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Comparison of Recombinant Cumulase with Bovine Derived Hyaluronidase for Oocyte Denudation before ICSI in Sibling Oocytes

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ABSTRACT

Assisted Reproductive Technology (ART) with Intra-cytoplasmic Sperm Injection (ICSI) commonly utilizes Bovine-Derived Hyaluronidase for oocyte denudation. The emergence of Recombinant Cumulase as an alternative merit an examination of its impact upon ICSI outcomes. We compared the effects of Recombinant Cumulase and Bovine-Derived Hyaluronidase on oocyte denudation during ICSI, investigating impact upon fertilization rates, blastulation rates, and euploidy rates, aiming to improve clinical practice and optimize ART for patient outcomes. This was a retrospective observational study involving 59 patients undergoing ICSI conducted at First IVF Fertility Centre, Abu Dhabi, United Arab Emirates. Oocytes from each patient were divided one group which employed Recombinant Cumulase, and the other group utilised Bovine-Derived hyaluronidase. Parameters such as age, number of oocytes retrieved, denudation outcomes, and euploidy rates were assessed to determine enzyme impact. Comparative analysis revealed no substantial difference in fertilisation rates or embryo development (blastocyst rate) between recombinant Cumulase and bovine-derived Hyaluronidase. However, the recombinant Cumulase group exhibited significantly higher ($p<0.05$) rates of normal euploid embryos (Recombinant Cumulase-48.5%; Bovine Derived Hyaluronidase- 38.0%), indicating a potential advantage. However, the results are from small cohort and need to be validated with large sample size. Selecting enzymes for ART procedures is challenging. Although no significant differences were observed in fertilisation rates, the Recombinant Cumulase group treated oocytes exhibited yielding higher normal euploid embryo rates. Not many studies have looked at the Euploidy rates with use of different enzymes for oocyte denudation. Further investigations are essential to refine best practices and advance patient outcomes in assisted reproduction.

INTRODUCTION

Reproductive health is a right of every citizen (Mukurunge *et al.*, 2023). Infertility impacts approximately 15% of couples and has been present in around 20-30 % of males worldwide (Al Khaldi *et al.*, 2023). Assisted Reproductive Technology (ART) has witnessed remarkable advancements, particularly with the Intra-Cytoplasmic Sperm Injection (ICSI) as a groundbreaking technique in addressing a myriad of infertility factor (Evison *et al.*, 2009). Within the intricate process of ICSI, a critical procedural step involves the denudation of oocytes, whereby the cumulus cells encapsulating the oocyte are removed, facilitating clear visualisation and assessment of oocyte maturity and quality (de Moura *et al.*, 2017). Hyaluronidase is an endoglycosidase that breaks down hyaluronic acid into monosaccharides by cleaving its glycosidic bonds; additionally, to some extent, it also breaks down other acid mucopolysaccharides in the connective tissue. (Jung *et al.*, 2020). Traditionally, bovine-derived Hyaluronidase has been the primary enzyme utilised for degrading the hyaluronic acid-rich cumulus cell matrix, coupled with mechanical pipetting for cell removal (de Moura *et al.*, 2017). However, concerns surround the purity of bovine-derived Hyaluronidase and the potential risk of pathogenic transmission associated with its use, prompting a quest for alternative enzymes for oocyte denudation in the ICSI procedure (Evison *et al.*, 2009). In the past, medical hyaluronidase was extracted from bovine or sheep testicles and used without purification. However, the mammalian hyaluronidase obtained in this way was low in purity and contained components that could cause an immune response. Subsequently, purification of mammalian hyaluronidase was implemented as a processing step, and microbial hyaluronidase obtained from Streptococcus agalactiae bacteria was also used to reduce side effects. (Jung *et al.*, 2020). The emergence of Cumulase, a recombinant form of Hyaluronidase, presents a promising alternative. Its manufactured origin potentially mitigates concerns related to pathogenic transmission and purity associated with bovine-derived Hyaluronidase (Furuhashi *et al.*, 2010). Given the significant impact that the choice of enzyme for oocyte denudation may have on successful fertilisation, subsequent embryo development, and clinical outcomes, an in-depth exploration into the comparative impacts of recombinant and bovine-derived Hyaluronidase is

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warranted (Tsampras *et al.*, 2022). Cumulase acts as a protease, breaking down the protein matrix enveloping the cumulus cells, while Hyaluronidase disintegrates the hyaluronic acid binding the cumulus cells together (de Moura *et al.*, 2017). Studies comparing the efficacy of recombinant Cumulase and bovine derived Hyaluronidase for denudation have yielded varying results. Some suggest that recombinant Cumulase is more effective in removing cumulus cells and improving fertilisation rates, while others report no significant differences between the two enzymes. Nonetheless, both enzymes are widely employed in clinical practice due to their demonstrated safety and effectiveness (Taylor *et al.*, 2006, de Moura *et al.*, 2017). Despite encouraging outcomes from the use of recombinant Cumulase® as part of assisted reproductive technology (ART) ICSI treatment program, it is pertinent to conduct a comparison with conventional bovine-derived Hyaluronidase to affirm its efficacy in UAE population. This study, conducted in the Fertility Centre in United Arab Emirates (UAE), aims to scrutinise the effectiveness and safety of two types of hyaluronidases currently available on the market: Cumulase, which is recombinant, and hyaluronidase (SAGE) derived from bovine source (Furuhashi *et al.*, 2010, Tsampras *et al.*, 2022). We focus on examining how these two forms of enzymes may impact not only fertilisation rates and blastocyst development rate (blastulation rate) but also whether these effects extend to variations in euploidy rates, potentially influencing the Clinical Pregnancy Rate (CPR) and Live birth rate (LBR). We examined the effects of these enzymatic agents on sibling oocytes, fertilisation rates, blastulation rates, and crucially, euploidy rates. The evaluation of these outcomes is vital for clinicians to make informed decisions and optimise the ART process for improved patient outcomes. This study endeavours to significantly contribute to the understanding of enzymatic impacts on the ICSI process, guiding clinical practices in ART for improving successful pregnancy. Understanding the effects of these enzymatic agents on fertilisation rates, blastulation rates, and, importantly, euploidy rates is imperative in optimising the ICSI process for improvement in clinical outcomes.

MATERIALS AND METHODS

It is a retrospective observational study at the First IVF Fertility Centre, Abu Dhabi, United Arab Emirates between September 2022 and August 2023.The data utilised in this research was sourced from the internal data management system, Meditex IVF (Germany). The dataset was acquired by filtering and selecting patients who fulfilled predetermined inclusion criteria from the Meditex database. We included patients aged between 23-45 years old, who underwent intracytoplasmic sperm injection (ICSI) pre genetic testing (PGTA) procedure as part of their planned ART treatment and had undergone oocyte denudation using recombinant cumulase or bovine derived hyaluronidase (same patient had half of its oocytes denuded with recombinant cumulase and the other half with bovine derived Hyaluronidase- performed by a single senior embryologist) with recorded data on fertilisation, embryo development with blastocyst (day 5/6 embryo) formation and embryo biopsy for genetic result with normal (euploid) and aneuploid (abnormal) embryos. We excluded patients with missing or incomplete data for the relevant outcome measures or key variables of interest or who received alternative enzymes or additives (e.g. calcium ionophore) that could confound the comparison between recombinant cumulase and recombinant hyaluronidase or oocytes treated with only one of the above denudation enzyme or patients who were subjected to non-standard IVF or ICSI protocols that might introduce variability in the outcomes. Procedure in embryology laboratory involved meticulous timed denudation process was used to eliminate the cumulus cells surrounding the oocytes. In this study, we used one of the two enzymes for the denudation process with either recombinant cumulase (Cooper surgical, USA) or bovine derived hyaluronidase (Cooper surgical, USA)., for sibling oocytes from the same patient. The process of denudation was conducted by a senior embryologist to ensure consistency and minimize variability as per ASRM guidance. The removal of the cumulus cell-oocyte complex (CCOC) was done using either bovine-derived hyaluronidase (Cooper surgical, USA) or Recombinant cumulase (Cooper surgical, USA). The oocytes underwent exposure to either recombinant cumulase or bivine derived hyaluronidase approximately three hours after collection, with the exposure lasting no longer than a minute. Subsequently, any remaining substances were gently aspirated using a glass pipette, followed by full denudation using a 140 µm flexipet (RI EZ-Tip 140) immersed in warm HEPES-buffered oocyte wash solution for approximately two minutes, with a maximum duration of five minutes. Post-denudation, the oocytes were transferred to a SAGE culture dish with media and four milliliters of oil. The maturity of the oocytes (with MII oocytes) was assessed before being returned to the incubator, and the Intracytoplasmic Sperm Injection (ICSI) procedure was carried out 30 minutes later only for mature MII oocytes. The evaluation of fertilisation status was conducted 14 to 18 hours post-ICSI, where the presence of pronuclei (PN) was examined. Normal fertilisation was identified when two separate pronuclei, each with nucleoli, were observed (2PN). Eggs were discerned by their cytoplasm appearing diffuse or non-indicating a breakdown in the vitelline membrane. All oocytes were inseminated by ICSI after 40 hrs. of trigger dose in Handling Origio media w/HEPES under magnification of x400 using an inverted microscope (Olympus IX-73, Japan) and micromanipulator (Narishigie, Japan). After ICSI insemination, oocytes were group cultured in pre-equilibrated droplets of 25 µL of Sage 1-step medium (Cooper Surgical Group, USA) overlaid with mineral oil (sage, Cooper Surgical, USA) in a MEA-tested dish. All embryos were incubated in a bench top incubator (K-System) at 37°C under atmosphere around 5.5% CO_2, 5.0% O_2 and 89.5% N_2, and pH of

7.28 to 7.32. Fertilisation was checked 16-18 h after ICSI. Embryo development was evaluated only on day 5. On day 5 embryos were graded according to Gardener's classifications (Gardner *et al.*, 2000, Gardner *et al.*, 2016) with blastocyst expansion graded on a numerical scale between 1 and 6. A trophectoderm biopsy was carried out on day 5 or 6 followed by vitrification (Cryotech, Japan). Blastocysts of grade 5 BB or better were biopsied and vitrified. Biopsied trophectoderm cells were lysed and DNA was amplified by Multiple Displacement Amplification (MDA) (Harper *et al.*, 2010;Cinnioglu *et al.*, 2019). Library preparation was performed from the amplified DNA then processed on Illumina's Next Generation Sequencer, the MiSeq. Analysis was performed using Bluefuse analysis software. Numerical and structural chromosomal abnormalities were reported. The primary endpoints of this process included assessing oocyte integrity post-denudation (with mature MII oocyte) as well as evaluating fertilisation rates, characterised by the presence of 2 pro nuclei (PN) and blastulation rates. The secondary endpoint involved determining the euploidy rate using next generation sequencing (NGS) on trophectoderm cells. All statistical analyses were carried out using SAS® software. Our approach involved conducting a comprehensive descriptive analysis to summarise and delineate the data succinctly. Essential summary statistics were computed for each enzyme group separately as well as for the entire dataset, encompassing measures including mean, median, and standard deviation for all collected variables. These calculations facilitated a comprehensive grasp of both the central tendencies and the spread of data within each group. Our primary objective was to ascertain whether a significant disparity existed in the rates of successful fertilisation between the two enzyme groups. A chi-square test of independence was employed to assess the relationship between the type of enzyme used and the definitive outcome of successful fertilisation. This statistical test allowed us to determine whether the observed variations in proportions were statistically significant or merely incidental.

RESULTS AND DISCUSSION

59 patients underwent oocyte denudation using either Recombinant cumulase or bovine-derived Hyaluronidase. The total number of oocytes denuded was 690 (350 recombinant cumulase group and 340 in bovine derived hyaluronidase group). Mean patient age was 36±6 years, with an average of 16±9 oocytes retrieved per patient (Table 1)

Table 1: Summary of Characteristics and Outcomes of Oocyte Denudation with Recombinant Cumulase and Bovine-Derived Hyaluronidase.

Characteristic Mean ± std	All patients (N=59)	Min-max
Age (years)	36 ± 6.0	23 – 47
Oocytes retrieved	16 ± 9	4 - 45

	Recombinant cumulase	Bovine derived hyaluronidase
	Mean ± std.	
Number of oocytes injected	6 ± 3.1	5.8 ± 3.2
Number of oocytes fertilised	4.2 ± 2.4	4.2 ± 2.8
Number of Blastocyst	2.3 ± 2.0	2.3 ± 2.2
Number of Euploid embryos	1.1 ± 1.2	0.9 ± 1.2

The table displays the mean ± standard deviation values for various parameters for all patients.

The comparison of fertilisation and embryo development outcomes between recombinant cumulase and bovine derived hyaluronidase used in oocyte denudation for Intracytoplasmic Sperm Injection (ICSI), are shown in Figure 1.

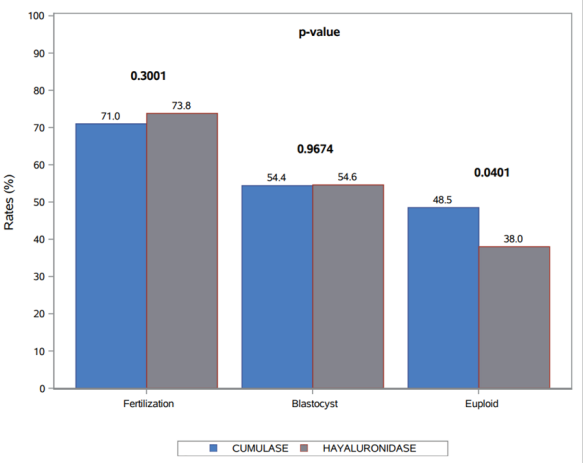

Figure 1: Comparison of Fertilisation and Embryo Development rates (%) between recombinant Cumulase and bovine-derived Hyaluronidase in Oocyte Denudation following ICSI.

The graph presents differences associated with various key parameters, including the number of oocytes injected, fertilised, resulting blastocysts, and normal embryos.

Each parameter's significance level is displayed, offering insights into the statistical significance or insignificance of differences observed between the two enzymatic treatments concerning fertilisation and subsequent embryo development outcomes during the ICSI procedure. Fertilisation rate (71.0% vs 73.8%) and blastocyst rate (54.4% vs 54.6%) between Recombinant cumulase and Bovine derived hyaluronidase treated oocytes respectively, was insignificant. However, Recombinant cumulase treated oocytes exhibited a significantly higher proportion ($p<0.05$) of euploid embryos compared to oocytes treated with Bovine derived hyaluronidase (48.5% vs 38.0%) (Figure 1; Table 2).

Table 2: Summary of fertilisation and embryo development of oocytes treated with Recombinant cumulase and Bovine derived hyaluronidase.

Recombinant Cumulase			
Injected	**Fertilised**	**Blastocyst**	**Euploid**
350	250	136	66
Bovine derived hyaluronidase			
Injected	**Fertilised**	**Blastocyst**	**Euploid**
340	251	137	52

Discussion

The cumulus-corona-oocyte complex, composed of cumulus granulosa cells embedded in a matrix of hyaluronan oligosaccharide chains cross-linked by hyaluronan binding proteins and proteoglycans, surrounds each oocyte and must be removed prior to intracytoplasmic sperm injection (ICSI). Oocyte denudation is a very important step in assisted reproductive technologies (ART). The purpose is to remove the surrounding cumulus cells from the oocyte to facilitate fertilization and subsequent embryo development. This was traditionally achieved using enzymatic digestion of the matrix with a bovine-derived hyaluronidase followed by mechanical denudation through pipetting. (Evison *et al.*, 2009). Additionally, animal-derived combinations might contain proteins impacting human oocytes differently, inducing varied responses between Bovine derived hyaluronidase types and batches. (Ishizuka *et al.*, 2014). Plant-derived preparations, like coronase were used as safer alternatives to minimize negative effects associated with bovine-derived enzymes (Parinaud *et al.*, 1998). A human recombinant hyaluronidase (Cumulase) has been developed with the intent of circumventing the problems and concerns associated with the animal origin and lack of purity of the bovine-derived form of the enzyme. (Evison *et al.*, 2009). In this study, oocyte denudation was compared between recombinant cumulase and bovine derived hyaluronidase with sibling oocytes form the same patient to see not only fertilization, blastocyst formation but importantly having euploid embryos which would increase the success of ART. The absence of variance in exposure time between both groups would strengthen the findings, highlighting the consistency and reliability of the findings. Successful denudation was confirmed by observing the extrusion of the body indicating maturation. The endpoints of this process included assessing oocyte integrity post-denudation, as well as evaluating fertilisation rates, characterized by the presence of 2 pro nuclei (PN) and blastocyst (blastulation) rates. But importantly to look at euploidy rates through NGS analysis, on trophectoderm cells. The utilization of Recombinant cumulase compared with bovine derived hyaluronidase showed higher euploidy rates, although the number of patients were small. Further elucidation on the underlying factors of bovine-derived hyaluronidase contributing to variations in euploidy rates is warranted for a comprehensive understanding of its impact on assisted reproduction outcomes. However, it's important to note that this observation is important but has to be assessed with caution based on the sample size. Further investigation with a larger sample size in a prospective study in the future is necessary to elucidate whether there is a notable difference in euploidy rates between the two-enzyme treatment. The inclusion of euploidy testing in the study holds considerable importance as it provides insights into the chromosomal integrity of embryos, thereby influencing clinical outcomes. However, the discussion concerning the results obtained from Next Generation Sequencing (NGS) is somewhat limited and warrants further elaboration. Delving deeper into the implications of euploidy rates derived from NGS data could shed light on their association with clinical pregnancy rates (CPR) or live birth rates (LBR), thus offering valuable insights into the overall effectiveness of the techniques employed in the study. As outlined in an earlier study reported that recombinant cumulase has a significant increase in fertilization compared to bovine derived hyaluronidase, (Evison *et al.*, 2009). In our study, fertilisation rates fertilization appeared slightly lower in the Recombinant Cumulase group (71.4%) compared to Bovine-Derived Hyaluronidase (73.8%), although the blastocyst formation rates showed negligible differences (54.4% for Recombinant Cumulase vs. 54.6% for Bovine-Derived Hyaluronidase). This study shows there was a significant difference in the effect of oocyte denudation time on embryo quality at assisted reproductive technology clinic. the denudation time of 3-4 hours group showed the highest result. There was no significant difference in the effect of intracytoplasmic sperm injection (ICSI) time on embryo quality at assisted reproductive technology clinic. (Tjahyadi *et al.*, 2022). We followed the denudation times as per this study. The longstanding use of Bovine-Derived Hyaluronidase in oocyte denudation raised concerns regarding low purity and variable concentrations, potentially risking prolonged exposure and consequent DNA damage to oocytes. (Jung *et al.*, 2020). Various studies comparing Recombinant Cumulase and Bovine-Derived Hyaluronidase highlighted Recombinant Cumulase safety and non-inferior efficiency, showcasing similar or improved parameters in fertilisation and embryo growth. (Taylor *et al.*, 2006, Vos *et al.*, 2008). This study emphasized Recombinant Cumulase 's efficacy and safety advantages over bovine derived forms. (Evison *et al.*, 2009) A study by on porcine oocytes confirmed the suitability of human recombinant Hyaluronidase for denudation without detrimental effects on oocyte quality. a human recombinant hyaluronidase, was found to be effective for oocyte denudation prior to microinjection, ICSI. Although animal-derived hyaluronidases are used to reduce the cost, they carry several risks from impurities as they are extracted from the testicles of animals and may contain various other proteins. (Lee *et al.*, 2021). Methodological variations across studies, including denudation techniques, enzyme concentrations, and exposure times, limit direct comparisons. Lower enzyme

concentrations potentially improved blastocyst rates and reduced costs, but further validation in laboratory settings is necessary3. Additionally, reports on increased Ca^{2+} levels and decreased embryonic potential associated with Bovine derived hyaluronidase require further investigation. (Ashibe *et al.*, 2021). Another study showed no significant differences in survival, fertilisation, or embryo development among varied Bovine derived hyaluronidase concentrations and mechanical denudation methods. (Van de Velde *et al.*, 1997). This review reported comparable fertilisation rates between recombinant and Bovine-Derived Hyaluronidase, aligning with our observations. (Rubino *et al.*, 2016). A systematic review and meta-analysis from three RCTs involving 2445 oocytes collected from 200 women were analysed, the available moderate to high quality trials found no statistical difference in fertilisation rate, embryo quality and live birth rate between the use of recombinant or bovine hyaluronidase for oocyte denudation before ICSI. (Tsampras *et al.*, 2022). However, none of the RCTs looked at Euploidy rates. Our study showed similar findings as the above systematic review and meta-analysis but we looked at the Euploid embryos. Interestingly, the percentage of normal euploid embryos was relatively higher in the Recombinant Cumulase group (48.5%) compared to Bovine-Derived Hyaluronidase (38.0%). While the results suggest promising trends favoring Recombinant cumulase in terms of higher normal euploid embryo rates, additional research with larger sample size is required to validate these findings and elucidate the underlying mechanisms. Our findings suggest that the choice of the enzyme may not significantly affect fertilisation rates or subsequent embryo development. However, ongoing research on denudation techniques, enzyme concentrations, and exposure times remains pivotal for each embryology laboratory and should constantly be monitored. Future studies should encompass confounding factors affecting oocyte quality and embryonic development, aiming to establish optimal enzyme concentrations, exposure durations, and cost-effective approaches towards higher live birth rates.

CONCLUSION

This comparative study compared the effects of recombinant cumulase and bovine-derived Hyaluronidase on oocyte denudation for ICSI in sibling oocytes from the same patient performed by a single senior embryologist. Results from the analysis of 59 patients showed no substantial differences in fertilisation rates or embryo development between the two enzymes. However, Recombinant cumulase demonstrated a statistically significant increase in normal euploid embryos, indicating a potential advantage over bovine-derived Hyaluronidase. Euploid rates had not been looked when comparing these two enzymes for denudation. However, the sample size of our study is small and findings have to be viewed with caution. A larger prospective study comparing these two enzymes for oocyte denudation should be performed with euploidy rate as an end point. These findings emphasise the complexity of enzyme selection in assisted reproduction and underline the necessity for ongoing research to optimise techniques for improving live birth rates in ART treatments. Further comprehensive investigations are imperative to ascertain subtle differences between enzymatic approaches and refine best practices in assisted reproduction for enhanced patient outcomes.

Strengths and limitations

The strength of this study is robust retrospective design with a substantial cohort with sibling oocytes of the same patient to have an accurate comparison, standardised denudation techniques, and assessment of euploidy rates. The procedure was performed by a single senior embryologist. The limitation is inherent confounders in retrospective analysis, smaller sample size and geographic-specific data

Ethics approval and consent to participate.

This study was conducted in strict adherence to ethical principles and guidelines. The research protocol and data collection procedures were approved by the Internal Research Ethics Committee of the First IVF Fertility Centre (Committee REC - FIVF-001) and the International Review Board of the Emirates of Abu Dhabi (ADHRTC-2023-114). All efforts were made to safeguard the confidentiality and privacy of their personal information throughout the study.

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Competing interest

The author(s) declares no conflict of interest.

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Data Availability Statement

The data supporting the findings of this study are stored in software Meditex IVF (Germany), which is designed specifically for In-Vitro Fertilization (IVF) clinics. Meditex IVF (Germany) serves as a tool for managing and documenting patient data within Assisted Reproduction organisations. However, it's important to note that there are restrictions on accessing this data due to licensing agreements for this study. Therefore, the data cannot be accessed publicly. Nevertheless, the authors of the study are willing to provide access to the data upon request.

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