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FERTILITY PRESERVATION

Evidence of metabolic activity during low-temperature ovarian tissue preservation in different media

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Abstract

Purpose Although ovarian tissue transportation has been validated for up to 24 h, there is no standard protocol to date. We aimed to elucidate how existing media currently used for ovarian tissue transportation affect ovarian tissue metabolism and cell viability.

Methods Cow ovarian fragments were immersed in 0.9% NaCl solution, IVF medium, Leibovitz 15 medium (L-15), or PBS for 1, 4, or 24 h at 4 °C. Media were analyzed for pH, lactate dehydrogenase (LDH) activity, and glucose, pyruvate, and lactate concentrations, while apoptosis was assessed by TUNEL assays in fixed fragments. Viability rates were assessed by flow cytometry (FACS).

Results There were lower pH levels in NaCl at all time points compared with other media. LDH activity increased with time and was lowest in NaCl at 1 and 4 h. There was no significant difference in glucose levels, but a significant pyruvate decrease in L-15 and a significant lactate increase in all media. TUNEL showed apoptosis rates ranging from 0 to 5%. FACS showed a mean of 4% necrotic cells and 15–19% apoptotic cells after 1 h of incubation, but less than 1% necrotic cells and 2–6% apoptotic cells after 24 h in all media.

Conclusion Our results indicate marked metabolic activity in ovarian tissue at 4 °C and suggest that cells use internal sources of energy, which may influence transplantation outcomes. This highlights the importance of better understanding whole tissue dynamics to develop a standard protocol for ovarian tissue transportation.

Keywords Metabolism · Assisted reproduction · Fertility preservation · Ovarian tissue transportation · Ovarian tissue cryopreservation

Introduction

Ovarian tissue cryopreservation and transplantation is a widely applied approach for fertility preservation in cancer patients [1]. More than 130 live births have been reported to date with use of this technique, yielding pregnancy rates of 30–60% and live birth rates of around 40% [2, 3].

While much emphasis is placed on improving ovarian tissue cryopreservation and grafting procedures and understanding events occurring before and after revascularization of grafted tissue [4–7], the importance of ovarian tissue transportation is progressively coming to the fore. In the early days of cryopreservation/transplantation, ovarian biopsies were usually collected and cryopreserved in the same hospital. In recent years, however, due to strict European directives on tissue banking and high costs involved in maintaining an ovarian tissue cryobank, only large hospitals can have such facilities.

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The concept of "the woman stays, the tissue moves," first established by the Danish network for transport of ovarian tissue for up to 4 h [8], is becoming increasingly accepted, steadily extending the duration of ovarian tissue transit. In order to reach larger numbers of patients, the *Ferti*PROTEKT network has now validated ovarian tissue transportation for up 24 h [9, 10].

Although pregnancies and live births have been reported after such a long period of transportation [10], it remains an empirical procedure and there is no standard protocol [9, 11]. Only three studies have evaluated the effect of different media on human ovarian tissue transport [12–14], but none of the studied media is used in clinical practice for ovarian tissue transportation [10, 15–17]. Moreover, we must bear in mind that the ovary is a highly complex organ with a diversity of cells and structures of vital importance to ensuring the survival and development of follicles [18], and available knowledge on ovarian tissue metabolism refers mainly to follicles [19], which are composed of two of the six different cell populations present in the ovary [20]. Transportation of this tissue is undoubtedly a challenging prospect. We therefore aimed to elucidate how existing media currently used for ovarian tissue transportation affect the metabolism of whole ovarian tissue during short- and long-term cold ischemia. Our results suggest that considerable metabolic activity was preserved in ovarian tissue samples at low temperatures.

Material and methods

Experimental design

To assess the effect of ovarian tissue transport at 4 °C, we used cow ovaries as a translational model for humans because of their physiological similarities [21], availability, and size, which allowed us to perform all media comparisons in the same animals. A total of 30 ovaries were obtained from 15 adult cows (Belgian Blue, 3–6 years of age) euthanized in a local slaughterhouse (Abattoir of Anderlecht, Brussels, Belgium). The ovaries were used in two different set of experiments. In the first study, we aimed to demonstrate metabolic activity in ovarian tissue transported at 4 °C. For this, ovarian fragments were incubated in three different media and, after 1, 4, or 24 h, both the media and tissue were analyzed. Based on results obtained from these first experiments, we carried out a second study to correlate metabolic activity and cell viability during ovarian tissue transportation. Modifications were made to transport media in order to avoid bias and store the tissue in similar conditions to those in clinical practice. Ovarian tissue fragments were incubated in one of the three media for 1 or 24 h at 4 °C, and media and isolated ovarian cells were then analyzed.

Experiment 1

Immediately after retrieval in the slaughterhouse, adipose tissue and ligaments were removed and the ovaries were cut into 10 fragments of 1 × 1.5 × 0.5 cm. One piece was immediately fixed in 4% paraformaldehyde (Alfa Aesar, ref. J61899, Karlsruhe, Germany), and the others were randomly immersed in 15-mL Falcon tubes containing 10 mL of one of the following solutions, with one fragment per tube (online resource 1: media composition) at 4 °C:

1) Universal IVF medium (Origio, Malov, Denmark, ref. #10310060A).
2) Leibovitz 15 medium (L-15) + GlutaMAX™ (Gibco, Bleiswijk, the Netherlands, ref. #31415-029) supplemented with 5% antibiotic-antimycotic (anti-anti, Gibco, Bleiswijk, the Netherlands, ref. #15240-062).
3) 0.9% sodium chloride (NaCl) solution (Merck, Darmstadt, Germany, ref. #1.06404.5000) supplemented with 5% anti-anti.

After 1, 4, or 24 h, the fragments were fixed in 4% paraformaldehyde and media were aliquoted for further analysis. A few aliquots of 1 mL were snap-frozen in liquid nitrogen to assess glucose, lactate, and pyruvate concentrations and lactate dehydrogenase (LDH) activity, and the remaining media were kept on ice for pH analysis.

Media analyses

Levels of pH were measured in each medium ($n = 9$ animals) using a pH meter (Consort™ C3010 multi-parameter analyzer). For glucose, pyruvate, and lactate, media were first thawed on ice, and then substrates and products of metabolism were evaluated in 24-h samples of each medium ($n = 6$ animals) using specific enzymatic assays on a CMA600 microdialysis analyzer (CMA Microdialysis, Kista, Sweden), as previously reported [22]. LDH quantification ($n = 9$ animals) was performed in each medium thawed on ice using an LDH assay kit (Abcam, ref. #ab102526) and read using a luminometer (2030 Multilabel Reader, Victor™ X4, PerkinElmer), according to the manufacturer's instructions.

Tissue analyses

Fixed ovarian fragments were dehydrated, embedded in paraffin, and cut into 5-μm-thick sections. To evaluate the apoptotic response, one sample of each medium was chosen from three different animals based on the highest concentrations of lactate measured (totaling nine samples). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed using the In Situ Cell Death Detection Kit, TMR Red (Roche, ref. 12 156 792

910, Mannheim, Germany), as described by Vanacker et al. [23] Paraffin sections were deparaffinized with paraffin-clearing solvent (X-Solv, Yvsolab NV/SA, Belgium) and rehydrated in propanol. DNAse was used to damage DNA in positive controls, and negative control sections were incubated with a label without enzyme solution. Slides were further stained with 4′,6′-diamidino-2-phenylindole (DAPI) and scanned with a Mirax digital slide scanner. Areas of TUNEL-positive cells and DAPI-stained cells were automatically analyzed using Visiopharm® software, and the percentage of TUNEL-positive/TUNEL-negative areas was calculated.

Experiment 2

To confirm cell viability after transportation, ovaries from 4 cows were obtained from a local slaughterhouse. Fragments of 1 × 1 × 0.5 cm were incubated in 20 mL of either phosphate-buffered saline without calcium chloride and magnesium chloride (PBS, Gibco, ref. 10010-023, Bleiswijk, the Netherlands) and IVF-medium or L-15 medium for 1 or 24 h at 4 °C. PBS was used in experiment 2 to avoid bias in the results due to pH alterations, as NaCl in experiment 1 was not a buffered solution.

Media analyses

Media were analyzed for pH, glucose, pyruvate, and lactate concentrations as previously described.

Viability assessment

Immediately upon arrival in the laboratory (1 h of transportation), to serve as a control group for each medium, one fragment of 5 × 5 mm was immersed in 5 mL McCoy 5A medium (Gibco, ref. 16600082) containing 100 μL Liberase™ DH (Merck, ref. 5401054001, Bornem, Belgium) and disrupted in the gentleMacs™ Dissociator (Miltenyi Biotec, Germany) for 1 h at 37 °C (program 37C_h_TDK_1). After digestion, the ovarian cell suspension was passed through a 100-μm filter and rinsed in PBS, before being centrifuged for 5 min at 1500 rpm. Supernatant was removed, and cells were stained with FITC Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher, ref. V13242) according to the manufacturer's instructions. Flow cytometry analysis was carried out with BD FACSCantoII (BD Biosciences) equipped with FACSDiva v8.0 software and analyzed by FlowJo software (BD Biosciences). At least 10,000 ovarian cells were analyzed. The same procedure was performed after 24 h of transportation.

Statistical analyses

Statistical analyses were carried out using IBM SPSS Statistics 22 software. Data were expressed as means ± SD. Continuous variables were evaluated for normal distribution using the Shapiro-Wilk test. Possible differences were investigated by one-way ANOVA followed by Tukey's post hoc test (normal distribution) or the Kruskal-Wallis test followed by the Mann Whitney *U* test (not normal distribution). $P < 0.05$ was considered statistically significant.

Results

Experiment 1

pH

First, we measured pH as an indicator of medium acidification [24–26] to obtain pH values ($n = 9$) in the different media over time (Fig. 1). We found a significantly lower pH in NaCl at all time points compared with IVF medium and L-15. While the pH increased with time in NaCl, it slightly but significantly decreased in L-15 after 24 h. It was not significantly altered in IVF medium.

Glucose, pyruvate, and lactate

We measured glucose, pyruvate, and lactate concentrations in media after 24 h ($n = 6$) as markers of metabolic activity for the longest period investigated (Fig. 2). Glucose levels were stable in IVF medium over 24 h of incubation. Pyruvate values were stable in all media except L-15, where a small

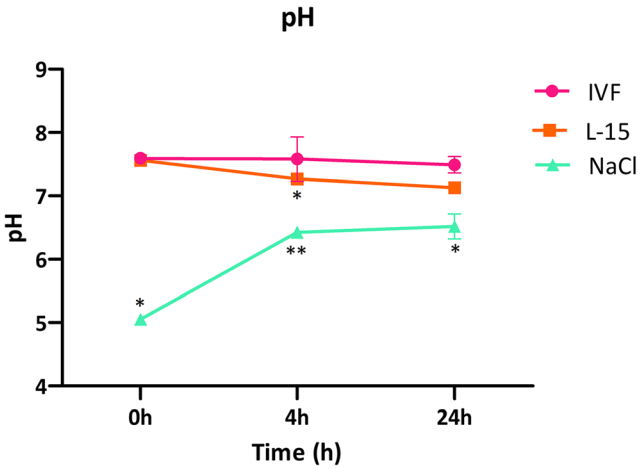

Fig. 1 pH variation over time in different media used for ovarian tissue transportation. Ovarian fragments of 1 × 1.5 × 0.5 cm were stored at 4 °C in 10 mL of IVF medium, L-15, or NaCl for the indicated times, at which pH values were measured using a pH meter ($n = 9$). Asterisk indicates $p < 0.05$ at the same time points

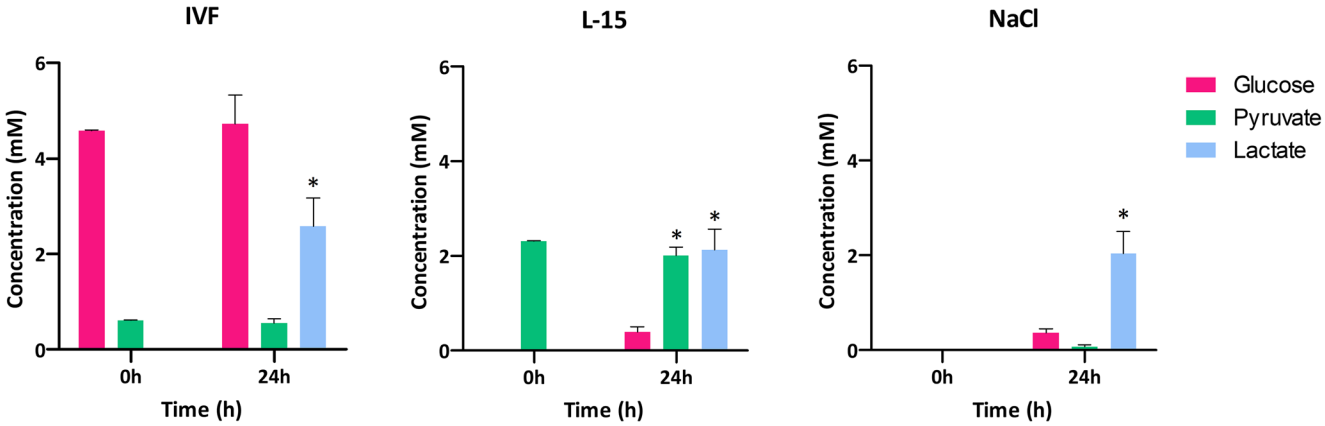

Fig. 2 Glucose, pyruvate, and lactate concentrations in different media used for ovarian tissue transportation. Ovarian fragments of 1 × 1.5 × 0.5 cm were stored at 4 °C in IVF medium, L-15, or NaCl for the indicated times, at which glucose, pyruvate, and lactate concentrations were determined in incubation media using a CMA600 enzymatic analyzer ($n = 6$). Asterisk indicates $p < 0.05$ for a given metabolite between 0 and 24 h

but significant decrease was noted at 24 h compared with 0 h. There was a significant release of lactate from ovarian samples in all media, with no significant difference between media.

LDH activity

LDH release is a marker of tissue damage, as only necrotic cells can release the enzyme [27]. In our experiments, LDH activity ($n = 9$) increased with time in all media (Fig. 3). Comparatively, however, it was significantly lower in NaCl than in IVF medium and L-15 at 1 h and 4 h of incubation. There was no difference in LDH activity at the 24-h time point between the three media.

Tissue analyses

As LDH release increased during storage, indicating necrosis, we finally evaluated apoptosis as a second major form of cell death. TUNEL-positive areas ($n = 3$) were 0.65 ± 0.35% in fresh tissue, 2.63 ± 1.39% after 24 h of storage in IVF medium, 5.47 ± 2.27% in L-15, and 1.27 ± 1.06% in NaCl (Fig. 4a). L-15 was the only medium displaying significantly higher apoptosis rates than fresh tissue. Upon closer inspection, apoptotic cells were mainly located in antral follicles (Fig. 4b), corpus albicans, and fibrotic tissue (Fig. 4c, d). No oocyte nuclei were observed on TUNEL slides.

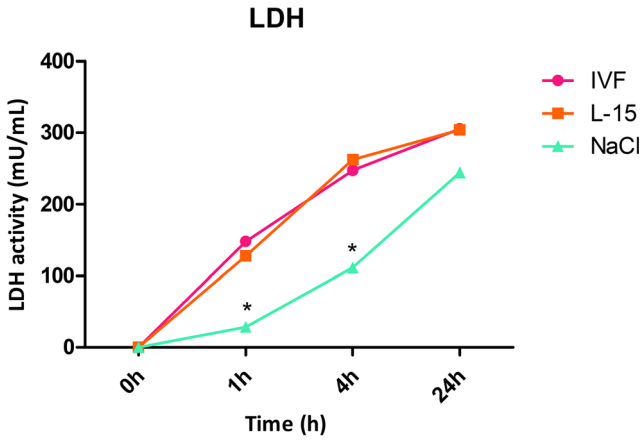

Fig. 3 LDH release from ovarian samples occurs at early time points in all storage media. Ovarian fragments of 1 × 1.5 × 0.5 cm were stored at 4 °C in IVF medium, L-15, or NaCl for the indicated times, at which LDH activity was calculated in incubation media ($n = 9$). Asterisk indicates $p < 0.05$ at the same time points

Experiment 2

Media analyses

No significant differences were observed in pH between media at 0, 1, and 24 h. There was no consumption of glucose or pyruvate in IVF and L-15 media after 24 h of incubation ($p > 0.05$), but there was significant lactate release in all media ($p < 0.05$) (Fig. 5).

Viability assessment

FACS showed a mean of 4% necrotic ovarian cells in tissue samples incubated in all media and 15–19% apoptotic ovarian cells after 1 h of incubation, but less than 1% necrotic ovarian cells and 2–6% apoptotic ovarian cells after 24 h in all media (Fig. 6). No significant differences were observed for necrosis or apoptosis in different media or time points ($p > 0.05$).

Discussion

To our knowledge, this is the first report to evaluate metabolic substrates in different media currently used for ovarian tissue transportation. Our results shed new light on the impact of different media on ovarian tissue during storage. We observed high lactate release in all tested media after 24 h of storage at

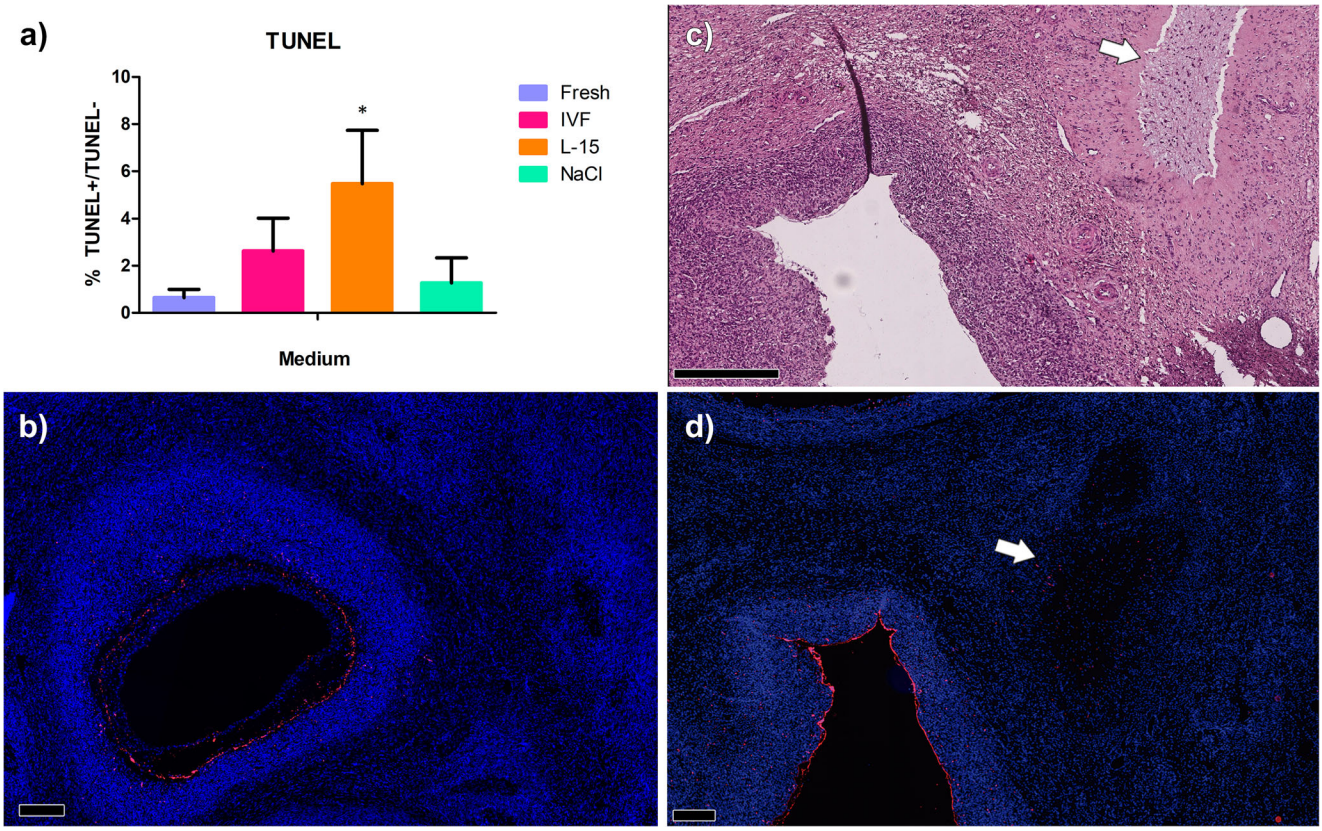

Fig. 4 Apoptosis was observed mainly in granulosa cells of antral follicles and fibrotic tissue after 24 h of storage in different media. Ovarian samples were stored at 4 °C in IVF medium, L-15, or NaCl solution for the designated times, after which apoptosis was evaluated using TUNEL assays (**a**). Asterisk indicates significantly different results ($p < 0.05$). Representative pictures show ovarian follicles in fresh tissue (**b**) and after 24 h of incubation in L-15 medium (**c**, **d**). Red = dead cells. Blue = DAPI. White arrows point to fibrotic tissue. **a**, **b**, **d** Scale bar = 200 μm. **c** Scale bar = 500 μm

4 °C, but no consumption of glucose and only low consumption of pyruvate. These results point to considerable metabolic activity in ovarian tissue during transportation.

This study evolved as a consequence of data obtained from the first set of experiments. Believing that storing ovarian tissue at 4 °C would not have a great impact on cell metabolism, in the first experiment, we evaluated only the longest period of transport. After analyzing our results, we decided to conduct a second series of experiments with an added control (1 h), in order to demonstrate the impact of 24 h of transport. Media used in the present study were chosen based on ovarian tissue transportation reports. IVF medium [15] and L-15 [16] are used in clinical practice in humans, while NaCl and PBS act as controls and have been used in ovarian transportation studies in different animal species [25, 26, 28–47].

Cell metabolism is intrinsically linked to acid-base balance. This balance is at risk, or lost, during severe ischemia, mainly because of lactic acid release [24, 48]. In this context, Tellado et al. [26] reported that follicular fluid pH was lower after porcine ovarian tissue storage in NaCl. This is consistent with the pH reduction we observed in L-15 medium in experiment 1 of the present study. On the other hand, the pH was altered in

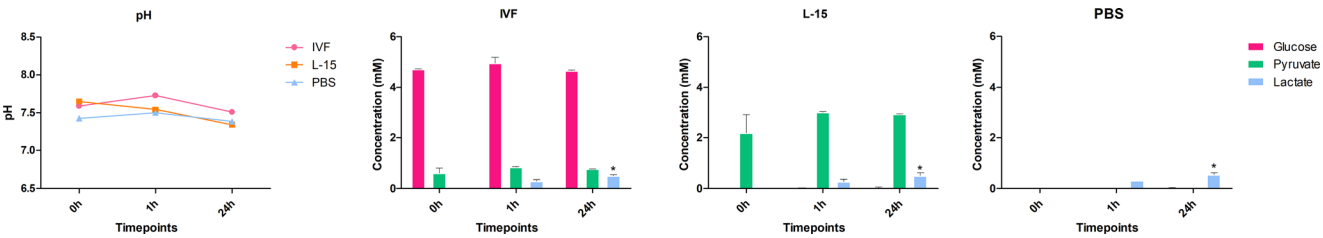

Fig. 5 pH and glucose, lactate, and pyruvate concentrations after ovarian tissue transportation in different media. Ovarian fragments of 1 × 1.5 × 0.5 cm were stored at 4 °C in 20 mL of IVF medium, L-15, or PBS for the indicated times, at which pH values were measured using a pH meter, and glucose, pyruvate, and lactate concentrations were determined in incubation media using a CMA600 enzymatic analyzer ($n = 4$). Asterisk indicates $p < 0.05$ for a given metabolite between 0, 1, and 24 h

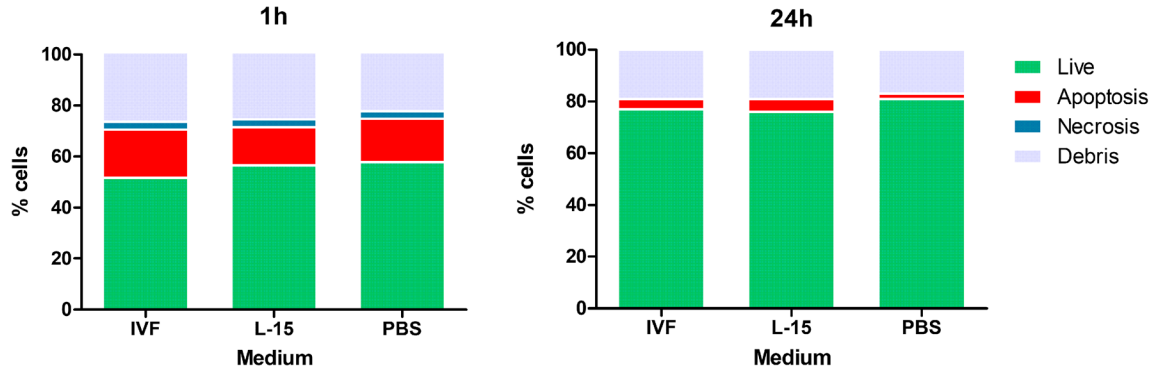

Fig. 6 Cell viability assessed by flow cytometry after ovarian tissue transportation in different media. Ovarian fragments of 1 × 1.5 × 0.5 cm were stored at 4 °C in 20 mL of IVF medium, L-15, or PBS for the indicated times. A piece of tissue was dissociated and cell viability was assessed with annexin V/propidium iodide with flow cytometry. Live cells are shown in green, apoptotic cells in red, necrotic cells in blue, and debris in gray

NaCl over time, possibly because it was the only medium that did not contain any buffer, since pH levels in PBS remained constant in experiment 2. Moreover, lactate concentrations were similar in all three media after 24 h of incubation in both experiments. Hence, the relatively acidic nature of NaCl at the beginning of the experiment did not appear to interfere with cell metabolism at 4 °C. In fact, cells can maintain their metabolism when extracellular pH levels are as low as 5.6 [24, 48]. Mild extracellular acidification may actually be beneficial to cells [49], inhibiting free radical generation and decreasing energy demands [24]. The levels of pH did not significantly alter in any media in experiment 2, possibly because the volume of media was higher and PBS contains phosphate buffer, avoiding great pH changes.

A lower pH might also affect LDH activity in NaCl [50]. Evaluating LDH activity in media is an indirect way of measuring necrosis [27], while TUNEL assays measure apoptosis. Our results showed higher apoptosis rates (5.47%) only in L-15 compared with fresh tissue (0.65%). The present study shows lower apoptosis rates than in sheep ovarian tissue stored in minimal essential medium (MEM) or Dulbecco's modified Eagle's medium (DMEM) for up to 24 h and cultured for 5 to 7 days [51, 52] and in mouse ovaries stored in DMEM for 24 h [53]. Our TUNEL findings and viability assessment by FACS support the hypothesis of increased lactate levels due to cell metabolism, since the percentage of apoptotic/necrotic cells was too low to affect lactate release in any media after 24 h. Experiment 1 revealed increasing LDH activity with time and similar LDH levels in all media after 24 h of storage, while experiment 2 showed less than 1% necrotic cells after 24 h. This discrepancy might be because media were not centrifuged before LDH assays. Curiously, we observed a higher percentage of viable cells after 24 h of transportation than after 1 h. This is consistent with reports by Isachenko et al. [54], who found more viable cells after storage of human ovarian tissue for 24 h at 5 °C, followed by in vitro culture (IVC) or culture in the chorioallantoic membrane of chick embryos. It may have been due to mitosis stimulation induced by the cold, as observed in neurons of rats exposed to low temperatures [55].

Glucose and lactate were quantified in porcine follicular fluid during transportation in NaCl [26] at 25 °C and showed decreased glucose and increased lactate concentrations, suggesting anaerobic glycolysis as a major metabolic event during transport. In contrast to these findings, we observed no glucose consumption in any media, low pyruvate consumption in L-15, but comparable lactate release across all media. Lactate concentrations were lower in experiment 2, undoubtedly because they were diluted in higher media volumes. Although glycine and glutamine present in L-15 might contribute to lactate release [56], there are no sources of energy in NaCl or PBS that could account for lactate production. Therefore, in our study, it is possible that ovarian cells used their internal energy sources rather than nutrients present in media to release lactate. Indeed, almost all mammalian cells, including ovarian cells, store fatty acids, which constitute sources of acetyl-CoA for lactate production [57], in lipid droplets [58, 59]. In oocytes and cumulus cells, triacylglycerol is the main lipid found in lipid droplets [60]. Palmitic, stearic, oleic, and linoleic fatty acids are most prevalent in oocytes of mammalian species, and saturated fatty acids make up the majority of lipids stored in oocytes [61]. Lipids are also an important source of energy during oocyte maturation; β-oxidation of fatty acids plays an essential role in oocyte maturation and developmental competence, as well as meiosis resumption and nuclear maturation in murine, bovine, and porcine oocytes [62, 63].

While the transport of vital organs is widely established, ovarian tissue transportation studies lag far behind [64]. Studies on organ preservation report that although cold ischemia during storage reduces cellular metabolism 12-fold, ATP and ADP are gradually depleted [65, 66]. In ovarian tissue, the pathway would then involve mitochondrial export of the tricarboxylic acid (TCA) cycle intermediate malate and its

cytosolic conversion to pyruvate and then lactate. Cells might also produce energy by autophagy, consuming cell components to survive [67]. Further studies are warranted to test these hypotheses, such as inhibition of fatty acid oxidation (FAO) [68] or LDH activity [69].

In addition, while follicle metabolism has been extensively studied (reviewed by Collado-Fernandez et al. [19]), follicles are just one of the numerous cell populations residing in ovarian cortex. These different cell populations are directly or indirectly involved in follicle survival and development. For instance, stromal cells make up 83% of the ovarian population [20] and play key roles in primordial follicle activation and further development [18, 70]. Indeed, our results may have been affected by the size of fragments [71], the presence of medullary tissue, and the volume of media used. Glucose, pyruvate, and lactate levels evaluated in the present study correspond to the metabolism of all ovarian cell populations, demonstrating the importance of investigating whole tissue dynamics.

Many effects of ischemia have been studied after ovarian tissue transplantation [5, 6, 72–75]. In particular, it has been reported that ovarian tissue hypoxia lasts for 3–5 days, with partial pressure of oxygen stabilizing around 10 days after transplantation of human ovary xenografts to mice [74]. Microdialysis assessments showed that anaerobic metabolism was maintained for up to 10 days after grafting human ovarian fragments to nude mice, namely until revascularization of the tissue [6]. When cells are engaged in anaerobic metabolism, they often consume their internal energy stores, accumulate metabolic byproducts, and export protons. Reperfusion triggers another series of events, generating reactive oxygen species (ROS) that cause more damage to cells [76]. In grafted ovarian tissue, the main consequences of ischemia revascularization include limited graft life span, empty follicles upon aspiration, and poor responder hormone profiles [77]. However, it is important to point out that ischemia does not begin after transplantation, but as soon as the tissue is removed from the patient, and continues until it is cryopreserved [9, 78]. Transportation of the ovary may take up to 24 h in clinical settings [9], and little is known about this process to date.

Studies in humans have assessed transportation by analyzing ovarian tissue after IVC [12–14]. While all of them concurred that low temperatures are useful when tissue needs to be transported for long distances, and observed little alteration to follicle morphology after transportation, evaluation by IVC may lead to bias, as this system is not well established for ovarian tissue and may not maintain cell interactions and follicle growth [79]. Investigating transportation protocols by techniques that resemble the physiological environment of ovarian tissue will provide us with more reliable information on understanding how this process affects fertility. Indeed, evaluating ovarian tissue transportation through transplantation, IVF, and embryo transfer, Kamoshita et al. [53] observed lower implantation rates after 24 h of transport, even though follicle morphology was similar to fresh controls. Our data indicate that ovarian cells might be consuming their own internal energy reserve in the first 24 h of transportation, which may play a role in the cascade of events that occur in the first 10 days post-grafting. To elucidate the impact of metabolic activity observed in the present study on fertility outcomes, xenotransplantation of transported tissue is recommended.

In conclusion, our results suggest marked metabolic activity in the ovaries at 4 °C, with ovarian cells apparently using internal sources of energy for lactate production rather than glucose or pyruvate present in some of the tested media. Since ovarian cells need their internal energy reserves for the post-grafting period prior to revascularization, this activity may have an impact on follicle and ovarian cell populations, influencing transplantation outcomes. Our findings highlight the importance of acquiring a better understanding of whole tissue dynamics in ischemia in order to develop a standard protocol that meets the needs of ovarian tissue during transport.

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Authors' contributions C.A.A. and J.M.V.V. conceived and designed the study. J.M.V.V., M.C.N.M.B., E.M., and C.A.A. acquired data. J.M.V.V., A.L.M.V., P.S., and C.A.A. analyzed and interpreted data. J.M.V.V. wrote the main manuscript text and prepared the figures. P.S., M.M.D., and C.A.A. edited the manuscript. All authors approved the final version to be published.

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Data availability Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval Since this study was performed with animals from a slaughterhouse, it was exempt from approval from an ethics board.

Consent to participate Not applicable.

Consent for publication Not applicable.

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