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Urea changes oocyte competence and gene expression in resultant bovine embryo *in vitro*

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Summary

Nutrition influences the microenvironment in the proximity of oocyte and affects early embryonic development. Elevated blood urea nitrogen, even in healthy dairy cows, is associated with reduced fertility and there is high correlation between blood urea levels and follicular fluid urea levels. Using a docking calculation (*in silico*), urea showed a favorable binding activity towards the ZP-N domain of ZP3, that of ZP2, and towards the predicted full-length sperm receptor ZP3. Supplementation of oocyte maturation medium with nutrition-related levels of urea (20 or 40 mg/dl as seen in healthy dairy cows fed on low or high dietary protein, respectively) dose-dependently increased: (i) the proportion of oocytes that remained uncleaved; and (ii) oocyte degeneration; and reduced cleavage, blastocyst and hatching rates. High levels of urea induced shrinkage in oocytes, visualised using scanning electron microscopy. Urea downregulated *NANOG* while dose-dependently upregulating *OCT4*, *DNMT1*, and *BCL2* expression. Urea at 20 mg/dl induced *BAX* expression. Using mathematical modelling, the rate of oocyte degeneration was sensitive to urea levels; while cleavage, blastocyst and hatching rates exhibited negative sensitivity. The present data imply a novel role for urea in reducing oocyte competence and changing gene expression in the resultant embryos.

Keywords: Blastocyst, Bovine oocyte, Molecular docking, Urea

Introduction

The chemical composition of follicular fluid plays an important role in oocyte fertilizability and also in the resultant embryo development because developing oocytes are in direct proximity to this microenvironment (Fortune, 1994; Józwik *et al.*, 2006; Revelli *et al.*, 2009). Urea can be transported by passive diffusion through the blood–follicle barrier (Józwik *et al.*, 2006), indicating a substantial correlation between urea concentration in follicular fluid and plasma in both humans and

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cows (Hammon *et al.*, 2005; Józwik *et al.*, 2006). Non-renal causes of increased blood urea nitrogen (BUN), therefore, include increased protein intake associated with higher urea levels in the female reproductive tract (FRT) of healthy dairy cows.

A body of evidence suggests a relationship between high levels of BUN and a reduction in fertility, even in healthy dairy cows (Ferguson *et al.*, 1993; Butler *et al.*, 1996; Sinclair *et al.*, 2000; De Wit *et al.*, 2001). Cumulus–oocyte complexes (COCs) collected from heifers offered a high-NH_3-generating diet (high levels of BUN) and had a reduced ability to develop to the blastocyst stage (Santos *et al.*, 2009). Urea in *in vitro* oocyte maturation impaired the segregation of chromatids and the extrusion of the polar body (De Wit *et al.*, 2001), resulting in decreased oocyte fertilizability, cleavage, and developmental rate.

Transcription factors such as CDX2 and OCT4 are necessary for the first cell fate decision to form a trophectoderm or inner cell mass (ICM) (Arnold & Robertson, 2009). Developmentally, these transcription factors should be strictly regulated to govern cell lineage allocation and the pattern of the early embryonic development in mammals (Wang *et al.*, 2000). For example, to sustain the pluripotent state *in vitro*, the precise level of OCT4 should be balanced (Wang *et al.*, 2000). Reduced *OCT4* expression results in induced trophectoderm differentiation, however its overexpression leads to differentiation into endoderm and mesoderm (Wang *et al.*, 2000). Any upregulation or downregulation of *OCT4* expression or any changes in the *OCT4*:*NANOG* ratio results in a divergent developmental programme (Wang *et al.*, 2000; Theunissen *et al.*, 2011; Muñoz-Descalzo *et al.*, 2012).

It has been shown that the zona pellucida (ZP), an extracellular matrix surrounding the mammalian oocyte, is involved in supporting communication between oocytes and follicle cells during oogenesis and also in protecting oocytes (Wassarman *et al.*, 1999; Gook *et al.*, 2008). Female mice that were null for ZP3 ($ZP^{-/-}$) showed retarded follicular development and their ovaries contained lower numbers of graafian follicles, suggesting a role for ZP proteins produced around growing oocytes in follicle development (Wassarman *et al.*, 1999; Gook *et al.*, 2008). In addition, ZP proteins ZP2 and ZP3 are shared in mammals (Pimenta *et al.*, 2013) and make up 80% of the total ZP (Wassarman, 2008). The presence of both ZP2 and ZP3 is critical for the general assembly of the ZP (Wassarman, 2008); sperm cells first bind to ZP3 to accomplish the acrosome reaction, then can bind to ZP2 (as the secondary receptor for acrosome-reacted sperm), penetrate the ZP, and fuse with the oocyte plasma membrane (Callebaut *et al.*, 2007).

Two domains of the ZP3 and ZP2 proteins, the N-terminal (ZP-N) and C-terminal (ZP-C), have been identified that are essential for dimerization of ZP proteins (Monné *et al.*, 2008; Han *et al.*, 2010). The tight association of ZP-C and ZP-N is essential to ensure the formation of a complete ZP (Callebaut *et al.*, 2007). Importantly, it has been shown that ZP-N is involved in other specific functions such as in zona pellucida filament formation (Jovine *et al.*, 2006; Wassarman, 2008; Monné *et al.*, 2008). Koyama *et al.* (1991) showed that using ZP2 antisera against the N-terminal of ZP-N domain, inhibited the penetration and binding of sperm into the oocyte. Many pathological mutations fall within the ZP-N domain (Monné *et al.*, 2008), it may work as an independent unit to show a specific function in reproductive processes (Jovine *et al.*, 2006).

In the current study, we used the ZP-N domain of the ZP3 or ZP2 protein for molecular docking with urea and primarily focused on the interaction of urea with the ZP-N domain of ZP3. Urea has a strong interaction with the backbone and side chains of proteins, causing denaturation (Caflisch & Karplus, 1999; Hua *et al.*, 2008). We, therefore, hypothesized a possible interaction between urea and the ZP-N domain of ZP3 or ZP-2. The supplementation of maturation medium with urea may alter oocyte competence and induce an imbalanced expression of transcription factors in the resultant blastocyst. Oocytes and early embryos, *in vivo* in contrast with *in vitro*, are exposed to various follicular and oviductal components such as hormones and cytokines that may interact with urea (Matoba *et al.*, 2014; Kowsar *et al.*, 2016).

Materials and methods

Computational and molecular docking (*in silico* study)

Using AutoDock4.2 via the genetic algorithm (GA) procedure (Morris *et al.*, 2009; Barzegar *et al.*, 2016), the urea molecule was docked into a PDB structure of: (1) the mammalian ZP-N domain of ZP3, crystal form I (PDB code: 3D4C); (2) the ZP-N domain of mouse ZP2 (PDB code: 5II6); (3) the full-length chicken sperm receptor ZP3 (PDB code: 3NK3) and (4) the predicted full-length mammalian sperm receptor ZP3. The files required to perform docking simulation were prepared utilizing the AutoDock Tools package 1.5.6 (Morris *et al.*, 2009). The binding sites of urea/protein complexes were regulated with a grid size that was set at 54 × 52 × 52 points for the urea/ZP-N domain of the ZP3 complex (PDB code: 3D4C), 58 × 52 × 78 points for the urea/ZP-N domain of mouse ZP2 complex (PDB code: 5II6), 82 × 56 ×110 points for the full-length chicken sperm receptor ZP3 (PDB code: 3NK3), and 68 × 82 × 66 points for the predicted full-length mammalian sperm receptor ZP3 along xyz axes

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and through a grid spacing of 0.375 Å, to cover the full active site of the ZP3 ZP-N domain and 1 Å for other proteins. To investigate the free energy and behaviour of urea binding to ZP-N, docking procedures were performed six consecutive times, such that each time the previous docked urea/ZP-N complex was used as the template (macromolecule) for the next docking run under the same conditions. To find the best free energy that indicated the strongest binding of urea to ZP-N, the total number of docking runs was set at 300.

Because the whole molecule of mammalian ZP3 (containing both ZP-N and ZP-C domains) was not determined, the full-length chicken sperm receptor ZP3 (PDB ID: 3NK3) was used to predict the whole mammalian ZP3 molecule using I-TASSER software (Roy *et al.*, 2010; Zhang 2007).

Production of bovine embryos (*in vitro* study)

Oocyte collection, *in vitro* oocyte maturation and fertilization, and *in vitro* embryo culture have been described previously (Hernandez-Ledezma *et al.*, 1992; Kimura *et al.*, 2004; Green *et al.*, 2016). Briefly, bovine ovaries were obtained from a local slaughterhouse, transported to the laboratory within 2 h at approximately 30°C in a thermos box containing 0.9% NaCl, 0.1% penicillin and streptomycin, washed in 30°C sterile 0.9% NaCl containing 0.1% penicillin and streptomycin, and stored in sterile saline until aspiration. Cumulus–oocyte complexes were only aspirated from follicles ranging from 4 to 8 mm in diameter as these oocytes, occupying the medium-sized (> 4–8 mm) follicles, have been shown to be influenced by a key nutritionally sensitive process (Sinclair *et al.*, 2000). Approximately 540 bovine COCs graded as class 1 or class 2 only according to a previous study (Leibfried & First, 1979) were selected under a stereomicroscope (Olympus, Tokyo, Japan) and washed in HEPES–TCM-199 (Invitrogen, Carlsbad, CA, USA) supplemented with 50 mg/ml kanamycin (Sigma-Aldrich), 50 mg/ml heparin (Sigma-Aldrich). Cumulus–oocyte complexes were then washed twice in HEPES–TCM-199 and randomly incubated in maturation medium in groups of 10 in bicarbonate-buffered TCM-199 supplemented with 10% (w/v) fetal bovine serum (FBS; BioWhittaker, Walkersville, MD, USA) and 10 IU/ml follicle-stimulating hormone (FSH; Sigma-Aldrich) in 35 mm cell culture dishes (Falcon brand, BD Biosciences, San Jose, CA, USA) for 24 h at 38.5°C under 5.5% CO_2 and 20% O_2, balanced N_2 and maximum humidity. The maturation medium was free from bovine serum albumin (BSA) to avoid any possible interaction with urea in the medium. To see how embryo development was affected by urea-exposed oocytes, urea (Sigma-Aldrich) was added to the maturation medium at 0 mg/dl (control group, $n = 143$ COCs, five replicates), 20 mg/dl ($n = 184$ COCs, five replicates), or 40 mg/dl ($n = 213$ COCs, five replicates). These urea concentrations were equivalent to 0, 9.3 and 18.7 mg/dl BUN, respectively. The urea levels used in our study are usually observed in serum of healthy dairy cows fed low or high protein diets (Ferguson *et al.*, 1993). After *in vitro* maturation, urea-exposed oocytes and control oocytes ($n = 10$/droplet) were fertilized with sperm from a single sire (Spermex, GmbH) at a final concentration of 1×10^6/ml in *in vitro* fertilization medium supplemented with amino acids (50 μl) in 35 mm cell culture dishes (Falcon brand, BD Biosciences, San Jose, CA, USA) at 38.5°C under 5% CO_2 in the air. Zygotes were recovered after 24 h, cumulus cells were manually stripped and the resultant embryos transferred into cell culture dishes ($n = 20$ per droplet) containing synthetic oviductal fluid (SOF) medium supplemented with amino acids and 4 mg/ml BSA. Embryos were then cultured in an incubator at 38.5°C under 5% O_2 and 6% CO_2 for 60 h. Developing embryos were scored using a stereomicroscope (Olympus, Tokyo, Japan) at the zygote, 8–16- or 2–8-cell stage, blastocyst, and hatching stages at days 1, 3, 7 or 9 post-insemination, respectively. Pools of seven embryos in five replicates were collected and stored in RNeasy lysis buffer (Qiagen) supplied with 2-mercaptoethanol (Sigma-Aldrich) at −70°C for qRT-PCR analysis. The criterion for the fertilization was the observation of two pronuclei (2-PN) in zygotes 18 h after insemination using a phase-contrast microscope (Nagano *et al.*, 2006; Son *et al.*, 2005). Oocytes with cytoplasmic degeneration by day 3 after insemination were considered as the degenerated oocytes (Makita *et al.*, 2016).

Scanning electron microscopy

For scanning electron microscopy analysis, COCs were cultured in maturation medium supplemented with different levels of urea (0, 20, 40 mg/dl). After 24 h, COCs were denuded from cumulus cells by pipetting in maturation medium. Then, oocytes were washed in phosphate-buffered saline to eliminate the culture medium. Oocytes were fixed for 2 h in 2.5% glutaraldehyde in 0.2 mol/l cacodylate buffer on 12 mm circular coverslips placed in Petri dishes containing a fixative solution. Coverslips were coated with poly-L-lysine solution (Sigma P-8920) (1:10 poly-L-lysine solution for 15–30 min). Next, using an ascending series of ethanol solutions, the oocytes were dehydrated and dried by immersing in hexamethyldisilazane (HMDS, Sigma-Aldrich) for 10 min and then air dried in a desiccator at room temperature (Jusman *et al.*, 2014). The dried samples were mounted on stubs, sputter-coated with a 10 nm layer of gold, and were checked under a Philips

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XL 30 scanning electron microscope (Eindhoven, The Netherlands).

RNA extraction, reverse transcription, quantitative analysis of transcripts by qRT-PCR

RNA extraction and reverse transcription were done as previously reported (Moulavi *et al.*, 2013). Briefly, total RNA was extracted using an RNeasy Micro kit (Qiagen, Mississauga, Ontario, Canada), then treated with DNase I (Ambion, Streetsville, Ontario, Canada) according to the manufacturer's manual. The WPA Biowave spectrophotometer (Cambridge, United Kingdom) was used to examine the quality and quantity of RNA. Reverse transcription was carried out at 25°C for 10 min, 42°C for 1 h and 70°C for 10 min. qRT-PCR was carried out using 1 µl of cDNA (50 ng), 5 µl of the SYBR Green/0.2 µl ROX qPCR Master Mix (2×) (Fermentas, Germany) and 1 µl of forward and reverse primers (5 pM) adjusted to a total volume of 10 ml using nuclease-free water. *ACTB* was used as the reference gene. The abundance of *ACTB* was constant throughout embryo developmental stages assessed. Each replicate of qRT-PCR was repeated three times to minimize technical errors. Primer sequences used for qRT-PCR were as follows: 5′-CCTTCTTTGAGTTCGGAG-3′, forward, and 5′-CCTTCAGAGACAGCCAG-3′, reverse, for *BCL2* (AC_000181.1, 121 bp, T_m: 60°C); 5′-AGCGAGTGTTC-TGAAGCG-3′, forward, and 5′-CCCAGTTGAAG-TTGCCGT-3′, reverse, for *BAX* (AC_000175.1, 182 bp, T_m: 61°C); 5′-GGTGTTTGGTGAACTCTC-3′, forward, and 5′-CATTGATTGTTCCAAGGCT-3′, reverse, for *NANOG* (AC_000162.1, 98 bp, T_m: 53°C); 5′-GGAAAGGTGTTCAGCCA-3′, forward, and 5′-TTCTCGTTGTTGTCAGC-3′, reverse, for *OCT4* (NC_000006.12, 131 bp, T_m: 62°C); 5′-GAAGCAGAA-TAAGAATCGG-3′, forward, and 5′-TTTGAAGAGT-CGTCTGGAA-3′, reverse, for *DNMT1* (AC_000164.1, 125 bp, T_m: 53°C); 5′-CCATCGGCAATGAGCGGT-3′, forward, and 5′-CGTGTTGGCGTAGAGGTC-3′, reverse, for *ACTB* (AC_000182.1, 146 bp, T_m: 60°C).

Evaluation of sensitivities of developmental parameters to urea

Linear and second-order polynomial basic functions were used to examine the sensitivity of developmental competence by the following relationships:

$$f(U) = A_0 + A_1U \quad (1)$$

$$f(U) = B_0 + B_1U + B_2U^2 \quad (2)$$

in which $f(U)$ is the predicated function for developmental competence based on urea (U). A_0 and A_1 are the unknown coefficients for the linear function and B_0–B_2 are the unknown coefficients for second-order polynomial function (non-linear function). The calibration of these unknown coefficients was implemented using MATLAB software (Version 7.10.0; The MathWorks Inc., Natick, Massachusetts, USA, 2010). It is defined as the sensitivity of developmental competence to urea using coefficient A_1 in a linear function:

$$a_i = \frac{A_1^i}{\sum_{i=1}^{n}\left(\sqrt{A_1^i}\right)^2} \quad (3)$$

where A_1^i is the coefficient of the linear function for i_{th} developmental competence index and n is the total numbers of developmental indexes ($n = 8$). The positive and negative values for the selectivity factor a_i are detonated by the increase or decrease of developmental parameters (cleavage rates, blastocyst rate, hatching rate, and degenerated oocytes) regarding the increasing urea concentration, respectively.

Statistical analysis

Using the Anderson–Darling test (EasyFit software, version 5.6, MathWave Technologies, Spokane, WA, USA), the normality of data, including gene expression data, was confirmed before analysis. Statistical analysis was performed using StatView 5.0 software (SAS Institute Inc., Cary, NC). The data were analyzed using one-way analysis of variance (ANOVA) using a PROC MIXED procedure; Tukey's multiple comparison *post hoc* test. Data are presented as mean ± SEM and all results were considered as statistically significant at $P < 0.05$. These data were collected by a minimum of five replicates with 35–40 embryos per replicate.

Results

Predicted urea/ZP docking

Molecular docking showed that urea molecules surround the active site of: (1) ZP-N domain of ZP3 (Fig. 1*A*); (2) ZP-N domain of ZP2 (Fig. 1*B*); (3) full-length chicken sperm receptor ZP3 (Fig. 1*C*); and (4) predicted full-length mammalian sperm receptor ZP3 (Fig. 1*D*). Also, docking procedures were run six or seven consecutive times. Each time the previous docked urea/ZP complex was used as the template (macromolecule) for the next docking run. The binding energy values for the interaction of each run achieved from 300 runs are shown in Table 1. The results showed that the binding energy values for urea interaction with all studied proteins, ZP-N domain of both ZP3 and ZP2 proteins, full-length chicken sperm receptor, and predicted full-length mammalian sperm receptor, were negative (Table 1).

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Table 1 Binding energies (Kcal/mol) obtained from docking of urea molecules with ZP proteins using AutoDock4.2 via the genetic algorithm procedure

	Energy, Kcal/mol			
Number of docked urea	ZP-N domain of mammalian ZP3	ZP-N domain of mouse ZP2	Full-length chicken ZP	Predicted full-length mammalian ZP
1	−3.54	−3.73	−3.23	− 3.41
2	−3.67	−3.35	−2.91	− 3.06
3	−3.39	−3.35	−2.65	− 2.93
4	−3.21	−3.02	−2.67	− 2.73
5	−3.23	−3.02	−2.65	− 2.83
6	−3.16	−3.02	−2.55	− 2.8
7	–	−2.94	−2.64	− 2.8

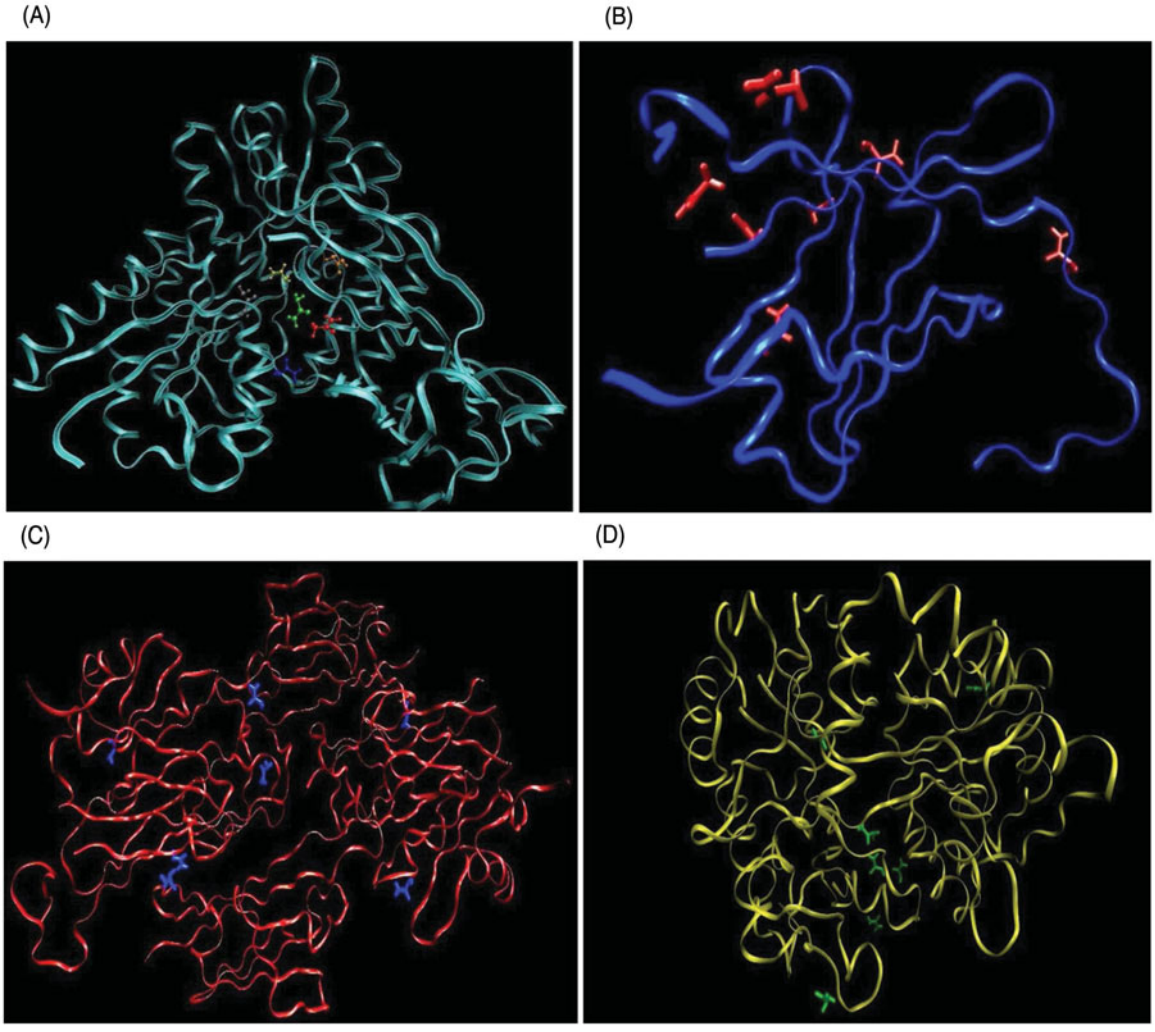


Figure 1 Molecular docking as a key approach is widely used in structural molecular biology to predict how a ligand binds to a protein (Morris & Lim-Wilby, 2008). AutoDock4.2 via the genetic algorithm (GA) procedure was conducted to predict urea binding to: (*A*) the zona pellucida (ZP)-N domain of ZP3 (PDB code: 3D4C); (*B*) the ZP-N domain of ZP2 (PDB code: 5II6); (*C*) the full-length chicken sperm receptor ZP3 (PDB code: 3NK3); and (*D*) the predicted full-length mammalian sperm receptor ZP3 (predicted using I-TASSER software). We ran the docking procedure six consecutive times such that each time we used the previous docked urea/ZP protein complex as the template macromolecule for the next docking run. As shown in (*A*), across six consecutive runs, the urea molecule is shown in black for the first run, then shown by blue, red, grey, orange, and green (the last run), respectively. Docking showed a favorable binding activity of urea towards the ZP-N domain of ZP3 and that of ZP2, the full-length chicken sperm receptor ZP3, and the predicted full-length mammalian sperm receptor ZP3.

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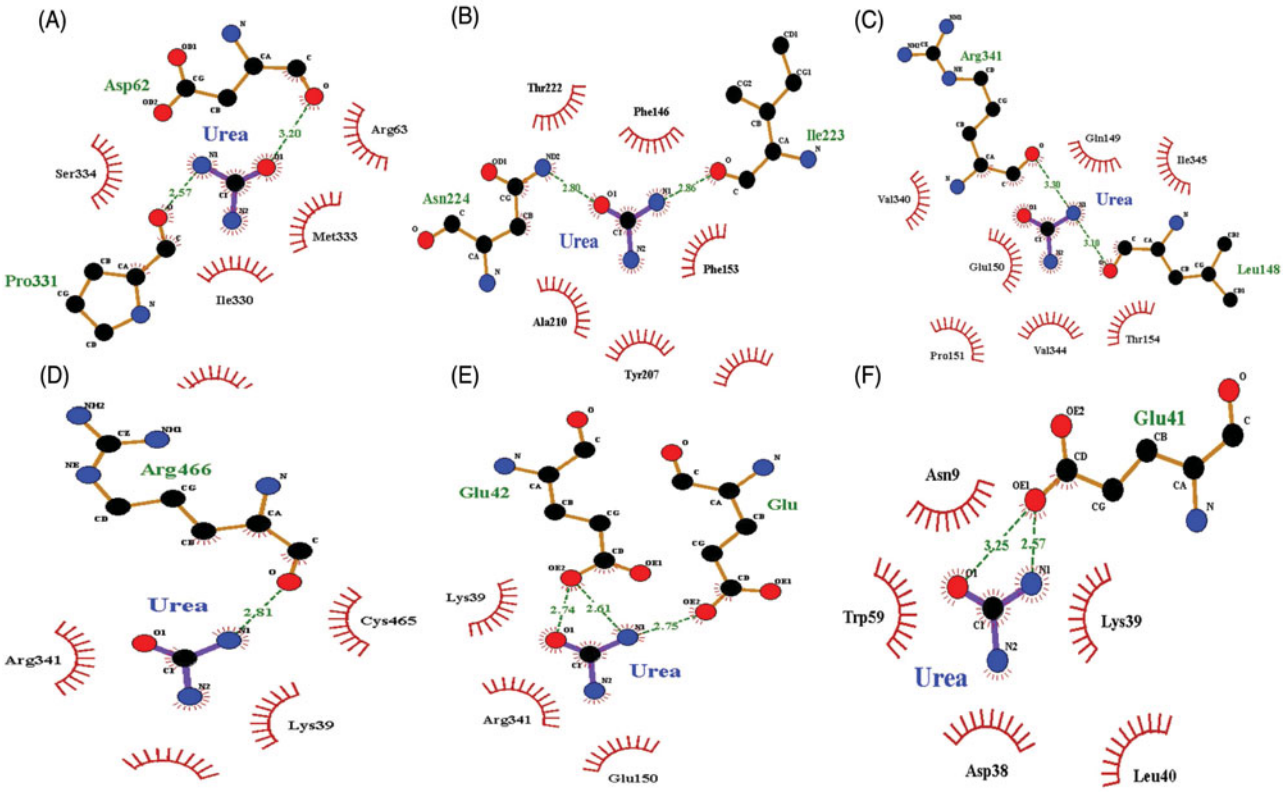


Figure 2 Two-dimension urea/ZP-N domain of ZP3 interaction diagrams. To investigate the free energy and behaviour of urea binding to ZP-N domain of ZP3 (PDB code: 3D4C), we ran docking procedure six consecutive times (*A*–*F*), such that we used the previous docked urea/ZP-N complex as the template macromolecule for the next docking run. LIGPLOT software was used to generate images and to visualize hydrophilic interactions, hydrogen bonding, and hydrophobic contacts between urea and ZP-N domain of ZP3 protein. The LIGPLOT shows the hydrogen bonds between urea and ZP-N domain of ZP3 at (*A*) Asp62 and Pro331; (*B*) Asn224 and Ile223; (*C*) Arg341and Leu148; (*D*) Arg466; (*E*) Glu42; and (*F*) Glu41 which are shown by dashed green lines. Also, the distance between acceptor and donor atoms is shown in the middle. Arcs that radiate spokes towards the urea molecule indicate hydrophobic interactions.

Atomic interactions between urea and ZP-N domain of ZP3

As LIGPLOT software showed, there were hydrophobic interactions between each urea and identified residues (Fig. 2*A*–*F*). The nitrogen atoms of the urea molecules mainly contributed to the hydrophobic interactions with the residues as shown in Fig. 2*A*–*F* (red arcs). The results showed the hydrogen bonds between urea molecules and some residues of ZP-N domain of ZP3 (green dashes) as shown in Fig. 2*A* Asp62 and Pro331, Fig. 2*B* Asn224 and Ile223, Fig. 2*C* Arg341and Leu148, Fig. 2*D* Arg466, Fig. 2*E* Glu42, and in Fig. 2*F* Glu41.

Scanning electron microscopy shows urea-induced shrinkage in oocyte

Scanning electron microscopy was used to investigate the effect of urea on the oocyte/zona pellucid a structure. As shown in Fig. 3, compared with the control group or urea-exposed group (20 mg/dl) (Fig. 3*A*, *B*, respectively), urea-exposed counterparts (40 mg/dl) showed a shrinkage in their structure (Fig. 3*C*).

Urea-exposed oocytes negatively show reduced developmental competence

Compared with the control group, the addition of 20 or 40 mg/dl urea to the oocyte maturation medium dose-dependently decreased fertilization rate (87.6, 81.1, and 75.1%, respectively) (Fig. 4*A*). Urea dose-dependently increased ($P < 0.01$) the rate of degenerated oocytes, by day 3 post-insemination (Fig. 4*B*). The presence of urea in the oocyte maturation medium dose-dependently reduced ($P < 0.01$) cleavage rate on day 3 (at the 6–18-cell stage but not the 2–8-cell stage) (Fig. 4*C*, *D*), blastocyst formation on day 7 (as a percentage of cultured or fertilized oocytes) (Fig. 4*E*, *F*), and rate of hatching on day 9 (as a percentage of cultured or fertilized oocytes or number of blastocyst on day 7) (Fig. 4*G*–*I*, respectively).

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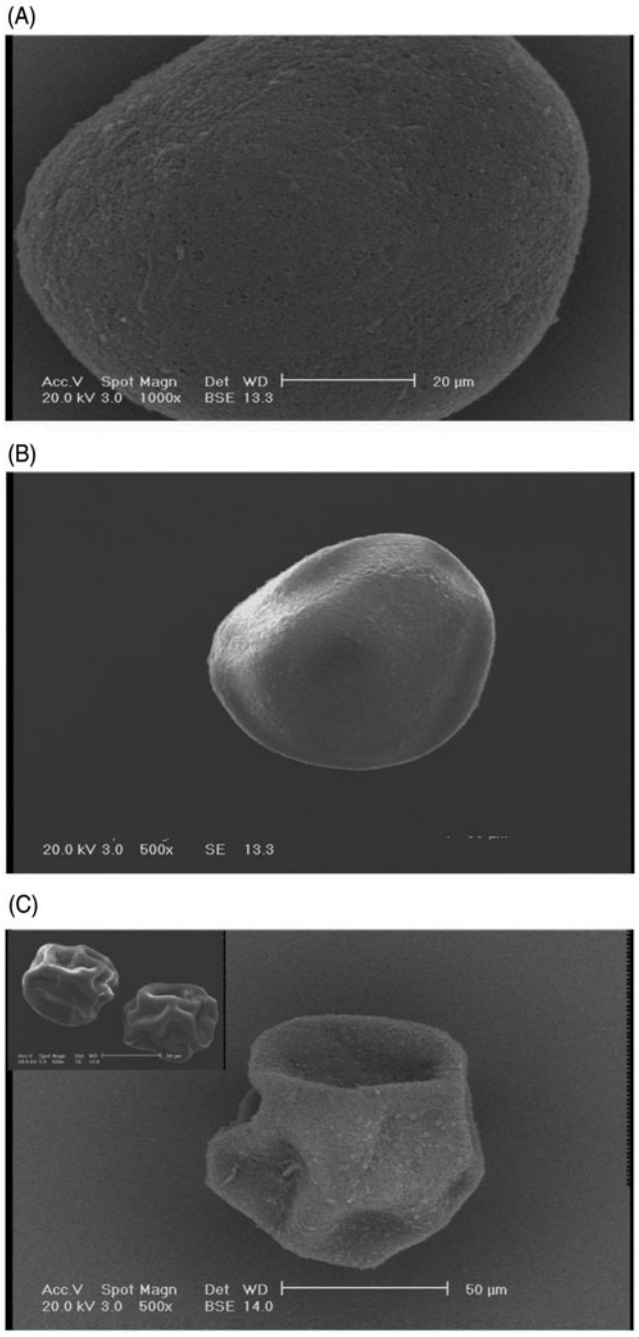


Figure 3 Scanning electron microscopy observation of bovine oocytes that were matured in the maturation medium supplemented with the different concentrations of (*A*) 0 mg/dl urea, the control group; (*B*) 20 mg/dl urea; and (C) 40 mg/dl urea for 24 h. Upon incubation of oocytes, 40 mg/dl urea induced shrinkage in oocytes.

Urea-exposed oocytes alter the expression of marker genes in resultant embryos

Urea at 20 mg/dl downregulated ($P < 0.01$) the expression of *NANOG* in blastocysts produced from urea-exposed oocytes. Blastocysts resulting from high urea-exposed oocytes (40 mg/dl) tended to reduce *NANOG* expression (Fig. 5*A*). Urea dose-dependently upregulated ($P < 0.01$) the expression of *OCT4* in blastocysts (Fig. 5*B*). Because of this altered expression pattern, the *OCT4*:*NANOG* ratio was significantly increased ($P < 0.01$) with increasing urea concentrations (Fig. 5C). mRNA expression of *OCT4* was over two-fold higher in blastocysts derived from urea-exposed oocytes (40 mg/dl) compared with the control group. Urea dose-dependently upregulated ($P < 0.01$) the expression of anti-apoptotic marker, *BCL2* in blastocysts produced from urea-exposed oocytes (Fig. 5*D*). Urea at 20 mg/dl upregulated ($P < 0.01$) and at 40 mg/dl down-regulated the expression of *BAX* compared with 20 mg/dl urea group (Fig. 5*E*). Urea dose-dependently upregulated ($P < 0.01$) the expression of *DNMT1*, an epigenetic marker, in blastocysts (Fig. 5*F*).

Sensitivity of developmental parameters to the presence of urea in maturation medium

Interestingly, statistical analysis predicted that the hatching rate on day 9 and blastocyst rate on day 7 had the highest negative sensitivity (−0.26) to urea (Table 2). Also, the rate of hatching or blastocyst per cultured oocytes (−0.26 and −0.25, respectively) showed a greater negative sensitivity than hatching or blastocyst rate per fertilized oocytes to urea (−0.25 and −0.18, respectively).

Discussion

To show urea contribution in reduced fertility, we recently reported that urea had the ability to disrupt *in vitro* the immunological and secretory response of oviduct epithelial cells (Kowsar *et al.*, 2016), implying a role for urea in disrupting the local environment of fertilization and early embryo development.

Importantly, urea has been able to induce protein denaturation and conformation by making hydrogen bonds with polar or charged groups (Caflisch & Karplus, 1999). AutoDock4 is widely used in the fields of biology and biomedicine and provides a quick estimation of bound conformations and free energies of binding small ligands i.e. urea with the target macromolecules, the ZP-N domain of ZP3 or ZP2 and full-length chicken ZP3, and predicted full-length mammalian ZP3 (Morris *et al.*, 2009; Barzegar *et al.*, 2016). Using AutoDock4 via the Lamarckian GA, molecular docking showed that urea had affinity for the ZP-N domain of both ZP3 and ZP2 proteins and for full-length chicken ZP3 and predicted full-length mammalian ZP3. These docking analyses suggested that urea, in general, favoured affinity with the ZP proteins.

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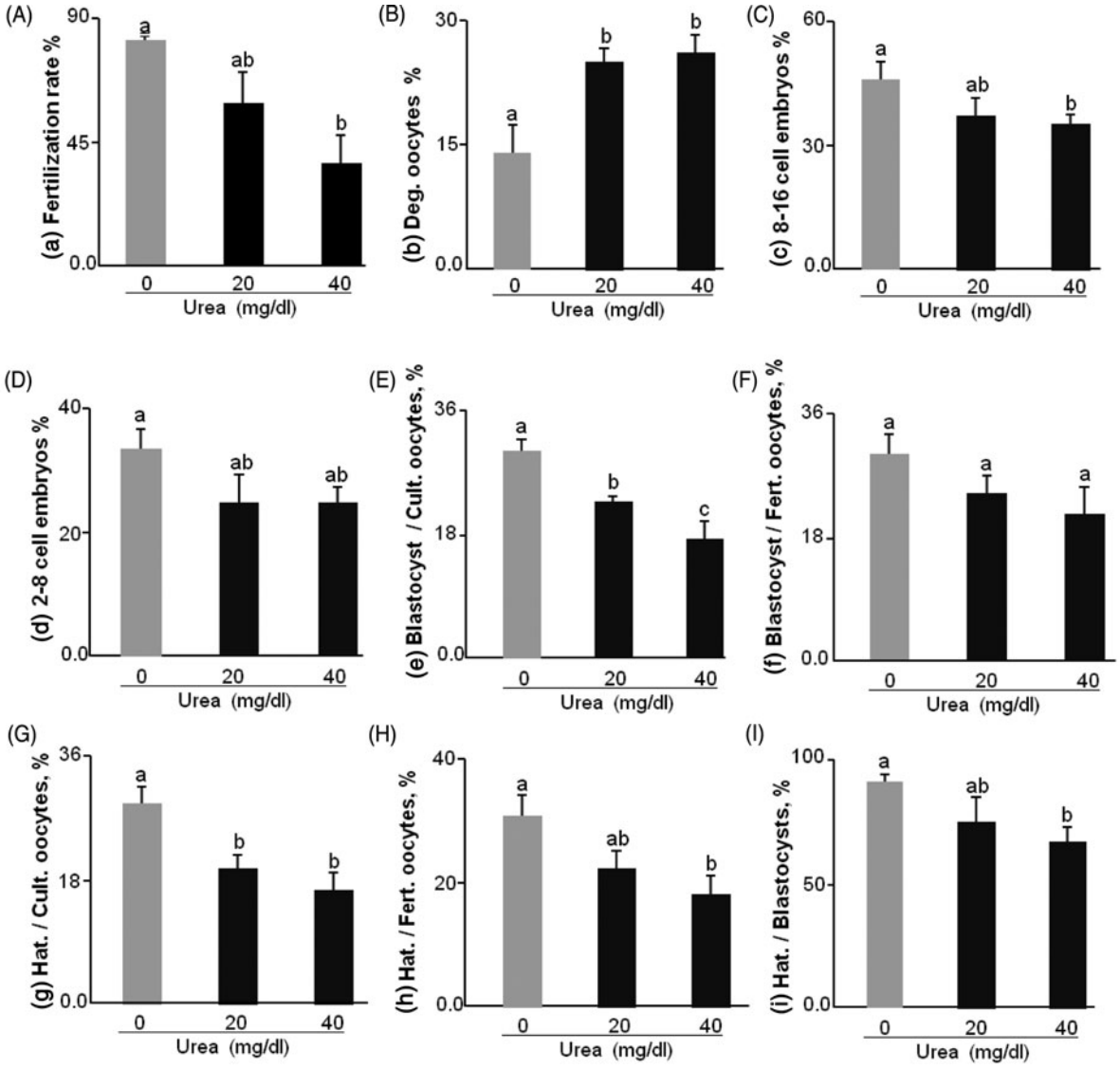


Figure 4 The effect of urea supplementation (0, 20, or 40 mg/dl) in maturation medium on oocyte competence. (*A*) Fertilization rate (%). (*B*) Degenerated oocytes (%). (*C*) 8–16-cell embryos (%). (*D*) 2–8-cell embryos (%). (*E*) Blastocysts/cultured oocytes (%). (*F*) Blastocysts/fertilized oocytes (%). (*G*) Hatching/cultured oocytes (%). (*H*) Hatching/fertilized oocytes (%). (*I*) Hatching/blastocysts (%). Blast, blastocyst; Deg, degenerated; Cult, cultured; Fert, fertilized; Hat, hatching. $n = 143–213$ oocytes per treatment across five replicates. Numerical values are presented as the mean ± standard error of the mean (SEM). [a,b,c]Different letters indicate significant differences between the treatments at $P < 0.05$.

The binding energy values for several molecular dockings of urea with the ZP-N domain of both ZP2 and ZP3 proteins, full-length chicken ZP3, and predicted full-length mammalian ZP3 were negative and showed no substantial changes. This result implies that the addition of urea did not decrease the total free energy, suggesting that ZP proteins especially the ZP-N domain of both ZP2 and ZP3 still had enough space and affinity to be docked by greater numbers of urea molecules. Regarding the important role of both ZP2 and ZP3 proteins for the general assembly of complete ZP (Callebaut *et al.*, 2007; Wassarman, 2008), the binding of urea with ZP-N domains of both ZP2 and ZP3 might interfere with the normal assembly of these ZP proteins. LIGPLOT analysis also showed that urea formed stable interactions with non-polar, acidic and basic residues of the ZP-N domain of ZP3 through hydrophobic and hydrogen bonding interactions, implying a change in the normal conformation of ZP3 sperm receptor. However, we could not confirm changes in ZP conformation under urea effect using the present molecular docking. Further studies such as molecular dynamic simulation are needed to confirm these changes in ZP protein conformation.

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Table 2 The sensitivity of the different developmental parameters to urea. The negative or positive values mean negative or positive sensitivity of different developmental parameters to urea. Negative sensitivity means that the parameter is decreased as urea concentration increases. Positive sensitivity means that the parameter is increased as urea concentration increases

	Hatching rate			Blastocyst rate		Cleavage rate		Degenerated oocytes by day 3 post-insemination
	Cultured	Fertilized	Blastocyst	Cultured	Fertilized	Cultured	Fertilized	
Sensitivity	−0.26	−0.25	−0.13	−0.25	−0.18	−0.13	−0.10	0.85

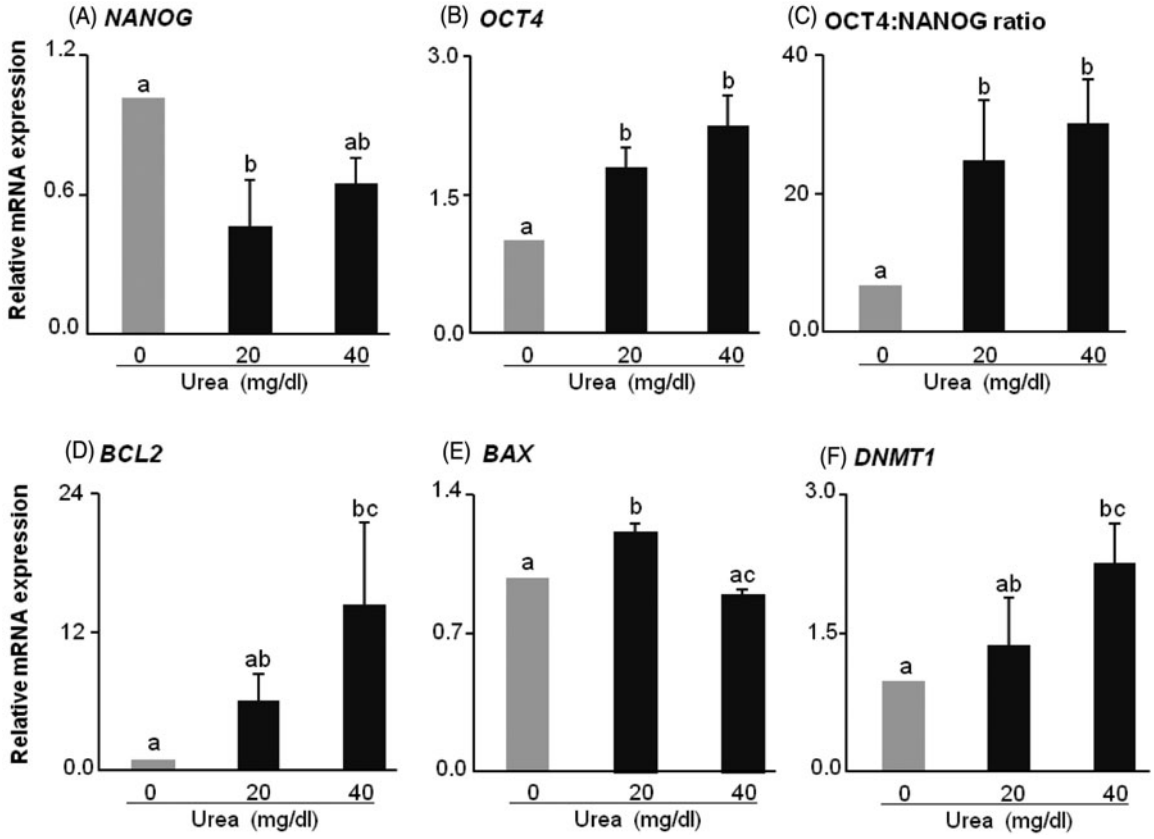


Figure 5 Relative mRNA expression of some marker genes in bovine blastocysts resulted from urea-exposed oocytes. (*A*) NANOG. (*B*) *OCT4*. (*C*) *OCT4:NANOG*. (*D*) *BCL2*. (*E*) *BAX*. (*F*) *DNMT1*. $n \geq 7$ blastocysts per treatment across five replicates. Each replicate of qRT-PCR was repeated three times to minimize the technical errors. Numerical values are presented as the mean ± standard error of the mean (SEM). [a,b,c]Different letters indicate significant differences between the treatments at $P < 0.05$.

To evaluate the possible effect of urea on bovine ZP, using scanning electron microscopy, we found that urea induced shrinkage in oocyte structure. Similarly, Zeuthen & MacAulay (2012) showed that urea may shrink *Xenopus* oocytes. Interestingly, Wagner *et al.* (2000) reported that urea induced a sustained shrinkage in vascular smooth muscle cells via inhibition of voltage-gated Ca^{2+} channels, which resulted in a decrease in cellular Ca^{2+}. Importantly, calcium channels have been shown to play a critical role in Ca^{2+} increase in the oocyte which is a pivotal process for meiosis re-initiation and consequently oocyte maturation (Tosti, 2006). It would be of interest to investigate the hypothesis that urea may negatively affect oocyte maturation via inhibition of Ca^{2+} channels and reduction of Ca^{2+} concentration in bovine oocytes.

In the present *in vitro* study, urea dose-dependently reduced oocyte fertilizability, cleavage rate on day 3, blastocyst rate on day 7, and hatching rate on day 9. De Wit *et al.* (2001) reported that 36 mg/dl (6 mM) of urea in the maturation medium reduced embryonic development as a percentage of cultured but not cleaved oocytes. Similarly, when the sensitivity of fertilizability or developmental competence of oocytes

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to the presence of urea in maturation medium was analyzed, the results showed that the hatching rate per cultured oocyte had the highest negative sensitivity to urea. Also, the blastocyst rate per cultured oocytes showed a greater negative sensitivity to urea than that per fertilized oocytes. These findings meant that as urea concentration increased, the rate of hatching or blastocyst per cultured oocytes was greatly decreased. In addition, the rate of degenerated oocytes by day 3 drastically showed a positive sensitivity to urea, meaning that the presence of urea in maturation medium may increase the rate of oocyte degeneration. These findings together suggested that dietary protein through elevation of BUN and consequent increase in urea concentration in the FRT may contribute to reduce the rate of cleaved oocytes and resultant embryo development in healthy dairy cows. However, further studies are needed to reveal the unknown interaction among urea, early embryo, and oviduct secretions as early embryonic development can be easily affected by the oviductal secretions that have also been influenced by urea (Kowsar *et al.*, 2016).

The expression of *OCT4* showing an important role in self-renewal, pluripotency and lineage commitment was dose-dependently up-regulated in blastocysts produced from urea-exposed oocytes. Conversely, *NANOG* expression, which maintains pluripotency in epiblast progenitors, was downregulated (Arnold & Robertson, 2009; Kalmar *et al.*, 2009; Madeja *et al.*, 2013). Consequently, the *OCT4/NANOG* ratio was elevated as urea concentration increased [over four-fold higher in blastocysts produced from urea-exposed oocytes (40 mg/dl) compared with their untreated counterparts]. It has been shown that cells co-expressing *NANOG* and *OCT4* are more stable in the pluripotent state than those that over-express these genes individually (Theunissen *et al.*, 2011). Indeed, the disruption of the *OCT4/NANOG* ratio leads to the inappropriate differentiation of ICM and embryonic stem cells to trophectoderm and extra-embryonic endoderm, respectively (Mitsui *et al.*, 2003; Boyer *et al.*, 2005). For example, from a toxicology point of view, ethanol has been shown to disturb the balance of these lineage specifiers (Sánchez-Alvarez *et al.*, 2013). Thus any imbalances may result in the loss of pluripotency or differentiation of embryonic stem cells (Boyer *et al.*, 2005). The higher *OCT4/NANOG* transcript ratio observed in the present study might also have resulted from *OCT4* expression in both ICM and trophectoderm while *NANOG* is poorly expressed in ICM in bovines (Berg *et al.*, 2011; Khan *et al.*, 2012). It seems in healthy dairy cows that have been fed high dietary protein diets to support their milk production, high urea concentrations in the follicular fluid may negatively affect oocyte competence and in turn early embryonic development. This idea was manifested by disrupting the normal and fine-tuned expression of key transcription factors.

Urea as a stress factor has cytotoxic effects on the growth and viability of cell populations, implying that cells exposed to increasing doses of urea may die via apoptosis (Glinos *et al.*, 1983; Kowsar *et al.*, 2016). Recently, we showed that high levels of urea may disrupt the oviduct epithelial monolayer, damaging the oviduct epithelium and disrupting sperm reservoir and fertilization site (Kowsar *et al.*, 2016). In the present study, the expression of *BCL2*, an anti-apoptotic gene, was dose-dependently elevated in blastocysts produced from urea-exposed oocytes. However, the *BAX* gene, a pro-apoptotic gene, was downregulated in blastocysts produced from urea-exposed oocytes (40 mg/dl). Importantly, *OCT4* can actively repress apoptotic pathways (Sánchez-Alvarez *et al.*, 2013; Kim *et al.*, 2015). Kim *et al.* (2015) reported that in porcine embryos, *BCL2* was up-regulated in *OCT4*-overexpressed fibroblast cells. Ogony *et al.* (2013) showed that ethanol as a toxic factor dose-dependently induced *OCT4* and downregulated apoptosis genes. These findings together may imply that, as a possible autonomous control system, blastocysts upregulate and downregulate anti-apoptotic and pro-apoptotic genes, respectively in response to urea, as a stressor.

DNMT1, one of the major DNA methyltransferase enzymes, regulates methylation status during DNA replication, genomic methylation and gametic imprinting (Grohmann *et al.*, 2005). Genomic hypomethylation and consequently early embryonic lethality can be expected due to a deficiency in *DNMT1* (Li *et al.*, 1993). *DNMT1* over-expression leads to the activation of the silent maternal allele and increased embryonic lethality (Biniszkiewicz *et al.*, 2002; Giraldo *et al.*, 2013). Importantly, during early embryonic development, the methylation level is drastically reduced, especially at the later pre-implantation stages (Messerschmidt *et al.*, 2014). In this study, in addition to an increase in oocyte degeneration by day 3 post-insemination, *DNMT1* expression was also increased in blastocysts produced from urea-exposed oocytes. Urea seems to interfere with the early embryonic development via alterations in the normal level of *DNMT1*.

Taken together, urea negatively interacts with the ZP-N domain of ZP3 and ZP2, and full length sperm receptor ZP3, induced oocyte shrinkage, reduced oocyte competence, and interferes with the normal expression of marker genes involved in the early embryonic development. Of importance, as proposed in Fig. 6, regarding the possible interactions between embryo, oviduct secretions and urea, these findings can be easily affected under physiological and pathophysiological conditions (Cebrian-Serrano *et al.*, 2013;

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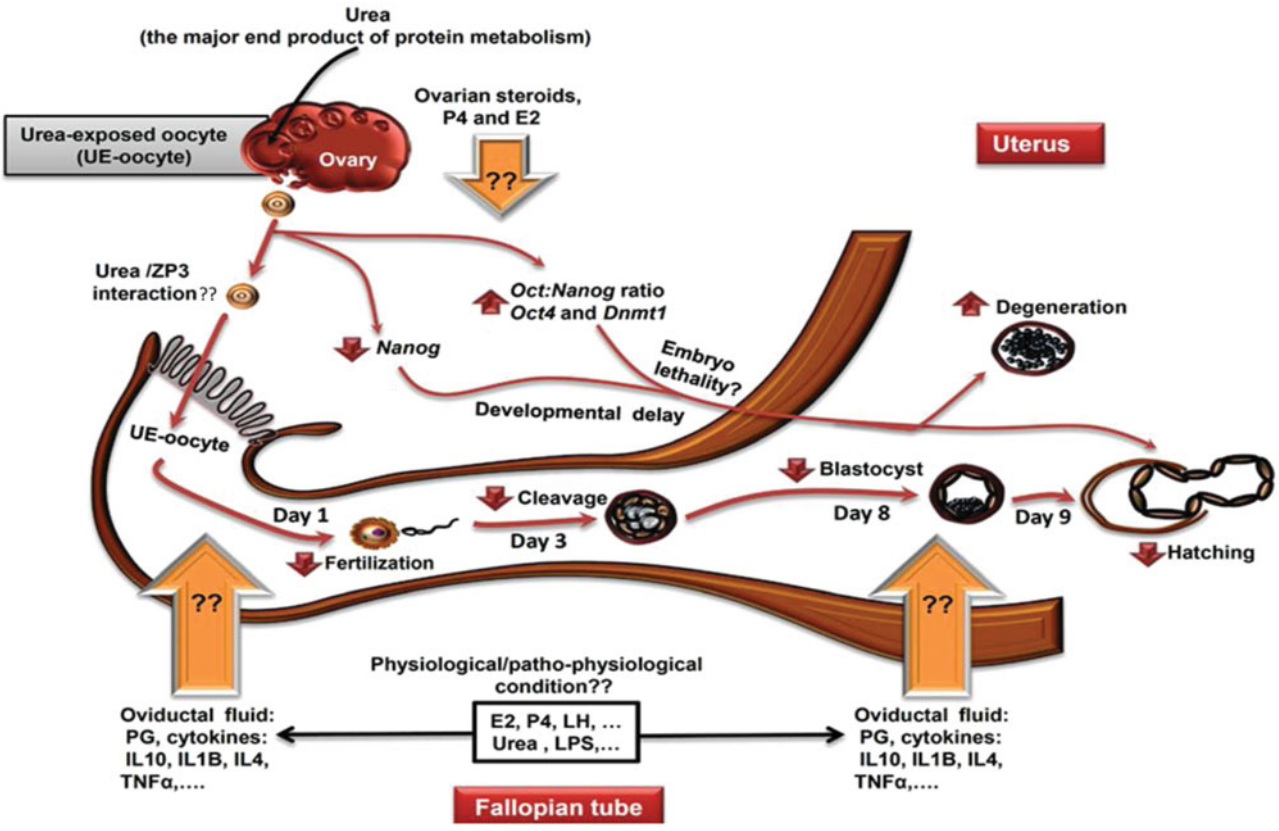


Figure 6 Schematic illustration of a proposal implying how urea may contribute to low fertility. Urea as a major end product of protein metabolism in dairy cows may contribute to the impaired early embryonic development via affecting oocytes before fertilization. Of great importance, regarding possible interactions between embryo, oviduct secretions conditions (Cebrian-Serrano *et al.*, 2013; Ibrahim *et al.*, 2015) and urea (Kowsar *et al.*, 2016), these *in vitro* findings physiologically and patho-physiologically can be easily affected. Thus further investigations are needed to unveil how oviductal secretions regulate the negative effect of urea, in particular during a high-protein diet in dairy cows. E2, estradiol; IL, interleukin; LH, luteinizing hormone; LPS, lipopolysaccharide; P4, progesterone; PG, prostaglandins.

Ibrahim *et al.*, 2015). Thus, further investigations are needed to unveil how these interactions regulate oocyte and embryo responses to the negative effect of urea, in particular during a high-protein diet.

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Ethical standards

Animal experiments were carried out according to the Guiding Principles for the Care and Use of Research Animals promulgated by the Isfahan University of Technology, Iran. The protocol and methods were approved by the Committee on the Ethics of Animal Experiments of the Isfahan University of Technology.

Conflict of interest

The authors have declared no conflict of interest.

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