Ultra-rapid freezing of mouse oocytes lowers the cell number in the inner cell mass of 5 day old in-vitro cultured blastocysts*

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We demonstrated previously that ultra-rapid freezing of mouse oocytes with 3.5 M dimethylsulphoxide (DMSO) decreased cell numbers in day 5 in-vitro cultured blastocysts. In the present study we counted cell numbers of trophectoderm (TE) and inner cell mass (ICM) separately following differential labelling of TE with propidium iodide (red) and ICM with bisbenzimide (blue). Blastocysts were from four groups of oocytes: (i) cumulus-enclosed; (ii) hyaluronidase-treated cumulus-free; (iii) cumulus-free and exposed to 3.5 M DMSO; and (iv) cumulus-free and ultra-rapidly frozen with 3.5 M DMSO. Mean (±SD) blastocyst cell numbers were 54.7 ± 22.0, 51.1 ± 17.3, 52.3 ± 13.1 and 40.4 ± 18.4, respectively. Mean TE cell numbers were 31.7 ± 18.2, 28.9 ± 13.3, 31.2 ± 13.3 and 26.2 ± 16.5 while mean ICM cell numbers were 23.0 ± 9.4, 22.2 ± 9.4, 21.1 ± 7.3 and 14.2 ± 7.3, respectively. Blastocyst and ICM cell numbers were significantly lower in the group derived from ultra-rapidly frozen oocytes compared with all other groups. Significantly more blastocysts had <32 cells and in blastocysts with >64 cells a lower mean percentage of ICM was found. Ultra-rapid freezing of mouse oocytes with 3.5 M DMSO can thus lead to day 5 in-vitro cultured blastocysts with significantly decreased cell numbers. The residual ICM cell number in affected blastocysts may not reach a critical mass sufficient for successful post-implantation development.

Keywords: blastocyst/inner cell mass/mouse/oocyte/ultra-rapid freezing

Introduction

Technological advances in assisted human reproduction have expanded faster than our knowledge of early embryology.

From 1978 on (Steptoe and Edwards, 1978), in-vitro fertilization (IVF) in combination with intracytoplasmic sperm injection (Palermo et al., 1992; Van Steirteghem et al., 1993a,b), embryo cryopreservation (Trounson and Mohr, 1983; Zeilmaker et al., 1984) and preimplantation diagnosis (Handyside et al., 1990) have become available as a battery of techniques in assisted reproduction. Fundamental questions concerning the effects of these techniques on preimplantation development can only be answered if we advance our knowledge of early embryology.

One of the most critical stages in early embryology is cell differentiation in the preimplantation embryo. At blastocyst formation two cell populations are formed: the outer layer of trophectoderm (TE) that surrounds the blastocyst cavity, and the inner cell mass (ICM) which is sequestered at one pole of the blastocyst. The TE is necessary for implantation and will form the placenta and extra-embryonic membranes. The ICM forms all three germ layers and all tissues of the embryo proper as well as providing complementary contributions to extra-embryonic membranes (Gardner, 1989).

We chose as a study model the effect of ultra-rapid freezing of mouse oocytes on the cell distribution between TE and ICM in the blastocyst. Freezing of oocytes was chosen as a study subject because survival rates, rates of IVF and blastocyst formation and pregnancy rates are still low in comparison with unfrozen controls (Bernard and Fuller, 1996; Karlsson et al., 1996; Van der Elst et al., 1997). We had demonstrated previously that ultra-rapid freezing with 3.5 M dimethylsulphoxide (DMSO) of mouse oocytes leads to an overall decrease in the number of Giemsa-stained nuclei in 5 day old in-vitro cultured blastocysts (Van der Elst et al., 1993). The aim of the present study was to investigate whether the reduced cell number in blastocysts derived from ultra-rapidly frozen mouse oocytes was due to a decrease in cell numbers in the TE, in the ICM or in both cell populations. To visualize cell differentiation we used a differential fluorescent labelling technique for TE and ICM in the blastocyst (Handyside and Hunter, 1984).

Materials and methods

Experimental design

Each experiment was performed on a single pool of mature oocytes which were randomly assigned to different subgroups: one test group of cumulus-free oocytes which underwent ultra-rapid freezing and three control groups: (i) cumulus-enclosed untreated oocytes; (ii) hyaluronidase-treated cumulus-free oocytes; and (iii) DMSO-exposed but unfrozen cumulus-free oocytes. Each experiment was repeated four times. Experiments were considered valid only when 5 day old
in-vitro cultured blastocysts in the control group contained the expected total number of cells (≥ 64) of which 30–40% represented ICM cells. One experiment had to be excluded from the series, so that data are the mean from three replicates.

**Oocyte collection, ultra-rapid freezing and oocyte survival**

Mature oocytes were collected from hyper-stimulated female F1 hybrid mice (C57Bl/6J×CBACA) 14 h after human chorionic gonadotrophin (HCG) injection. Ultra-rapid freezing was initiated by dehydrating cumulus-free mouse oocytes in consecutive solutions of 0.25 and 0.5 M sucrose in HEPES-buffered Earle’s medium with 20% fetal bovine serum (FBS) for 5 min each. Oocytes were then exposed to the freezing solution of 3.5 M DMSO and 0.5 M sucrose for 2–3 min in the freezing straw. All manipulations were carried out at 22°C. Thawing was performed by agitating the straw for 5–6 s in a 37°C water bath. Dilution of the cryoprotectant and re-hydration of oocytes was carried out by exposure to 0.5 and 0.25 M sucrose solutions at 22°C for 5 min each followed by transfer and rinsing in sucrose-free medium. Survival of oocytes was checked under an inverted microscope at ×400 magnification. Surviving oocytes had a clear cytoplasm, the perivitelline space was not enlarged and there was no sign of zona damage, cytoplasm leakage or clumping of cellular organelles.

**In-vitro fertilization**

Oocytes from the control groups and surviving frozen-thawed oocytes underwent insemination for 4 h in Whittingham’s medium with 3% (w/v) bovine serum albumin (BSA). Resulting embryos were cultured in Earle’s medium with 0.5% (w/v) BSA. The IVF rate was defined as the number of 2-cell embryos present 24 h after insemination compared to the number of oocytes undergoing culture after insemination. The developmental rate was defined as the number of blastocysts present 120 h after insemination compared to the number of 2-cell embryos 24 h after insemination. Blastocysts were collected at 120 h after insemination and underwent differential staining of TE and ICM.

**Differential staining of ICM and TE**

The cell nuclei in TE and ICM were labelled differentially with polynucleotide-specific fluorochromes. Outer TE cells were stained red with propidium iodide (PI; Sigma P-4170, Bornem, Belgium) following selective antibody-mediated complement lysis. Blastocysts were preincubated for 30 min at 22°C with whole rabbit anti-mouse serum (Sigma M-5774) and then exposed to a 1:5 guinea-pig complement solution (Sigma S-1639) containing 10 mg PI per ml at 37°C for 5 min. ICM cells are protected from lysis since the antibodies cannot pass through the junctions between the cells of the trophectoderm. Next, blastocysts were fixed and counterstained in absolute ethanol containing 20 mg per ml of the blue fluorochrome bisbenzimide (Sigma B-1155). Blastocysts were washed in absolute ethanol overnight and mounted in glycerol on glass slides under light pressure. Under UV illumination TE cells appear pink because of the dual red and blue labelling while ICM cells appear blue. Cell counting was performed blindly by one investigator under direct fluorescent microscopy at ×250 magnification. Approximately 70% of blastocysts were recovered following staining and mounting. Unambiguous total cell counting was possible for 95% of blastocysts and unambiguous differential cell counting for 65% of these. In cases where counting was not possible, the blastocysts were not sufficiently well spread or, occasionally, were floating in the glycerol. The numbers of blue and red nuclei per blastocyst were counted three times and a mean of the counts per blastocyst was registered. For each experimental group

**Table I. Survival, fertilization and day 5 blastocyst formation rates of four groups of mouse oocytes exposed to different treatment protocols**

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of oocytes</th>
<th>No. surviving treatment (%)</th>
<th>No. of 2-cell embryos (%)</th>
<th>No. of blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>168</td>
<td>135 (80%)$^d$</td>
<td>91 (67%)$^d$</td>
<td></td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>124</td>
<td>82 (66%)$^f$</td>
<td>58 (71%)$^f$</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>140</td>
<td>140 (92%)$^g$</td>
<td>79 (81%)$^g$</td>
<td></td>
</tr>
<tr>
<td>Frozen</td>
<td>290</td>
<td>252 (87%)$^h$</td>
<td>173 (69%)$^h$</td>
<td></td>
</tr>
</tbody>
</table>

Untreated: untreated cumulus-enclosed oocytes.
Hyaluronidase: hyaluronidase-treated cumulus-free oocytes.
DMSO: dimethylsulphoxide-exposed hyaluronidase-treated cumulus-free oocytes.
Frozen: hyaluronidase-treated cumulus-free oocytes ultrarapidly frozen with DMSO.

Superscripts indicate comparisons between groups: $^aP = 0.03$, $^b\chi^2 = 4.7$, $^c\chi^2 = 9.5$, $^d\chi^2 = 6.8$, $^e\chi^2 = 0.01$, $^f\chi^2 = 6.5$, $^g\chi^2 = 0.008$, $^h\chi^2 = 11.8$, $^i\chi^2 = 6.8$, $^j\chi^2 = 2$, $^k\chi^2 = 4.9$, $^l\chi^2 = 0.005$, $^m\chi^2 = 8.1$, $^n\chi^2 = 2$. [$^{\chi^2}$ with Yates’ correction for continuity was used.]

mean and SD values were calculated on the basis of the values for the individual blastocysts.

**Statistics**

Fertilization and developmental rates and allocation of blastocysts to different cell number categories were compared by hierarchical $\chi^2$ tests. Mean cell numbers and mean ICM:TE ratios were compared using one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparisons test.

**Results**

**Oocyte survival and developmental rates**

The mean survival rates of 290 ultra-rapidly frozen oocytes and of 140 DMSO-exposed oocytes were 87% (range 82–95%) and 94% (range 91–100%) respectively ($P = 0.03$ (Table I).

The fertilization rate per inseminated oocyte was 80% for 168 cumulus-enclosed untreated control oocytes and was significantly lower for 124 cumulus-free oocytes treated with hyaluronidase (66%) and for the 252 oocytes which survived ultra-rapid freezing (69%) ($P = 0.02$, $\chi^2 = 9.5$, $df = 3$). The fertilization rate for 132 surviving DMSO-exposed oocytes was not different from that of the untreated control group.

The blastocyst formation rate per 2-cell embryo was 67% in the untreated control group and was significantly higher for DMSO-exposed oocytes (81%) and for ultra-rapidly frozen oocytes (82%) ($P = 0.008$, $\chi^2 = 11.8$, $df = 3$). The blastocyst formation rate for hyaluronidase-treated oocytes was 71%.

**Cell numbers in ICM and TE of day 5 in-vitro cultured blastocysts**

The mean cell number ± SD in 5 day old in-vitro cultured blastocysts derived from cumulus-enclosed untreated control oocytes was $54.7 ± 22.0$ with $31.7 ± 18.2$ cells in the TE and $23.0 ± 9.4$ in the ICM (Table II). In the group of hyaluronidase-treated oocytes the mean cell number was $51.1 ± 17.3$ with $28.9 ± 13.3$ cells in the TE and $22.2 ± 9.4$ cells
Oocyte freezing and blastocyst differentiation

Table II. Mean cell numbers in trophectoderm (TE) and inner cell mass (ICM) of 5 day old blastocysts derived from four groups of mouse oocytes exposed to different treatment protocols

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of blastocysts</th>
<th>Total cell number</th>
<th>TE cell number</th>
<th>ICM cell number</th>
<th>%ICM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>33</td>
<td>54.7 ± 22.0b</td>
<td>31.7 ± 18.2</td>
<td>23.0 ± 9.4f</td>
<td>45 ± 17%</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>30</td>
<td>51.1 ± 17.3c</td>
<td>28.9 ± 13.3</td>
<td>22.2 ± 9.4f</td>
<td>45 ± 16%</td>
</tr>
<tr>
<td>DMSO</td>
<td>41</td>
<td>52.3 ± 13.1d</td>
<td>31.2 ± 13.3</td>
<td>21.1 ± 7.3b</td>
<td>42 ± 16%</td>
</tr>
<tr>
<td>Frozen</td>
<td>46</td>
<td>40.4 ± 18.4h,c,d</td>
<td>26.2 ± 16.5</td>
<td>14.2 ± 7.3c</td>
<td>38 ± 17%</td>
</tr>
</tbody>
</table>

For explanation of treatment groups see Table I. Results are expressed as mean ± SD. Superscripts indicate comparisons between groups: *P = 0.002, one-way ANOVA; b,P = 0.01, c,P = 0.05, Newman–Keuls multiple comparisons test; dP < 0.0001, one-way ANOVA; e,fP = 0.001, Newman–Keuls multiple comparisons test.

Table III. Cell number categories in 5 day old blastocysts derived from four groups of mouse oocytes exposed to different treatment protocols

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of blastocysts</th>
<th>% of blastocysts with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>≤32 cells</td>
</tr>
<tr>
<td>Untreated</td>
<td>33b</td>
<td>21</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>30c</td>
<td>10</td>
</tr>
<tr>
<td>DMSO</td>
<td>41d</td>
<td>2.5</td>
</tr>
<tr>
<td>Frozen</td>
<td>46e,f,g,h</td>
<td>48</td>
</tr>
</tbody>
</table>

For explanation of treatment groups see Table I. Superscripts indicate comparisons between groups: *P < 0.0001, χ² = 36.6, df = 6; b,P = 0.03, χ² = 6.9, df = 2; c,P = 0.002, χ² = 12.1, df = 2; dP < 0.0001, χ² = 24.5, df = 2.

in the ICM. In the group of DMSO-exposed oocytes the mean cell numbers were 52.3 ± 13.1 with 31.2 ± 13.3 in the TE and 21.1 ± 7.3 in the ICM. In the group of ultra-rapidly frozen oocytes the total mean cell number was 40.4 ± 18.4 which was significantly lower than in all other groups (P = 0.002, one-way ANOVA). The mean cell number in the TE was 26.2 ± 16.5 which was similar to that in other groups, whereas the mean ICM cell number was 14.2 ± 7.3 which was significantly lower than in all other groups (P = 0.001, one-way ANOVA). The ICM:TE ratio was not significantly different between blastocysts of different treatment groups.

Categories of blastocysts according to cell number

In the group of untreated control oocytes 21% of 5 day old in-vitro cultured blastocysts had ≤32 cells, 49% had 33–64 cells and 30% had >64 cells (Table III). In the hyaluronidase-treated group 10% of blastocysts had ≤32 cells, 73% had 33–64 cells and 17% had >64 cells. In the DMSO-exposed group 2.5% of blastocysts had ≤32 cells, 85% had 33–64 cells and 12.5% had >64 cells. In the ultra-rapidly frozen group 48% of blastocysts had ≤32 cells, 39% had 33–64 cells and 13% had >64 cells. This last distribution was significantly different from those of all other groups (P < 0.0001, χ² = 36.6, df = 6).

Ratio of ICM:TE in categories of blastocysts with different cell numbers

Categories of blastocysts with increasing cell numbers were defined according to the expected cell number before the fifth, between the fifth and sixth and following the sixth cleavage division, being respectively ≤32, 33–64 and >64 cells (Table IV). In each category the lowest percentage of ICM cells was consistently recorded in the group of blastocysts derived from ultra-rapidly frozen oocytes such that in blastocysts having >64 cells a significantly lower ICM:TE ratio was noted (P < 0.05, Newman–Keuls multiple comparisons test).

Discussion

The present study confirms and extends our previous work (Van der Elst et al., 1993), where we showed that ultra-rapid freezing of mouse oocytes with 3.5 M DMSO can lead to a significant decrease in the mean total number of cells of 5 day old in-vitro cultured blastocysts. By using a differential fluorescent labelling technique for ICM and TE we were able to show here that the decrease in the total mean number of cells in the blastocyst was due to a decrease in the mean absolute number of cells in the ICM. This may be explained partly by the fact that a high proportion of day 5 blastocysts were small (≤32 cells) and that the ICM:TE ratio tended to be lower in blastocysts completing the sixth cleavage division. It is equally important to mention that a proportion of blastocysts from ultra-rapidly frozen oocytes were normal with respect to total cell number and ICM:TE ratio.

The decrease in cell numbers in 5 day old blastocysts derived from ultra-rapidly frozen oocytes was a consistent finding in all three replicate experiments. The validity of our data is supported by the fact that all oocytes of the four experimental subgroups in each replicate experiment were derived randomly from a single pool of oocytes. Ultra-rapid freezing has the advantage of allowing the investigator to freeze, thaw and fertilize oocytes in the same time span as

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>% ICM in blastocysts with</th>
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<tbody>
<tr>
<td></td>
<td>≤32 cells</td>
</tr>
<tr>
<td>Untreated</td>
<td>49</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>70</td>
</tr>
<tr>
<td>DMSO</td>
<td>57</td>
</tr>
<tr>
<td>Frozen</td>
<td>40</td>
</tr>
</tbody>
</table>

For explanation of treatment groups see Table I. aP < 0.05, Newman–Keuls multiple comparisons test.
that in which control oocytes are handled. In addition, the survival rate of ultra-rapidly frozen oocytes in this study (87%) was higher than in our previous study (78%) (Van der Elst et al., 1993). The fertilization rate of ultra-rapidly frozen oocytes (69%) was significantly lower than the controls (80%), as it was in our previous study (59% as compared to 69%, respectively). The high rate of blastocyst formation (82%) from 2-cell embryos derived from ultra-rapidly frozen oocytes implied that the culture conditions were appropriate.

The mean number of cells present in 5 day old blastocysts (51–55) and the presence of 40% ICM cells are similar to previous estimates of mouse blastocyst cell numbers. Using serial sectioning, Copp (1978) reported 30–150-cell blastocysts with 32–41% ICM; Chisholm et al. (1985) reported early mouse blastocysts with 35 cells and 31% ICM. Using both spreading and sectioning techniques, Kiessling et al. (1991) found a mean of 55–58 cells with 30% ICM in 5 day old blastocysts. Handyside (1978, 1981) reported 45% of ICM in 29–37-cell early blastocysts after differential staining.

The mean number of cells in the control 5 day old blastocysts in this study (51–55) indicates that they had almost completed the sixth cleavage division (64 cells) which corresponds morphologically to the expanded blastocyst stage which was visible under the microscope. Blastocyst formation commences with cavitation, which coincides with the completion of the fifth cleavage division at the 32-cell stage (Smith and McLaren, 1977). Although the blastocysts derived from ultra-rapidly frozen oocytes had only just completed the fifth cleavage division (mean of 40 cells) they already had the appearance of fully cavitated blastocysts. It has been documented that cavitation is not necessarily dependent on the number of cells (Smith and McLaren, 1977; Chisholm et al., 1985) and occurs as part of a clockwork programme.

Decreased cell numbers in blastocysts may be due to delayed cleavage and/or disturbed allocation of cells to inner and outer layers. If delayed cleavage were the only cause of decreased cell numbers, then an equivalent cleavage delay would be found in TE and ICM cells, since the ratio of ICM to TE is fairly constant throughout blastocyst development. This type of delay has been described recently by Ray et al. (1995), who demonstrated that female human blastocysts contain fewer cells than male human blastocysts because of a 4.5 h early cleavage delay. In our study fertilization rates were decreased in ultra-rapidly frozen oocytes as well as in hyaluronidase-treated oocytes, yet only the ultra-rapidly frozen oocytes formed smaller blastocysts with reduced cell numbers in the ICM.

Allocation of cells to inner and outer layers of the compacted embryo must occur in a spatially and temporally synchronized way (Kelly et al., 1978; Lehtonen et al., 1988). Thus, if a blastomere is not positioned correctly or is positioned too late during the operation of the embryonic ‘clockwork programme’ it may not reach its destination. The origin of outer TE and inner ICM has been traced back to the late 8-cell stage when compaction occurs (Ducibella and Anderson, 1975) and when each cell forms a stable microvillus pole on its free outer surface (Handyside, 1980). Depending on the orientation of subsequent divisions, the polarized cells typically produce either one polar and one apolar daughter or two polar daughters (Johnson and Ziomek, 1981). Polar cells remain external in the morula whilst apolar cells come to reside internally (Johnson and Maro, 1986). It is the descendants of these inner cells that will give rise to the ICM when cavitation starts, marking the transition from morula to blastocyst.

Embryos with reduced numbers of cells are still able to give rise to fetuses (Willadsen and Polge, 1981; Papaioannou et al., 1990; Loskutoff et al., 1993; Tao et al., 1995). The limits of tolerable cell loss have yet to be fully understood. Embryos produced from 1/8 blastomeres often fail to develop an ICM (Loskutoff et al., 1993). It therefore appears that the ICM must have a certain critical mass to be able to form the three germ layers of the embryo. If too little ICM is present then the embryonic endoderm cannot be formed. Although only the embryonic ectoderm gives rise to the embryo proper, the interactions between the three germ layers are necessary for full embryonic and fetal development. Major defects in the ICM may lead to the formation of a trophoblastic vesicle, or the presence of too few cells in the ICM may result in preclinical abortions or blighted ova (Edwards, 1986). Iwasaki et al. (1990) suggested that the reduced ICM in bovine embryos following IVF as compared to those obtained by fertilization in vivo may be the cause of low pregnancy rates. It has also been shown that both biopsied 3/4 mouse embryos (Somers et al., 1990) and halved 2-cell mouse embryos (Papaioannou and Ebert, 1995) have a significant reduction of the ICM. These findings might also have implications for embryo freezing where blastomeres are often damaged and lower pregnancy rates than from fresh embryos are found (Hartshorne et al., 1990; Van der Elst et al., 1995).

Further studies are needed to investigate the developmental potential of 5 day old in-vitro cultured blastocysts derived from ultra-rapidly frozen oocytes. ICM cell death is another possible cause of decreased ICM cell numbers and can be investigated by counting the number of dead cells present in TE and ICM (Handyside and Hunter, 1986). If the problem is oocyte-related, then cryopreservation of later embryonic stages should be more able to support normal development. If fertilization delay is responsible, then ICSI could be helpful since it enables timed fertilization. However, experiments in which embryos were cultured for longer than 5 days did not restore the cell number, but led to a further decrease in absolute and relative numbers of cells in the ICM (Kiessling et al., 1991). In-vitro implantation experiments may provide insight into the ability of these blastocysts to reach the gastrula and early egg-cylinder stages where interaction between the germ layers comes into operation. Pregnancy data following embryo transfer into pseudopregnant females are obviously essential to determine the effect of this freezing strategy on the functional quality of the embryos. As such the present study does not demonstrate any adverse effect upon outcome of implantation and pregnancy. However, this particular experimental model using mouse oocytes in combination with DMSO and ultra-rapid freezing should not be extrapolated to other situations.

In conclusion, the relevance of this study lies in the demonstration of the possible impact of assisted reproduction techniques upon early embryology. We have shown that ultra-
rapid freezing of the mouse oocyte with 3.5 M DMSO can lead to the formation of a proportion of 5 day old in-vitro cultured blastocysts with a significant decrease in the number of cells in the ICM. This raises the question of whether the residual ICM cell number in affected blastocysts reaches the critical mass for successful post-implantation embryo development.

Acknowledgements
Special thanks go to Mr F. Winter of the Language Education Centre who corrected the manuscript. Mrs M. Van der Linden is acknowledged for her skilful technical support. During this study J.V.D.E. was a Postdoctoral Fellow of the Belgian National Fund for Scientific Research (N.F.W.O.), Brussels, Belgium. This study was supported by grant nos. 3.0028.91 and 3.0181.95 from the Belgian Fund for Medical Scientific Research.

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Received on December 9, 1996; accepted on March 5, 1998