Restoration of fertility by orthotopic transplantation of frozen adult mouse ovaries

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BACKGROUND: Successful thawing and orthotopic transplantation of ovarian tissue has produced live offspring in mice, but until now has only been successful for very young ovary donors. METHODS: Whole and half ovaries from adult C3H/HeNCrlBR (C3H) and whole ovaries from B6129SF1/J were frozen-thawed and then grafted orthotopically into B6C3F1/CrlBR (B6C3F1) and B6129SF1/J recipients, respectively. In bilateral transplant groups (bilateral), recipients underwent a bilateral ovariectomy, followed by orthotopic grafting. In unilateral groups recipients either underwent bilateral ovariectomy followed by unilateral grafting (unilateralovx) or had only one ovary removed and replaced with a graft (unilateral) along with complete transection of the remaining oviduct. RESULTS: Ovary size and number of follicles decreased dramatically in grafted compared with control groups, but the loss in the unilateralovx group was significantly less than in the unilateral group. Similar numbers of litters and litter size were obtained in bilateral and unilateral grafts of fresh ovary. However, a much lower number of litters and litter size were derived from unilateral grafts than from unilateralovx grafts of frozen ovary. CONCLUSIONS: Normal fertility can be restored by orthotopic grafting of fresh or frozen adult mouse ovaries and no significant difference between fresh and frozen ovaries was found. Grafting of half ovaries does not alter the overall fertility rate. Unilateralovx grafting is an efficient procedure to produce live pups and removes the negative effect of recipient native ovaries on post-grafting fertility.

Keywords: cryopreservation; ovary; fertility; transplantation; mouse

Introduction

Mouse genetics is set to play a pivotal role in the study of mammalian gene function. Large-scale mutagenesis projects using N-ethyl-N-nitrosourea (ENU) have provided a large number of mutant mice to elucidate genome function (Justice et al., 1999; O’Bien and Frankel, 2004; Sakuraba et al., 2005). Cryopreservation of both mouse embryos and spermatozoa is currently widely employed for the archiving of mouse stocks and provides a “mutant mouse libarary” that allows researchers to conduct reverse genetics in any particular target gene (Glenister et al., 2000; Sakuraba et al., 2005). Moreover, frozen ovaries may offer a valuable addition to the current cryopreservation approaches. Cryopreservation of ovaries can provide an opportunity for long-term preservation of early stage oocytes. Subsequent thawing and surgical engraftment of ovaries can preserve important sources of experimental, domestic and wild animals, as well as restore fertility in young women who have undergone cancer treatment with chemo- and/or radiotherapy. The cryopreservation of ovaries was developed initially in the mouse and rat (Deanesley et al., 1954; Parkes et al., 1954, 1957). It has now been established that the slow cooling protocol developed for oocytes and embryos can successfully be applied to ovarian tissue (Karrow et al., 1997; Shaw et al., 1999). The possibility of grafting frozen-thawed mouse ovaries into host female animals was demonstrated with live offspring arising from grafted tissue by Parrott (1958, 1960). To date, orthotopic grafting has proved to be the most efficient method of restoring fertility of cryopreserved ovaries. Frozen-thawed ovaries trans-planted orthotopically have been able to restore normal ovarian cycling in the mouse (Harp et al., 1994), rat (Wang et al., 2002), sheep (Baird et al., 1999) and human (Grischenko et al., 1987). The recipients of orthotopic grafts of frozen ovaries are capable of becoming pregnant, and live offspring have been born in the mouse (Parrott et al., 1960; Candy et al., 1997, 2000; Gunasena et al., 1997; Sztein et al., 1998; Liu et al., 2002), sheep (Gosden et al., 1994) and human (Donnez et al., 2004). However, in mouse studies, only young ovary donors (10–21 days old) have been used and the number of live births has been low (Sztein et al., 1998; Candy et al., 2000; Glenister et al., 2000; Snow et al., 2003; Torrents et al., 2003). Practically, many rare and valuable mutant mice are old adults, thus cryopreservation of adult ovaries and transplantation into young mice is a valuable tool.
Although some of the follicles may be lost during cryopreservation, post-grafting ischaemia is thought to be the key factor responsible for the shortened fertile lifespan and reduced litter size in ovarian grafts (Torrents et al., 2003). Early studies suggested that around half of the primordial follicles are lost due to initial ischaemia (Nugent et al., 1997; Baird et al., 1999 and Gosden et al., 2000). Rapid revascularization of ovarian grafts is likely to be influenced by a several host factors including fibroblast growth factor, transforming growth factor and nerve growth factor as well as the levels of gonadotrophins and ovarian steroids (Torrents et al., 2003). Gonadotrophins may be particularly important as they can up-regulate angiogenic growth factors in the ovary (Imthurn et al., 2000) and pre-grafting gonadotrophin treatment of the host improves the survival of growing follicles in the mouse (Wang, 2002). In contrast, Nugent (1998) reported that the administration of gonadotrophins to ovary recipients for 3 days post-grafting did not have an influence on follicle survival. Thus, the effect of exogenous gonadotrophins is influenced by the timing of the injections, but the effect of endogenous ovarian hormones on the survival of grafted ovaries is not currently known.

In the present study, whole fresh or frozen adult mouse ovaries, or half ovaries, were orthotopically transplanted into host mice bilaterally or unilaterally. In the case of unilateral transplants, the host’s native ovary may play an important role in regulating the gonadotrophin environment in the grafted ovary, and thus the surgical success and fertility of the graft. To explore this, recipients either underwent complete ovariectomy before unilateral grafting (unilateral unovx) or had only the graft side ovary removed before grafting (unilateral) along with transection of the contralateral oviduct.

Materials and Methods

Animals
All mice used in this experiment were ordered from Charles River Canada (Montreal, Quebec, Canada) and the Jackson Laboratory (Maine, USA). Donor ovaries were collected from C3H/HeNcrlBr (6–8 weeks old) and B6129SF1/J (C57BL/6d × 129S1/SvimJ) (6–8 weeks old) females. The recipients of ovarian grafts were 6–8-week-old female B6C3 (C57BL/6NcrlBR × C3H/HeNcrlBR) F1 and B6129SF1/J hybrid mice. One cohort of grafted mice were mated to C3H/HeNcrlBR and C57BL/6NcrlBR males, respectively (8–10 weeks old) and housed in specific-pathogen-free facilities at the Hospital for Sick Children (Toronto, Ontario, Canada). Another cohort of grafted mice was allowed to recover for 2 weeks and then sacrificed for quantification of follicle numbers.

Cryopreservation
Intact ovaries from C3H and B6129SF1/J females were dissected free of fat and mesentery. Ovaries were used either for freezing (within 30 min) or for transplant into the ovarian bursae of recipient mice (within 1 h). Some intact ovaries collected from C3H were cut in half and then frozen (HFH) and others were frozen and then cut in half (FrH) after thawing. All manipulations were carried out at room temperature in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA). A standard slow-freezing rapid-thawing protocol was used for cryopreservation. Briefly, four ovaries were placed in a 1.8 ml cryovial (Corning, Corning, NY, USA) with 0.3 ml of 1.5 mol/l dimethylsulphoxide (DMSO, Sigma, St Louis, MO, USA) in MEM and held at room temperature for 10 min. The vials were sealed and placed in a programmable rate freezer (Thermo Forma 7452, Marietta, OH, USA) set at 0°C for 20 min and then cooled at a rate of 2°C/min to −7°C. Ice crystal nucleation (seeding) was induced manually 5 min later by touching the side of the cryovial with forceps previously cooled in liquid nitrogen. After a further 5 min, the cryovials were cooled at 0.3°C/min to −40°C and then at 10°C/min to −150°C. The cryovials were then plunged into liquid nitrogen and stored for at least 2 weeks.

Thawing
The cryovials were warmed in air at room temperature for 40 s and then immersed in 30–35°C water until the ice melted. The ovaries were removed from the cryovials, washed twice and held in MEM at room temperature. The ovaries were grafted into the ovarian bursae of recipient mice within 1 h of thawing.

Transfer to the ovarian bursa
Fresh and frozen ovaries collected from C3H were transplanted into the ovarian bursae of B6C3F1 mice and frozen ovaries from B6129SF1/J were transplanted into the bursae of B6129SF1/J mice. Briefly, the ovarian bursa was exteriorized through an abdominal incision over the lumbar fossa. A small incision was made just below the fat pad and the bursal membrane was reflected over the bursa. A pair of fine watchmaker’s forceps was used to grip the hilum and a second pair was used to sever the native ovary from the hilum. Pressure was applied to control bleeding. The donor ovary was placed within the bursal cavity and the bursal membrane replaced over it and sutured before returning it to the peritoneal cavity. As outlined in Table I, for bilateral grafting, both ovaries of the recipient animal were removed and replaced with donor ovaries. For unilateral grafting, the left ovary of the recipient was replaced with the donor ovary. The remaining right ovary either had its oviduct transected (unilateral) or was removed (unilateral unovx). Recipient mice with both ovaries removed served as bilateral surgical controls, and mice with their left ovary removed and right oviduct transected served as unilateral surgical controls.

Breeding of the recipients
The recipients were allowed to recover for 2 weeks after surgery and were then paired continuously with sexually mature, fertile males (C3H × C3H and B6129SF1/J × C57BL/6). The date of birth and number of pups in each litter was recorded. The pups were removed from their parents 3–4 days after birth and euthanized.

Histology
C5H ovaries were collected fresh (unmanipulated), frozen-thawed, or 2 weeks after grafting, and were placed in formalin. Ovary size was measured 30 min later and after 24 h of fixation, ovaries were embedded in paraffin wax, and the entire ovary was serially sectioned every 10 μm, and stained with haematoxylin and eosin (Fisher, Ottawa, Ontario, Canada). Follicles were counted in every second section where the nucleolus was present within the nucleus of the oocyte in order to obtain an estimate of the number of follicles in each ovary. Follicles were classified as follows: (i) primordial follicles with up to one layer of flattened granulosa cells surrounding the oocyte; (ii) primary follicles with one or two layers of cuboidal granulosa cells; (iii) preantral follicles with three or more layers of granulosa cells and no antrum and (iv) antral follicles with an antral...
cavity. Only normal follicles were counted, and they had to contain intact granulosa cell layers, occasional isolated granulosa cells with pyknotic nuclei and unfragmented oocytes with a germinal vesicle.

**Statistical analysis**

All follicle counting data are expressed as mean ± SEM. An ANOVA test was used to compare the means and the Tukey post-test was used to compare one group with another and a Chi-squared test was used to compare the proportion of fertile females. Differences were considered significant when \( P < 0.05 \).

**Results**

**Ovary size**

Ovaries collected immediately at euthanasia (fresh control), after freezing–thawing (freezing control) and 2 weeks after grafting were measured 30 min after formalin fixing. The ovary size was calculated by length \( \times \) width \( \times \) height. As shown in Fig. 1, there were no significant differences in size between fresh and frozen-thawed groups in both control and grafted groups \( (P > 0.05) \). Ovary size dramatically decreased in both grafting groups (both fresh and frozen) compared with control groups \( (P < 0.01) \). Ovary size in the unilateral group was significantly smaller than in the bilateral and unilateral\(^{\text{ovx}}\) groups of either fresh or frozen ovaries \( (P < 0.01) \). No significant difference was found between the bilateral and unilateral\(^{\text{ovx}}\) groups \( (P > 0.05) \).

**Distribution of classified follicles post-grafting**

In Fig. 1, it is clear that there is a dramatic loss of ovary tissue following grafting. To further understand this tissue loss, ovaries collected immediately at euthanasia (fresh control), after freezing–thawing (freezing control) and 2 weeks after grafting were fixed and sectioned for histological analysis. As shown in Figs. 2–5, there were no significant differences in the number of follicles of each classification between fresh and frozen-thawed groups \( (P > 0.05) \). The number of follicles of each classification in the unilateral group was significantly lower than in controls in both fresh and frozen-thawed groups \( (P < 0.05) \), except the fresh antral follicle group. When expressed as a proportion of the total number of follicles identified in serially sectioned controls, 52.1 ± 19.7% and 19.0 ± 7.7% of follicles in the bilateral and unilateral groups, respectively, survived in fresh ovary grafts, and 28.6 ± 2.2% and 7.8 ± 3.4%, respectively, survived in frozen-thawed ovary grafts.

**Effect of recipient ovary**

The results above indicated that there was no significant difference between fresh and frozen groups in either ovary size or distribution of follicles (Figs. 1–5). In this experiment, frozen-thawed ovaries were grafted bilaterally, unilaterally and unilaterally\(^{\text{ovx}}\). Histological analysis indicated that a significantly higher number of primordial follicles survived the grafting processing in the unilateral\(^{\text{ovx}}\) group compared with the unilateral group (Fig. 2, \( P < 0.01 \)). No significant differences were found among fresh (letters) and frozen (*) groups. Values are significantly different between columns with different letters or number of stars.
differences were found in the distribution of primary, preantral and antral follicles between the unilateral ovx and unilateral groups (Figs. 3–5, \(P > 0.05\)). In total, 43.7 ± 16.0% of the follicles in the unilateral ovx group survived the grafting, which was significantly higher than 7.8 ± 3.4% in the unilateral group (\(P < 0.01\)).

Breeding and fertility

Recipients carrying fresh and frozen-thawed transplanted ovaries, either whole or half, from C3H or B6129, were mated beginning 2 weeks after grafting. There was no difference in the proportion of fertile grafts in the fresh (70.0%) and frozen-thawed (87.1%) whole ovary groups (\(P > 0.05\)). For half ovary grafts, HFr unilateral ovx showed a lower percentage of fertile grafts compared with HFr bilateral grafts (40% versus 80%). None of the surgical controls showed signs of pregnancy or produced offspring, indicating that bilateral ovariectomy was complete, and in the unilateral ovariectomy groups, transection of the remaining oviduct was successful. As shown in the C3H groups in Table II, a similar number of litters and litter size was derived from bilateral and unilateral grafts of fresh C3H ovaries (\(P > 0.05\)). However, a significantly lower number of litters and litter size was derived from frozen-thawed unilateral grafts compared with unilateral ovx C3H whole ovaries (\(P < 0.05\)). More pups per ovary were produced from unilateral ovx grafts compared with unilateral grafts (\(P < 0.05\)). Overall the number of litters and litter size obtained in the C3H half ovary grafts were not significantly different. On average, a higher number of pups per ovary was obtained in unilateral ovx (12.8) and HFr unilateral ovx (16.4) grafts of frozen-thawed C3H ovaries and unilateral ovx (27.2) grafts of frozen-thawed B6129SF1/J ovaries, compared with all other groups (\(P < 0.05\)). Compared with the HFr bilateral ovary grafts, FrH bilateral and HFr unilateral ovx ovary grafts had lower numbers of pups per ovary (\(P < 0.05\)). Compared with the unilateral ovx C3H whole ovary grafts, unilateral ovx B6129SF1/J grafts produced significantly higher numbers of pups per ovary (\(P < 0.05\)).

No difference was observed between any groups in the latency between pairing and birth of the first litters (\(P > 0.05\)). Some recipient animals with grafts of fresh and frozen-thawed ovaries reproduced continuously for up to 6 litters over a course of 6–8 months. The interval between litters was similar in both fresh and frozen-thawed ovary grafts (\(P > 0.05\)).

Discussion

It has previously been demonstrated that the fertility of mice can be restored after orthotopic transplantation of fresh or frozen ovaries, but in most of these studies, the fertility rate is still low, and the number of litters and total pups produced is much lower than that of control animals (Parrett et al., 1996; Candy et al., 1997; Gunasena et al., 1997; Sztein et al., 1998; Liu et al., 2002). Candy et al. (2000) first reported that grafts of frozen 10-day-old C57BL/6 mouse ovaries restored normal reproductive lifespan after orthotopic transplantation. In this study 80% of recipients of fresh and frozen ovarian grafts were fertile. Recipients of fresh and frozen ovaries had a mean number of litters of 6.2 and 8.4,
Table II. Breeding of the grafts accepted fresh and frozen-thawed ovaries.

<table>
<thead>
<tr>
<th></th>
<th>Fresh C3H ovaries</th>
<th>Frozen-thawed C3H ovaries</th>
<th>Frozen-thawed half C3H ovaries</th>
<th>Frozen-thawed B6129</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bilateral</td>
<td>Unilateral</td>
<td>Unilateral&lt;sup&gt;inx&lt;/sup&gt;</td>
<td>HF bilateral</td>
</tr>
<tr>
<td>No. of recipients</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>No. of ovaries</td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>No. (%) of fertile grafts</td>
<td>4 (80.0)</td>
<td>3 (60.0)</td>
<td>3 (100.0)</td>
<td>3 (60.0)</td>
</tr>
<tr>
<td>Days between pairing and first litter (mean ± SEM)</td>
<td>33.0 ± 5.3</td>
<td>38.7 ± 9.5</td>
<td>39.4 ± 8.0</td>
<td>33.8 ± 5.5</td>
</tr>
<tr>
<td>Days between litters (mean ± SEM)</td>
<td>25.6 ± 1.1</td>
<td>26.8 ± 3.2</td>
<td>26.2 ± 1.7</td>
<td>29.8 ± 3.2</td>
</tr>
<tr>
<td>Total litters (mean ± SEM)</td>
<td>14 (3.5 ± 0.9)</td>
<td>9 (3.0 ± 1.0)</td>
<td>18 (3.6 ± 0.6)</td>
<td>16 (4.0 ± 0.7)</td>
</tr>
<tr>
<td>Litter size (mean ± SEM)</td>
<td>5.9 ± 0.7</td>
<td>2.9 ± 0.6</td>
<td>5.5 ± 0.6</td>
<td>5.1 ± 0.6</td>
</tr>
<tr>
<td>Total pups</td>
<td>83</td>
<td>26</td>
<td>99</td>
<td>82</td>
</tr>
<tr>
<td>Pups/ovary transferred</td>
<td>8.3</td>
<td>5.2</td>
<td>9.9</td>
<td>16.4 b*</td>
</tr>
<tr>
<td></td>
<td>10.4 ± 2.6</td>
<td>8.7 ± 1.2</td>
<td>9.9 ± 2.6</td>
<td>16.0 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>10.3 ± 2.4</td>
<td>23.0 ± 15.0</td>
<td>2.9 ± 1.1</td>
<td>34.0 ± 5.1</td>
</tr>
</tbody>
</table>

*P < 0.05 (letters represent comparison between paired columns and * between C3H and B6129).
respectively, and a mean litter size of 5.4 and 6.3, respectively. The high success rate of Candy et al.’s study might be explained by the use of 10-day-old donors, whose ovaries contain more early stage follicles, and the use of young hybrid mice as recipients, which have a higher breeding capability. In the present study, 6–8 week-old adult C3H and B6129SF1/J females were used as ovary donors because our goal is to rescue adult mutant female mice. Ovaries were frozen in DMSO and were orthotopically grafted into bilaterally or unilaterally ovariectomized recipients after thawing. After orthotopic transplantation, similar percentages of the recipients of frozen mature whole ovaries in both strains were subsequently fertile. Lower mean number of litters in fresh, frozen-thawed and half C3H ovary grafts (3.3, 2.9 and 2.9), and the mean litter sizes (4.7, 4.4 and 4.2), respectively, were obtained compared with Candy’s report (2000). The number of each class of follicle was lower in mature females (Figs. 2–5) than the literature values for 10 day old females (Figs. 3 and 4, Candy et al., 1997). We found that smaller follicles had a better survival rate after freezing–thawing and grafting (Fig. 4).

Unilateral transplantation of frozen ovaries resulted in lower number of litters and litter size than did bilateral and unilateral\(^{ovx}\) frozen groups (Table II). More pups per ovary were obtained in unilateral\(^{ovx}\) than in unilateral groups (12.8 versus 1.6, Table II). This result is consistent with our observation that more follicles survived the processing of freezing and grafting in the unilateral\(^{ovx}\) group than in the unilateral group (Fig. 4). The reasonable conclusion may be that the host native ovary had a negative effect on the viability and fertility of grafted ovaries.

Compared with C3H, hybrid B6129SF1 gave a significantly higher litter size (7.6, \(P < 0.01\)) and pups per ovary (27.2, \(P < 0.05\)) although the average number of litters were similar (4.5, Table II). The present results agree with previous observations that hybrids have a higher breeding capability than inbred mice (Candy et al., 2000).

With respect to half ovary grafting, the FrH bilateral group had a lower number of litters and pups per ovary than the HFr bilateral group. This suggests that cutting ovaries when fresh may cause less damage to the tissue. HFr unilateral grafts had a lower fertility rate than HFr bilateral grafts. On average, 16.4 pups were produced in HFr half ovaries grafted bilaterally, which is similar to whole ovaries grafted unilaterally (12.8), but higher than HFr half ovaries grafted unilaterally (9.2). It is clear that half ovary grafting does not alter the overall fertility rate; each ovary produces roughly the same number of living pups regardless of whether it is cut in half. This makes banking of half ovaries a viable option to increase the overall chances of obtaining a live pup, since the surgical risks are spread over two recipients.

Two weeks after transplantation of mature mouse ovaries, ovary size dramatically decreased by 70, 90 and 60% in bilateral, unilateral and unilateral\(^{ovx}\) groups compared with the control, respectively. No difference in ovarian tissue loss was found between fresh and frozen groups. Histological examination showed that the number of follicles at all stages of development was decreased by half after grafting compared with controls in either fresh or frozen groups (Figs. 2–4). A similar follicle survival rate was found between fresh and frozen groups regardless of whether grafting was bilateral or unilateral\(^{ovx}\). In terms of the total number of follicles, frozen ovaries in the unilateral\(^{ovx}\) group (43%) had a better survival rate than in the unilateral group (10%). These results confirmed that, as previously reported, the major loss of follicles occurs after grafting rather than after freezing (Liu et al., 2002; Torrents et al., 2003).

In the mouse, ovarian tissue is transplanted orthotopically into host animals without vascular anastomosis. The majority of transplanted follicles are lost during an ischaemic phase that persists until neovascularization (Candy et al., 1997; Gunasena et al., 1997; Nugent et al., 1997; Baird et al., 1999; Gosden et al., 2000; Kim, 2003; Torrents et al., 2003; Revel et al., 2004). It is known that gonadotrophins can up-regulate angiogenic factors, such as fibroblast growth factor, transforming growth factor, and nerve growth factor, which benefit rapid revascularization of ovarian grafts. Histological examination has confirmed that administration of exogenous gonadotrophins improves the viability of murine ovarian tissue grafted to the body wall by pre-treatment of the donor or treatment of the recipient before and after grafting (Imthurn et al., 2000). Gonadotrophin treatment of recipients before subcutaneous grafting improved the survival of growing follicles (Wang et al., 2002). In contrast, a previous report did not support this conclusion (Nugent et al., 1998) and suggested that treatment with PMSG for 3 days after grafting did not impact the growth of grafted ovarian follicles. The magnitude of the hormone’s effect was influenced by the timing of the injections relative to the time of grafting (Imthurn et al., 2000). The present study demonstrates that the host native ovary plays a negative effect on the survival and fertility of the grafted ovarian tissue. This is evident by the greater tissue loss in unilateral compared with unilateral\(^{ovx}\) transplantation, as well as the lower litter size, number of litters, and pups per ovary in unilateral versus unilateral\(^{ovx}\) groups. A possible explanation for this is negative feedback from the host hypothalamic–pituitary–ovary axis. The remaining host ovary in the unilateral group could produce enough oestrogen to sustain negative feedback compared with the unilateral\(^{ovx}\) group, resulting in decreased FSH and LH secretion. Since both host ovaries were removed in the bilateral and unilateral\(^{ovx}\) groups, an immediate lack of negative feedback after grafting could facilitate the production of GnRH, FSH and LH leading to enhanced revascularization and survival of the grafted ovarian tissue. The smaller follicles may benefit from this protection more than the larger ones as Fig. 2 indicates that more primordial follicles survived in the unilateral\(^{ovx}\) group.

In summary, fertility can be restored by orthotopic grafting of frozen-thawed adult mouse ovaries. The follicles of ovaries transplanted unilaterally survive the processing of grafting better and produce more pups per ovary when the remaining host ovary is surgically removed.

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References


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