In vitro Development of Murine Embryos in presence of *Campylobacter fetus*

Desarrollo de Embriones Murinos *in vitro* en Presencia de *Campylobacter fetus*

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SUMMARY: Bovine campylobacteriosis caused by *Campylobacter fetus* is associated with reproductive losses. The knowledge about the mechanisms of bacterial pathogenesis is limited, then a murine experimental model is proposed. BALB/c females and males were used. Two-cell embryos were cultured in Ham-F10 as control group (CG). Treatment groups were constituted by the addition of Cfv 1 and 3, or Cff 2 and 5. Morulae were placed in Ham-F10 (CG); treatment groups were constituted by the addition of Cfv27, CFF (cell-free filtrate) and *Brucella* broth (BB). Blastocysts were cultured in MEM (CG); challenge group were constituted by the addition of Cfv 27. Differentiation, hatching, hatched, adhesion and expansion were evaluated. Results were analyzed by Chi^2 test. In two-cell embryo, the differentiation rate was not modified when the study strains were added ($p > 0.05$). The differentiation rate at 24 h for embryos at the morula stage was lower for BB, Cfv, and CFF, compared with CG ($p < 0.05$). After 48 h culture, no differences were observed in blastocyst formation for Cfv and BB, compared to CG ($p > 0.05$). However, the differentiation rate for the CFF group was lower than for CG ($p < 0.05$). At 48 and 72 h, the hatching rate was higher in CFF and Cfv groups than in CG ($p < 0.05$). Differences were not detected in blastocyst cultures. In conclusion, under these experimental conditions, Cf was not detrimental to the development of murine embryos. Efforts will be intensified to establish in vitro infection models that reproduce their pathogenicity.

KEY WORDS: Campylobacteriosis; Blastocyst; Morulae; *in vitro*; Mouse.

INTRODUCTION

Bovine genital campylobacteriosis is one of the most important venereal diseases in Argentina (Campero, 2002). *Campylobacter fetus* venerealis (Cfv) and *Campylobacter fetus* fetus (Cff) are the causative agents of this disease, which is characterized by producing embryonic death and occasional abortion in cattle (Clark, 1971; Hoffer, 1981; Garcia *et al*., 1983; Hum, 1987). *Campylobacter fetus* (Cf) is highly adapted to mucosal surfaces (Hu & Kopecko, 2000) and bacterial population structure is clonal. Cfv is thought to represent a bovine clone of Cf (Gorkiewics *et al*., 2010). It is considered that Cf pathogenic mechanisms are related not only to the effects of bacterial virulence but also to the participation of the host immune response. Several reports suggest that Cf virulence factors are the cause of embryonic mortality. Some virulence factors that have been associated with the generation of changes in the uterine as well as in the oviductal environment are the microaerophilic property of the bacteria and the production of mucinases. Such changes interfere with the normal embryo development during free life, causing a delay in the implantation and, at a later stage, they lead to embryo death (Hoffer; Garcia *et al*.; Hum; Joens *et al*., 2010). However, several works on in vitro fertilization of bovine oocytes carried out in the presence of Cfv show that fertilization and early embryonary development would not be affected (Bielanski *et al*.).

Campylobacter fetus produces direct or indirect damage to the host cell through different mechanisms such as adhesion (Chiapparrone *et al*., 2011), invasion (Hu & Kopecko, 2008; Larson *et al*., 2008) and production of cytotoxins (Casadevall & Pirofski, 2000; Catena *et al*., 2010). Although these virulence factors have been studied on strains of Campylobacter genus isolated from human beings. No evidence is available to indicate whether Cf employs similar virulence strategies. The presence of exotoxins has been confirmed in cytotoxicity assays (Johnson & Lior, 1987;

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Misawa *et al.*, 1995; Tresierra *et al.*, 1995). In 1993, cytolethal toxins were demonstrated in cultures of CHO cells infected with Cff strains of from bovines, establishing or opening the question on the effect of such cytotoxins on embryos (Ohya *et al.*, 1993).

The absence of genetic tools to manipulate Cf and the difficulty to carry out studies to investigate the molecular basis of host-bacterial interaction lead to the continuous search for new experimental models (Corbeil, 1980; McCardell *et al.*, 1986; O'Sullivan *et al.*, 1988; Young *et al.*, 2000).

Experimental infection assays were carried out in mice in an attempt to find an alternative experimental model. However, the disease could not be reproduced, Catena *et al.* (unpublished data). Considering Animal Welfare legislations and the economic cost of the susceptible hosts (bovines) such model could not be implemented. For that reason it would be important to develop an ex vivo study model to reproduce the natural niche conditions for Cf.

This work proposes to find a mouse model that allows ex vivo study of the *Campylobacter fetus* pathogenesis at different stages of the early embryonic development.

MATERIAL AND METHOD

Animals. Female and male mice from our own breeding colony went through a lighting regimen consisting of 12 h of exposure to light and 12 h in darkness, for seven days before treatment. Mice were placed in plastic boxes with free access to tap water and were handled according to the Animal Welfare Act. (Resol. 087/02, Faculty of Veterinary Sciences, UNCPBA). Five to eight weeks old, C57BL6 X BALB/c females, weighing 20-25 g were superovulated by intraperitoneal injection of 10 IU of equine chorionic gonadotropin (eCG - Novormon ®, Laboratorios Syntex S.A, Argentina) and 48 h later were injected with 10 IU of human chorionic gonadotropin (hCG, Profasi ®, Laboratorios Serono, Méjico). Females were mated with 12-14 weeks old, proven fertility BALB/c males. Day one of pregnancy was determined when vaginal plugs were first observed.

Embryos collection. Two-cell embryos were recovered from the oviducts of pregnant females euthanized by cervical dislocation on day 2 of pregnancy. Pre-implantation embryos at morula stage were recovered from the oviducts and uterus of pregnant females euthanized on day 3 of pregnancy and blastocysts were collected from the uterus on day 4 of pregnancy. Embryos were obtained from the oviducts and uterus by flushing with Ham F10 (Gibco BRL, Life Technologies NY, USA) supplemented with 0.4% of bovine serum albumin (BSA - Sigma Chemical Company, St. Louis, MO, USA) using a 30G needle on a 1-cc syringe.

Bacterial strains and growth conditions. Cfv 1, Cfv 3 and Cfv 27, and Cff 2 and Cff 5 isolated from the cervical mucus of natural infected heifers were provided by the culture collection of the Laboratory of Clinical and Experimental Microbiology at the Faculty of Veterinary Sciences, UNCPBA. The strains were grown in an anaerobic jar at 37°C on Skirrow medium (FA Merck) with Campylobacter selective supplement (Oxoid, USA) and incubated for 3 days in an atmosphere of 10% CO2, 5% O_2 and saturation of N2. The bacterial colonies were swept and washed with PBS (pH 7.2), centrifuged and re-suspended in minimum essential medium (MEM-EAGLE, M0643 Sigma-Aldrich, USA) to a final cell concentration of 4.5 (OD480). Gram staining was used to examine the morphology and purity of bacteria; only curved, spiral rods were selected. Spherical shaped bacteria were discarded since this morphology is considered a sign of loss of Campylobacter viability. Stock cultures were maintained at -70°C in 15% (vol/vol) glycerol-tryptose phosphate broth (Merck 13811).

Cell-free filtrate (CFF) was obtained from the supernates of centrifuged (30 min at 3000 g) of Cfv 27 culture in Brucella broth. To eliminate bacteria remaining in the supernates, these were either passed through cellulose filters (0.22 micron pore size Millipore Corp., Bedford, MA, USA), or gentamicin was added to a final concentration of 200 mg/L.

Culture procedures. Two-cell embryos were placed into cell culture dishes (Falcon # 3002 - 60x15 mm) in groups of 15, in 60 µL micro-drops of Ham-F10 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% of calf bovine serum, as control group (n: 45). Treatment groups were constituted by the addition of 1 x 108/mL of Cfv 1 (n: 45) and Cfv 3 (n: 45), or Cff 2 (n: 45) and Cff 5 (n: 45). Embryos were cultured for 72 hours at 37°C under silicon oil (Parafarm®, Argentina) in a humidified atmosphere of 5% CO2/95% air. To evaluate the progress of development and embryonic morphology, embryos were checked every 24 h.

Murine morulae were placed into cell culture dishes (Falcon # 3002 - 60x15 mm), in groups of 5, in micro-drops (25µl) of Ham-F10 supplemented with 0.4% of BSA, as control group (CG, n: 97), with Cfv 27 added at 1x108 /mL (Cfv, n: 129), with 0.25 µL of their cell-free filtrate (CFF, n: 119) and with 0.25 µL of Brucella broth (BB, n: 94); as treatment groups. Embryos were cultured for 72 h at 37°C under silicon oil (Parafarm®, Argentina) in a humidified

atmosphere of 5% CO_2/95% air. To evaluate the progress of development and embryonic morphology, embryos were checked every 24 h. Fifty nine good to excellent quality blastocysts were cultured in 6-well cell culture plates (Greiner Bio-One, Cellstar® TC-plate N° 657160). Blastocysts were randomly divided in groups of 4, and cultured in 2 mL of MEM supplemented with 10% of calf bovine serum, as control group (n: 24). Blastocysts were cultured in 1.75 mL of MEM and a suspension of 0.25 mL of Cfv (1x108/mL of Cfv 27), as challenge group (n: 35), was added. Blastocysts were cultured for 96 h at 37°C in a humidified atmosphere of 5% CO_2/95% air. To evaluate the in vitro development, embryos were observed daily and sequentially photographed.

Evaluation of in vitro embryo development. In cultures starting with two-cell embryos, the percentage of cell differentiation (number of embryos which reached the blastocyst stage/number of cultured embryos at the two-cell stage per 100) was determined. In embryo cultures started at the morula stage, the percentages of cell differentiation and hatching (number of zona-escaping blastocysts/number of cultured embryos at the morula stage per 100) were determined. To study differentiation rates, morulae were evaluated after culture periods of 24 and 48 h. To evaluate the hatching rate, the embryos were scored after 48 and 72 h of culture. In cultures started as blastocysts, the percentages of hatched embryos (blastocyst that protruded trough the zona pellucida), adhesion and cellular expansion were determined after 24, 48, 72 and 96 h of culture. Adhesion was considered when it was not possible to remove the

embryos from the bottom of the well and expansion, when multiplication of adhered embryonic cells was observed.

Statistical analysis. Percentages of differentiation, hatching, hatched, adhesion and expansion were analyzed by Chi^2. Significant differences were considered when $p < 0.05$.

RESULTS

Embryos cultured from two-cell stage did not suffer any modification in the differentiation rate at 72 h, when the strains in study were added: Cff 2 (90%); Cff 5 (100%); Cfv 1 (100%); Cfv 3 (90%). The differentiation rate for the control group was 95% ($p > 0.05$).

The differentiation rate at 24 h for embryos cultured at the morula stage was lower for BB, Cfv, and CFF, compared with CG ($p < 0.05$). After 48 h of incubation no significant differences were observed in blastocyst formation for Cfv and BB, with respect to CG ($p > 0.05$). However, the differentiation rate for the CFF group was lower than for CG ($p < 0.05$) (Table I).

At 48 h and 72 h, the hatching rate was higher in CFF and Cfv groups than in CG ($p < 0.05$). Differences were not detected between BB and CG ($p > 0.05$) (Table I).

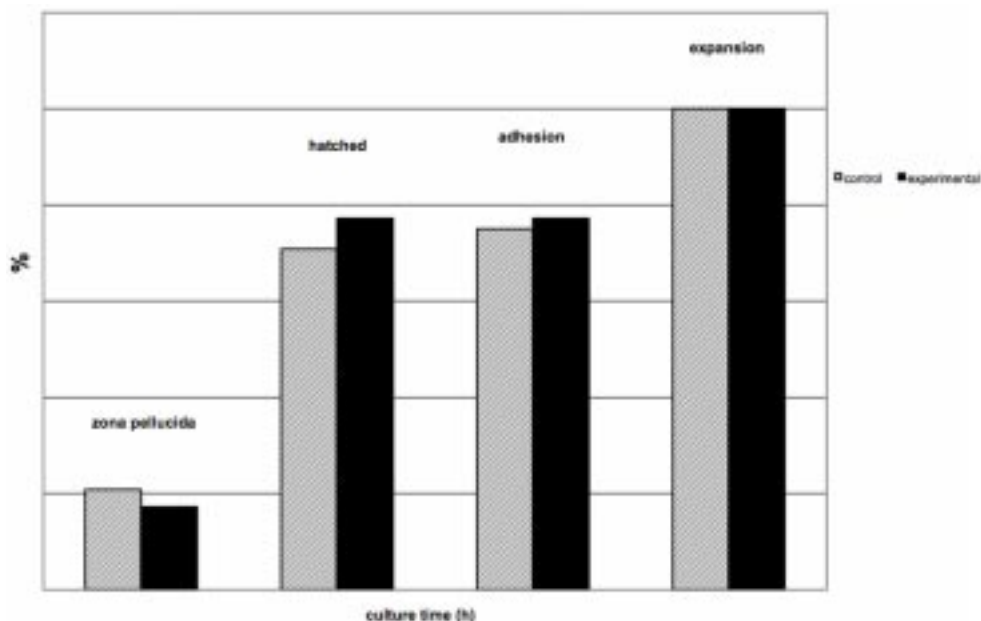Differences were not detected for the evaluated parameters in blastocyst cultures ($p > 0.05$) (Figs. 1 and 2).

Fig. 1. Development of murine blastocysts cultured in presence or absence of Cfv.

Table I. Differentiation and hatching rates in murine embryos (at morula stage) cultured in presence or absence of Cfv.

Groups	Parameters			
	Differentiation (%)		Hatching (%)	
	24 h	48 h	48 h	72 h
CG	82.5[a]	89.6[a]	49.4[a]	65.5[b]
BB	67.0[b]	76.2[ab]	44.5[a]	66.2[ab]
Cfv	66.6[b]	78.3[ab]	71.2[b]	79.2[ac]
CFF	65.3[b]	70.7[b]	70.3[b]	83.5[c]

CG: control group; BB: Brucella broth; Cfv: addition of *Campylobacter fetus* at 1 x 108 Cfv/mL; CFF: cell-free filtrate. Different letters within the same column, show significant differences ($p < 0.05$)

DISCUSSION

The host and tissue tropism of Cf subspecies offers a unique opportunity to understand the molecular bases of habitat selection. The direct effect of Cf on the bovine embryo is one of the aspects of the pathogenesis which has not even elucidated (Garcia & Brooks, 1993). According to this, we have developed models that allow us to study the effect of Cff and Cfv on their target cells, mainly bovine endometrial and vaginal cells (Chiapparrone *et al.*, 2011).
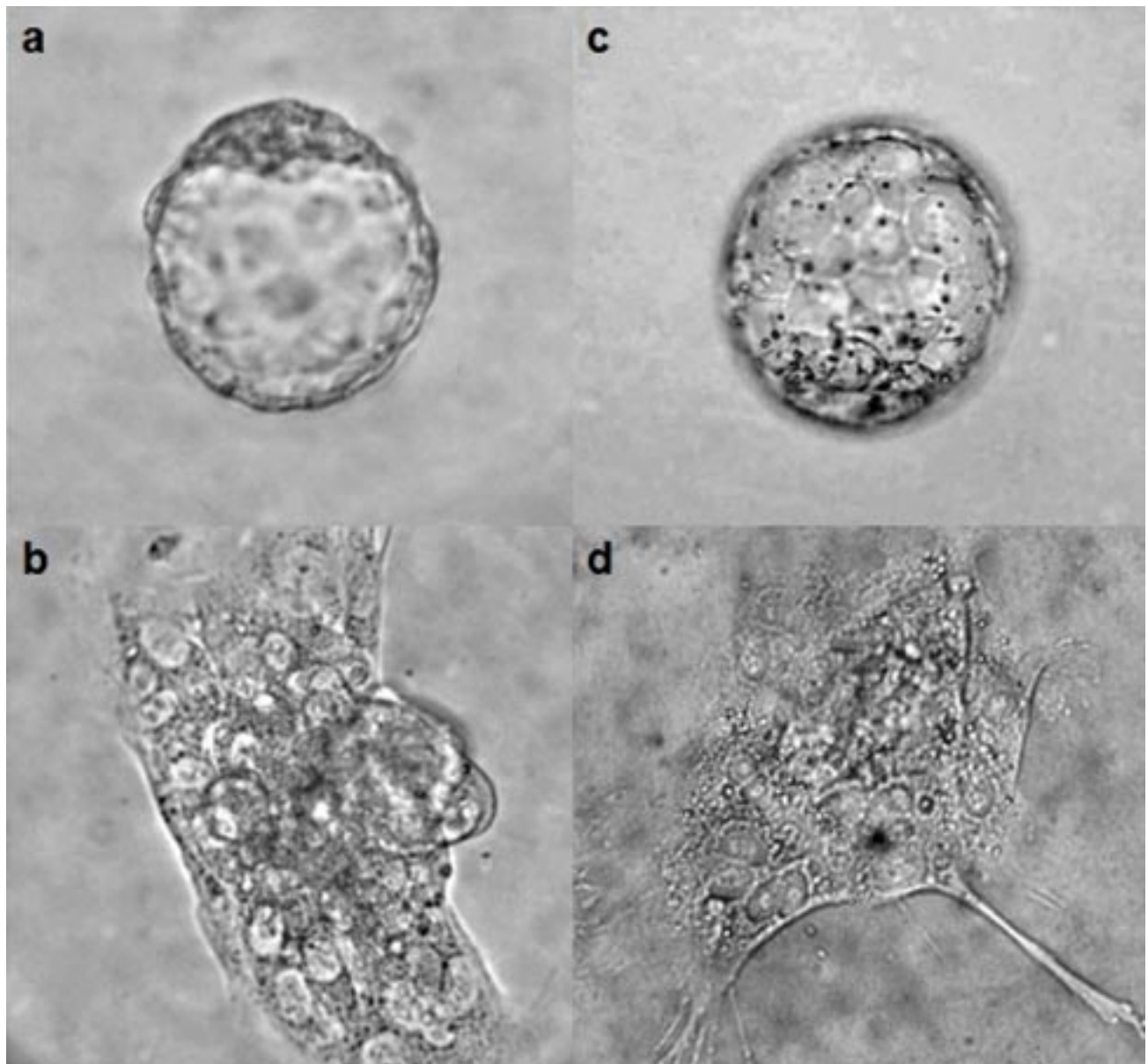

Fig. 2. Photomicrographs of adhesion and cellular expansion in blastocysts at starter culture and 96 h later. Control embryo: a) 0 h and b) 96 h of culture; challenge embryo: *Campylobacter fetus* added at 1 x 108 Cfv/mL, c) 0 h and d) 96 h of culture.

The development of an ex-vivo model, such as the in vitro murine embryo culture allowed us to evaluate the action of Cf at the different stages of embryo development. Our results show that, in the presence of Cfv, there is a decrease in the differentiation rate during the first 24 culturehours. However, at later time-points, differences with the control group are not detected. On the basis of this observation, it can be inferred that the formation of the embryonic tissues derived from the internal cell mass, as well as those constituting the fetal placenta derived from the trophectoderm, is not affected by Cfv. It is possible to relate these findings with those from assays carried out with experimentally infected female bovines which were diagnosed as non-pregnant by ultrasonography; although the presence of trophoblastic cells in uterine histological sections (indicating mother-embryo interaction) was observed (Catena *et al.*, 2001). However, when the embryos were cultured in Ham F10 with the addition of cell-free filtrate, the differentiation rate resulted negatively affected at 24 and 48 culture hours. This may be a consequence to the effect of bacterial toxins present in the filtrate, as it was observed in bovine endometrial and vaginal cells (Chiapparrone *et al.*, 2011) and in cell line cultures (Catena *et al.*, 2003; Chiapparrone *et al.*, 2009).

The presence of Cf or the cell-free filtrate was not detrimental to the hatching process. This information is valuable considering that this process is an indicator of embryo viability and it is closely related to the embryo implantation capacity (Gordon & Dapunt, 1993).

The analysis of the relation between the presence of Cfv and embryo expansion once the zona pellucida was lost, revealed that the virulence factors of the strain used did not produce any evident interference in the development of the embryo. This is in agreement with in vitro studies on bovine oocytes, which determined that the presence of Cfv did not affect the fertilization or the early embryo development (Bielanski *et al.*, 1994; Joens *et al.*), as it was observed for other reproductive pathogens (Bielansky & Dubuc, 1994; Bielanski *et al.*, 2004).

The results obtained from studies with ex-vivo models and pregnant bovine females (Catena *et al.*, 2003) suggest that the determining factor of embryo death would be the bacteria-host interaction and not a direct effect of the bacteria alone (Beer & Billingham, 1979; Croy, 2001).

In conclusion, under these experimental conditions, Cfv and Cff were not detrimental to the early development of murine embryos cultured in vitro. Efforts will be intensified to understand the effects of CFF, specifically to establish in vitro infection models that reproduce the pathogenicity of Cf in order to identify the virulence factors that affect embryo cells.

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CATENA, M.; TERUEL, M.; MORÁN, P.; CHIAPPARRONE, M.; ECHEVARRIA, H. & SOTO, P. Desarrollo de embriones murinos in vitro en presencia de *Campylobacter fetus*. *Int. J. Morphol., 31(4)*:1168-1174, 2013.

RESUMEN: La campilobacteriosis bovina caudada por *Campylobacter fetus* produce pérdidas reproductivas existiendo poca información de los mecanismos de patogenicidad de dicha bacteria, por lo cual se propone un modelo utilizando ratones BALC/c. Embriones de dos células fueron cultivados en Ham-F10: grupo control (GC), los grupos experimentales fueron adicionados con las cepas Cfv 1, Cfv 3, Cff 2 y Cff 5. Mórulas fueron cultivadas en Ham-F10 (GC); los grupos tratados recibieron Cfv27, CFF (filtrado libre de células) y caldo Brucella (BB). Blastocistos fueron cultivados en MEM (GC) y MEM más Cfv 27 (grupo desafiado). Se evaluó: diferenciación, "hatching", "hatched", adhesión y expansión. Los resultados fueron analizados por Chi2. En embriones de dos células, la diferenciación no fue modificada por acción de las cepas evaluadas (p > 0,05). Para embriones en estadío de mórula, la diferenciación a las 24 h de cultivo fue menor para BB, Cfv, y CFF, comparado con el GC (p < 0,05). Luego de 48 h de cultivo, no hubo diferencias entre Cfv, BB, y CG (p > 0,05), no obstante para el grupo CFF la diferenciación fue menor al CG (p < 0,05). El porcentaje de "hatching" (48 y 72 h de cultivo), fue mayor en los grupos CFF y Cfv comparado con el GC (p < 0,05). La adición de Cfv 27 no modificó el desarrollo de blastocistos. En el modelo propuesto, Cf no afectó negativamente el desarrollo embrionario. Futuros trabajos serán necesarios para establecer un modelo de infección in vitro en pos de reproducir su patogenicidad.

PALABRAS CLAVE: Campylobacteriosis; Blastocisto; Mórula; *in vitro*; Ratón.

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