Teratogenicity of *Staphylococcus aureus* L-forms using a mouse whole-embryo culture model

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Our previous studies have suggested that *Staphylococcus aureus* L-forms are able to pass through the placental barrier of mice from the maternal side to the fetal body and affect fetal growth and development, but little is known about the direct influence of *S. aureus* L-forms on embryos during the critical period of organogenesis. Mouse embryos at gestational day 8.5 were cultured *in vitro* for 48 h with 0, 50, 100, 200 or 400 c.f.u. *S. aureus* L-forms ml⁻¹. At the end of the culture period, the mouse embryos were assessed morphologically for viability, growth and development. Bacteriological and immunohistochemical staining were used to determine the existence of *S. aureus* L-forms in embryonic tissues. We found that both crown–rump length and head length of mouse embryos exposed to *S. aureus* L-forms at a concentration of 50 c.f.u. ml⁻¹ were reduced. When the mouse embryos were exposed to 100, 200 or 400 c.f.u. *S. aureus* L-forms ml⁻¹, the total morphological score, number of somites, dry embryo weight, yolk sac diameter, crown–rump length and head length were significantly lower than those of the control group. With the increased concentration of *S. aureus* L-forms in the culture medium, there were fewer normally developed embryos and more embryos with abnormalities or retardation in growth. *S. aureus* L-forms detected by Gram-staining and immunohistochemical detection of antigen were found in the tissues of embryos infected by *S. aureus* L-forms. These data suggest that *S. aureus* L-forms exert a direct teratogenic effect on cultured mouse embryos *in vitro*.

**INTRODUCTION**

*Staphylococcus aureus*, a common and major human pathogen, is carried by 25–35% of the population as resident bacterial flora, and can cause severe infection in pregnant women and their fetuses and in infants in the perinatal period (Dancer & Noble, 1991; Pinter et al., 2009). More recently, *S. aureus* has been reported as an emerging problem among pregnant women (Andrews et al., 2009; Me´ndez et al., 2010; Chen et al., 2007; Laibl et al., 2005). Such colonization among pregnant women facilitates the vertical transmission of *S. aureus* from a colonized mother to her infant. Such vertical transmission also has been reported among post-partum women (Saiman et al., 2003). Although several lines of study have reported that *S. aureus* colonization among third trimester pregnant women and post-partum women places the neonate or infant at increased risk for vertically transmitted infection, little is known about the influence of such *S. aureus* colonization on the fetus in the uterus during the first trimester of pregnancy.

It is known that wild-type *S. aureus* is not able to pass through the placental barrier. However, a *S. aureus* variant strain, a cell-wall-deficient bacterium named *S. aureus* L-form, that can pass through the placental barrier has been shown to emerge spontaneously *in vivo* (Michailova et al., 2000; Mattman, 2001) under certain conditions unfavourable to the organisms. Some clinical and experimental data have indicated that these bacterial L-forms frequently contribute to longevity and persistence *in vivo* (Acar & Sabath, 1978; Watanakunakorn, 1979) and are associated with atypical, chronic or latent infection (Domingue & Woody, 1997; Mattman, 2001). In contrast to the wild-type strain, *S. aureus* L-forms have a number of characteristics such as small size and a plastic shape (Mattman et al., 1961; Mattman, 2001) that contribute to their ability to pass through the placental barrier from the maternal side to the fetus and to cause vertical fetal infection similar to what occurs in viral infections. Our previous studies (Xia et al., 1999; Xu et al., 2007) have suggested that *S. aureus* L-forms are able to pass through the placental barrier in this way and affect fetal growth and development, but little is known about the direct influence of *S. aureus* L-forms on
embryos during the critical period of organogenesis. To investigate this influence, a mouse whole-embryo culture system was chosen as the experimental approach because of the necessity of eliminating maternal influences (e.g., maternal toxicity or metabolism) in the study design, whilst retaining the structural and functional integrity of the embryo (New, 1978). This system allows whole embryos to grow and develop in vitro to approximately the same extent as they do in vivo (Tam, 1998).

METHODS

**S. aureus L-forms.** S. aureus Cowan I strain was purchased from the National Institutes for Food and Drug Control (Beijing, China) and induced into S. aureus L-forms as described previously (Mattman et al., 1961; Chatterjee et al., 1967). S. aureus L-forms were prepared at a concentration of $1 \times 10^4$ c.f.u. ml$^{-1}$.

**Animals.** BALB/c mice were purchased from the Anhui Provincial Center for Medical Experimental Animals (Anhui, China). Mice were housed under controlled conditions of temperature (25 °C), humidity (40–60 %) and light (12/12 h light/dark cycle). Rodent chow and filtered tap water were available *ad libitum*. Female mice were housed at two to four per cage, and male mice were housed individually. Female mice were mated with male mice (three females to one male) overnight in our facilities. Gestation was confirmed the following morning by the presence of sperm in a vaginal smear and this was considered gestational day (GD) 0. At GD 8.5, pregnant dams were anesthetized with ethyl ether and the uterus was removed from each dam to prepare the embryos for whole-embryo culture. The Animal Research Ethics Committee of Bengbu Medical College of China approved this study.

**Whole-embryo culture.** Early somite-stage conceptuses (three to six somites) were prepared for culture as described previously (Sadler, 1979). Briefly, embryos were explanted at GD 8.5 in Hanks’ solution. The maternal connective tissue and myometrium were removed, leaving the conceptus surrounded by decidua. The decidua was removed along with the parietal yolk sac and the Reichert’s membrane was opened, leaving the conceptus intact. Up to three embryos were cultured in 30 ml serum bottles with a Teflon stopper containing 1 ml serum per embryo. Embryos from a given litter were distributed randomly among different doses of *S. aureus* L-forms. The culture medium was male rat serum, which was immediately centrifuged, heat inactivated (56 °C for 30 min), filter sterilized and supplemented with 50 U penicillin G ml$^{-1}$ and 50 μg streptomycin ml$^{-1}$. The bottles were gassed with a mixture of 5 % O$_2$, 5 % CO$_2$ and 90 % N$_2$ for 3 min, sealed with a Teflon stopper and placed on a rotating wheel (40 r.p.m.) in an incubator at 37 °C. After 24 h of culture, the bottles were regassed with 20 % O$_2$, 5 % CO$_2$ and 75 % N$_2$, followed by 8 h of culture, and the bottles were then further gassed with 40 % O$_2$, 5 % CO$_2$ and 55 % N$_2$ for another 16 h of culture before harvesting the embryos. The different types of gas mixtures were pre-mixed and prepared commercially.

**Experimental groups.** For evaluation of *S. aureus* L-form-induced dysmorphogenesis, the GD 8.5 embryos were assigned randomly to one of five study groups. Group A was the control group of mouse embryos treated without *S. aureus* L-forms. The GD 8.5 embryos in groups B–E were exposed to *S. aureus* L-forms added to the medium of rat serum at a concentration of 50, 100, 200 or 400 c.f.u. ml$^{-1}$, respectively. In each group, 18 embryos were used to study the effect of *S. aureus* L-forms.

**Morphological assessment.** After 48 h of culture, at the equivalent of GD 10.5, the embryos were examined for viability, growth and development by a researcher who was not aware of the study group assignment. Mean yolk sac diameter (YSD), crown–rump length (CRL) and head length (HL) were measured, and dry embryo weight (EW) was determined with an electronic balance after the mouse embryos had been dried for 24 h in an oven at 80 °C. Embryonic morphology was studied according to a standard morphological scoring system (Van Maele-Fabry et al., 1990), which gives a numerical score (0–5) to each of 17 morphological features based on their stage of development, with higher scores for more-developed fetuses. The score of each feature was added to get the total morphological score for the 17 features. Embryos with a total morphological score of <$2$ were most probably damaged as a result of explantation and were excluded from the analysis.

**Histology.** To evaluate whether *S. aureus* L-forms could go through the barrier of the visceral yolk sac from the medium to the embryo, Gram-staining and immunohistochemistry were used to determine the presence of *S. aureus* L-forms in embryonic tissues. After culture for 48 h in the presence or absence of 400 c.f.u. *S. aureus* L-forms ml$^{-1}$, at least five conceptuses were removed from the assay bottles, rinsed three times with 1 mM PBS at room temperature to eliminate the effect of the remnant *S. aureus* L-forms from the medium and transferred individually into 8 cm culture dishes. The visceral yolk sac surrounding the embryo was cut slightly and the embryo exteriorized. These mouse embryos were then fixed in 3 % paraformaldehyde, dehydrated in increasing concentrations of alcohol, cleared in xylene, embedded in paraffin and serially sectioned (8 μm sections). For the bacteriological determination of *S. aureus* L-forms, the embryonic tissue sections were stained using the method of Brown & Hoppes (1973), a technique for Gram-staining in paraffin-embedded tissues. For the antigenic detection of *S. aureus* L-forms, immunohistochemistry was carried out using a Vectastain Elite ABC kit (Vector Laboratories) according to the instructions provided by the manufacturer. Briefly, the embryonic tissue sections were dehydrated and hydrated through successive dilutions of ethanol into PBS. Following this procedure, endogenous peroxidases were quenched using 0.3 % H$_2$O$_2$ in methanol solution (30 min) and the sections were blocked with normal goat serum for 20 min to prevent non-specific binding. The sections were incubated with a primary rabbit anti-L-form antibody (prepared by our institute) for 1 h at room temperature, followed by 30 min each with secondary goat anti-rabbit antibody conjugated with biotin and avidin–biotin complex reagent. After incubation with each antibody, a single PBS rinse was performed for 5 min. The chromogen Nova Red was used to localize the antigen of *S. aureus* L-forms. The sections were counterstained with haematoxylin, dehydrated with ethanol, cleared with xylene and mounted in mounting medium.

**Statistical analysis.** All results, except for the percentages of normality, are expressed as means ± SD. To assess the significance of differences between experiments, Turkey’s b test in a one-way analysis of variance was used. Statistical significance was defined as $P<0.05$.

RESULTS

**Teratogenicity of S. aureus L-forms**

At 48 h after culture of the whole mouse embryos with *S. aureus* L-forms at different concentrations, the level of teratogenicity was determined. With increasing concentrations of *S. aureus* L-forms, fewer embryos developed normally and more embryos were shown to have abnormalities or...
retardation of growth (Fig. 1). Embryonic differentiation in circulation formation around the yolk sac, heart, caudal neurotube, fore/mid/hindbrain and branchial bars was clearly showed to be affected in the embryo group infected with 400 c.f.u. *S. aureus* L-forms ml$^{-1}$ (Fig. 2).

**Influence of *S. aureus* L-forms on the morphological development of cultured whole embryos**

The difference in development of the embryos was measured at 48 h after explantation. Table 1 shows that both total morphological score and the number of somites of mouse embryos exposed to 100, 200 or 400 c.f.u. *S. aureus* L-forms ml$^{-1}$ were significantly lower than those of the control group, but this was not true of the embryos exposed to 50 c.f.u. *S. aureus* L-forms ml$^{-1}$. Fig. 3 shows that HL and CRL in the embryos infected with 50 c.f.u. *S. aureus* L-forms ml$^{-1}$ were shorter than those of the control group ($P<0.01$). In the groups exposed to 100, 200 or 400 c.f.u. *S. aureus* L-forms ml$^{-1}$, YSD, HL and CRL (Fig. 3) and dry EW (Fig. 4) differed significantly from those of the control group ($P<0.01$).

### Table 1

<table>
<thead>
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<th><em>S. aureus</em> L-forms (c.f.u. ml$^{-1}$)</th>
<th>Normality (%)</th>
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<tr>
<td>0</td>
<td>100</td>
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<tr>
<td>50</td>
<td>80</td>
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<td>100</td>
<td>60</td>
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<td>200</td>
<td>40</td>
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<td>400</td>
<td>20</td>
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Fig. 1. Normality rate of *in vitro*-cultured mouse embryos exposed to different concentration of *S. aureus* L-forms. The percentage of normality was calculated using the ratio of the number of normal and total embryos in each group, based on numerical scores according to a standard morphological scoring system (Van Maele-Fabry *et al.*, 1990). Values were calculated with data from three experiments carried out in triplicate ($n=18$ in each group).

Fig. 2. Examples of abnormalities of *in vitro*-cultured mouse embryos exposed to different concentrations of *S. aureus* L-forms for 48 h. (a) Control mouse embryo showing a ‘G’-shaped body position. (b) Embryo exposed to 50 c.f.u. *S. aureus* L-forms ml$^{-1}$ displaying a ‘C’-shaped body position. (c) Embryo exposed to 100 c.f.u. *S. aureus* L-forms ml$^{-1}$ displaying abnormalities of the mid/hindbrain (thin arrows) and heart (thick arrow). (d) Embryo exposed to 200 c.f.u. *S. aureus* L-forms ml$^{-1}$ displaying abnormalities of the fore/mid/hindbrain (thin arrows), heart (thick arrow) and hindlimb (dotted arrow). (e) Embryo exposed to 400 c.f.u. *S. aureus* L-forms ml$^{-1}$ displaying an open fore/mid/hind neural tube (open arrow), pericardial effusion (arrowhead), a reversed ‘C’-shape body position and abnormality of the hindlimb (dotted arrow) and branchial bars.
Determination of *S. aureus* L-forms in embryonic tissues

To investigate whether *S. aureus* L-forms could pass through the barrier of the yolk sac from the medium to the embryo, cultured conceptuses were removed from assay bottles and rinsed three times with PBSto eliminate the remnant *S. aureus* L-forms from the medium. The visceral yolk sac surrounding the embryo was then removed and the embryo exteriorized. Using the method of Brown and Hopps (1973), we found spherical Gram-positive bacteria in the tissues of mouse embryos incubated with 400 c.f.u. *S. aureus* L-forms ml\(^{-1}\) (Fig. 5), indicating that *S. aureus* L-forms passed through the barrier of the yolk sac from the cultured medium to the embryos.

Detection of *S. aureus* L-form antigen in embryonic tissues by immunohistochemistry

Embryonic tissues treated with 400 c.f.u. *S. aureus* L-forms ml\(^{-1}\) as described above were subjected to immunohistochemistry using anti-L-form antibody and *S. aureus* L-form antigens were detected in the mouse embryo tissues (Fig. 6).

**DISCUSSION**

In the present study, a whole-embryo culture model was chosen to study the teratogenicity of *S. aureus* L-forms. Mice embryos in this model were cultured *in vitro* from GD 8.5 to 10.5, which is the critical period of organogenesis in the mouse, equivalent to 3–6 weeks after fertilization in human embryos (Tanaka *et al.*, 1991). This model has been used extensively in studies in the field of teratogenesis and related mechanisms (Longo *et al.*, 2010; Guo *et al.*, 2011). Unlike other *in vivo* animal models, this model enables the direct assessment of external factors on embryogenesis and is not affected by any metabolic and kinetic differences between humans and other animals (Webster *et al.*, 1997). Of course, fundamental biological differences exist between humans and other animals;

<table>
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<th>Characteristic</th>
<th>Concentration of <em>S. aureus</em> L-forms (c.f.u. ml(^{-1}))</th>
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<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Total morphological score</td>
<td>49.7 ± 0.5</td>
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<tr>
<td>Number of somites</td>
<td>28.5 ± 0.3</td>
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*P < 0.01 compared with the control group.
†P < 0.05 compared with the control group.
therefore, results generated from animal studies should be interpreted with caution.

We investigated the direct effect of *S. aureus* L-forms on mouse embryos during the critical period of organogenesis. Our results showed that *S. aureus* L-forms had a significant effect on the morphogenesis of the embryos. When mouse embryos were exposed to *S. aureus* L-forms at a concentration of 50 c.f.u. ml$^{-1}$, both embryonic CRL and HL decreased but the total morphological score, number of somites, EW and YSD remained the same. However, a significant reduction in all of the above indices was found in mouse embryos exposed to *S. aureus* L-forms at concentrations of $\geq 100$ c.f.u. ml$^{-1}$, and appeared in a dose-dependent manner. We also found that there were fewer normally developed embryos, and more embryos were shown to have abnormalities or retardation in growth with the increase in concentration of *S. aureus* L-forms in infection. Furthermore, we found the presence of *S. aureus* L-forms and its antigen in the cultured embryos incubated with *S. aureus* L-forms. These data suggested that *S. aureus* L-forms can directly affect the growth and development of mouse embryos and are teratogenic, consistent with some results in an *in vivo* study using *S. aureus* (Xia et al., 1999).

A series of studies has shown that apoptosis plays an important role in embryonic growth and development, and mediates the teratogenicity of some factors (Nakajima et al., 2008; Tung & Winn, 2011). Therefore, further studies are required to elucidate whether *S. aureus* L-forms can induce the apoptosis of mouse embryos.

To our knowledge, this study is the first to investigate the teratogenicity of a microbe using a mouse whole-embryo model. *S. aureus* L-forms, compared with the original wild-type *S. aureus*, present the characteristics of slower growth and proliferation (Mattman et al., 1961; Mattman, 2001), so that they fail to cause the serum medium to become too poor in terms of nutrition to meet the growth of mouse embryos in the *in vitro* culture system. Furthermore, *S. aureus* is a clinically common residential pathogen and causes increased risk of vertical infection in the perinatal period. Therefore, *S. aureus* L-forms were chosen to study their teratogenicity on mouse embryos. In this study, we found direct effects of *S. aureus* L-forms on the *in vitro* cultured mouse embryos. These results showed that *S. aureus* L-forms could retard the growth of mouse embryos. Although the results from such animal teratogenicity studies may not reflect the circumstances in humans, our findings suggest that women during their first trimester of pregnancy should be cautious about infections caused by *S. aureus* and its L-forms.

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