RESEARCH ARTICLE

Sexual dimorphic expression and release of transcription factors in bovine embryos exposed to oxidative stress

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Abstract
Sexually dimorphic differences in genome activity, which is orchestrated by transcription factors (TFs), could explain the differential response of male and female embryos to environmental stressors. To proof this hypothesis, the expression of cellular and extracellular TFs was investigated in male and female bovine embryos in vitro cultured either under low (5%) or high (20%) oxygen levels. The intracellular reactive oxygen species (ROS), total cell number, expression of nuclear factor (erythroid-derived 2) factor 2 (NFE2L2), Krüppel-like factor 4 (KLF4), notch receptor 1 (NOTCH1), E2F transcription factor 1 (E2F1), and SREBF2 along with extracellular vesicles (EVs) biogenesis genes were assessed at the blastocyst stage and their released EVs. Low blastocyst rate in both sexes due to oxidative stress (OS) was accompanied by increased ROS accumulation and reduced cell number in female embryos. The messenger RNA and protein levels of NFE2L2 as well as KLF4 expression, were higher in male embryos exposed to OS compared with female embryos. However, the expression of NOTCH1 and E2F1 was higher in female embryos cultured in high oxygen level. Male embryos exposed to OS released more EVs enriched with NFE2L2, superoxide dismutase 1, and NOTCH1 accompanied by elevated expression of EVs biogenesis genes. Accordingly, differential expression of TFs and their release into spent media could partially explain the sexual dimorphic response of bovine embryos to environmental stresses.

KEYWORDS
EVs, oxidative stress, sexual dimorphism, transcription factors

1 | INTRODUCTION

Early embryogenesis involves various morphological, cellular, and biochemical changes related to genome activity (Stanton, Macgregor, & Green, 2003), which could be orchestrated by several transcription factors (TFs). TFs are involved in several biological functions such as embryo development, apoptosis, differentiation, and stress response (Leite et al., 2017). The exposure of embryos to the in vitro culture conditions is known to alter an array of TFs involved in cell death, lipid metabolism, cellular growth, proliferation, and oxidative stress (OS) response (Gad et al., 2012; Leite et al., 2017). OS is triggered by an imbalance between reactive oxygen species (ROS) and its scavenger of antioxidant molecules (Martindale & Holbrook, 2002), due to culture media, temperature, pH, and oxygen tension (Cagnone...
& Sirard, 2016; Wale & Gardner, 2016). For instance, the oxygen levels in oviduct and uterus of most mammalian species (1.5–8.7%) are less than half of atmospheric oxygen level (20%; Fischer & Bavister, 1993), which is widely used in in vitro fertilization (IVF) laboratories and consequently lead to elevation of ROS (Amin et al., 2014; Leite et al., 2017; Yoon et al., 2014). Excess accumulation of ROS damages cellular bioactive molecules such as DNA, protein, and lipid (Martindale & Holbrook, 2002), subsequently compromising the mitochondrial integrity and activity (Amin et al., 2014; Kadenbach, Arnold, Lee, & Hüttemann, 2004) as well as cell number (Amin et al., 2014; Kelley & Gardner, 2016; Leite et al., 2017), resulting in abnormal embryonic development or apoptosis (Amin et al., 2014; Kadenbach et al., 2004; Leite et al., 2017).

The molecular defense mechanisms involve the nuclear factor 2 (erythroid–derived 2) (NRF2 or NFE2L2) signaling pathway and the antioxidants machinery, which is reported to be vital for the survival of embryos under OS conditions (Amin et al., 2014; Kelley & Gardner, 2016; Leite et al., 2017). The NFE2L2 as TF can bind and regulate other TFs such as sterol regulatory element-binding transcription factors (SREBFs; Amin et al., 2014; Kamisako et al., 2014), notch receptor 1 (NOTCH1; Zhao et al., 2016), and pluripotency-related genes (Jang, Wang, Kim, Lalli, & Kosik, 2014).

The sex ratio is believed to be affected by numerous factors either in vivo or in vitro including ovarian factors, maternal diets, and culture conditions (Gutiérrez-Adán, Granados, Pintado, & La Fuente, 2001; Hylan et al., 2009; Iwata, 2012; Rosenfeld & Roberts, 2004). Furthermore, male and female embryos are known to being differ in their transcription (Bermejo-Alvarez, Rizos, Rath, Lonergan, & Gutierrez-Adan, 2010), epigenetic profile (Bermejo-Alvarez, Rizos, Rath, Lonergan, & Gutierrez-Adan, 2008), and metabolite consumption (Gómez et al., 2018), which may be associated with their differential response to environmental insults. So far, this sexual dimorphic response to stress between male and female embryos is controversial and not fully understood. Some argue that female embryos are more tolerant to heat stress than the male embryos (Pérez-Crespo et al., 2005). This could be attributed to the transcriptional dimorphism derived from unaccomplished X-chromosome inactivation during early embryo development (Bermejo-Alvarez et al., 2010). This phenomenon is not only correlated with the upregulation of several genes linked to sex chromosome but also to autosomal chromosomes (Pérez-Cerézales et al., 2018) like glucose-6-phosphate dehydrogenase (G6PD), which is an X-linked gene related to oxygen-free radicals. In bovine, the G6PD has been found to be upregulated in female morula than male counterparts exposed to OS accompanied by enhanced developmental components (Iwata et al., 2002). Contrastingly, others reported that the bovine female embryos were more susceptible to apoptosis than male counterparts (Ghys et al., 2016; Oliveira et al., 2016) following fertilization either with sex-sorted or unsorted semen and cultured under two different culture conditions (Ghys et al., 2016).

Extracellular vesicles (EVs) are small vesicles (30 nm to 5 µm) originated in cell lumen of most cells and secreted into the extracellular space (H. C. Anderson, Mulhall, & Garimella, 2010; Chiang & Chen, 2019; Lawson, Vicencio, Yellon, & Davidson, 2016; Stähl, Johansson, Mossberg, Kahn, & Karpman, 2019) including biological fluids such as plasma, milk, saliva, semen, urine (Lawson et al., 2016), follicular fluid (Hung, Hong, Christenson, & McGinnis, 2015) and uterine aspirates (Campoy et al., 2016). On the basis of their size, EVs can be subdivided into apoptotic bodies (1,000–5,000 nm), microvesicles (100–1,000 nm), and exosomes (30–200 nm; Lawson et al., 2016; Sun et al., 2010; de la Torre Gomez, Goreham, Bech Serra, Nann & Kussmann, 2018). They carry a cargo of bioactive molecules such as DNA, RNAs, proteins to be transferred into the extracellular environment to maintain cellular homeostasis (Takahashi et al., 2017) and/or transfer genetic material to neighbor or distant recipient cells (Saeed-Zidane et al., 2017). In addition, its cargo content varies depending on the cell origin (de la Torre Gomez et al., 2018), physiological status (Hung et al., 2017; Navakanitworakul et al., 2016), inflammation (Jolly, Thompson, & Winchester, 1975) and stress status (Beninson et al., 2014; Bewicke-Copley et al., 2017) of the donor cells.

However, the effect and regulation of EVs biogenesis and secretion in bovine embryos during OS condition is not yet fully understood. Hence, the current study was designed to understand the cellular and extracellular defense mechanisms of male and female bovine embryos exposed to OS under in vitro culture condition.

## 2 RESULTS

### 2.1 Effect of OS on cleavage and blastocyst rate of male and female embryos

The culture of male and female embryos under 5 or 20% oxygen levels revealed no significant difference in cleavage rate (Figure 1a). However, blastocyst rates at Days 7 and 8 were significantly reduced under 20% compared with 5% oxygen level in both male and female embryos (Figure 1b,c). Interestingly, Day 8 blastocyst rate of male embryos was significantly higher compared to female counterparts at both oxygen concentrations. The sex of embryos produced using sexed semen was validated using a combination of primers and results are indicated in Figure S1.

### 2.2 Higher atmospheric oxygen-induced ROS accumulation in both male and female embryos

Embryos cultured under 20% oxygen level showed significantly elevated intracellular ROS accumulation irrespective of the sex of embryo (Figure 2). However, male embryos exhibited a significantly higher accumulation of ROS when cultured under 5% oxygen level compared with female counterparts. Nonetheless, there were no significant differences in ROS accumulation between male and female embryos cultured under 20% oxygen level.

### 2.3 OS resulted in reduced total cell number

OS due to high oxygen level reduced the blastocyst cell number in both male and female embryos (Figure 3). Moreover, the impact of higher oxygen level was pronounced in female blastocysts, as evidenced by a significant reduction in total cell number compared with male embryos cultured under 20% oxygen level. The blastocyst
cell number did not differ between male and female embryos cultured under 5% oxygen level.

2.4 Oxygen level altered the expression of TFs in male and female embryos

Analysis on the differential response of male and female bovine embryos in terms of expression of TFs related to OS response (NFE2L2), differentiation and apoptosis (Krüppel-like factor 4 [KLF4], NOTCH1), cell cycle (E2F transcription factor 1 [E2F1]), and cholesterol biosynthesis (SREBF2) was performed at the blastocyst stage. Results showed that the expression pattern of NFE2L2 was significantly higher in male blastocysts cultured under 20% oxygen level compared with those cultured under 5% oxygen level (Figure 4). Similarly, NFE2L2 tended to be higher in female embryos cultured under 20% compared with 5% oxygen level. Likewise, the transcript abundance of KLF4 was significantly higher in male embryos exposed to higher oxygen tension compared to the low oxygen level. However, no significant difference was observed in the expression level of NOTCH1 in

**FIGURE 1**  The cleavage (a) and blastocyst rates at Day 7 (b) and Day 8 (c) of male and female preimplantation embryos cultured under 5% (white bar) or 20% (black bar) oxygen levels. Data are represented as mean ± SEM of four independent biological replicates.

**FIGURE 2**  Intracellular ROS accumulation (a) and fluorescence intensity of ROS signal (b) of male and female preimplantation embryos derived from 5% (white bar) and 20% (black bar) oxygen levels. Scale bar = 50 μm, values of fluorescence intensity are represented as means ± SEM (*p ≤ .05, **p ≤ .01, ***p ≤ .001). ROS, reactive oxygen species.
both male and female embryos cultured under 20% oxygen level compared with 5% oxygen level. On the other hand, NOTCH1 was significantly higher in female embryos cultured under 20% oxygen level compared with their male counterparts cultured under the same oxygen level. The expression of E2F1 did not show any significant difference between male embryos cultured under 5 or 20% oxygen levels. Contrary to this, E2F1 was significantly higher in female blastocysts derived from 20% oxygen level compared with those from 5% or male embryos cultured under 20% oxygen level. Male embryos cultured under both 5 and 20% oxygen level showed elevated SREBF2 expression pattern compared to female blastocysts cultured under both oxygen levels. Interestingly, exposure of male embryos to elevated oxygen level led to significant upregulation of SREBF2 compared to those cultured under lower oxygen level. On the other hand, the female embryos did not show any significant difference after culture at 5 and 20% oxygen levels in terms of SREBF2 expression.

2.5 | OS-induced expression of NFE2L2 and KLF4 proteins

The protein expression of selected TFs in male and female blastocysts cultured under 5 or 20% oxygen levels revealed that NFE2L2 protein was significantly higher and proportionally localized in the nucleus of both male and female embryos cultured under 20% oxygen level than those cultured under 5% oxygen level. As opposed to the messenger RNA (mRNA) level, the protein level of NFE2L2 was higher in male embryos cultured under 20% oxygen than their female counterpart (Figure 5), and no significant difference was noticed between male and female embryos cultured under 5% oxygen level.

Even though the KLF4 protein level tended to be higher in embryos cultured under 20% compared with 5% counterparts, male and female embryo did not show a statistically significant difference in the KLF4 protein abundance irrespective of the oxygen level (Figure 6).

2.6 | Dysregulation of antioxidant and differentiation genes after induced OS

Exposure of embryos to higher oxygen level significantly increased the expression of catalase (CAT) and superoxide dismutase 1 (SOD1) in male embryos (Figure 7). However, only female embryos showed a significantly higher abundance of CAT in response to high oxygen level, as opposed to SOD1, which was indifferent in female embryos cultured under higher or lower oxygen levels. Similarly, the expression of differentiation-related gene POU class 5 homeobox 1 (POU5F1) was significantly higher in male and female embryos cultured under 20% oxygen level compared with the 5% counterparts. However, no

FIGURE 3 | Total cell number of male and female blastocysts derived from 5% (white bar) and 20% (black bar). Data are represented as means ± SEM (**p ≤ .01, ***p ≤ .001)

FIGURE 4 | The messenger RNA expression pattern of transcription factors (NFE2L2, KLF4, NOTCH1, E2F1, and SREBF2) in male and female blastocysts cultured in 5 or 20% oxygen level. Expression was compared between 5% (white bar) and 20% oxygen level within and between sexes. Data are represented as means ± SEM (*p ≤ .05, **p ≤ .01, ***p ≤ .001). The arbitrary units were multiplied by 100. E2F1, E2F transcription factor 1; KLF4, Krüppel-like factor 4; NFE2L2, nuclear factor (erythroid-derived 2) factor 2; NOTCH1, notch receptor 1
significant differences in transcript abundance of CAT, SOD1, and POU5F1 were observed between male and female bovine embryos cultured under the same oxygen level. The expression of GATA binding protein 4 (GATA4) was not different within the sex of embryo cultured under either 5 or 20% oxygen level. However, under both oxygen levels, male embryos exhibited elevated expression of GATA4 compared with female embryos.

2.7 Characterization of EVs derived from male and female embryos spent media

The presence of EVs in the spent culture media of male and female embryos was confirmed using EV marker protein namely CD63 and their purity or absence cellular contamination was checked by the absence of cytochrome c (CYCS) in the isolated EVs (Figure 8a). Subsequently, the concentration and size of particles were determined using nanoparticle tracking analysis (NTA) and the morphology of the isolated EVs was assessed using transmission electron microscopy (Figure 8b,c). The size distribution of EVs was ranging from 117 ± 2.1 to 145.2 ± 1.1 nm irrespective of the embryo sex and oxygen level. The concentration of EVs was higher in male embryos cultured under 20% oxygen level compared with those derived from 5% oxygen level. However, the EVs released into cultured media of male embryos exposed to OS had a higher concentration and bigger average size than EVs released from female embryos.

2.8 Expression of EV biogenesis and secretion-related genes under OS condition

To evaluate whether the difference in the concentration of EVs is attributed to the impact of OS, the expression levels of genes related to EVs biogenesis (ALIX, vacuolar protein sorting 4 homolog B [VPS4B], and STEAP3 metalloreductase [TSAP6]) and secretion (RAB11 family interacting protein 2 [RAB11FIP1], RAB35, and RAB27A) were investigated. Results revealed that the expression levels of ALIX, VPS4B, RAB11FIP1, and RAB27A were significantly higher in male embryos cultured under 20% oxygen level compared with the 5% counterparts (Figure 9). However, only the expression of ALIX was higher in female embryos cultured under 20% oxygen level. Exposure of embryos to higher oxygen level led to elevated expression of EV biogenesis related-gene VPS4B and secretion-related genes RAB11FIP1 and RAB27A in male embryos compared with female embryos cultured under the same oxygen level. However, the expression of TSAP6 and RAB35 was not affected by both the sex of embryo and oxygen level.
In an attempt to determine whether the changes occurred in the embryos due to OS could affect the communication between embryos via the release of stress indicators loaded in EVs, the mRNA content of released EVs was isolated and quantified for mRNA level of stress-related candidate TFs. Results revealed that the NFE2L2 (Figure 10) carried by EVs released from male embryos cultured under 20% oxygen level was higher compared to EVs released from embryos cultured under 5% oxygen level.

**FIGURE 6** Detection and immunolocalization of KLF4 protein in male and female blastocyst-stage embryos derived from 5 or 20% oxygen levels. Red color reveals the localization of the KLF4 protein, while the blue color, is nuclear staining (DAPI). Scale bar = 50 µm. The bars represent the means ± SEM of fluorescence intensity of the protein as quantified by the ImageJ software. KLF4, Krüppel-like factor 4

**FIGURE 7** The messenger RNA expression pattern of genes related to the antioxidant system (CAT and SOD1) and differentiation (POUSF1 and GATA4) in male and female blastocysts cultured under 5 or 20% oxygen levels. Expression was compared between 5% (white bar) and 20% oxygen level under the same sex and between sexes. Data are represented as means ± SEM (*p ≤ .05, **p ≤ .01, ***p ≤ .001). The arbitrary units were multiplied by 100. CAT, catalase; GATA4, GATA binding protein 4; POUSF1, POU class 5 homeobox 1; SOD1, superoxide dismutase 1

### 2.9 EV-coupled transcript releases due to OS

In an attempt to determine whether the changes occurred in the embryos due to OS could affect the communication between embryos via the release of stress indicators loaded in EVs, the mRNA content of released EVs was isolated and quantified for mRNA level of stress-related candidate TFs. Results revealed that the NFE2L2 (Figure 10) carried by EVs released from male embryos cultured under 20% oxygen level was higher compared to EVs.
released from male embryos cultured under 5% oxygen level and female embryos cultured under 20% oxygen. Similarly, SOD1 was significantly higher in EVs released from male embryos exposed to OS compared with those cultured under normal conditions. The transcript level of CAT, KLF4, and E2F1 did not show significant differences among all investigated groups. It is noteworthy that the EVs released from male embryos exposed to OS showed a significantly higher amount of NOTCH1 transcript than those cultured under 5% oxygen level and the EVs released from female embryos exposed to OS. EVs released from female embryos under 5 and 20% oxygen levels did not show significant differences in terms of NOTCH1 transcript level.

3 | DISCUSSION

The suboptimal culture conditions of the in vitro production system are partly responsible for the reduction in number and quality of blastocysts (Farin & Farin, 1995; Gad et al., 2012). In addition to the culture media, the higher oxygen level in in vitro system is responsible for contributing to the suboptimal environment (Amin et al., 2014; Kelley & Gardner, 2016; Leite et al., 2017). However, previous evidence indicated that there was no significant difference observed in terms of embryo development between maturation followed by fertilization at high oxygen tension and maturation at low oxygen followed by fertilization at high oxygen level, but a dramatic effect was observed in case of fertilization at low oxygen level (Bermejo-Alvarez, Lonergan, Rizos, & Gutiérrez-Adan, 2010).

Accordingly, the maturation and fertilization were done at high oxygen tension in the current study. Furthermore, here we showed that irrespective of the sex of embryo, those embryos cultured under 5% oxygen level showed higher development rate of blastocyst than those under 20% oxygen level, as it has been reported before (Amin et al., 2014; Leite et al., 2017; Yoon et al., 2014). Embryos cultured under either 5 or 20% oxygen levels (Figure 1) were skewed toward the male embryos when cultured under 5% oxygen level and the black bars indicate 20% oxygen level.
under higher oxygen tension (20%) revealed elevated intracellular ROS accumulation irrespective of the sex of the embryos (Figure 2). However, male embryos exhibited higher ROS accumulation than female embryos at lower oxygen level, which may be attributed to higher metabolism rate of male embryo compared with female counterparts (Tiffin, Rieger, Betteridge, Yadav, & King, 1991). Consistently, metabolite analysis of the spent culture media of male and female bovine embryos cultured under low oxygen tension revealed extensive metabolite exchange of male embryos than the female counterpart (Gómez et al., 2018). However, this difference was faded out under high oxygen tension, which may be associated with the potential role of glucose in ROS scavenging process (Andrisse et al., 2014).

**FIGURE 9** The messenger RNA expression pattern of extracellular vesicles biogenesis (ALIX, VPS4B, and TSAP6) and secretion (RAB11FIP1, RAB35, and RAB27A) genes in male and female blastocysts cultured under 5 or 20% oxygen levels. Expression was compared between 5% (white bar) and 20% oxygen levels under the same sex and between sexes. Data are represented as means ± SEM (*p ≤ .05, **p ≤ .01, ***p ≤ .001). The arbitrary units were multiplied by 100. RAB11FIP1, RAB11 family interacting protein 2; TSAP6, STEAP3 metalloreductase; VPS4B, vacuolar protein sorting 4 homolog B.

**FIGURE 10** The messenger RNA (mRNA) expression pattern of extracellular vesicles-coupled transcription factors (NFE2L2, KLF4, NOTCH1, and E2F1) and antioxidant genes (CAT and SOD1) released into culture media of male and female embryos cultured under 5 or 20% oxygen levels. Expression was compared between 5% (white bar) and 20% (black bar) oxygen levels within and between sexes. The mRNA level was measured by quantitative reverse transcription-polymerase chain reaction and normalized by the geometric means of three housekeeping genes (ACTB, GAPDH, and 18S). Data are represented as means ± SEM (*p ≤ .05, ***p ≤ .001). The arbitrary units were multiplied by 100. CAT, catalase; E2F1, E2F transcription factor 1; KLF4, Krüppel-like factor 4; NFE2L2, nuclear factor (erythroid-derived 2) factor 2; NOTCH1, notch receptor 1; SOD1, superoxide dismutase 1.
The higher oxygen tension is also reported to compromise the cell proliferation, as evidenced by subsequent reduction in total cell number of embryos (Amin et al., 2014; Leite et al., 2017; van Soom et al., 2002; Yoon et al., 2014). Likewise, we found that embryos exposed to high oxygen level had a reduced total cell number (Figure 3) independent of the sex of embryo, which could be associated with dysregulation of development and apoptosis-related genes (Leite et al., 2017). The negative effect of high oxygen level in terms of cell number was more pronounced in female embryos than male counterparts. This fact is in agreement with Dallemagne et al. (2018), who showed that exposing bovine embryos to OS from Days 5 to 7 in presence of FCS but not bovine serum albumin supplemented with insulin, transferrin and selenium (BSA-ITS) resulted in a reduction of total cell number and increasing apoptosis in female embryos compared with male counterparts. Moreover, higher cell number was observed in male embryos than female cultured under 5% oxygen level in presence FCS or BSA-ITS, which was not in agreement with the current study (Dallemagne et al., 2018). Supporting to our results, no significant difference in total cell numbers was observed between male and female bovine embryos cultured under low oxygen tension (Siqueira & Hansen, 2016), which indicates the contribution of culture media in the variation between male and female embryos under either normal or stress conditions.

In fact, one of the cellular mechanisms in response to suboptimal conditions is activation/suppression of transcription to maintain cellular homeostasis. This has been elucidated by the fact that higher intracellular ROS level induces the expression of NFE2L2 in bovine granulosa cell (Saeed-Zidane et al., 2017) and preimplantation embryos (Amin et al., 2014). NFE2L2 is a TF found to be sequestrated in the cytoplasm via Kelch-like ECH-associated protein 1 (KEAP1). During stressor-induced NFE2L2 activation, the NFE2L2 disengages from KEAP1 and translocated in the nucleus to bind with a specific DNA motif called antioxidant response element to induce the antioxidant machinery (Bryan, Olayanju, Goldring, & Park, 2013; Zhang, 2006). In consistent with this, we found that the mRNA expression (Figure 4) and protein pattern (Figure 5) of NFE2L2 were higher in male embryos cultured under 20% oxygen level compared with 5% oxygen counterparts. However, female embryos showed the only elevation of their protein. This was further followed by increased expression of antioxidant genes namely CAT and SOD1 (Figure 7), which was clearly pronounced in male embryos than the female ones (Figure 4a). Besides to its role in the activation of antioxidant machinery, NFE2L2 has been implicated in the regulation of other cellular function such as lipid metabolism (Amin et al., 2014; Kamisako et al., 2014), via regulation of SREBFs genes. The SREBFs family has three subtypes namely SREBF1a, SREBF1c, and SREBF2, which are involved in the regulation of cellular fatty acids and cholesterol biosynthesis through binding to sterol regulatory element (SRE: Daemen, Kutmon, & Evelo, 2013; Shimano, 2001). Our results revealed that the expression of SREBF2 was increased under high oxygen tension (Figure 4), which may be occurred in response to endoplasmic reticulum stress-induced cholesterol accumulation via increasing SREBP2 cleavage (Colgan, Tang, Werstuck, & Austin, 2007). Interestingly, the expression of SREBF2 was significantly higher in male compared with female counterparts either cultured in 5 or 20% oxygen levels, which may be attributed to developmental dimorphism in favor of male embryos (Xu et al., 1992). Recent studies also evidenced that bovine male embryos release higher lipids and lipid-like molecules into culture media than female counterparts (Gómez et al., 2018).

NOTCH1 is one of NOTCH signaling pathway receptors, which is branching in various cell function including proliferation, differentiation, and apoptosis. Various combinations of NOTCH signaling pathway receptors and ligands might lead to different cellular response (E. R. Andersson, Sandberg, & Lendahl, 2011). In the present study, the expression of NOTCH1 was higher in those embryos exposed to OS coupled with elevated NFE2L2 transcript, as previously has been shown (Zhao et al., 2016). The upregulation of NFE2L1 under high oxygen level is attributed to cell apoptosis prevention via suppression of apoptosis signal-regulating kinase 1 (ASK1), and then preventing the p38 mitogen-activated protein kinases signaling pathway (Mo et al., 2013). This was more pronounced in embryos produced in vitro (Heras et al., 2016). Consistent with this finding, in the current study higher expression of NOTCH1 was found in female embryos exposed to OS than male counterparts (Figure 4). This phenomenon is believed to be deemed a reaction to the high level of apoptosis induced by the expression of E2F1. E2F1 is one isoform of E2Fs family involved in the regulation of cell cycle and apoptosis (Crosby & Almasan, 2004). Consequently, the reduction in total cell number (Figure 3) in female embryos derived from high oxygen level was coupled with upregulation of E2F1 compared with male ones. On the contrary, Rivero, Montagnani, and Stecca (2017) reported the positive impact of E2F1 in promoting cancer cell proliferation via direct binding and regulation of KLF4.

KLF4 belongs to KLFs TF family that are involved in the regulation of proliferation, apoptosis, and differentiation of various cell types (Miao, Wu, & Shi, 2017) as well as stem cell fate in coordination with NANOG and POU5F1 (Chan et al., 2009). In the current study, we found that the higher expression of KLF4 was accompanied by differential expression of KLF4 target genes under high oxygen tension namely: POU5F1 and GATA4 (Figure 7). This could explain a disturbed embryonic stemness pattern at higher oxygen level (Jang et al., 2014), which was remarkable in male embryos. Irrespective of the sex of the bovine embryo, transcript analysis of blastocysts showed upregulation of pluripotency-related genes (NANOG and SOX2), but not POU5F1 in response to OS (Leite et al., 2017). However, the mRNA level of GATA4 was upregulated in male embryos cultured either in 5 or 20% oxygen level compared with female counterparts.

The EVs are supposed to be involved in several biological functions including embryo-maternal communication (Saadeldin, Oh, & Lee, 2015; Szekeres-Bartho, Šućurović, & Mulac-Jerčević, 2018). Their size, as well as concentration, could vary according to cellular physiological status. Accordingly, our results indicated that the higher number and bigger size EV (Figure 8c) were found in spent media of male embryos cultured under high oxygen level. Similar results were obtained in our previous study in granulosa cells exposed to OS (Saeed-Zidane et al., 2017). In
contrast, the female embryos exhibited a lower number and bigger size EVs upon exposure to OS. A possible explanation for this sexual dimorphic phenotype can be differences in the expression of genes involved in biogenesis and release of EVs (Hessvik & Llorente, 2018; Kowal, Tkach, & Théry, 2014). Accordingly, the mRNA expression pattern of those genes revealed that ALIX and VPS4B were significantly increased in male embryos exposed to OS condition (Figure 9). However, female embryos showed higher expression of ALIX transcript only. In addition, the mRNA expression level of RAB11FIP1 and RAB27A, responsible for EVs secretion (Kowal et al., 2014), showed an upregulation in male embryos exposed to OS compared with 5% oxygen level and/or female counterparts. This may indicate the sexual dimorphic physiological need in biogenesis and secretion of EVs.

It is not only the number of EVs released into extracellular space but also the molecular cargo of those EVs, which was different between male and female embryos exposed to OS. As shown in Figure 10, EVs from male embryos at higher oxygen level contained higher level of NFE2L2 and SOD1 transcripts compared to those cultured under low oxygen level, which might be beneficial of development of embryos (Pavani et al., 2018). The role of EVs is not only in cell–cell communication (Saeed-Zidane et al., 2017) but also in the removal of undesirable cellular molecules (Takahashi et al., 2017). Contrary to cellular expression, the NOTCH1 was increased in EVs derived from male embryos exposed to OS compared with female counterparts, which may indicate the selectivity of cargo molecules to be exported by EVs (Bhome et al., 2018; Hinger et al., 2018) to facilitate cell–cell communication or maintain of cellular homeostasis. Herein, we can summarize that exposing preimplantation bovine embryos to OS compromised the transformation of blastocysts irrespective of embryo sex, due to increasing intracellular ROS accumulation, which in turn leads to increase apoptosis and delay of differentiation in response to dysregulating of the TFs related to stress response, development, differentiation, and apoptosis. Interestingly, the reduction in blastocyst formation and total cell count was more pronounced in female embryos exposed to OS compared with male counterparts, which was accompanied with alteration in cellular expression and subsequent release of TFs through EVs. Taken together, exposure either male or female embryos to OS altered their TFs expression pattern coupled with a reduction in development and delaying in differentiation. However, the male embryos are more tolerant to OS than female counterparts via activation of NFE2L2 and their downstream antioxidant genes at the cellular and extracellular levels, which partially regulate other TFs involved in apoptosis, differentiation, and development leading to skewed sex ratio toward male embryos.

4 | MATERIALS AND METHODS

4.1 | Oocyte collection and maturation

Ovaries were obtained from a nearby slaughterhouse and transported to the laboratory in 0.9% physiological saline solution (NaCl) within 2–3 hr. Upon arrival, the ovaries were rinsed with prewarmed (37°C) 70% ethanol followed by two to three times washing in fresh phosphate buffer saline (PBS), and cumulus-oocyte complexes (COCs) were then aspirated from small follicles (2–8 mm), using sterilized syringe attached to 18G needle. Oocytes with homogenous cytoplasm and surrounded by multiple layers of compacted cumulus cells were then selected for in vitro maturation. Groups of 50 COCs were transferred to TCM 199 modified culture media (Sigma-Aldrich, Munich, Germany) supplemented with 4.4 mM HEPES, 33.9 mM NaCHO3, 2 mM pyruvate, 2.9 mM calcium lactate, 55 mg/ml gentamicin and 12% (vol/vol) heat-inactivated estrus cow serum (Schellander, Fuhrer, Brackett, Korb, & Schleger, 1990) and then cultured for 22–24 hr at 39°C under 5% CO2 in air with maximum humidity.

4.2 | In vitro embryo production

Following maturation, COCs were coincubated for 20 hr under 20% oxygen level with 2 × 106 sperms/ml sex-sorted sperm in F-TALP medium supplemented with 20 mM penicillamine, 10 mM hypotaurine, 2 mM noradrenaline, 6 mg/ml bovine serum albumin (BSA), 50 mg/ml gentamicin, and 1 mg/ml heparin (Parrish et al., 1986). Subsequently, presumptive zygotes were denuded by repeated pipetting and transferred to 400 μl of synthetic oviductal fluid (SOF) culture medium (Holm, Booth, Schmidt, Greve, & Callesen, 1999) supplemented with 10% exosomes-depleted fetal bovine serum in four-well dishes (Thermo Fisher Scientific, Roskilde, Denmark). Presumptive zygotes were incubated in two different oxygen level 5% CO2 in air (20% supraphysiological level) or 5% CO2, 5% O2, and 90% N2 (5% physiological level) until the blastocyst stage (Day 8). After recording developmental data, the male and female embryos were collected and washed twice in PBS and then snap-frozen in cryotubes containing lysis buffer (40 U/ml RNasin [Promega, WI], 0.8% Igepal [Sigma-Aldrich, MO], 5 mM dithiothreitol [Promega]). The validation of the sex of pooled IVP embryos (10 blastocyst per each replicate) using sex-sorted semen was performed using multiplex polymerase chain reaction (PCR) as described before (Rattanasuk, Pampai, & Ketudat-Cairns, 2011). Briefly, a combination of two primers, bovine specific primer (Forward: 5′−TTTACCTTAGAAACAACCGGAGGCACGACACGACCC′ and Reverse: 5′−TACGGAAAGGAAAGATGACCTGACC′ with amplicon size 538 bp) and Y-specific primer (Forward: 5′−CTCGAAAAGCAACCAACAGAC3′ and Reverse: 5′−GAAGTTTCAGGAGGACGGCC−3′ with amplicon size 300 bp), was used to determine the sex of embryos. A total of 20 µl amplification reaction for each replicate was subjected to thermal cycle program of initial preheating with 95°C for 2 min, followed by 35 cycles of 20 s at 95°C, 45 s at 52°C, and 50 s at 72°C followed by final extension for 10 min at 72°C. Finally, PCR products were electrophoresed on 2% agarose gel and the image was visualized under ultraviolet using ChemiDoc™ XR+ system (Bio-Rad Laboratories GmbH, Munich, Germany).

4.3 | Intracellular ROS detection

To investigate the effect of OS (20% O2) on intracellular ROS accumulation in male and female bovine embryos, the ROS level in blastocyst was detected using the 6-carboxy-2,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) fluorescent probe (Life Technologies,
Darnstadt, Germany). Fifteen blastocysts from each group were incubated with 400 µl of 5 µM H2DCFDA for 20 min in the dark at 37°C. Thereafter, the embryos were washed twice in PBS-PVA and the images were immediately acquired under an inverted microscope (Leica DM IRB, Germany), using a green fluorescence filter. The fluorescent intensity was analyzed as a single embryo for each group using ImageJ 1.48v software (National Institutes of Health, http://image.nih.gov).

4.4 | Total cell count

Total blastocyst cell number of male and female bovine embryos cultured under low (5%) or high (20%) oxygen levels were quantified using nuclear fluorescence staining with the glycerol-based Hoechst 33342 (Sigma-Aldrich) according to the manufacturer’s procedure. For that, 10 blastocysts from each group were fixed for 5 min in a solution containing 2% formalin and 0.25% glutaraldehyde. The fixed blastocysts were mounted and stained for 10 min with glycerol–water–acetone–sulfuric acid (12.5 mg/ml) solution on clean glass slides. The stained nuclei were visualized using a fluorescent microscope (Olympus, Tokyo, Japan) with a blue (excitation: 330–385 nm; emission: 420 nm; dichromatic: 400 nm) and orange (excitation: 490–520 nm; emission: 510 nm) filter. The cell number was recorded for individual blastocysts from each cultured group using Zen 2.3V (blue edition; https://www.zeiss.com/microscopy/int/products/microscope/software/zen-lite.html).

4.5 | RNA extraction and complementary DNA synthesis

Four biological replicates (pool of 10 blastocysts/each) embryos from each group were used for RNA extraction. Total RNA was extracted using a PicoPure RNA isolation kit (Arcturus, Munich, Germany) according to the manufacturer’s instruction. Briefly, a combination of lysis buffer was added to certain volume of PBS-CMF (calcium-magnesium-free) containing EVs followed by vortex and incubation for 10 min at room temperature, respectively. After incubation, 500 µl of absolute ethanol was added to the mixture and mixed well by vortexing. Appropriate volume from mixture was transferred to a mini spin column and then centrifuged at 3,300g. After the withdrawal of the flow through, the mini spin column was washed twice with washing buffer each time followed by centrifugation at 3,300g for 30 s. Afterward, the mini spin column was dried with centrifugation at 13,000g for 1 min at room temperature. Finally, the RNA was eluted in appropriate amount of elution buffer and then stored at −80°C till further application.

RNA concentration was measured (Tables S1 and S2) using 8000 NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Germany) and equal quantities of RNA input was used for complementary DNA (cDNA) synthesis. The cDNA synthesis was performed using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Briefly, an equal amount of RNA was incubated with 0.5 µl of oligo-dT and 0.5 µl random primer, followed by incubation at 65°C for 5 min. Besides this, a master mix containing 4 µl of 5X reaction buffer, 1 µl of RiboLock RNase inhibitor, 2 µl of 10 mM dNTP mix, and 2 µl of M-MuLV reverse transcripts were prepared and gently mixed by pipetting. At the end of incubation, a total of 9 µl was added to each specimen, and then incubated at 25°C for 5 min, then 37°C for 60 min followed by 70°C for 5 min to terminate the reaction. After the incubation time, the cDNA was kept at −20°C for gene expression analysis.

4.6 | Quantitative real-time PCR

The transcript level of developmental and stress-related TFs namely, NFE2L2, KLF4, NOTCH1, SREBF2, and E2F1; TFs target genes namely, CAT, SOD1, POU5F1, and GATA4 as well as genes associated with EVs biogenesis and secretion namely, programmed cell death 6 interacting protein (PDCD6IP/AUX), VPS4B, TSAP6, RAB11FIP1, RAB35, member RAS oncogene family (RAB35), and RAB27A, member RAS oncogene family (RAB27A) was investigated in male and female bovine embryos cultured under 5 and 20% oxygen level using iTaq™ primaQUANT real-time PCR (qRT-PCR) Master Mixes (Steinbrenner Laborsysteme GmbH, Wiesenbach, Germany) in Applied Biosystem® StepOnePlus™ (Applied Biosystems, Foster City, CA). Primers were designed using Primer3 online software (http://bioinfo.ut.ee/primer3-0.4.0/; Table 1). The master mix containing cDNA, optimized amount of forward and reverse primers and SYBR green master mix was run in a thermal cycler program of 95°C for 3 min initial denaturation, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. Melting curve was generated to verify the amplicon specificity. The expression level of ACTB and GAPDH was used for normalization for embryonic transcripts, while 18S was used as a third housekeeping gene for EV-coupled transcript profiling. The relative abundance of gene transcript was expressed as arbitrary unit calculated by

$$2^{-\Delta C_{\text{target gene}} - \Delta C_{\text{housekeeping gene}}}$$

(Livak & Schmittgen, 2001).

4.7 | Immunohistochemistry

To quantify and localize the NFE2L2 and KLF4 proteins in male and female bovine embryos cultured under 5 and 20% oxygen levels, immunohistochemistry assay was conducted. For this, 10 embryos from each group were fixed using 4% paraformaldehyde and kept at 4°C overnight. Thereafter, the fixed embryos were washed four times for 5 min each with glycerine in PBS, followed by permeabilization step using 0.5% (vol/vol) Triton X-100 (Sigma-Aldrich) in PBS for 3 hr at room temperature. After the incubation time, the samples were incubated with 4% donkey serum for 1 hr at room temperature, followed by incubation at 4°C overnight with primary rabbit polyclonal antibody for NFE2L2 (1:100; Biornbyt, UK) and KLF4 (1:100, Thermo Fisher Scientific, Germany) dissolved in 4% donkey serum. Following incubation, the embryos were washed four times with PBS-PVA, followed by incubation with Alexa fluor goat anti-rabbit secondary antibody (1:350 dilution; LifeSpan Biosciences) for 3 hr at 37°C. Afterward, the specimens were mounted on the slide.
with VECTASHIELD mounting medium containing DAPI (Vector Laboratories, Burlingame, CA). Finally, samples were visualized under confocal microscope CLSM LSM-780 and the signals were analyzed with ImageJ 1.48v (National Institutes of Health, http://imagej.nih.gov).

### 4.8 EVs isolation and characterization

Four biologicals replicated of a total of 350 µl culture media from male and female bovine embryos cultured under 5 and 20% oxygen level were collected for EVs isolation. Briefly, the spent media were subjected to differential centrifugation at 4°C starting from 300g, 10 min to remove cells followed by 2,000g, 15 min to remove dead cells; and finally, 10,000g, 30 min to remove cell debris, macroparticles, and apoptotic bodies. Then, the supernatant was collected and subjected to ultracentrifugation at 120,000g for 70 min at 4°C in a Beckman SWTi55 rotor to isolate EVs. Afterward, the isolated EVs were washed in PBS-CMF and then ultracentrifuged using the aforementioned

| Gene   | Accession number | Primer sequences | Product size (bp) |
|--------|------------------|------------------|-------------------|
| ACTB   | NM_173979        | F: 5'-TGTCACCTTCCAGCAGAT-3' R: 5'-TCACCTTACCCGTTGACAGT-3' | 249              |
| GAPDH  | NM_001034034     | F: 5'-CCCAAGATATCATCCCTGCT-3' R: 5'-CTGGTTACCCCTTCTCTGTA-3' | 185              |
| 18S    | NR_036642        | F: 5'-CGGACGCTAGAATAATTGGA-3' R: 5'-CTGGATCGTCTCGAAGACCTC-3' | 210              |
| KLF4   | NM_001105385.1   | F: 5'-CAAACCAAGAGGGGAAGAC-3' R: 5'-AAACTTCCACCAAAACCAT-3' | 175              |
| NOTCH1 | XM_024999642.1   | F: 5'-AGCAGATCTGAGCGGCA-3' R: 5'-TCCAGGTTGATCTCGAAG-3' | 125              |
| E2F1   | NM_001206079.1   | F: 5'-CTTGGGTAATACGTGAGCCGC-3' R: 5'-CTGGGTAATACGTGAGCCGC-3' | 209              |
| SOD1   | NM_174615        | F: 5'-CAGCCGTTGGAGGCTCTGTA-3' | 189              |
| CAT    | NM_001035386.1   | F: 5'-TGGGACGCAAATCCCTTGAC-3' R: 5'-AAAGTGGGTCTGTGTTCCAG-3' | 178              |
| SREBF2 | NM_001205600.2   | F: 5'-TTCCCTGCTGCTCTTTAAC-3' R: 5'-CACGATCATGACATTACCA-3' | 183              |
| NFE2L2 | NM_001011678     | F: 5'-CCCAGCTTCCAGGCTCCTC-3' R: 5'-TCAGCGTAATCCCTTCTTGT-3' | 165              |
| GATA4  | NM_001192877.1   | F: 5'-CTGGCTGCTGCTGGGAATGAC-3' R: 5'-GACTTCTCGTCTCTTGTGCG-3' | 228              |
| ALIX   | XM_005222590.4   | F: 5'-GAGTACCCAGTCTATCCT-3' R: 5'-AGGGTCCAGATCCTTTAAGGT-3' | 225              |
| VPS4B  | NM_001076156.1   | F: 5'-TTATAGAGCCGACCCCAATGTG-3' R: 5'-GAGTTATATTCTTCTGTTGCC-3' | 208              |
| TSAP6  | NM_001304338.1   | F: 5'-CAAGGATGGAAAATAGGCAG-3' R: 5'-CAAAAGGTTTGAGCCGTAAGAAG-3' | 222              |
| RAB11FIP1 | XM_005226137.4 | F: 5'-TCGTCGAGCTGAGACTGAC-3' R: 5'-GTTGTCGATGTAGTCTGAT-3' | 176              |
| RAB35  | NM_001098127.1   | F: 5'-ATCACCTCCAGCAGATGATCG-3' R: 5'-CTGGTCCTCAAACAACCTGAT-3' | 233              |
| RAB27A | NM_001101270.1   | F: 5'-CTATGCGGCTTTCTTGCTT-3' R: 5'-ACTCCGATATTCTGTCGAAAG-3' | 196              |
| POU5F1 | NM_174580.3      | F: 5'-GATAACCCAGCAGCGATG-3' R: 5'-CTGGTTTCTCGAATCACTGTC-3' | 240              |

Abbreviations: F, forward; PCR, polymerase chain reaction; R, reverse.

**TABLE 1** The list of primers sequence used for quantitative real-time PCR
procedure. Finally, the isolated EVs were suspended in PBS-CMF and kept at –80°C for further investigation.

The purity of the isolated EVs was examined using immunoblotting for the presence of EV marker CD63 protein and for the absence of mitochondrial cellular marker, CYCS. Briefly, an equal amount of EVs solution from each group was incubated with 2X sodium dodecyl sulfate (SDS) loading buffer at 95°C for 5 min and then loaded onto 12% SDS-PAGE gel. Following electrophoresis, the proteins were transferred to nitrocellulose membranes (Protran® Schleicher & Schuell Bioscience) using a semi-dry blotting system (Bio-Rad Laboratories GmbH). Subsequently, the membrane was blocked using 1X blocking solution (Carl Roth GmbH) for 1 hr at room temperature followed by incubation overnight at 4°C with the primary rabbit polyclonal antibody against CD63 (1:1,000; System BioSciences) and goat polyclonal against CYCS (1:350; Santa Cruz Biotechnology Inc, Germany). After the incubation time, the membrane was washed three times with diluted Tris-buffer saline with Tween 20 (TBST) followed by incubation for 1 hr at room temperature with goat anti-rabbit (1:5,000) (System BioSciences) and donkey anti-goat (1:5,000; Santa Cruz Biotechnology Inc) secondary antibodies. Afterward, the membrane was washed with diluted TBST followed by incubation with Clarity ECL Substrate (Bio-Rad Laboratories GmbH). Finally, the bands were visualized and the images were picked up using the ChemiDoc™ XRS+ system (Bio-Rad Laboratories GmbH).

The concentration and size of EVs were evaluated using NanoSight NS300 following manufacturer protocols (Malvern Instruments, Malvern, UK). Briefly, a total of 50 µl from isolated EVs were diluted in 1 ml PBS-CMF, and then five consecutive videos were recorded. The recorded videos for each sample were analyzed using NTA software to obtain mean, mode of the particles size and concentration per ml of volume.

4.9 | Electron microscopy

A total of 30 µl from isolated EVs were used for visualization under electron microscopy. Briefly, the sample was dropped on parafilm and coincubated with Formvar/carbon-coated grids for 5 min at room temperature. Afterward, the Formvar/carbon-coated grid was washed with PBS and subsequently stained with 2% uranyl acetate followed by washing with PBS. Finally, the grids were dried and the images were acquired using electron microscopy.

4.10 | Statistical analysis

The data were statistically analyzed using two-way analysis of variance (ANOVA) followed by multiple pair-wise comparisons using the Tukey post hoc test (GraphPad Prism Version 7). The data are presented as mean ± SEM of biological quadruplicate. Statistical significance was determined at p ≤ .05.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

REFERENCES

Amin, A., Gad, A., Saililew-Wondim, D., Prastowo, S., Held, E., Hoelker, M., ... Tesfaye, D. (2014). Bovine embryo survival under oxidative-stress conditions is associated with activity of the NRF2-mediated oxidative-stress-response pathway. Molecular Reproduction and Development, 81, 497–513.

Anderson, H. C., Mulhall, D., & Garimella, R. (2010). Role of extracellular membrane vesicles in the pathogenesis of various diseases, including cancer, renal diseases, atherosclerosis, and arthritis. Laboratory Investigation, 90, 1549–1557.

Andersson, E. R., Sandberg, R., & Lendahl, U. (2011). Notch signaling: Simplicity in design, versatility in function. Development, 138, 3593–3612.

Andrisse, S., Koehler, R. M., Chen, J. E., Patel, G. D., Vallurupalli, V. R., Ratliff, B. A., ... Fisher, J. S. (2014). Role of GLUT1 in regulation of reactive oxygen species. Redox Biology, 2, 764–771.

Beninson, L. A., Brown, P. N., Loughridge, A. B., Saludes, J. P., Maslanik, T., Hills, A. K., ... Fleschner, M. (2014). Acute stressor exposure modifies plasma exosome-associated heat shock protein 72 (Hsp72) and microRNA (miR-142-5p and miR–203). PLOS One, 9, e108748.

Bermejo-Alvarez, P., Lonergan, P., Rizos, D., & Gutierrez-Adan, A. (2010). Low oxygen tension during IVF improves bovine oocyte competence and enhances anaerobic glycolysis. Reproductive BioMedicine Online, 20, 341–349.

Bermejo-Alvarez, P., Rizos, D., Rath, D., Lonergan, P., & Gutierrez-Adan, A. (2008). Epigenetic differences between male and female bovine blastocysts produced in vitro. Physiological Genomics, 32, 264–272.

Bermejo-Alvarez, P., Rizos, D., Rath, D., Lonergan, P., & Gutierrez-Adan, A. (2010). Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. Proceedings of the National Academy of Sciences of the United States of America, 107, 3394–3399.

Bewicke-Copley, F., Mulcahy, L. A., Jacobs, L. A., Samuel, P., Akbar, N., Pink, R. C., & Carter, D. R. F. (2017). Extracellular vesicles released
following heat stress induce bystander effect in unstressed populations. Journal of Extracellular Vesicles, 6, 1340746.

Bhome, R., Del Vecchio, F., Lee, G.-H., Bullock, M. D., Primrose, J. N., Sayan, A. E., & Mirnezami, A. H. (2018). Exosomal microRNAs (exomiRs): Small molecules with a big role in cancer. Cancer Letters, 420, 228–235.

Bryan, H. K., Olayanju, A., Goldring, C. E., & Park, B. K. (2013). The Nrf2 cell defense pathway: Keap1-dependent and -independent mechanisms of regulation. Biochemical Pharmacology, 85, 705–717.

Cagnone, G., & Sirard, M.-A. (2016). The embryonic stress response to in vitro culture: Insight from genomic analysis. Reproduction, 152, R247–R261.

Campoy, I., Lanau, L., Altadill, T., Sequeiros, T., Cabrera, S., Cubo-Abert, M., ... Colas, E. (2016). Exosome-like vesicles in uterine aspirates: A comparison of ultra-centrifugation-based isolation protocols. Journal of Translational Medicine, 14, 180.

Chan, K. K.-K., Zhang, J., Chia, N.-Y., Chan, Y.-S., Sim, H. S., Tan, K. S., ... Choo, A. B.-H. (2009). KLF4 and PBX1 directly regulate NANOG expression in human embryonic stem cells. Stem Cells, 27, 2114–2125.

Chiang, C., & Chen, C. (2019). Toward characterizing extracellular vesicles at a single-particle level. Journal of Biomedical Science, 26, 9.

Colgan, S. M., Tang, D., Werstuck, G. H., & Austin, R. C. (2007). Endoplasmic reticulum stress causes the activation of sterol regulatory element binding protein-2. The International Journal of Biochemistry & Cell Biology, 39, 1843–1851.

Crosby, M. E., & Almasan, A. (2004). Opposing roles of EZFs in cell proliferation and death. Cancer Biology & Therapy, 3, 1208–1211.

Daemen, S., Kutmon, M., & Evelo, C. T. (2013). A pathway approach to investigate the function and regulation of SREBP1s. Genes and Nutrition, 8, 289–300.

Dallemagne, M., Ghys, E., De Schrevel, C., Mwaema, A., De Troy, D., Rasse, C., & Donnay, I. (2018). Oxidative stress differentially impacts male and female bovine embryos depending on the culture medium and the stress condition. Theriogenology, 117, 49–56.

Farin, P. W., & Farin, C. E. (1995). Transfer of bovine embryos produced in vivo or in vitro: Survival and fetal development. Biology of Reproduction, 52, 676–682.

Fischer, B., & Bavister, B. D. (1993). Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. Reproduction, 99, 673–679.

Gad, A., Hoelker, M., Besenfelder, U., Havliceck, V., Cinar, U., Rings, F., ... Tesfaye, D. (2012). Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions. Biology of Reproduction, 87, 100.

Ghys, E., Dallemagne, M., De Troy, D., Sauvegarde, C., Errachid, A., & Donnay, I. (2016). Female bovine blastocysts are more prone to apoptosis than male ones. Theriogenology, 85, 591–600.

Gutiérrez-Adán, A., Granados, J., Pintado, B., & De La Fuente, J. (2001). Influence of glucose on the sex ratio of bovine IVM/IVF embryos cultured in vitro. Reproduction, Fertility, and Development, 13, 361–365.

Gómez, E., Carrocera, S., Martin, D., Herrero, P., Canela, N., & Muñoz, M. (2018). Differential release of cell-signaling metabolites by male and female bovine embryos cultured in vitro. Theriogenology, 114, 180–184.

Heras, S., De Coninck, D. I. M., Van Poucke, M., Goossens, K., Bogado Pascottini, O., van Nieuwerburgh, F., ... van Soom, A. (2016). Suboptimal culture conditions induce more deviations in gene expression in male than female bovine blastocysts. BMC Genomics, 17, 72.

Hessvik, N. P., & Llorente, A. (2018). Current knowledge on exosome biogenesis and release. Cellular and Molecular Life Science, 75, 193–208.

Hinger, S. A., Cha, D. J., Franklin, J. L., Higinbotham, J. N., Dou, Y., Ping, J., ... Patton, J. G. (2018). Diverse long RNAs are differentially sorted into extracellular vesicles secreted by colorectal cancer cells. Cell Reports, 25, 715–725.e4.

Holm, P., Booth, P. J., Schmidt, M. H., Greve, T., & Callesen, H. (1999). High bovine blastocyst development in a static in vitro production system using soaaf medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology, 52, 683–700.

Huang, W.-T., Hong, X., Christenson, L. K., & McGinnis, L. K. (2015). Extracellular vesicles from bovine follicular fluid support cumulus expansion. Biology of Reproduction, 93, 117.

Huang, W.-T., Navakanitworakul, R., Khan, T., Zhang, P., Davis, J. S., McGinnis, L. K., & Christenson, L. K. (2017). Stage-specific follicular extracellular vesicle uptake and regulation of bovine granulosa cell proliferation. Biology of Reproduction, 97, 644–655.

Hylan, D., Giraldo, A. M., Carter, J. A., Gentry, G. T., Bondlioni, K. R., & Godke, R. A. (2009). Sex ratio of bovine embryos and calves originating from the left and right ovaries. Biology of Reproduction, 81, 933–938.

Iwata, H. (2012). Factors affecting the primary sex ratio in cows. Journal of Mammalian Ova Research, 29, 96–102.

Iwata, H., Kimura, K., Hashimoto, S., Ohta, M., Tominaga, K., & Minami, N. (2002). Role of G6PD activity on sex ratio and developmental competence of bovine embryos under oxidative stress. Journal of Reproduction and Development, 48, 447–453.

Jang, J., Wang, Y., Kim, H.-S., Lalli, M. A., & Kosik, K. S. (2014). Nrf2, a regulator of the proteasome, controls self-renewal and pluripotency in human embryonic stem cells. Stem Cells, 32, 2616–2625.

Jolly, R. D., Thompson, K. G., & Winchester, B. G. (1975). Bovine mannosidosis—a model lysosomal storage disease. Birth Defects Original Article Series, 11, 273–278.

Kadenbach, B., Arnold, S., Lee, I., & Hüttemann, M. (2004). The possible role of cytochrome c oxidase in stress-induced apoptosis and degenerative diseases. Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1655, 400–408.

Kamisako, T., Tanaka, Y., Kishino, Y., Ikeda, T., Yamamoto, K., Masuda, S., & Ogawa, H. (2014). Role of Nrf2 in the alteration of cholesterol and bile acid metabolism-related gene expression by dietary cholesterol in high fat-fed mice. Journal of Clinical Biochemistry and Nutrition, 54, 90–94.

Kelley, R. L., & Gardner, D. K. (2016). Combined effects of individual culture and atmospheric oxygen on preimplantation mouse embryos in vitro. Reproductive BioMedicine Online, 33, 537–549.

Kowal, J., Tkach, M., & Théry, C. (2014). Biogenesis and secretion of exosomes. Current Opinion in Cell Biology, 29, 116–125.

Lawson, C., Vicencio, J. M., Yellon, D. M., & Davidson, S. M. (2016). Microvesicles and exosomes: New players in metabolic and cardiovascular disease. Journal of Endocrinology, 228, R57–R71.

Leite, R. F., Annes, K., Ispada, J., de Lima, C. B., dos Santos, É. C., Fontes, P. K., ... Milazzotto, M. P. (2017). Oxidative stress alters the profile of transcription factors related to early development on in vitro produced embryos. Oxidative Medicine and Cellular Longevity, 2017, 1–14.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods, 25, 402–408.

Martindale, J. L., & Holbrook, N. J. (2002). Cellular response to oxidative stress: Signaling for suicide and survival. Journal of Cellular Physiology, 192, 1–15.

Miao, X., Wu, X., & Shi, W. (2017). MicroRNA-346 regulates neural stem cell proliferation and differentiation by targeting KLF4. American Journal of Translational Research, 9, 5400–5410.

Mo, J.-S., Yoon, J.-H., Ann, E.-J., Ahn, J.-S., Baek, H.-J., Lee, H.-J., ... Park, H.-S. (2013). Notch1 modulates oxidative stress induced cell death through suppression of apoptosis signal-regulating kinase 1. Proceedings of the National Academy of Sciences of the United States of America, 110, 6865–6870.
Navakanitworakul, R., Hung, W.-T., Gunewardena, S., Davis, J. S., Chotigaeat, W., & Christenson, L. K. (2016). Characterization and small RNA content of extracellular vesicles of follicular fluid of developing bovine antral follicles. *Scientific Reports*, 6, 25486.

Oliveira, C. S., Saraiva, N. Z., Lima, M. R., Oliveira, L. Z., Serapião, R. V., Garcia, J. M., ... Camargo, L. S. A. (2016). Cell death is involved in sexual dimorphism during preimplantation development. *Mechanisms of Development*, 139, 42–50.

Parrish, J. J., Susko-Parrish, J. L., Leibfried-Rutledge, M. L., Critser, E. S., Eyestone, W. H., & First, N. L. (1986). Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology*, 25, 591–600.

Pavani, K., Hendrix, A., van den Broeck, W., Couch, L., Szymanska, K., Lin, X., ... Brackett, B. G., Korb, H., & Schlegel, W. (1990). In Shimano, H. (2001). Sterol regulatory element binding proteins (SREBPs): Transcriptional regulators of lipid synthetic genes. *Progress in Lipid Research*, 40, 439–452.

Pérez-Ceresales, S., Ramos-Ibeas, P., Rizos, D., Lonergan, P., Bermejo-Alvarez, P., & Gutiérrez-Adán, A. (2018). Early sex-dependent differences in response to environmental stress. *Reproduction*, 155, R39–R51.

Pérez-Crespo, M., Ramírez, M. A., Fernández-González, R., Rizos, D., Lonergan, P., Pintado, B., & Gutiérrez-Adán, A. (2005). Differential sensitivity of male and female mouse embryos to oxidative induced heat-stress is mediated by glucose-6-phosphate dehydrogenase gene expression. *Molecular Reproduction and Development*, 72, 502–510.

Rattanakus, S., Parnpai, R., & Ketudat, F. (2017). Multiplex polymerase chain reaction used for bovine embryo sex determination. *Journal of Reproduction and Development*, 57, 539–542.

Rivero, M., Montagnani, V., & Stecca, B. (2017). KLF4 is regulated by RAS/RAF/MEK/ERK signaling through EZF2 and promotes melanoma cell growth. *Oncogene*, 36, 3322–3333.

Rosenfeld, C. S., & Roberts, R. M. (2004). Maternal diet and other factors affecting offspring sex ratio: A review. *Biology of Reproduction*, 71, 1063–1070.

Saadeldin, I. M., Oh, H. J., & Lee, B. C. (2015). Embryonic-maternal cross-talk via exosomes: Potential implications. *Stem Cells and Cloning: Advances and Applications*, 8, 103–107.

Saeed-Zidane, M., Linden, L., Salliew-Wondim, D., Held, E., Neuhoff, C., Tholen, E., ... Tesfaye, D. (2017). Cellular and exosome mediated molecular defense mechanism in bovine granulosa cells exposed to oxidative stress. *PLOS One*, 12, e0187569.

Schellander, K., Fuhrer, F., Brackett, B. G., Korb, H., & Schlegel, W. (1990). In vitro fertilization and cleavage of bovine oocytes matured in medium supplemented with estrous cow serum. *Theriogenology*, 33, 477–485.

Shimano, H. (2001). Sterol regulatory element-binding proteins (SREBPs): Transcriptional regulators of lipid synthetic genes. *Progress in Lipid Research*, 40, 439–452.

Ståhl, A., Johansson, K., Mossberg, M., Kahn, R., & Karpman, D. (2019). Exosomes and microvesicles in normal physiology, pathophysiology, and renal diseases. *Pediatric Nephrology*, 34, 11–30.

Sun, D., Zhuang, X., Xiang, X., Liu, Y., Zhang, S., Liu, C., ... Zhang, H.-G. (2010). A novel nanoparticle drug delivery system: The anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Molecular Therapy*, 18, 1606–1614.

Szekeres-Bartho, J., Šužurović, S., & Mulac-Jerčević, B. (2018). The role of extracellular vesicles and PIBF in embryo-maternal immune-interactions. *Frontiers in Immunology*, 9, 2890.

Takahashi, A., Okada, R., Nagao, K., Kawamata, Y., Hanyu, A., Yoshimoto, S., ... Hara, E. (2017). Exosomes maintain cellular homeostasis by excreting harmful DNA from cells. *Nature Communications*, 8, 15287.

Tiffin, G. J., Rieger, D., Betteridge, K. J., Yadav, B. R., & King, W. A. (1991). Glucose and glutamine metabolism in pre-attachment cattle embryos in relation to sex and stage of development. *Reproduction*, 93, 125–132.

de la Torre Gomez, C., Goreham, R. V., Bech Serra, J. J., Nann, T., & Kuusmann, M. (2018). Exosomes*—A review of Biophysics, Biology and Biochemistry of exosomes with a focus on human breast milk. *Frontiers in Genetics*, 9, 92.

Wale, P. L., & Gardner, D. K. (2016). The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Human Reproduction Update*, 22, 2–22.

Xu, K. P., Yadav, B. R., King, W. A., & Betteridge, K. J. (1992). Sex-related differences in developmental rates of bovine embryos produced and cultured in vitro. *Molecular Reproduction and Development*, 31, 249–252.

Yoon, S. B., Choi, S. A., Sim, B. W., Kim, J. S., Mun, S. E., Jeong, P. S., ... Chang, K. T. (2014). Developmental competence of bovine embryos depends on the coupled response between oxidative and endoplasmic reticulum stress. *Biology of Reproduction*, 90, 104.

Zhang, D. D. (2006). Mechanistic studies of the Nrf2-Keap1 signaling pathway. *Drug Metabolism Reviews*, 38, 769–789.

Zhao, Q., Mao, A., Yan, J., Sun, C., Di, C., Zhou, X., ... Hang, H. (2016). Downregulation of Nrf2 promotes radiation-induced apoptosis through Nrf2 mediated Notch signaling in non-small cell lung cancer cells. *International Journal of Oncology*, 48, 765–773.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.