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Distribution Pattern of E-Cadherin in Parthenogenetic Sheep Embryos (*Ovis aries*) and Its Relationship with Fragmentation

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Abstract

The objective of this study was to determine the distribution pattern of E-cadherin in parthenogenetic sheep embryos (*Ovis aries*) and its relationship with fragmentation. Mature oocytes were activated with calcium ionophore (8 μg·mL^{-1} in TCM-199 with Hepes and 2% NCS) for 5 min, washed in TCM-199 with 20% NCS for 3 min, incubated in BO-IVC medium with 6-DMAP (2 mM) for 4 h, washed and cultured in 100 μL of the same medium at 38˚C and 5% CO_2. At 36 h, the development rate was assessed and stained for E-cadherin. Parthenogenetic embryos were fixed (methanol-PBS 1:1 and 2:1, 2 min each) and permeabilized in 1% Triton X100 for 5 min. Blocked in PBS with 1% albumin-fraction V for 1 h. Embryos were incubated in anti-E-cadherin primary antibody (1:50) for 24 h and in anti-IgG-FITC secondary antibody (1:50) for 24 h, determining the fluorescence intensity in the cytoplasmic region and at cell junctions in a confocal microscope. Results showed that cytoplasmic E-cadherin was highest in fragmented embryos (79.73 ± 5.3) and 4-cell embryos (57.6 ± 5.1), and lowest in 6-cell embryos (27.8 ± 3.0). At the cell junctions, progressively increased from 4-cell (45.8 ± 5.1) to 8-cell embryos (58.7 ± 6.2). It is concluded that E-cadherin exhibits a developmental stage-dependent localization with cytoplasmic predominance in early stages and a progressive accumulation at cell junctions, which coincides with that reported in embryos obtained by *in vitro* fertilization, while in fragmented embryos the

pattern suggests alterations in the normal redistribution of E-cadherin, for example, aberrant E-cadherin localization may serve as molecular marker for poor embryo quality or impending developmental failure.

Keywords

E-Cadherin, Embryos, Parthenogenetic, Sheep

1. Introduction

Parthenogenesis is defined as the generation of an individual without the participation of the paternal genome; this occurs naturally in some species. The use of parthenogenetic embryos has shed light on some molecular mechanisms of embryonic development [1].

A parthenogenetic embryo is obtained when the oocyte is artificially activated [2]. Oocyte activation can be performed using compounds such as ethanol, calcium ionophore, Tyrode's solution, and puromycin. Electric activation is also available [3]. The production of parthenogenetic embryos is used to study the mechanisms that regulate embryo development [4] or as a control group in *in vitro* mammalian embryo culture systems, due to their close resemblance to mammals and because they allow for obtaining precise information on the behavior of *in vitro* embryo cultures [5].

E-cadherin is a transmembrane glycoprotein that regulates epithelial cell adhesion and is calcium (Ca^{2+}) dependent [6]. It is produced in both oocytes and embryos and has an important role in the regulation of morphogenesis through the formation of adherent junctions, the termination of cell shape and polarity, and cell signaling during the embryo compaction process. It is also expressed in germ cells and on the surface of blastomeres during the early stages of embryonic development [7] [8].

In a 6- to 8-cell embryo, or even a 16-cell embryo, depending on the animal species, the blastomeres increase the cell-cell contact surface area due to the formation of adherent junctions. This process marks the beginning of the embryonic compaction [9].

Cell contacts are due to E-cadherin, which is progressively distributed from the cytoplasm to the cell membrane, producing increasingly tighter cell contacts and adherent junctions as the embryo compacts [10].

Embryo compaction is characterized by a mechanical flattening of the blastomeres that changes the organization of the membranes and the cytoplasm, causing cell polarization by cell contact and the appearance of tight junctions that divide the plasma membrane of the external blastomeres into apical and basolateral membrane domains, Ca^{2+}-dependent cell adhesion and cell-cell communication mediated by gap junctions [7] [9].

Loss or alteration of E-cadherin can alter the regulation of cell adhesion and a

decreased capacity for organization, which can lead to embryonic fragmentation [11]. Studies that have addressed this issue indicate that E-cadherin dysfunction can cause embryonic cell dissociation and the accumulation of cytoplasmic fragments, which is an indication that cellular interactions are not occurring properly [12].

Embryo cells with E-cadherin defects tend to experience an increased rate of apoptosis and necrosis, which also contributes to embryo fragmentation [13].

Fragmentation occurs in most embryos, and its origin is not fully established [14]. Some theories indicate that the fragments originate from cellular remains without a nucleus or that they are the result of the decomposition of one or more cells of the embryo itself during its divisions, which share space with the cells themselves [15]. Poor oocyte quality can result in embryos with a high degree of fragmentation [16]. Fragmentation can be classified into four degrees, depending on the percentage of space occupied by the fragment: Grade 1: less than 10% of the free space between cells; Grade 2: fragments occupy between 10 and 25%; Grade 3: fragments occupy between 25 and 30%; and Grade 4: fragments occupy more than 35%. Its presence at high levels (>15% - 20%) is associated with a lower rate of development to blastocyst and implantation [17].

Cytoplasmic fragmentation is the most common cause of embryo loss after *in vitro* fertilization (IVF), and is associated with the presence of several abnormalities, such as low cell counts, unequal blastomere size, impaired cell-cell contact, and multinucleated blastomeres [18].

This process can compromise embryo viability and reduce implantation and blastocyst development rates, as it impedes cell-to-cell contact for compaction and the initiation of blastulation [15].

The failure of cell-cell adhesion by E-cadherin promotes the extrusion and detachment of blastomeres, leading to embryonic fragmentation.

Therefore, the objective of this study was to determine the location and distribution pattern of E-cadherin in parthenogenetic sheep (*Ovis aries*) embryos and its relationship to fragmentation.

2. Materials and Methods

All reagents used were from Sigma Aldrich unless otherwise stated. The animal study protocol was approved by the Institutional Ethics Committee, "Ethics Commission of the Biological and Health Sciences Division of the Universidad Autónoma Metropolitana Iztapalapa (protocol code CECBS23-13 and date of approval: 4 April 2023)" for studies involving animals, and it is in agreement with the ARRIVE 2.0 (Animal Research: Reporting of *In Vivo* Experiments; E&E, Explanation and Elaboration) guidelines (PLOS Biology| https://doi.org/10.1371/journal.pbio.3000410).

2.1. *In Vitro* Maturation of *Ovis aries* Oocytes

Based on the methodology of Hernández-Martínez *et al.* [19] and Vazquez-Avendaño *et al.* [20] with some modifications, for *in vitro* maturation (IVM) of oocytes,

ovaries were collected weekly during spring to summer seasons at a local slaughterhouse from adult domestic sheep (*Ovis aries*) creole type coming from States surrounding Mexico City, and transported to the laboratory for 1 hour in 100 mL of physiological solution (0.9% NaCl and 1% antibiotic-antifungal, *In Vitro*, S.A., CDMX, México) at 30˚C - 35˚C. Cumulus-oocyte complexes (COC) were aspirated from ovarian follicles (2 - 5 mm in diameter) by puncture with an 18 - 20 G hypodermic needle and a 10 mL syringe containing 1 mL of aspiration medium based on TCM-199 with Hepes (*In Vitro*, S.A., CDMX, Mexico) supplemented with 100 IU/mL of heparin sodium salt. The COCs were recovered in a 35 mm Petri dish and selected according to their morphology and number of granulosa cell layers [21]. Subsequently, they were incubated for 22 h in a 4-well dish (Nunc, Thermofisher, Massachusetts, USA) containing 500 µL of *in vitro* maturation medium (IVM) based on Hepes-free TCM-199 (*In Vitro*, SA, CDMX, Mexico) supplemented with cysteine [0.57 mM], D-glucose [3.05 mM], polyvinyl alcohol [PVA] [0.1%], sodium pyruvate [0.91 mM], 10% newborn calf serum (NBCS) (Biowest, Nuaillé, France), 0.1 IU of FSH-LH (Pluset, Calier, Italy), gentamicin [50 µg/mL] and epidermal growth factor [EGF, 10 ng/mL], under mineral oil. Then they were incubated at 38˚C and 5% CO_2 in high humidity for 24 h.

After IVM, oocytes were denuded from cumulus cells in Eppendorf tubes containing 1.5 mL of TCM-199 with Hepes supplemented with hyaluronidase (0.5 mg/mL) and incubated for 8 min under the described conditions. Subsequently, gentle and constant pipetting was performed with a 200 µL automatic micropipette. The denuded oocytes were washed twice in 100 µL of TCM-199 with Hepes and 2% NBCS, transferred to 50 µL droplets of the same medium, and selected based on the presence of the first polar body, as a sign that they were mature, that is, in Metaphase II (MII) of the cell cycle [20].

2.2. Oocyte Activation to Produce Parthenogenetic Embryos

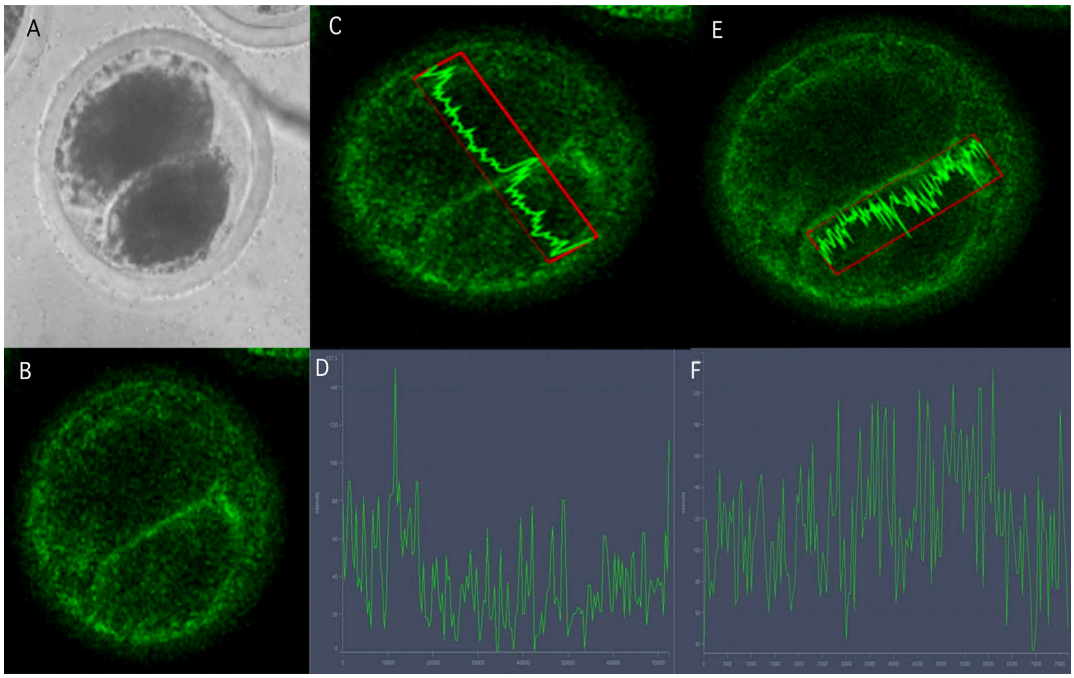
According to Vazquez-Avendaño *et al.* [21], with some modifications, for the development of parthenogenetic embryos, MII oocytes were activated with calcium ionophore A23187 (8 $\mu g \cdot mL^{-1}$) in TCM-199 with Hepes and 2% NBCS, for 5 min. Subsequently, they were washed 3 times in TCM-199 with Hepes and 20% NBCS, for 3 min, and incubated in BO-IVC medium (IVF-Biosciences) containing 6-DMAP (2 mM), for 4 h under the conditions described. The activated oocytes were washed 3 times and cultured for *in vitro* development (IVD) in 100 µL of the same medium covered with mineral oil, under the same conditions. At 36 h of culture, the embryos developmental stage was determined, and they were processed for E-cadherin immunostaining.

2.3. E-Cadherin Immunostaining

E-cadherin immunostaining was performed following the technique proposed by Barcroft *et al.* [22], for which parthenogenetic *O. aries* embryos were fixed using two concentrations of methanol-PBS solution, the first at a 1:1 ratio and the sec-

ond at 2:1 (v:v), each for 2 min. The embryos were then permeabilized in 1% Triton X100 for 5 min. Next, they were incubated in PBS with 1% albumin-fraction V for 1 h, then incubated with the primary antibody (1:50) anti-E-cadherin (cat. ab287970- 100 ug, anti-e cadherin antibody (36/e-cadherin, abcam) overnight. Finally, the embryos were incubated with the secondary antibody (1:50) anti-IgG labeled with FITC (Cat. ab150117- 500 ug, goat polyclonal secondary antibody to mouse IgG H&L, abcam) overnight.

To determine the distribution pattern of E-cadherin, embryos were evaluated using a confocal microscope (Zeiss LSM T-PMT, FITC filter). Images were analyzed using ZEN Life software (Zeiss), and fluorescence intensity was determined in fluorescence units (fu) in the cytoplasm and at cell junctions (**Figure 1**).



A) Bright field. B) Dark field. E-cadherin immunostaining, FITC filter. C) Evaluation of E-cadherin distribution in the cytoplasm, E) Evaluation of E-cadherin distribution in the adherent junctions. 40X magnification seen on a Zeiss LSM T-PMT confocal microscope. D and F) E-cadherin uf graphics.

Figure 1. Evaluation of E-cadherin in a 2-cell parthenogenetic embryo of domestic sheep (*Ovis aries*).

An analysis of variance was applied to compare the fluorescence intensity (given in fluorescence units—fu, evaluated by the ZEISS ZEN lite software) between the different stages of embryonic cleavage and between cytoplasmic E-cadherin and adherent junctions [23].

To ensure data consistency and reproducibility, the regions of interest (cytoplasmic region vs. adherent junctions) were defined and standardized as follows: In the first step, to determine cytoplasmic E-cadherin, the fluorescence intensity of the cytoplasm in blastomeres was measured transversely (**Figure 1(C)**). While in the second, the junctions between blastomeres were measured longitudinally to obtain the fluorescence intensity of the E-cadherin (**Figure 1(E)**).

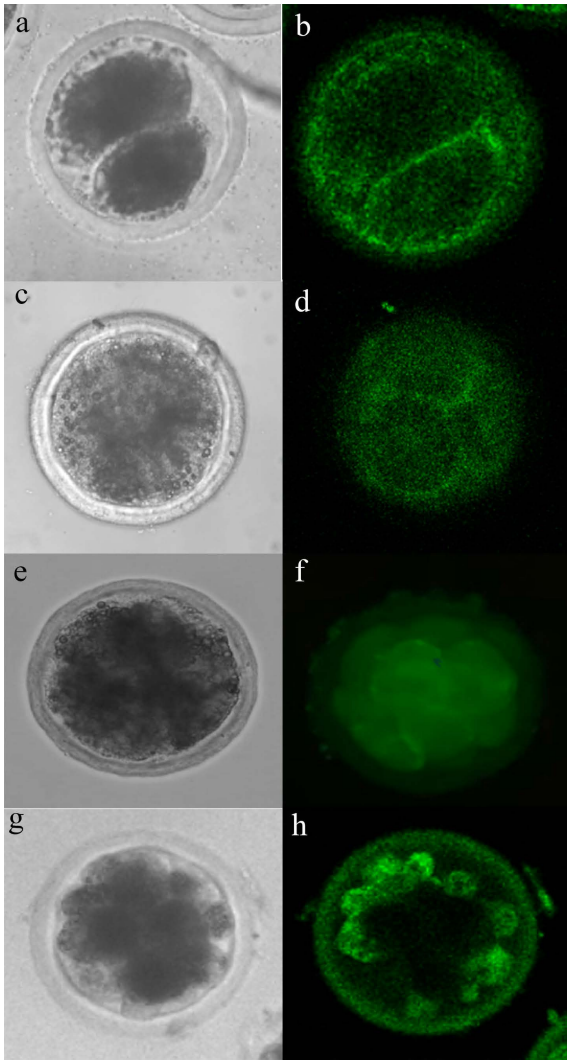
3. Results

A total of 40 *O. aries* parthenogenetic embryos that developed to different cleavage stages were evaluated: 2 to 4-cell (n = 9), 5 to 6-cell (n = 6), 7 to 8-cell (n = 10), and fragmented (n = 15) (**Table 1**, Figure 2).

Table 1. *In vitro* development rate of *O. aries* parthenogenetic embryos.

Oocyte number	IVM N (%)	Cleavage N (%)	2 - 4-cell N (%)	5 - 6-cell N (%)	7 - 8-cell N (%)	Fragmentated N (%)
100	54 (75)	40 (74)	9 (16.6)	6 (11.1)	10 (18.5)	15 (27.7)

The percentage of each column was obtained from the oocyte number.



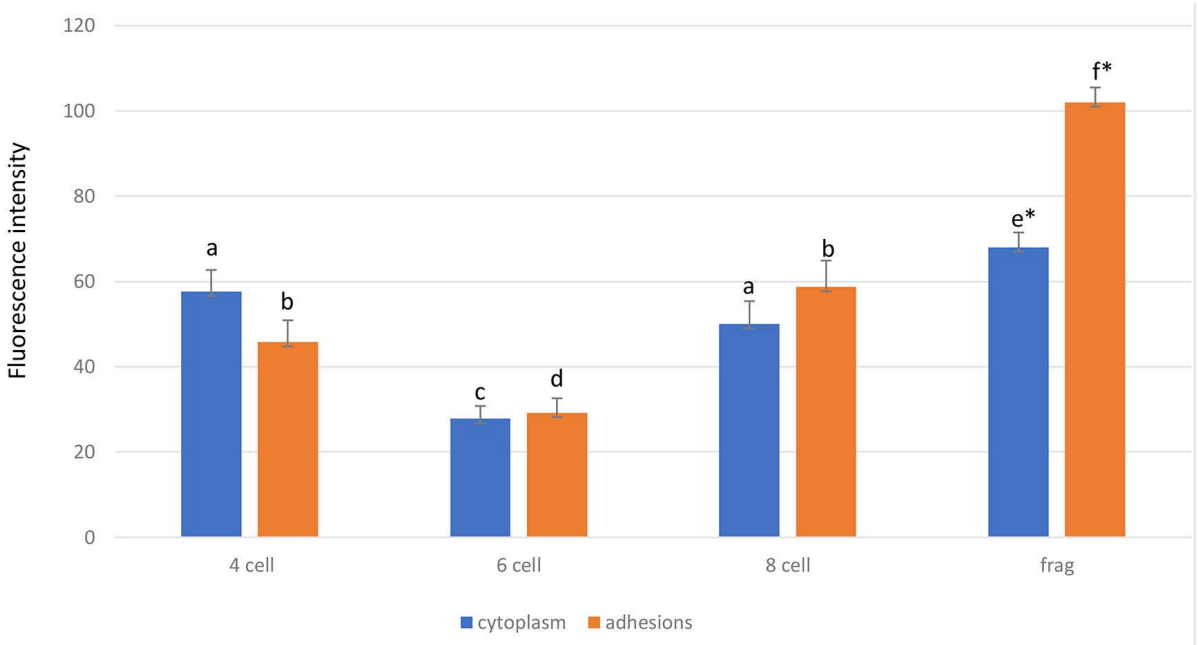
Left column, images in bright field. Right column, images in dark field. a) and b) 2- to 4-cell embryo. c and d) 5- to 6-cell embryo. e and f) 7- to 8-cell embryo. g and h) Fragmented embryo. Magnification 40X, Zeiss LSM T-PMT confocal microscope.

Figure 2. Distribution pattern of E-cadherin in cytoplasmic and adherent junctions between blastomeres, in *O. aries* parthenogenetic embryos at different development stages and fragmented, after 36 h of *in vitro* culture.

The results of the fluorescence intensity of E-cadherin in cytoplasmic and adherent junctions are shown in Figure 3. For the 4-cell embryos it was 57.6 ± 5.1 fu and 45.8 ± 5.1 fu, for the 6-cell embryos it was 27.8 ± 3.0 fu and 29.2 ± 3.4 fu, and for 8-cell embryos it was 50.0 ± 5.9 fu and 58.7 ± 6.2 fu, respectively.

From the 4- to 6-cell stage, E-cadherin significantly decreased in embryos in the cytoplasmic region, whereas from the 6- to 8-cell stage it significantly increased in the adherent junctions ($p < 0.05$).

E-cadherin did not show significant differences between its localization in cytoplasmic and adherent junction in the 4-cell, 6-cell, and 8-cell embryo groups. However, in the fragmented embryos, there were significant differences, with preferential localization in the cytoplasm ($p < 0.5$) (Figure 3). Figure 3 shows a high standard deviation in the distribution of E-cadherin in adherent junctions; this can be explained by the higher fluorescence observed in fragmented embryos compared to normal ones.



Different literals a, b, c, d, e, f, show significant differences in E-cadherin in cytoplasmic and adherent junctions between embryo cleavage stages. *Significant differences between E-cadherin in cytoplasmic and adherent junctions ($p < 0.05$).

Figure 3. Fluorescence units (fu) of E-cadherin in cytoplasmic and adherent junctions, in *O. aries* parthenogenetic embryos at different development stages.

4. Discussion

The results show that ovine parthenogenetic embryos exhibit a stage-dependent E-cadherin distribution pattern. At early stages (2- to 4-cell), E-cadherin is present in a higher amount in the cytoplasm, whereas at the 8-cell stage, E-cadherin is concentrated mainly at cell-cell junctions. These results are consistent with Alikani [18], who observed that E-cadherin is mainly localized in the cytoplasmic regions, whereas at advanced stages, its localization is concentrated in cell-cell

contact regions in early-stage embryos obtained by IVF.

The E-cadherin distribution pattern in fragmented embryos had previously been described by Alikani [18]. The author points out that E-cadherin presents a diffuse and pronounced cytoplasmic fluorescence at the cell margins. The embryo fragmentation rate of 27.7% observed in the present study (**Figure 2(h)**), corresponds to the cleavage stages prior to embryonic genome activation as pointed out by Alikani [18]. In sheep, embryo genome activation occurs between 8 and 16-cell stages [24].

The origin of embryo fragmentation has not been fully established. Factors such as culture conditions, oocyte quality, abnormal cell divisions, and excessive manipulation are considered [25].

It is possible that in fragmented embryos, the abnormalities in E-cadherin distribution are due to the manipulation carried out for the *in vitro* production of parthenogenetic embryos and to parthenogenetic activation with 6-DMAP, preventing the activation of the maturation-promoting factor (MPF) [26], which could have led to early divisions due to defective spindles. 6-DMAP is a serine-threonine kinase inhibitor that mimics the events following sperm fertilization, initiating DNA replication as if the oocyte had been fertilized, causing it to divide and develop as an embryo (parthenote). Furthermore, as an inhibitor of cyclin-dependent protein kinases (CDKs) crucial for cell cycle regulation and proper mitotic spindle formation, it can lead to defective spindle formation and abnormal cell division [27].

Pelzer *et al.* [28] point out that embryo fragmentation may be due to prolonged contact between the chromosomal material and the cell cortex, a process like meiosis, in which Rho-GTPases, specifically RhoA, participate Kale *et al.* [29], point out that RhoA generates tension in the cytoskeleton for its organization, necessary for E-cadherin to activate, which is consistent with the fact that once E-cadherin forms homotypic junctions, it negatively regulates RhoA expression.

Fragmentation is linked to apoptosis [27]. The marked relationship between E-cadherin and apoptotic processes has become evident in studies against different carcinomas [30]. Research focuses on the process of epithelial-to-mesenchymal transition (EMT), where E-cadherin plays a key role.

According to Lu *et al.* [31], E-cadherin is involved in embryo differentiation, including apoptotic events, and can induce, at high concentrations, the programmed cell death-inducing signaling complex (DISC) by activating the apoptotic receptors CDR4/CDR5, Fas (CD95), or TNF-R1. This links it to fragmentation events, which could explain the higher intensity of E-cadherin fluorescence observed in the fragmented embryos and its localization at the periphery of the zona pellucida. The DISC assembly mechanism begins with the ligation of DR5 and DR4, followed by stable assembly with E-cadherin mediated by α-catenin. To complete the assembly of a DISC complex, the Fas adaptor protein-associated death domain (FADD) and the apoptosis-initiating protease caspase-8 are recruited. This indicates that E-cadherin can mediate both indirect apoptosis (initiating the recruitment of DR5

and DR4) and direct apoptosis (organizing the actin cytoskeleton) [31].

E-cadherin proteolysis by caspases or metalloproteinases has also been described to generate fragments that can act as intracellular signals to induce apoptosis [32]. Thus, when E-cadherin loses its function, an apoptotic cascade is activated, since cells depend on stable intercellular junctions for their viability. This loss of adhesion can be interpreted by the cell as irreparable structural damage [33].

On the other hand, embryos receive extracellular stimuli in the form of soluble molecules (growth factors, cytokines, and hormones) that interact with cell surface receptors, adherent interactions with the extracellular matrix, and cell-cell adhesion. These stimuli generate changes in the actin cytoskeleton at specific sites, through the Rho proteins [34].

Arnold *et al.* [35] linked the Rho GTPases family to E-cadherin function due to their role in cytoskeletal remodeling. This is relevant because Rho GTPases are closely related to embryogenesis, and their deficiency disrupts epithelial morphogenesis, tubulogenesis, and the development of the central nervous system and limbs [36].

E-cadherin activates GTPases that regulate cell adhesion mediated by junctional proteins of the Rho family, with interference in cell morphology and migration [34]. Rho GTPases are 20 to 30 kDa proteins of the Ras superfamily, which include: Rho A, B, C, D, E, Rac1 and 2, RacE, Cdc42Hs and TC10. Particularly, RhoA, Rac1 and Cdc42 regulate the organization of the actin cytoskeleton, allowing cell-cell adhesion of the cell membrane mediated by E-cadherin. This relationship between E-cadherin and GTPases can be positive or negative. A negative regulation of RhoA will allow the arrangement of the cytoskeleton and the consequent union to E-cadherin. While being active, RhoA will decrease the concentration of E-cadherin [7], which could be another cause of embryo fragmentation.

Finally, although E-cadherin is an adhesion molecule, it participates in different signaling pathways directly or indirectly because it is closely related to catenins (α, β, γ), particularly with β-catenin in the actin cytoskeleton [37]. This, in turn, is related to the Wnt/β-catenin signaling pathway (Wingless-related integration site), which expresses totipotential genes for nuclear reprogramming, such as the octamer-binding transcription factor 4 (Oct4). For signaling to occur, an intermediate physiological level of β-catenin must be present [7] [38]. Therefore, the E-cadherin adequate localization and distribution is decisive for embryo development.

The β-catenin is a molecule that binds to E-cadherin and plays a role in regulating the balance between proliferation and apoptosis. When E-cadherin/β-catenin binding is lost, the latter can translocate to the nucleus and modify the gene expression, activating or repressing pro- or anti-apoptotic genes [39].

The results obtained in this study support the hypothesis raised, showing that the distribution of E-cadherin in *O. aries* parthenogenetic embryos is significantly related to embryo fragmentation, affecting the quality of embryo development,

with emphasis on the group of fragmented embryos, where its fluorescence by itself causes a high standard deviation, that agrees with the literature referring to the relationship of E-cadherin with apoptosis [31].

This study evaluated parthenogenetic embryos at 36h of culture, limiting the evaluation of later stages of embryonic development; however, this time was selected to simplify the evaluation of the images obtained by confocal microscopy, because in a previous study (Data not shown), we observed that the greater the number of blastomeres in the embryo, the less clarity in the intensity of fluorescence in the adherent junctions between blastomeres and the difficulty of being analyzed by the confocal microscope.

In conclusion, the E-cadherin localization depends on the embryo development stage, with cytoplasmic predominance in early stages (2- to 4-cell) and progressive accumulation at cell junctions (8-cell), a pattern consistent with that reported for IVF-derived embryos. On the other hand, fragmented embryos showed diffuse and intense cytoplasmic fluorescence, suggesting alterations in the normal redistribution of E-cadherin. This alteration may be linked to apoptotic processes, as E-cadherin has been proposed to participate in the activation of programmed cell death pathways such as DISC. Finally, E-cadherin is not only a marker of embryo compaction but also a potential molecular indicator of embryo viability and fragmentation.

Author Contributions

Conceptualization: A.T.-C., D.A.A.-G. and M.d.C.N.-M.; data curation: Y.A.G-D.; D.S.-A.; J.R.V.-A.; formal analysis: Y.A.G-D.; D.S.-A.; J.R.V.-A and D.A.A.-G.; funding acquisition: M.d.C.N.-M.; investigation: J.R.V.-A., A.T.-C. and M.d.C.N.-M.; methodology: Y.A.G-D.; D.S.-A.; A.T.-C.; Y.V.M.-d.l.S. and J.R.V.-A.; project administration: M.d.C.N.-M.; resources: D.A.A.-G., A.T.-C.; and M.d.C.N.-M.; supervision: A.T.-C.; J.R.V.-A. and M.d.C.N.-M.; validation: A.T.-C.; E.S.-B. and M.d.C.N.-M.; visualization: A.T.-C. and M.d.C.N.-M.; writing—original draft: Y.A.G-D.; D.S.-A.; A.T.-C. and M.d.C.N.-M.; writing—review and editing: Y.A.G-D.; D.S.-A.; J.R.V.-A., D.A.A.-G., A.T.-C., and M.d.C.N.-M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The animal study protocol was approved by the Institutional Ethics Committee, "Ethics Commission of the Biological and Health Sciences Division of the Universidad Autónoma Metropolitana Iztapalapa (protocol code CECBS23-13 and date

of approval: 4 April 2023)".

Data Availability Statement

The data are available from the first author, Alfredo Trejo Córdova (atrejo109@hotmail.com; atrejo@xanum.uam.mx), upon request.

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Conflicts of Interest

The authors declare no conflicts of interest.

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