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The Massive Impact of Ram's Sperm Starvation on the Fertilization and Blastocyst Rates in Terms of Sperm Quality and Capacitation

Saif AL-HAFEDH, Fatin CEDDEN

Department of Animal Science, Faculty of Agriculture, Ankara University, Ankara, Türkiye

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Corresponding Author: Saif AL-HAFEDH, E-mail: alhadithy@ankara.edu.tr
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ABSTRACT

During sperm incubation *in vitro*, the sperm capacitation medium must play a key role in establishing conditions that perfect the required physiological requirements for sperm metabolic activities to obtain a high *in vitro* fertilization rate ensured by the ideal development of the embryos. Therefore, sperm collected from the caudal epididymis of rams should acquire progressive motility and fertilization. This process occurs through physiological and biochemical changes known as capacitation, a prerequisite for fertilization. In this work, we have studied a new way to incubate sperm, applied for the first time in ram, using four different treatments in terms of energy substrates and different incubation methods. In this sperm energy restriction and recovery treatment, sperm was let starving for 50 min until it lost its capacity for motility, and then was provided with glucose and incubated for 60 min. In the other three treatments, the sperm was not exposed to starvation and was incubated by the standard methods of maturation and capacitation by evaluating different energy substrates. According to the obtained results, the sperm was hyperactive and showed a significant increase in the ability to fertilize oocytes in the treatment that involved starvation and then re-activation of the sperm by adding glucose, compared to other treatments. In conclusion, the effects of this treatment persisted until after fertilization, which led to the production of a high percentage of blastocysts.

Keywords: Sheep, Sperm Starvation, Blastocyst, IVF Capacitation, Rescue Protocol

1. Introduction

The use of assisted reproductive technologies in small ruminants, such as sheep, allows for rapid and sustained growth in animals of genetic quality, increasing sheep production efficiency (Zhu et al. 2018). However, the in vitro embryo production (IVP) system in sheep lags behind its *in vivo* counterpart and *in vitro* systems produced in other mammalian species in terms of efficiency and quality, limiting the advancement of this promising technology (Ledda & Gonzalez 2018; Ledda et al. 2019). Many essential aspects limit sheep IVP, including the environment supplied by sperm capacitation, in vitro fertilization (IVF), and poor embryo culture conditions, resulting in low numbers of viable blastocysts for transfer (Amiridis & Cseh 2012; de Souza et al. 2014; Souza et al. 2021). IVF in sheep occurs through several steps. First, sperm for IVF are obtained from the epididymis of slaughtered rams, as slaughterhouses are one of the cheapest sources of biological material collection (Hajihassani et al. 2019; Merati & Farshad 2020); secondly, sperm should undergo a process known as capacitation, characterized by a sequence of physiological and biochemical processes (sperm membrane destabilization, motility changes, and acrosome reaction). Although in vitro capacitation can be accomplished by incubating sperm in a simple medium containing a capacitation-supporting component at a specific temperature and pH level, however, the media currently utilized for sperm capacitation is not entirely appropriate for IVF (García-Álvarez et al. 2015; Umehara et al. 2018; Roldan 2019). Cross-reactivity between metabolic and signaling pathways is required for sperm capacitation (Goodson et al. 2012). Consequently, in a sperm capacitation medium (CAP), energy substrates and chemicals that increase sperm motility, capacitation are required; these typically include heparin (García-Álvarez et al. 2015), glucose (Leahy et al. 2016), inositol (Vazquez-Levin et al. 2020), caffeine plus heparin (El-Shahat et al. 2016).

An appropriate supply of adenosine triphosphate (ATP) for sperm is a prerequisite for many processes required for successful fertilization (Visconti et al. 2011). Sperm motility consumes over 70% of total ATP (Bohnensack & Halangk 1986), making sperm more energetically demanding than other cell types (Garrett et al. 2008). Several investigations in several species have found strong links between sperm ATP concentration and sperm motility and swimming velocity (Tourmente et al. 2015), and reductions in specific sperm characteristics are linked to decreases in internal ATP levels (Tourmente et al. 2019). Thus, ATP consumption rate is essential to sperm performance (Sansegundo et al. 2022). Studies in primates (humans and chimpanzees) (Anderson et al. 2007), felids (Terrell et al. 2011), and strains of laboratory mice (Odet et al. 2013) found the main pathway used for ATP production in sperm differs between species. Different methods for improving mice sperm functionality have recently been reported. The first method required transient exposure to the Ca2+ ionophore A23187 (Sánchez et al. 2021), while the second required sperm incubation without energy nutrients (starvation) (Navarrete et al. 2019). Both methods were linked to a loss of motility followed by a rescue step involving the removal of ionophores or the addition of energy nutrients.

IVF requires crucial capacitation changes, which by utilizing intracytoplasmic sperm injection can be overcome, but there are some extremely obstacles in applying this technique in sheep-like it being economically expensive. This study aimed to design new strategies of capacitation by starving ram's sperm *in vitro* and estimate the effects of its quality. The absence of previous studies of ram sperm in this area boosts our study goals by using a CAP devoid of energy nutrients and glucose reintroduction after the sperm has ceased to move to determine how these starvation and rescue protocols help them to become more fertile and enhance embryonic development.

2. Material and Methods

The study was conducted in the Reproductive Biology and Animal Physiology Laboratory at Ankara University, Faculty of Agriculture, Department of Animal Science. From February 1, 2021, to January 1, 2021. The experiment was repeated seven times, in which 225 ovine ovaries were collected randomly from 120 slaughtered ewes and 27 fresh testes rams in the slaughterhouse. Although the sheep were of different ages, their reproductive status was unknown before slaughtering.

2.1. Sample collection

2.1.1. Testes collection

Fresh testes-epididymides were recovered in the scrotal sacs of mature rams slaughtered at an abattoir in Ankara, Türkiye. Individually packed testes were placed on ice and brought to the laboratory in an ice chest 1 to 2 h post-mortem. The epidermis tail was rinsed with phosphate-buffered saline (PBS) (P-5493, Sigma) to eliminate blood clots and debris upon arrival at the laboratory. The epididymis samples were then kept refrigerated at 4 °C until processing. Then ram epididymides were manipulated 18 h after the post-mortem.

2.1.2. Collection of ovaries

Ovaries were also collected from an abattoir in Ankara, Türkiye. They were placed in a cooling box containing 0.9% saline solution at 4° C and transferred to the laboratory within 1-2 h. First, the ovaries were separated from all the surrounding ligament tissues. Then, all were washed for the first time in a PBS (P5493, Sigma) to remove blood clots and remaining dirt, wash twice with distilled water, and finally put in a sterile glass container containing the oocyte collection medium (OCM).

2.2. Sperm Collection and preparation

After 18 h of transport to the laboratory, the epididymis was placed in a sterile Petri dish. HEPES-TL was injected into the epididymal tail by using a G-18 needle. The surface of the epididymis tail was removed via a sharp scalpel, and epididymis contents were withdrawn by using a sterile syringe of 5 mL (Lone et al. 2011). For sperm maturity, cauda epididymides contents were collected and disseminated in four different treatments (Table 1). The first treatment was 100 µL of sperm was diluted with 2 mL of the non-CAP containing glucose (G7021, Sigma); placed for 60 minutes in the incubator at 37 °C, 5% CO_2, then heparin (P4562, Sigma) was added to it and placed in the incubator for 50 minutes at 37 °C, 5% CO_2. The second treatment was performed by adding 100 µL of sperm to the CAP, which contains bovine serum (A3311, Sigma) and glucose, and the sperm was incubated as in the first treatment. In the third treatment 100 µL the contents of the epididymis were added to media devoid of all nutrients (which do not contain glucose). Once sperm became motionless, 2 mL of the sperm energy recovery medium (RSE) (glucose and heparin) were added to it and placed in the incubator for 50 minutes at 37 °C, 5% CO_2. Finally, in the fourth treatment, the contents of the epididymis were added to the minimum essential medium (MEM) culture medium, and the sperm has incubated as in the first treatment. The quality of the sperm was assessed by individual and

collective sperm movement. Sperm with an individual motion of less than 60% were rejected. The presence of the protoplasm droplet at the end of the sperm tail was estimated as evidence of sperm maturity and capacitation.

Table 1- Composition of sperm maturity and capacitation media

Component (g)	***Non-CAP***	***CAP***	***RSE***	***MEM***
NaCl	6.976	6.976	6.976	10%
KCl	0.356588	0.356588	0.356588	-
$CaCl_2 \cdot 2H_2O$	0.2513871	0.2513871	0.2513871	-
KH_2PO_4	0.161959	0.161959	0.161959	-
$MgSO_4 \cdot 7H_2O$	0.2933052	0.2933052	0.2933052	-
$NaHCO_3$	2.1061	2.1061	2.1061	-
Na pyruvate	0.11	0.11	-	-
*Supplements				
Glucose	1.0017	1.0017	-	-
HEPES	4.766	4.766	4.766	-
BSA	-	0.005	-	-

*On the day of use, supplements were added capacitation media

2.3. Oocytes collection

The ovaries were collected by long and sterile forceps and placed in an OCM. The surface of each ovary was sliced with a sharp blade. After that, the ovaries were transferred to a sterile glass container containing OCM. Oocytes were assessed under the microscope (LEICA DM IL LED; Wetzlar, Germany) to select oocytes to conduct our research. A total of 800 oocytes were cultured in this research; 200 oocytes were distributed per treatment.

2.4. Oocytes evaluation

Based on the number of cumulus cells and cytoplasmic homogeneity, the quality of the collected oocytes was graded as good (class A), fair (class B), and poor, as described by Wani et al. (2000).

2.5. Oocytes maturation

After collection, assessment and classification, all oocytes from class A and B were selected. Oocytes were washed twice in OCM before being incubated for 24 h in TCM199 media supplemented with 10 µL/mL Na Pyruvate, 4 µL/mL Gentamycin, 100 µM Glutamax, 1 IU/mL follicle-stimulating hormone, 1 IU/mL luteinizing hormone, 10 mg/mL Estradiol, and 10% fetal calf serum covered with mineral oil at 38.5 °C, 5% CO_2, and 90% humidity. At the end of the maturation period, plates were examined via an inverted microscope (LEICA DM IL LED; Wetzlar, Germany), then the appearance of the first polar body was estimated as an indication of oocyte maturation.

2.6. In Vitro fertilization

After capacitation, the sperm were centrifuged for 6 min at (500 g), the supernatant was removed, and the sperm were resuspended in 1 mL of sperm CAP. Then IVF drops containing mature oocytes (20-45 oocytes) were subjected to IVF by four treatments for the sperm capacitation media, each treatment separately, with sperm concentrations of $10x10^6$/mL. In an incubator with 5% of CO_2, fertilization dishes were kept at 38.5 °C for 18-22 hours after fertilization, after that fertility rates were obtained by dividing total number of fertilized oocytes by total number and multiplying by one hundred.

2.7. In Vitro culture

Zygotes were transferred into SOF media. The media containing zygotes were incubated at 38.5 °C, 5% CO_2, and 90% humidity. The embryonic development was monitored every 24 hours (Figure 1) with the replacement of 50% of the media with a new sterile medium every 24-hour. During the monitoring time, undeveloped zygotes were removed in order to maintain only developing embryos in the media.

2.8. Assessment of sperm (live/dead)

The integrity of the sperm plasma membrane was assessed using a combination of Hoechst 33,342 and propidium iodide (PI) staining (Sutradhar et al. 2010). 5µL/mL of Hoechst 33,342 was added to each tube containing sperm suspension in the medium and was incubated at 37 °C for 15 minutes. After that, the suspension was given 50 µL of PI and incubated for 5 minutes at 37 °C. Then, a 20 µL sperm suspension was put in a 1:1 glycerol/PBS solution on a glass slide and covered with a cover slip (Eskandari & Momeni 2016). The slides were then examined under a microscope (LEICA DM IL LED; Wetzlar, Germany) with the proper excitation and emission filters. PI red staining on the head of sperm indicates a damaged plasma membrane, whereas Hoechst blue staining indicates a healthy membrane (Figure 2).

2.9. Statistical analysis

Experiment was replicated seven times. Data were analyzed by one-way ANOVA using SPSS version 23.0 statistical software and results are presented as the mean (± SEM). Duncan's multiple range test was carried out for comparing means, and p-value <0.01 was considered significant.

3. Results and discussion

The study results showed in Table 2 a significant increase in the percentage of fertilization ($p<0.01$) in RSE compared to the other treatments (79.95%). There were no significant differences between CAP and non-CAP in the fertilization percentage of oocytes with 59% and 53%, respectively. These were followed by MEM with a 34% fertilization rate.

Table 2- The impacts of sperm capacitation treatment on fertilization rate (%)

Treatment	*Number of oocytes*	*Number of fertilized oocytes*	*Fertilization rate (%)*
non-CAP	200	106	53.00±3.63[b]
CAP	200	118	59.00±3.84[b]
RSE	200	159	79.95±7.25[a]
MEM	200	68	34.00±6.69[c]

[a],[b]Different superscripts indicate significant differences between sperm capacitation treatments ($p<0.01$)

As shown on Table 3, a significant superiority was observed ($p<0.01$) for RSE regarding cleavage rate with 76.72%. The other three treatments (CAP, non-CAP and MEM) have shown 50.84%, 45.28%, and 19.11% of cleavage rate, respectively. The treatment MEM exhibited the lowest cleavage percentage (19.11%) compared to the others.

Table 3- The impacts of sperm capacitation treatment on embryonic development rate (%)

Treatment	*Number of fertilized oocytes*	*Cleavage rate (%)*	*Morula rate (%)*	*Blastocysts rate (%)*
non-CAP	106	45.28±2.30[b]	27.35±1.78[bc]	7.43±2.07[b]
CAP	118	50.84±3.082[b]	38.13±2.00[b]	11.11±3.34[b]
RSE	159	76.72±8.79[a]	59.74±7.64[a]	27.70±4.00[a]
MEM	68	19.11±1.14[c]	2.94±0.54[c]	0

[a],[b]Different superscripts indicate significant differences between sperm capacitation treatments ($p<0.01$)

Morulae rate was a higher ($p<0.01$) in RSE treatment (59.74%) compared to the other treatments. CAP has shown similar result with non-CAP treatment. However, the rate for non-CAP was not different from MEM treatment, which was found as 27.35 %, and 2.94%, respectively. However, MEM showed a significantly lower Morulae rate (2.94%) in comparison with RSE ($p<0.01$).

A significant superiority ($p<0.01$) was observed in RSE treatment regarding the rate of blastocysts conformation (27.70%). Moreover, results indicate that the rate of the blastocyst formation was similar in CAP and non-CAP, which were found as 11.11% and 7.43%, respectively.

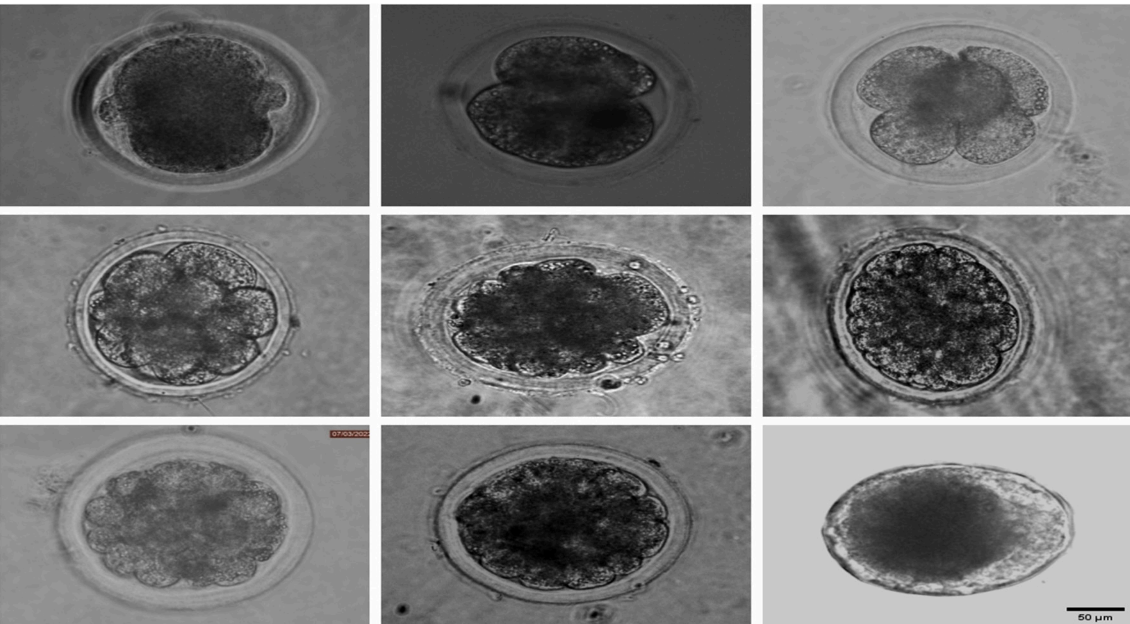

Figure 1- Representative images of IVF-derived embryos development

For well-known substances, such as glucose, the role and the mechanism on capacitation are still poorly understood (Jin & Yang 2017). Although some publications report that a lack of glucose affects negatively capacitation (Hidalgo et al. 2020; Qiu et al. 2016), others claims that glucose per se has no effect on capacitation *in vitro* (Zhu et al. 2019). Glucose is carried into the sperm cell by glucose transporter and helps generate ATP through glycolysis. ATP is used for sperm hyperactivation motility and permeability transition pore (Bucci et al. 2011; Jin & Yang 2017). During the capacitation, different conditions for sperm incubation have an impact on the development of an embryo (Küçük et al. 2020; Ferré et al. 2017). Energy substrates, ions, and a cholesterol-binding source in a particular medium can be used to achieve capacitation *in vitro* (Sajeevadathan et al. 2019; Visconti et al. 2011). A TYH medium containing only pyruvate and glucose as energy substrates can achieve capacitation and IVF in mice (Toyoda & Yokoyama 2016).

In this study, it was observed that ram sperm becomes immotile when cultured without some exogenous nutrients such as glucose and pyruvate for less than 50 min and motility can be restored by adding these energy sources.

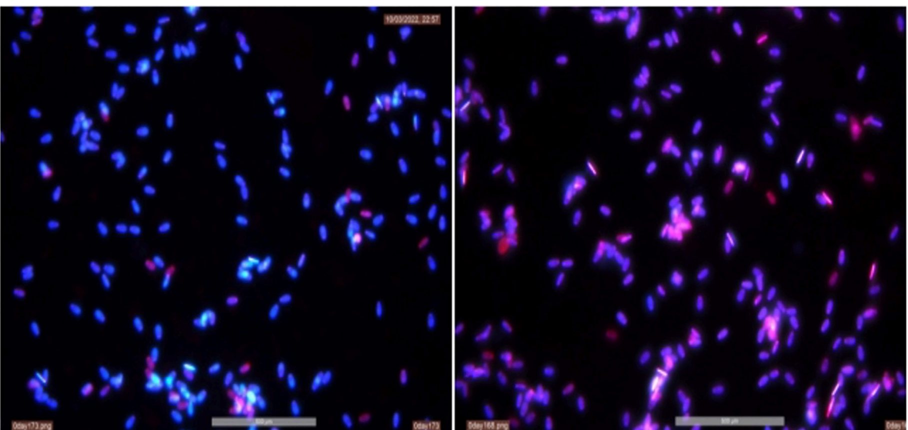

Figure 2- Ram sperm stained fluorescently. On a merged image, sperm cells with red-colored heads are dead sperm

Once re-activated, starved sperm performed higher IVF rates and hyper activation after being recovered. This increase in sperm functional properties has an unclear molecular-based mechanism. Two possible explanations may be suggested: the molecules that impair capacitation efficiency are consumed during the starving phase owing to a shortage of energy sources, or molecules that improve capacitation are increased after rescue. Another hypothesis is that the starvation followed by the salvage procedure produces metabolite changes, increasing specific metabolites that may encourage the formation of hyperactive sperm motility and fertilization (Otasevic et al. 2020). Surprisingly, oocytes fertilized with the RSE treatment produced a higher proportion of two-celled embryos that reached the

blastocyst stage. Moreover, blastocysts obtained with the RSE treatment showed an excellent growth rate and a higher number of total cells at 192 h after fertilization, both of which are indicators of optimal implantation potential (Binder et al. 2015). It has been suggested that sperm incubation conditions for IVF can alter embryonic development, which aligns with our findings (Li et al. 2006; Gu et al. 2013; Zhao et al. 2017; Zheng et al. 2018). The molecular basis for embryo post-fertilization effects, on the other hand, is unknown. The post-fertilization effects observed after RSE treatment may be attributed to epigenetic modifications in the male gamete (Figure 3). Epigenetic markers such as histone modifications and DNA methylation, essential in embryo development, are distributed differently in male and female gametes, indicating that environmental factors can alter epigenetic processes, affecting gene expression and having a significant impact on development (Zeng & Chen 2019; Canovas et al. 2017). Small non-coding RNAs in sperm may play a function in non-genomic paternal trait transfer (Gross et al. 2019).

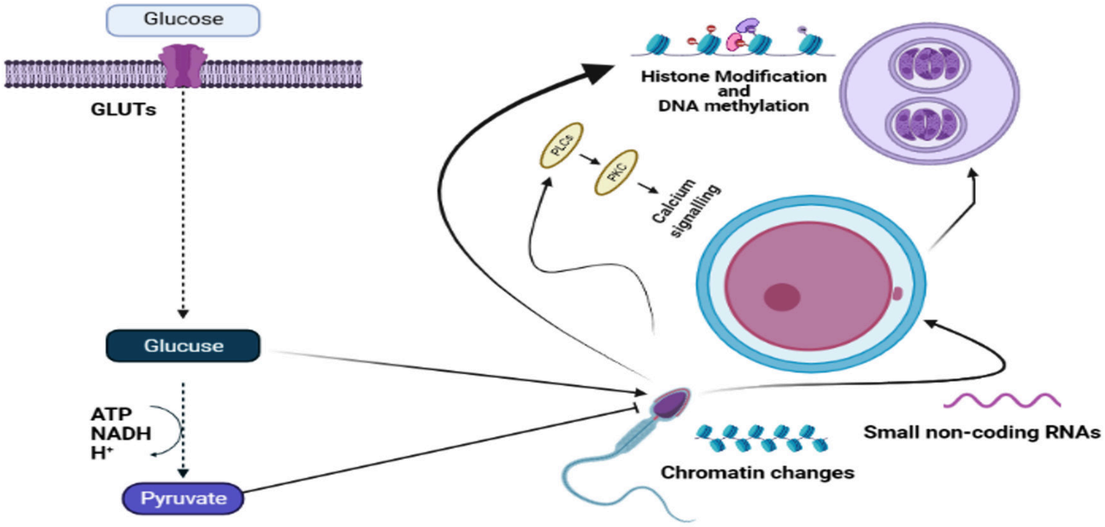

Figure 3- Possible molecular basis for the effect of glucose on capacitation and post-fertilization

One theory is that RSE treatment influences post-fertilization development by altering RNA levels, specifically the pool of non-coding RNAs, and another theory is that RSE treatment causes chromatin changes in sperm before fertilization, which has a direct impact on embryonic development (Gannon et al. 2014). These results imply that as ram sperm moves through the layers of cumulus cells and ZP, they undergo modification that assists or promotes nuclear reorganization, allowing phospholipase C zeta (PLCζ) to be exposed and activated (Saleh et al. 2020; Kashir et al. 2013). Our data has shown that treatment of ram sperm by RSE improved their capacity to activate oocytes after fertilization. We suppose that RSE circumstances cause modification of the head in ram sperm, promoting nuclear decondensation and PLCζ release, probably similar to what happens during natural sperm entrance and fertilization. In all assisted reproductive technologies, viable embryos which are capable for implantation are the most crucial step for a successful pregnancy. Implantation failure is more familiar with poor-quality embryos. These findings show that sperm quality before fertilization affects embryo growth. Similarly, sperm treated with RSE should provide improved embryo development in early stage; however, the treatment of RSE should improve overall fertilizing sperm quality boosting the likelihood for producing embryos of higher quality, rather than optimum sperm selection. Generally, sperm energy restriction and recovery RSE is a new technique that improves sperm fertilization and embryo growth after IVF.

4. Conclusions

Our findings highlight several crucial yet unanswered problems in reproductive biology and embryo development disciplines that require additional investigation. It was also demonstrated that sperm capacitation conditions persist after fertilization, opening new avenues for research into the association between sperm capacitation and embryo development in the early stages. Overall, sperm function become improved due to treatment with RSE before and following fertilization, which could be applied in other mammals, including humans, with substantial implications for ART operations.

Data availability: Data are available on request due to privacy or other restrictions.

Authorship Contributions: Concept: S.A-H., F.C., Design: S.A-H., F.C., Data Collection or Processing: S.A-H., F.C., Analysis or Interpretation: S.A-H., F.C., Literature Search: S.A-H., F.C., Writing: S.A-H., F.C.,

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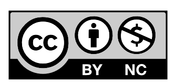

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