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Effectiveness of near-infrared spectroscopy as a non-invasive tool to discriminate spectral profiles of *in vitro* cultured oocytes from goats

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Abstract

Here, we aimed to discriminate between the spectral profiles of spent culture media after oocyte *in vitro* maturation (IVM) and culture (IVC) from goats of different ages subjected to repeated hormonal treatments. The profiles were discriminated using near infrared (NIR) spectroscopy combined with multivariate methods. A total of 19 goats (young = 10; old = 9) were subjected to serial hormonal stimulation (HS) with gonadotropins. Cumulus oophorus complexes (COCs) were collected using laparoscopic ovum pick-up (LOPU) and subjected to IVM and parthenogenetic activation. The initial embryos were subjected to IVC. Spent culture media were collected after oocyte IVM and on day 2 of IVC and analyzed using NIR spectroscopy. NIR spectral data were interpreted through chemometric methods, such as principle component analysis (PCA) and partial least square discriminant analysis (PLS-DA). The results of PCA analysis clearly showed a separation in the spectral profiles between the experimental groups (HS sessions; young and old animals) both after IVM and IVC. Overall, the main absorption bands were attributed to the C-H group second overtone, first overtone of O-H and N-H, and C-H combinations and may serve as molecular markers. On the other hand, the spectral data obtained using PLS-DA models provided a better classification of the groups. The results showed the possibility of discriminating young and old groups as well as the three HS sessions with high specificity, sensitivity, and accuracy using NIR spectra. Thus, the culture medium analysis using NIR spectroscopy combined with multivariate methods indicated the dissimilarities between the groups and provided an insight into the *in vitro* development of goat oocytes. This technique serves as an efficient, objective, rapid, and non-invasive method to discriminate spectral profiles.

Keywords: near infrared; spectroscopy; culture medium; oocyte; goat.

Introduction

Goats are important experimental models for *in vitro* embryo production (IVEP) and offer great advantages in the generation of transgenic animals as well as cloning and conservation studies of animals that are at risk of extinction (Koeman et al., 2003; Mariano et al., 2015). Subjecting young animals to IVEP may help reduce generation interval (Paramio, 2010) and increase the recovery rate of oocytes per ovary (Koeman et al., 2003). Despite its great potential, IVEP in goats may not be in compliance with other domestic species, leading to

variations in success rates associated with embryos produced *in vivo* (Paramio, 2010; Freitas et al., 2017), especially among animals of different ages (Leoni et al., 2009, 2015).

The ability of an oocyte to successfully develop into an embryo is critical during the capacitation process, which precedes ovulation. During capacitation, the oocyte accumulates a set of information that can be used in the successive stages of development. The heterogeneity of oocytes collected for *in vitro* maturation (IVM) is still a limiting factor to the success of embryonic development in goats (Souza-Fabjan et al., 2014a). Some aspects or conditions such as oocyte source (Souza-Fabjan et al., 2014a), ovarian status and stimulation (Gibbons et al., 2007; Avelar et al, 2012), age (Souza-Fabjan et al., 2013), and season (Souza-Fabjan et al., 2014b) may contribute to oocyte heterogeneity and affect its developmental competence. The effects of age on IVEP in goats have been investigated and differences in some reproductive aspects between young and old goats were reported (Paramio, 2010; Baldassarre et al., 2007). Furthermore, the use of laparoscopic ovum pick-up (LOPU) technique and hormonal stimulation (HS) followed by IVEP has been effective in improving the poor reproductive performance of animals at very young age (Currin et al., 2017), especially in the animals that had their fertility affected with advanced age (Baldassarre et al., 2007).

Although morphological evaluation is very useful, it is highly subjective and may be unreliable for predicting the viability of these structures upon cultivation under *in vitro* conditions (Seli et al., 2008; Vergouw et al., 2008). Therefore, several studies have focused on the development of an improved method for gamete evaluation as the primary objective (Li et al., 2015). To address the main pitfalls in IVEP, a metabolomic profiling technology was proposed as an alternative to invasive techniques to enable simultaneous and high performance investigation of metabolic profiles based on the metabolites present in biological samples (McLennan et al., 2020; Leary and Sturmey, 2020). This technique evaluates the entire metabolic profile as a pattern and may be used for sample classification (Seli et al., 2010; Muñoz et al., 2014; Li et al., 2015).

Several technologies analyze spent culture media and suggest the presence of metabolic differences between oocytes or embryos, which mainly determine the reproductive potential (Ahlström et al., 2011; Muñoz et al., 2014; Rubessa et al., 2016). Optical spectroscopies as analytical technologies have been employed to investigate the complex metabolic profiles of culture media, including Raman, Fourier transform infrared (FT-NIR), and near infrared (NIR) spectroscopy (Botros et al., 2008, Ishigaki et al., 2014). Of these, the NIR spectroscopy has gained wide acceptance in different fields (Xiaobo et al., 2010), and is recognized as a primary and simple instrumentation that exposes the target sample to light of different wavelengths. It measures the characteristic absorption spectrum of the sample (Botros et al., 2008) and stands out owing to very fast and highly reproducible measurements, high sensibility, short and simple sample processing methods, easy maintenance and operation, and cost effectiveness (Botros et al., 2008). In this way, NIR technology provides a new and useful approach to oocyte and embryo evaluation using the spent medium culture in a non-invasive, non-toxic, and rapid analysis and processes small volumes compatible with the *in vitro* culture (IVC) system without preparation or prior separation.

NIR spectroscopy, in combination with chemometric tools, has demonstrated good results in the extraction of hidden information from simple spectra that may be difficult to interpret using computation of multivariate models (Rødgaard et al., 2015). These interpretations can detect minimal variations and accurately identify alterations in the metabolic spectra between oocytes (Nagy et al., 2009) from young and old animals, based on the differences in maturation rates and embryo production (Souza-Fabjan et al., 2013) as well as the changes in the quality of oocytes recovered by LOPU after repeated hormonal treatments (Gibbons et al, 2007; Avelar et al., 2012). Several studies have described the use of spectroscopy on spent culture media for human (Vergouw et al., 2008), mouse (Ishigaki et al., 2017), cattle (Muñoz et al., 2014; Li et al., 2018), fish (Ishigaki et al., 2016, 2017; Bik et al., 2020) and goat (Zhang et al., 2018) models. Thus, the use of NIR spectroscopy combined with multivariate analysis may accurately determine a metabolic pattern based on the spectral analysis of the oocyte maturation medium, that can be linked to oocyte subsequent metabolism and viability and improve *in vitro*

culture system for assisted reproduction. In this way, predicting the potential of implantation and consequent pregnancy may be possible (Nagy et al., 2009).

Herein, we propose the use of NIR spectroscopy in combination with multivariate analysis as a technique for the non-invasive discrimination between the spectral profiles of spent culture media after IVM and IVC of oocytes obtained from goats of different ages subjected to repeated hormonal treatments.

Materials and methods

Animals

A total of 19 mixed-breed goats were selected as oocyte donors, 10 aged between 1.3 ± 0.2 years and weighing 26.5 ± 2.0 kg, and nine non-lactating goats, aged between 7.0 ± 1.0 years and weighing 42.8 ± 2.1 kg. Both groups exhibited homogenous body condition scores (2.8 ± 0.1) on a scale of 1 to 5 adapted from Morand–Fehr and Gall (1981). The goats were allocated in the Experimental Farm of School Veterinary Medicine, State University of Ceará (UECE), located in Guaiúba, CE, in the equatorial zone (4º2′23″S and 38º38′14″W) and subjected to 30–45 days of adaptation to environmental and food conditions with balanced diet as per their nutritional requirements.

Hormonal treatment

Estrus synchronization and ovarian stimulation were performed according to protocols described by Gibbons et al. (2007).

Estrous synchronization

Briefly, the ovarian status was synchronized in all goats with an intramuscular administration of 1 ml (0.075 mg) of a prostaglandin F2α analogue (PGF2α; Prolise®; ARSA S.R.L., Buenos Aires, Argentina), which was followed by the insertion of an intravaginal progesterone insert (CIDR®, InterAg®, Hamilton, New Zealand) after 48 h. The intravaginal insert was removed after the last oocyte recovery.

Hormonal stimulation

Follicular development was stimulated by the simultaneous intramuscular administration of a single dose of 60 mg follicle-stimulating hormone (FSH; Folltropin®; Vetrepharm, London, ON, Canada) along with a single dose of 300 UI equine chorionic gonadotropin (eCG; Novormon 5000®; Syntex, Argentina). The first FSH/eCG dose was administered 48 h after the dispositive insertion and repeated every 4 days to complete three laparoscopic LOPU sessions.

LOPU and oocyte evaluation

The oocyte donors were divided into four groups (4-5 animals per group) and subjected to three sessions of HS and subsequent LOPU with a 4-day time interval between the sessions (Gibbons et al., 2007). LOPU was performed 24 h after each FSH/eCG treatment and 36 h of fasting. The follicular fluid from each animal was separately aspirated into collection tubes and warmed to 38°C in Dulbecco's phosphate-buffered saline (D-PBS; Nutricell®, Campinas, SP, Brazil), which was supplemented with 5% fetal bovine serum (FBS; Sigma Chemical Co., St Louis, MO, USA), antibiotics (100 IU/ml penicillin plus 0.1 mg/ml streptomycin; Sigma Chemical Co., St Louis, MO, USA), and 0.05 mg/ml heparin (Liquemine®, Campinas-SP, Brazil). The follicular fluid sediment was used to recover cumulus oophorus complexes (COCs) that were classified according to the method described by Baldassarre et al. (2003) with minor modifications. Briefly, the assessment of the quality of COCs was

based on visual criteria observed using a stereomicroscope (SMZ-645; Nikon®, Tokyo, Japan) and classified into four different grades as follows: Grade 1 (GI; oocyte with multilayered compact cumulus cells and evenly homogeneous cytoplasm); Grade 2 (GII; oocyte with 2-3 layers of cumulus cells and evenly homogeneous cytoplasm); Grade 3 (GIII; oocyte with 1 layer of cumulus cells, partially or totally denuded with homogeneous cytoplasm); and degenerated (DEG; denuded oocyte with heterogeneous cytoplasm or cumulus expansion).

IVM, parthenogenetic activation, and IVC

Selected COCs, segregated as viable by grade (G-I to G-III) according to morphological quality and experimental groups, were subjected to IVM, parthenogenetic activation and IVC procedures according to Fernandes et al. (2014), with minor modifications. Briefly, COCs were subjected to IVM at 38.5°C and 5% CO_2 in petri dishes (Corning®, USA) under mineral oil-containing drops of 100 µL of maturation medium. The medium comprised TCM 199® medium supplemented with 0.022 µg/ml sodium pyruvate, 10,000 IU penicillin, 10,000 µg/ml streptomycin sulfate, 10% FBS, 10 ng/ml epidermal growth factor (EGF), 5 µg/ml FSHp (Folltropin®; Bioniche, Belleville, Ontario, Canada), 10 µg/ml luteinizing hormone (LH; Lutropin®; Bioniche), 1 µg/ml 17β-estradiol, and 100 µM cysteamine. After 24 h of IVM, oocytes were mechanically denuded with successive pipetting and observed under a stereomicroscope to determine the maturation rate through the careful visualization of the presence of the first polar body (metaphase II-MII). The in vitro-matured oocytes were parthenogenetically activated after exposure to 5 mM ionomycin for 5 min, followed by incubation in 2 mM 6-dimethylaminopurine (6-DMAP) in G1 (Vitrolife®) medium for 4 h. After activation, the presumed zygotes were cultured in G1 medium in an incubator at 38.5°C and 5% CO_2 in a humidified atmosphere for 72 h. The embryos were subsequently incubated in 100-µL droplets of TCM199-HEPES with 10 M Hoechst 33342 (Sigma®, Deisenhofen, Germany) at 38.5°C for 30 min and individually examined under a fluorescence microscope (Nikon®, Eclipse 80i, Tokyo, Japan) to visualize cellular DNA and determine the cleavage rate.

Sample collection and NIR spectroscopy analysis

After IVM of oocytes and IVC of presumed zygotes, 25 µL aliquots of the spent culture medium from each animal and blank controls (medium cultured without oocytes or embryos) were individually stored in correctly identified cryovials at −80°C for further analysis using NIR spectroscopy equipment (Perten DA 7200©). The medium was analyzed as microdrops covered with mineral oil in a petri dish. Before analysis, samples were thawed at room temperature (25°C ± 1°C) and evaluated in triplicate for spectral identification using NIRS. These spectra were recorded in the wavelength range between 950 and 1,650 nm (10526-6061 cm^{-1}) at room temperature and collected at 5 nm intervals. The equipment was set to measure the raw transmittance and a signal average of 30 measurements with an integration time of 100 ms for each measurement. The analysis time was approximately 5 min per sample. The background spectrum (i.e., blank controls) was also obtained using the same acquisition time and power.

Statistical analysis

The data were transformed into Excel 2010 and imported into the R programming environment (R Core Team, 2019). The spectral data were subjected to transformation with the second derivative to adjust baseline errors. Principal component analysis (PCA) was used to reduce the dimensionality of the data. The first three main components (uncorrelated independent variables) were used for sample clustering. Thus, it was possible to visualize all the spectra through the scores in a multidimensional space. We used the partial least square-discriminant analysis (PLS-DA) to predict the different classes. The PLS-DA algorithm maximizes the covariance between the spectra and the sample groups (age and HS sessions) through the successive selection of orthogonal latent variables (LVs). Moreover, variable importance in the projection (VIP) values obtained from supervised statistical PLS-DA was used to quantify the

contribution of each wavelength in a PLS-DA model. According to the criteria of a VIP value > 1.0 in the PLS-DA model (Gosselin et al., 2010).

Cross-validation was performed 100 times in an iterative process to estimate the classification error rate to evaluate the performance of model by function perf, into the mixOmics package (Rohart et al., 2017). The samples were randomly divided (k-fold = 10) into 10 groups of equal sizes (MIV = 11 and CIV = 12), where nine were used to the training set and one to the test set. Moreover, to determine the predictive capacity of the model, the receiver operating characteristic (ROC) curve was used to evaluate the cutoff value by combining the highest specificity and sensitivity, which allowed classification of age groups and serial hormonal stimulation (HS) groups through the area under the curve (AUC).

Ethics approval

All procedures used in this study were conducted as per the Brazilian Directive on the Care and Use of Animals in Teaching or Scientific Research Activities (DBCA (Chapter VI, item 6.3.10). The study was approved by the Ethics Committee for the use of animals of the State University of Ceará under registration order number 6497021/2017.

Results

Figure 1 shows the raw and second derivative spectral profiles after IVM (Figure 1A and 1B, respectively) and IVC (Figure 1C and 1D, respectively) obtained using NIR spectroscopy. The results were obtained from the analysis of culture spent media of young and old animals. In Figure 2, the raw and second derivative NIR spectra are shown after IVM (Figure 2A and 2B, respectively) and IVC (Figure 2C and 2D, respectively) in response to the three HS sessions. The spectra obtained showed six main absorption bands, 1,150, 1,220, 1,400, 1,425, 1,445 and 1,650 nm, attributed to the vibration of C-H group second overtone, first O-H and N-H overtone, and C-H combinations. Evaluation of the second derivative-treated spectra revealed more sharp peaks and valleys at 1,130-1,225, 1,610-1,625, and 1,355-1,460 nm range. The spectral profile of young and old animals as well as the samples from different HS sessions showed different absorption patterns in different regions.
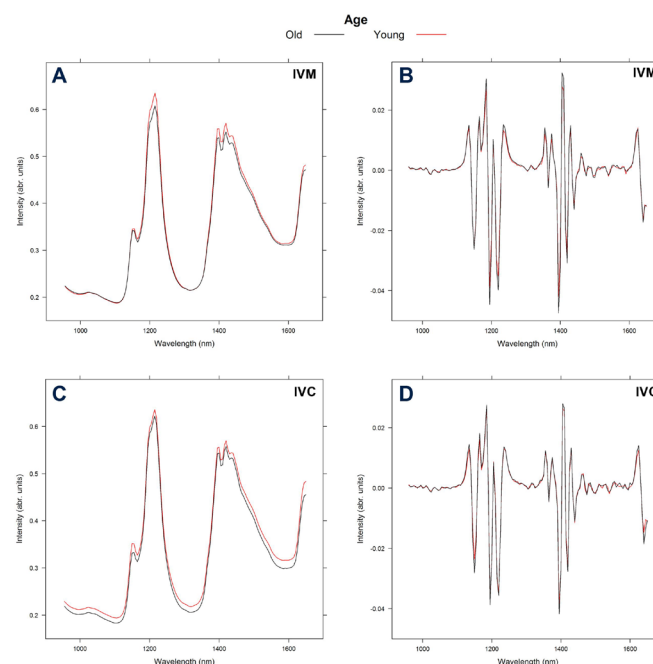

Figure 1. Raw (A) and second derivative (B) NIR spectra of spent culture medium samples after IVM. Raw (C) and second derivative (D) NIR spectra of spent culture medium after IVC of oocytes from old (black line) and young (red line) goats.
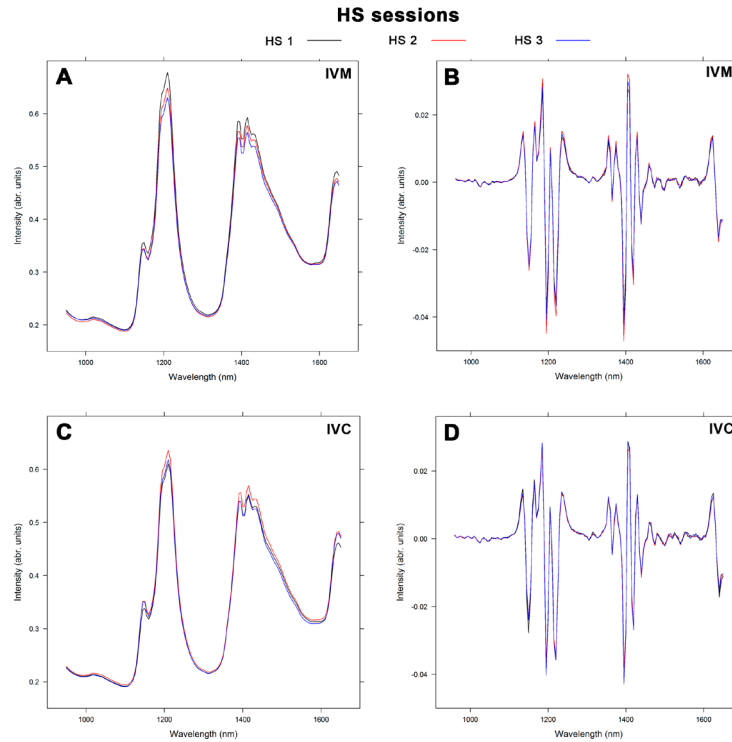
Figure 2. Raw (A) and second derivative (B) NIR spectra of spent culture medium samples after IVM. Raw (C) and second derivative (D) NIR spectra of spent culture medium after IVC of oocytes from the first (black line), second (red line), and third (blue line) hormonal stimulation (HS) session.

Figure 3 presents the three-dimensional score plot of the first three PCs of the different age groups (young and old) for IVM (Figure 3A) and IVC (Figure 3B) phases obtained through PCA. Figure 4 shows the score plot of the three HS sessions for IVM (Figure 4A) and IVC (Figure 4B). Analysis of multivariate was capable to segregate the groups in age and HS effects. Multivariate retained for the first, second and third principal components respectively 17%, 12%, and 11% of the total variance in IVM model and for IVC 18%, 13%, and 12% of variance, accounting for respectively 40% and 43% of the cumulative variance together.
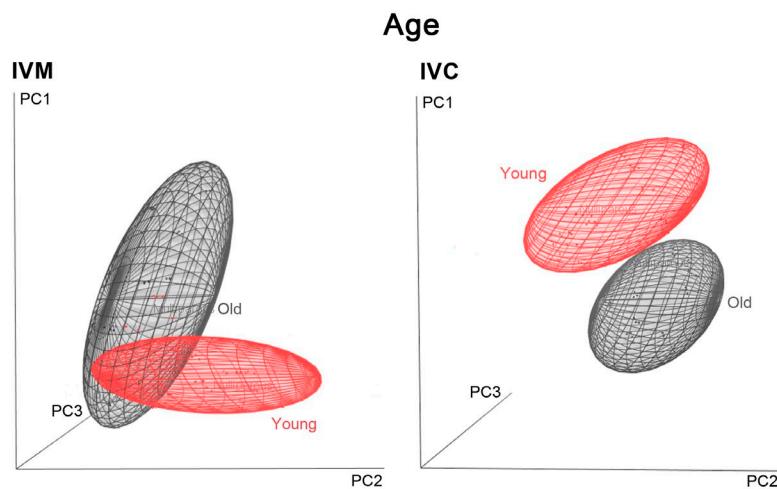

Figure 3. Score plot of PCA model with three principal components of spent culture medium spectra of oocytes from the young (red) and old groups (black) after IVM (left) and IVC (right).
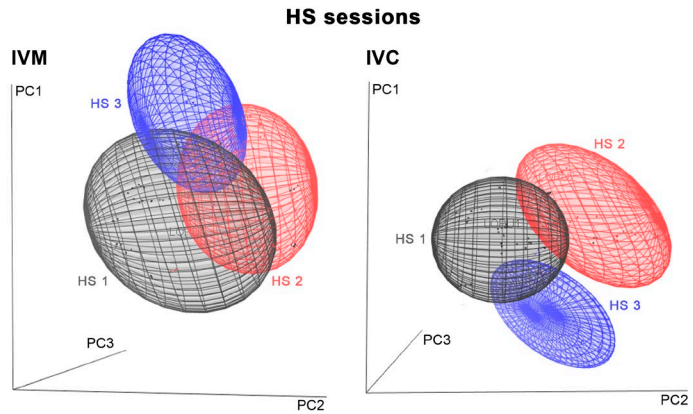
Figure 4. Score plot of PCA model with three principal components of spent culture medium spectra of oocytes obtained from the first (black line), second (red line), and third (blue line) hormonal stimulation (HS) session after IVM (left) and IVC (right).

In Figure 5, were illustrated the values of variable importance in the projection (VIP) for IVM (Figure 5A) and IVC (Figure 5B). The IVM VIPs shows a distinct pattern distribution for age and hormonal stimulation sessions. In HS curve there was a highest value (1.78) at 1,340 nm wavelengths and VIPs range distributed from 950 nm to 1,615nm. On the contrary, the age curve shows VIPs from 1,145 nm to 1,650 nm, with two peaks located at 1,200 nm and 1,400 nm but both always showing VIP below 1.4.
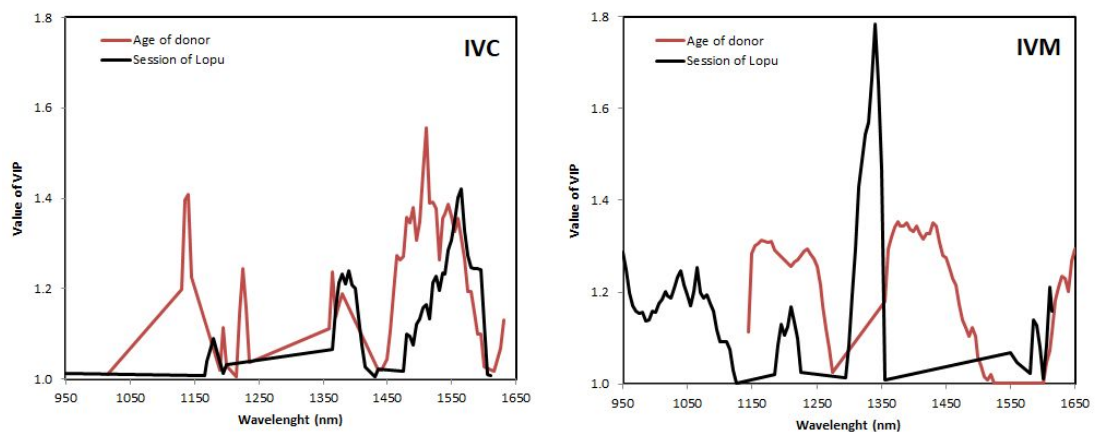
Figure 5. Distribution curves of variable importance in the projection (VIP) values > 1.0 obtained from the PLS-DA multivariate model of spent culture medium spectra of oocytes obtained from goats with different ages (red) and hormonal stimulation sessions (black) after IVM (A) and IVC (B).

The distribution curves of the VIPs in the IVC showed values below 1.6 and with similar patterns. Due to areas of larger peaks, they can be identified between 1,360 nm and 1,400 nm and successively between 1,460 nm and 1,600 nm. The age of the animals also registered a smaller peak between 1,135 nm and 1,145 nm.

Considering the groups of young and old animals, the error rates of PLS-DA with five LVs were 0.21 and 0.04 for IVM and IVC, respectively. The AUC for IVM was 86%, while that for IVC was 100%. The loading vectors of the first two LVs for the PLS-DA model considering the age group are provided in Figure 6. The main regions that contributed to this result included 1,075, 1,085, 1,370, 1,395 and 1,525 nm for IVM phase and 1,045, 1,055, 1,110, 1,385 and 1,535 nm for IVC.
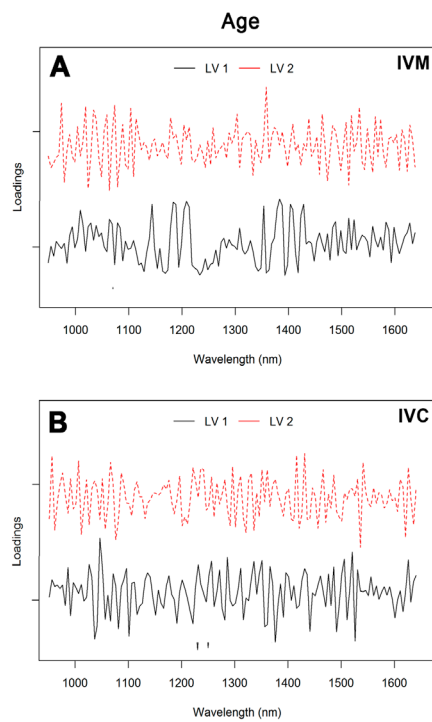
Figure 6. Loading plot of the first two latent variables (LVs) for the PLS-DA model of spent culture medium spectra of oocytes obtained from the young and old groups after IVM (A) and IVC (B).

For HS sessions, error rates were 0.39 and 0.19 for IVM and IVC, respectively. The AUC was 94% (HS 1 versus others), 96% (HS 2 versus others) and 82% (HS 3 versus others) for IVM and 96% (HS 1 versus others), 98% (HS 2 versus others) and 100% (HS 3 versus others) for IVC. The loading plot (Figure 7) showed the main bands, which allowed the discrimination between groups subjected to different HS sessions. The main regions that contributed to the separation of HS sessions were 1,070, 1,305, 1,325, 1,555 and 1,560 nm for IVM and 960, 985, 1,230, 1,520 and 1,610 nm for IVC.
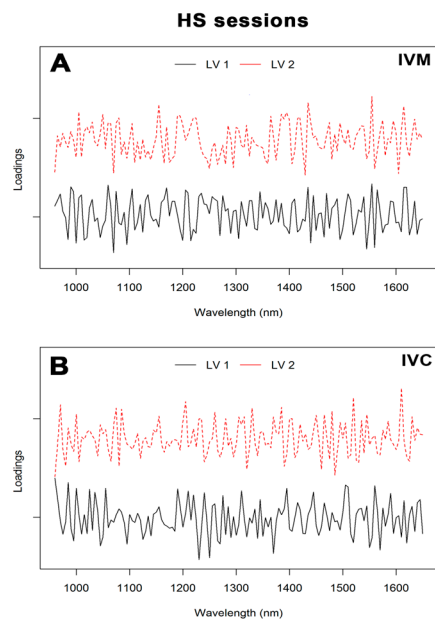
Figure 7. Loading plot of the first two latent variables (LVs) for the PLS-DA model of culture medium spectra of oocytes obtained from hormonal stimulation (HS) sessions after IVM (A) and IVC (B).

Discussion

One of the known advantages of NIR technology is the ability to construct characteristic spectra, which serve as the "fingerprint" of the sample (Currà et al., 2019). Here, we used NIR analysis for overall spectral profile comparisons, with an aim to distinguish between young and old groups and first, second and third HS session without further metabolic investigation. We found that the technique in conjunction with multivariate analysis was capable of separating the experimental groups. During *in vitro* development, oocytes and embryos may secrete/absorb certain levels of substances into/from the medium culture, such valine, lysine, glutamine, glycine, phenylalanine, phytosphingosine, lactate, glucose, pyruvate and phosphate (Zhang et al., 2018; Wiweko et al., 2020). NIR spectroscopy is not suitable for structure elucidation; therefore, band attribution is recommended for spectral interpretation and for a better understanding of the results. This includes the use of band characteristics such as intensity and wavelength position for correlating chemical knowledge about the sample in an attempt to identify the corresponding compound (Pasquini, 2003).

In our study, young and old animals were subjected to sequential HS for the evaluation of oocyte and embryo quality. The effectiveness of ovarian stimulatory protocols on maturation and cleavage rates is well known (Baldassarre and Karatzas, 2004; Paramio, 2010; Baldassarre et al., 2018), and studies have evaluated the effects of repeated hormonal treatments on the morphological quality of recovered oocytes (Gibbons et al., 2007).

Overall, the NIR spectrum provides information about the spectral signatures of biological samples (Chen et al., 2015). The wavelength range evaluated in this work comprises the region between 950 and 1,650 nm, wherein four functional groups (C-H, N-H, O-H, and C-C) can be monitored. The spectra were different in some regions, reflective of the vibration, overtone, and combinations of functional groups such as O-H, N-H, and C-H. These overtones are anharmonic, leading to very broad, complex spectra that are not directly interpretable (Nagy et al., 2009). In addition, molecules have different vibration patterns, leading to the requirement of highly complex and sensitive equipment for the identification of a large number of molecules. Previous studies have reported differences in the absorbance patterns in NIR spectra by evaluating culture media obtained from oocytes at different maturation stages (Nagy et al 2009) and single embryo culture for viability assessment (Seli et al., 2007; Li et al, 2015).

In the present study, the NIR spectra were pretreated with the second derivative to extract important information and minimize variability (Chen et al., 2015). The second derivative technique is a common filter used in practice to focus on the variation in the spectra associated with the chemical composition of the sample (Kirdar et al., 2010). Therefore, this technique was considered as a suitable choice for the pre-treatment of NIR spectra for subsequent multivariate calibration. The second derivative spectra in the range of the wavelength 1,130–1,225, 1,355–1,460, and 1,610–1,625 nm demonstrated changes in the peak bands. According to Chen et al. (2015), the absorption range between 1,111 and 1,250 nm is associated with C-H second overtones, while the range between 1,333 and 1,666 nm is attributed to the first overtone of O-H and N-H, and C-H combinations. As observed in Figure 1B and 1D, several overlapping vibrational bands were detected in the two groups of spectra (young and old animals) at both times of evaluation (after IVM and IVC). Furthermore, several sharp peaks and valleys were detected, which were not observed with raw spectra. This observation is indicative of the high information content in the NIR spectra, which may explain the variation in the absorption bands that may not be easy to distinguish or attribute to certain functional groups. According to Huang et al. (2010), the derivative technique can prove difficult for spectral interpretation and may possibly lead to more noisy spectra. It is worth noting that the peak wavelengths were similar between age groups, and that some absorbance peaks in culture medium spectra after IVC were more pronounced than those after IVM (Fig. 1).

The use of multivariate statistical techniques such as PCA and PLS-DA enabled the extraction of useful information from NIR spectra. PCA was conducted to review the data from spectral profiles of the investigated groups and to examine the possible clustering in samples. Our results demonstrated a good separation between different age groups in the culture

media after both IVM and IVC. Differentiated metabolism can be influenced by significant deficiencies in the number and organization of organelles in young animal oocytes, wherein reduction in the amount of endoplasmic reticulum has been observed (Damiani et al., 1996). According to Baldassarre et al. (2018), multiple applications of gonadotrophins for follicular development, especially in young animals, improved the cytoplasmic conditions of competent oocytes and consequently the cleavage rates. This result reveals the complexity of the physiological and biochemical mechanisms involved in the *in vitro* development of goat oocytes. Nevertheless, a more careful assessment using NIR analysis would reveal the distinct patterns of secretions in the experimental groups based on spectral evaluations of spent culture media and uncover previously unseen differences (Kirkegaard et al., 2014).

The first three PCs showed about 43% of total variation in the NIR spectra. These results needed to be further analyzed using PLS-DA to maximize the separation between the groups and provide a better understanding for classification (i.e., dimensionality reduction) and predicting model construction (i.e., discriminant analysis) (Lee et al., 2018). We applied the classification error rate, ROC curve and AUC as figures of merit and constructed an ROC curve to help select a threshold (cutoff value), which was used to obtain high sensitivity, specificity, and overall accuracy. ROC-AUC is considered a powerful tool for the detection of small differences between groups and accurately finds the main variables responsible for discrimination between two classes (Szymanska et al., 2012). As a categorical model, these tools are valid for describing tlhe predictive ability of the PLS-DA model (Gromski et al., 2015). In our findings, the PLS-DA model showed consistent AUC values, which were higher than 0.8. For the IVC phase, excellent AUC value was obtained (1.0: perfect discrimination). The figures of merit indicated that the classification model established herein was characterized with high accuracy and acceptable values of errors. Therefore, it can be stated that PLS-DA was an efficient and reliable model with excellent discriminatory power for the comparison of the culture media of young and old animal oocytes after IVC. Moreover, the loading plot in Figure 6A shows that LV1 focused on the second overtone of C-H in –C-H$_2$, O-H, and N-H first overtones, and C-H combination regions, while LV2 focused on the second overtone C-H stretching and H$_2$O bands.

Oocytes from young animals subjected to hormonal treatments had greater developmental capacity and may potentially combine the effect of older age and multiple gonadotropin stimulations (Baldassarre et al., 2018). Our results showed six main absorption peaks in NIR spectra, 1,150, 1,220, 1,400, 1,425, 1,445, and 1,645 nm, for the three groups, that may be attributed to the vibration of the C-H group second overtone, first O-H and N-H overtone, and C-H combinations. PCA showed that the three HS sessions were well clustered (Figure 4), and PC1 resolved most of the variance between them. Furthermore, the results of the PLS-DA procedure to discriminate the experimental groups achieved the highest sensitivity and specificity, best accuracy values, and low error rates. These efficient classification results suggest that the procedure was reliable, and that the classification models were robust. Our loading plots support these results (Figure 6), revealing the dominating or influencing variables. As shown in Figure 6B, LV1 focused on the water bands, C-H second overtone, and O-H first overtone. The region between 1,070, 1,305, and 1,325, and 1,555 and 1,560 nm for IVM and 960 and 985, and 1,230, 1,520, and 1,610 nm for IVC contributed to the differentiation of various classes. It is worth noting that some absorption bands were difficult to assign, mostly owing to the absence of previous identification or band overlaps.

Conclusion

We conclude that NIR spectroscopy is a rapid and non-invasive technique for the analysis of culture media used for *in vitro* goat oocyte maturation and development. In combination with multivariate methods, NIR spectroscopy serves as a useful tool for the classification and separation of mature and cleaved oocytes from young and old goats as well as from those subjected to repeated HS at each session. PCA enabled the separation of the experimental groups, while the PLS-DA model enabled the prediction of sample classes with high accuracy.

The results of this work show that the NIR spectra may provide relevant information on IVM and IVC and contribute to the high efficiency of the technique.

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