

Our results proved that this technique is able to detect biological damage in mouse oocytes. As demonstrated by our in vitro model of oxidative stress, RMS is effective in the identification of molecular oxidative injuries and is successful in monitoring oxidative damage during postovulatory aging or ovarian aging.

PCA analysis clearly discriminates the spectra of young oocytes from those of aged-oocytes and of oocytes chemically treated to induce oxidative damage. PCA also revealed similarities between the spectra of aged oocytes and oxidative stressed oocytes indicating that the molecular changes caused by ageing have similar characteristics to chemically induced oxidative damage.

The Raman data, therefore, represent a further confirmation of the link between aging and oxidative stress, confirming the notion that the reduced biological competence of aged-oocytes and their developmental abnormalities are associated with chemical alterations induced by oxidative injuries. [10, 11, 33].

The RMS profile of aged and oxidative damaged oocytes showed major changes in the peak attributable to lipid molecules compared to young oocytes. In particular, a decrease in the intensity of the peaks at 1,659 and 1,450 cm^{-1}, which have been attributed to the C=C stretching vibration and the methylene deformation associated with endogenous lipids, respectively, highlights a perturbation of the lipids components. Finding changes in lipid content is consistent with

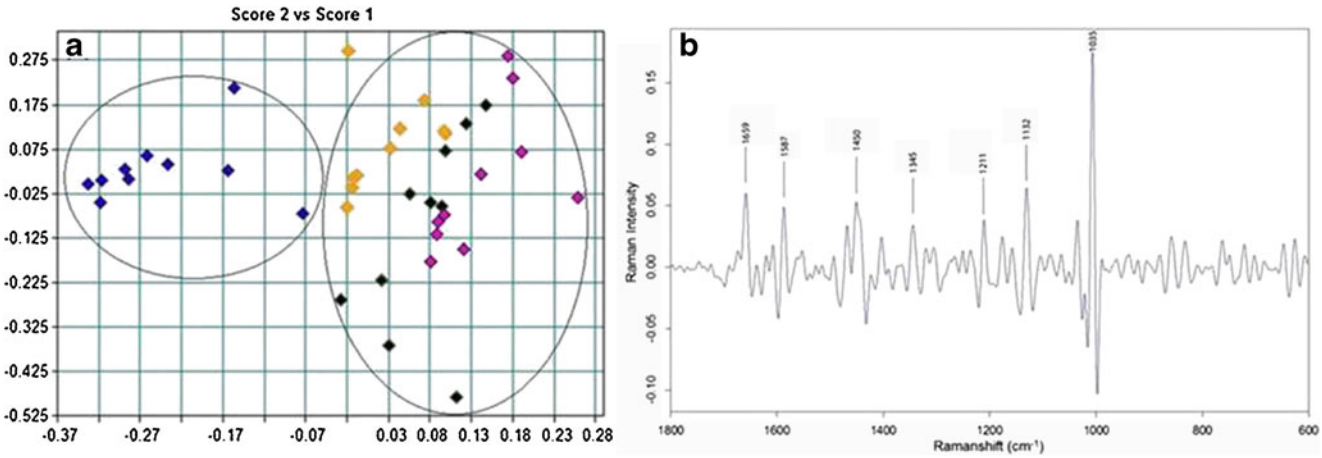

Fig. 3 PCA score plot (PC2 vs PC1) applied to the second derivative spectra of young (*blue dots*), in vitro aged (*black dots*), oxidative damaged (*pink dots*) and old (*orange dots*) oocytes in the 1,800–600 cm^{-1} spectral range; each dot represents the average spectrum of each oocyte (**a**). PCA loading plot (PC2) applied to the four experimental classes in the 1,800–600 cm^{-1} spectral range (**b**)

lipid peroxidation, a well-known marker of oxidative damage [9]. Unsaturated fatty acids of oocyte membranes are highly susceptible to oxidative attack. Hydroxyl radicals initiate a free-radical chain reaction mechanism and remove an hydrogen atom from one of the carbon atoms in polyunsaturated fatty acids and lipoproteins, causing lipid peroxidation [4, 15]. The damage in membrane phospholipids decreases membrane fluidity, causing severe structural changes, and reduces the activity of membrane-bound enzymes, thereby affecting cellular signalling pathways [15].

The Raman spectra of aged and oxidative damaged oocytes also exhibited significant modifications with regard to the protein component compared to young oocytes as suggested by the decrease of α-helix structures. Enhancement in ROS, in aged oocytes, is associated with a modification in proteins crucial to maintain viability and integrity of various organelles and cytoskeleton, and thus may affect their activity, organization and distribution [10]. Oxidative damage of the proteins is characterized by structural modification of side chains by ROS, including oxidation of sulfhydryl groups, oxidative adducts on amino acid residues near metal-binding sites, cross-linking, unfolding, and protein fragmentation. Direct ROS attack on the amino acid side chains of proline, arginine, lysine, and threonine results in protein carbonyl formation, which eventually changes the structure of a protein and results in alterations of protein function [15].

In conclusion, our findings demonstrate that RMS may provide a highly informative means for the assessment of the biological changes, which occur in the oocyte under oxidative stress.

However, our study also suggests that the current procedures of RMS can be further optimized.

In order to improve the usefulness of RMS for selecting good quality oocytes in assisted reproductive technology further work will be needed to assess the sensitivity of RMS for monitoring, and so quantifying, slight oxidative injury changes in oocytes (e.g. applying different doses of oxidants or after oocyte exposition to in vitro culture for different duration time).

Moreover, as underlined in methods, RMS imaging of the oocyte requires long acquisition times because of the diameter of the cell and the non-directional light scattering pattern limiting the application for living systems. This limitation could be overcome by line scans or analysis of selected areas of the oocytes which may avoid cell deterioration. In addition, a detailed interpretation and analysis of the spectra which demand specialist knowledge and the use mathematical procedures, could allow setting spectral features that could be used as biomarkers of oocyte status.

Finally, to make the RMS applicable in the human reproductive field, it is imperative to implement the safety of this procedure by using controlled conditions during oocyte analysis (e.g. temperature, sterile/single-use supports, appropriate holding medium) and also what type of laser and time course are safer for scanning live oocyte should be confirmed.

Once optimized, Raman microspectroscopy could represent a useful diagnostic tool for proper assessment of the oocyte and for the selection of good quality female gametes, leaving them unaltered and thus suitable for use in assisted reproductive techniques.

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