Influence of *Campylobacter fetus* subsp. *fetus* on ram sperm cell quality

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Campylobacter fetus subsp. *fetus* infection can occur in female sheep, causing infertility or abortion. Despite extensive research on the effect of these bacteria on female fertility, little research has been done on the influence of *C. fetus* subsp. *fetus* on the male factor. Our objective was to examine the influence of *C. fetus* subsp. *fetus* on ram sperm. Motility index, percentage of live spermatozoa, mean α t value (an indication of the chromatin stability of the sperm cell) and percentage of sperm cells expressing the FAS receptor were measured in sperm incubated in the presence or absence of *C. fetus* subsp. *fetus*. The motility index and viability of sperm incubated with the bacteria were lower than those of untreated sperm samples after 5 h. In bacteria-incubated sperm cells, the percentage expressing FAS receptor was already significantly elevated at 15 min. Bacteria-incubated sperm showed a greater prevalence of morphological damage. The bacteria were attached to tail and acrosome regions, and the sperm damage was concentrated in both the motility and chromatin regions. Bacteria-infected sperm cells showed a decrease in motility, increase in early acrosome reaction and chromatin damage. Similar effects were induced by incubation of the sperm with supernatants from *C. fetus* subsp. *fetus* cultures. Thus this study demonstrates that *C. fetus* subsp. *fetus* has a detrimental effect on the quality of ram sperm.

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INTRODUCTION

Campylobacter fetus is a microaerophilic Gram-negative bacterium and a recognized veterinary and human pathogen (Garcia *et al.*, 1983; Penner, 1988). This bacterium is divided into two closely related subspecies, *Campylobacter fetus* subsp. *venerealis* and *Campylobacter fetus* subsp. *fetus*, the latter of which is the main focus of this study. *C. fetus* subsp. *fetus* is associated with sporadic epizootic abortion in cattle and sheep (Brooks *et al.*, 2001; Grogono-Thomas *et al.*, 2000; Schulze *et al.*, 2006; van Bergen *et al.*, 2006). In Denmark, it has been reported that more than 60 % of fetal loss in sheep is associated with *C. fetus* subsp. *fetus* infection (Agerholm *et al.*, 2006). Similarly, in New Zealand, *C. fetus* subsp. *fetus* is the leading cause of diagnosed abortions in sheep (Mannering *et al.*, 2006). Marked tropism for the placenta, exhibited by *C. fetus* subsp. *fetus*, was suggested to be the main cause of these abortions (Garcia *et al.*, 1983; Steinkraus & Wright, 1994).

In humans, *C. fetus* subsp. *fetus* has been reported to be an aetiologic agent in human abortions in general (Hood & Todd, 1960), and of septic abortion with intact fetal membranes in particular (Steinkraus & Wright, 1994). To the best of our knowledge, no association between infection by *C. fetus* subsp. *fetus* and male fertility potential has been reported. Therefore, the aim of this study was to

determine the possible effect of infection by *C. fetus* subsp. *fetus* on sperm quality and fertility potential in rams.

We show here that incubation of ram sperm with *C. fetus* subsp. *fetus* or with bacterial supernatant from *C. fetus* subsp. *fetus* cultures causes defects in the sperm that were previously associated with decreased sperm quality and viability.

METHODS

All animals in this study were handled under the supervision of and according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Bar-Ilan University, Ramat-Gan, Israel.

Sperm and bacteria preparation. Using a sterile artificial vagina, ram semen was collected from Assaf breed rams after male stimulation by a rutted female. The sample was then washed twice and diluted in Ringer's phosphate medium with glucose (pH 5).

Bacterial cultures of *C. fetus* subsp. *fetus* were a kind gift from Dr M. Bernstein of the Department of Bacteriology, Israeli Veterinary Institute in Beit Dagan, Israel. Three strains of isolated bacteria from aborted sheep fetus were obtained. Bacteria were maintained on blood agar plates under anaerobic conditions (Lancaster & Simon, 2002).

Before each experiment, the bacteria were transferred to grow on trypticase soy agar with 5% DSB plates (Novamed) for 2 days at 37 °C. After that, the bacteria were harvested and transferred to the medium (suspension).

Other bacteria, including *Escherichia coli* and *Erwinia amylovora*, were cultured in the same media.

Using spectrophotometry, we found that a 0.1 OD₆₆₀ bacterial suspension equalled a bacterial concentration of $27 \times 10^8 \text{ ml}^{-1}$. To examine the influence of the bacteria on sperm quality, we incubated the 0.1 OD bacteria with $50 \times 10^6 \text{ ml}^{-1}$ sperm cells, which created a bacteria:sperm ratio of 54:1. This ratio was determined to be the lowest concentration of the bacteria that caused a sperm motility decrease after 5 h of incubation. Moreover, it is a similar infection ratio to that in Reichart *et al.* (2001). A similar concentration of the bacterium *Ureaplasma urealyticum* was used in a study by Reichart *et al.* (2000). After 2 days of bacterial growth, bacterial supernatant was produced by centrifugation and the bacterial suspension was passed through a 0.2 µm filter.

Sperm morphology analysis was performed using scanning electron microscopy as described previously (Evenson & Wixon, 2005).

Parameters of sperm viability. The parameters examined included motility, viability, morphology, sperm chromatin structure and FAS receptor expression, as these have been shown to predict sperm cell damage (Reichart *et al.*, 2000). The motility parameter indicates a tail defect, while all the other parameters are indicative of a head defect.

The mean αt value (an indication of the chromatin stability of the sperm cell) and %COMP αt (percentage of cells outside the main peak) were assessed by testing 10 aliquots of fixed sperm nuclei by FACS analysis (Reichart *et al.*, 2000).

The sperm were incubated in Ringer's glucose phosphate with 0.5 % glucose at room temperature (25 °C) for the duration of the experiment after dilution to $50 \times 10^6 \text{ cells ml}^{-1}$.

Sperm motility was determined using the sperm quality analyser (SQA; Gameta). The SQA has a photoelectric cell that detects fluctuations in the optical density of the suspension caused by the motility of sperm and converts them to a sperm motility index value (as described by Reichart *et al.*, 2000).

FAS receptor expression. FAS receptor expression was determined by immunofluorescence. Rabbit polyclonal anti-FAS IgG [$2 \mu\text{l}$ (ml sperm suspension) $^{-1}$ (MACS antibody; Miltenyl)] was added to each sample, which was then incubated at room temperature for 1.5 h. The samples were then washed and secondary antibody [bovine anti-rabbit IgG FITC, $1 \mu\text{l}$ (ml sample) $^{-1}$; Santa Cruz Biotechnology] was added for 30 min at 37 °C. The labelled cells represented damaged sperm. The cells were analysed by FACS at an excitation wavelength of 488 nm and emission wavelength of 535 nm (Xu *et al.*, 2001).

Statistical analysis. All statistical analyses were performed using SPSS for Windows, version 10.0. The sperm samples analysed in this study included spermatozoa incubated with bacteria and untreated sperm cells. Therefore, the type of treatment (bacteria/control) was defined as a between-sample factor. The sperm parameters motility index, percentage of live spermatozoa, mean αt value and percentage of sperm cells with FAS receptor were measured at two time points: 15 min and 5 h after the start of the experiment. Fifteen minutes is the minimum time to determine whether the bacterial influence is immediate, and 5 h reflected a time between capacitation and acrosome reaction of the sperm, as in mammals it is known that the time before fertilization with the oocyte is 4–5 h (Takahashi *et al.*, 1992). Therefore, the time of evaluation was defined as a within-sample factor. Time changes in the sperm parameters of the treated versus untreated samples were analysed using ANOVA with repeated measures. The between-sample changes in these parameters were analysed by ANOVA and the within-sample changes were analysed using a paired *t*-test. With regard to sperm morphology, one-way ANOVA was used since these variables were assessed only once, after 5 h of incubation.

RESULTS AND DISCUSSION

To evaluate the effects of *C. fetus* subsp. *fetus* on sperm, changes in four parameters indicative of sperm quality and fertility, i.e. motility index, percentage of live spermatozoa, percentage of sperm cells expressing FAS receptor and mean αt value, were examined in the control group of untreated sperm (C) and in samples incubated with *C. fetus* subsp. *fetus* bacteria (B) at 15 min and 5 h. After 5 h of incubation, these parameters in both the treated and control sperm were significantly lower compared to their mean values at 15 min (for example, sperm motility index was 524 ± 21.4 vs 463 ± 24.4 , $t=7.2$, and 519 ± 16.4 vs 333 ± 48.1 , $t=13.9$, respectively; $P \leq 0.01$, Table 1). Comparison of control sperm and those incubated with the bacteria revealed that the two groups were statistically similar in their 15 min motility-index values; however, after 5 h of incubation, the bacteria-treated samples exhibited significantly lower values of this variable compared to controls ($F=46.9$, $P \leq 0.01$; Table 1).

Similar results were obtained with regard to sperm viability. The percentage of live spermatozoa was significantly

Table 1. Comparison of parameters of sperm quality and fertility in control sperm samples and samples incubated with *C. fetus* subsp. *fetus*

Sperm characteristics	No. of experiments	Control: time of incubation (h)		With bacteria: time of incubation (h)	
		0.25	5	0.25	5
Motility index	8	523.9 ± 21.4	$463.4 \pm 24.4^*$	519.2 ± 16.4	$332.7 \pm 48.1^{*\ddagger}$
Viability (%)	6	85.6 ± 5.8	$76.5 \pm 7.2^*$	82.0 ± 5.2	$62.2 \pm 10.1^{*\ddagger}$
FAS (%)	6	16.8 ± 4.8	21.0 ± 5.3	$36.2 \pm 7.1^\dagger$	$61.6 \pm 11.9^{*\ddagger}$
αt	10	213.1 ± 47.5	217.2 ± 46.9	214.4 ± 47.0	215.5 ± 49.7

*Significantly different from the value at 15 min ($P \leq 0.01$).

†Significantly different from the control value ($P \leq 0.01$).

‡Significant interaction between type of treatment (C/B) and time (0.25/5).

decreased after 5 h of incubation in both bacteria-incubated and control samples compared to their values after 15 min ($85.6 \pm 5.8\%$ vs $76.5 \pm 7.2\%$, $t=12.2$, and $82.0 \pm 5.2\%$ vs $62.2 \pm 10.1\%$, $t=8.0$, respectively, $P \leq 0.01$; Table 1).

Both samples exhibited similar sperm viability after 15 min; however, after 5 h of incubation, the sperm co-incubated with bacteria exhibited significantly lower viability compared to the control ($F=8.0$, $P \leq 0.02$; Table 1).

No change over time was observed in the percentage of control sperm cells expressing the FAS receptor; however, in sperm incubated with the bacteria, the degree of FAS expression after 5 h of incubation was significantly higher compared with expression after only 15 min incubation ($61.6 \pm 11.9\%$ vs $36.2 \pm 7.1\%$, $t=-8.1$, $P \leq 0.01$; Table 1).

It is notable that the percentage of bacterially treated sperm cells expressing FAS receptor was already significantly higher than in the controls after 15 min ($36.2 \pm 7.1\%$ vs $16.8 \pm 4.8\%$, $F=30.7$, $P \leq 0.01$). A similar increase in FAS expression was observed after 5 h of incubation [$61.6 \pm 11.9\%$ (+ bacteria) vs $21.0 \pm 5.3\%$ (control), $F=58.1$, $P \leq 0.01$; Table 1]. No significant changes within or between samples were observed with regard to the α t values (Table 1).

Of the five sperm morphological defects examined after 5 h of incubation with or without bacteria, i.e. percentages of abnormal acrosome, disconnected heads, agglutinated heads, broken tails and twisted tails, four of these occurred at a significantly higher prevalence in the samples incubated with bacteria (abnormal acrosome: $23.4 \pm 8.8\%$ vs $14.3 \pm 5.8\%$, $t=-6.4$; disconnected heads: $28.1 \pm 9.8\%$ vs $16.7 \pm 4.1\%$, $t=-4.2$; agglutinated heads: $40.6 \pm 10.7\%$ vs $22.5 \pm 7.0\%$, $t=-7.0$, $P \leq 0.01$; and broken tails: $25.6 \pm 10.2\%$ vs 14.6 ± 7.8 , $t=-3.0$, $P \leq 0.03$; Table 2). The percentage of twisted tails was not statistically different between groups (Table 2; Fig. 1).

To determine whether the bacteria act through a secreted factor, changes over time in motility index, percentage of live spermatozoa, percentage of sperm cells with FAS receptor and mean α t values were examined in the control sperm samples and compared to those in sperm samples incubated with the supernatant of *C. fetus* subsp. *fetus* cultures. As above, in both treated and control groups, the motility index was significantly decreased after 5 h of

incubation compared to the initial state (518.7 ± 12.8 vs 442.6 ± 31.3 , $t=8.6$; and 367.3 ± 45.3 vs 131.2 ± 45.3 , $t=17.0$, respectively, $P \leq 0.01$; Table 3). Comparison of supernatant and control groups demonstrated that at both time points, 15 min and 5 h, the mean motility index in the treated sperm was significantly lower compared to that in the control ($F=123.7$ and $F=383.3$, respectively, $P \leq 0.01$; Table 3). In both groups, the percentage of live spermatozoa was significantly decreased after 5 h of incubation compared to the 15 min time point ($84.2 \pm 9.0\%$ vs $72.6 \pm 8.6\%$, $t=2.9$, $P \leq 0.04$; and $76.2 \pm 11.3\%$ vs $40.2 \pm 4.5\%$, $t=7.6$, $P \leq 0.01$, respectively; Table 3).

There was no significant difference at 15 min between the sperm samples in their sperm viability values; however, after 5 h of incubation, sperm viability in the supernatant-incubated samples was significantly lower compared to that in the controls ($F=102.0$, $P \leq 0.01$; Table 3).

As above, no significant changes over time were observed for FAS receptor expression in the control group; however, for sperm treated with bacterial supernatant, the mean value of this parameter after 5 h of incubation was significantly higher compared to that at 15 min ($94.0 \pm 3.1\%$ vs $83.7 \pm 2.6\%$, $t=-12.8$, $P \leq 0.01$; Table 3). At both points tested (15 min and 5 h), the percentage of sperm cells expressing FAS receptor in supernatant-treated cultures was significantly higher compared with the control ($83.7 \pm 2.6\%$ vs $14.0 \pm 2.4\%$; and $94.0 \pm 3.1\%$ vs $17.7 \pm 6.3\%$, respectively, $F=2674.5$ and $F=779.0$, respectively, $P \leq 0.01$). No significant changes were observed for the mean α t values.

After 1 h of incubation in bacterial supernatant, all five morphological defects studied occurred at a significantly higher prevalence in samples treated with bacterial supernatant relative to the control (Table 4) ($t=-5.8$, $t=-6.2$, $t=-7.3$, $t=-4.3$ and $t=6.9$, respectively, $P \leq 0.03$; Table 4).

To determine whether the effect of *C. fetus* subsp. *fetus* on the sperm cells is unique to this bacterial species, we compared its effects to those of other Gram-negative bacteria, *E. coli*, commonly found in the natural flora of the intestine, and *Erwinia amylovora*, which exists on growing plants. While *C. fetus* subsp. *fetus* had a significant effect on motility ($P \leq 0.01$), viability ($P \leq 0.05$) and FAS

Table 2. Comparison of different morphological malformations in control sperm samples and samples incubated with *C. fetus* subsp. *fetus*, after 5 h

Values are the percentage of sperm exhibiting malformation. Seven experiments were performed, with 100 sperm analysed in each experiment.

Sperm samples	Abnormal acrosome	Disconnected heads	Agglutinated heads	Broken tails	Twisted tails
<i>C. fetus</i> subsp. <i>fetus</i>	$23.4 \pm 8.8^*$	$28.1 \pm 9.8^*$	$40.6 \pm 10.7^*$	$25.6 \pm 10.2^*$	18.6 ± 6.8
Control	14.3 ± 5.8	16.7 ± 4.1	22.5 ± 7.0	14.6 ± 7.8	17.6 ± 6.4

*Significantly different from the control value ($P \leq 0.01$).

Fig. 1. (a) Scanning electron micrograph of non-infected sperm (in Ringer's glucose phosphate, pH 6.3) (control); (b) scanning electron micrograph of bacteria-infected sperm cells (with *C. fetus* subsp. *fetus*, 1 : 54 ratio of sperm cell to bacterial cells, in Ringer's glucose phosphate, pH 6.3). Bar, 5 µm. The figure shows different types of damage to the sperm cell mediated by the bacteria, including cut-off head, damage to the acrosome, and cells in various stages of apoptosis.

expression ($P \leq 0.05$), no significant effect was induced by the other bacterial species (Table 5). This shows that *C. fetus* subsp. *fetus* has a unique effect on ram sperm quality.

Over recent decades, the phenomenon of infertility and early abortion in sheep with a background of *C. fetus* subsp. *fetus* infection in the female tract has become known

(Agerholm *et al.*, 2006). Brooks *et al.* (2001) have examined the effect of *C. fetus* subsp. *fetus* on the female genital tract. The present study focused on the possible contribution of the bacteria to subfertility mediated by male factors. Our results suggest that the occurrence of subfertility in sheep may also be related to the presence of the bacteria in the semen, in agreement with Eaglesome *et al.* (1995). We have

Table 3. Comparison of parameters of sperm quality and fertility for control sperm samples and samples incubated with *C. fetus* subsp. *fetus* supernatant

Sperm characteristics	No. of samples	Control: time of incubation (h)		With bacterial supernatant: time of incubation (h)	
		0.25	5	0.25	5
Motility index	12	518.7 ± 12.8	442.6 ± 31.3*	367.3 ± 45.3†	131.2 ± 45.3*†‡
Viability (%)	5	84.2 ± 9.0	72.6 ± 8.6*	76.2 ± 11.3	40.2 ± 4.5*†‡
FAS (%)	7	14.0 ± 2.4	17.7 ± 6.3	83.7 ± 2.6†	94.0 ± 3.1*†‡
αt	12	203.5 ± 48.4	204.7 ± 48.2	221.3 ± 39.3	228.0 ± 41.1

*Significantly different from the value at 15 min ($P \leq 0.01$).

†Significantly different from the control value ($P \leq 0.01$).

‡Significant interaction between type of treatment (C/B) and time changes (0.25/5) in the variable.

Table 4. Comparison of morphological malformations of control sperm samples and samples incubated for 1 h with *C. fetus* subsp. *fetus* supernatant

Values are the percentage of sperm exhibiting malformation.

Sperm samples	Abnormal acrosome	Disconnected heads	Agglutinated heads	Broken tails	Twisted tails
Incubated with supernatant	28.8 ± 9.8*	36.8 ± 10.6*	34.2 ± 8.7*	24.7 ± 7.3*	24.7 ± 6.9*
Control	12.3 ± 3.2	14.5 ± 2.7	22.2 ± 7.9	17.3 ± 6.1	15.0 ± 5.2

*Significantly different from the control value ($P \leq 0.01$).

shown the occurrence of characteristics previously related to sperm subfertility induced by the infection of sperm with *C. fetus* subsp. *fetus*. Following exposure to the bacteria, sperm cells introduced into the female (even by artificial insemination) can lose their viability and motility, thus reducing fertility potential and the chance of the semen to fertilize (Berkovitz *et al.*, 2004).

Bacterial infection may cause occlusion of the male tract or the accessory glands and thus lead to a decrease in the amount of sperm cells. It may even be related to a decrease in the quality of the sperm cells secreted into the semen, as expressed by a decrease in sperm cell motility and an increase in the number of sperm cells exhibiting abnormal morphology (Diemer *et al.*, 1996).

Vizzier-Thaxton *et al.* (2006) reported that horizontal and vertical transmission of *Salmonella* and *Campylobacter* can occur in broiler breeder flocks. Although the mechanism of this transmission is still unclear, scanning electron microscopy showed that *Salmonella* was associated with all three segments (head, midpiece and tail) of the spermatozoa and was, apparently, equally distributed. *Campylobacter* was mainly associated with the midpiece and tail segments; few isolates were located on the head segment (Vizzier-Thaxton *et al.*, 2006). In this study, we found that *C. fetus* subsp. *fetus* was attached to two main regions: the tail (close to the mitochondrial area) and the acrosome, especially the post-acrosome region (Fig. 1). The

damage to sperm was manifested in characteristics associated with both the motility and chromatin regions. Bacteria-infected sperm cells showed a decrease in motility and an increase in premature acrosome reaction as well as chromatin damage. *C. fetus* subsp. *fetus* caused the sperm head to separate from the tail, a significant increase in the development of apoptosis, and acrosome damage (Fig. 1).

Sperm morphology is considered disturbed in the presence of more than 20 % abnormal sperm cells (Liu *et al.*, 2004). Incubation (up to 5 h) of sperm cells with *C. fetus* subsp. *fetus* caused up to 30 % of cut-off heads, early acrosome reaction and agglutination. Recent reports show that the presence of bacteria in semen may lead to early abortions as a result of damage to sperm cells (Reichart *et al.*, 2000). Thus the bacteria can cause damage to sperm that will only be manifested following fertilization. This could explain why the bacteria have been recognized more as a trigger of abortion than a reason for infertility (Agerholm *et al.*, 2006).

The nature of the agent secreted by *C. fetus* subsp. *fetus* causing toxicity to sperm is not known. LPS has been suggested as mediating the toxic effects of the bacterium (Brooks *et al.*, 2002). Kajihara *et al.* (2006) found that LPS-treated mice demonstrate an increase in FAS-positive cells in many germ cell populations, especially in spermatocytes and spermatids. Their study suggests that the Fas/FasL system mediates apoptosis of germ cells in the testis of LPS-

Table 5. Comparison of parameters of sperm quality in samples incubated with *C. fetus* subsp. *fetus* versus *Erwinia amylovora* or *E. coli*

Sperm characteristics	No. of experiments	Time of incubation (0.25 h)				Time of incubation (5 h)			
		Control	<i>C. fetus</i>	<i>Erwinia amylovora</i>	<i>E. coli</i>	Control	<i>C. fetus</i>	<i>Erwinia amylovora</i>	<i>E. coli</i>
Motility index	5	523.9 ± 21.4	519.2 ± 16.4	531.7 ± 73.4	518.9 ± 94.3	463.4 ± 24.4	332.7 ± 48.1†	445.71 ± 46.4‡	396.5 ± 33.6†
Viability (%)	5	85.6 ± 5.3	76.5 ± 7.2	86.2 ± 5.8	83.7 ± 8.4	76.5 ± 7.2	62.2 ± 10.1*	78.4 ± 8.5‡	73.6 ± 2.1†
FAS (%)	3	16.8 ± 4.8	36.2 ± 7.1*	18.1 ± 4.8†	26.7 ± 7.9†	21.0 ± 8.3	61.6 ± 11.9†	37.6 ± 9.4†	45.1 ± 7.2*
αt	2	213.1 ± 47.5	214.4 ± 47.0	211.4 ± 38.1	216.4 ± 92.1	217.2 ± 46.9	215.5 ± 49.7	212.4 ± 75.5	218.1 ± 33.8

*Significantly different from the control ($P \leq 0.05$).

†Significantly different from the control ($P \leq 0.01$).

‡No significant difference from the control.

treated mice (Kajihara *et al.*, 2006). Nevertheless, it has been shown that different species of bacteria produce LPS with different structures and toxicity (Braun *et al.*, 2005). The synthesis of toxic LPS is regulated by environmental and nutritional factors (such as sugar). *C. fetus* subsp. *fetus* demonstrates significantly greater toxicity than other bacteria such as *Erwinia amylovora* and *E. coli*. It is possible that this difference reflects the production by *C. fetus* subsp. *fetus* of higher levels of toxic LPS than other bacteria.

This study provides an important basis for understanding the mechanism of bacterial effects on the male and female genital tracts by showing that *C. fetus* subsp. *fetus* can be toxic to the sperm cells.

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