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Equivalent outcomes of human oocytes after vitrification or slow freezing with a modified rehydration protocol

Krzysztof Papis[1,2*], Karolina Hardej[1], Ewa Stachowiak[1], Krystyna Żyżyńska-Galeńska[3], Piotr Lewandowski[1] and Katarzyna Kozioł[1]

Abstract

Background According to data from numerous research studies and reviews, the efficiency of the oocyte slow-freezing method is believed to be compromised. Here, we attempt to challenge this notion by showing our retrospective evaluation of the efficiency of the traditional vs. successfully modified method of slow-frozen oocyte recovery compared with that of vitrified oocytes. Specifically, we compared the efficiency of a modified thawing/rehydration system applied to oocytes that had already been slow-frozen with the effects of oocyte vitrification. Moreover, we verified this comparison using chemical activation of slow-frozen vs. vitrified oocytes and parthenogenetic embryo development.

Results Twenty-two and 73 thawing cycles of slow-frozen oocytes were performed using traditional and modified rehydration methods, respectively. For comparison, 105 warming cycles of vitrified oocytes were analyzed. The survival rate of oocytes subjected to the traditional rehydration method was 65.1%. In contrast, significantly higher ratios of 89.8% and 89.7% of oocytes survived the thawing/warming procedure performed according to the modified rehydration procedure or vitrification, respectively ($P \leq 0.0001$). Clinical pregnancy and implantation rates tended to be higher after a transfer of embryos developed in the modified rehydration group vs. traditional rehydration group (33.8% and 25.5% vs. 23.5% and 13.8%, respectively) and were comparable to vitrification effects (30.1% and 26.6%). Transfer of embryos developed after modified post-thawing rehydration method resulted in 23 births with 25 healthy and one preterm baby, not significantly different from 28 births reported after oocyte vitrification. Slow-frozen oocytes that were chemically activated after the superior modified rehydration method gave similar survival (91.9% vs. 99.0%), activation (76.0% vs. 64.6%) and blastocyst rates (15.2% vs. 9.4%) in comparison with vitrified oocytes, respectively.

Conclusions The modified post-thawing rehydration method applied to slow-frozen oocytes offers benefits in terms of higher oocyte survival, fertilization, and development or activation rates, comparable to the respective measures of vitrified oocytes and, in clinical settings, high pregnancy, implantation, and birth rates. It may bring new hope to patients who have slow-frozen oocytes stored in IVF clinics.

*Correspondence:
Krzysztof Papis
krzysztof.papis@novum.com.pl

Full list of author information is available at the end of the article

Keywords Human oocyte, Slow-freezing, Propanediol, Rehydration method, Vitrification, Chemical activation

Introduction

For years, slow freezing has been a well-known and widely used method of oocyte and embryo cryopreservation in reproductive medicine and animal reproduction. Since 1985, when the vitrification method was introduced [1], some modifications have become more popular in these applications, notably its minimum volume or microvolumetric (MV) variant. MV vitrification is the only acceptable means of long-term cryogenic storage for oocytes of certain animal species, such as cattle or swine [2–5]. Human oocyte slow freezing, although assessed as less efficient than MV vitrification [6–8], apparently still remains in use in several clinics. Moreover, presumably, many slow-frozen human oocytes are currently in storage, still waiting for future use by women who decided to freeze them, or for possible donation [9].

Initially, the most popular approach to human oocyte freezing was derived directly from cleavage-stage embryo freezing methods [10, 11]. Supplementation of 1.5 M 1,2 propanediol (PrOH) solution with 0.1 M sucrose has led to an increased ratio of surviving embryos as well as a higher pregnancy rate after frozen embryo transfer [11, 12].

Compared to embryos, human oocytes are more sensitive to freezing: the direct implementation of an embryo freezing protocol resulted in post-thaw survival rates, which remained unacceptably low for many years. Raising the concentration of sucrose in the oocyte freezing medium from 0.1 M to 0.2 or 0.3 M increased the oocyte survival rate from 34 to 60% and 82%, respectively [13]. In spite of the progress in post-thaw survival of these modified-approach oocytes, clinical pregnancy (CPR) and implantation (IR) rates remained lower compared to those obtained from vitrified oocytes in a majority of available reports [6–8, 14–17].

Unlike the freezing step of the oocyte cryopreservation system, which has undergone successful modifications, the thawing method and PrOH post-thawing removal based on cleavage embryo thawing/rehydration procedures have remained largely unchanged. The few modifications proposed [18–20] showed little improvement in terms of efficacy and, apparently, did not deserve wider use. On the other hand, a successful modification of the rehydration method has already been proposed [21, 22] for frozen cleavage-stage embryos. It is based on a three-step use of sucrose solutions, which reduces cell swelling to an acceptable level, resembling procedures commonly used for vitrified embryos or oocytes. Parmegiani et al. [9, 23]. adapted this approach to oocytes, comparing the traditional PrOH-sucrose rehydration to the method used for vitrified oocytes, i.e., sucrose-only approach starting at 1.0 M concentration, the latter resulting in higher oocyte survival, activation, and parthenogenetic development rates. Our preliminary, auspicious retrospective data based on an original, modified approach to oocyte rehydration showed a real possibility of obtaining high survival, development, and pregnancy rates of slow-frozen oocytes [24].

Parthenogenetic activation of human oocytes bypasses the fertilization process, artificially initiating cleavage of embryos, some of which can develop to the blastocyst stage [25], providing a surrogate model for embryo development following cryopreservation [9, 26–28]. This approach has already been tested as a cryopreservation efficiency measure.

The current article presents our retrospective data of oocyte survival, clinical pregnancy, and birth rates obtained after thawing or warming procedures performed between 2007 and 2022, including two distinct methods of slow frozen oocyte rehydration compared to vitrified oocytes. To verify and support retrospective observations, we performed the chemical activation procedure of slow frozen or vitrified oocytes followed by the in vitro culture up to Day 7.

Materials and methods

Patients and procedures

Since 2004, our clinic has offered oocyte cryopreservation to IVF patients. The first successful thawing cycles were attempted in March 2007. This retrospective analysis is based on laboratory and clinical data obtained from 22 + 73 + 105 consecutive thawing or warming cycles performed between March 2007 and December 2022.

Chemical activation was applied to oocytes donated for research that underwent cryopreservation between 2007 and 2016 by slow freezing or vitrification. Enrolled patients signed an informal consent in accordance with the requirements specified by the regional ethical committee (project KB/1120/17, decision No 51/17).

Ovarian stimulation and fertilization procedures

Patients underwent one of two types of ovarian stimulation. Urinary gonadotropins (MENOPUR, Ferring Pharmaceuticals, Warsaw, Poland) or recombinant FSH (PUREGON, Organon, Warsaw, Poland; Gonal-F, Merck Serono, Warsaw, Poland) in combination with GnRH antagonist (Orgalutran, Organon or Cetrotide, Merck Serono) were used in antagonist cycles. Alternatively, GnRH agonist (Diphereline, Ipsen Pharma, Warsaw, Poland or Decapeptyl, Ferring Pharmaceuticals) was employed in the long agonist cycle co-treatment. Final oocyte maturation and ovulation were induced

by injecting 10,000 IU hCG (Pregnyl, Merck Serono) or 6,500 IU recombinant hCG (Ovitrelle, Merck Serono) as soon as three follicles of 17 mm were observed ultrasonographically. Oocyte retrieval was performed using vaginal ultrasound-guided puncture of ovarian follicles 36+/-1 h after hCG administration.

Two to three hours post-thawing/warming procedure, surviving oocytes were subjected to fertilization by intracytoplasmic sperm injection (ICSI), which was carried out according to standard methods. Fertilization was evaluated under an inverted microscope 16–20 h later. Sage (SAGE, CT, USA) or Vitrolife (Sweden) G1/G2 culture media were used for fertilization and subsequent embryo culture. The embryos were transferred to patients on days 2, 3, or 5/6 of culture.

Slow freezing procedures

The slow freezing/rapid thawing method, according to Fabbri et al. [13]. with minor modifications, was performed from 2004 to 2013 using Oocyte Freeze solution (MediCult/Origio, Denmark), based on Dulbecco's phosphate-buffered saline (PBS) containing PrOH and 0.3 M sucrose (refers to 269 oocytes subjected to thawing) until it was withdrawn by the producer. Afterward, oocytes were frozen using a PrOH solution supplemented with 0.2 M sucrose (FreezeKit Cleave, Vitrolife, Sweden), of which 311 were thawed and included in the retrospective analyses.

Three to 8 h after collection, oocytes devoid of cumulus cells were subjected to cryopreservation. After a 5–10 min incubation in base solution (Embryo Freezing Pack Vial 1, MediCult/Origio), oocytes were transferred directly to a freezing solution containing 1.5 M PrOH and 0.3 M (OocyteFreeze, MediCult/Origio) or 0.2 M (Vitrolife) sucrose for a total of 15 min of incubation (including the straws' loading period). All pre-freezing incubations were performed at room temperature (20–22°C) in Petri dishes, containing 1.5 ml of fluid each. One to 5 oocytes were loaded into single straws (Crio Bio System, France), which were heat sealed and put into an automated Kryo 10 series III biological horizontal freezer (Kryo 10/1.7, Planer, UK). Starting at 20 °C, the temperature was reduced to – 6.5°C at a rate of – 2 °C/min. After 5 min of soaking time, ice crystals nucleation was induced manually at – 6.5 °C. After an additional 10-min hold time, the straws were cooled to – 30 °C at a rate of – 0.3 °C/min and then rapidly to – 150 °C at a rate of – 50 °C/min. After an additional 10 min of temperature stabilization, the straws were transferred into liquid nitrogen for storage.

For thawing, all slow-frozen oocytes were warmed in the air for 30 s and then plunged into a 30 °C water bath for 30 s regardless of the rehydration system employed thereafter.

Slow-frozen oocytes rehydration in PrOH-sucrose solutions

In the first analyzed period, a traditional system of rehydration was employed [13, 29] with minor modifications. Briefly, the cryoprotectant was removed at room temperature by four-step rehydration approach in the successive thawing solutions (OocyteThaw, MediCult/Origio), containing 1.0 M PrOH + 0.2 M sucrose, 0.5 M PrOH + 0.2 M sucrose and 0.2 M sucrose (w/o PrOH), respectively. The final, rinsing step was performed in a base, isotonic solution. All steps were performed in Petri dishes containing 1.5 ml of fluid, for 3 min each. Finally, oocytes were warmed up and placed in warm IVF culture medium at 37 °C in an atmosphere of 5% CO2 in air.

Slow-frozen oocytes rehydration in sucrose-only solutions

In the second period, a modified, sucrose-mediated rehydration procedure was performed [24]. The specific components of vitrification warming media (Vitrification Warming Kit, SAGE, USA or Vitrification Thaw Kit, Fujifilm Irvine Scientific, USA) were employed for this purpose. For the first dilution step, the 0.5 M sucrose solution was used. For the second step, a 0.1 M sucrose solution was prepared by mixing one volume of 0.5 M sucrose with four volumes of base warming solution devoid of sucrose (20% dissolution). The entire rehydration procedure was performed in 50 µl droplets of successive solutions, prepared in a Petri dish, which was subjected to warming on a warming plate set up at 38°C during the course of the dilution procedure. This warming step was intended to stepwise return oocytes to optimal temperatures while avoiding excessive evaporation and osmotic changes in droplets of working solutions. Following a thawing procedure identical to that described for the first period, the contents of the straw were evacuated to an empty Petri dish, and within 1.5–2 min, oocytes were collected and moved with a small portion of fluid to a 50 µl droplet of 0.5 M sucrose solution. After 1 min, oocytes were moved to a 50 µl droplet of 0.1 M sucrose for 2 min and after that, to an already fully warmed base solution. After an additional 1–2 min, oocytes were transferred to the warmed IVF medium.

Oocyte vitrification procedure

The closed variant of the oocyte vitrification method was performed throughout the study. Oocytes were equilibrated in Kitazato (Japan) or Fujifilm Irvine Scientific (USA) solution. In both cases, the equilibration solution contained 7.5% DMSO and 7.5% ethylene glycol. After 10–12 min in the equilibration solution, oocytes were placed for up to 1 min in a vitrification solution containing 15% DMSO, 15% ethylene glycol, and 0.5 M sucrose or trehalose. Oocytes suspended in vitrification solution were placed on a High-Security Vitrification carrier (Crio Bio, France) in the smallest possible droplet of

fluid. Subsequently, carriers were inserted into protective straws, which were sealed and plunged immediately into liquid nitrogen. Sealed straws were placed in goblets and moved to liquid nitrogen tanks for storage.

Chemical activation

For the activation experiment, slow-frozen oocytes were subjected to the most efficient modified sucrose-only rehydration method. The traditional rehydration method was omitted in the research due to its low efficiency.

After thawing or warming, donated oocytes underwent the process of chemical activation with calcium ionophore (CultActive, Gynemed, Germany). Thawed/warmed oocytes were incubated in a 5μM ionophore for 15 min. Oocytes were incubated at 37°C, 5% O_2, 6% CO_2. After the activation, oocytes were washed in the G-IVF medium (Vitrolife, Sweden), placed in 2mM 6-DMAP in Multipurpose Handling Medium-Complete (Fujifilm Irvine Scientific) and incubated for 3 h at 37°C, and then washed in Continuous Single Culture medium (Fujifilm Irvine Scientific). Culture of activated oocytes was performed in a time-lapse EmbryoScope incubator. Dedicated EmbryoScope dishes had been prepared in advance by filling wells with Continuous Single Culture medium, subsequently covered by mineral oil. Oocytes were placed separately in each well and cultured for 7 days.

Activation was evaluated 18–20 h post-treatment. The number of activated oocytes, 2-cell embryos on Day 2 day, 4-cell embryos on Day 3 day and blastocysts on Day 7 was recorded and analyzed.

Endometrial preparation and embryo transfer

Embryo transfers (ETs) were performed in natural or artificial cycles after hormonal preparation of the endometrium. For natural cycles, the ovarian follicle's growth was monitored until an ultrasound examination confirmed follicle rupture. One day after ovulation, 300–400 mg/day of intravaginal progesterone (Luteina, Adamed, Poland) was applied. For artificial cycles, 2 mg of estradiol valerate (Progynova, Merck, Poland) was orally administered three times daily, starting on the first day of the cycle. When the thickness of the endometrium reached at least 7 mm (preferably 9–10 mm), 600 mg/day of intravaginal progesterone (Luteina) was administered. The estradiol and progesterone administration was continued for 10 weeks of pregnancy until the placental shift occurred.

Statistical evaluation

Morphological survival of thawed oocytes was evaluated under a dissecting microscope 2–3 h post-thawing and is expressed as a ratio of thawed oocytes. The normal fertilization rate was calculated as a percentage of oocytes that survived thawing procedures, whereas the Day 2 or Day 3 cleavage rate (referred to as development rate) was calculated out of fertilized oocytes. The clinical pregnancy rate (CPR) was presented as a ratio of gestational sacs observed during transvaginal ultrasound examination at least five weeks after embryo transfer and calculated out of the total number of performed ETs. The implantation rate was the ratio of gestational sacs to the total number of transferred embryos.

The age of patients at the time of oocyte collection is presented as a mean (± SEM) and compared between groups. Continuous variables were compared using a Kruskal-Wallis test for independent samples or an unpaired *t*-test with Welch's correction where applicable.

Differences between the proportion of oocytes that survived, were fertilized, and developed were analyzed for all treatment groups using the Chi-square test with Yates's correction (InStat 3.01, GraphPad Software INC., CA, USA). Values were considered statistically significant when $P \leq 0.05$.

Analysis of chemical activation data and parthenogenetic embryo development rates was performed with a non-parametric one-way ANOVA test using Statistica 12.0. Statistically significant results underwent a post-hoc Tukey's test. Values were considered statistically significant when $P \leq 0.05$.

Results

Retrospective analyses of laboratory outcomes

A total of 1203 oocytes thawed or warmed in 200 thawing/warming cycles were analyzed retrospectively in this report. It was observed that thawed oocytes subjected to the modified sucrose-mediated rehydration method and those warmed after vitrification (423/471, 89.8% and 559/623, 89.7%, respectively) had a significantly higher survival rate in comparison to the oocytes thawed and rehydrated traditionally (71/109, 65.1%; $P \leq 0.0001$) (Table 1).

The fertilization rate was found superior for the vitrified oocytes group in comparison with traditionally treated oocytes in period 1 ($P \leq 0.05$). On the other hand, Day 3 development rate of embryos derived from fertilized eggs was inferior in the vitrification group in comparison with both groups of slow-frozen oocytes (64.9 vs. 87.2 or 75.4%, $P \leq 0.05$, Table 1). However, the development rate of embryos calculated out of the oocytes that survived thawing/warming procedures was not different between the groups (Table 1).

Retrospective clinical results

Both implantation and pregnancy rates obtained after the transfer of embryos developed due to the modified post-thawing rehydration procedure or after vitrification tended to be higher in comparison with the traditional rehydration approach (Table 2). Four children were born in the first, traditional treatment period of

Table 1 Laboratory outcomes of slow frozen and vitrified oocytes

Cryopreservation method	Slow freezing		Vitrification	*P*-value
Rehydration method	Traditional, PrOH - sucrose	Modified, 0.5 M sucrose	1.0 M sucrose	-
Thawing/warming cycles	22	73	105	-
Oocytes treated (mean ± SE)	109 (4.95 ± 0.692)	471 (6.45 ± 0.546)	623 (5.93 ± 0.432)	-
Oocytes survived and subjected to ICSI (%)	71/109 (65.1) [A]	423/471 (89.8) [B]	559/623 (89.7) [B]	$P \leq 0.0001$
Oocytes fertilized (%)	39/71 (54.9) [c]	280/423 (66.2) [cd]	390/559 (69.8) [d]	$P \leq 0.05$
Embryo development rate of cryopreserved oocytes (%)	34/109 (31.2) [g]	211/471 (44.8) [h]	253/623 (40.6)	$P \leq 0.05$
Embryo development rate of survived oocytes (%)	34/71 (47.9)	211/423 (49.9)	253/559 (45.3)	NS
Embryo development rate of fertilized oocytes (%)	34/39 (87.2) [e]	211/280 (75.4) [e]	253/390 (64.9) [f]	$P \leq 0.05$

Values with different superscripts differ at: [A] vs. [B] $P \leq 0.0001$, [c] vs. [d]; [e] vs. [f]; [g] vs. [h]- $P \leq 0.05$, test chi-square with Yates's correction

Table 2 Clinical outcomes of transfer of embryos developed within 3 distinct oocyte treatment groups

Cryopreservation/rehydration method	Slow freezing/traditional	Slow freezing/modified	Vitrification/1.0 M sucrose	*P*-value
Patient's age (mean ± SEM)	33.7 (± 1.121)	34.1 (± 0.766)	31.44 (± 0.297)	NS
No. of thawing cycles	22	73	105	-
Number of ET	17	68	93	-
No. of embryos transferred (mean ± SE)	29 (1.71 ± 0.143)	106 (1.56 ± 0.074)	128 (1.38 ± 0.053)	*(P = 0.078)**
No. of pregnant patients (Clinical pregnancy rate)	4 (23.5%)	23 (33.8%)	28 (30.1%)	NS**
Implantation rate	4/29 (13.8%)	27/106 (25.5%)	34/128 (26.6)%	NS**
Miscarriage rate	-	1/27 (3.70%)	1/34 (2.94%)	NS*
No. of deliveries	4	23	28	-
Delivery rate (%)	4/17 (23.5)	23/68 (33.8)	28/93 (30.1)	NS**
No. of live births	4	26	33	-
Live birth rate/ ET (%)	4/17 (23.5)	26/68 (38.2)	33/93 (35.5)	NS**

*Kruskal-Wallis test; **Chi-square test

Table 3 Outcome following chemical activation of slow frozen or vitrified oocytes

Cryopreservation method	Slow freezing/ sucrose-only rehydration	Vitrification/ 1.0 M sucrose rehydration	*P*-value
Number of thawed/ warmed oocytes	136	97	-
Oocytes survived (survival rate)	125/136 (91.9%)	96/97 (99.0%)	NS
Oocytes activated (activation rate)	95 (76.0%)	62 (64.6%)	NS
Embryos developed to ≥ 2 blastomeres at day 2	92 (96.8%)	34 (54.8%)	$P \leq 0.01$*
Embryos developed to ≥ 4 blastomeres at day 3	70 (73.7%)	24 (38.7%)	$P \leq 0.01$*
Number of blastocysts	19	9	-
Blastocyst rate of survived oocytes	19/125 (15.2%)	9/96 (9.4%)	NS
Blastocyst rate of activated oocytes	19/95 (20%)	9/62 (14.5%)	NS

*ANOVA

the procedure before it was ceased due to its lower efficiency, whereas twenty-three births (including four sets of twins) occurred after a modification of the rehydration method. In the second period of the procedure, twenty-six children were born, as one fetus of a twin pregnancy was miscarried, resulting in the delivery of a single preterm baby. The remaining 25 children born in the second period were healthy and free of congenital malformations. A similar situation was observed in the vitrification group, where one twin pregnancy had a miscarriage of one of the fetuses. All remaining 33 children born after oocyte vitrification were healthy as well.

Chemical activation effects

A total of 233 oocytes were used for analyses of survival and development rates after slow freezing vs. vitrification by means of chemical activation.

One hundred and thirty-six slow-frozen oocytes were thawed and treated using the modified 3-step sucrose-only rehydration method, giving 125 survived oocytes (91.9%). Of 97 vitrified/warmed oocytes, 96 survived (99.0%) (Table 3). Successful activation and further development of activated oocytes were verified at days 2 and 3 after activation and at the blastocyst stage (Table 3). Out of 125 survived slow-frozen oocytes, 95 were successfully activated (76.0%), and 19 blastocyst-stage embryos were obtained (15.2%). Of 96 vitrified/warmed oocytes, 62 (64.6%) were activated, and only 9 blastocysts developed in vitro (9.4%). However, respective values were statistically not different (NS).

Discussion

In this paper, we present a retrospective analysis of data, showing that slow-frozen oocytes subjected to the modified, sucrose-only mediated rehydration approach demonstrated a significant increase in survival rates and a clear tendency for higher pregnancy and implantation rates in comparison with a traditional rehydration method. Additionally, post-thawing/warming chemical oocyte activation results support the notion that the modification of the slow-frozen oocytes rehydration method may lead to better laboratory outcomes.

In our experience, the vast majority of the slow frozen/rapid thawed oocytes were intact immediately after retrieval from the freezing straws. Thereafter, in a substantial proportion of thawed oocytes, ooplasm darkening and subsequent degeneration were observed, mainly during rehydration steps (unpublished data). Admittedly, the relatively low survival rate of frozen-thawed oocytes obtained in our clinic remained within the range of results presented by others, either in reported clinical [13, 18, 30, 31] or experimental trials [32] as well as in recommendations referring to similar freezing and thawing protocols [33]. However, the successful use of sucrose-only rehydration of frozen-thawed cleavage stage embryos [22] or blastocysts [34], as well as vitrified cleavage embryos or blastocysts [35–37] convinced us to modify our conservative approach to thawed oocyte treatment.

Sucrose as an osmotic buffer in cryoprotectant removal and rehydration procedures began to be used more than forty years ago [38, 39], enabling the fast expansion of frozen embryo transfer in bovine reproduction. Since then, solutions of disaccharides (e.g. sucrose, trehalose) have been widely used for the safe rehydration of vitrified oocytes and/or embryos, providing a limit of swelling of cells subjected to removal of permeated cryoprotectants [2, 40, 41]. On the other hand, the excessive post-thawing or post-warming shrinkage of cells has typically been considered harmless, though this aspect of rehydration procedures has rarely been examined [42]. Indeed, severe, post-thawing oocyte shrinkage might have been the reason for the much lower survival rate (60–62%) of human oocytes treated with two concentrations (0.5 and 0.2 or 0.3 M) of sucrose for 10 min each [19, 20]. On the contrary, we have already shown the efficacy of a sucrose-free approach to the rehydration of vitrified bovine oocytes utilizing a warm, serum-supplemented TCM 199 medium [3, 43]. Avoiding the unnecessary post-warming shrinkage of vitrified bovine oocytes treated usually with 1.0–0.25 M of sucrose, trehalose, or galactose [2, 5, 44, 45] was considered one of the key factors enabling the high developmental performance of oocytes vitrified in these experiments [3, 43].

The first step of the modified approach presented here – 0.5 M sucrose rehydration - resulted in moderate oocyte swelling suppression. Due to the time limit of this step (1 min), the approach does not allow severe, long-lasting shrinkage of the cells to occur. A second step of the procedure performed in a diluted 0.1 M sucrose solution supports cells' rehydration, preventing, however, further cell swelling. The efficacy of this approach seems to be fully confirmed by the high survival rate of oocytes and by the embryological and clinical data presented here. Our results mirror those of a study on mouse oocyte vitrification [46], which showed a great impact of rehydration stress on total (accumulated) osmotic damage of vitrified oocytes. Our data confirm formerly published observations on the efficacy of a sucrose-only rehydration procedure [9, 24]. It is essential from a practical point of view that the whole procedure can be performed conveniently using specific components of vitrification-warming kits. However our data strongly suggest the possibility of omitting the most concentrated, 1.0 M sucrose solution step in a rehydration procedure described and recommended by Parmegiani group [9, 23]. That being said, our results, obtained without any modification of the thawing procedure itself, contradict a part of Parmegiani et al.'s conclusions suggesting the necessity of "fast warming" to overcome several problems that these authors suspect would happen during "traditional" thawing [9, 23].

Encouraging seem to be the clinical data reported here, such as the relatively high pregnancy and "take home baby" rates. Of great importance is the good health of the children born: in only one case of a twin pregnancy did a single premature stillbirth occur. Moreover survival, fertilization and embryo development rates of slow-frozen oocytes subjected to the modified rehydration system gave very similar outcomes in comparison with vitrified/warmed oocytes both in clinical (retrospective data) and experimental settings (chemical activation). Pregnancy, implantation and birth rates were not significantly different either (Table 2). The examined retrospectively new rehydration procedure resulted in the births of 25 healthy babies, one pre-term infant, and one stillbirth, which seems to confirm no danger of this approach to the health of new-born children. Still, prospective randomized clinical trials would be essential for the final evaluation of the benefits resulting from this modified rehydration procedure in clinical practice.

It should be emphasized that the data presented here were obtained under typical IVF clinic conditions, which, at least in terms of time between oocyte retrieval and patients' decision about their eventual cryopreservation, were far from optimal. This period was usually much longer than the two hours indicated in some reports [29] and recommendations [47] and, obviously, longer than those

obtainable in clinics collecting donor's oocytes. On the other hand, Ubaldi et al. [48]. did not find this two-hour recommendation meaningful, having observed no impact on clinical effects of up to an 8-hour vitrification delay. However, the imperfections mentioned above might have had an impact on the relatively low fertilization rate observed in all analyzed groups, reaching approximately 70%. Moreover, possible bias in collected data should be considered due to the long observation time, corresponding with unavoidable variations in specific cryopreservation solutions, culture media, and culture conditions used throughout the entire period. The other limitation of this study was the low number of oocytes treated initially in a traditional manner, but we decided to cease this approach as soon as we realized its inefficiency. Nevertheless, the high survival rate and promising clinical data after launching the modified rehydration procedure of frozen oocytes were obtained (and still are) regularly and seem much closer to those reported for vitrified than for slow-frozen oocytes.

The retrospective data discussed above was strongly supported by our chemical activation trial, which was performed thereafter. It was confirmed that oocytes can be thawed with high efficiency when treated by our modified 3-step rehydration method. In chemical activation experiments, the parthenogenetic day 3 embryo development rate was even significantly higher after activation of slow-frozen oocytes than of vitrified counterparts (Table 3), which was an unexpected phenomenon.

As has been highlighted very recently, a relatively high proportion of patients who have decided to cryopreserve their oocytes are about to give up on their further storage due to high costs of storage and/or a dropping level of optimism about the real chances of getting pregnant using those oocytes [49]. The current common belief in lower outcomes from slow-frozen oocytes undoubtedly strengthens this pessimistic tendency. However, it should never deprive of hope for maternity of fertility preservation patients, specifically those treated for cancer who need to be sure that their former attempts to keep oocytes for future use were not useless. In light of our analyses, their hope may be ascertained. Current recommendations referring to numerous reports describing inferior results of the "traditional" slow freezing method indicate unanimously a preferential use of oocyte vitrification as the most potent cryopreservation approach [15, 17, 47]. Obviously, the opposite effect described here needs to be confirmed before any real rehabilitation of human oocyte slow-freezing approach could be considered. On the other hand, a rising concern over hidden side effects of oocyte vitrification has recently been observed. It was suggested that certain epigenetic modifications altering fertilization and/or post-fertilization performance of oocytes and/or embryos may be considered a potential source of health problems sometimes found in babies born from vitrified oocytes [50]. The problem of whether slow freezing methods may be equally or to a lesser extent involved in this kind of risk has already been raised [51]. In light of this, it might be advisable to screen and compare epigenetic consequences detectable in embryos developed from slow-frozen vs. vitrified oocytes, at least by means of an inclusion of slow-frozen oocytes in meta-analyses undertaken currently, to prevent abandoning the perhaps safer cryopreservation method, which would be simply injudicious.

Probably due to specific Polish legal considerations, cryopreservation of oocytes has not been very popular in our country until recently. Hence, the number of patients and, consequently, the number of oocytes subjected to thawing and included in this analysis were relatively low. Still, it seems evident that the presented method and data deserve wide dissemination as they provide an optimistic view of the fate of oocytes already collected by traditional slow freezing in hundreds of IVF clinics worldwide.

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Author contributions
K.P. designed and performed the procedures, analyzed and interpreted the data, wrote, revised and finally approved the manuscript; K.H. contributed to performing the procedures, acquisition and analyzing of data; E.S. contributed to performing the procedures, acquisition and analyzing of data, revised and approved the manuscript; K.Ż.G. revised and approved the manuscript; P.L. interpreted the data, critically revised and approved the manuscript, K.K. performed the procedures, interpreted the data, revised and finally approved the manuscript. All authors reviewed and approved the manuscript.

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Data availability
The data collected and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
According to the regional bioethical committee, no written informed consent was required for participation due to the retrospective nature of the first part of this study. Patients enrolled in the oocyte chemical activation study signed an informal consent following the requirements specified by the regional bioethical committee (project KB/1120/17, decision No 51/17).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Clinical trial number
Not applicable.

Author details
[1]"nOvum" Fertility Clinic, Bociania 13, Warsaw 02-807, Poland
[2]Center for Translational Medicine, Warsaw University of Life Sciences, Nowoursynowska 100, Warsaw 02-797, Poland

[3]International Institute of Molecular and Cell Biology, Księcia Trojdena 4, Warsaw 02-109, Poland

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