

Actions of Tumor Necrosis Factor- α on Oocyte Maturation and Embryonic Development in Cattle¹

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PROBLEM: Infertility can accompany mastitis in cattle. Involvement of tumor necrosis factor- α (TNF- α) in this phenomenon is suggested by observations that circulating concentrations of TNF- α are elevated after intramammary infection or infusion of endotoxin. It was hypothesized that (1) TNF- α acts on the oocyte during maturation to decrease the percent of oocytes that cleave and develop following fertilization; (2) exposure of embryos to TNF- α after fertilization reduces development to the blastocyst stage; and (3) TNF- α increases the proportion of blastomeres that undergo apoptosis in a stage-of-development dependent manner.

METHOD OF STUDY: In one experiment, oocytes were matured with various concentrations of TNF- α and then fertilized and cultured without TNF- α . In another study, embryos were cultured with TNF- α for 8 days beginning after fertilization. Finally, embryos were collected at the two or four-cell stage (at 28–30 hr after insemination) or when ≥ 9 -cells (at day 4 after insemination) and cultured \pm TNF- α for 24 hr. The proportion of blastomeres undergoing apoptosis was then determined by the TUNEL procedure.

RESULTS: Addition of TNF- α to maturation medium did not affect the proportion of oocytes that cleaved. However, the percent of oocytes that developed to the blastocyst stage at day 8 after insemination was reduced ($P = 0.05$) at all TNF- α concentrations tested (0.1–100 ng/mL). When added during embryo culture, there was no significant effect of TNF- α on the proportion of oocytes that became blastocysts. In addition, TNF- α did not induce apoptosis in two and four-cell embryos. For embryos ≥ 9 -cells, however, 10 and 100 ng/mL TNF- α increased ($P < 0.05$) the percent of blastomeres labeling as TUNEL-positive.

CONCLUSION: TNF- α can have deleterious actions on oocyte maturation that compromise development of the resultant embryo. While exposure of fertilized embryos to TNF- α did not inhibit development to the blastocyst stage, TNF- α increased the percentage of blastomeres undergoing apoptosis when exposure occurred for embryos ≥ 9 -cells. Increased blastomere apoptosis could conceivably compromise subsequent embryo survival.

Keywords: Apoptosis, embryo, oocyte, tumor necrosis factor- α

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INTRODUCTION

Early embryonic death is among the most common causes of pregnancy termination; in cattle, most embryo losses occur during the first 2 weeks of

gestation.¹ The mechanisms mediating embryo survival and death are incompletely understood. One possible determinant of embryonic survival or failure is inappropriate signaling by maternally-derived cytokines. There is evidence that activation of immune responses

via vaccination in sheep² as well as mastitis in cattle^{3,4} can cause infertility. In rats, embryonic loss associated with diabetes has been associated with excessive uterine production of tumor necrosis factor- α (TNF- α)⁵ and it is possible that this cytokine could mediate effects of peripheral immune activation on embryonic survival. Circulating concentrations of TNF- α in cattle are elevated after intramammary *Escherichia coli* infection or infusion of endotoxin.⁶⁻⁹

The actions of TNF- α on embryonic development are not well understood. Exposure of rat blastocysts to TNF- α decreased cell proliferation¹⁰ and increased the rate of blastomere apoptosis.¹¹ Exposure of mouse blastocysts to TNF- α reduced the average number of cells in the inner cell mass (ICM) and, upon transfer to recipients, TNF- α pre-treated blastocysts had a higher rate of resorption.¹² In contrast to these effects on blastocysts, cleavage rate and the proportion of *in vitro*-fertilized oocytes or two-cell embryos developing to the expanded blastocyst stage were unaffected by the presence of TNF- α during fertilization and subsequent embryo culture.¹³ In this same study, the combination of TNF- α and endotoxin reduced development to the expanded blastocyst stage without affecting cleavage rate.

For the present study, it was hypothesized that (1) TNF- α acts on the oocyte during maturation to decrease the percent of oocytes that cleave and develop following fertilization; (2) exposure of embryos to TNF- α after fertilization reduces the ability of embryos to develop to the blastocyst stage; and (3) TNF- α causes an increase in the proportion of blastomeres that undergo apoptosis in a stage-of-development dependent manner.

MATERIALS AND METHODS

Murine recombinant TNF- α was purchased from United States Biological (Swampscott, MA, USA). The TNF- α was dissolved in 5 mM Tris-HCl, pH 8.0 and then diluted 1:1000 in culture medium so that the final concentration of Tris was 5 μ M. In all experiments, control medium (i.e., 0 ng/mL TNF- α) also contained 5 μ M Tris.

Modified Tyrode's solutions were obtained from Cell and Molecular Technologies (Lavallete, NJ, USA) to prepare 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-tyrode albumin lactate pyruvate (TALP), *in vitro* fertilization (IVF)-TALP and sperm-TALP as described.¹⁴ Essentially fatty-acid free bovine serum albumin (EFAF-BSA) and bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO, USA). Pituitary-derived FSH (Folltropin-V) was obtained from Vetrepharm

Canada Inc. (London, Canada). Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Frozen semen from various bulls was donated by Southeastern Semen Service (Wellborn, FL, USA). Oocyte collection medium was tissue culture medium (TCM)-199 with Hank's salts without phenol red (Sigma or Atlanta Biologicals, Norcross, GA, USA) and with the addition of 2% (v/v) bovine steer serum, 0.04 U heparin/mL, 100 U/mL penicillin-G, 0.1 mg/mL streptomycin, and 1 mM glutamine. Oocyte maturation medium was TCM-199 with Earle's salts (Cell and Molecular Technologies) supplemented with 10% (v/v) steer serum, 2 μ g/mL estradiol 17- β , 20 μ g/mL follicle stimulating hormone, 22 μ g/mL sodium pyruvate, 50 μ g/mL gentamicin and an additional 1 mM glutamine. Two modifications of potassium simplex optimized medium (KSOM) called KSOM-bovine embryo 1 (BE1) and KSOM-BE2, respectively, were prepared as described elsewhere.¹⁵

The *in situ* cell death detection kit was purchased from Roche Diagnostics Corporation (Indianapolis, IN, USA) and propidium iodide from Sigma. The Prolong® antifade kit was purchased from Molecular Probes (Eugene, OR, USA). Polyvinylpyrrolidone (PVP) was obtained from Eastman Kodak Company (Rochester, NY, USA). The RQ1 RNA-free DNase and RNase A were purchased from Promega (Madison, WI, USA) and Qiagen (Valencia, CA, USA), respectively. Other reagents were obtained from Fisher (Pittsburgh, PA, USA) or Sigma.

Oocyte Maturation, Fertilization and Embryo Culture

The procedures for embryo production were performed as described earlier.¹⁵ Briefly, ovaries from a mixture of beef and dairy cattle were obtained from slaughtered cows and used to obtain cumulus-oocyte complexes (COCs) by slicing 2–10 mm follicles. Groups of 10 COCs were transferred to pre-equilibrated 50 μ L drops of oocyte maturation medium and matured for 22 hr at 38.5°C in an atmosphere of 5% (v/v) CO₂ in humidified air. After maturation, COCs were washed in HEPES-TALP once and placed in groups of 30 per well into 600 μ L of IVF-TALP. For IVF, 25 μ L PHE [0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μ M epinephrine in 0.9% (w/v) NaCl] and ~ 0.3 – 1×10^6 Percoll-purified spermatozoa were placed in each well. Fertilization was allowed to proceed for 8–10 hr at 38.5°C and 5% (v/v) CO₂ in humidified air. Presumptive zygotes were then removed from fertilization wells, denuded of cumulus cells by vortexing in ~ 40 μ L of HEPES-TALP, washed two to three times in HEPES-TALP, and cultured in pre-equilibrated 50 μ L microdrops of modified KSOM-BE1 or KSOM-BE2 overlaid with mineral

oil. Culture was at 38.5°C and 5% (v/v) CO₂ in humidified air. Embryos were harvested from microdrops according to the requirements of specific experiments. Embryos at the two-cell and four-cell stage were harvested at 28–30 hr after insemination. Embryos \geq 9-cells were harvested on Day 4 after insemination.

Effects of TNF- α during Oocyte Maturation on Cleavage Rate and Embryo Development to the Blastocyst Stage

Cumulus-oocyte complexes were matured in groups of 10 in oocyte maturation medium containing 0, 0.1, 1, 10 or 100 ng/mL TNF- α . The embryos receiving 0 ng/mL TNF- α were cultured in KSOM containing 5 μ M Tris-HCl, pH 8.0 as this concentration of Tris was present in TNF-containing media. After maturation, COCs were washed and fertilized in medium without TNF- α . Presumptive zygotes from each treatment were collected after fertilization and placed in one or more groups of up to 30 in 50 μ L drops of KSOM-BE2 without TNF- α . The experiment was replicated seven times using a grand total of 172–199 embryos per group (i.e. summed across the seven replicates).

Effects of 24-hr Culture of two-cell, four-cell, and \geq 9-cell embryos with TNF- α on Embryo Cell Number and Apoptosis

In the first experiment, embryos at the two and four-cell stage were collected 28–30 hr after insemination and placed in a new drop of modified KSOM (either KSOM-BE1 or KSOM-BE2) containing 0, 0.1, 1, 10 or 100 ng/mL TNF- α for 24 hr at 38.5°C and 5% (v/v) CO₂ in humidified air. Embryos were then processed for the terminal deoxynucleotidyl transferase mediated X-dUTP nick end labelling (TUNEL) reaction as described below. The experiment was replicated three times using a grand total of 15–28 two-cell embryos per group and seven to 19 four-cell embryos per group. In the second experiment, embryos that were \geq 9-cells were collected on Day 4 after inseminated and transferred to a new drop of modified KSOM (KSOM-BE1 or KSOM-BE2). Embryos were then subjected to the same treatments as in the first experiment. The experiment was replicated five times using a grand total of 33–46 embryos per group.

Effects of TNF- α during Embryo Culture on Development to the Blastocyst Stage

Cumulus-oocyte complexes were matured and fertilized in medium without TNF- α . Following fertilization, presumptive zygotes were cultured in one or more groups of up to 30 in 50 μ L drops of embryo culture medium (KSOM-BE1) containing concentrations of 0–100 ng/mL TNF- α . Development was recorded at day 8 after insemination. For each replicate, approximately

equal numbers of presumptive zygotes were distributed across treatments. The experiment was replicated six times using a grand total of 131–167 embryos per group.

Determination of Apoptotic Cells Using TUNEL Reaction

Embryos were washed twice in 50 μ L drops of PBS [10 mM potassium phosphate, 0.9% (w/v) NaCl, pH 7.4] containing 1 mg/mL polyvinylpyrrolidone (PBS-PVP). Subsequently, embryos were fixed for 1 hr at room temperature in a 50 μ L drop of 4% (w/v) paraformaldehyde in 100 μ M phosphate, pH 7.4 with 0.9% (w/v) NaCl, washed twice for 2 min each in 50 μ L microdrops of PBS-PVP, and stored in 600 μ L PBS-PVP in four-well plates at room temperature until TUNEL staining (up to 4 weeks). Embryos were placed in 50 μ L drops of permeabilization solution [0.5% (v/v) Triton X-100 in 0.1% (w/v) sodium citrate] for 1 hr at room temperature. Positive-control embryos were incubated in RQ1 RNase free DNase (50 U/mL) for 1 hr at 37°C. Embryos were washed twice in PBS-PVP and incubated in 25 μ L microdrops of TUNEL mixture (fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase prepared as directed by the manufacturer) for 1 hr at 37°C in the dark. For negative controls, terminal deoxynucleotidyl transferase was absent. After reaction with the TUNEL mixture, embryos were transferred to 50 μ L drops of RNase A (50 μ g/mL) for 1 hr at room temperature, followed by incubation for 30 min at room temperature in 50 μ L drops of 0.5 μ g/mL propidium iodide. Embryos were washed four times in 50 μ L drops of PBS-PVP to remove excess propidium iodide, transferred to a poly-L-lysine coated slide, and allowed to dry. Coverslips were mounted with mounting medium containing Prolong Antifade® (Molecular Probes, Eugene, OR, USA).

Fluorescence was examined using a Zeiss Axioplan 2 fluorescence microscope with dual filter. The total number of nuclei and number of TUNEL-labeled nuclei were recorded for each embryo. Some embryos were also examined using a Bio-Rad 1024ES laser scanning confocal microscope (Carl Zeiss, Göttingen, Germany and Bio-Rad, Richmond, CA, USA). For fluorescein, an argon-ion laser adjusted to less than 560 nm was used, and for propidium iodide, a helium-neon laser adjusted to more than 560 nm was used. Images were obtained using a 303 objective, 10% power, and Z-steps of 0.5–1.0 μ m.

Statistical Analysis

Within each replicate, the percent of oocytes that cleaved and the percent of oocytes and cleaved embryos that developed to the blastocyst stage was

calculated for all embryos within the same treatment. Data on percent cleavage and percent development (untransformed and after arcsin transformation) were analyzed by least-squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Replicate was considered random and other main effects were considered fixed. Data are reported as least-squares means \pm S.E.M. of the untransformed data while probability values are derived from analyses of transformed data.

Differences between various levels of a main effect were determined using the pdiff mean separation test of SAS and by partitioning variance associated with treatment effects into individual orthogonal contrasts. The orthogonal contrasts were control versus all concentrations of TNF- α ; 0.1 and 1 versus 10 and 100 ng/mL; 0.1 versus 1 ng/mL; and 10 versus 100 ng/mL.

For apoptosis experiments, data were calculated for each embryo as total cell number and percent cells

undergoing apoptosis (i.e. positive for TUNEL reaction). Data were analyzed by least square analysis of variance using the General Linear Models procedure of SAS. Replicate was considered random and other main effects were considered fixed. Percentage data were transformed using the arcsin transformation as described above. Differences between treatments were determined by the pdiff mean separation test of SAS and by separating treatment variance using individual orthogonal contrasts described previously.

RESULTS

Effect of TNF- α during Oocyte Maturation

Addition of TNF- α to oocyte maturation medium had no effect on the proportion of oocytes that cleaved (Fig. 1, top panel). As compared with control oocytes, however, the proportion of oocytes that developed to the blastocyst stage at day 8 was reduced ($P < 0.05$) at

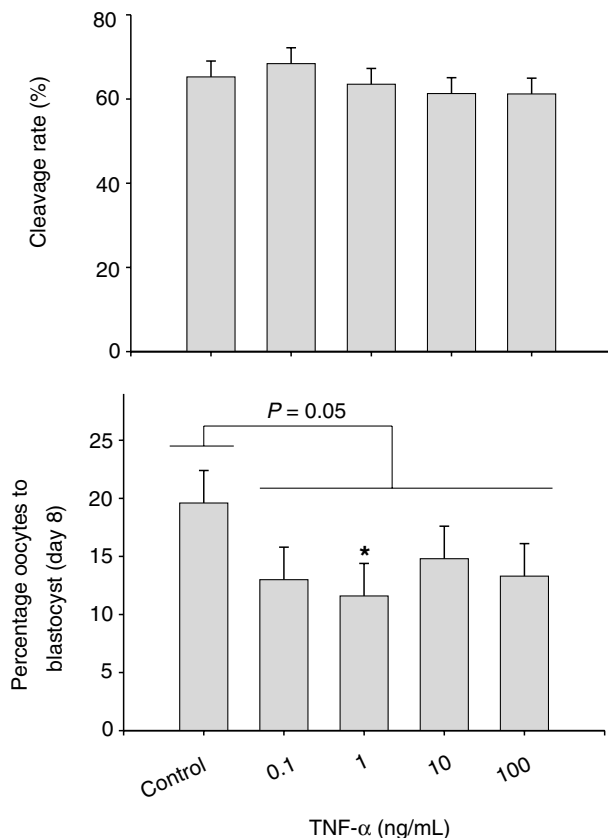


Fig. 1. Effects of tumor necrosis factor- α (TNF- α) during oocyte maturation on the percentage of oocytes that cleaved (top panel) and developed into blastocyst on day 8 after insemination (bottom panel). Results are least-squares means \pm S.E.M. The experiment was replicated seven times using 172–199 oocytes per group. Means that differ ($P < 0.05$) from control oocytes (0 ng/mL) are indicated by asterisks. Significant orthogonal contrasts are indicated by horizontal lines over the bars.

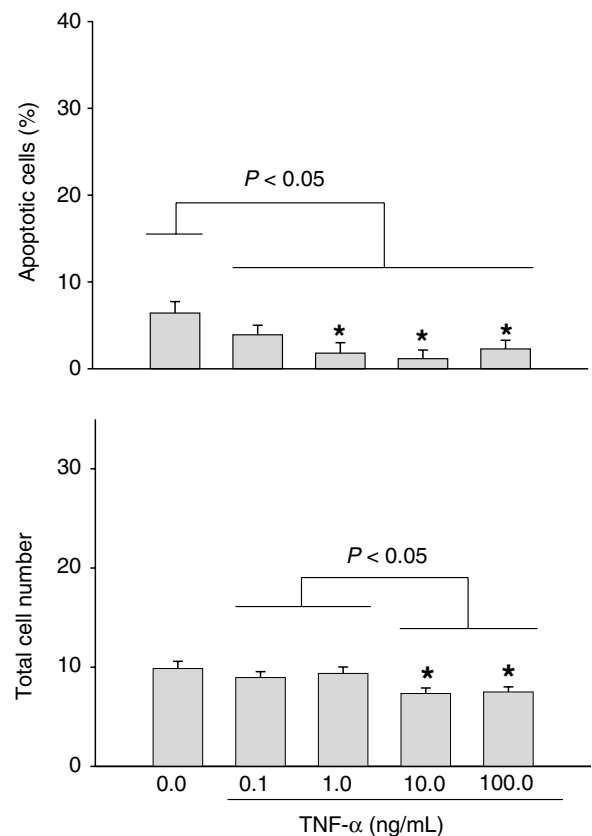


Fig. 2. Effect of 24-hr culture with tumor necrosis factor- α (TNF- α) on percent blastomeres undergoing apoptosis (top panel) and cell number (bottom panel) for two-cell embryos treated 28–30 hr after insemination. The experiment was replicated three times using 15–28 embryos per group. Results are least-squares means \pm S.E.M. Means that differ ($P < 0.05$) from control oocytes (0 ng/mL) are indicated by asterisks. Significant orthogonal contrasts are indicated by horizontal lines over the bars.

all concentrations of TNF- α (Fig. 1, bottom panel). The magnitude of the reduction in development was similar for all concentrations of TNF- α tested.

Actions of TNF- α on Embryonic Apoptosis and Development

A series of experiments was performed to characterize effects of 24 hr incubation with TNF- α on embryonic growth (as determined by cell number) and apoptosis (as determined by TUNEL reaction). At the two-cell stage, there was a reduction ($P < 0.05$) in cell number 24 hr after culture for embryos treated with 10 and 100 ng/mL TNF- α (Fig. 2, bottom panel). Representative images of two-cell embryos analyzed by the TUNEL reaction are shown in Fig. 3 (panels A and B). There was no evidence for TNF- α induced apoptosis. Indeed, the percent of cells labeled as apoptotic was very low at this stage in all treatments and was lower ($P < 0.05$) for embryos cultured with 1, 10, and 100 ng/mL TNF- α as compared with control embryos (Fig. 2, top panel). At the four-cell stage, culture with TNF- α did not affect total cell number 24 hr after culture (Fig. 4, bottom panel) or the percent of blastomeres undergoing apoptosis (Fig. 3, panels C and D and Fig. 4, top panel). For embryos ≥ 9 -cells at

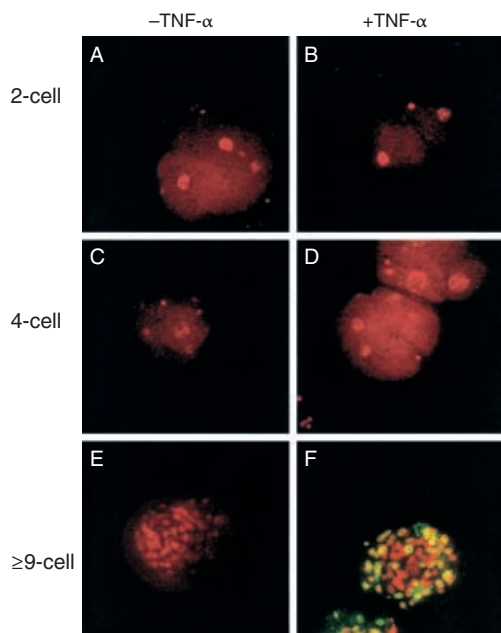


Fig. 3. Representative confocal images of embryos illustrating the effect of tumor necrosis factor- α (TNF- α) on frequency of apoptotic nuclei as analyzed by the TUNEL reaction. Embryos were labeled with propidium iodide (red channel) and fluorescein isothiocyanate-conjugated dUTP (green channel). Each panel illustrates one or two (panels D and F) embryos. Panels A and B: embryos treated at the two-cell stage. Panels C and D: embryos treated at the four-cell stage. Panels E and F: embryos treated at ≥ 9 -cells. Embryos in panels A, C, and E were not exposed to TNF- α while embryos in panels B, D, and F were cultured with 100 ng/mL TNF- α .

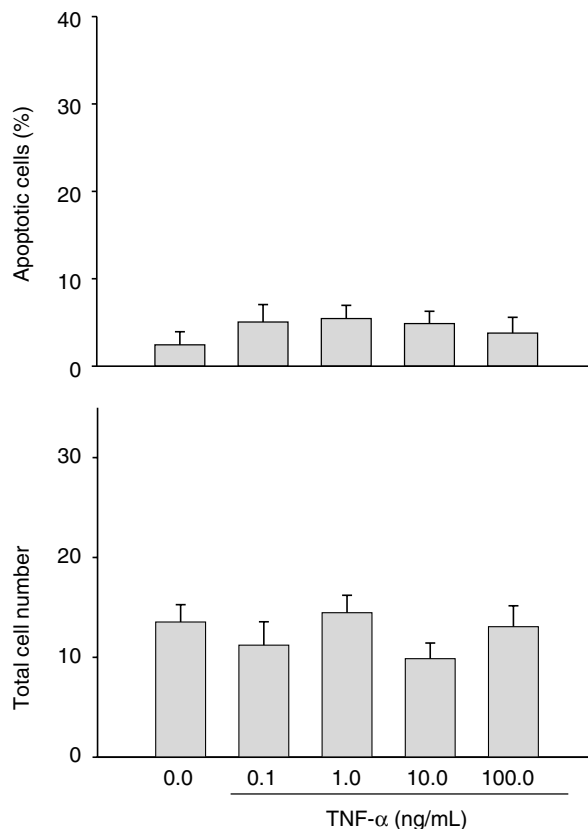


Fig. 4. Effect of 24-hr culture with tumor necrosis factor- α (TNF- α) on percent blastomeres undergoing apoptosis (top panel) and cell number (bottom panel) for four-cell embryos treated 28–30 hr after insemination. Results are least-squares means \pm S.E.M. The experiment was replicated three times using seven to 19 embryos per group.

day 4 after insemination, TNF- α had no effect on cell number (Fig. 5, bottom panel) but TNF- α induced apoptosis at concentrations of 10 and 100 ng/mL ($P < 0.05$) (Fig. 3, panels E and F and Fig. 5, top panel).

Prolonged incubation of embryos with TNF- α beginning after fertilization until day 8 after insemination had no effect on cleavage rate or the proportion of presumptive zygotes that developed to the blastocyst stage at day 8 (Fig. 6).

DISCUSSION

Results indicate that TNF- α can compromise the oocyte during maturation in a way that reduces potential of the resultant embryo to develop to the blastocyst stage. In addition, while not affecting development when added after fertilization, TNF- α does cause apoptosis in a stage-of-development dependent manner. Such an action by TNF- α could conceivably affect embryonic survival.¹²

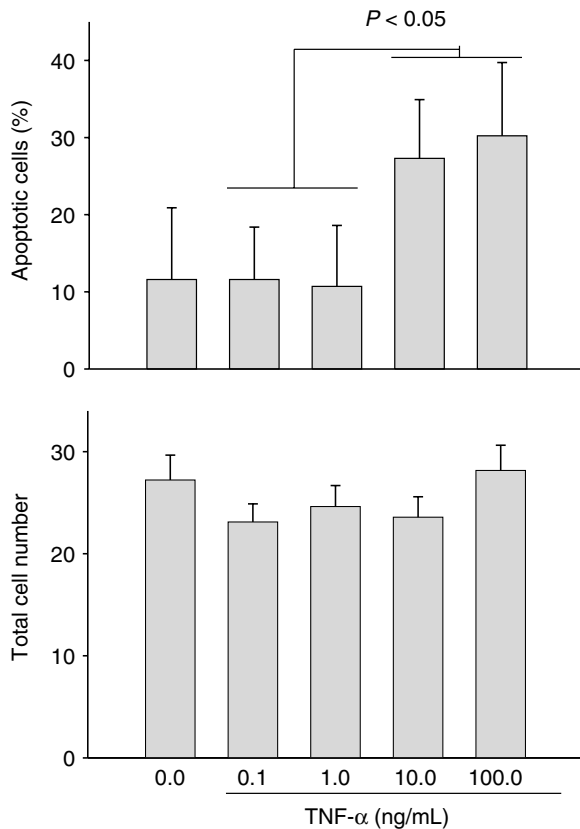


Fig. 5. Effect of 24-hr culture with tumor necrosis factor- α (TNF- α) on percent blastomeres undergoing apoptosis (top panel) and cell number (bottom panel) for embryos ≥ 9 -cells treated at day 4 after insemination. Results are least-squares means \pm S.E.M. The experiment was replicated five times using 33–46 embryos per group. Significant orthogonal contrasts are indicated by horizontal lines over the bars.

The effect of TNF- α on oocyte maturation could reflect direct actions on the oocyte or effects mediated via cumulus cells that are also in culture. It is not known whether the oocyte has TNF- α receptors but bovine granulosa cells had specific ^{125}I -TNF- α binding sites.¹⁶ One possibility is that some actions of TNF- α are mediated by molecules induced by TNF- α . For example, TNF- α stimulates secretion of prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) in many tissues including bovine endometrial cells.^{17,18} It is not known whether TNF- α induces $\text{PGF}_{2\alpha}$ secretion by cumulus cells. If so, however, such an action by TNF- α could be a cause for disruption of oocyte maturation by $\text{PGF}_{2\alpha}$. Addition of $\text{PGF}_{2\alpha}$ to oocyte maturation medium decreased the proportion of bovine oocytes that developed to the blastocyst stage.¹⁵

As addition of TNF- α during maturation did not affect cleavage rate, it did not disrupt maturational events required for the oocyte to become capable of fertilization. However, embryos formed following fertilization were less likely to develop to the blastocyst

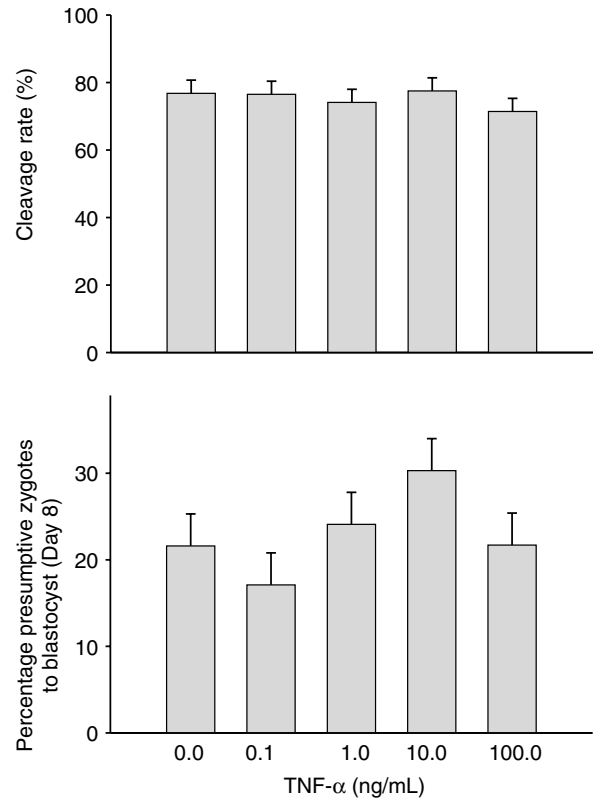


Fig. 6. Effects of continuous exposure to tumor necrosis factor- α (TNF- α) beginning after fertilization on the percentage of oocytes that cleaved (top panel) and developed into blastocyst on day 8 after insemination (bottom panel). Results are least-squares means \pm S.E.M. The experiment was replicated six times using 131–167 embryos per group.

stage if the oocyte from which the embryo was derived was exposed to TNF- α . Thus, TNF- α is affecting some aspect of oocyte physiology that is important for subsequent developmental potential. A variety of events occur during maturation of the oocyte including nuclear maturation of the oocyte as well as cytoplasmic maturation involving molecular and structural changes that include redistribution of cortical granules, mitochondria, golgi and endoplasmic reticulum and the acquisition of the ability to undergo cortical granule exocytosis.^{19,20} One factor related to an oocyte's capacity for sustained development after fertilization is post-transcriptional and post-translational mechanisms to prevent maternal mRNA and protein degradation during storage in the oocyte and regulation of its temporal expression.²¹ Indeed, oocyte competence for producing an embryo capable of sustained development was related to the length of the poly(A) tail of oocyte mRNAs.²² Many of the mRNAs in the oocyte are masked or kept in a non-translatable state through binding of specific ribonucleoproteins.²³ Perhaps, TNF alters one or more of these events in the oocyte to reduce its potential for successful development.

In the current study, TNF- α induced apoptosis in embryos ≥ 9 -cells in development. Such a result is consistent with the well-known ability of TNF- α to activate the apoptosis cascade through interactions with the TNF receptor.^{24,25} It is not surprising that TNF- α did not induce apoptosis in two-cell and four-cell embryos because embryos at this stage lack the capacity for apoptosis as has been shown for apoptosis induced by heat shock²⁶ and arsenic.²⁷ It is also possible that developmental changes in embryonic responses to TNF- α involve changes in receptor expression in the embryo. While TNF- α receptors were demonstrated in the mouse embryo at the blastocyst stage of development,¹⁰ no receptors were found on mouse morulae.²⁸ The pattern of expression of TNF- α receptors in bovine embryos is not known but the observation that TNF- α caused a slight but significant reduction in cell number in two-cell embryos suggests some receptors are present at this early stage in development. The minimal effective concentration of TNF- α was higher for embryos (10 ng/mL) than for COCs (1 ng/mL), however, and some component of the TNF signaling pathway may be compromised in embryos at the stages of development examined.

While a reduction in cell viability caused by apoptosis might be expected to reduce the competence of embryos to develop to the blastocyst stage, this in fact did not occur. Rather, there was no effect of TNF- α treatment beginning after fertilization on the proportion of embryos that developed to the blastocyst stage of development. Perhaps at this early stage of development, the loss of cells through apoptosis does not compromise development. Massive loss of cell number caused by surgical bisection of bovine morula does not prevent development to the blastocyst stage.²⁹ It is possible, however, that embryonic survival beyond the blastocyst stage can be compromised by TNF-induced apoptosis. Transfer of bisected embryos (i.e. with lower cell numbers) to recipient females can result in lower embryonic or fetal survival in mice,³⁰ cattle,³¹ and sheep.³² Exposure of mouse blastocysts to TNF- α decreased the average number of cells in the ICM lineage and reduced pregnancy rates following transfer to recipients.¹²

One question that formed the rationale for these studies was whether TNF- α could be a potential mediator of effects of mastitis^{3,4} or immunization² on fertility. Previous studies demonstrated an increase in plasma concentrations of TNF- α in cattle after endotoxin administration to 0.25–5 ng/mL and after coliform mastitis to 5–6 ng/mL.^{6–9} Concentrations within this range were sufficient to affect oocyte maturation in the present study but were lower than the 10 and 100 ng/mL TNF- α concentrations necessary to induce

apoptosis in embryos ≥ 9 -cells. Unfortunately, effects of 5 ng/mL TNF- α on induction of apoptosis were not evaluated.

The anabolic protein hormone, somatotropin, has been reported to increase pregnancy rate in cattle.^{33,34} While this effect appears to involve direct stimulatory effects of somatotropin and IGF-1, a somatotropin-induced hormone, on embryonic development,³⁵ it is also possible that somatotropin counteracts deleterious effects of TNF- α on the oocyte or embryo. Administration of somatotropin decreased plasma concentrations of TNF- α , thromboxane B₂ and cortisol after endotoxin challenge in calves and reduced TNF- α binding capacity of hepatic microsomal membranes.³⁶

In conclusion, TNF- α can have deleterious actions on oocyte maturation which compromise the ability of the resultant embryo to develop to the blastocyst stage. While exposure of fertilized embryos to TNF- α did not inhibit the percentage of embryos developing to the blastocyst stage, the cytokine did increase the percentage of blastomeres undergoing apoptosis when exposure occurred after embryos reached nine cells or greater. Further studies will be required to elucidate the mechanism by which TNF- α acts during oocyte maturation to compromise oocyte developmental potential and to determine whether increased blastomere apoptosis caused by TNF- α reduces embryo survival.

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