Culture Supernatant of *Lactobacillus acidophilus* Stimulates Proliferation of Embryonic Cells

W. I. Li,*[,1] Benjamin G. Brackett,* and Jaroslava Halper†

*Department of Physiology and Pharmacology; and †Department of Pathology, College of Veterinary Medicine, The University of Georgia, Athens, Georgia 30602

Our previous report showed that supernatants of *Lactobacillus acidophilus* (LS) cultures possessed chemotactic and angiogenic properties. Specifically, LS stimulated gene expression and the secretion of tumor necrosis factor-α (TNF-α), the proliferation of immune cells *in vitro*, and blood vessel formation. Chemotaxis and proliferation of inflammatory cells *in vivo* were also stimulated by LS. In the current study, we hypothesized that LS stimulates the growth and development of other rapidly dividing cells, including embryonic cells. The stimulatory effects of LS on a neuroblastoma cell line (Neuro-2a), chicken embryos, and bovine embryos were examined. The addition of LS to Neuro-2a cultures caused a proliferation of cells in a concentration-dependent manner. Pretreatment of LS at 56°C for 30 mins did not affect its stimulatory activity. The administration of LS to the chorioallantoic membrane (CAM) of chicken-embryonated eggs for 1–2 days resulted in extensive thickening of the membrane. The thickening was due to the influx and proliferation of fibroblasts and inflammatory cells, the accumulation of loose connective tissue composed primarily of mucopolysaccharides, and/or the formation of blood vessels. Stimulatory effects of LS on bovine embryos were also observed. The treatment with LS significantly promoted the development of zygotes to the four-cell stage and from the four-cell stage to blastocysts. These results have confirmed our hypothesis that LS exerts a stimulatory effect on the cells of embryonic stages including neuroblastoma cells, the CAM of chicken embryos, and bovine embryos from zygotes to blastocysts. Exp Biol Med 230: 494–501, 2005

Key words: *Lactobacillus* supernatant; Neuro-2a cells; chicken chorioallantoic membrane; bovine embryos

Probiotic microorganisms have beneficial effects on human, as well as animal health. The primary benefits of probiotics are in the digestive system and include the control of diarrhea, the decrease of lactose intolerance, and the reduction of cholesterol absorption (1–3). The mechanisms by which probiotics exert their beneficial effects on the host are still speculative. Probiotic bacteria may inhibit growth of several undesirable microorganisms by producing antimicrobial substances (4, 5), reduce gut pH by secreting lactic acid, improve immune activities by enhancing cytokine production (6), or compete with pathogens for available growth factors and other nutrients (7).

One of the predominant probiotic bacteria in the gastrointestinal tract is *L. acidophilus*. Studies have indicated that *L. acidophilus* exerts a wide range of health-promoting effects, including modulation of immune activities, *in vivo* and *in vitro*, on humans and animals (8). Treatment with *L. acidophilus* can modify the concentrations of gut microbial populations, thus controlling gut bacterial overgrowth (9, 10). In addition, it has been well documented that *L. acidophilus* interacts with immune cells *in vivo* and *in vitro*. Oral administration of *L. acidophilus* enhanced mitogen-induced murine lymphocyte proliferation and serum levels of IgG and IgM (11) and gut mucosal IgA-secreting cells (12). Kitazawa *et al.* (13) reported that isolated murine macrophages produced IFN-α/-β when exposed to living or heat-killed *L. acidophilus*. Utilizing the murine macrophage cell line RAW264.7, Rangavajhyala *et al.* (14) demonstrated that *L. acidophilus* stimulated the production of interleukin-1α (IL-1α) and TNF-α. The production of IL-6, IL-10, IL-12, and TNF-α in the murine J774.1 macrophage cell line was also found to be enhanced by *L. acidophilus* (15).

Our previous data showed that *L. acidophilus* supernatants (LS) possessed chemotactic and angiogenic properties. Specifically, LS stimulated the proliferation of macrophages and lymphocytes *in vitro* and the chemotaxis and proliferation of fibroblasts, endothelial cells, and inflammatory cells *in vivo* (16). The sc injection of LS into the ear lobes of young rodents elicited the proliferation of blood vessels and the influx of neutrophils. Based on these

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[1] To whom correspondence should be addressed at Department of Physiology and Pharmacology, College of Veterinary Medicine, The University of Georgia, Athens, GA 30602. E-mail: oliverli@vet.uga.edu

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observations, we hypothesized that LS is also capable of inducing the proliferation and differentiation of other rapidly dividing cells, such as embryonic cells. We chose chicken embryos, a neuroblastoma cell line that mimics an early stage of neuronal development, and bovine embryos to test our hypothesis.

Materials and Methods

Materials. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

Preparation of Supernatants from *Lactobacillus* Cultures. The procedure for the preparation of LS has been previously reported (16). Briefly, *L. acidophilus* (ATCC strains 4356 and 43121) were grown in MRS broth (pH 5.5; Difco Laboratories, Detroit, MI) at 37°C for 24 hrs under microaerophilic conditions. This medium contains a rich nutrient base as well as polysorbate, acetate, magnesium, and manganese, which are known to promote the growth and proliferation of lactobacilli. Overnight bacterial cultures contained 2.5×10^8 colony-forming units, and these cultures were centrifuged at 10,000 *g* for 15 mins at 4°C. The resulting supernatants were filtered through a 0.2-μm membrane filter to remove the remaining bacteria and debris, lyophilized, and then stored at –20°C. At the time of the experiments, the lyophilized LS was reconstituted with deionized water, filtered with Puradiscs (0.22-μm pore size; Whatman Inc., Ann Arbor, MI) and termed LS. The pH of LS was 2.35 + 0.15. The presence of lipopolysaccharide (LPS) in LS was excluded with a diagnostic kit from Cambrex Corporation (East Rutherford, NJ). The kit is based on the *Limulus* amebocyte lysate test, which is specific for LPS diglucosamine backbone (16).

Neuroblastoma Cell Proliferation Assay. The murine neuroblastoma cell line Neuro-2a (CCL-131, ATCC; Manassas, VA) was used for its neuroblast-like differentiation during the embryonic stage (17). Cells were cultured at 37°C in Eagle's minimum essential medium (MEM) with 2 m*M* L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/l sodium bicarbonate, 0.1 m*M* nonessential amino acids, 1.0 m*M* sodium pyruvate, and 10% fetal bovine serum. To test for heat resistance, LS in air-sealed tubes was heated in 56°C water for 30 mins (designated as LS-56C) or in boiling water (100°C) for 30 mins (designated as LS-100C). To study the mitogenic activity of LS, LS-56C, and LS-100C, 5000 Neuro-2a cells were plated in 96-well, flat-bottom, microtiter plates containing 50 μl medium per well. After a 6-hr incubation, cells were treated with serial dilution of LS, LS-56C, or LS-100C in culture medium together with 1 μCi/well ^{3}H-thymidine (methyl-^{3}H-thymidine, specific activity 6.7 Ci/nmol; NEN Life Sciences Products, Inc., Boston, MA) in eight replicates and incubated at 37°C under 5% CO_2 in air for 96 hrs. All cultures were harvested onto fiberglass filter discs with an automated cell harvester (Skatron; Sterling, VA). Filter discs were then placed in scintillation vials containing 3-ml of scintillation cocktail, and the ^{3}H-thymidine incorporated into DNA was determined in a Beckman liquid scintillation counter. Each experiment was repeated three times. The least-square ANOVA using the General Linear Models procedure of SAS was used for statistical analysis.

CAM Assay. The use of embryonated chicken eggs was approved by the Animal Use Committee at The University of Georgia. We followed the procedure described by Carter and Halper (18). Briefly, a window was cut into the shell of eggs with 8-day-old chicken embryos. After the LS was suspended in 2% methylcellulose in phosphate-buffered saline (PBS) at 5–10 μl/20 μl methylcellulose, it was placed on top of the CAM and under the window, away from visible blood vessels. Control eggs received hydrochloric acid dissolved in methylcellulose to approximate the acidic milieu of the extract. The window was closed with the eggshell or tape to prevent drying out and contamination. Following 24–48 hr incubation at 37°C, the treated membranes were excised, fixed in formalin, and embedded in paraffin. Three to five embryonated eggs were examined per data point, and the experiment was repeated three times. One to three 5-μm-thick sections per embryo were stained with hematoxylin/eosin for light microscopy. The thickness of LS–treated and control CAMs was evaluated under light microscopic control using a micrometer, and statistical evaluation was performed using a two-tail *t* test.

Selected sections were stained with Masson's trichrome for collagen, Gomori's silver impregnation method for reticular fibers, and alcian blue for acid and sulfated mucopolysaccharides. Certain sections were stained with periodic acid-phenylhydrazine (i.e., Schiff reagent, also known as PAS) for neutral mucopolysaccharides.

Growth and Development of Bovine Embryos. Bovine embryos were produced *in vitro* under chemically defined conditions using previously described procedures (19). This involved three major components: maturation of oocytes, fertilization, and embryo culture.

In Vitro *Maturation (IVM) of Oocytes.* Ovarian follicles were aspirated from ovaries within 15 mins after slaughter of cows in a commercial slaughterhouse. Cumulus oocyte complexes (COCs) were harvested from the follicles of surface diameters less than 6 mm. Follicular fluids were pooled and maintained at 33°C for 3 hrs during transit to the laboratory. Oocytes with at least two compact layers of cumulus were used for IVM. The COCs were washed twice with maturation medium and covered with light mineral oil. The maturation medium was TCM-199 supplemented with 50 μg/ml sodium pyruvate, 2.2 mg/ml $NaHCO_3$, 1 mg/ml polyvinyl alcohol (PVA), 0.25 m*M* glutamine, 0.1 m*M* cystine, 0.1 m*M* cysteamine, 10 m*M* HEPES, and 50 μg/ml gentamicin sulfate plus 0.1 IU/ml of recombinant human follicle-stimulating hormone (FSH; 1.7 IU/μg; Ares Advanced Technology Inc., Randolph, MA) and 5 ng/ml of recombinant human insulin-like growth factor-I (IGF-I; Promega, Madison, WI). The COCs were incubated under

moist 5% CO_2, 5% O_2, and 90% N_2 in a modular incubator chamber (Billups-Rothenberg Inc.; Del Mar, CA) for 24 hrs. The same atmosphere, incubator, and temperature of 39°C were employed for all IVM cultures.

In Vitro *Fertilization.* Swim-up selected spermatozoa were prepared in modified-defined medium (mDM) without hypotaurine or epinephrine. Frozen semen that originated from Holstein and Brahman bulls was used. Spermatozoa in frozen straws were thawed at 37°C for 30 secs, after which 110–150 μl of semen were layered under 1.5 ml of mDM in each of several 12-mm × 75-mm tubes. The tubes were held at a 45-degree angle for 45 mins at 39°C under moist 5% CO_2 in air. The uppermost 850 μl aliquots from each tube were then pooled in a 15-ml tube and centrifuged at 320 *g* for 10 mins. The resulting sperm pellet was resuspended to 380- and 20-μl mDM containing heparin (100 μg/ml final concentration for Brahman semen or 200 μg/ml final concentration for Holstein semen) and incubated for 15 mins before insemination. Matured COCs were then added to each drop, with minimal amounts of medium for co-incubation with spermatozoa. A concentration of 2×10^6 motile spermatozoa per ml in 100 μl for 18 hrs allowed the initiation of the fertilization process.

In Vitro *Culture of Fertilized Oocytes and Embryos.* At 18 hrs postinsemination, loosely associated cumulus cells and spermatozoa were removed from zonae by gentle pipetting of the oocytes. Presumptive zygotes were cultured in groups of 20 in 50 μl of synthetic oviductal fluid (SOF) modified to contain 0.1 m*M* of nonessential amino acids (NEA), 0.5 m*M* of glutamine, 0.4 m*M* of threonine, 3 mg/ml of PVA instead of bovine serum albumin, and no glucose. In the initial trial experiment, serial dilutions of LS were co-incubated with embryos, and a ratio of 1:800 dilution with medium was determined to be the most effective. As such, this dilution of LS was used in all subsequent experiments. Thus, treatments with 1:800 dilution of LS were applied to embryo cultures. At 72 hrs postinsemination, embryos with at least four cells were selected for further culture, and any cumulus cells remaining were completely removed. From 72–144 hrs postinsemination, embryos were cultured in a citrate-supplemented, SOF-based medium (c-SOF + NEA; Ref. 19), 0.4 m*M* of threonine, plus treatment of 1:800 dilution of LS. At 144 hrs postinsemination, embryos were cultured in the maturation medium devoid of FSH and IGF-I. Proportions of embryos reaching blastocyst stages (i.e., early, full, expanding) were recorded at 192 hrs post-insemination.

For all embryo-culture steps, recombinant human epidermal growth factor (EGF; 5 ng/ml) and vehicle (culture medium of the same volume as LS or EGF) were used as positive and negative controls, respectively. Data were recorded as percentages of cleaved oocytes at the four-cell stage from total number of oocytes applied to the fertilization process and the percentages of embryos at the four-cell stage that developed into blastocysts. The experiment was repeated four times. The SEM represented the variation of data. Chi-square analysis was employed to detect the difference between treatments, and significant differences were assigned at $P < 0.05$.

Results

Stimulatory Effect of *L. acidophilus* on the Proliferation of Neuroblastoma Neuro-2a Cells. The number of Neuro-2a cells increased when cultured in Eagle's MEM for 4 days, as indicated by the amount of ^{3}H-thymidine uptake by the cells. The addition of LS to Neuro-2a cells caused an increase in the rate of proliferation in a concentration-dependent manner (Fig. 1). The concentration-dependent curve for LS pretreated at 56°C for 30 mins was similar to that of LS without heat treatment, indicating that the stimulatory effect of LS on Neuro-2a cell proliferation was at least partially heat resistant. On the other hand, heat treatment of LS at 100°C for 30 mins basically abolished the concentration-dependent curve of LS although the levels of ^{3}H-thymidine uptake remained still higher than those of the control (Fig. 1). The difference between the effect of nonheated LS and LS heated at 100°C ($P < 0.01$) and between LS heated at 56°C and LS heated at 100°C ($P < 0.01$) was statistically significant. There was no significant difference between the effect of nonheated LS and LS heated at 56°C ($P < 0.4667$).

Stimulatory Effects of LS on CAM. Untreated CAM consists of a thin layer of connective tissue interspersed with fibroblasts and occasional blood vessels (Fig. 2A). The membrane is lined with epithelial cells that are chorionic cells on one side and allantoic cells on the other. Focal thickening of the membrane where LS was applied was observed on inspection of the eggs 1–2 days later. Examination of histologic sections by light microscopy revealed that the CAMs removed from embryonated eggs were thickened due to several phenomena not necessarily present in the same membrane. The average thickness of the LS–treated membranes was 222.7 μm with an SD of 120.8, whereas the average thickness of control membranes was 52.9 μm with an SD of 43. This difference in thickness was statistically significant ($P < 0.001$).

Many membranes contained numerous blood vessels, which varied in size and were almost always engorged with blood (Fig. 2B and C). Blood vessels in several membranes contained white blood cells admixed with red blood cells (not shown). Only occasionally were white blood cells present in surrounding loose connective tissue. This loose connective tissue led to the thickening of some membranes and was due to the accumulation composed primarily of so-called mucopolysaccharides (Fig. 3). Because this tissue stained more intensively with alcian blue than with PAS, and because of its embryonic origin, we hypothesize that this material is mostly hyaluronic acid (Fig. 3B). Masson's trichrome and silver-impregnation methods revealed the presence of minimal amounts of collagen and reticulin fibers (i.e., young collagen fibers), respectively (data not shown).

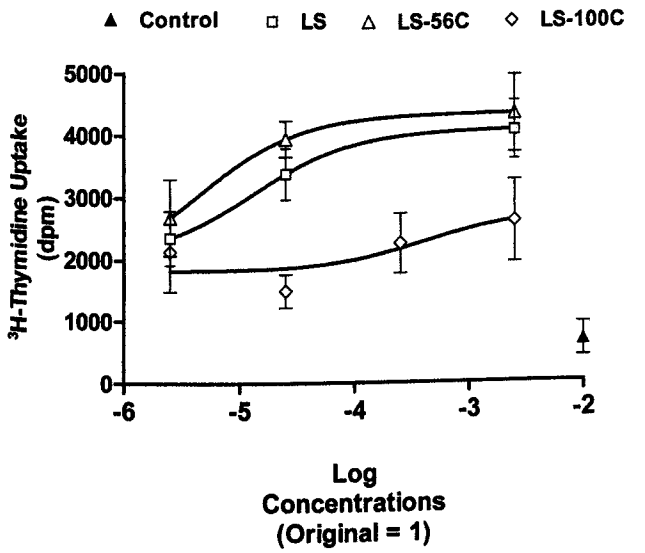

Figure 1. Proliferation of neuroblastoma cells as indicated by levels of ^{3}H-thymidine uptake. Murine neuroblastoma cells (Neuro-2a) were grown in 96-well, flat-bottom, microtiter plates in the presence of serial dilutions of LS (△), LS heated to 56°C for 30 mins (□), or LS heated to 100°C for 30 mins (◇). Several control wells did not receive any LS (▲). After co-incubating with a preparation of LS and 1 μCi/well ^{3}H-thymidine for 96 hrs, cells were harvested and the ^{3}H-thymidine incorporated into DNA was determined. The difference between the effect of nonheated LS and LS heated to 100°C ($P < 0.01$), and between LS heated to 56°C and LS heated to 100°C ($P < 0.01$) was statistically significant. There was no significant difference ($P < 0.4667$) between nonheated LS and LS heated to 56°C.

Effects of LS on the Growth and Development of Bovine Embryos *In Vitro*. Results from this experiment are shown in Table 1. Exposure to LS at 1:800 dilution significantly promoted the development of presumptive zygotes to the four-cell stage (47.1 + 2.3%) compared with the control (42.7 + 2.3) and the EGF-treated group (42.9 + 3.2; $P < 0.05$). The proportion of blastocysts developing from the four-cell stage in the LS–treated group (61.2 + 3.7%) was significantly higher than that of the control (49.8 + 4.1) and was similar to the EGF-treated group (61.7 + 3.3% $P < 0.05$). The proportion of blastocysts developing from oocytes was significantly higher in the LS–treated group (28.8 + 2.9%) compared with the control (21.3 + 3.3% $P < 0.05$). The experiments were performed four times, each time with similar results.

Discussion

Our findings indicate that LS stimulates the proliferation of murine neuroblastoma Neuro-2a cells in a concentration-dependent manner. The stimulatory effects of LS on Neuro-2a cells appear to be highly potent, as the range of effective concentrations was between the dilutions of 10^6-fold and 10^4-fold. At the present time, the identity of this mitogenic factor(s) remains unknown. However, there are several lines of evidence suggesting that this mitogenic factor(s) may not be a large protein or even a protein to begin with. First, the protein concentration of undiluted LS (i.e., <3.0 μg/ml) is low. Within the effective dilution range of 10^6-fold and 10^4-fold, a large protein would yield concentrations at subnanomolar levels. It is unlikely that a protein can exert stimulatory activity in such low concentrations. Second, our preliminary experiments of digesting neutralized LS with trypsin did not inactivate the stimulatory activity of this mitogenic factor(s). Third, heat treatment of LS at 56°C for 30 mins had no effect, whereas treatment at 100°C for 30 mins significantly reduced its stimulatory activity. Because the integrity of proteins with large molecular weights usually cannot withstand heat treatment of 56°C for 30 mins, it can be deduced that the putative mitogenic factor(s) may not be a protein at all but, rather, a polysaccharide. Further investigations, including determination of the molecular weight, will be conducted in our laboratory in the near future.

The mitogenic effect of LS on Neuro-2a cells led us to further examine our hypothesis that LS may stimulate other rapidly dividing cells. In the CAM assay, LS was shown to stimulate the influx and proliferation of several types of cells including fibroblasts, neutrophils, macrophages, and lymphocytes. The proliferation of lymphocytes is comparable to our previous findings that LS augments pokeweed mitogen-induced lymphocyte proliferation *in vitro* (16). However, because the inflammatory cells were present predominantly in blood vessels, we surmise that the primary effect of LS on these cells is chemotactic, rather than inducing cell proliferation. The extent of cellular proliferation stimulated by LS is more profound than that of many other growth factors routinely tested in our laboratory. In addition to its effect on cellular influx and proliferation, LS also enhanced the accumulation of extracellular matrix and edematous fluid in the chicken embryos. To reach such strong responses during the 48-hr treatment, intercellular communications in the 8-day-old chicken embryos must be quite pronounced. In this aspect, cytokines may have played an important role. The release of cytokines on exposure to lactobacilli or other lactic-acid bacteria such as TNF-α (14, 15, 20–24), ILs-2, -5, -6, -8, 10, and -12 (14, 20, 21, 22, 25–28), and interferons (13, 28–30) has been reported by many research groups. Similarly, we have observed that LS treatment increases the gene expression and secretion of TNF-α in a murine macrophage cell line (16).

The chicken chorioallantoic membrane forms blood vessels during its growth and development. In our study, treatment of the membrane with LS accelerated the process of angiogenesis. Parallel to this are findings that in the ear lobes of young rats and mice, LS stimulated the formation of blood vessels, possibly mediated through an increase of receptors of vascular endothelial growth factor (16). Taking the observations of its induction of cytokine secretion and angiogenesis into consideration, it is likely that LS acts as a chemotactic agent to trigger a local inflammation-like reaction. This reaction includes the proliferation and chemoattraction of fibroblasts, neutrophils, macrophages,

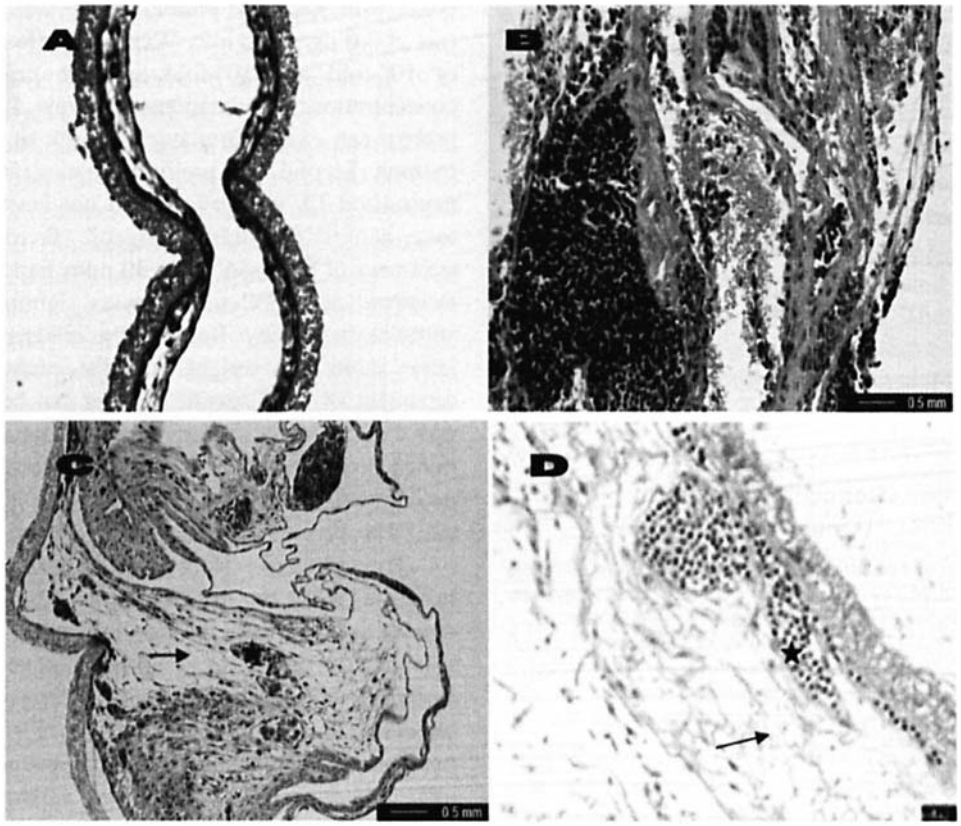

Figure 2. Angiogenesis and CAM thickening. In chicken CAM, LS stimulates blood vessel proliferation. For 1–2 days, LS was applied to the CAM of 8-day-old chicken embryos (see Materials and Methods). Membranes were collected 1–2 days later, fixed in formalin, processed for histology, and stained hematoxylin/eosin. (A) Control CAM treated only with PBS formed by a fine layer of extracellular matrix interspersed with a few fibroblasts. Magnification: ×400. (B and C) The CAM that was treated with LS is thickened due to the presence of engorged blood vessels (*). Magnification: ×400 and ×200, respectively. (C) Some areas of CAM were thickened due to the presence of connective tissue, which was very loose at times (→) and interspersed with fibroblasts. (D) Engorged blood vessels containing white blood cells (*) are separated by loose connective tissue (→).

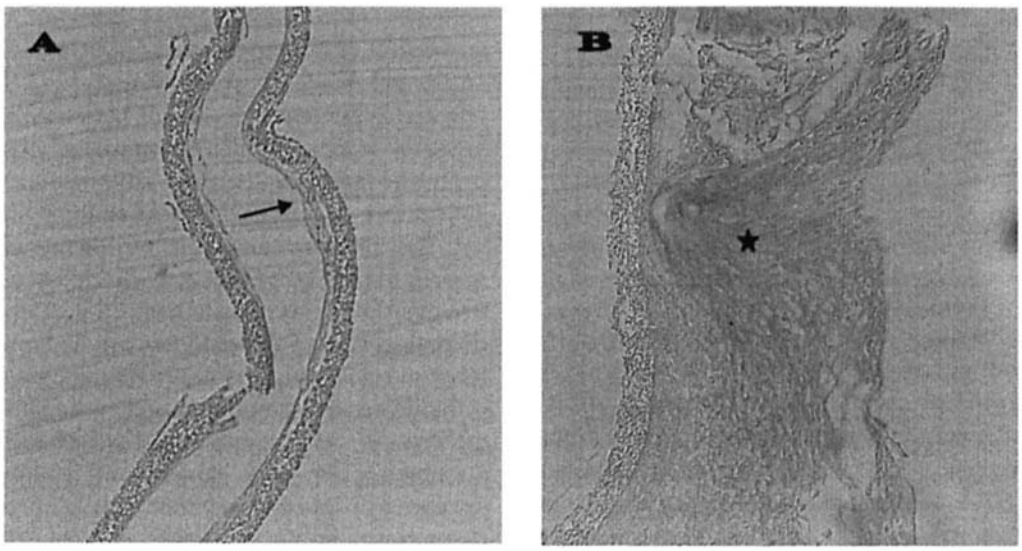

Figure 3. The accumulation of mucopolysaccharides in CAM. (A) Thin, untreated CAM contains only small amounts of alcian blue material (→). (B) The CAM that was treated with LS is thickened due to the accumulation composed primarily of tissue intensively stained with alcian blue (*).

Table 1. Effects of LS on Bovine Embryos from 18–192 hrs Postinsemination (1:800× dilution) or EGF on Bovine Blastocyst Production *In Vitro*[a]

Treatment	Oocytes	Blastocysts	% four-cell/oocytes	Day 8 blastocysts	% blastocysts/four-cell	% blastocysts/oocytes
Control	483	206	42.6 ± 2.3[a]	102	49.5 ± 4.1[c]	211 ± 3.3[e]
L. acidophilus	259	122	47.1 ± 2.0[b]	75	61.4 ± 3.7[d]	28.9 ± 2.9[f]
EGF (5 ng/ml)	269	115	42.7 ± 3.2[a]	71	61.7 ± 3.3[d]	26.4 ± 3.3[e,f]

[a] This analysis was done on numbers of oocytes and blastocytes combined from four experiments. Different letters within columns denote significant differences among different data points ($P < 0.05$).

and lymphocytes; the accumulation of extracellular matrix or fluid; and the formation of blood vessels.

Whether the LS–induced, inflammation-like reaction is beneficial to the living organism was indirectly answered by the results from experiments involving bovine embryos. An important finding in the current study is that co-culturing of LS with bovine embryos increased the percentage of oocytes that developed to the blastocyst stage. Embryonic development was enhanced by LS to both the four-cell and blastocyst stages. This is in contrast to EGF, which exerts its enhancing activity after the four-cell stage. The mechanism by which LS is stimulatory toward embryonic growth and development *in vitro* is yet to be determined. However, it is possible that LS owing to its stimulatory effect on cytokine secretions, facilitates a better intercellular communication among the bovine embryonic cells. As a consequence, the growth of cells within each embryo is better orchestrated which, in turn, results in higher success rates of embryonic survival. Our novel finding that the growth of chicken and bovine embryos can be stimulated by LS may pave the way for using LS as a tool to further investigate the mechanism of embryonic growth and development.

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