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Title of the Article: "*in vitro* Bovine Embryo Production using medium's supplemented with recombinant human galectin-1 buffered"

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1. Abstract

As recommended in the ICH Guidelines (S5-R2 and S6-R1), and based on bioethical concerns, we chose bovine embryos (BE) to check the *in vitro* embryo development considering the use of different amounts of rHGAL-1 as supplementations of *in vitro* embryo culture (IVP) mediums. Based on procedures for commercial BE *in vitro* production, using oocytes aspirated from slaughterhouse ovaries, the rHGAL-1 supplementation performed in two experiments (#01 on the oocyte maturation - IVM medium supplemented and experiment #2 on culture step IVC, supplemented SOF medium). There were IVP commercial procedures done, with 3 IVP batches per experiment and distributed the oocytes in four groups of treatment (one control group and three different dosages of rHGAL-1 to supplement both IVM and SOF mediums, using (2, 20 and 40µg.mL-1 respectively). A total of 962 (experiment 1) and 1,213 (experiment 2) oocytes were aspirated and submitted to IVP procedure. There was no damage to in vitro bovine embryos growth, considering cleavage percentage (%CLE), blastocysts development on day 7 (BlD7, BxD7, BhD7), or hatching blastocysts maturation on day 8 (BhD8%), regardless of rHGAL-1 supplementation. The immunohistochemistry assay with D8 embryos cultivated with rHGAL-1 supplementation on the culture medium (SOF medium) could demonstrate the presence of exogenous GAL-1, distributed in mass cell and trophoblastic cells, and the profile observed is dependent of exogenous supplementation and it was more evident in hatched embryos. The findings reassure the use of a reasonable amount of rHGAL-1 for in vitro embryonic development and make using rHGAL-1 in assisted reproduction in humans more reliable and safer.

Keywords

Tolerana®, Pregnancy, Fetal-Maternal Recognition, Reproduction, Reproductive Safety Toxicology

2. Abbreviations

AI – artificial insemination procedure

BE – bovine embryos

Bl – blastocysts

BlD7 – blastocysts on day 7

BlD8 – blastocysts on day 8

Bh – hatched blastocysts

BhD7 - hatched blastocysts on day 7

BhD8 - hatched blastocysts on day 8

Bx – expanded blastocysts

BxD7 – expanded blastocysts on day 7

BxD8 – expanded blastocysts on day 8

CLE – cleavage

COCs - *cumulus*–oocyte complexes

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D2 – day two of embryo development
D7 – day seven of embryo development
D8 – day eight of embryo development
GAL-1 - Galectin-1
IVC – *in vitro* embryo culture step
IVF – in vitro fecundation step
IVM – *in vitro* oocyte maturation step
IVP – in vitro embryo production procedure
rHGAL-1 – human recombinant Galectin-1
SOF – SOF medium, used on the IVC

3. Introduction

Galectin-1 (GAL-1) has been cited as a mediator involved in preventing early embryonic death in mammals and in maternal-fetal tolerance (both innate and adaptive), associated with regulation and modulation of the immunological responses and adherence to the endometrium. It also contributes to placentation, controlling the development, migration, and trophoblastic invasion, essential in early gestational development [3, 4, 5, 6, 7, 8]. GAL-1 is a 14kDa lectin, with a high affinity to carbohydrates (beta-galactoside and lactose), expressed different tissues but with a considerable amount of mRNA and protein expression in endometrium and placenta (in trophoblasts, stromal cells, villous endothelium, syncytiotrophoblast apical membrane, and villous stroma, maternal decidua, and fetal membranes), and in fetal membranes (amnion, chorioamniotic mesenchyma, and chorion), considering different mammals' species, including humans.

GAL-1 can be detected in the endoplasm of mouse oocytes and preimplantation embryos at all stages, including the zygotes, 2-cell embryos, 4-cell embryos, 8-cell embryos, and blastocysts (mainly at trophectoderm cells) [10]. Gal-1 expression was observed in 3–5 days human embryos, potentially increasing trophoblast attachment to the uterine epithelium [5, 9]. It is remarkable the importance of Gal-1 as a contributor to feto-maternal tolerance, and it has been described by many investigators and has been extensively reviewed [3, 5]. Several immune cells with essential roles in the establishment and maintenance of pregnancy synthesize and respond to GAL-1, e.g., CD4+ CD25+ regulatory T-cell, which play a vital role in tolerating the immunogenic paternal alloantigens [5, 1, 11], and the regulation of the expression of human leukocyte antigen (HLA-G) in extravillous trophoblast, contributing to the tolerance via its interaction with immune and trophoblast cells [9].

Our research group recently evaluated the effect of a single intrauterine dose of human GAL-1 buffered on pregnancy rate in inseminated cows. The findings suggested that one dose of eGAL-1 was reasonable in the beef cattle artificial insemination routine and considerably improved the pregnancy rate [1, 2]. Thinking about a

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future possibility of using the exogenous rHGAL-1 administration in human reproduction procedures (artificial insemination, embryo transfer, ex.), we are spending a lot of effort in studies to comprehend pharmacokinetics, toxicology, and pharmacological security, all based on *ICH Topic S5-R2* (1994)[13] and *ICH Guideline S6R1* (2011)[14], and this article is part of them. This paper complements the Reproductive Safety Toxicology Studies and verifies the embryo development when there were supplemented mediums of *in vitro* embryo Culture (IVP) with rHGAL-1 in three different dosages of supplementation. Herein, we elected bovine embryos because of the significant availability and bioethical concerns.

4. Materials and Methods

4.1. rHGAL-1 production and purification - The method for obtaining rHGAL -1 is determined by the manufacturing process of Inprenha Biotecnologia® and is previously wholly described [1, 2]. Aliquots of *E. coli* strains transformed with the vector insertion containing the GAL-1 gene (pET-29a(+)+lgals-1 gene[1]) were grown in systems with LB Broth Base medium containing kanamycin sulfate until obtaining optimal bacterial growth rate, demonstrated by optical density. Induction of expression is done by adding Isopropyl-D-Thiogalactopyranoside (Sigma-Aldrich) to the culture. After the induced growth period, the bacterial suspension is retained by microfiltration on a Hollow Fiber membrane (0.22 µm, Cytiva) and centrifuged at 5000 g for 15-20 minutes at 4°C, always with the supernatant being discarded and the "bacterial crude = pellet," which were then subjected to bacterial lysis. For Bacterial lysis, the crude or bacterial pellet was resuspended in Phosphate Saline Lysis buffer (1X PBS - 136.8 mM NaCl, 2.7 mM KCl, 6.4 mM Na2HPO4, 0.9 mM KH2PO4, pH 7.4), containing 14 mM Mercaptoethanol, protease inhibitor EDTA-free, lysozyme-1, RNAse A-Type 3A, and DNAse I Type IV-10. All components are Sigma-Aldrich. The pellet diluted in Lysis buffer (Chemical Lysis) was subjected to constant homogenization for 70 minutes and then sonicated for three cycles of 15 seconds each in a Vibra-Cells Sonicator, Sonics (Mechanical Lysis), with intervals of 20 seconds between each cycle. The bacterial lysate was then clarified by centrifugation at 7,000 g for 20 minutes at 4°C and filtered through a 1.0 µm filter (Whatman) with a peristaltic pump (maximum pressure of 4 BAR). The lysate was submitted to 3 steps for protein purification by chromatography in an AKTA Protein Purification System (Cytiva) to obtain a buffered protein solution containing only GAL-1. The first step is based on affinity chromatography on agarose-lactose columns (Sigma-Aldrich), followed by a "size exclusion" chromatography (Sephadex G-25, Cytiva) and another affinity chromatography (PIERCE High-Capacity Endotoxin Removal Resin column - Thermo Scientific) to the removal of bacterial endotoxins (LPS). After all the chromatographic steps, the protein concentration was determined by spectrometry (Abs 280 nm) and expressed in milligrams of protein per milliliter ($mg.mL^{-1}$) and

[1] Subcloning of G*AL*l-1 into pET-29a(+) expression vector. G*AL*-1 Consensus Coding Sequence (CCDS) CCDS13954.1 (length 408nt) was synthesized and subcloned, with juxtaposed insertion of the desired sequence, immediately after the RBS Ribosome binding site sequence of a pET-29a (+) expression vector cut in NdeI / HindIII (GenScript®). This construct was then used for competent transformation of the Rosetta strain of Escherichia coli, maintained in a cell bank

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were submitted to sterilizing filtration (0.22 µm PES membrane). Purified protein batches were submitted to the last stage of industrialization only if they reached compliance with the quality standard predetermined by the company, including protein concentration (1.05 ± 0.05 mg.mL^{-1}), microbiological status, protein bioactivity (Hemagglutination test), molecular weight analysis by SDS-PAGE, and SEC (size exclusion chromatography), protein secondary structure analysis (Circular Dichroism Analysis), aggregate detection and molecular size by DLS (Dynamic Light Scattering) analysis and endotoxin quantification (LPS). Protein identity was confirmed by LCMS (Liquid Chromatography-Mass Spectrometry) and nucleotide sequence confirmation of human galectin-1 cDNA - galectin-1 (Homo sapiens) Consensus Coding Sequence (CCDS) CCDS13954.1 (https://www.ncbi.nlm.nih.gov/projects/CCDS/CcdsBrowse.cgi?REQUEST=ALLFIELDS&DATA=CCDS13954.1&ORGANISM=0&BUILDS=CURRENTBUILDS).

4.2. Experiment 1 – IVM medium supplementation - IVP of bovine embryos procedure were done considering four groups of treatments, one controlled group (no supplementation) and others three groups with rHGAL-1 supplementation on BIOK IVM medium, been 2 µg/mL of rHGAL-1 (group 2); 20 µg/mL of rHGAL-1 (group 3); 40 µg/mL of rHGAL-1 (group 4). The IVM mediums used for each group were prepared, excluding the water amount (QS) and adding the QS amount of volume of protein solution (this, to do not exchange the amount of the other medium components). The protein solution was added on the day of use of the IVM medium in the IVP routine. Other's mediums used in the IVP routine were not supplemented. Three different IVP batches with equal distribution of the aspirated oocytes per group per batch were done.

4.3. Experiment 2 – SOF medium supplementation - IVP of bovine embryos procedure were done considering 4 groups of treatments, one controlled group (no supplementation) and others three groups with rHGAL-1 supplementation on BIOK SOF medium, been 2 µg/mL of rHGAL-1 (group 2); 20 µg/mL of rHGAL-1 (group 3); 40 µg/mL of rHGAL-1 (group 4). The SOF mediums used for each group were prepared, excluding the water amount (QS) and adding the QS amount of volume of protein solution (this to do not exchange the amount of the other medium components). The protein solution was added on the use day of SOF medium in the IVP routine (used during the development and feedings). Other's mediums used in the IVP routine were not supplemented. Three different IVP batches with equal distribution of the aspirated oocytes per group per batch were done.

4.4. Dose Selection and Justification – The selection of rHGAL-1 doses used on IVP experiments was based on the proportion of product (adequate amount already established for artificial insemination procedures in cattle, equal to 200 ± 10 µg of rhGAL-1) over the size of the uterus lumen of a bovine female (100 ± 20 mL). The in vitro oocyte environment is 100 µL, which means 1000X less than the uterine lumen volume. Therefore, a 1000X lower dose in the IVP drops will be used (ex. 2 µg.mL^{-1} of medium or 0.2 µg in 100 µL of medium). This will be the lowest dose employed (group 2). We will consider the medium dose (group 3) 10X plus and the high dose (group 4) 20X plus more protein/mL in each medium.

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4.5. Animals – cows sent for slaughter in commercial slaughterhouses, duly registered with the competent Agency. Breeds, age, or nutritional are parameters not controlled. The slaughterhouse decided the type of animals is accepted for slaughter. Ovaries from cows slaughtered on the same day were all grouped and sent to the PIV laboratory, and they are considered one batch of PIV. All ovaries were kept in an isothermal box until be manipulated in the laboratory. The distance between the slaughterhouse and PIV laboratory is 180km approximately.

4.6. IVP – all procedures to in vitro bovine embryo Culture were performed according to manufacturer's instructions, using commercial Mediums, as BIOK WASH Medium - to wash the oocytes removing the follicular fluid and cell *debris*; BIOK MIV Medium – to incubated the selected oocytes during the maturation step procedure; BIOK TL Medium used to set up the Percoll gradient, and in the first washes of oocytes when transferring them to IVF step, BIOK PERCOLL Medium is used in the processing of semen after thawing, in this way, it is possible to obtain the live sperm pellet that will be used in fertilization; BIOK IVF is used in the processing of semen after passage through the Percoll Gradient, in the correction of the sperm concentration of the inseminating dose, in the washing of oocytes when transferring them to the IVF plate and in the fertilization plates.; BIOK DESNUD was developed to help in the denudation of the zygotes as it facilitates the detachment of the granulosa cells, without the need for excessive pipetting, preventing lesions in their zona pellucida; BIOK SOF is used for washing zygotes when transferring them to IVC plates and in embryonic development culturing. For each IVP batch, the identical medium batches were used in all treatment groups, and it was used only one semen batch for all experiments.

4.6.1. *Ovum-pick-up* and COCs selection - on the IVP laboratory reception, the ovaries from cows slaughtered of each batch were washed with warmed physiological solution (37 ^{o}C) using a sieve repeated times until remove all blood. After that, the cleaned ovaries were incubated at 37 ^{o}C into a glass bottle until all ovaries were aspirated. The ovum pick up was done by 30 x 10mm needle and syringe aspiration, aspirating all visible follicles, excluding only the cystic ones (bigger than 30 mm). The follicular fluid and oocytes picked were grouped in 50 mL conical tubes kept on 37 ^{o}C incubation until the oocyte selection. Using a stereomicroscope with an 80X magnification, the aspirated oocytes were transferred to a 90 mm plate. After morphological selection, they were transferred to BIOK WASH Medium twice and moved to IVM plates. Traditional methods, as described by [15, 16], for morphological evaluation of oocyte quality are based on scoring systems and classification of cumulus-oocyte complexes (COCs), PBS, and spindles were used. The COCs collected from follicles are usually classified according to the compactness of the cumulus investment and ooplasm characteristics.

4.6.2. IVM – 05 micro drops (90 µL) of BIOK IVM medium were distributed in 35 mm plates, all drops covered by autoclaved mineral oil (Irvine Scientific co. 9305). Plates were pre-equilibrated in a 5.5 ± 0.5% CO_2 and 38,5 ± 1.0 ^{o}C atmosphere for at least 2hs before transferring the previously selected and washed oocytes. Maximum of 25 oocytes per drop, adding 10 µL of washed aspirated oocytes per drop, were kept on maturation for 24 to

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26 hours at 5.5 ± 0.5% CO_2 and 38,5 ± 1.0 °C atmosphere.

4.6.3. IVF - 05 micro drops (90 µL) of BIOK IVF medium were distributed in 35 mm plates, all drops covered by autoclaved mineral oil (Irvine Scientific co. 9305). Plates were pre-equilibrated in a 5.5 ± 0.5% CO_2 and 38,5 ± 1.0°C atmosphere for at least 2hs before transferring the maturated oocytes. The IVM oocytes were transferred from IVM plates, washing the oocytes twice in BIOK TL-Semen medium and twice in BIOK IVF medium; Transfer the washed oocytes to the IVF plates previous prepared (up to 25 oocytes for each drop) and kept the IVF plates in an incubator until the moment of fertilization, while processing the semen. The semen preparation consisted of a Percoll short gradient to recover the viable sperms (in a sterile conical bottom microtube, added 135 µL of BIOK TL Medium to 135 µL of 90% BIOK Percoll and homogenize (creating the 45% Percoll); Slowly deposit 260 µL of 90% Percoll on the bottom of the microtube, under the 45% Percoll, and immediately after thawing the semen (37 °C 20 seconds in a water bath), deposited it in the microtube, on the surface of the Percoll gradient; and centrifuged 7 min 2700 G, removing the supernatant and add 1 mL of IVF medium to the formed pellet; centrifuge again 5 min 950 G, and collect 40 µL of the pellet developed, added to the 40 µL BIOK IVF medium in another microtube; perform semen evaluation (motility and vigor) and correction of concentration of sperm/drop. Minimal of 60% motility of viable sperms, at 10 x 10^6/mL of sperm concentration, were used in all experiments, and 8 to 10 µL of processed semen, diluted on BIOK IVF medium, were transferred to previous prepared IVF drops containing matured and washed oocytes. The IVF plates were kept at incubation for 16 to 18 hours at 5.5 ± 0.5% CO_2 and 38,5 ± 1.0 °C atmosphere.

4.6.4. IVC - 05 micro drops (90 µL) of BIOK SOF medium were distributed in 35 mm plates, all drops covered by autoclaved mineral oil (Irvine Scientific co. 9305). Plates were pre-equilibrated in a 5.5 ± 0.5% CO_2 and 38,5 ± 1.0 °C atmosphere for at least 2 hrs before transferring the zygotes. The zygotes were transferred from IVF plates, washing then twice in BIOK DENUD medium and twice in BIOK SOF medium using repeated pipetting to remove the granulosa cells, both previously equilibrated in a 5.5 ± 0.5% CO_2 and 38,5 ± 1.0 °C atmosphere for at least 2 hrs before; Transfer the denude zygotes to the IVC plates previous prepared and equilibrated (up to 25 oocytes for each drop) and kept the IVC plates in an incubator for eight days. On Day 3 and 5, half-volume of BIOK SOF medium volume (50 µL) in each drop was substituted for the same volume of pre-equilibrated new SOF medium (same batch medium always). On day2, the cleaved zygotes (%CLE) percentage was determined, counting how many zygotes have more than two cells, using a stereomicroscope at 80X of amplification. On day 7, the rate of total blastocysts (%BD7), considering the sum of blastocysts (%BlD7), expanded blastocysts (%BxD7 and hatched (%BhD7) considering morphological characteristics of each step of development. On day 8, the percentage of hatched blastocysts (%BhD8), considering morphological characteristics, hatched embryos. Morphological characteristics were evaluated according to Wang and Sun (2007) [15], and it was deemed counted, for statistical analysis, just embryos classified as degree one and two.

4.6.5. Statistical Analysis - the means and standard deviation results for each embryo development and parameters for each treatment group in experiment 1 were analyzed using descriptive statistical analysis and

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PROC GLM / ANOVA Method (by SAS Systems). Parameters of embryo development considered for statistical analysis were CLE, BlD7, BxD7, BhD7, BD7, BlD8, BxD8, BhD8, and BD8. The same was done for experiment 2 (SOF medium supplementation).

4.6.6. Immunohistochemistry – some embryos, in different stages and obtained at experiment 2 (rHGAL-1 supplementation in the SOF medium) were submitted to immunohistochemistry to detect rHGAL-1 (exogenous protein). The embryos were fixed according to the previous protocol [17]. After the culture finished, on the 8th day, the embryos were transferred to PBS and fixed in freshly prepared methanol/DMSO (4:1) at 4°C overnight. After that, they were transferred into freshly prepared methanol/DMSO/H_2O_2 (4:1:1) at room temperature for 5-10 hrs. The embryos were stored in groups in 100% methanol at -20°C for later usage. On the day of the assay the followed steps were: (i) the embryos were rehydrated in microtubes containing 200 µL 50% methanol in PBS at room temperature for 30 minutes; (ii) after tube spin (3minutes) the solution was discharged and added 200 µL 0.1%BSA in PBS at room temperature for 30 minutes; (iii) discharged the solution and added the coat solution (PBS containing 2% BSA and 1.:100 Biotin-Linked Polyclonal Antibody to human GAL-1 – Cloud Clone Corp, LAA321Hu71), overnight at 4 degrees; (iv) the coating solution was substituted by blocking solution (PBS containing 2% BSA) for 2 hours at room temperature; (v) executed three wash steps in 100µL drops with PBS 0.2% BSA; (vi) transfer the embryos to 100 µL drops with PBS 0.2% BSA and 1:500 NeutrAvidin® biotin-binding protein (Thermo Scientific™, A2666) for 2 hours at room temperature; (vii) executed three wash steps in 100 µL drops with PBS 0.2% BSA; (viii) transfer the embryos, individually, into 2 µL of PBS to a glass slide; (ix) after dried, drop 20 µL of EnVision™ FLEX DAB and 20 µL Substrate Chromogen System (DAKO, Denmark) and cover using a cover glass; (x) digital photos were took in contrast microscope (200X), to demonstrate yellow-darked points indicating the rHGAL-1 detected in embryos cells.

4.7. Bioethics Committee Approval - The installation of the Sponsoring Institution is registered for reproduction and experimentation on animals with the CEUA ("Pre-clinical studies – compliance with registration requirements of Tolerana® as a human biopharmaceutical product," under the coordination of Dr. Eríka C. Morani / CEUA.RI; Issue: 01/08/2020; Revision: 01, was approved at the CEUA meeting on 01/15/2020).

5. Results

The results obtained with embryonic development (n = 967 aspirated oocytes) with the IVP procedure plus supplementation of the IVM medium (experiment 1) can be seen in Table 1. It details the IVF parameters (%CLE, observed 36-48 hours after IVF) obtained in the 03 batches performed according to the design of experiment 1. On average, 79% of %CLE and no statistical difference between groups ($p<0.05$) when the IVM medium was supplemented with the test item (eGAL-1). Figure 1 helps to illustrate such results, but the CLE means were 79.47 ± 3.05 % for G1, 88.66 ± 4.38% for G2, 66.73 ± 43.50% for G3, and 81.66 ± 17.84% for G4. The SD found was considerable, most likely due to the batch IVP effect (1, 2, and 3), characterizing the absence of

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significatively results in the parameter evaluated for IVP embryos (supplementation IVM medium). To explain the SD found in the G3, the medium drop of this group had contamination that compromised the oocyte maturation. Figure 1 illustrates the cleaved zygotes evaluated on the D2 of embryo development, considering the four treatment groups.

Table 2 details the specific parameters of embryonic development (%BD7; %BlD7; %BxD7 and %BhD7) in the IVC stage of the procedure, with evaluation at D7 obtained, detail the medium and SD per group in the three IVP batches performed according to the design of experiment 1. There was no statistically significant difference ($p<0.05$) between the treatment groups for the cited parameters. Considering the sum of all embryonic stages (BD7), the mean and SD of BD7 were 26.0 ± 7.6 % for G1, 33.9 ± 9.2% for G2, 18.5 ± 8.8% for G3, and 18.5 ± 10.6% for G4, when the IVM medium was supplemented with the rHGAL-1 solution and SOF medium was not supplemented (Figure 2). Figures 3 to 5 help illustrate results considering other parameters of embryonic development (BlD7, BXD7, BhD7).

Table 3 details the same parameters of embryonic development (%B; %Bl; %Bx and %Bh), but now, considering the D8 of IVC in the three IVP batches designed as experiment 1. Again, there was no statistically significant difference ($p<0.05$) between the treatment groups for the cited parameters. Considering the sum of all embryonic stages (BD8), the mean and SD of BD8 were 29.3 ± 12.3 % for G1, 37.7 ± 11.1% for G2, 24.0 ± 12.4% for G3, and 23.2 ± 6.7% for G4, when the IVM medium was supplemented with the rHGAL-1 solution and SFO medium was not supplemented (Figure 6). Figures 7 to 9 help illustrate results considering other parameters of embryonic development (BlD8, BXD8, BhD8). Figure 10 can demonstrate the quality of *in vitro* bovine embryos developed in each treatment group (G1, G2, G3, and G4) and may explain no evident morphological damage happens when there is the rHGAL-1 supplementation of the IVM medium.

The results obtained with the rHGAL-1 supplementation of the SOF medium (experiment 2) can be seen in table 4 (oocyte number n= 1,213). It details the %CLE observed 36-48 hours after IVF obtained in the 03 batches performed according to the design of experiment 2. On average, 79,30% of %CLE and no statistical difference between groups ($p<0.05$) when the SOF medium was supplemented with the test item (rHGAL-1). Figure 11 helps to illustrate such results, but the CLE means were 84.4 ± 7.6 % for G1, 81.6 ± 2.6% for G2, 78.3 ± 4.5% for G3, and 72.9± 0.7% for G4. Comparing the %CLE at experiment 1 (IVM medium supplemented with rHGAL-1) and experiment 2 (SOF medium supplemented with rHGAL-1) is noted %CLE different between IVP batches and groups but not detectable in statistics. Figure 11 illustrates the results of cleaved zygotes evaluated on the D2 of embryo development, considering the four groups of treatment in experiment 2. Still, it is valid to remember that the item test supplementation was done only on the SOF medium, after the IVF step.

Table 5 details the bovine *in vitro* embryonic development (BlD7; BxD7; BhD7 and BD7) evaluated at D7 of Culture, considering three IVP batches performed according to the experiment 2 design. There was no statistically significant difference ($p<0.05$) between the treatment groups for the BD7, the mean and SD of BD7 were 33.4 ± 3.9 % for G1, 34.7 ± 6.8% for G2, 21.5 ± 19.4% for G3, and 22.5 ± 4.4% for G4, when the SOF medium was supplemented with the rHGAL-1 solution other IVP mediums were not supplemented (Figure 12). Figures 13 to 15 help illustrate results considering other parameters of embryonic development (BlD7, BXD7, BhD7) in

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experiment 2. The %BxD7 on this second experiment demonstrated statistical difference ($p<0.05$) between groups (G1 and G2 with better results compared with G3 and G4, been 12.7 ± 3.0 and 11.5 ± 2.70 for G1 and G2 versus; 3.4 ± 3.2 and 5.0 ± 5.1 for G3 and G4 respectively – Figure 14). Parameters such as BlD7 and BhD7 demonstrated no statistical difference between groups on experiment 2 (Figure 13 and 15).

Looking for the embryonic development parameters results evaluated at D8 (BlD8, BxD8, BhD8, and BD8), detailed in Table 6 (mean and SD of 4 groups in three IVP batches performed according to the design of experiment 2), observed 30,15% of BD8 development in average, and 36.7 ± 4.7 in G1, 36.7 ± 6.5 in G2, 23.1 ± 20.2 in G3 and 24.1 ± 3.3 in G4, with no statistical difference, again, between groups of treatment (Figure 16). Analyzing BlD8, BxD8, and BhD8, they didn't show any statistical difference in experiment 2, as demonstrated in Figures 17 to 19. Figure 20 can illustrate the quality of *in vitro* bovine embryos developed in each treatment group (G1, G2, G3, and G4), and no morphological damage was evident with the rHGAL-1 supplementation of the SOF medium.

The immunohistochemistry assay with embryos cultivated with rHGAL-1 supplementation on the culture medium (SOF medium) could demonstrate the presence of exogenous GAL-1, distributed in mass cell and trophoblastic cells. Figures 22 to 24 can illustrate the presence of the yellow-dark spots detected after DAB stained and its relationship with the amount of rHGAL-1 used in the medium' supplementation (G2, G3, and G4), suggesting that how much more protein, more dark spots detected. Exogenous GAL-1 could penetrate the zona pellucida (as can see in blastocysts with intact zona pellucida), but it was clear and more evident, the detection of spots in trophoblastic cells of hatched embryos. Another important observation was in the Control Group, where it was detected yellow-dark spots too, in a lower amount, but detected (Figure 21).

6. Discussion

This study aimed to complement the pharmacological knowledge, specifically Reproductive Safety Toxicology of rHGAL-1, as an active ingredient in products and potential products intended for assisted reproduction, whether in production animals or humans.

The hypothesis of using exogenous GAL-1 as a tool to increase fertility was supported by the authors of this article and has been the subject of study since 2008, always considering the supplementation of an adequate amount of this protein into the lumen uterus would bring the desired effect on reproduction, and to know the impact of this protein on embryonic development can help a lot.

The scientific support to initiate the use of an administration of exogenous rHGAL-1 started reading the article published by [4], who demonstrated a higher rate of pregnancy loss in female knockout mice for the LGALS1 gene, the gene responsible for the expression of GAL-1, and others theoretical support and literature reports, demonstrating intrinsic participation of GAL-1 in important physiological events related to maternal recognition of pregnancy, either by the immunological aspect, or by biological aspects such as embryonic elongation and adhesion, and trophoblastic development and placentation, [3, 4, 5, 6, 7, 19, 20, 21].

There are so many particularities between placentation and embryo elongation of different mammals' species but, based on [20, 21, 22], that described that GAL-1 has a high degree of structural conservation, dimerization,

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and binding properties with carbohydrates and integrins (adhesion proteins), suggesting that these properties are conserved among vertebrates and that they maintain a pattern of gene expression among the different types of the placenta (deciduous or not), supported the decision to use a human gene to produce the recombinant protein for other species. Previous studies have demonstrated the biological functionality of rHGAL-1 to improve the pregnancy rate in cows, suggesting the potential use in other species [1, 2].

As part of the Non-Clinical Studies needed to start Clinical Trials in humans (Phase-1 onwards), the authors published pharmacokinetics study results [12] and as complementation, this article presents pharmacological aspects (Reproductive Safety Toxicology) of rHGAL-1 tested in three different dosages, as a supplementation of IVM and SOF mediums during the IVP procedures. The choice of bovine *in vitro* embryos was based on bioethical questions, and because the possibility to test a larger number of oocytes and embryos disponible in IVP procedures. It was used bovine ovaries originated by slaughterhouse ovaries ovum pick-up and, aiming to meet the recommendations of the ICH Guides, as Tests Preclinical Safety Evaluation of Biotechnology-derived pharmaceuticals [13 and 14], as is the case presented here - recombinant protein-based product.

The form of administration of exogenous rHGAL-1 in assisted reproduction procedures, suggested by the authors, is the administration of the protein solution directly into the uterine lumen during the insemination act or embryo inovulation. Hence the importance of the present study, which helps to understand the effect of exogenous protein presence and contact on embryonic development. Even recognizing the particularities of embryonic development *in vivo* and *in vitro*, it was possible to verify that no harmful effects were detected in the embryonic development of bovine embryos produced *in vitro* when we supplemented embryo maturation and culture mediums (experiments 1 and 2), according to the embryonic development parameters evaluated at D2, D7 or D8 of Culture, considering CLE, Bl, Bx, Bh and B taxes. The immunohistochemistry assay could demonstrate the protein detection in D8 embryonic structures, in different degree of detection, suggesting that according with the amount of exogenous protein used to supplement the SOF medium (experiment 2) more spots of immunoreaction was detected. It was reported that Lgals1 expression is observed in 3–5 days human embryos, and potentially increasing trophoblast attachment to the uterine epithelium [9]. Then, the immunohistochemistry assay results may suggest that the bigger amount of immunoreactive spots detected in latter structure embryonic structures (hatched and expanded), may be because there was a complementation of protein detected, with an endogenous expression of GAL-1 (bovine). The cross-reaction between IgG anti-human GAL-1 and bovine-GAL1 is high, and the authors detected those in ELISA Assays too. The degree of similarity between them is bigger than 89%.

The mean ± SD of development embryonic parameters observed at D7 and D8 in groups that were supplemented with a larger amount of rHGAL-1 (G3 and G4) were a little lower, but not statistically different, from G1 (placebo) and G2 (2 $\mu g.mL^{-1}$). The means observed between the IVP batches can explain the SD values. Still, there is a consensus that in IVP commercial protocols is normal to find variation between embryonic development, reflecting on the embryonic parameters measured. Microbiological contamination in some drops may further amplify this issue (fact happens on some drops of G3 on experiment 1). However, the statistical analysis was able and concise to demonstrate that the "rHGAl-1 supplementation mediums (IVM or SOF) effect”

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did not interfere with embryonic development, considering blastocysts development taxes and no morphological evidence was observed in the embryos of different groups of treatment.

Although the results have not shown interference of the test item on bovine *in vitro* embryo development, it is essential to note that the effectiveness of rHGAL-1 on fertility has been verified with small doses of the protein (0.200 mg for cows, whose average weight is 400 kg, that means 0.0005 $mg.kg^{-1}$). For another species, the idea is to keep that dosage proportion. In both experiments with IVP embryos, G3 and G4 extrapolated the amount of protein used in the efficacy tests in cows by 10 and 20X more protein, creating an exciting range of possible doses to test.

This study obtained essential answers to questions intrinsic to recombinant protein-based biopharmaceuticals. It is worth noting that the number of aspirated oocytes and the Culture were considered in each of the two experiments, more than 2.000 in total, reinforcing the accuracy of the objective of this study.

The experiments still leave an open question - is it possible for rHGAL-1 to interact with different stages of embryonic development (especially the steps with differentiated trophoblasts), capable of altering the effectiveness of the embryo in maternal recognition of pregnancy? or is the role of rHGAL-1 in increasing reproductive efficiency-related only on the uterine endometrium? New experiments are underway to elucidate this issue and working with rHGAL-1 supplementation. Another experiment in focus is a Reproductive Toxicity Studies in female rats that received a single dose of rHGAL-1, administrated Intra uterus by surgical procedures, based on [13], which goals is to get the information in different phases of the pregnancy – 1st (Conception to implantation, evaluating adult female reproductive functions, and comparing the number and evaluating the gestational sacs); 2nd (Implantation to the closure of the hard palate, assessing adult female reproductive functions, embryonic development, major organ formation); 3rd (Closure of the hard palate to the end of pregnancy, evaluating mature female reproductive functions, fetal development, and growth, organ development, and growth); 4th (Birth to weaning, evaluating adult female reproductive functions, neonate adaptation to extrauterine life, preweaning development and growth until 14 days). With both experiments, we can better discuss a single and intrauterine rHGAL-1 dose and its interaction with embryos and consequences to embryo and fetus development, and of course, with the mothers' health. Studies reported support that GAL-1 plays a critical role in regulating embryo implantation and in maintaining early placenta function (trophoblast differentiation, migration, and selective gene expression in placentation) [9]. The GAL-1 expression increases significantly during pregnancy and several studies indicate the potential use of Lgals1 as a biomarker for miscarriage, recurrent fetal loss and preeclampsia (PE) [5]. It was showed a decreased expression of GAL-1 in the trophoblastic cells from women with early pregnancy loss (using proteomic analysis) [19]. A study demonstrated increase expression of GAL-1 in the invasive extravillous trophoblasts in first-trimester human placenta, which has been shown to promote syncytium formation [9, 24]. They suggested that blockage of GAL-1-mediated angiogenesis or lectin can disturb the processes associated with good placentation. Data from the authors (the improvement of the amount of GAL-1 into the bovine uterus) can be cited as a good example of impact of this protein in the pregnancy rate (1, 2).

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7. Conclusion

Based on these results, it was observed that rHGAL-1 does not interfere with bovine IVP embryo development, measured by CLE evaluated on D2, and Bl, Bx, and Bh evaluated on D7 and D8 od the Culture and based on the amount of protein used (2, 20 and 40 $\mu g.mL^{-1}$). There was GAL-1 immunodetection on D8 embryonic structures, and the profile is dependent of exogenous supplementation.

8. Patent

The company, assisted by specialized lawyers, has already patented this innovation in several countries due to this highly innovative and unique technological content. This pa-tent was co-participated with the University of São Paulo (FFRP). World patent WO/2012/083396.

9. Author Contributions:

Conceptualization, M.R. and E.d.S.C.M.; methodology,M.R., G.C.F..; software, G.C.F..; validation, M.R.; formal analysis, G.C.F..; investigation, E.d.S.C.M, M.R. and N.A.A.d.S..; resources, E.d.S.C.M and M.R..; data curation, E.d.S.C.M.; writing—original draft preparation, M.R..; writing—review and editing, G.C.F..; visualization, E.d.S.C.M.; supervision, M.R.; project administration, M.R.; funding acquisition, M.R.; and E.d.S.C.M. All authors have read and agreed to the published ver-sion of the manuscript.

10. Funding:

Yoni Group/Inprenha Biotecnologia conducted the research, based on FAPESP grants, process #2019/09/722-5.

11. Institutional Review Board Statement:

The present study complied with the ethical requirements for animals' use in experiments and was approved by CEUA.RI; Emission: 08.01.2020; Revision: 01, approved at 15th January of 2020.

12. Acknowledgements

Thanks to the technical team at the Animal Reproduction Department of FCAV UNESP Jaboticabal, their unstinting efforts to support this research work. And special thanks to the inventors of the mentioned patents CAMILLO DEL CISTIA ANDRADE, LÍLIAN CATALDI RODRIGUES, MARCELO DIAS BARUFFI, MARCELO RONCOLETTA, and ERIKA DA SILVA CARVALHO MORANI who ceded/granted the transfer, without reservations, all rights, ownership, action, and interests of the invention to USP (University of Sao Paulo) and Inprenha Biotecnologia. This acknowledgment is extended to USP, co-author of the patent, which transferred to Inprenha Biotecnologia the rights of exploitation of the patent.

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13. Conflict of interest

We declare that is any conflict of interest.

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Table 1 – Number (n) and percentage of cleaved embryos (%CLE), evaluated on D2 of culture (36 to 48h after IVF) in relation to the number of oocytes submitted to IVM for the 04 treatment groups (G1, G2, G3 and G4) and 3 IVP batches (1, 2 and 3) performed in experiment 1 (IVM medium supplementation with test item at different concentrations for each treatment group).

Groups	IVP batch #	IMV oocytes number	CLE (D2)			
			n	%	$\overline{X}$	SD
G1	1.1	40	33	82,50%	79,47[a]	3,05
	1.2	110	84	76,36%		
	1.3	88	70	79,55%		
G2	1.1	33	30	90,91%	88,66[a]	4,38
	1.2	116	97	83,62%		
	1.3	82	75	91,46%		
G3	1.1	34	30	88,24%	66,73[a]	43,50
	1.2	127	121	95,28%		
	1.3	84	14	16,67%		
G4	1.1	34	30	88,24%	81,66[a]	17,84
	1.2	127	121	95,28%		
	1.3	96	59	61,46%		
total		967	764	79.00%		

a = no statistical difference (p< 0.05) between groups

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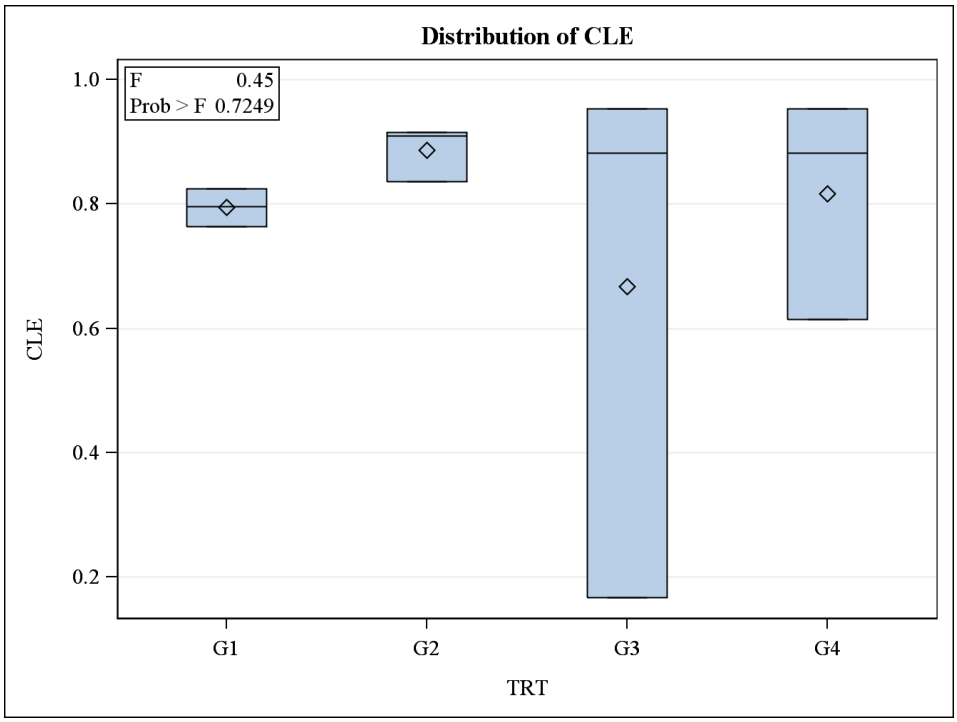

Figure 1. Illustrative figure of the result of N zygotes cleaved (CLE) evaluated on D2 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 1 (IVM medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

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Table 2 – Number (n) and percentage of blastocysts (BlD7), expanded blastocysts (BxD7) and hatched blastocysts (BhD7), and the sum of blastocysts generated (BD7), evaluated on D7 of culture, always in relation to the number of oocytes submitted to IVM, considering the 04 treatment groups (G1, G2, G3 and G4) and the batches of IVP (1, 2 and 3) performed in experiment 1 (IVM supplementation with test item at different concentrations for each group of treatment), with IVF and IVC performed in conventional media.

Groups	IVP batch #	Number of IVM Oocytes	BlD7				BxD7				BhD7				BD7			
			n	%	$\overline{X}$	SD	n	%	$\overline{X}$	SD	n	%	$\overline{X}$	SD	n	%	$\overline{X}$	SD
G1	1.1	40	6	15,0	14,5[a]	1,6	6	15,0	11,1[a]	5,7	0	0,0	0,4[a]	0,7	12	30,0	26,0[a]	7,6
	1.2	110	14	12,7			5	4,6			0	0,0			19	17,3		
	1.3	88	14	15,9			12	13,6			1	1,1			27	30,7		
G2	1.1	33	7	21,2	19,9[a]	3,0	4	12,1	11,5[a]	6,0	2	6,1	2,6[a]	3,1	13	39,4	33,9[a]	9,2
	1.2	116	19	16,4			6	5,2			2	1,7			27	23,3		
	1.3	82	18	22,0			14	17,1			0	0,0			32	39,0		
G3	1.1	34	8	23,5	14,4[a]	9,4	0	0,0	4,1[a]	4,4	0	0,0	0,0[a]	0,0	8	23,5	18,5[a]	8,8
	1.2	127	19	15,0			11	8,7			0	0,0			30	23,6		
	1.3	84	4	4,8			3	3,6			0	0,0			7	8,3		
G4	1.1	32	2	6,3	11,3[a]	4,5	0	0,0	6,7[a]	6,3	0	0,0	0,6[a]	1,0	2	6,3	18,5[a]	10,6
	1.2	120	18	15,0			9	7,5			2	1,7			29	24,2		
	1.3	96	12	12,5			12	12,5			0	0,0			24	25,0		
Total		962	141	15.02			82	8.32			7	0.88			230	24.21		

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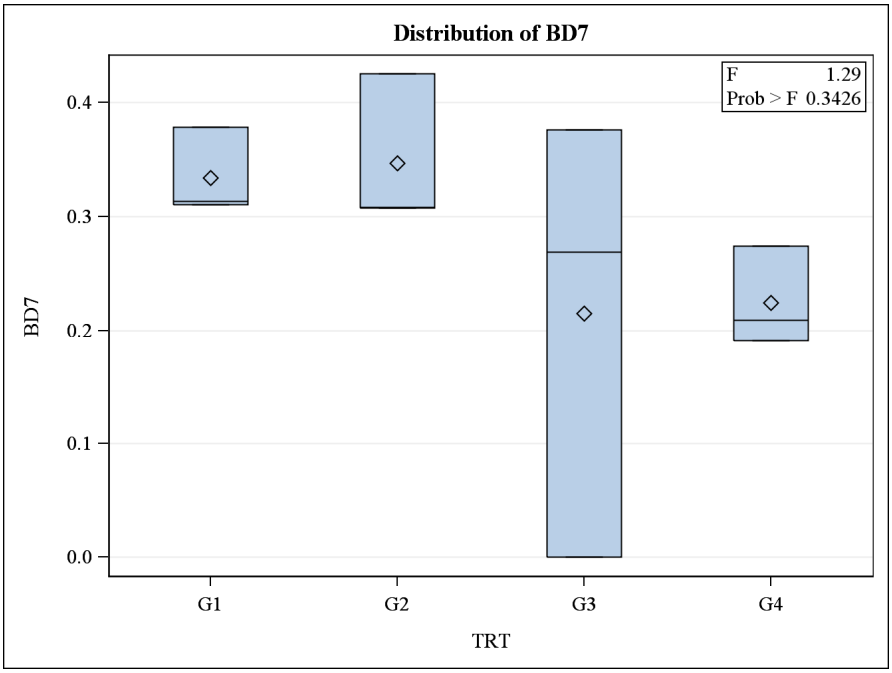

Figure 2. Illustrative figure of the result of total blastocysts (BD7) evaluated on D7 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 1 (IVM medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

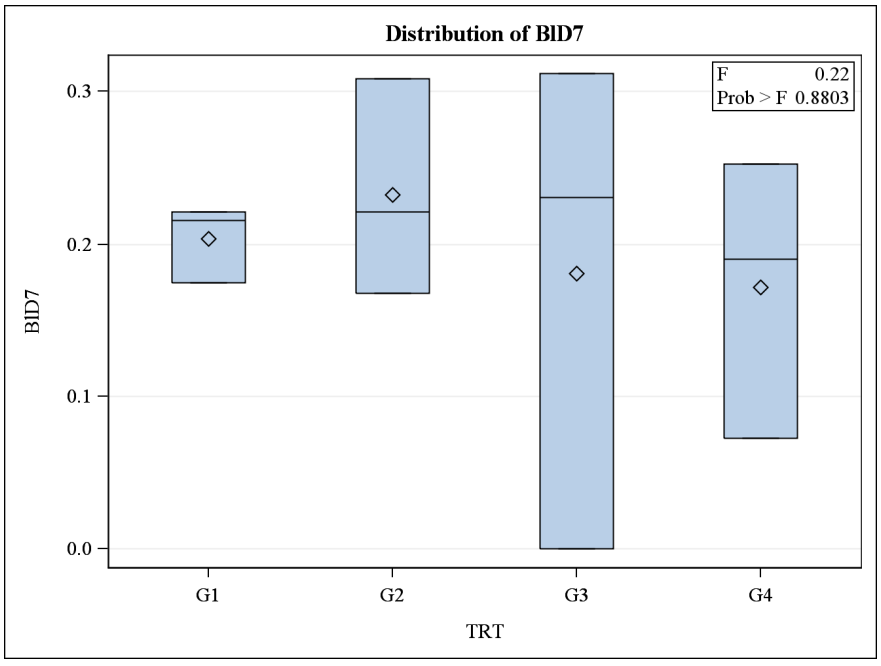

Figure 3. Illustrative figure of the result of blastocysts (BlD7) evaluated on D7 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 1 (IVM medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

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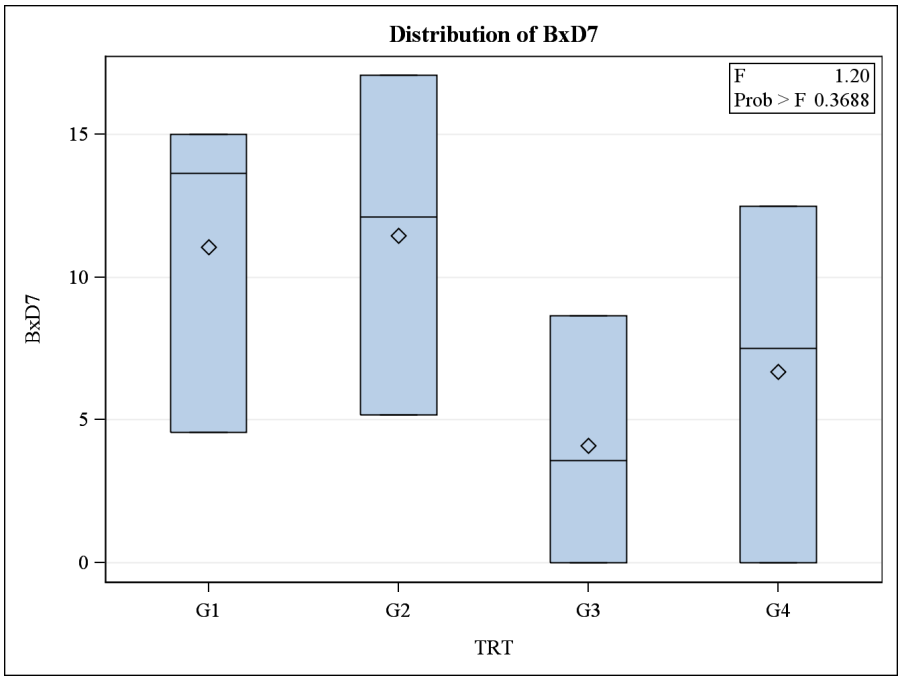

Figure 4. Illustrative figure of the result of expanded blastocysts (BxD7) evaluated on D7 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 1 (IVM medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

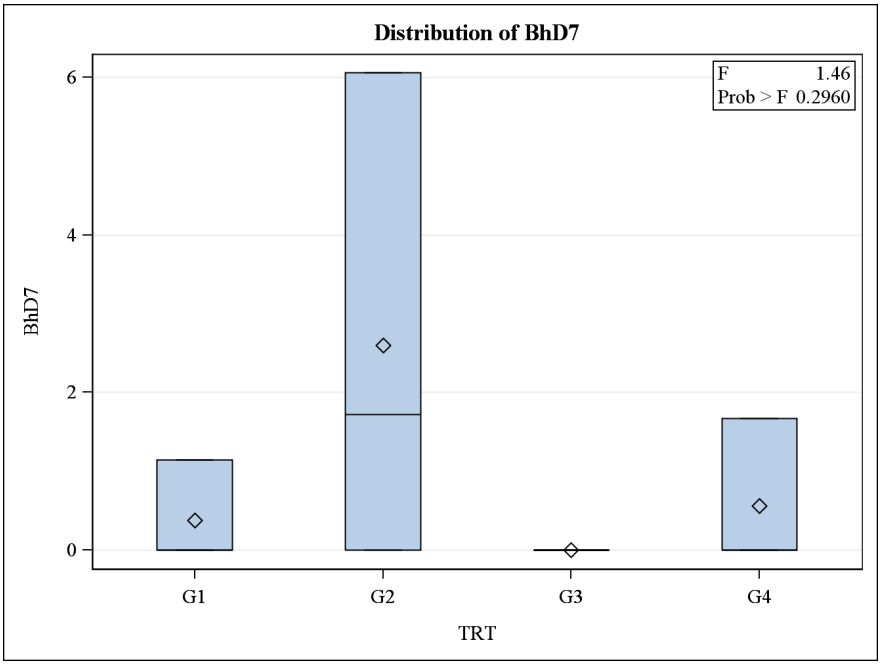

Figure 5. Illustrative figure of the result of hatched blastocysts (BhD7) evaluated on D7 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 1 (IVM medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

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Table 3. Number (n) and percentage of blastocysts (BlD8), expanded blastocysts (BxD8) and hatched blastocysts (BhD8), and the sum of blastocysts generated (BD8), evaluated on D8 of culture, always in relation to the number of oocytes submitted to IVM, considering the 04 treatment groups (G1, G2, G3 and G4) and the batches of IVP (1, 2 and 3) performed in experiment 1 (IVM supplementation with test item at different concentrations for each group of treatment), with IVF and IVC performed in conventional media.

Groups	IVP batch #	Number of IVM Oocyte	BlD8				BxD8				BhD8				BD8			
			n	%	$\overline{X}$	SD	n	%	$\overline{X}$	SD	n	%	$\overline{X}$	SD	n	%	$\overline{X}$	SD
G1	1.1	40	3	7,5	7,4[a]	1,8	8	20,0	13,4[a]	5,7	6	15,0	8,6[a]	6,2	17	42,5	29,3[a]	12,3
	1.2	110	6	5,5			11	10,0			3	2,7			20	18,2		
	1.3	88	8	9,1			9	10,2			7	8,0			24	27,3		
G2	1.1	33	3	9,1	10,4[a]	2,6	4	12,1	11,9[a]	6,6	8	24,2	15,5[a]	7,6	15	45,5	37,7[a]	11,1
	1.2	116	10	8,6			6	5,2			13	11,2			29	25,0		
	1.3	82	11	13,4			15	18,3			9	11,0			35	42,7		
G3	1.1	34	4	11,8	6,9[a]	4,3	5	14,7	10,8[a]	6,3	3	8,8	6,2[a]	2,6	12	35,3	24,0[a]	12,4
	1.2	127	7	5,5			18	14,2			8	6,3			33	26,0		
	1.3	84	3	3,6			3	3,6			3	3,6			9	10,7		
G4	1.1	32	0	0,0	4,1[a]	3,7	2	6,3	9,0[a]	3,2	3	9,4	10,1[a]	2,2	5	15,6	23,2[a]	6,7
	1.2	120	6	5,0			15	12,5			10	8,3			31	25,8		
	1.3	96	7	7,3			8	8,3			12	12,5			27	28,1		
total		962	68	7.19			104	11.28			85	10.08			257	28.55		

a = no statistical difference (p<0.05) between groups

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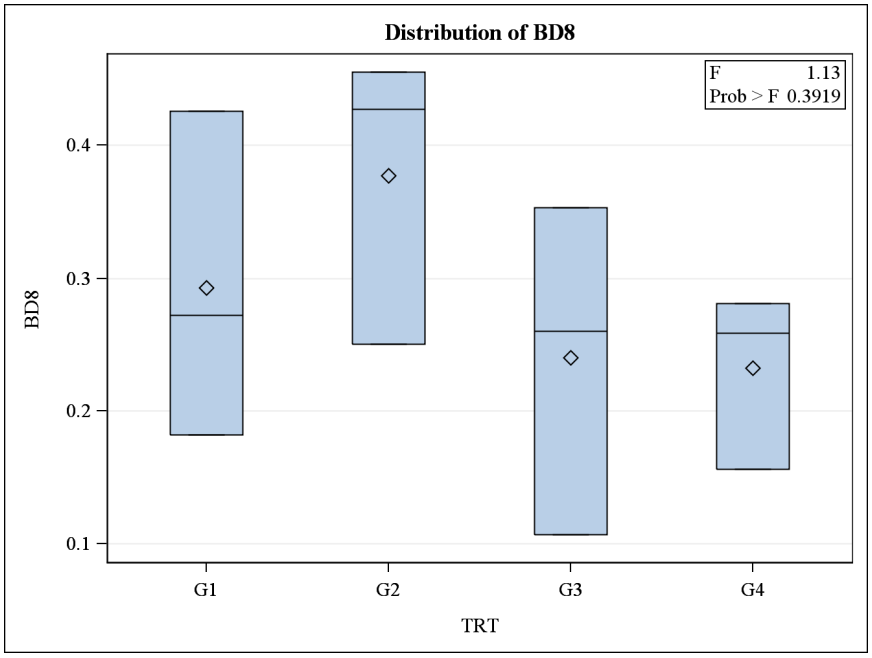

Figure 6. Illustrative figure of the result of hatched blastocysts (BhD8) evaluated on D8 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 1 (IVM medium supplementation).◇= average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

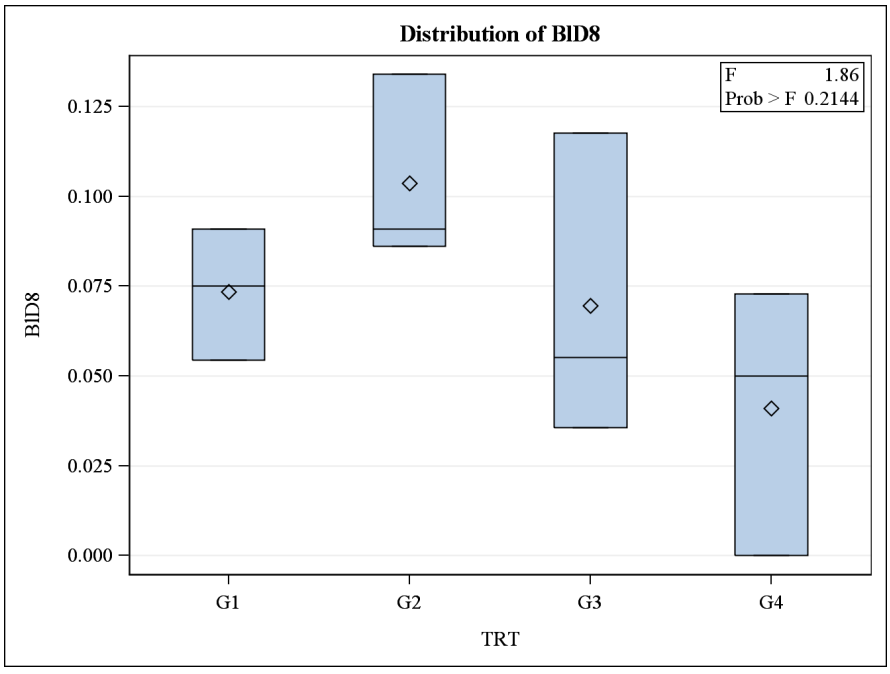

Figure 7. Illustrative figure of the result of blastocysts (BlD8) evaluated on D8 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 1 (IVM medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

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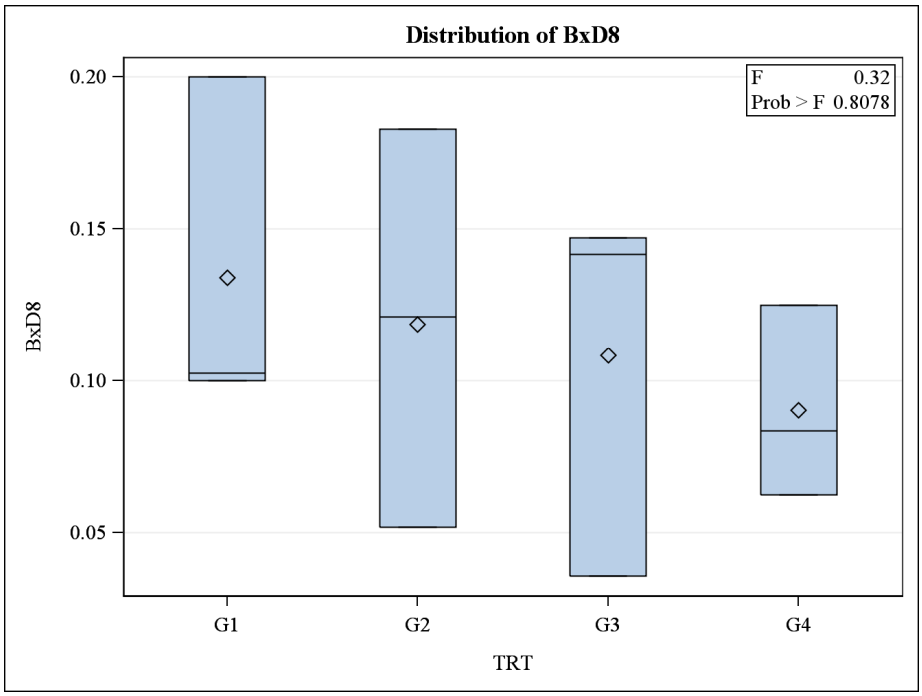

Figure 8. Illustrative figure of the result of expanded blastocysts (BxD8) evaluated on D8 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 1 (IVM medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

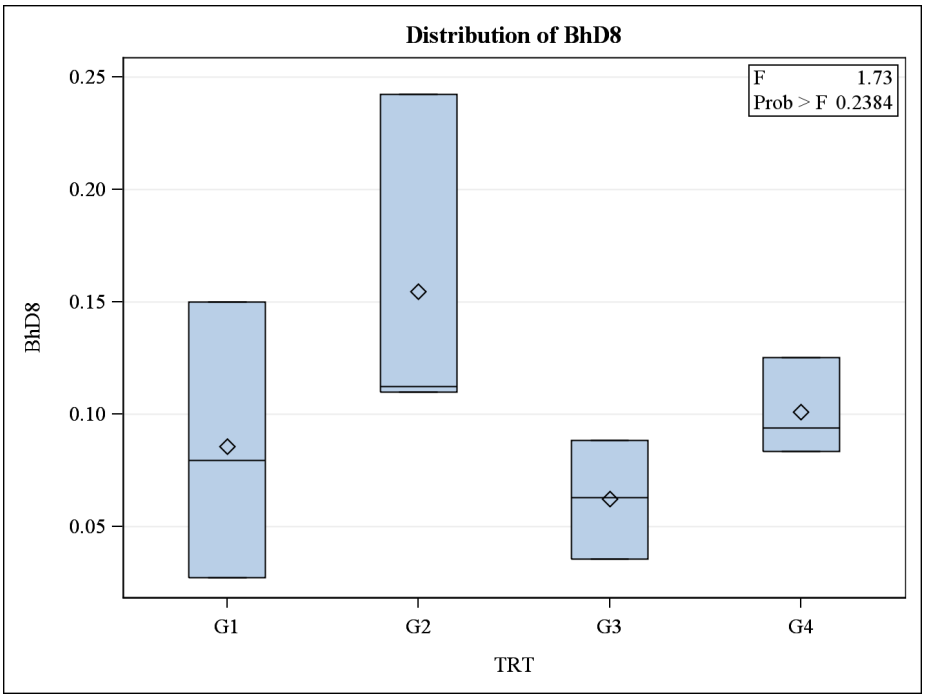

Figure 9. Illustrative figure of the result of expanded blastocysts (BxD8) evaluated on D8 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 1 (IVM medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

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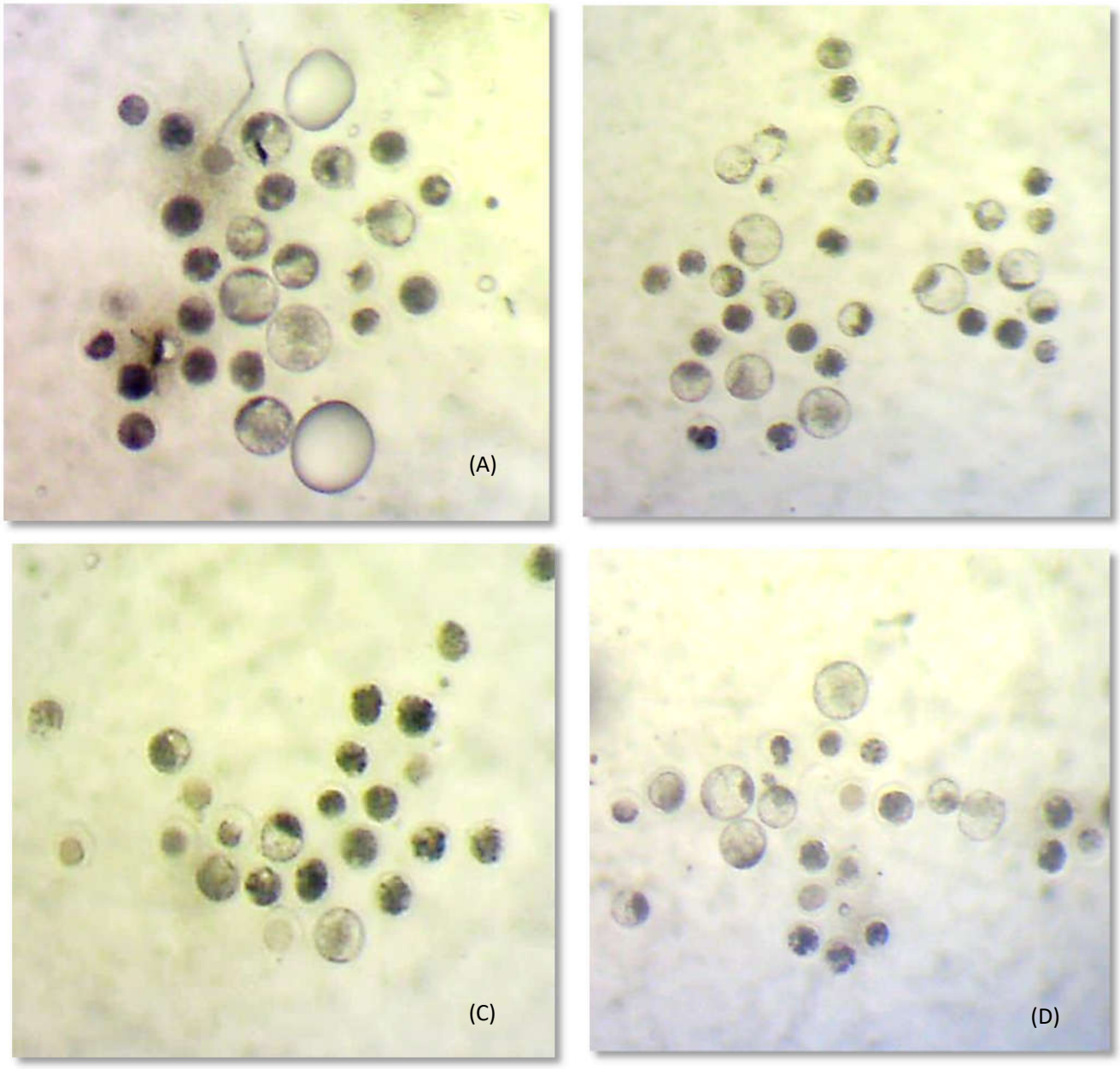

Figure 10. Illustrations of the *in vitro* bovine embryos developed in one of the Cultive drops (experiment 1 / IVP batches 1.3) of each treatment Groups, (A)= group ; (B) = group 2; (C) = group 3; (D) = group 4, respectively. Disregard the size difference observed between the structures, as it is due to the enlargement/reduction of the photo, depending on the number of embryonic structures present in each photo. The objective here is illustrate the morphological characteristics and degrees of the embryos obtained.

(B)

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Table 4 – Number (n) and percentage of cleaved embryos (%CLE), evaluated on D2 of culture (36 to 48h after IVF) in relation to the number of oocytes submitted to IVM for the 04 treatment groups (G1, G2, G3 and G4) and 3 IVP batches (1, 2 and 3) performed in experiment 2 (SOF medium supplementation with test item at different concentrations for each treatment group).

Groups	IVP batch #	IMV oocytes numbe	CLE (D2)			
			n	%	?	SD
G1	2.1	102	89	87,25	84,4[a]	7,6
	2.2	103	93	90,29		
	2.3	95	72	75,79		
G2	2.1	107	90	84,11	81,6 [a]	2,6
	2.2	104	82	78,85		
	2.3	94	77	81,91		
G3	2.1	105	85	80,95	78,3 [a]	4,5
	2.2	104	84	80,77		
	2.3	93	68	73,12		
G4	2.1	110	81	73,64	72,9 [a]	0,7
	2.2	105	76	72,38		
	2.3	91	66	72,53		
Total		1213	963	79.30		

a = no statistical difference (p<0.05) between groups

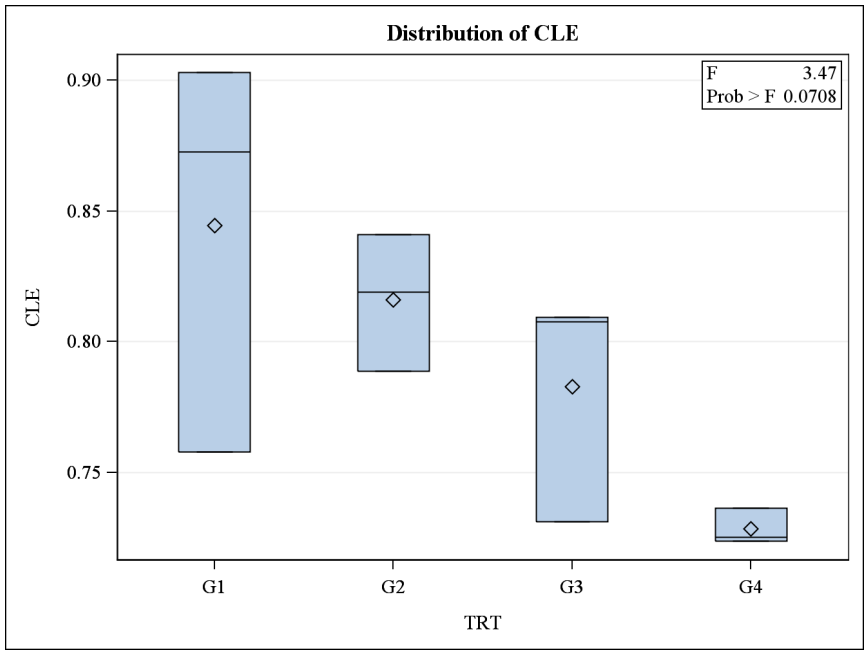

Figure 11. Illustrative figure of the result of N zygotes cleaved (N CLE) evaluated on D2 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 2 (SOF medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method (p<0.05).

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Table 5 – Number (n) and percentage of blastocysts (BlD7), expanded blastocysts (BxD7) and hatched blastocysts (BhD7), and the sum of blastocysts generated (BD7), evaluated on D7 of Cultive, always in relation to the number of oocytes submitted to IVM, considering the 04 treatment groups (G1, G2, G3 and G4) and the batches of IVP (1, 2 and 3) performed in experiment 2 (SOF supplementation with test item at different concentrations for each group of treatment), with IVM and IVF performed in conventional medium.

Groups	IVP batch #	Number of IVM Oocyte	BlD7				BxD7				BhD7				BD7			
			n	%	$\overline{X}$	SD	n	%	$\overline{X}$	SD	n	%	$\overline{X}$	SD	n	%	$\overline{X}$	SD
G1	2.1	102	22	21,5	20,4[a]	2,5	10	9,80	12,7[a]	3,0	0	0,00	0,3[a]	0,6	32	31,37	33,4[a]	3,9
	2.2	103	18	17,5			13	12,62			1	0,97			32	31,07		
	2.3	95	21	22,1			15	15,79			0	0,00			36	37,89		
G2	2.1	107	18	16,8	23,3[a]	7,1	15	14,02	11,5[a]	2,7	0	0,00	0,0[a]	0,0	33	30,84	34,7[a]	6,8
	2.2	104	23	22,1			9	8,65			0	0,00			32	30,77		
	2.3	94	29	30,9			11	11,70			0	0,00			40	42,55		
G3	2.1	105	0	0,00	18,1[a]	16,2	0	0,00	3,4[b]	3,2	0	0,00	0,0[a]	0,0	0	0,00	21,5[a]	19,4
	2.2	104	24	23,10			4	3,85			0	0,00			28	26,92		
	2.3	93	29	31,20			6	6,45			0	0,00			35	37,63		
G4	2.1	110	8	7,30	17,2[a]	9,1	12	10,91	5,0[a,b]	5,1	1	0,91	0,3[a]	0,5	21	19,09	22,5[a]	4,4
	2.2	105	20	19,10			2	1,90			0	0,00			22	20,95		
	2.3	91	23	25,30			2	2,20			0	0,00			25	27,47		
total		1213	235	19.76			99	8.16			2	0.16			336	28.05		

a = no statistical difference (p<0.05) between groups.

a is different of b at p<0.05.

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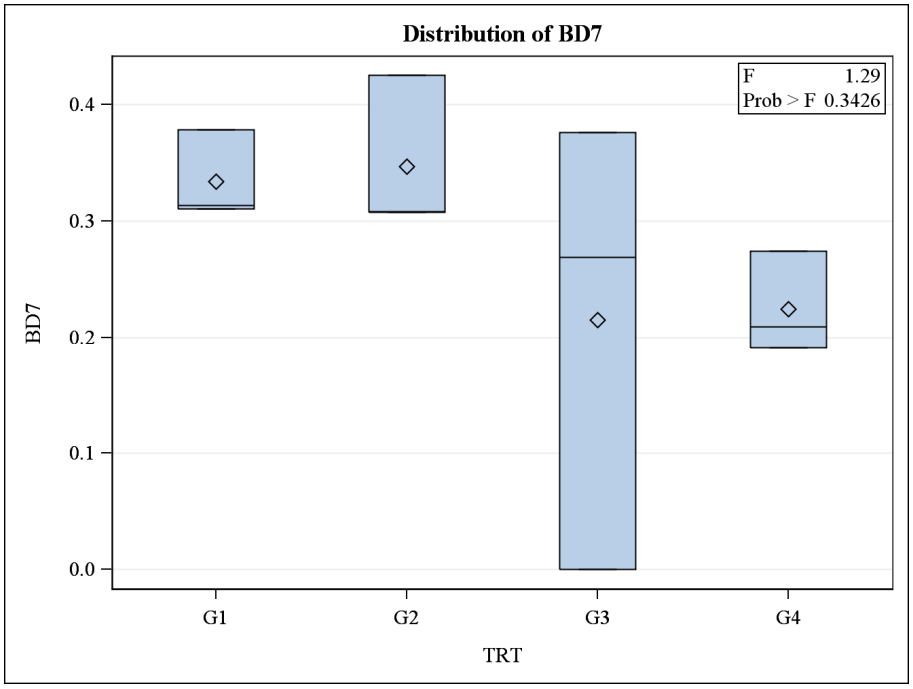

Figure 12. Illustrative figure of the result of total blastocysts (BD7) evaluated on D7 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 2 (SOF medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

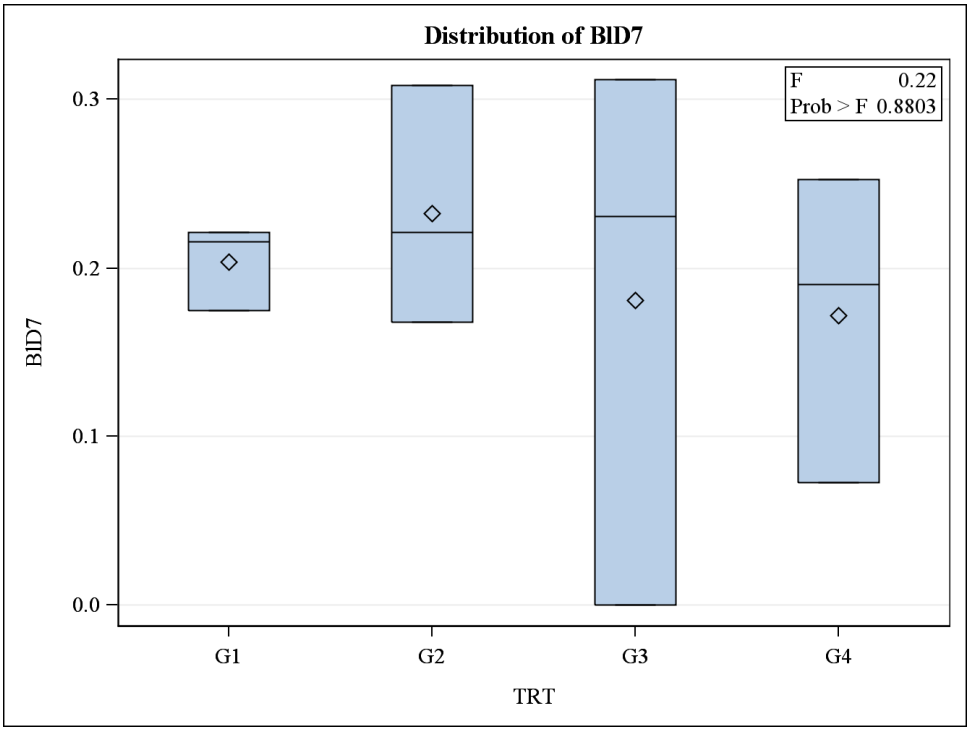

Figure 13. Illustrative figure of the result of blastocysts (BlD7) evaluated on D7 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 2 (SOF medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

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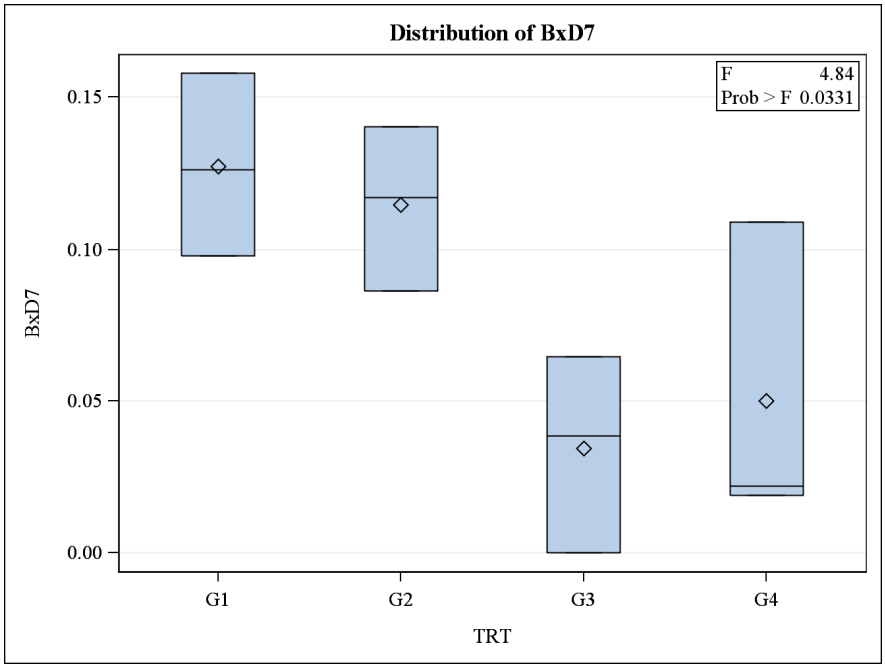

Figure 14. Illustrative figure of the result of expanded blastocysts (BxD7) evaluated on D7 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 2 (SOF medium supplementation). ◇ = average; bar = SD. There was significant difference between G3 and other groups, regarding the parameter analyzed by ANOVA method ($p<0.05$).

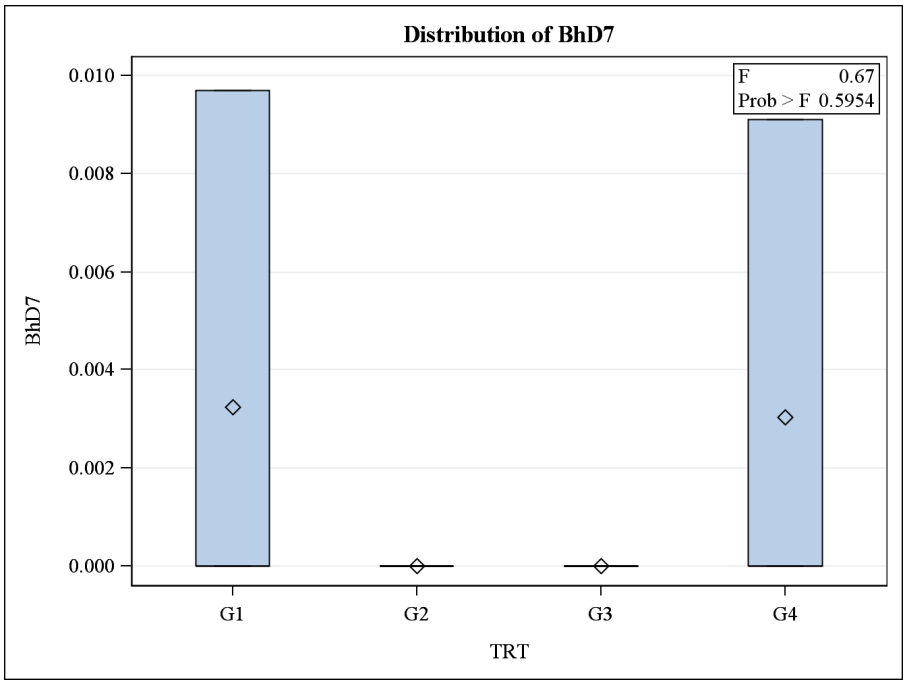

Figure 15. Illustrative figure of the result of hatched blastocysts (BhD7) evaluated on D7 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 2 (SOF medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

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Table 6. Number (n) and percentage of blastocysts (BlD8), expanded blastocysts (BxD8) and hatched blastocysts (BhD8), and the sum of blastocysts generated (BD8), evaluated on D8 of culture, always in relation to the number of oocytes submitted to IVM, considering the 04 treatment groups (G1, G2, G3 and G4) and the batches of IVP (1, 2 and 3) performed in experiment 2 (SOF supplementation with test item at different concentrations for each group of treatment), with IVM and IVF performed in conventional media.

Groups	IVP batch #	Number of IVM Oocyte	BlD8				BxD8				BhD8				BD8			
			n	%	$\overline{X}$	SD	n	%	$\overline{X}$	SD	n	%	$\overline{X}$	SD	n	%	$\overline{X}$	SD
G1	2.1	102	5	4,90	6,0[a]	1,6	19	18,63	20,7[a]	1,9	8	7,84	10,1[a]	3,1	32	31,37	36,7[a]	4,7
	2.2	103	8	7,77			23	22,33			9	8,74			40	38,83		
	2.3	95	5	5,26			20	21,05			13	13,68			38	40,00		
G2	2.1	107	4	3,74	4,0[a]	1,2	17	15,89	19,2[a]	3,2	12	11,21	13,5[a]	2,4	33	30,84	36,7[a]	6,5
	2.2	104	3	2,88			20	19,23			14	13,46			37	35,58		
	2.3	94	5	5,32			21	22,34			15	15,96			41	43,62		
G3	2.1	105	0	0,00	3,6[a]	4,5	0	0,00	12,0[a]	11,0	0	0,00	7,5[a]	7,1	0	0,00	23,1[a]	20,2
	2.2	104	9	8,65			15	14,42			9	8,65			33	31,73		
	2.3	93	2	2,15			20	21,51			13	13,98			35	37,63		
G4	2.1	110	0	0,00	4,7[a]	6,5	12	10,91	10,7[a]	1,8	11	10,00	8,7[a]	1,8	23	20,91	24,1[a]	3,3
	2.2	105	2	1,90			13	12,38			10	9,52			25	23,81		
	2.3	91	11	12,09			8	8,79			6	6,59			25	27,47		
total		1,213	54	4.56			188	15.62			120	9.97			362	30.15		

a = no statistical difference (p<0.05) between groups.

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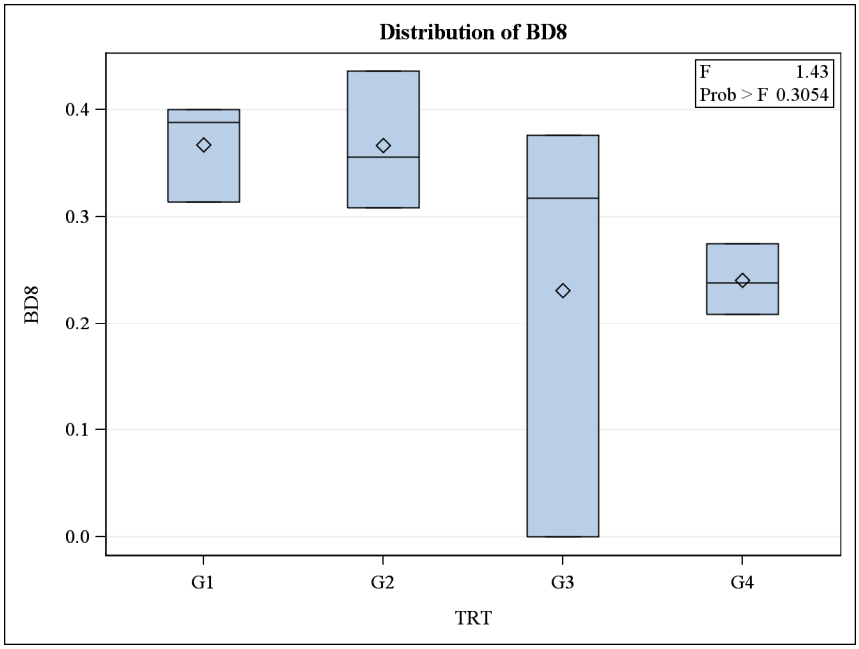

Figure 16. Illustrative figure of the result of hatched blastocysts (BhD8) evaluated on D8 of embryo development, in the four different treatment groups (G1, G2, G34 and G4) – experiment 2 (SOF medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

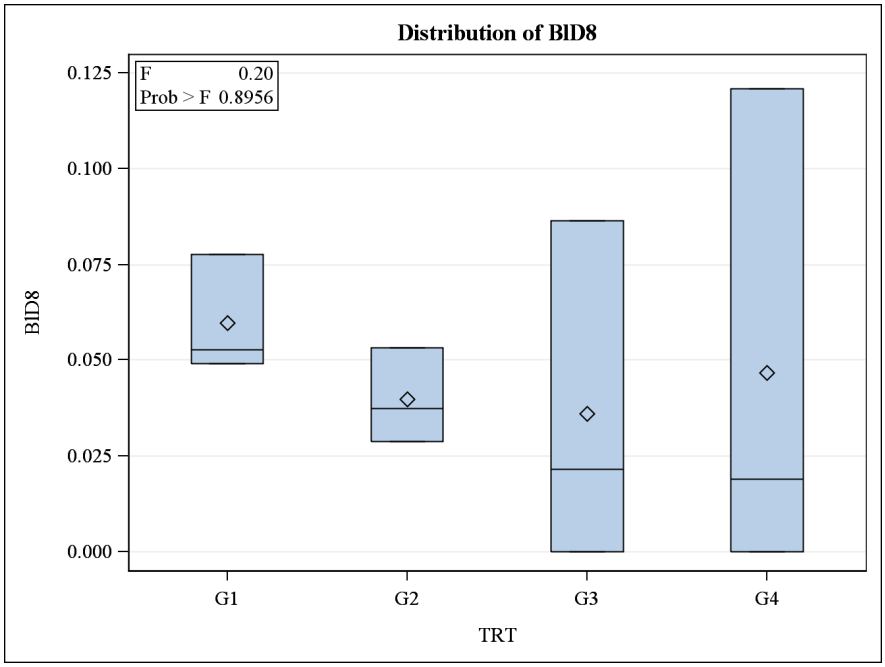

Figure 17. Illustrative figure of the result of blastocysts (BlD8) evaluated on D8 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 2 (SOF medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

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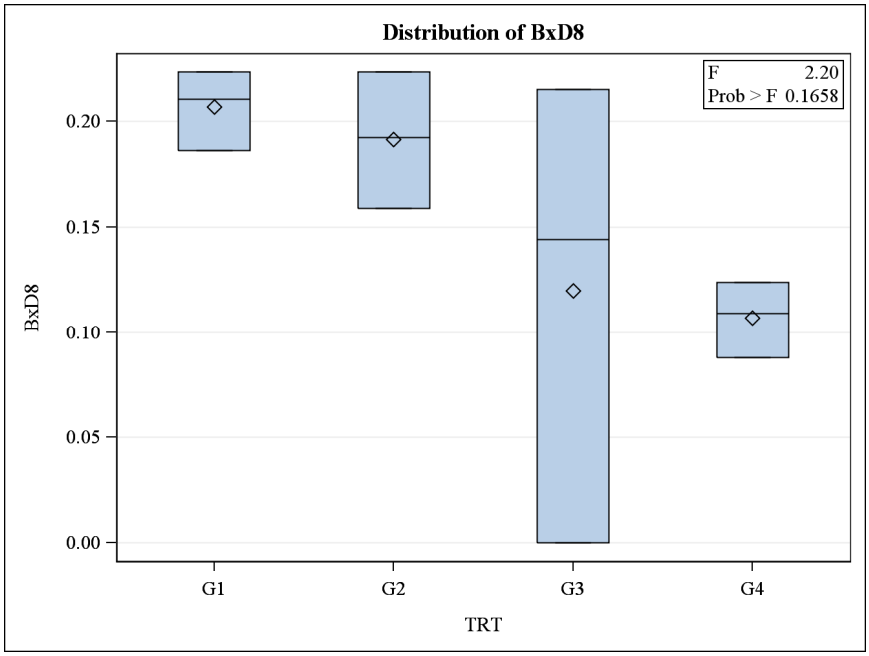

Figure 18. Illustrative figure of the result of expanded blastocysts (BxD8) evaluated on D8 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 2 (SOF medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

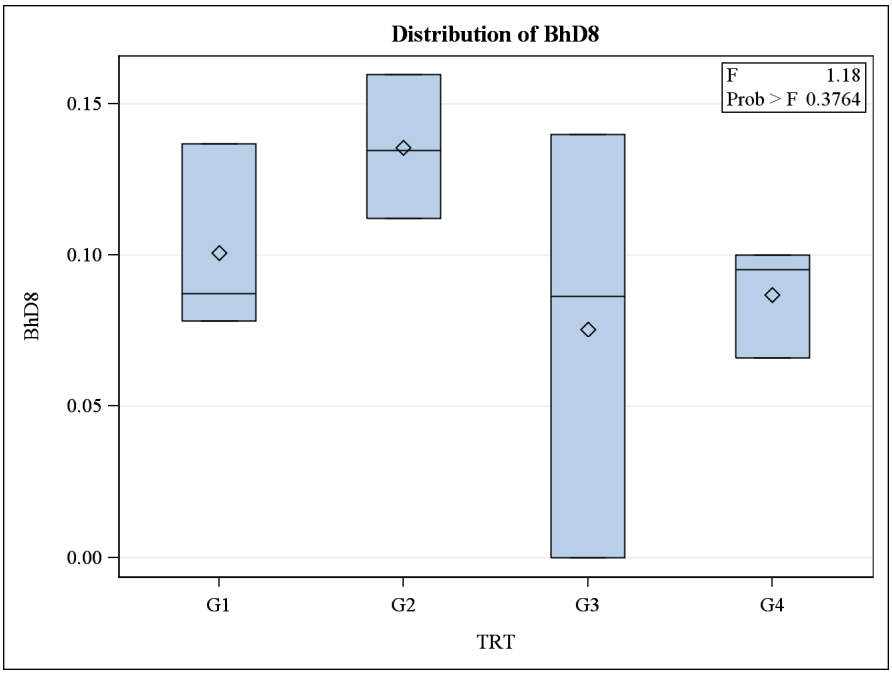

Figure 19. Illustrative figure of the result of hatched blastocysts (BhD8) evaluated on D8 of embryo development, in the four different treatment groups (G1, G2, G3 and G4) – experiment 2 (SOF medium supplementation). ◇ = average; bar = SD. There was no significant difference between the groups regarding the parameter analyzed by ANOVA method ($p<0.05$).

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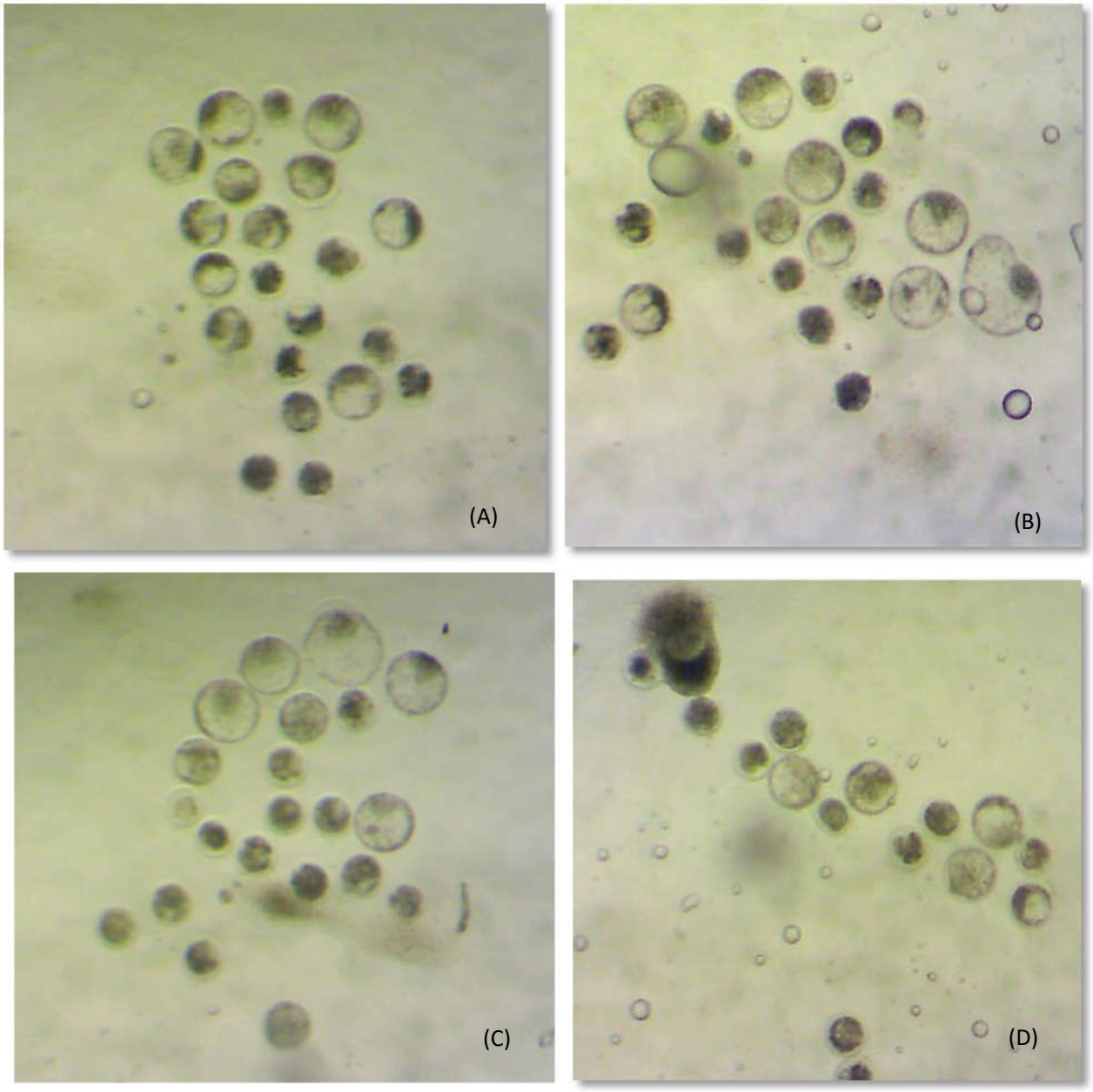

Figure 20. Illustrations of the *in vitro* bovine embryos developed in one of the Cultive drops (experiment 2 / IVP batches 2.1) of each treatment Groups, (A)= group ; (B) = group 2; (C) = group 3; (D) = group 4, respectively. Disregard the size difference observed between the structures, as it is due to the enlargement/reduction of the photo, depending on the number of embryonic structures present in each photo. The objective here is illustrate the morphological characteristics and degrees of the embryos obtained.

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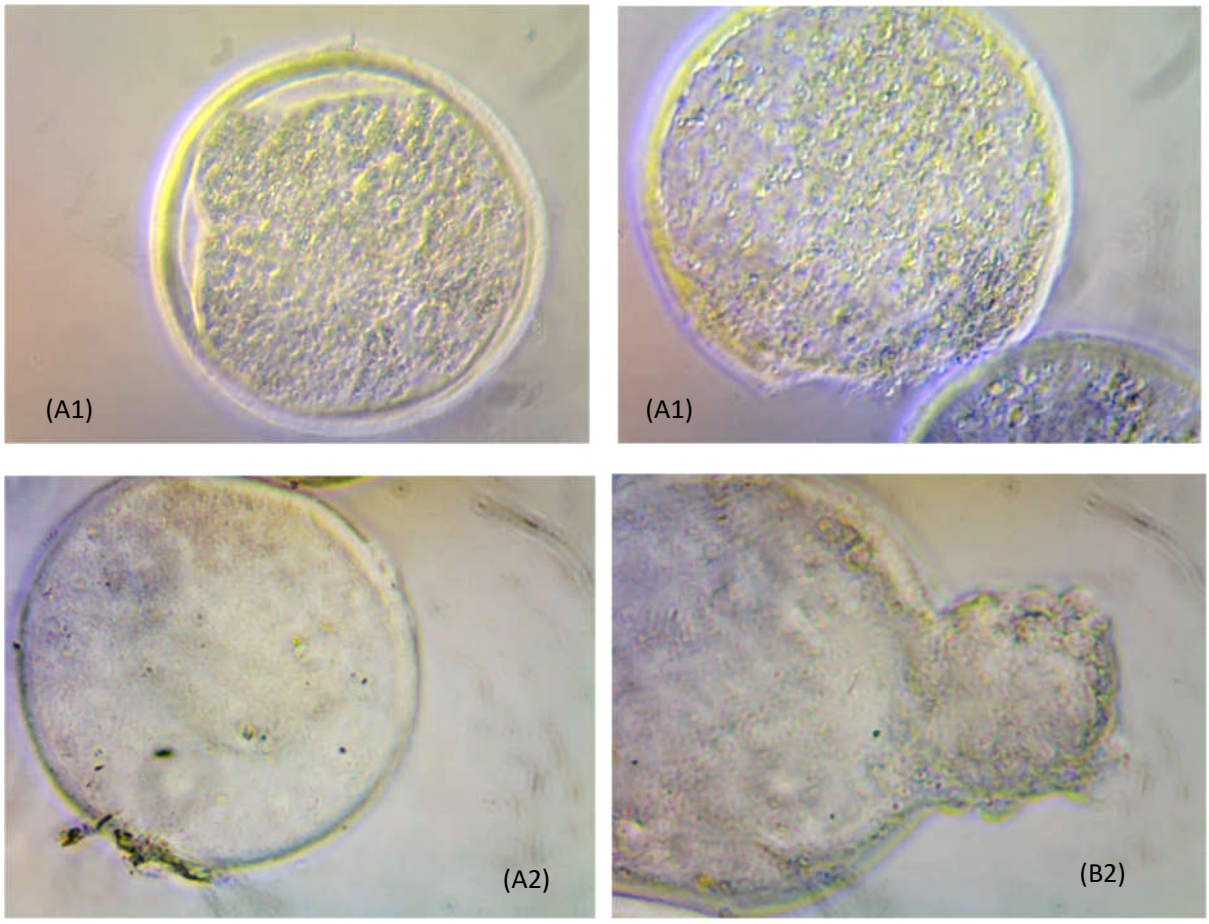

Figure 21. Illustrations of contrast microscope 200X, (A1) blastocysts of the experiment #1; (A2) expanded blastocysts of the experiment #2; (B) hatched Blastocyst of the experiment #2, all DAB stained *in vitro* bovine embryos cultivated without rHGAL-1 supplementation (Control Group).

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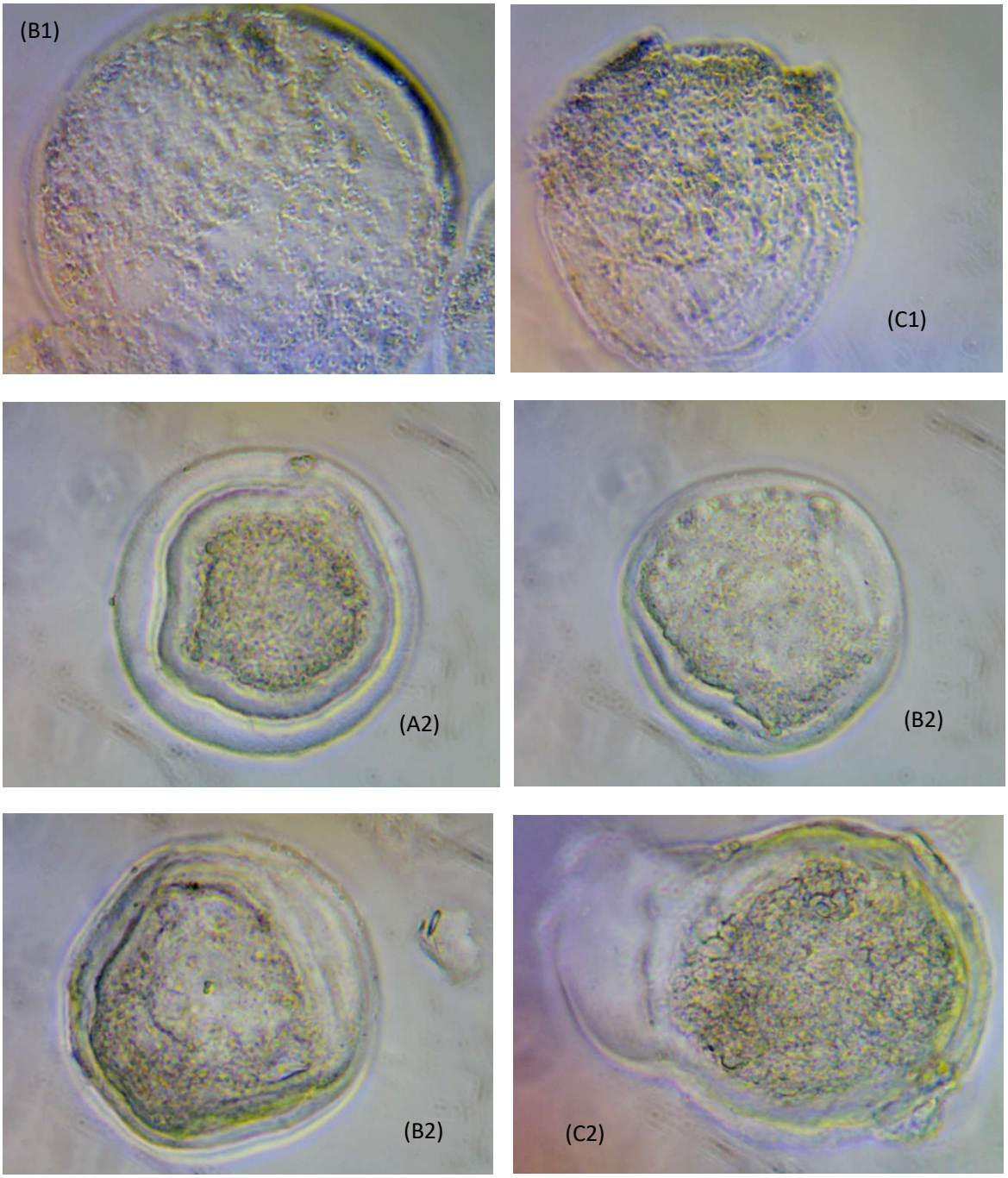

Figure 22. Illustrations of contrast microscope 200X, (B1) expanded blastocyst of the experiment #1; (C1) hatched blastocyst of the experiment #1; (A2) blastocyst of the experiment #2; (B2) expanded blastocysts of the experiment #2; (C2) hatched Blastocyst of the experiment #2; all DAB stained *in vitro* bovine embryos and with 2µg/mL of rHGAL-1 supplementation (Group 2).

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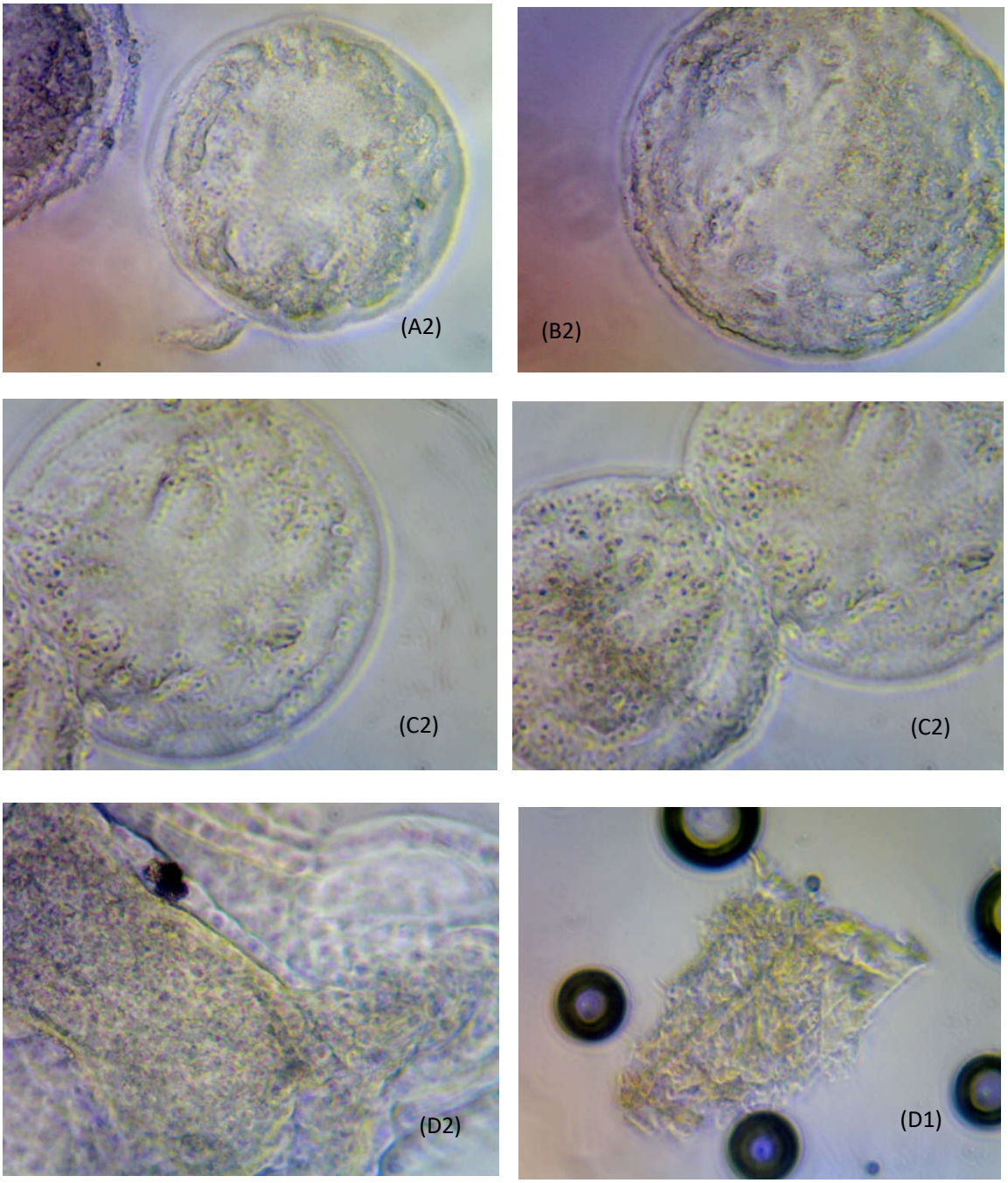

Figure 23. Illustrations of contrast microscope 200X, (A2) blastocyst; (B2) expanded blastocyst; (C2) hatched Blastocysts; (D2) trophoblast of a hatched blastocyst (bent structure when fixing to the blade), all DAB stained *in vitro* bovine embryos both DAB stained *in vitro* bovine embryos (experiment 2 / IVP batch 3) and with 20µg/mL of rHGAL-1 supplementation (Group 3).

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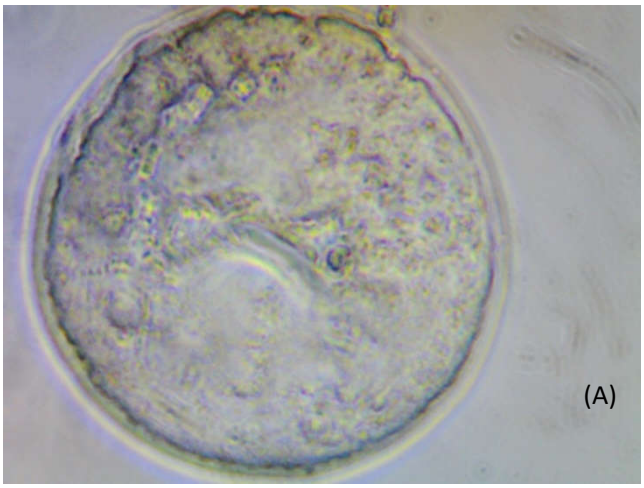

Figure 24. Illustrations of contrast microscope 200X, (A) blastocysts; (B) expanded blastocyst; (C) hatched Blastocysts;, all DAB stained *in vitro* bovine embryos both DAB stained *in vitro* bovine embryos (experiment 2 / IVP batch 3) and with 40µg/mL of rHGAL-1 supplementation (Group 4).