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**Ovarian tissue cryopreservation in girls undergoing haematopoietic stem cell transplant:
Experience of a single centre**

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(Article begins on next page)

**Title: OVARIAN TISSUE CRYOPRESERVATION IN FEMALE CHILDREN
UNDERGOING HAEMATOPOIETIC STEM CELL TRANSPLANT: EXPERIENCE OF A
SINGLE CENTRE**

Running title: Ovarian tissue cryopreservation in children

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Abstract

Fertility after childhood cancer is a major concern. The risk of subfertility depends on the type of malignant disease and its treatment. Conditioning regimens before haematopoietic stem cell transplant (HSCT) present a high risk (>80%) of ovarian failure. Since 2000 we proposed to female patients undergoing HSCT at our Centre cryopreservation of ovarian tissue to preserve future fertility. After clinical and haematological evaluation the patients underwent ovarian tissue collection by laparoscopy. The tissue was analyzed by histologic examination to detect any tumor contamination and then frozen following the slow freezing procedure and cryopreserved in liquid nitrogen.

Since August 2000 to September 2013, 47 patients planned to receive HSCT, underwent ovarian tissue cryopreservation. The median age at diagnosis was 11.12 years and at the time of procedure was 13 years. Twenty-four patients were not pubertal at time of storage, while 23 patients had already experienced menarche. Median time between laparoscopy and HSCT was 25 days. Twenty-five out of 27 evaluable patients (93%) developed hypergonadotropic hypogonadism at a median of 23.30 months after HSCT. One patient requested autologous orthotopic transplantation that resulted in one live birth. Results show a very high rate of iatrogenic hypergonadotropic hypogonadism highlighting the need for fertility preservation in these patients.

Introduction

1 Survival after childhood cancer has substantially improved during the last decades and is
2 now up to 80% considering all diseases, and nearly 75% of the patients will be living 10
3 years after diagnosis (1). Even more, long-term survival rate of children undergoing
4 haematopoietic stem cell transplant (HSCT) is constantly increasing.

5 It is now well known that improving in survival presents, on the other side, an increase in
6 mortality and morbidity in long term survivors (2-3-4).

7 Among all the late effects, infertility is reported as a major concern, especially in female
8 cancer survivors (5).

9 Cancer treatment often involves aggressive radiotherapy or chemotherapy, which may
10 permanently impair reproductive function. Ovarian damage after HSCT is determined by
11 conditioning regimen that can include chemotherapy and/or radiotherapy. This effect could
12 be increased by previous exposure to gonadotoxic treatment (6).

13 In particular, total body irradiation (TBI) and older age at time of HSCT can negatively
14 affect the persistence of ovarian function and the onset of premature ovarian failure (POF)
15 (7-9). When administered before puberty, TBI is less gonadotoxic, with 40-60% of patients
16 experiencing spontaneous recovery versus 10-14% in post-pubertal girls (6). The
17 protective effect of younger age might be related to the higher number of nongrowing
18 follicles (10), to the higher resistance of primordial follicles to vascular phenomena and
19 fibrosis or to paracrine factors (11).

20 Moreover, a model has been evaluated to predict the age of onset of menopause
21 according to radiation dose and age at irradiation (12).

22 Loss of ovarian function after chemotherapy that includes an alkylating agent
23 (cyclophosphamide, busulfan) could result in both sterilization and endocrine function
24 deficiency as ovarian hormonal production is closely related to the presence
25 of oocytes and maturation of the primary follicles (13-14).

26 Due to all these factors the risk of infertility in patients undergoing conditioning regimen for
27 HSCT has been defined as >80% (10).

28 Fertility preservation is a key component of POF management in young people and should
29 be considered for all young people undergoing potentially gonadotoxic cancer treatments
30 or at high risk for ovarian failure.

31 Cryopreservation of ovarian tissue is the main option available to preserve fertility in
32 women who require cancer treatment but cannot delay the chemotherapy and in
33 prepubertal patients (15). The advantage is that it requires just few days to plan and
34 perform the laparoscopic surgery and, as the retrieval of ovarian tissue is not dependent
35 on the menstrual cycle, no delay in treatments is required. Moreover this technique allows
36 the storage of a great number of primordial follicles that are relatively resistant to
37 cryodamage (about 70%–80% survival) (16).

38 In our Centre, since 2000, we enrolled female patients at high risk for subsequent infertility
39 in the “Fertisave” protocol, consisting of laparoscopic ovarian tissue cryopreservation.

40 **Patients and methods**

41 After obtaining the approval of the ethical committee we proposed to female patients
42 candidate to HSCT who were at high risk of subsequent infertility, ovarian tissue
43 cryostorage to preserve their fertility.

44 Informed consent was signed by patients or parents/legal guardians. Data on previous
45 chemo/radiotherapy exposure, conditioning regimen, gonadal function have been
46 collected. After clinical and hematological evaluation the patients were referred to surgery.

47 Ovarian tissue retrieval was frequently scheduled at the same time of another procedure
48 such as bone marrow harvest or placement of a central line for chemotherapy

49 administration. Ovarian tissue collection was performed by laparoscopic surgery. We
50 collected mono or bilateral ovarian cortex biopsies. The amount of cortex to cut is a

51 compromise between the need to cryopreserve as much tissue as possible and the need

52 to maintain an ovarian volume permitting the future transplantation: usually about 50% of
53 ovarian cortex was removed (17). Atraumatic scissors were used to perform the ovarian
54 cortex explant, and electro-coagulation was avoided as much as possible in order to
55 preserve the tissue to be cryopreserved as well as the remnant ovary. After retrieval the
56 ovarian tissue was immediately rinsed in in vitro fertilization (IVF) buffered medium and
57 transported in ice to the IVF laboratory, where the freezing procedure took place.
58 At the same time we performed histological examination before storage, to detect any
59 tumor contamination in all patients affected by malignant disease.

60 In our Centre rapid transport of the removed tissue to the laboratory was performed,
61 anyway it has been demonstrated that transport from the place of removal to the tissue
62 bank is also possible over a longer period of time (18). Once the sample reached IVF
63 laboratory ovarian cortex was enucleated from medullary compartment with sharp scalpel
64 dissection, and then it was cut in small thin cubes and placed in Petri dishes containing
65 slow freezing media (19). Then ovarian cortex was stored in liquid nitrogen following slow-
66 freezing procedure (20). Cryoprotectants used for the freezing procedures were the same
67 used for oocyte cryopreservation although time of exposure was significantly increased, as
68 cryoprotectant penetration into ovarian strips requires several minutes. After completing
69 partial dehydration of the fragments, they were placed in 1.5 ml cryovials (4/5 each),
70 loaded in a programmable vertical freezer (Kryo Planer) and frozen at -196°C. While
71 ovarian tissue was prepared for cryopreservation, a small piece of the ovarian cortex was
72 sent to pathologist in order to evaluate the number and density of primordial follicles and
73 the possible presence of tumoral cells (21-23).

74 Prior to perform reimplantation, a small piece of frozen-thawed tissue has been analyzed
75 to check the presence and density of morphologically normal primordial follicles. At the
76 time of transplantation a small fraction of the bilateral remnant atrophic ovaries were
77 collected in order to detect possible presence of follicles. Thawing procedure required

78 removal of cryoprotectant from the ovarian strips. Cryovials were exposed to room
79 temperature for few minutes, plunged in a 30°C water bath and exposed to decreasing
80 stepwise solutions of cryoprotectants. The fragments were placed in a Petri dish filled with
81 IVF buffered medium equilibrated at room temperature and carried to the operating room.
82 Transplantation took place into the pelvic cavity (orthotopic transplant). The advantages of
83 orthotopic transplantation include the possibility of natural conception, the favorable
84 environment for follicular development and the proven efficacy in restoring fertility (24).

85 **Results**

86 Since August 2000 to September 2013, 228 female patients underwent HSCT in our
87 Center. Forty-seven (20.61%) underwent ovarian tissue cryopreservation. The other
88 patients were excluded due to parents' refusal, poor prognosis or lower risk of subsequent
89 infertility.

90 Patients' characteristics are summarized in Table 1.

91 Patients were affected by: Blackfan Diamond Anemia (n=1), Ewing Sarcoma (n=3),
92 immunodeficiency (n=2), Acute Myeloid Leukemia (n=11), Acute Lymphoblastic Leukemia
93 (n=14), Chronic Myelogenous Leukemia (n=5), Non Hodgkin Lymphoma (n=2),
94 Myelodysplastic Syndrome (n=2), Thalassemia (n=7).

95 The median age at diagnosis was 11.12 years (range: 0-17.49 years). The median age at
96 the time of procedure was 13 years (range: 2.7-20.3 years).

97 Twenty-four patients (51%) were not pubertal at time of storage, while 23 patients (49%)
98 had already experienced menarche.

99 Laparoscopic surgery resulted in no acute or chronic complications.

100 Histological examination revealed no tumor contamination in all patients affected by
101 malignant disease (n=36).

102 Eleven patients (23.4%) had cryopreservation before undergoing any treatment except for
103 iron chelation treatment in thalassemic patients (n=7) while 36 patients (76.6%) had
104 already received chemotherapy.

105 Median time between laparoscopy and HSCT was 25 days (range: 14-595 days).

106 Conditioning regimens were TBI-based (12 Gy) in 23 patients (48.9%), Busulfan-based in
107 21 patients (44.7%) and without TBI or Busulfan in 3 patients (6.4%).

108 Five patients (10.6%) received additional treatment after HSCT: basin radiotherapy 54Gy
109 (n=2), lower limb radiotherapy 54 Gy (n=1), imatinib (n=1), second HSCT (n=1).

110 Median follow up time was 6.54 years (range: 0.30-13.68 years), median age at last follow
111 up was 18.6 years (range: 5.46-29.36 years).

112 Forty patients (85.1%) were alive at last follow up while 7 (14.9%) were dead due to
113 progression of disease or transplant related mortality.

114 Hypogonadism

115 Thirteen patients were not evaluable for hypogonadism due to early age (10 patients), lost
116 to follow up (2 patients) and continuous treatment with estroprogestinic after transplant (1
117 patient).

118 Twenty-five out of 27 evaluable patients (93%) developed hypergonadotropic
119 hypogonadism at a median of 23.30 months (range: 1.13-123.37 months) after the end of
120 treatment. Twenty-four patients (96%) are in estroprogestinic therapy.

121 The median age at diagnosis was 10.59 years (range: 0-16.86 years). The median age at
122 cryopreservation was 13.6 years (range: 4.39-20.31 years). Fourteen patients (56%) who
123 developed hypogonadism were pubertal at time of cryopreservation.

124 The therapy before cryostorage consisted of polichemotherapy in 20 patients (80%),
125 tyrosine kinase inhibitor treatment in 2 patients (8%), iron chelation in 2 patients (8%) and
126 no therapy in 1 patient (4%).

127 Conditioning regimen was TBI-based in 13 patients (52%) and Busulfan-based in 11
128 patients (44%).
129 Two patients have not developed, to date, hypogonadism, but no evaluation could be done
130 on subsequent development of premature ovarian failure.
131 One patient is affected by Chronic Myeloid Leukemia, diagnosed at the age of 13.74
132 years, treated with oncocarbide before HSCT, and with a TBI-based HSCT. She is now
133 23.78 years old, in treatment with tyrosine kinase inhibitors, due to a molecular relapse of
134 the disease.
135 The second patient is affected by thalassemia and underwent a Busulfan-based HSCT at
136 the age of 7.66 years. She is now 15.9 years old with regular menses.

137 Follicles

138 The median of collected follicles was 25/mm² (range: 0-120/mm²).
139 Evaluating the pubertal status at time of ovarian tissue collection the median of collected
140 follicles was 20/mm² (range: 4-45/mm²) and 35/mm² (range: 0-90/mm²) in pubertal and
141 pre-pubertal patients, respectively.
142 The median of collected follicles was 25/mm² (range: 3-120/mm²) in patients that
143 underwent only chelation treatment or tyrosine kinase inhibitor treatment and 26/mm²
144 (range: 0-90/mm²) in patients that underwent gonadotoxic treatment before collection.
145 The median in patients who subsequently developed hypogonadism is 25/mm² (range: 0-
146 85/mm²).

147 Pregnancies

148 One patient requested autologous orthotopic transplantation of ovarian cortical tissue that
149 resulted in one live birth (25).

150 **Conclusions**

151 Fertility after childhood cancer has become a topic of major concern in the last few years.
152 Wallace et al. have defined the risk of subfertility related to the type of malignant disease

153 and its associated treatment (12). According to these criteria the conditioning regimen (TBI
154 and chemotherapy) before HSCT presents a high risk (>80%) of subsequent infertility (7,
155 9, 12).

156 TBI and older age at treatment are well known risk factors for subsequent hypogonadism
157 as well as busulfan administration but no prediction can be made of the real risk for
158 developing infertility (7, 12, 26).

159 Since 2000, in our Centre, we proposed an experimental protocol for ovarian tissue
160 cryostorage to female patients undergoing HSCT, to preserve future fertility.

161 Different series of ovarian tissue cryopreservation in female children have been recently
162 reported (27-32). The number of patients ranged between 23 and 58 for each study. The
163 surgical technique varied from whole ovary collection to multiple biopsies of the cortical
164 tissue. The main goal was to evaluate the feasibility of the procedure.

165 To date almost 30 live births have been reported worldwide after orthotopic autologous
166 ovarian transplant (24, 26, 33-48) whereas heterotopic graft has led to one twin
167 pregnancy (49), a biochemical pregnancy (50) and four spontaneous pregnancies with
168 three live births were described as a result of a reactivation of the native ovary (51).

169 The analysis of the recovery of ovarian function is difficult because of the lack of reports in
170 the literature which indicate how many patients in the world have been subjected to
171 transplantation of ovarian tissue, anyway the recovery of ovarian function has been
172 described in all published cases of ovarian transplantation, both orthotopic and
173 heterotopic. Donnez et al. describes an average time of approximately 3-4 months from
174 graft to the recovery of ovarian function, in agreement with the timing of folliculogenesis
175 (34).

176 At our Centre all the patients addressed to ovarian tissue cryopreservation presented a
177 high risk to develop future infertility. The results showed a very high percentage of
178 hypergonadotropic hypogonadism (93%) occurrence on the evaluable patients, and no

179 current evaluation of POF could be done. This is a confirmation that the patients we
180 selected were actually good candidates for ovarian tissue cryostorage thus balancing
181 possible surgical complications. Nonetheless, in our series we reported no surgical
182 complications and the patients underwent subsequent HSCT only after a median time of
183 25 days from surgery.

184 Some of the previous reports also included patients with a low-medium risk of subsequent
185 infertility, who went through spontaneous pregnancies after treatments. In our opinion
186 ovarian tissue cryopreservation should not be offered to patients with a moderate risk of
187 gonadotoxicity as surgical risk is not compensated with a real advantage in this group of
188 patients.

189 No evaluation could be done on the follicle density of the collected tissue and any
190 correlation to previous therapy because of the low number of patients with the same
191 characteristics.

192 The report of a live birth among our patients gave us a confirmation of the effectiveness of
193 our technique of ovarian tissue retrieval, cryopreservation and transplantation (25).

194 Two problems should be argued: the possibility to restore hormonal ovarian function and
195 the tumor contamination of the cryopreserved tissue.

196 It has been reported that there are individual variations in the duration of endocrine
197 function after transplantation; ovarian function has been demonstrated to persist up to 7
198 years after transplantation with a mean duration of 4-5 years if follicular density is well
199 preserved (24). Ovarian tissue transplantation can represent an option to induce
200 spontaneous puberty in pre-pubertal patients undergoing primitive hypogonadism, as it
201 has been recently reported (52-53), but this approach should be validated in the future.

202 Many authors recently debate about the risk of reimplanting ovarian tissue with tumor cells
203 contamination (54). Sommerzer et al have previously defined the risk of ovarian

204 metastases according to cancer type with the higher risk for leukemia, neuroblastoma and
205 Burkitt Lymphoma (55).

206 In particular ovarian metastases have been found in up to 30% of ALL patients at autopsy,
207 even if they are rarely clinically detected (56). Recently, using disease specific PCR
208 techniques, Dolmans et al found contamination of ovarian tissue in 33% of CML patients
209 and in 70% of ALL patients (57). At the same time, Greve et al. reported that ovaries from
210 leukemia patients in complete remission do not appear to contain viable malignant cells, in
211 contrast to ovarian tissue retrieved before chemotherapy (58-59).

212 As far as our series of patients, no disease could be detected by histological examination
213 of the tissue before cryostorage. Furthermore, we experimentally evaluated by RT-PCR
214 the expression of molecular translocations, when present at the diagnosis of the disease
215 (n=7), and no positivity has been found (data not shown).

216 However it is now recommended that ovarian tissue autotransplantation should be
217 discouraged when there is any risk of reimplanting malignant cells (51). Other techniques
218 such as isolated follicles transplantation (60), *in vitro* follicular culture (61-63) and isolation
219 of primordial follicles followed by transplantation of an alginate matrigel matrix containing
220 isolated ovarian cells (58) require additional research before becoming available for
221 humans.

222 In conclusion even if ovarian tissue cryopreservation is still to be considered
223 investigational, it offers very encouraging results and represents the only option to
224 preserve fertility in pre-pubertal girls.

225 **Conflict of interests:**

226 The authors declare no conflicts of interest.

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