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Effects of Feeding OmniGen-AF® during Superovulation on *in Vitro* Development of Embryos Recovered from Donor Beef Cows

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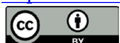

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Abstract

Embryo quality is crucial when selecting embryos for transfer. Variation in quality may be attributed to poor oocytes, semen, stress, inflammation, and potential immune system dysregulation. OmniGen-AF® (OG) feeding supports immune system function and animal health. Our laboratory recently reported lower percent degenerate embryos recovered and increased plasma progesterone in beef cattle donors fed OG during superovulation. *In vitro* development of embryos recovered from donor cows fed OG prior to collection is presented here. Embryos were recovered from 24 beef cows assigned to four treatment groups: 0 g OG/hd/d and 200 mg Folltropin®-V (FSH) (0/200); 0 g OG/hd/d and 400 mg FSH (0/400), 56 g OG/hd/d, 200 mg FSH (56/200) and 56 g OG/hd/d and 400 mg FSH (56/400). Good to excellent quality early blastocysts were cultured for 8 d. and development through hatching, embryonic volume and plasminogen activator (PA) production were quantified. The complete protocol was repeated 90 - 120 d later as Replicate 2. Optimal development was observed by embryos recovered from 0/200 cows where percent blastocysts hatching was greater ($P < 0.05$) compared to 56/200 and 0/400 cows and embryonic volume was greatest ($P < 0.05$) in Replicate 1. However, percent blastocysts hatching from 0/200 cows was similar ($P > 0.10$) to 56/400 cows and embryos recovered from 56/400 cows in Replicate 1 produced more ($P < 0.05$) PA compared to all other groups. For cows superovulated with the standard 400-mg FSH dose, feeding OG supported *in vitro* embryo development similar to that observed for 0/200 cows.

Keywords

Bovine, OmniGen, Follicle Stimulating Hormone, Embryo Development

1. Introduction

Assisted reproductive technologies are commonly used to improve overall herd genetics and female reproductive performance [1]. Embryo collection and transfer is a common assisted reproductive technology used in beef and dairy cattle. The key to the success of embryo transfer is recovering a high number of good to excellent quality embryos from donor cows for transferring to suitable recipients to achieve optimal pregnancy rates [2]. Numerous studies have been conducted employing nutritional strategies in feeding protocols during donor superovulation in attempts to increase both the number and quality of embryos recovered [3]. As an example, Yaakub *et al.* [4] fed dairy heifers increased concentrates during a superovulation protocol and recovered more embryos of poor quality compared to restricted-fed counterparts. Indeed, diet has been shown to impact gene expression in cattle embryos, specifically genes associated with blastocoel formation and oxidative stress [5] [6]. OmniGen-AF® (OG; Phibro Animal Health Corporation, Teaneck, NJ) is a nutritional supplement shown to benefit overall animal health. OG is a specialty blend of ingredients that supports animal health during periods of stress, such as heat stress, by mitigating the negative physiologic effects associated with stress [7] [8] [9]. Feeding OG has also been shown to support immune system regulation during exposure to stressors [10] [11]. Dairy producers have reported a benefit in reproductive performance with the addition of OG into their herds [12] and incorporating OG into the ration of dairy cows has shown to reduce the number of days open [13]. Recently, our laboratory reported feeding OG to beef cow donors provided the beneficial effects of more transferrable embryos, fewer percent degenerate embryos and increased plasma progesterone on the day of embryo recovery [14]. The effects of feeding OG on donor embryo survivability have not been reported. Hormone dose is also an important factor to examine, as exogenous follicle stimulating hormone (FSH) can rescue follicles beginning to undergo atresia [15]. Reducing the FSH dose can lower the total number of embryos recovered but perhaps increase the number of transferrable embryos more likely to establish a pregnancy [16]. Good to excellent quality embryos may also produce more plasminogen activator (PA) during the hatching process [17]. PA is produced by blastocyst trophoblast and is proposed to participate in the breakdown of the zona pellucida via a zonalytic mechanism with uterine plasminogen [18]. Previous reports have demonstrated blastocysts completing the hatching process release significantly more PA than blastocysts failing to escape the zona pellucida [17]. Therefore, the objective of this research was to evaluate *in vitro* development and PA production as measures of viability in embryos recovered from donor beef cows fed OG and superovulated with 200 or 400 mg FSH.

2. Material and Methods

2.1. Animal Care and Use

All animals were humanely treated and cared for in compliance with an approved

protocol in accordance with Oregon State University IACUC Guidelines.

2.2. Animal Housing and Feeding

Detailed descriptions of the animal housing and feeding and estrous synchronization, superovulation, artificial insemination and embryo collection protocols used in the study have been reported in Snider *et al.* [14] hence a brief description is presented here. Twenty-four cross-bred Angus cows were housed in a free stall barn at the Oregon State University Beef Center in Corvallis, OR with access to *ad libitum* grass hay and water. Cows were blocked for age, and randomly assorted into Control or OG-supplemented groups. All cows received a mixture of 0.2 kg ground corn and 6 oz molasses mix once a day. For cows fed OG, the supplement was hand-added to the corn/molasses mixture. All OG treatments were fed for 49 d with the superovulation protocol initiated on d 28 of the feeding period.

2.3. Estrous Synchronization, Superovulation and Artificial Insemination

Cows selected for Control or OG-feeding were randomly assigned to receive either 200 or 400 mg FSH (Folltropin®, Bioniche, Athens, GA) in the superovulation protocol. Treatment groups were fed and injected with: 0 g OG/hd/d and 200 mg Folltropin®-V (FSH) (0/200); 0 g OG/hd/d and 400 mg FSH (0/400), 56 g OG/hd/d and 200 mg FSH (56/200) and 56 g OG/hd/d and 400 mg FSH (56/400), respectively. Estrous synchronization was started on day 28 of OG feeding with 25-mg prostaglandin $F_{2\alpha}$ (PGF; Lutalyse®, Zoetis, Florham Park, NJ) injected i.m. followed by a second 25-mg PGF i.m. injection 12 days later. Ten days after the first PGF injection, cows received a 4-day regimen of FSH within their respective groups. FSH was administered at twice daily doses of 25 or 50 mg per injection for cows receiving 200 or 400 mg FSH, respectively. Cows were observed for estrus at 12-h intervals starting 24 h after the last PGF injection. Cows displaying estrus were artificially inseminated with 0.5 ml of frozen-thawed semen at 0, 12, and 24 h post-estrus. Cows not displaying estrus by 36 h after the last PGF injection were artificially inseminated at 48, 60 and 72 h following this injection. All cows received 100 μg GnRH (Factrel®, Zoetis, Florham Park, NJ) at the time of the first artificial insemination. All cows were entered in a second replicate 90 - 120 d after the first embryo collection. Cows remained in their original treatment groups and all procedures conducted were identical with the first replicate.

2.4. Embryo Collection and Grading

Embryos were non-surgically collected 7 d after estrus onset. The flush medium consisted of Dulbecco's phosphate buffered saline containing 10% antibiotic/antimycotic solution (Sigma-Aldrich, St. Louis, MO) and 0.2% heat-treated bovine fetal calf serum (Sigma-Aldrich, St. Louis, MO). Recovered embryos were

scored for developmental stage and quality using the four-rank grading system described by Lindner and Wright [19].

2.5. Embryo Culture and *in Vitro* Development

Early blastocysts of good to excellent quality were cultured singly in 15-µl microdrops of Ham's F-12 (Sigma-Aldrich, St. Louis, MO) containing 1.5% BSA (Sigma-Aldrich, St. Louis, MO) under paraffin oil on 15 × 60 mm plastic tissue culture dishes in a humidified atmosphere of 5% CO_2 in air at 39˚C for 8 d. Embryos were transferred to a fresh microdrop every 24 h and 10 µl of culture medium were collected and stored at −20˚C for PA analysis. Prior to transfer, embryos were examined for stages of development: early blastocyst, blastocyst, expanded blastocyst (a visibly thinned zona pellucida is observed), initiating hatching blastocyst (when rupture of the zona pellucida is first observed) and hatched blastocyst (when the blastocyst is completely free of the zona pellucida).

2.6. Embryo Volume

After 192 h in culture, embryos were measured for volume using an ocular micrometer. Measurements were recorded either in diameter or length x width depending on the size and shape of the embryo and all measurements were recorded in micrometers. The volume of a prolate ellipsoid was used to determine embryo volume if the shape of the embryo deviated from that of a sphere (**Figure 1**). The final value in the equation is given in cubic microns and was converted to nanoliters (1 nl = 1 × 10^6 cubic microns).

2.7. Plasminogen Activator Assay

PA concentrations in embryo-conditioned medium were determined using the caseinolytic agar gel assay described by Kaaekuahiwi and Menino [17]. To determine PA activity, 10 µl of conditioned medium or human urokinase standard (Sigma-Aldrich, St. Louis, MO) were combined with 15 µl of 120 ng/µl human plasminogen (Sigma-Aldrich, St. Louis, MO) and incubated for 15 min at 39˚C. Twenty microliters of the mixture were pipetted into wells cut into the caseinolytic agar gel plate and incubated for 24 h at room temperature to determine plasmin activity. The reaction was stopped by incubating the plates in 3% acetic acid for 15 min. Zones of caseinolysis were measured using an electronic digital caliper and PA concentrations were determined from standard curves of caseinolytic

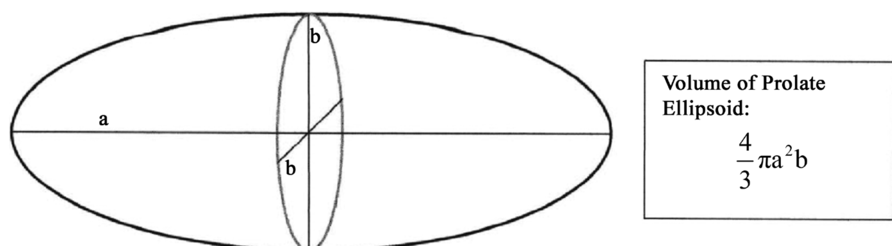


Figure 1. Volume of prolate ellipsoid.

diameter by log urokinase concentration.

2.8. Statistical Analyses

Chi-square analysis was used to determine differences in percent embryos developing to the expanded, hatching and hatched blastocyst stages as a result of FSH dose, OG feeding and Replicate [20]. Analyses of variance (ANOVA) for 2 × 2 × 2 factorial designs were used to identify differences due to treatments in times required for embryos to develop to a specific cell stage and embryo volumes. Sources of variation in the ANOVA were FSH (200 or 400 mg), OG (0 or 56 g/hd/day), Replicate (1 or 2) and the interactions. Repeated measures ANOVA for a 2 × 2 × 2 factorial design were used to evaluate differences in PA concentrations during culture. Sources of variation in the ANOVA were FSH, OG, Replicate, time in culture (Time) and the interactions. In analyses where Replicate was not a significant effect data were pooled. If significant effects were observed in the ANOVA differences between means were evaluated using the Fisher's least significant differences procedures. All analyses were performed using the NCSS statistical software program (Number Cruncher Statistical System; 2007, Jerry Hintze, Kaysville, UT).

3. Results

Fifty-three and 44 good to excellent quality early blastocysts recovered from cows in Replicates 1 and 2, respectively, were selected for culture (**Table 1**). Three to five cows within each treatment group and replicate contributed to the pool of embryos.

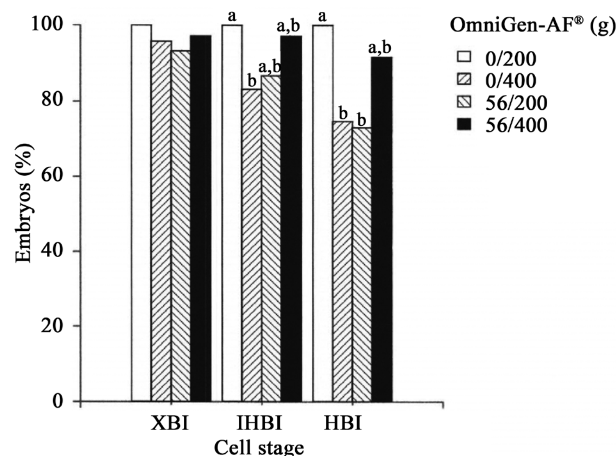
Replicate did not affect ($P > 0.10$) percent embryos developing to the expanded (Replicate 1, 98% compared with Replicate 2, 96%), hatching (Replicate 1, 92% compared with Replicate 2, 93%) and hatched blastocyst (Replicate 1, 87% compared with Replicate 2, 86%) stages. Development to the expanded blastocyst stage was similar ($P > 0.10$) for embryos recovered from cows superovulated with either dose of FSH (200 mg, 97% compared with 400 mg, 97%) and fed either 0 or 56 g OG (0 g, 98% compared with 56 g, 98%) (**Figure 2**). Percent embryos initiating hatching was similar ($P > 0.10$) for embryos recovered from cows superovulated with either dose of FSH (200 mg, 95% compared with 400 mg, 92%) and fed either 0 or 56 g OG (0 g, 91% compared with 56 g,

Table 1. Numbers of embryos selected for culture recovered from cows fed 0 or 56 g OmniGen-AF® (0, 56) and superovulated with 200 or 400 mg FSH (200, 400).

Treatment	Replicate 1	Replicate 2	Total
0/200	12	10	22
0/400	13	11	24
56/200	9	6	15
56/400	19	17	36
Totals	53	44	97

94%). However, percent embryos initiating hatching was greater ($P < 0.05$) from 0/200 compared to 0/400 cows but similar ($P > 0.10$) to either group fed 56 g OG (Figure 2). Percent embryos completing the hatching process was similar ($P > 0.10$) for embryos recovered from cows superovulated with either dose of FSH (200 mg, 89% compared with 400 mg, 85%) and fed either 0 or 56 g OG (0 g, 87% compared with 56 g, 86%). Percent embryos completing hatching was greater ($P < 0.05$) from 0/200 compared to 0/400 and 56/200 cows (Figure 2). Similar to initiation of hatching, percent embryos completing hatching from 0/200 cows did not differ ($P > 0.10$) from 56/400 cows (Figure 2).

Times required for embryos to develop to the expanded blastocyst stage were not affected ($P > 0.10$) by FSH (200 mg, 33 ± 4 h vs. 400 mg, 35 ± 3 h), OG (0 g, 34 ± 3 h vs. 56 g, 34 ± 3 h) or Replicate (1, 34 ± 4 h vs. 2, 35 ± 3 h). However, the FSH X OG interaction was significant where onset of expansion was slower ($P < 0.05$) in embryos recovered from 0/400 compared to 0/200 cows (Table 2). Rates of development to the expanded blastocyst stage were similar ($P > 0.10$) among embryos recovered from 0/200 cows and cows fed 56 g OG.



[a,b]Percents within a cell stage without similar superscripts differ ($P < 0.05$).

Figure 2. Percent embryos developing to the expanded (XBl), initiating hatching (IHBl) and hatched blastocyst (HBl) stages *in vitro* recovered from cows superovulated with 200 or 400 mg FSH and fed 0 or 56 g OmniGen-AF®.

Table 2. Onset of expansion (XBl) and initiation of hatching (IHBl) and completion of hatching (HBl) *in vitro* for embryos recovered from cows fed 0 or 56 g OmniGen-AF® (0, 56) and superovulated with 200 or 400 mg FSH (200, 400).

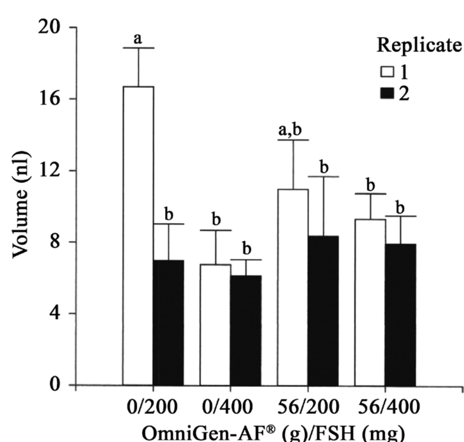
Treatment	XBl		IHBl		HBl	
	n	Time (h)[a]	n	Time (h)	n	Time (h)
0/200	22	28 ± 4[b]	22	63 ± 5	22	82 ± 5
0/400	23	41 ± 4[c]	20	60 ± 6	18	79 ± 5
56/200	14	36 ± 5[b,c]	13	61 ± 7	11	72 ± 7
56/400	35	30 ± 3[b,c]	35	62 ± 4	33	79 ± 4

[a]Values reported are means ± SE. [b,c]Means without similar superscripts differ ($P < 0.05$).

Initiation of the hatching process was not affected ($P > 0.10$) by FSH (200 mg, 64 ± 6 h vs. 400 mg, 60 ± 5 h), OG (0 g, 62 ± 6 h vs. 56 g, 63 ± 5 h) or Replicate (1, 59 ± 3 h vs. 2, 66 ± 3 h). Embryos recovered from all treatments initiated hatching at similar ($P > 0.10$) times in culture (Table 2). Times required for embryos to complete hatching were also not affected ($P > 0.10$) by FSH (200 mg, 77 ± 3 h vs. 400 mg, 78 ± 3 h), OG (0 g, 80 ± 3 h vs. 56 g, 75 ± 3 h) or Replicate (1, 82 ± 3 h vs. 2, 74 ± 4 h). Completion of hatching occurred at similar ($P > 0.10$) times in culture for embryos recovered from cows across treatments (Table 2).

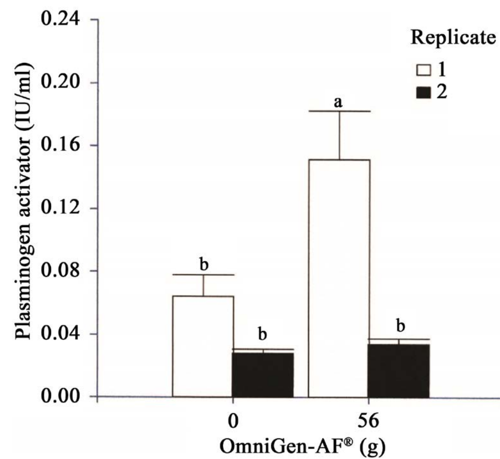
Embryo volumes at the end of culture were not affected ($P > 0.10$) by FSH (200 mg, 10.8 ± 1.6 nl vs. 400 mg, 7.5 ± 1.3 nl) or OG (0 g, 9.1 ± 1.4 nl vs. 56 g, 9.2 ± 1.4 nl). However, embryos recovered from cows in Replicate 1 attained larger ($P > 0.05$) volumes compared to embryos recovered in Replicate 2 (10.9 ± 0.8 nl vs. 7.4 ± 0.9 nl, respectively). There was a significant FSH X Replicate interaction where volumes were greater ($P < 0.05$) for embryos harvested from cows superovulated with 200 compared to 400 mg FSH in Replicate 1 and both doses in Replicate 2 (Figure 3).

Plasminogen activator production by cultured embryos was not affected ($P > 0.10$) by FSH (200 mg, 0.055 ± 0.024 vs. 400 mg, 0.074 ± 0.019 IU/ml) or OG (0 g, 0.046 ± 0.022 vs. 56 g, 0.082 ± 0.021 IU/ml). Plasminogen activator production by embryos recovered in Replicate 1 was greater ($P < 0.05$) compared to Replicate 2 (0.099 ± 0.012 vs. 0.029 ± 0.014 IU/ml, respectively). The OG X Replicate interaction was significant where PA production was greatest (P < 0.05) from embryos recovered from cows fed 56 g OG in Replicate 1 (Figure 4). The FSH X OG X Replicate interaction was also found to be significant where PA production was greatest ($P < 0.05$) from embryos recovered from 56/400 cows in Replicate 1 compared to all other treatments in Replicates 1 and 2 (Figure 5). Plasminogen activator production differed ($P < 0.05$) by Time in culture and a significant Replicate X Time interaction was observed (Figure 6). Peak PA production



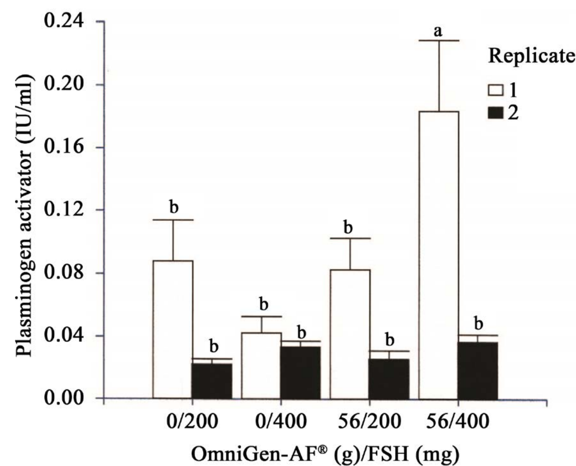
[a,b]Means without similar superscripts differ ($P < 0.05$).

Figure 3. Embryonic volumes (means ± SE) determined at the last day of culture for embryos recovered from cows superovulated with 200 or 400 mg FSH and fed 0 or 56 g OmniGen-AF® in Replicates 1 and 2.



a,bMeans without similar superscripts differ ($P < 0.05$).

Figure 4. Plasminogen activator production (means ± SE) *in vitro* by embryos recovered from cows fed 0 or 56 g OmniGen-AF® in Replicates 1 and 2.



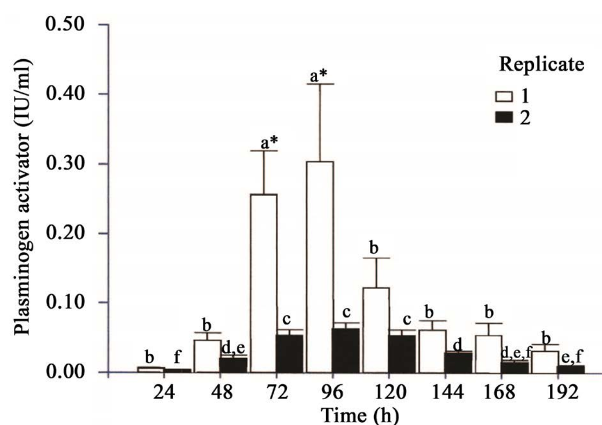
a,bMeans without similar superscripts differ ($P < 0.05$).

Figure 5. Plasminogen activator production (means ± SE) *in vitro* by embryos recovered from cows superovulated with 200 or 400 mg FSH and fed 0 or 56 g OmniGen-AF® in Replicates 1 and 2.

($P < 0.05$) was measured during 72 - 120 h of culture and production was greater ($P < 0.05$) by embryos recovered in Replicate 1 at 48 - 192 h compared to Replicate 2 (**Figure 6**).

4. Discussion

Embryo quality can be affected by many variables during the superovulation process [1]. Transferring an embryo of optimal quality is critical to ensure successful hatching, attachment and pregnancy establishment in recipient cows [2] Certain factors involved with superovulation, such as FSH dose, handling of donor cows and overall animal health can contribute to a stress response affecting embryo quality [21] [22]. In a recent report from our laboratory, feeding donor beef cows OG during superovulation provided more transferrable embryos and



a,bMeans without similar superscripts differ ($P < 0.05$) within Replicate 1. c,d,e,fMeans without similar superscripts differ ($P < 0.05$) within Replicate 2. *Different ($P < 0.05$) from corresponding mean in Replicate 2.

Figure 6. Plasminogen activator production (means ± SE) over time in culture by embryos recovered from cows superovulated with 200 or 400 mg FSH and fed 0 or 56 g OmniGen-AF® in Replicates 1 and 2.

decreased percent degenerate embryos recovered [14]. Results presented in this report demonstrate how feeding OG, a health-supporting supplement, in conjunction with a superovulation protocol can influence embryo development *in vitro*. Embryos collected from 0/200 cows exhibited greater *in vitro* development with respect to percent blastocysts initiating and completing hatching, earlier onset to blastocoelic expansion and embryonic volume attained compared to most other treatment combinations. The poorer development by embryos recovered from 0/400 cows may be due to a greater proportion of suboptimal oocytes being rescued with this higher dosage [23]. However, when cows were superovulated with 400 mg FSH and fed OG, development approached, if not exceeded with respect to PA production, which observed for embryos from 0/200 cows. Indeed, the earlier onset of blastocoelic expansion, albeit not perpetuated through initiation and completion of hatching with regards to timing, may have supported the greater hatching rates observed in embryos from 0/200 and 56/400 cows. Somewhat unexpected, embryos from cows superovulated with 200 mg FSH and fed 56 g OG did not complete hatching as well as their 0 g OG-fed counterparts in spite of similar onset to blastocoelic expansion, embryonic volume and PA production. The 56/200 cows provided the least number of embryos in both replicates hence failure of a fewer embryos to complete hatching would have a greater impact on the percentage. Timing of development to a specific embryonic stage is important because embryos developing asynchronously, as in delayed hatching from the zona pellucida, may result in the blastocyst missing the critical window for signaling pregnancy [24]. From these results there appears to be a benefit for embryos recovered from donor cows superovulated with 400 mg FSH and receiving OG feeding. Perhaps modulation of the immune system or inflammation by feeding OG is indirectly affecting follicular dynamics or uterine physiology [9] [25].

Embryo volume was measured at the end of the culture period, equivalent to 15 d of development. Embryos recovered from 0/200 cows in Replicate 1 achieved the greatest volumes compared to all other treatment groups, including those in Replicate 2, with the exception of embryos recovered from cows superovulated with the same dose but fed 56 g OG in Replicate 1. Embryo volume, because of its connection with cell number, can relate to a healthier embryo with improved opportunity for development.

Plasminogen activator production was quantified to provide a measure of embryo vitality. Increased PA production has been linked with successful embryo hatching *in vitro* and potentially healthier embryos [17] [18]. Interestingly, embryonic PA production was greater by embryos recovered from cows in Replicate 1 compared to Replicate 2 and greatest by embryos recovered from 56/400 cows in Replicate 1. Peak PA production was also observed at 72 and 96 h and 72 to 120 h *in vitro* for embryos cultured in Replicates 1 and 2, respectively, and is similar to previous reports [17] [18]. The peak in PA production corresponds to the period when embryos are actively engaged in the hatching process. The replicate difference observed in overall PA production could be related to different cows, albeit within the same treatment, contributing to the two pools of embryos used for culture. Cow source can be a source of variation for embryonic PA production *in vitro* [17]. It is also possible the pool of embryos generated in the first cycle of superovulation were more robust than the second. This is somewhat evidenced by the greater mean volume observed in Replicate 1 compared to 2 and may be due to the quality of the selected oocyte pool. There is also an apparent incongruity with embryonic volume and PA production as embryos with the greatest volume did not have the greatest PA production. Kaaekuahiwi and Menino [17] reported significant correlation coefficients for bovine embryo diameter and cell number with PA production of 0.40 and 0.35, respectively, hence some deviation from this relationship would not be unexpected. In this same study, the correlation coefficient for bovine embryo diameter with cell number was 0.79 [17]. Clearly, feeding OG to cows superovulated with 400 mg had an impact on overall development, including PA production.

Overall, the results suggest a positive effect of feeding OG to cows superovulated with 400 mg FSH on early embryonic development when evaluated *in vitro*. Whether this benefit extends to a greater likelihood of pregnancy establishment in recipients requires additional research as well as determining how OG induces its biological effect. Feeding cows OG has been linked to lowering the stress response and supporting immune function [8] [11] [26]. Perhaps through these mechanisms, OG is exerting positive effects on follicular maturation and embryo development during superovulation protocols in donor cows.

5. Conclusion

Development *in vitro* was greatest by embryos recovered from cows fed 0 g OG and treated with 200 mg FSH suggesting the most favorable treatment regimen.

However, when cows were fed OG during treatment with 400 mg FSH, embryo development approached the response of 200 mg FSH. As the higher FSH dose may yield more embryos, these observations suggest feeding OG may ameliorate negative effects on embryo development associated with higher FSH doses. The negative impact of high-dose FSH inducing super-physiological folliculogenesis and ovulation may include ovarian damage due to inflammation and tissue scarring, down-regulation of hormone feedback due to high hormone concentrations and shifts in uterine secretions resulting in an intrauterine environment that is unsupportive for embryo survival or growth. Feeding OG may provide immune and physiological support during this process for ovarian repair and uterine environment modulation to support embryo survival and growth. Further research is needed to elucidate the physiologic mechanisms and target organs defining how feeding OG provides a beneficial effect on follicular maturation and embryo development. Overall, the results support a potential benefit for embryo viability when donor cows are fed OG during a superovulation protocol using the standard dose of 400 mg FSH.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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