SHORT REPORT

Effect of cryo preservation on *Mycobacterium leprae* growth in the footpads of non-immunosuppressed mice

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**Summary**

**Objective** To investigate using the mouse footpad system, whether the use of cryopreservants help in retaining the viability of *Mycobacterium leprae* samples stored at three different temperatures of 4°C, −20°C and −70°C for 30 days.

**Design** Biopsies from eight untreated lepromatous leprosy cases were homogenised and inoculated into footpads of normal Swiss White mice within 24 hours (control) and remaining homogenates in each case was divided and stored at 4°C, −20°C and −70°C respectively for 1 month, using either 10% skimmed milk (SM) or Roswell Park Memorial Institute media + 10% glycerol (RPMI) (test). Homogenates adjusted to contain 1 × 10^4 M. leprae/footpad was inoculated into 10 mice per set. Harvestings were done at 6th, 7th, 8th and 12th months. Footpad counts showing > 1 × 10^5 M. leprae at 6th month or later were considered as positive yield.

**Results:**

**Control** All the cases showed 100 fold growth and 100% take. Viability at 4°C: Only one case (SM) showed a 100 fold increase and 23% take.

Viability at −20°C: Two cases showed fold growth that was 40–60 fold less with takes of 63% (SM) and 71% (RPMI) respectively.

Viability at −70°C: Positivity was 45% but the fold increase was less as compared to control and takes were between 80–20%, except one RPMI where take was 100%.

**Conclusion:** The viability assessed using the mouse footpad was best and consistent in the inoculas that were injected within 24 hours of harvest from the host tissue (control group). None of the storage temperatures used matched with the controls with respect to bacterial yield or % takes. Among the three storage temperatures, −70°C appeared to be better with 45% of the samples showing growth. There was no significant difference noted between the two preservatives used.

**Introduction**

It is becoming increasingly clear that viability of *Mycobacterium leprae* (*M. leprae*) is highly compromised by a variety of physical, physiological, temperature and storage conditions.1

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There are no easy tools that can discern the dead from the viable cells. The only definitive measure of \textit{M. leprae} viability is its ability to replicate \textit{in vivo}.2

There are a number of studies in the past that have investigated the survival of \textit{M. leprae} outside the host and the effect of various storage and temperature conditions on its viability. In one of the earliest studies, capacity of \textit{M. leprae} in nasal discharge to survive outside the body was tested in three cases, by allowing the nasal discharge to dry in the dark, at room temperature. Sampling for mouse inoculation was undertaken at 1, 1.75, 3, 7 and 10 days. Full survival was found after 24 hours, and 10% after 1.75 days, and none after 3, 7 and 10 days.3

The effects of rapid and slow rates of freezing in liquid nitrogen, storage in liquid nitrogen for 12 months, and the rate of subsequent thawing on the viability and growth of \textit{M. leprae} in Mouse Footpad (MFP) were studied by Colston and Hilson.4 They noted that slow freezing was less deleterious than quick freezing, with loss of viability of 90% compared with 98%. Shepard and McRae5 detected viability in samples stored at 0\,8°C and 31\,8°C up to 2 weeks. Viability was maintained better in suspensions stored in 10% glycerol and kept at 4°C overnight before freezing down to −60°C. Rees6 has shown that for optimum preservation of \textit{M. leprae}, one of the essential requirements is dimethyl sulfoxide as cryopreservant with critical cooling of −15°C to −60°C with a cooling rate of less than 1 degree C/min. Suspensions of \textit{M. leprae} stored in 10% glycerol and 0.1% bovine serum albumin were quick frozen in an ethanol-dry ice bath and stored at −60°C for 1–50 weeks were subjected to MFP tests, immediately after quick freezing and at different time intervals. Though decrease in number of viable organisms was noted, viability did not decrease progressively during prolonged storage, suggesting that the observed decrease in viability resulted from freezing rather than from storage.7 In a study by Desikan and Sreevatsa,8 viable \textit{M. leprae} were detected by MFP method in samples stored in saline at room temperature for 60 days as well as in Hank’s balanced salt solution at 4°C and −20°C for 60 days and at −70°C for 28 days. They were able to isolate viable bacilli from samples exposed to adverse conditions like drying in shade for up to 5 months, in wet soil for 46 days and daily exposure of 3 hours to direct sunlight for 7 days. Sreevatsa and Katoch9 noted a decrease in the viability of \textit{M. leprae} when decontaminated with 4% NaOH as compared to 2% NaOH. Results of MFP experiments show that the bacilli survived for one day at 37°C, 7 days at 20°C to 30°C, for 90 days in lyophilized conditions and samples stored at 4°C with single exposure to room temperature remained viable up to 14 days. These findings can be explained on the grounds that in the footpads, the minimum infecting dose of \textit{M. leprae} could be as low as one or two viable cells, as indicated in the serial dilution experiments.10 Decrease in the viability of lyophilized \textit{M. leprae} suspended in 10% fetal calf serum as against those suspended in 10% skim milk has been recorded by Kohsaka \textit{et al}.11 In a study on cultivable mycobacterial cultures, it was noted that the cultures frozen in skimmed milk and stored at −20°C survived for 4 years, whereas half of the lyophilized cultures were no longer viable at 4 years.12

With these backgrounds the present study was undertaken to investigate whether the use of a cryopreservant will help in retaining the viability and yield in the footpad, of stored \textit{M. leprae} suspensions. We have tested the effect of storage with preservatives at three different temperatures for 30 days, on the growth of \textit{M. leprae} in the footpads of normal Swiss White (SW) mice. Eight human derived \textit{M. leprae} isolates were subjected to mouse footpad testing as follows: a) within 24 hours of harvest (control), and b) on storage for 30 days under three different temperatures i.e. 4°C, −20°C and −70°C using two different cryopreservatives i.e. 10% skimmed milk (Hi Media) [SM] and Roswell Park Memorial Institute media (GIBCO) + 10% glycerol [RPMI]. It was noted that the best and a consistent yield was obtained only with the inocula that was injected within 24 hours of harvest from the host tissue.
Materials and Methods

Incisional biopsies were obtained from eight untreated lepromatous cases after taking informed consent. Biopsies were homogenised, suspended in sterile saline and the bacterial load per gram weight was determined using the standard protocol.\textsuperscript{13} Homogenates thus obtained were divided into four parts. One part was inoculated into the hind footpads of normal SW mice within 24 hours and served as the control. The other three parts, in each case was stored at 4°C, –20°C and –70°C respectively for 1 month, using either SM or RPMI (test group). Cooling down of samples were done manually i.e. 15 minutes each at 4°C, –20°C and later transferred to –70°C. Of these eight cases, four were preserved in only SM, one only in RPMI and the rest in both. In all the cases, inocula size was maintained at $1 \times 10^4$ M. leprae/footpad and 10 mice per set were used. Footpad harvests were done at 6, 7, 8 and 12 month intervals. Two counts per intervals were obtained between the 6th and 8th month and all the remaining mice were harvested at 12th post. One or more footpad counts showing $> 1 \times 10^5$ M. leprae in the harvests carried out at the 6th month or later were considered as a positive yield.

Skimmed milk (10%) freshly prepared using sterile distilled water and RPMI medium to which 10% glycerol was added was used as the preservant. One hundred millilitres of M. leprae suspension containing bacterial load between $10^6$ –$10^8$ was added to 0.5 ml of either preservants and stored at 4°C, –20°C and –70°C respectively for 1 month.

Results

**Viability in the Control Group**

In all, except one case, the first peak with $> 100$ fold growth was seen between 6th–8th months and a second peak at the 12th month (see Table 1). One case (no 3) showed an increase of 40 fold only. All the footpads harvested in all the cases showed a positive yield and 100% take (where take is defined as number of footpads showing positive yield/total number of foot pads harvested).

**Viability at 4°C**

Of the 11 inoculas tested, only one showed a 100 fold increase at 12 months and take was 23%.

**Viability at –20°C**

Of the 11 inoculas tested, 2/11 (1 each SM and RPMI) showed fold growth that was 40–60 fold less as compared to the control. The takes were 63% (SM) and 71% (RPMI) respectively.

**Viability at –70°C**

Of the 11 inoculas tested, 5/11 (45%) that includes three of SM and two of RPMI showed fold increase. In all the five cases, the fold increase was significantly lower as compared to control. The takes were also less, ranging between 80–20% except one RPMI case where the take was 100%.
Table 1. Results of mouse foot pad harvests in control and after storage at 4°C, −20°C and −70°C for 1 month using either skimmed milk (SM) or Roswell Park Memorial Institute media + 10% glycerol (RPMI) as cryopreservants

<table>
<thead>
<tr>
<th>Case no.</th>
<th>BI and MI (%) of inocula</th>
<th>Inoculated within 24 hrs</th>
<th>Preserved in SM or RPMI and stored for 1 month at</th>
<th>4°C</th>
<th>−20°C</th>
<th>−70°C</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>SM</td>
<td>RPMI</td>
<td>SM</td>
</tr>
<tr>
<td>1</td>
<td>4 + (2·5)</td>
<td>191 ± 41 (100)</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
<td>52 ± 8 (71)</td>
</tr>
<tr>
<td>2</td>
<td>4 + (4·7)</td>
<td>195 ± 76 (100)</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>3 + (0·7)</td>
<td>40 ± 39 (100)</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>4 + (2)</td>
<td>139 ± 42 (100)</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>4 + (5)</td>
<td>209 ± 173 (100)</td>
<td>0</td>
<td>ND</td>
<td>31 ± 36 (63)</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>5 + (6)</td>
<td>97 ± 22 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>7</td>
<td>4 + (8)</td>
<td>80 ± 89 (100)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>3 + (6)</td>
<td>210 ± 48 (100)</td>
<td>119 ± 42 (23)</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>8/8</td>
<td>1/7</td>
<td>0/4</td>
<td>1/7</td>
<td>1/4</td>
</tr>
</tbody>
</table>

ND – Not done, BI – Bacterial index, MI – Morphological index (%), SM – 10% Skimmed milk, RPMI – Roswell Park Memorial Institute media + 10% glycerol, AFB – Acid fast bacilli.
Discussion and Conclusions

Viability assessed using the footpads of non immunosuppressed SW mice show that the best results with regard to bacterial yield and take were obtained with the inocula that was injected within 24 hours of harvest. In this case all the eight samples showed a good fold growth with 100% take. Regardless of the preservatives, none of the storage temperatures used matched with the controls with respect to bacterial yield or % take. Among the three storage temperature conditions, −70°C appeared to be better with 45% of the samples showing growth. However, total yields as well as % take were significantly lower as compared to the corresponding controls. There was no significant difference noted between the two preservatives. Samples stored at 4°C for 30 days virtually failed to grow in the footpads while only 2/11 samples stored at −20°C showed some growth (1 SM and 1 RPMI).

Truman and Krahenbuhl studied the association between ability of M. leprae to oxidize 14C palmitate in BACTEC 7 H 12 B medium (Radiorespirometry) and the MFP growth results of the suspensions stored at −80°C, 4°C, 25°C, 33°C and 37°C for up to 3 weeks. Suspensions stored in 7H12 liquid medium retained <1% viability within 3 weeks of harvest. Freezing bacillary preparations or incubating them at 37°C resulted in nearly an immediate equivalent loss in metabolic activity and viability. M. leprae viability was maintained best when bacilli were stored for only short periods of time at 4°C–33°C. Our study findings also show that time interval between harvest from host tissues and its usage in experiments is critical. Secondly, there is a need for more innovative programme protocol that might improve the maintenance of M. leprae viability during shipment and storage.

References