Research Article

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Regenerative Medicine

First transplantation of isolated murine follicles in alginate

Aim: Our aim is to develop an artificial ovary allowing survival and growth of isolated follicles and ovarian cells, to restore fertility in women diagnosed with pathologies at high risk of ovarian involvement. **Materials & methods:** For this, alginate beads containing isolated preantral follicles and ovarian cells were autografted to immunocompetent mice. One week after grafting, the beads were invaded by proliferating murine cells (12.1%) and capillaries. **Results:** The recovery rate of follicles per graft ranged from 0% to 35.5%. Of the analyzed follicles, 77% were Ki67-positive and 81%, TUNEL-negative. Three antral follicles were also identified, evidencing their ability to grow in the matrix. **Conclusion:** Our results suggest that an artificial ovary is now conceivable, opening new perspectives to restore fertility in women.

Keywords: alginate • artificial ovary • cancer • degradation • isolated preantral follicles • ovarian cells

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In recent years, we have seen an increase in available fertility preservation options and they are now more commonly proposed. While embryo and oocyte cryopreservation can yield good results in terms of fertility restoration, cryopreservation and transplantation of ovarian tissue is the only method for patients of prepubertal age or those who require immediate cancer treatment. Indeed, this approach can restore ovarian function, with 22 live births reported to date [1]. Even if frozen–thawed ovarian tissue transplantation is generally considered a safe procedure, reintroducing cancer cells that may be present in the cryopreserved tissue remains a risk, especially in the case of leukemia [2–4]. For patients suffering from this type of cancer, a safer option would be isolation and grafting of preantral follicles, since these entities are surrounded by a basement membrane that separates them from blood vessels, white blood cells and nerves [5].

To ensure survival, development and safe handling of isolated follicles, it is essential to encapsulate them in an artificial ovary. Follicles need to maintain their 3D structure in order to avoid breakdown of the metabolic link between granulosa cells (GCs) and oocytes, which could lead to uncoordinated growth and differentiation of somatic and germ cells [6]. Another advantage of a 3D artificial ovary would be the ability to effectively mimic physiological conditions, since many cellular processes in organogenesis occur exclusively in 3D [7]. The artificial ovary would also need to be biodegradable, since during folliculogenesis there is exponential growth of follicles from the primordial (0.03 mm) to the antral stage (18–24 mm) and massive recruitment of cells and vessels to support follicle development [8].

Because hematological malignancies represent one of the most common malignant diseases for which ovarian tissue needs to be cryobanked, an increasing population of young female patients would benefit from artificial ovary grafting [9]. Unfortunately, this procedure is not yet available. The first steps towards its development were taken in 1993 by Carrol and Gosden [10], who grafted a plasma clot containing a reorganized frozen–thawed suspension of digested

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murine ovaries to the ovarian bursa of mice and obtained live births. In the pilot study of Dolmans *et al.* [11], in which a plasma clot was used to encapsulate isolated preantral human follicles, we reported follicular development up to the antral stage after long-term grafting to severe combined immunodeficient (SCID) mice [11]. However, low recovery rates after grafting and the undefined composition of plasma led us to look for a more standardized and adapted matrix [8]. We therefore conducted a preliminary study [12], which showed that an alginate–matrigel matrix was able to degrade after 1 week of autografting in mice, allowing murine ovarian cells (OCs), a mix between stromal and endothelial cells, to survive and proliferate. Moreover, vascularization and a low inflammatory response were also observed [12].

In the present study, our aim was to develop a transplantable artificial ovary suitable for clinical applications. To this end, after testing different types of alginate, NovaMatrix 1% sterelized alginate (SLM) was chosen and used to autograft encapsulated isolated murine follicles and OCs to a peritoneal pocket that was created on the inner side of the peritoneum. After one week of grafting, we evaluated OC proliferation and survival, follicle survival, development and health status, and alginate matrix degradation, vascularization and inflammatory reaction.

Materials & methods

Ovariectomy procedure

The Committee on Animal Research of the Université Catholique de Louvain (Brussels, Belgium) approved the guidelines for animal welfare. For this study, we used six female NMRI mice aged 36–40 weeks, which were housed and ovariectomized as described [12].

Isolation of murine follicles & OCs

A tissue chopper (McIlwain Tissue Chopper, Mickle Laboratory, Guildford, UK) adjusted to 0.5 mm was used to mince the ovaries. The cutting procedure was swift (<5 min) and uniform-sized pieces were obtained. The minced ovarian tissue was then placed in 50 ml conical tubes containing 10 ml Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Bornem, Belgium) and resuspended several times with a Pasteur pipette in order to further disrupt the tissue to collect as many follicles as possible. The cell suspension was centrifuged at 40G for 10 min at 4°C and the supernatant was discarded, except for 5 ml placed in two sterile plastic Petri dishes. The follicles were picked up by two operators under a stereomicroscope (Leica, Van Hopplynus Instruments, Brussels, Belgium) over the course of 1 h maximum using a polycarbonate micropipette (Flexipet™ micromanipulation pipettes, 130 µm inner diameter; Cook Ob/Gyn, IN, USA), and placed in droplets of PBS without calcium and magnesium supplemented with 10% FBS at 4°C, where they were left until the process of follicular retrieval was completed. The two Petri dishes were then pooled together in a 50 ml conical tube and rinsed with 2 ml of PBS. The suspension was filtered through sterilized 80 µm and 11 µm nylon net filters (Millipore, Brussels, Belgium) and centrifuged at 231G for 5 min at 4°C. OCs were counted using Trypan Blue (Sigma-Aldrich) and a Bürker chamber (VWR, Leuven, Belgium) and resuspended in PBS to obtain a final concentration of 50,000 OCs/3 µl of medium. The remaining cells were fixed in 4% formaldehyde for 1 h, and cytospin slides were prepared using the Thermo Electron Shandon Cytospin 2 Cytocentrifuge (Thermo Scientific, Erembodegem-Aalst, Belgium).

Calcium alginate embedding

NovaMatrix 3% SLM alginate (NovaMatrix, Sandvika, Norway) was diluted to 1% with 3-(N-morpholino)propanesulfonic acid buffer (MOPS, Sigma-Aldrich). A 3 µl droplet containing isolated OCs and another of 2 µl containing 37–45 isolated follicles were transferred to 20 µl droplets of alginate solution. A solution of 0.1 M $CaCl_2$ (Sigma-Aldrich) was slowly poured around the alginate droplets to form the beads, which were incubated for 7 min and then moved to a small Petri dish containing MOPS buffer for 5 min.

Autotransplantation procedure

To graft the artificial ovary, the animals were reanesthetized using the protocol previously described for ovariectomy. As the alginate bead was larger than the ovarian bursa, we decided to create a pocket in the peritoneum to accommodate the bead. To this end, a circle was sutured on the anterior wall of the peritoneum at the level of the bladder using nonabsorbable 6/0 Prolene (Ethicon, Johnson & Johnson Medical NV/SA, Diegem, Belgium). The bead was then placed in the center of this circle and both ends of the thread were pulled together and tied to close the pocket. To induce angiogenesis before placing the alginate bead, the pocket was scratched with a scalpel blade. In case of bead fragmentation, pieces of the alginate bead were grafted, and those remaining were recovered in order to establish their follicular content. One bead was grafted per mouse, except for mouse 5, in which two beads could be grafted due to the high number of recovered isolated follicles.

The abdominal wall and skin were then closed with non-absorbable 6/0 and 4/0 Prolene (Ethicon), respectively. After surgery, injection of atipamezole (1 mg/kg; Antisedan, Pfizer) was used to reverse anesthesia. After 1 week, the animals were asphyxiated with CO_2, and the grafts were recovered under visual control, embedded in agarose 2% (UltraPure™ Agarose, Invitrogen, Merelbeke, Belgium) and fixed in formalin.

Histological analysis

After fixation in formalin, the grafts were embedded in paraffin for histological analysis. They were then cut into 5 µm serial sections and every fourth section was stained with hematoxylin–eosin for histological analysis in order to first identify the alginate matrix. To identify and evaluate isolated follicles and OCs, histological analysis was performed on recovered alginate beads.

Immunohistochemistry

Cell characterization

To characterize isolated OCs to be grafted, vimentin and CD34 immunohistochemical stainings were performed. Vimentin is a cytoskeleton protein mainly expressed in the cytoplasm of cells of mesenchymal origin, such as stromal and endothelial cells, and strongly expressed in ovarian stromal connective tissue [13]. On the other hand, CD34 protein is expressed in early hematopoietic and vascular tissue.

Vimentin and CD34 immunostainings were carried out according to a previously described protocol [12].

To quantify vessels (group of CD34-positive cells), one slide per graft, located in the middle of the alginate bead, was scanned by a Mirax Scan apparatus (Zeiss, Germany) and visualized using Mirax Viewer software. Around each alginate bead edge, a zone of 1 mm was defined. All vessels present in this area were counted and delineated, and the total vessel surface area was calculated.

Cell proliferation

OC and GC proliferation were analyzed by immunohistochemistry against Ki67, a nuclear protein present during G1, S, G2 and M replication phases, as previously described [12]. The only difference was that the primary antibody used overnight at 4°C was arat anti-mouse Ki67 antibody (1:50 dilution; M7249, Dako, Glostrup, Denmark).

For OC proliferation, one slide per graft, located in the middle of the alginate bead, was scanned by a Mirax Scan apparatus and visualized using Mirax Viewer software. Positive and negative cells present inside the alginate matrix were counted in order to calculate the proportion of proliferating cells. For follicular growth, follicles with at least one Ki67-positive GC were considered to be growing [14–17].

Apoptosis

Apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) to detect DNA fragmentation, according to a previously described protocol [12]. Tonsil tissue was used as a positive control.

TUNEL-positive surface areas were morphometrically analyzed to quantify apoptosis. To this end, sections were examined at 10 or 20× magnification, and all high-power fields (HPFs) were digitized, either for TUNEL staining or DAPI counterstaining, using a Leica DFC320 camera and IM50 program (Leica, Van Hopplynus Instruments, Brussels, Belgium). After grafting, TUNEL-positive and -negative cells present inside and on the edge of the alginate matrix were counted. For follicle apoptosis, TUNEL-positive and negative GCs were counted in order to calculate the proportion of positive GCs. Follicles were classified as undamaged when both the oocyte and all GCs were alive, moderately damaged when up to 50% of GCs were alive and highly damaged when both the oocyte and more than 50% of GCs were dead [18–20].

Analysis of the inflammatory reaction after grafting

Immunohistochemistry for CD45 was used to evaluate inflammation around grafted material, as CD45 is a marker of inflammatory cells, including leukocytes, macrophages and T lymphocytes [21]. For this purpose, the protocol described by Vanacker *et al.* [12] was followed.

One slide per graft, located in the middle of the alginate bead, was scanned by a Mirax Scan apparatus and visualized using Mirax Viewer software. A zone of 1 mm around each alginate bead edge was defined. All CD45-positive cells present in this area were counted in order to assess the CD45-positive cell population in each mouse.

Health status of the follicles

Inhibin alpha immunostaining was used to characterize GC health status. Inhibin alpha is secreted by GCs of murine and human follicles in order to inhibit FSH secretion by the pituitary gland. Inhibin alpha staining was assessed to clearly identify follicular structures without visible oocytes on hematoxylin–eosin slides. Histosafe was used to deparaffinize the sections, which were rehydrated in 2-propanol. Endogenous peroxidase activity was blocked and a demasking step was performed for 75 min at 98°C with citrate buffer and Triton 20%. Incubation for 30 min with goat serum

Table 1. Analysis of follicle recovery, vessel formation and CD45-positive cells around and infiltrating the alginate matrix after one week of grafting.

Mouse	Before grafting		After grafting									
			Follicles			Vessel formation				CD45-positive cells		
	Number of grafted OCs	Number of grafted follicles	Number of follicles found	Number of inhibin alpha-positive follicular structures	Total number of vessels analyzed	Total surface area of vessels (μm^2)	Total surface area analyzed (μm^2)	Vessel surface area/total surface area (%)	Number of vessels/ μm^2	Total number of CD45+ cells	Total surface area analyzed (μm^2)	Number of CD45+ cells/μm^2
1	50,000	44	4	5	559	57,951	6,328,810	0.92	8.83×10^{-5}	619	6,638,145	9.32×10^{-5}
2	50,000	40	4	2	279	55,305	5,633,357	0.98	4.95×10^{-5}	364	5,578,243	6.53×10^{-5}
3	50,000	40	1	3	537	141,368	9,224,862	1.53	5.82×10^{-5}	443	9,701,324	4.57×10^{-5}
4	50,000	37	7	1	622	79,700	8,692,227	0.92	7.16×10^{-5}	275	8,998,666	3.06×10^{-5}
5 Right	50,000	45	3	5	439	79,886	9,240,487	0.86	4.75×10^{-5}	441	10,727,992	4.11×10^{-5}
5 Left	50,000	45	7	9	482	210,981	9,181,593	2.30	5.25×10^{-5}	257	5,387,791	4.77×10^{-5}
6	50,000	43	5	4	547	87,237	10,996,376	0.79	4.97×10^{-5}	43	9,400,833	4.57×10^{-6}

OC: Ovarian cell.

and BSA was performed to block nonspecific binding sites, before incubation with mouse anti-human inhibin alpha antibody (1:200 dilution; MCA951S, Serotec) overnight at 4°C. The slides were incubated for 30 min at room temperature with streptavidin-HRP (1:2 dilution; K4001, Dako, Glostrup, Denmark). The chromogen used was diaminobenzidine (SK4100, Vector, Laboratories, Peterborough, UK). The slides were then counterstained with hematoxylin and mounted with aqueous mounting medium (Aquatex, Merck, Darmstadt, Germany). Negative (dilution solution without any anti-inhibin alpha antibody) and positive (mouse ovary) controls were carried out.

Groups of GCs with no visible oocyte on hematoxylin–eosin slides stained for inhibin alpha were considered as follicles.

Results

Follicle population characterization before grafting

In order to avoid follicular viability impairment, isolated follicles could not be staged before grafting. A supplementary isolation procedure was therefore performed to assess which follicle stages were grafted in our model. Non-grafted control isolated murine preantral follicles were retrieved with a polycarbonate micropipette (130 µm inner diameter) and were found at primordial to secondary stages, ranging from 17 to 101 µm in diameter.

OC population characterization before grafting

Following the isolation procedure, non-grafted OCs were fixed and cytospin slides prepared. After anti-vimentin staining, 5029 cells were counted and 90.5% of them were vimentin-positive. Anti-CD34 immunostaining was subsequently performed on these fixed isolated OCs and 10% of the 12,762 counted cells were CD34-positive. We can therefore assume that a large number of isolated cells encapsulated in the alginate matrix were stromal cells of mesenchymal origin.

Graft recovery rate & macroscopic aspect

After 1 week of autografting of isolated murine follicles and OCs, all seven peritoneal bursae could be clearly identified thanks to sutures in the six mice after dissecting the skin from the peritoneum with blunt scissors. All seven matrices were easily recovered from the intraperitoneal cavity. No adhesions with intraperitoneal organs or major inflammatory reactions around the grafting site were noted. The peritoneal scar was satisfactorily healed in all cases. Alginate beads occupied a mean of 314 sections after grafting (292 in graft 1, 404 in graft 2, 280 in graft 3, 228 in graft 4, 312 in graft 5 [right], 352 in graft 5 [left] and 336 in graft 6).

Follicle recovery, survival, growth & health status

Follicles were observed in all beads after one week of grafting. They were present either inside the alginate matrix or integrated in the tissue surrounding the remaining matrix. The overall recovery rate was 12% (30 out of 294 follicles), with an individual recovery rate varying between 0% and 19% per graft. However, after inhibin alpha staining was used to identify follicular structures without visible oocytes on hematoxylin–eosin slides, the recovery rate increased to 20% (59 out of 294 follicles) (Table 1).

All follicles showed a slightly stained round oocyte, with no sign of degeneration or retraction, surrounded by different numbers of well-organized GCs in close contact with each other, and no pyknotic bodies (Figure 1A–C).

Follicles were found at different stages of development after grafting, ranging from primordial to antral. Different growing follicles are shown in Figure 1A–C. Their growth was confirmed by Ki67 immunostaining (Figure 1D). At least three follicles were analyzed per graft, giving a total of 26 follicles altogether, and 77% of this number showed Ki67-positive GCs, demonstrating their proliferative status.

Follicle apoptosis was investigated by TUNEL to detect DNA fragmentation. A total of 16 follicles were analyzed by TUNEL, and 81% of them were classified as undamaged, as no TUNEL-positive cells were found. Three of the 16 analyzed follicles showed a maximum of two TUNEL-positive GCs out of 70. This demonstrates a low apoptosis rate in follicles after isolation, encapsulation and grafting in the artificial ovary.

Inhibin alpha immunostaining was used to characterize GC health status (Figure 1E) and evidence follicles when the oocyte was not visible on hematoxylin–eosin slides. A total of 34 follicles were evaluated after grafting (at least two follicles per graft) and 91.2% of them showed faint to strong brown staining in GCs, indicating that a high proportion of follicles were healthy and still able to produce inhibin alpha in the artificial ovary.

OC survival & proliferation

After 1 week of grafting, OCs were found in clusters inside and around the alginate matrix (Figure 2A). Vimentin immunostaining revealed the mesenchymal origin of these cells (Figure 2B). The healthy status of the OCs was confirmed by Ki67 immunostaining, which showed a mean percentage of 12.1%

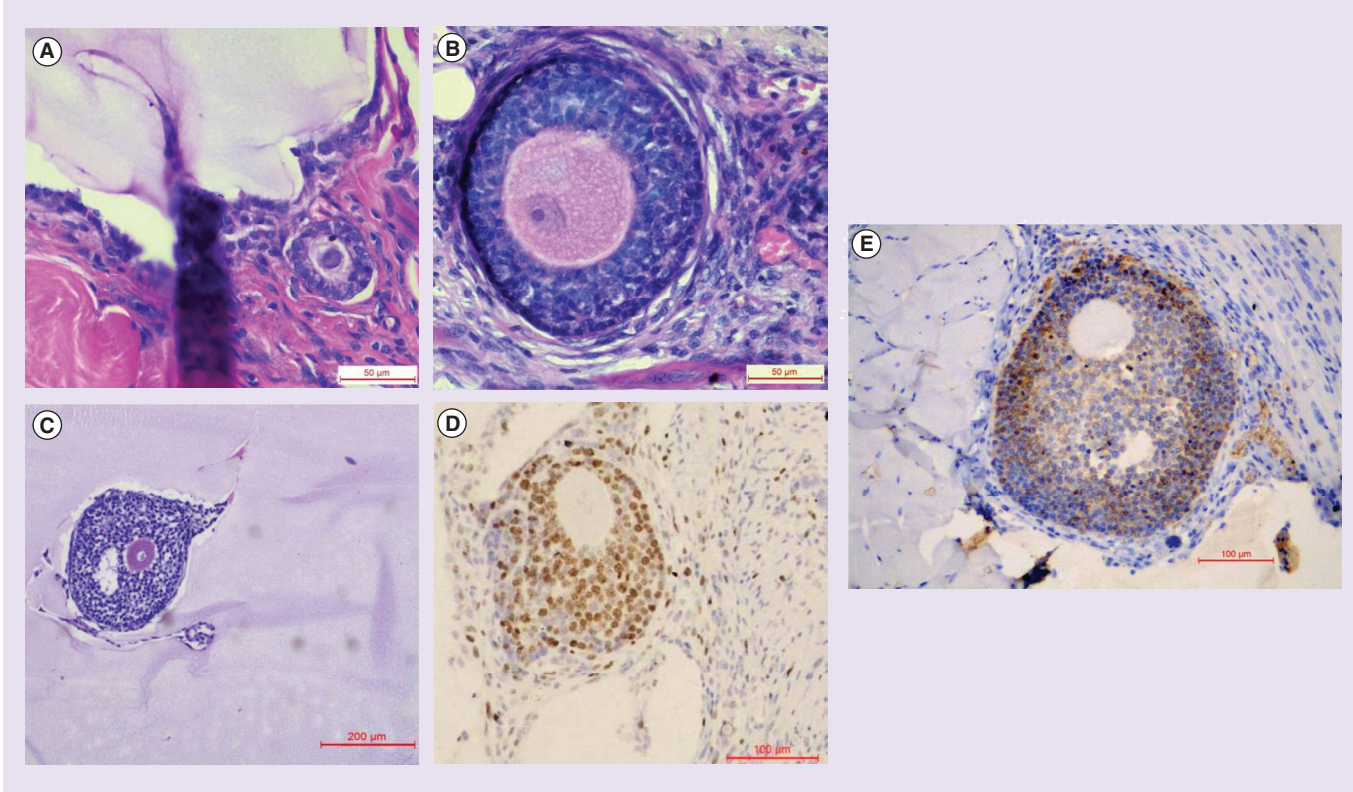

Figure 1. Isolated murine follicles recovered after 1 week of grafting in an alginate matrix. Primary **(A)**, secondary **(B)** and antral **(C)** follicles were recovered from inside the matrix or surrounding tissue. Proliferating follicles were evidenced by Ki67 immunostaining (proliferating granulosa cells in brown) **(D)**. Follicles were also able to produce inhibin alpha, as shown by brown cells **(E)**.

proliferating OCs (Figure 2C). DNA fragmentation was assessed by TUNEL, which evidenced 2.3% of dead cells (Figure 2D).

Scaffold degradation, revascularization & inflammatory response

Although five beads broke up into fragments during the grafting procedure, the pieces did not entirely disappear after 1 week of grafting. Thus, while degradation was found to have started after such a short period, it was not complete. In all grafts, beads and bead fragments were populated by proliferating OCs grouped in clusters and isolated growing follicles, as shown in Figures 1 & 2.

Blood capillaries and endothelial cells were observed around and inside the alginate matrix. Indeed, CD34-positive cells were found to surround and infiltrate the matrix (Figure 3A). Moreover, as indicated by the presence of red blood cells, functional blood vessels were also seen inside the alginate matrix after 1 week of grafting (Figure 3B, arrows). Table 1 shows the number of grafted follicles and OCs, and the number of vessels per surface unit, which varied from mouse to mouse but remained within the same range.

The inflammatory reaction, evidenced by CD45 immunostaining, was limited to the matrix periphery and no fibrous capsule was observed around the grafts. Some occasional CD45-positive cells were also found infiltrating the matrix (Figure 4). The small and globoid shape of these cells (stained in brown) suggests a leukocyte-type reaction. As shown in Table 1, a small number of CD45-positive cells per surface unit were identified in the grafts. Moreover, no mice died as a result of the procedure. They were all healthy after one week of grafting, proving that no major reaction was initiated by the alginate matrix.

Discussion

The present study shows promising results, indicating that it may well be feasible to develop an artificial ovary to graft isolated follicles and OCs. Indeed, previous pilot studies [10,11] have demonstrated survival

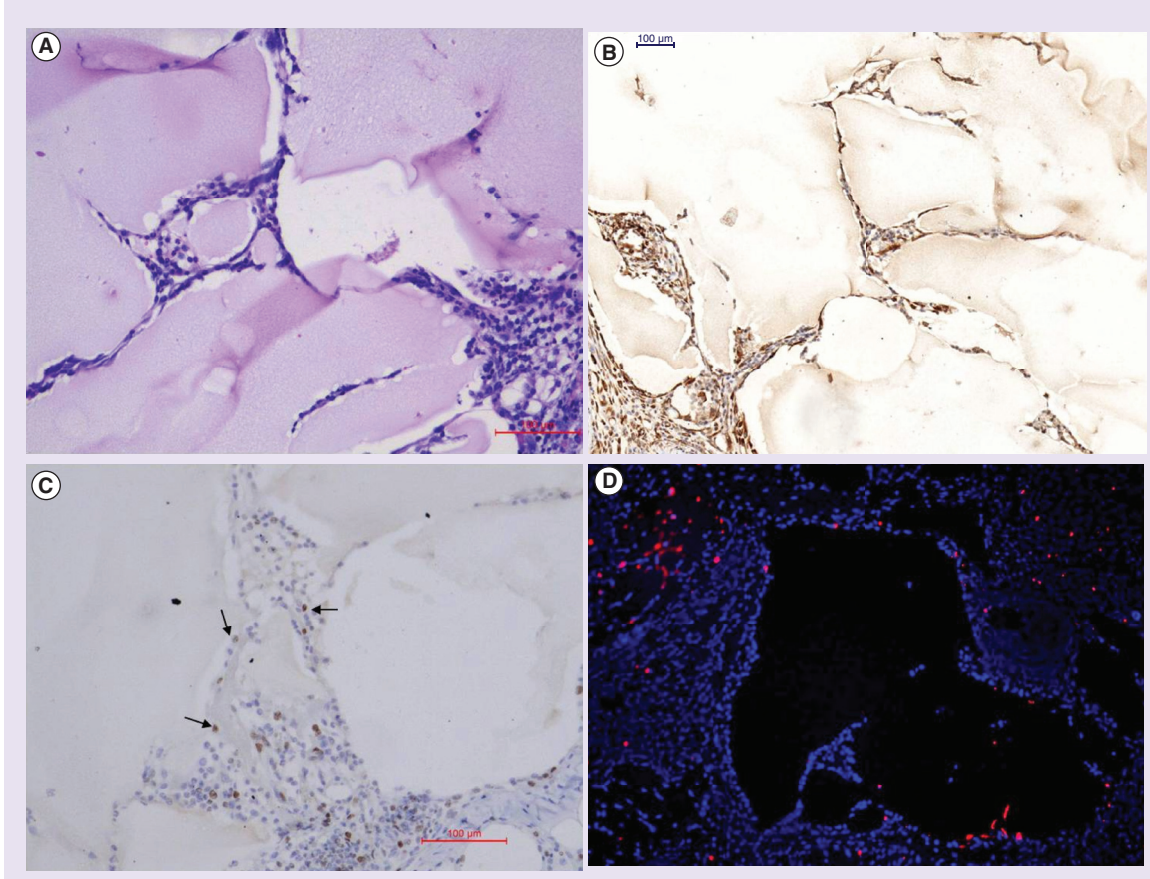

Figure 2. Grafted murine ovarian cells were alive and proliferating after 1 week of grafting. Ovarian cancers are shown grouped in clusters within the alginate matrix **(A)**. The mesenchymal origin of these cells was evidenced by anti-vimentin immunostaining **(B)**. Ovarian cells were able to proliferate inside the matrix, as shown by Ki67-positive brown cells **(C)**. DNA strand breaks were analyzed by TUNEL in isolated ovarian cells encapsulated in the alginate matrix: DAPI counterstaining (cells in blue) and TUNEL staining (cells in red) images are merged **(D)**.

and development of mouse and human follicles after grafting in a plasma clot. Here, we identified a GMP-grade alginate matrix (NovaMatrix 1% SLM) able to overcome the drawbacks encountered with plasma clots, such as low recovery rates or undefined plasma composition. Moreover, this matrix meets all the criteria to support isolated follicle and OC grafting, as it is degradable, biocompatible and easy to handle, while allowing follicle growth and development, stromal tissue restructuring and angiogenesis. This study is also the first to analyze grafting of isolated follicles and OCs together.

The follicle recovery rate after one week of grafting in an alginate matrix was similar to results previously obtained after transplantation of human ovarian follicles encapsulated in plasma clots to the ovarian bursa of SCID mice [11]. However, while all alginate beads and 7.5–35.5% of grafted follicles were recovered in our study, Dolmans *et al.* [11] recovered approximately 20% of grafted follicles, but less than 60% of grafted clots. Our higher graft recovery rate may be partially explained by the grafting procedure, as the plasma clot could be lost due to the hole made in the ovarian bursa. Regarding the 80% of follicles that disappeared after grafting, we believe that this was due to ischemic injury that occurs in the first days after transplantation [22]. Indeed, follicle loss after autografting of ovarian tissue has been reported to be between 60 and 80% [23,24].

Follicles were found either inside the alginate matrix or in tissue around the bead periphery. We believe this was due to the peripheral localization of some follicles within the matrix at the time of grafting. After 1 week, degradation and colonization of peripheral parts of the beads by grafted murine OCs and peritoneal tissue can be assumed, integrating follicles within tissue.

Interestingly, three antral follicles were found after 1 week of grafting. This may have been due to pick-up of secondary follicles, which is very common in suspensions of isolated murine preantral follicles. It is, thus, very likely that these secondary follicles grew to the antral stage during the week of grafting, as murine ovarian folliculogenesis is known to be completed within 3 weeks [25].

A high follicle survival rate was obtained within the artificial ovary after one week of grafting, as evidenced by histology, TUNEL analysis and Ki67 immunostaining. Indeed, most of the recovered follicles were morphologically normal and developing, as demonstrated by proliferating GCs. After TUNEL analysis, only three follicles showed less than 3% of atretic GCs. According to some authors, GC death is not only related to follicle atresia, but can also occur naturally, to a certain extent, during normal follicle development [26–30]. Moreover, follicles were able to produce inhibin alpha after isolation, encapsulation and 1 week of grafting, proving that they were healthy.

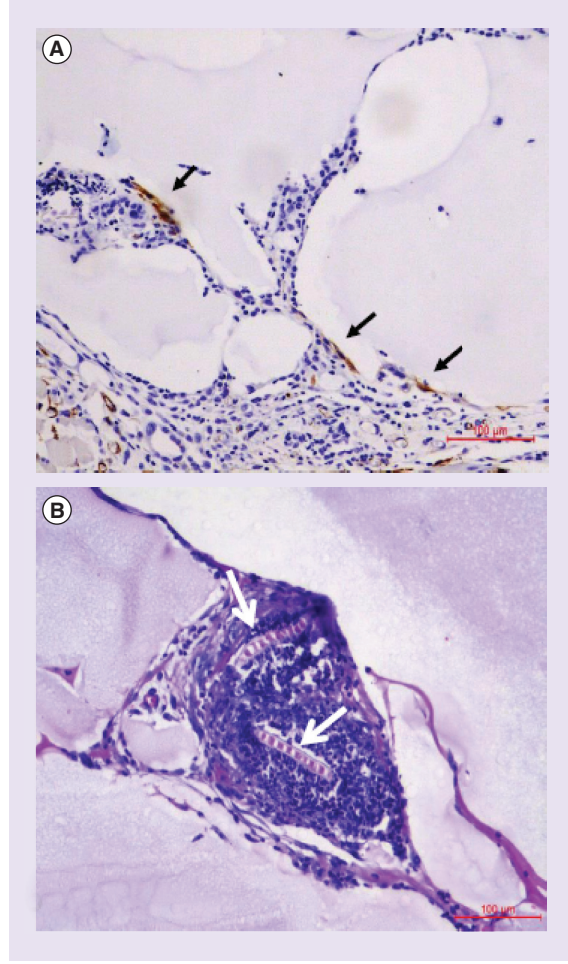

Figure 3. Blood vessels surrounding and infiltrating the alginate matrix after 1 week of grafting. Blood vessels and endothelial cells, stained in brown owing to anti-CD34 immunostaining (arrows), were located at the bead periphery or inside the matrix **(A)**. Some vessels were already functional after 1 week of grafting, as indicated by the presence of red blood cells (**B**, arrows).

In a previous study [31], we set up a follicle isolation protocol adapted for human ovarian tissue using an enzyme called Liberase Dispase High (DH). Since murine ovarian tissue is softer than human tissue, we did not use this enzymatic digestion method in the present study, as there was a risk of destroying the murine follicles. We performed a filtration step through 80 and 11 µm nylon net filters, as previously developed for human stromal cell isolation [32]. We believe this procedure is the reason why we grafted a smaller number of endothelial cells than in our previous study [12]. Indeed, vessels may not be disrupted by a mechanical isolation procedure alone. However,

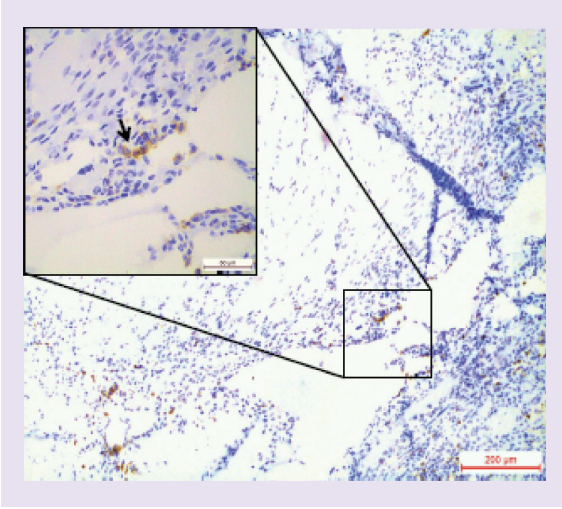

Figure 4. CD45-positive cells (in brown) were observed mainly surrounding the alginate beads, but some cells were also found inside (arrows).

healthy and proliferating follicles were found after 1 week of grafting and only a small percentage of OCs were apoptotic, demonstrating high survival rates of these cells in the alginate matrix. Moreover, the rate of proliferating OCs observed after 1 week of grafting was similar to what we obtained in our previous study [12], suggesting reorganization of stromal and endothelial cells into ovary-like tissue.

Despite the small proportion of endothelial cells grafted in the alginate beads, CD34-positive cells and infiltrating blood capillaries were identified surrounding and infiltrating the matrix after 1 week of grafting. Two hypotheses can thus be drawn: either stromal cells are able to differentiate into endothelial cells or endothelial cells are recruited to the grafting site by stromal cells. Both scenarios have already been described [33–36], and we believe these phenomena may contribute to the development of new ovarian tissue, as blood vessels containing red blood cells, a sign of normal functioning, were also seen within the alginate matrix after only 1 week of grafting. Moreover, the number of vessels per surface unit was also similar to our previous study [12], in which more endothelial cells were grafted.

Since NovaMatrix alginate contains only negligible levels of endotoxins and has already been applied in anti-acid compositions and for dental implants [37–41], we hypothesized that the inflammatory reaction to the grafted material would be low. Indeed, CD45-positive cells were fewer in number than in our previous study with an alginate–matrigel matrix [12]. On the other hand, we believe that to precipitate matrix degradation and angiogenesis, consequently improving follicle and cell survival, some degree of inflammation is necessary. Therefore, to encourage this process, we scratched the peritoneum [17], inducing granulation tissue formation. Donnez *et al.* showed that preparing the vascular bed can improve graft vascularization by increasing local angiogenesis [42,43].

Unfortunately, most of the alginate beads broke during the grafting procedure, so it is not possible to calculate their degradation. However, since isolated follicles were found inside tissue surrounding the bead periphery, marginal bead degradation can be assumed as soon as 1 week after grafting. No correlation was observed between the number of follicles found within tissue and the broken alginate beads, but it is noteworthy that the lowest follicle recovery rate (7.5%) was obtained after grafting of a broken bead and the highest (35.5%) after grafting of an intact bead. These broken beads highlight the fragility of the matrix during the surgical procedure. Matrix handling and composition are important factors that need to be enhanced in our model. Combining alginate with another polymer could improve not only handling, but also degradation. Shikanov *et al.* obtained live offspring after grafting of mouse ovarian tissue enclosed in a fibrin matrix [44]. They reported that fibrin mimics physiological angiogenesis, as it acts as a bridge facilitating host endothelial cell migration and proliferation.

In order to obtain a transplantable artificial ovary that can be used for clinical application, our team have already developed protocols to isolate human preantral follicles [31] and freeze them within alginate beads [45,46]. These protocols could be slightly modified to meet criteria for use in a clinical setting. However, although we grafted fresh isolated follicles and OCs in this study, once the artificial ovary is applied in a clinical setting, our goal is to proceed with isolation of follicles and OCs in two different steps. Prior to cancer treatment, an ovarian biopsy would be collected for freeze–thawing and preantral follicle isolation [46]; after treatment, a second biopsy would be harvested to isolate OCs, avoiding the risk of contaminating the artificial ovary with malignant cells [47].

Conclusion

This study demonstrates that alginate is a promising material for the creation of an artificial ovary. This matrix allowed isolated follicles to survive, develop and maintain their 3D structure. Isolated OCs were also able to survive and proliferate, and vascularization was observed around and inside the alginate beads. Moreover, this matrix is biocompatible, as evidenced by the low inflammatory response after 1 week of grafting.

Future perspective

To our knowledge, this is the first attempt to develop a biodegradable artificial ovary *in vivo* containing

isolated murine follicles and OCs suitable for clinical applications. We plan to extend our transplantation period in order to assess the final stages of follicle development and study degradation kinetics of the matrix. It will also be necessary to test xenografting of isolated human follicles and OCs in mice.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Approval for this study was obtained from the Ethics Committee of the Faculty of Medicine of the Catholic University of Louvain (ref. 2014/UCL/MD/007). The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

Executive summary

Grafting of frozen–thawed ovarian tissue is not safe in case of leukemia

- Hematological malignancies, such as leukemia, represent one of the most common malignant diseases for which ovarian tissue needs to be cryobanked. Unfortunately, these are known to disseminate in ovaries.
- A safer alternative would be isolation and grafting of preantral follicles in a biodegradable artificial ovary, which would preserve their 3D structure and mimic the physiological environment.
- A preliminary study conducted with an alginate–matrigel matrix containing murine stromal and endothelial cells showed encouraging results.

Materials & methods

- Primordial to secondary isolated murine follicles and a mix of stromal (90%) and endothelial (10%) murine cells were grafted for 1 week to the peritoneal wall of mice (n = 7). All the grafts were recovered after 1 week.

Results

- Follicle recovery, survival, growth & health status
  - Follicles from the primordial to the antral stage were observed in all beads (recovery rate between 7.5 and 35.5%). In total, 77% of them were growing and 81% were not apoptotic after isolation, encapsulation and grafting in the artificial ovary. Moreover, 91.2% showed anti-inhibin alpha staining in granulose cells, indicating that they were healthy.
- Ovarian cell survival & proliferation
  - After 1 week of grafting, ovarian cells were healthy, as 12.1% of them were proliferating and only 2.3% were apoptotic.
- Scaffold degradation, revascularization & inflammatory response
  - Alginate beads and fragments were surrounded and invaded by functional blood vessels and endothelial cells. No major inflammatory reaction was observed around the alginate matrix.

Conclusion

- An alginate matrix containing healthy preantral follicles and ovarian cells is a promising candidate for creation of an artificial ovary.

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