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Evaluation of the Effect of Microoperation on Porcine Parthenogenetic Embryo Competence by Raman Spectroscopy

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JLS, LB, XGY and YQL conceived and designed the experiments. JLS performed the experiments and analyzed the data. SSL and KHL contributed reagents/materials/ analysis tools. JLS, SSL and YQL wrote the paper.

Key words
Raman spectroscopy, Porcine parthenogenetic embryos, Microoperation, Mechanical damage, Embryo culture media.

ABSTRACT

Intracytoplasmic sperm injection (ICSI), as the savior of male infertility, has been applied to artificial assisted reproduction (IVF). The oocytes are inevitably ruptured by the microinjector in the process of microoperatin. To study the effects of mechanical damage from microopetations on embryo development, we analyzed 60 the spent culture media of porcine parthenogenetic embryos experienced different types of microopetation using Raman spectroscopy. The results showed that Group II with the puncture of egg zona and plasma membrane had the highest cleavage rate and blastocyst rate of porcine parthenogenetic embryos, which was significantly higher than Group I with intact oocytes ($P<0.05$) and Group III with the puncture of zona and plasma membrane and aspiration egg cytoplasm ($P<0.05$) on the cleavage rate, and was extremely significantly higher than Group III on the blastocyst rate ($P<0.001$). Raman spectroscopy of the spent culture media of these parthenogenetic embryos revealed that there were obvious reduced in the intensity of Raman characteristic peaks at 810 cm^{-1}, 1371 cm^{-1} and 1563 cm^{-1} in the microoperation groups (Group II and Group III). These changes of Raman characteristic peaks suggested that ICSI had influenced the metabolism of embryonic membrane lipids, membrane proteins and nucleic acids. The PCA analysis also exhibited that these three groups were well distributed in different areas. In conclusion, the microoperation of ICSI caused some changes in the metabolism of embryos. Raman spectroscopy is a valuable technology for assessing embryonic metabolism.

INTRODUCTION

Since the first successful implementation of intracytoplasmic sperm injection (ICSI) in 1992 (Palermo *et al*., 1992), ICSI has been applied in artificial assisted reproduction as the savior of male infertility and remedial measure of fertilization failure in conventional *in vitro* fertilization (IVF) (Steptoe and Edwards, 1978), which has a tendency to increase. During the microsurgery of ICSI, the microinjector penetrates the zona and plasma membrane, and injects a single sperm into the egg. However, this would disorder the organization of cellular internal structures. This kind of mechanical damage can disturb the normal life of oocytes, and even cause the death of oocytes. In fact, ICSI is the fertilization of compulsive behavior, and disobeys the biological laws of natural insemination. This is more invasive than IVF, and gives rise to the mechanical damage of oocytes. To date, little attention has been given to the mechanical damage caused by the microinjection system on embryonic development. Liu *et al*. (2015) developed a computational model based on dissipative particle dynamics to simulate the cellular structure of a cell, and quantitatively analyze the damage that a cell experiences during microinjection. No desired method can provide a full and fair conclusion with respect to the effects of mechanical damage on embryonic development.

Some recent studies have suggested that embryo quality and viability is correlated with the changes in chemical composition of the culture medium (Vergouw *et al*., 2011, 2012; Muñoz *et al*., 2013; Munoz and Uyar, 2014; Nadal-Desbarats *et al*., 2013; Li *et al*., 2015; Nagy *et al*.,

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2008). The culture medium is the direct microenvironment of an *in vitro* cultured embryo (Zhao *et al*., 2013; Shen *et al*., 2012). The embryonic development requires the uptake of certain substances from the surrounding culture medium and discharge of metabolites in culture media. Metabolomics, unlike genomics and proteomics, provides important information on the expected function, and offers an immediate snapshot of all present biological functions, reflecting up-to-the-minute events that have occurred. Therefore, the investigators conceived to assess the effects of mechanical damage on embryonic development based on the metabolic fingerprinting analysis of embryo culture media using Raman spectroscopy.

Several technologies have been used to evaluate culture media, such as near-infrared spectroscopy (NIR) (Seli *et al*., 2007), Fourier transform infrared spectroscopy (FT-IR) (Muñoz and Uyar, 2014), Raman spectroscopy (Zhao *et al*., 2013) and nuclear magnetic resonance (NMR) (Nadal-Desbarats *et al*., 2013). At present, Raman spectroscopy has been considered as a very promising tool to detect the structure information of biomolecules in aqueous solution and embryo culture media due to its high sensitivity to detect tiny biochemical and molecular variations in tissues or biological samples, and the weak Raman signal of water molecules (Wu *et al*., 2011).

In the present study, the microsurgery of ICSI was simulated, and three groups of porcine parthenogenetic embryos with different mechanical damage levels were constructed. Then, Raman spectroscopy was employed to analyze the metabolomic profiling of these embryo culture media, in order to explore the effect of mechanical damage on embryo development.

MATERIALS AND METHODS

Animal ethics

All animal procedures and protocols used in the present study were carried out in accordance with the Guide for Care and Use of Laboratory Animals (8th edition, released by the National Research Council, USA), and were approved by the Institutional Animal Care and Use Committee (IACUC) of Guangxi University.

Source of materials and experimental design

Porcine cumulus oocyte complexes (COCs) were obtained from slaughterhouse ovaries, and selected COCs with at least three layers of cumulus cells for *in vitro* maturation. According to the design scheme, three groups of embryos with different types of microsurgeries were prepared. Group I was assigned as the control group, which comprised of intact oocyte parthenogenetic embryos. Groups II and III were assigned as two different microsurgery groups, respectively. The oocytes in Group II were developed parthenogenetic embryos, which were punctured into the zona pellucida and plasma membrane. In Group III, the oocytes were punctured to the zona pellucida and plasma membrane, and the cytoplasm was aspirated 2-3 times.

Production of porcine parthenogenetic embryos with different mechanical damages

After 42-44 h of *in vitro* maturation culture of porcine COCs, cumulus cells were removed using hyaluronidase. According to the presence of the first polar body, the oocytes in the metaphase II arrested stage were picked out and randomly divided into three groups. The metaphase II oocytes in the two groups were used for the microsurgeries in a droplet of TL-Hepes-PVA medium containing 10% fetal bovine serum and 7.5 μg/ml CB using the traditional ICSI method. All oocytes, including intact and microsurgery oocytes, were recovered at 30 min in PZM-3. Then, these oocytes were activated by electro-activation with the parameters of 1.2 kV/cm and 30 μs for two times to form the embryos. Each 15 embryos were transferred into 30-μL droplets of PZM-3 balanced over four hours, and placed in an incubator at 37°C with 5% CO_2 in air and maximum humidity.

Spent culture media and embryo development for analysis

After 24 and 144 h of embryo culture, the number of divided cells and blastocysts were counted, respectively. At the fifth day of *in vitro* culture, the early blastocysts and blastocysts were individually identified and cultured in 20-μL droplets of PZM-3 for four hours. The spent culture media (15 μL per droplet) from the three groups and blank droplets incubated without embryos (three blank mediums per batch of embryos simultaneously cultured) were collected after four hours of single embryo culture, and stored at -80°C until the Raman spectroscopy analysis. Four biological replicates were implemented. Each replicate comprised of five spent culture media of blastocysts and three blank culture media. A total of 72 culture media (60 spent culture media, and 12 blank culture media) were collected.

Raman spectroscopy

The details of the laser Raman spectroscopy was according to a previous publication (Huang *et al*., 2010). The Raman system used in the present study was the Spectra Pro 2300i (Acton, USA) equipped with an inverted microscope (Nikon TE 2000u) and CCD detector (Spec-10, Princeton Instruments). The excitation laser was 785

nm, which was focused on a spot with a power of 15 mW. The spectral resolution was approximately 6 cm^{-1}, and optical grating was 600 line/mm.

Spectra acquisition

Before acquiring the Raman spectrum of the spent culture media, three spent culture media were randomly selected to confirm the integration time measured by Raman. The integration time was initially set at 120 sec to acquire the Raman spectra of the spent culture media. When the spectrum below the 500-cm^{-1} Raman shift got saturated, the integration time was downregulated or upregulated, accordingly. After 5-6 adjustments, the ideal integration time were determined to be 100 sec.

The spent culture media and blank sample (15 µL) were thawed at room temperature (25°C) for 30 min, and shifted in Raman-compatible 6-mm diameter quartz sample cells for spectral measurement. A total of 72 sample media (60 spent culture media, 20/group; 12 blank media) was randomly analyzed by Raman spectroscopy using a spectral integration time of 100 sec per sample. The spectra were recorded from 300 to 2200 cm^{-1} at room temperature (25 ± 1°C).

Spectra analysis

The spectra were acquired using the WinSpec32 software, and converted to ASCII data. These experimental data were first subtracted by the average of the blank data using Origin 8.0 software (OriginLab). Then, baseline correction and removal of the fluorescence background were performed through minimax maximum adaptive scaling using MatLab2018 software (trial version) (Gao *et al*., 2013). The spectra and loading plot were analyzed by principal component analysis (PCA) with MATLAB 2018. Vector normalization was performed for the analysis conducted through PCA, and the PCs were obtained from the correlation matrix.

RESULTS

Development of porcine parthenogenetic embryos using different types of microsurgery

In the present study, the different types of microinjection during ICSI were employed to induce mechanical damage to the oocytes, and these oocytes were underwent the activation of parthenogenesis using the same parameters. After *in vitro* culture, the results revealed that group II (the punctured egg plasma membrane) had the highest cleavage rate and blastocyst rate of porcine parthenogenetic embryos. Furthermore, the cleavage rate was significantly higher than that in group I (intact oocyte; 98.34% *vs.* 93.43%, $P<0.05$) and group III (punctured egg plasma membrane and aspiration cytoplasm; 98.34% *vs.* 92.17%, $P<0.05$), while the blastocyst rate was extremely significantly higher than that in group III (65.8% *vs.* 52.20%, $P<0.001$) (Table I; Fig. 1).

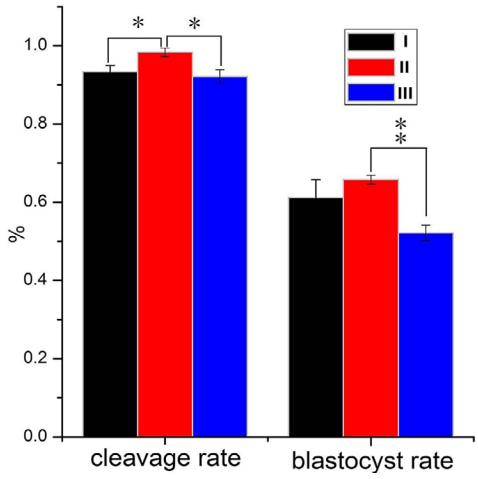

Fig. 1. Development of porcine parthenogenetic embryos with the different types of microoperation. I, intact oocytes pathenogenesis; II, the puncture of zona and plasma membrane of oocytes parthenogenesis; III, the puncture of zona and plasma membrane and aspiration cytoplasm of oocytes parthenogenesis.

Table I.- The effect of coming from mechanical trauma to the cleavage rate and blastocyst rate, and the blastocyst cell number of parthenogenesis.

Type of experiments	No. of manipulated oocytes	No. of cleavages (% of manipulated oocytes)	No. of blastocysts (% of manipulated oocytes)	Cell number of blastocyst
Intact oocytes parthenogenesis (Group I)	169	158 (93.43±3.00) [a]	103 (61.22±9.03)	44.17±11.03
Mechanical trauma oocyte parthenogenesis (Group II)	181	178 (98.34±2.20) [b]	119 (65.8±2.31) [a]	40.75±11.44
Mechanical trauma oocyte parthenogenesis(Group III)	174	161 (92.17±3.43) [a]	90 (52.20±3.91) [b]	41.00±11.21

The significant differences represent with different superscripts in the same column ($P<0.05$).

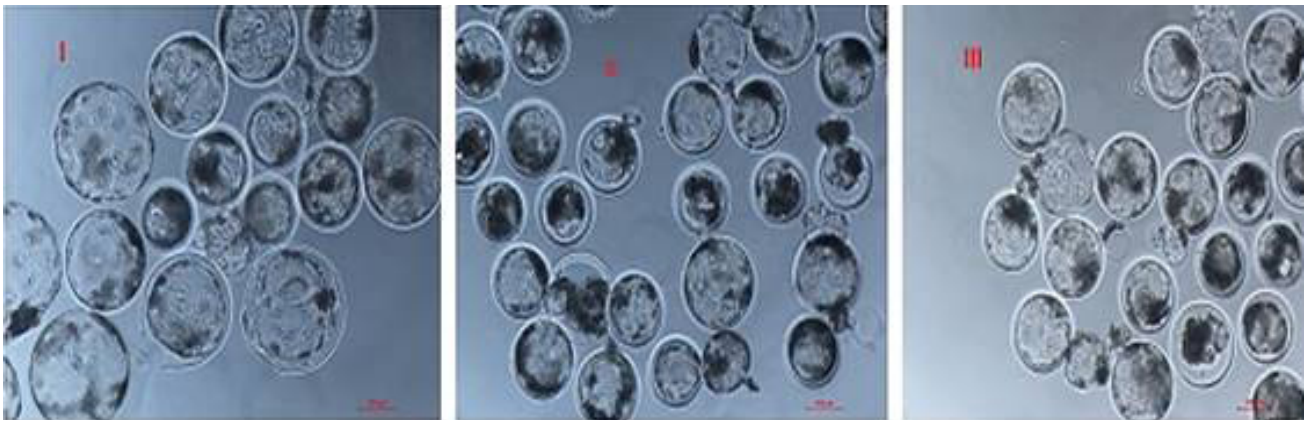

Fig. 2. The shape of parthenogenetic blastocysts generated from intact oocytes and different microoperated oocytes

Table II.- Raman frequency from spent culture media of porcine parthenogenic embryos and tentative assignments.

Raman frequency (cm^{-1})	Tentative assignments
810	O-P-O symmetrical stretching vibration of phosphodiester linkage (lipids)
1003	The symmetrical breathing vibration of phenylalanine (protein)
1371	C, A, T, Trp protein, lipids
1563	COO- antisymmetric strenching, Guanine, adenine, Trp protein
1616	C=C stretching mode of tyrosine, phenylalanine and tryptophan
1058	C-C skeleton stretching vibration (lipid)
1411	COO- symmetrical stretch of glycine (protein)
1437	CH2 deformation (lipid)
980-1112	Breathing vibration of Phenylalanine substituting benzene ring (protein)
1458	CH2 bending mode of DNA, CH2 deformation (thymine), C=C bending mode of adenine and guanine
1586	C=C, C=N stretching vibration of Guanine, adenine ring

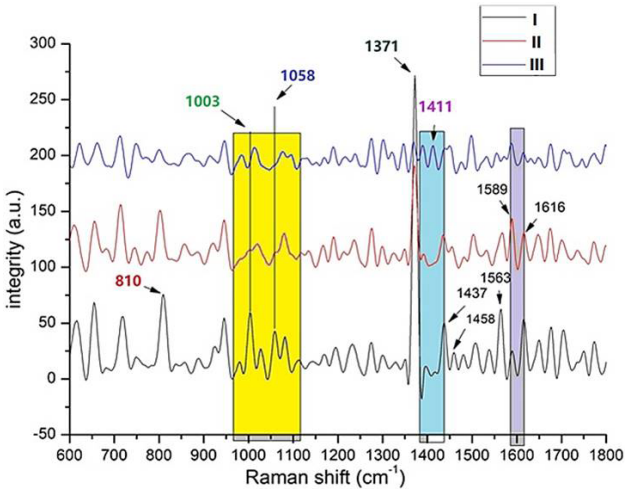

Fig. 3. Raman spectra of the spent culture media from porcine parthenogenetic blastocysts by difference types of microoperation

The total number of cells in blastocysts was counted by staining with Hochest 33342. Ten blastocysts were selected from each group. The average number cells for each parthenogenic blastocyst in the three groups were 44.17, 40.75 and 41.00, respectively. No significant difference was spotted among these three groups.

The embryonic morphological analysis revealed that the parthenogenetic blastocysts in group I was larger and more transparent, when compared to the other groups (groups II and III) that underwent microsurgery, and had a more uniform cell distribution (Fig. 2). However, there was no significant difference between group II and group III. These blastocysts were smaller in diameter, darker in color and more easily hatched at the break of the zona pellucida and plasma membrane.

Raman spectroscopy analysis

Microsurgery has had an impact on embryonic development. Thus, Raman spectroscopy has been used to analyze the composition changes of culture medium, which directly reflects the metabolic differences of embryos from different types of microsurgeries. A total

of 60 Raman spectra of spent culture medium were acquired. From the average spectra (Fig. 3) and pairwise subtraction spectra (Fig. 4) of the different microsurgery groups, there was an obvious intensity reduction in Raman characteristic peaks at 810 cm^{-1}, 1371 cm^{-1} and 1563 cm^{-1} in the microsurgery groups (groups II and III), especially at 1371 cm^{-1}. Moreover, the weakening extent of Raman intensity was correlated to the degree of mechanical damage. The characteristic peaks intensities in group III were significantly weaker than that in group II. In the Raman spectra of the microsurgery groups, there was the disappearance of characteristic peaks at 1,058 cm^{-1} and 1,003 cm^{-1}, and the decline was at a ratio of 1,437/1,458, while the increase was at a ratio of 1,586/1,616. A novel characteristic peak at 1,411 cm^{-1} was presented in Raman spectra of group III. The Raman spectra sufficiently revealed the fact that the microsurgery seriously disturbed the metabolism of porcine parthenogenic embryos. According to the checked published information between Raman frequency and tentative assignments, the tentative assignments of changing characteristic peaks are presented in Table II (Carey, 1982; Karvaly and Loshchilova, 1977; Xu *et al*., 1998). The metabolic differences of embryos with different types of microsurgeries mainly occurred during the construction of membrane lipids, membrane proteins and nucleic acid. The symmetrical breathing vibration of phenylalanine at 1,003 cm^{-1}, COO- antisymmetric strenching, Guanine, adenine, Trp protein at 1,563 cm^{-1}, C=C stretching mode of tyrosine, phenylalanine and tryptophan at 1,616cm^{-1}, Breathing vibration of Phenylalanine substituting benzene ring at 980-1,112 cm^{-1}, and COO- symmetrical stretch of glycine at 1,411 cm^{-1}, are all part of the member protein. Membrane lipids were altered at the phosphodiester linkage, CH_2 deformation, and C-C skeleton stretching vibration at 1,058 cm^{-1}. Nucleic acid mainly occurred at 1,458 and 1,586 cm^{-1}, which attributed to the CH_2 bending mode, CH_2 deformation, C=C bending mode of adenine and guanine and C=C, C=N stretching vibration of guanine, and the adenine ring, respectively.

Principal component analysis (PCA)

In order to visualize the optimal distinction of the three experimental groups, PCA was employed to generate effective diagnostic algorithms. The PCA scatter plots were drafted based on the first, second and third principal components (PCs). This exhibited that the three groups were well-distributed in different areas (Fig. 5). The distribution area of group I was much wider than that of groups II and III. The distribution areas of two microsurgery groups (groups II and III) tended to be consistent, which was caused by the damage of microscopic operation on the embryos. However, it was manifested that the greater the mechanical damage, the more concentrated the distribution was.

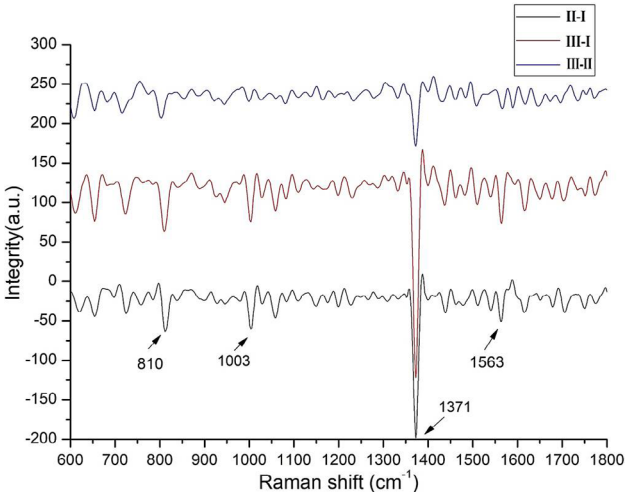

Fig. 4. Pairwise subtraction Raman spectra of spent culture media with difference types of microoperation.

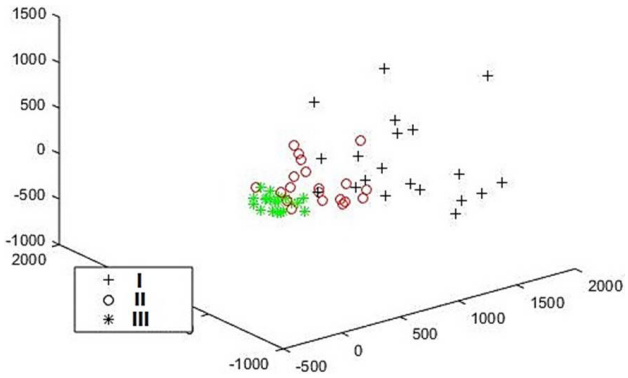

Fig. 5. PCA 3D scatter plots of porcine parthenogenetic blastocysts with difference types of microoperarion.

DISCUSSION

Mechanical damage to cells caused by microinjection

With the rapid development of biology, microinjection has been widely performed to introduce foreign materials into living cells using a glass microinjector (Tesson *et al*., 2011; Wefers *et al*., 2013), such as for gene delivery (Mellott *et al*., 2013; Perry *et al*., 1999; Wong *et al*., 2014), drug development (Zema *et al*., 2012), and *in vitro* fertilization (Bongso *et al*., 1989; Garcia-Rosello *et al*., 2009). During the microinjection, the soft cell is inevitably ruptured by the microinjector. Some literatures have reported that this not only punctures the pronucleus, but also damages the cytoplasm. Furthermore, this markedly

reduced the viability of rabbit embryos on transgenesis using microinjection. There have also been some contradictory reports that the damage to the cytoplasm of zygotes did not affect their preimplantation development in murine zygotes after microinjection (Andreeva and Serova, 1992; Popova *et al*., 2002). ICSI, as one of the applications of microinjection, is an important assisted reproductive technique for serving male infertility in humans, livestock and endangered species. Some research has shown that ICSI embryos are different from the spontaneous embryos on gene expression, epigenetics and viability (Pan *et al*., 2014). However, ICSI did not increase the risks on adverse obstetric outcomes, birth defects and long-term sequelae, when compared with IVF (Lie *et al*., 2005; Wen *et al*., 2012). Furthermore, several reports of serious flaws in the birth of ICSI have raised concerns about the use of ICSI (Peltoniemi *et al*., 2011; Schuffner *et al*., 2006; Sweet *et al*., 2014; Xiao *et al*., 2009). In the present experiment, it was found that the mechanical rupture of membranes was beneficial to the activation of porcine oocytes and the development of parthenogenetic embryos, while the aspiration of the cytoplasm of oocytes after sperm injection is a significant disadvantage to the development of parthenogenetic embryos. Furthermore, the metabolomics analysis of different types of microsurgery groups was inconsistent with above conclusions. The changes of the Raman shift suggest that mechanical damage does generate an adverse effect on the metabolism of embryonic membrane lipids and membrane proteins, which is consistent with the fact that microsurgery really broke the cytomembrane and transparent zone. However, minor mechanical damages of microsurgery have no significant effect on embryo development. Therefore, it is worth rethinking that some scholars even advocate to aspirate the cytoplasm of oocytes with the injection needle after sperm injection for promoting the activation of oocyte reprogramming (Ma and Ho-Yuen, 2000; Gianpiero *et al*., 1995).

Egg cell membranes, which are also called egg plasma membrane, are biomembranes that are similar to any other cell membranes, which have the functions of selective material transportation, information transmission, enzymatic reaction and cell recognition. In addition, a layer of egg membranes (oolemma) are also coated around the plasma membrane. In mammals, the oolemma is known as the zone pellucida, while in other animals, it is called the yolk membrane. The oolemma, and not the biomembrane, is made of glycoproteins. Its role is to protect the egg from foreign sperm invasion. In addition, it should not be involved in embryo metabolism. Therefore, the puncture of the egg plasma membrane and cytoplasm should be the principal cause of embryo metabolic changes in the present experiment. At present, it could not be determined how long these metabolic changes would last, or whether these would affect the metabolism of the fetus and even the individual. Some retrospective studies on ICSI-derived individuals had found that these were susceptible to obesity in early youth and some metabolic diseases in adulthood (Belva *et al*., 2012a, b, c). Furthermore, it was reported once that the puncture of the pronucleus of murine zygotes in the process of microinjection adversely affected embryo implantation, and increased the frequency of birth of inviable progeny and various defects, such as the hypoplasia, hyperplasia and dysplasia of tissues and atresia in some organs (Popova *et al*., 2002). Hence, it was considered that the metabolic diseases of ICSI-derived individuals are likely to be caused by mechanical damage through ICSI.

Application of Raman spectroscopy in biological research

Metabolomics is the study of the qualitative and quantitative analysis of complete small molecular weight metabolites of an organism or cell during a specific physiological period. Recently, various reports have employed Raman spectral techniques to evaluate the metabolomic profiling of culture media (Scott *et al*., 2008; Huang *et al*., 2013; Santos *et al*., 2015; Seli *et al*., 2007). Raman spectroscopy, similar to infrared spectroscopy, was used to analyze the structure and composition of materials, according to the vibration of the molecular chemical groups and bonds. Each sample presents its own unique Raman spectrum (Wu *et al*., 2011). Raman spectra, as a non-invasive and non-destructive technique, have been applied for disease prediction, cell identification, drug poisoning, embryonic development potential evaluation and individual identification (Dong *et al*., 2015; Fabian *et al*., 2016; Zhu *et al*., 2018). It has potential for commercial application, and has a series of advantages such as: the high sensitivity to detect tiny biochemical and molecular variations in tissues or biological samples; the equipment is not expensive and easy to handle; the results can be immediately obtained (Santos *et al*., 2015; Scott *et al*., 2008); it uses a small sample volume (only a small amount in μL) and there is no need for pretreatment or sample preparation; the weak Raman signal in water molecules. Therefore, Raman spectroscopy can be considered as a promising technique for the evaluation of embryonic metabolomic profiling based on the analysis of the spent culture medium.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Andreeva, L.E. and Serova, I.A., 1992. The effect of the micromanipulations used for transgenosis on mouse development. *Ontogenez,* **23**: 637-643.

Belva, F., Painter, R., Bonduelle, M., Roelants, M., Devroey, P. and De Schepper, J., 2012. Are ICSI adolescents at risk for increased adiposity? *Hum. Reprod.,* **27**: 257-264. https://doi.org/10.1093/humrep/der375

Belva, F., Roelants, M., Painter, R., Bonduelle, M., Devroey, P. and De Schepper, J., 2012. Pubertal development in ICSI children. *Hum. Reprod.,* **27**: 1156-1161. https://doi.org/10.1093/humrep/des001

Belva, F., Roelants, M., De Schepper, J., Roseboom, T.J., Bonduelle, M., Devroey, P. and Painter, R.C., 2012. Blood pressure in ICSI-conceived adolescents. *Hum. Reprod.,* **27**: 3100-3108. https://doi.org/10.1093/humrep/des259

Bongso, T.A., Sathananthan, A.H., Wong, P.C., Ratnam, S.S., Ng, S.C., Anandakumar, C. and Ganatra, S., 1989. Human fertilization by micro-injection of immotile spermatozoa. *Hum. Reprod.,* **4**: 175-179. https://doi.org/10.1093/oxfordjournals.humrep.a136867

Carey, P., 1982. *Biochemical applications of Raman and resonance Raman spectroscopes*. Academic Press.

Dong, R., Weng, S., Yang, L. and Liu, J., 2015. Detection and direct readout of drugs in human urine using dynamic surface-enhanced Raman spectroscopy and support vector machines. *Anal. Chem.,* **87**: 2937-2944. https://doi.org/10.1021/acs.analchem.5b00137

Fabian, D., Kacmarova, M., Kubandova, J., Cikos, S. and Koppel, J., 2016. Raman spectroscopy analysis of differences in composition of spent culture media of *in vitro* cultured preimplantation embryos isolated from normal and fat mice dams. *Reprod. Biol.,* **16**: 120-129. https://doi.org/10.1016/j.repbio.2016.02.002

Gao, G.M., Li, X., Qin, Z.D., Wei, K.L., Huang, H.M., Liu, J.X. and Yao, H.L., 2013. New method for elimination background fluorescence of Raman spectrum and its application. *Acta Optica Sin.,* **33**: http://en.cnki.com.cn/Article_en/CJFDTotal-GXXB201302042.htm

Garcia-Rosello, E., Garcia-Mengual, E., Coy, P., Alfonso, J. and Silvestre, M.A., 2009. Intracytoplasmic sperm injection in livestock species: An update. *Reprod. Domest. Anim.,* **44**: 143-151. https://doi.org/10.1111/j.1439-0531.2007.01018.x

Gianpiero, D., Palermo, M.D., Cohen, J., Alikani, M. and Adler, A., 1995. Intracytoplasmic sperm injection: A novel treatment for all forms of male factor infertility. *Fertil. Steril.,* **63**:1231-1240. https://doi.org/10.1016/S0015-0282(16)57603-1

Huang, W.E., Li, M., Jarvis, R.M., Goodacre, R. and Banwart, S.A., 2010. Shining light on the microbial world the application of Raman microspectroscopy. *Adv. appl. Microbiol.,* **70**: 153-186. https://doi.org/10.1016/S0065-2164(10)70005-8

Huang, Z., Sun, Y., Wang, J., Du, S., Li, Y., Lin, J., Feng, S., Lei, J., Lin, H., Chen, R. and Zeng, H., 2013. Rapid and nondestructive method for evaluation of embryo culture media using drop coating deposition Raman spectroscopy. *J. Biomed. Opt.,* **18**: 127003. https://doi.org/10.1117/1.JBO.18.12.127003

Karvaly, B. and Loshchilova, E., 1977. Comments on the quantitative interpretation of biomembrane structure by Raman spectroscopy. *Biochim. Biophys. Acta,* **470**: 492-496. https://doi.org/10.1016/0005-2736(77)90140-7

Li, X., Xu, Y., Fu, J., Zhang, W.B., Liu, S.Y. and Sun, X.X., 2015. Non-invasive metabolomic profiling of embryo culture media and morphology grading to predict implantation outcome in frozen-thawed embryo transfer cycles. *J. Assist. Reprod. Genet.,* **32**: 1597-1605. https://doi.org/10.1007/s10815-015-0578-z

Lie, R.T., Lyngstadaas, A., Orstavik, K.H., Bakketeig, L.S., Jacobsen, G. and Tanbo, T., 2005. Birth defects in children conceived by ICSI compared with children conceived by other IVF-methods; a meta-analysis. *Int. J. Epidemiol.,* **34**: 696-701. https://doi.org/10.1093/ije/dyh363

Liu, F., Wu, D., Wu, X. and Chen, K., 2015. Analyses of the cell mechanical damage during microinjection. *Soft Matter,* **11**: 1434-1442. https://doi.org/10.1039/C4SM02773F

Mellott, A.J., Forrest, M.L. and Detamore, M.S., 2013. Physical non-viral gene delivery methods for tissue engineering. *Annls. Biomed. Eng.,* **41**: 446-468. https://doi.org/10.1007/s10439-012-0678-1

Ma, S. and Ho-Yuen, B., 2000. Assessment of maximal fertilization rates with intracytoplasmic sperm injection. *J. Assist. Reprod. Genet.,* **17**: 80-86. https://doi.org/10.1023/A:1009410331774

Muñoz, M., Uyar, A., Correia, E., Díez, C., Fernandez-Gonzalez, A., Caamaño, J.N., Trigal, B., Carrocera, S., Seli, E. and Gomez, E., 2013. Non-invasive assessment of embryonic sex in cattle by metabolic fingerprinting of *in vitro* culture medium. *Metabolomics,* **10**: 443-451.

Muñoz, M. and Uyar, A., 2014. Metabolomic prediction of pregnancy viability in superovulated cattle embryos and recipients with fourier transform infrared spectroscopy. *Biomed. Res. Int*., **2014**: 608579. https://doi.org/10.1155/2014/608579

Nadal-Desbarats, L., Veau, S., Blasco, H., Emond, P., Royere, D., Andres, C.R. and Guerif, F., 2013. Is NMR metabolic profiling of spent embryo culture media useful to assist *in vitro* human embryo selection? *Magma,* **26**: 193-202. https://doi.org/10.1007/s10334-012-0331-x

Nagy, Z.P., Sakkas, D. and Behr, B., 2008. Symposium: Innovative techniques in human embryo viability assessment. Non-invasive assessment of embryo viability by metabolomic profiling of culture media (metabolomics). *Reprod. Biomed. Online*, **17**: 502-507. https://doi.org/10.1016/S1472-6483(10)60236-2

Palermo, G., Joris, H., Devroey, P. and Van-Steirteghem, A.C., 1992. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *The Lancet,* **340**: 17-18. https://doi.org/10.1016/0140-6736(92)92425-F

Pan, X., Kong, D., Liu, L., Gao, F., Zhang, X., Tang, B. and Li, Z., 2014. Development block of golden hamster ICSI embryos is associated with decreased expression of HDAC1, HSPA1A and MYC. *Cell Biol. Int.,* **38**: 1280-1290. https://doi.org/10.1002/cbin.10319

Peltoniemi, O.M., Kari, M.A. and Hallman, M., 2011. Repeated antenatal corticosteroid treatment: A systematic review and meta-analysis. *Acta Obstet. Gynecol. Scand.,* **90**: 719-727. https://doi.org/10.1111/j.1600-0412.2011.01132.x

Perry, A.C., Wakayama, T., Kishikawa, H., Kasai, T., Okabe, M., Toyoda, Y. and Yanagimachi, R., 1999. Mammalian transgenesis by intracytoplasmic sperm injection. *Science,* **284**: 1180-1183. https://doi.org/10.1126/science.284.5417.1180

Popova, E.A., Krivokharchenko, A.S. and Vil'ianovich, L.I., 2002. *In vitro* development of murine embryos using different types of microinjections. *Ontogenez,* **33**: 107-110.

Santos, E.C., Martinho, H.S., Annes, K., Leite, R.F. and Milazzotto, M.P., 2015. Rapid and noninvasive technique to assess the metabolomics profile of bovine embryos produced in vitro by Raman spectroscopy. *Biomed. Opt. Express,* **6**: 2830-2839. https://doi.org/10.1364/BOE.6.002830

Schuffner, A., Centa, L., Reggiani, C. and Costa, S., 2006. Acral and renal malformations following ICSI. *Arch. Androl.,* **52**: 145-148. https://doi.org/10.1080/01485010500379863

Scott, R., Seli, E., Miller, K., Sakkas, D., Scott, K. and Burns, D.H., 2008. Noninvasive metabolomic profiling of human embryo culture media using Raman spectroscopy predicts embryonic reproductive potential: A prospective blinded pilot study. *Fertil. Steril.,* **90**: 77-83. https://doi.org/10.1016/j.fertnstert.2007.11.058

Seli, E., Sakkas, D., Scott, R., Kwok, S.C., Rosendahl, S.M. and Burns, D.H., 2007. Noninvasive metabolomic profiling of embryo culture media using Raman and near-infrared spectroscopy correlates with reproductive potential of embryos in women undergoing in vitro fertilization. *Fertil. Steril.,* **88**: 1350-1357. https://doi.org/10.1016/j.fertnstert.2007.07.1390

Shen, A.G., Peng, J., Zhao, Q.H., Su, L., Wang, X.H., Hu, J.M. and Yang, Q., 2012. Accurate and noninvasive embryos screening during *in vitro* fertilization (IVF) assisted by Raman analysis ofembryos culture medium. *Laser Physics Lett.,* **9**: 322-328. https://doi.org/10.1002/lapl.201110134

Steptoe, P.C. and Edwards, R.G., 1978. Birth after the reimplantation of a human embryo. *The Lancet,* **312**: 366. https://doi.org/10.1016/S0140-6736(78)92957-4

Sweet, D., Carnielli, V., Greisen, G., Hallman, M., Ozek, E., Plavka, R., Saugstad, O.D., Simeoni, U., Speer, C.P. and Halliday, H.L., 2014. European consensus guidelines on the management of neonatal respiratory distress syndrome in preterm infants--2013 update. *Zhonghua Er Ke Za Zhi,* **52**: 749-755.

Tesson, L., Usal, C., Menoret, S., Leung, E., Niles, B.J., Remy, S., Santiago, Y., Vincent, A.I., Meng, X., Zhang, L., Gregory, P.D., Anegon, I. and Cost, G.J., 2011. Knockout rats generated by embryo microinjection of TALENs. *Nat. Biotechnol.,* **29**: 695-696. https://doi.org/10.1038/nbt.1940

Vergouw, C.G., Kieslinger, D.C., Kostelijk, E.H., Botros, L.L., Schats, R., Hompes, P.G., Sakkas, D. and Lambalk, C.B., 2012. Day 3 embryo selection

by metabolomic profiling of culture medium with near-infrared spectroscopy as an adjunct to morphology: A randomized controlled trial. *Hum. Reprod.,* **27**: 2304-2311. https://doi.org/10.1093/humrep/des175

Vergouw, C.G., Botros, L.L., Judge, K., Henson, M., Roos, P., Kostelijk, E.H., Schats, R., Twisk, J.W., Hompes, P.G., Sakkas, D. and Lambalk, C.B., 2011. Non-invasive viability assessment of day-4 frozen-thawed human embryos using near infrared spectroscopy. *Reprod. Biomed. Online*, **23**: 769-776. https://doi.org/10.1016/j.rbmo.2011.08.015

Wefers, B., Meyer, M., Ortiz, O., Hrabe de Angelis, M., Hansen, J., Wurst, W. and Kuhn, R., 2013. Direct production of mouse disease models by embryo microinjection of TALENs and oligodeoxynucleotides. *Proc. natl. Acad. Sci. U.S.A.,* **110**: 3782-3787. https://doi.org/10.1073/pnas.1218721110

Wen, J., Jiang, J., Ding, C., Dai, J., Liu, Y., Xia, Y., Liu, J. and Hu, Z., 2012. Birth defects in children conceived by *in vitro* fertilization and intracytoplasmic sperm injection: A meta-analysis. *Fertil. Steril.,* **97**: e1331-1334. https://doi.org/10.1016/j.fertnstert.2012.02.053

Wong, F.K., Haffner, C., Huttner, W.B. and Taverna, E., 2014. Microinjection of membrane-impermeable molecules into single neural stem cells in brain tissue. *Nat. Protoc.,* **9**: 1170-1182. https://doi.org/10.1038/nprot.2014.074

Wu, Y., Liu, K., Song, K. and Pan, S., 2011. Three powerful research tools from single cells into single molecules: AFM, laser tweezers, and Raman spectroscopy. *Appl. Biochem. Biotechnol.,* **165**: 485-496. https://doi.org/10.1007/s12010-011-9267-2

Xiao, W.L., Zhang, D.Z., Hou, J.W., Sun, J. and Jia, M.Y., 2009. Multiple orofacial malformations in a boy who was conceived by intracytoplasmic sperm injection. *J. Plast. Reconstr. Aesthet. Surg.,* **62**: e298-300. https://doi.org/10.1016/j.bjps.2007.10.085

Xu, Y., Yang, H. and Zhang, Z., 1998. Raman spectroscopic study of space structure of membrane proteins and membrane lipids in photodamaged human erythrocyte sensitized by hypocrellin B. *Sci. China C Life Sci.,* **41**: 608-616. https://doi.org/10.1007/BF02882902

Zema, L., Loreti, G., Melocchi, A., Maroni, A. and Gazzaniga, A., 2012. Injection Molding and its application to drug delivery. *J. Contr. Release,* **159**: 324-331. https://doi.org/10.1016/j.jconrel.2012.01.001

Zhao, Q., Yin, T., Peng, J., Zou, Y., Yang, J., Shen, A. and Hu, J., 2013. Noninvasive metabolomic profiling of human embryo culture media using a simple spectroscopy adjunct to morphology for embryo assessment in *in vitro* fertilization (IVF). *Int. J. mol. Sci.,* **14**: 6556-6570. https://doi.org/10.3390/ijms14046556

Zhu, Q., Yu, X., Wu, Z., Lu, F. and Yuan, Y., 2018. Antipsychotic drug poisoning monitoring of clozapine in urine by using coffee ring effect based surface-enhanced Raman spectroscopy. *Anal. Chim. Acta,* **1014**: 64-70. https://doi.org/10.1016/j.aca.2018.02.027