

## The Benefits of Density Gradient Centrifugation for Sperm Preparation for ICSI, as Well as the Many Forms of Density Gradient Preparation and their Applications in Bacteriospermia and Contaminated Samples Study in Male and Female Infertile Couples

Bezar Ghan VV\*, Deepthi, Vineeth Reddy Lekkala, Geetha Reddy, Ram Reddy and Raja Gopal K

Amrutha Fertility Centre, Yenapoya Medical College, Yenapoya University, India

\*Corresponding Author: Bezar Ghan VV, Amrutha Fertility Centre, Yenapoya Medical College, Yenapoya University, India.

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### Abstract

Human sperm is very diverse, varying not just from person to person but also from sample to sample. According to the World Health Organization, spermatozoa quality is determined by the motility, concentration, and shape of the sperm in each given sample (WHO).

**Keywords:** Density Gradient; ICSI; Bacteriospermia

### Introduction

A popular method adopted to separate sperm from seminal fluid and other debris is by adopting centrifugation techniques. Centrifugation involves the production of centrifugal forces by rotating about a fixed point. There are three kinds of centrifugation techniques: differential, density gradient, and ultracentrifugation [1]. The principal behind separating sperm from the seminal fluid is to get a high concentration of motile sperm in the pellet which can be used for assisted reproductive techniques (ART). This mini-review aims to look at density gradient centrifugation (DGC), its different types, and its use in infectious diseases.

### Principal

Density gradient centrifugation (DGC) acts on the principle of separating particles based on their different densities or specific gravity by centrifugal forces. This is achieved by using a centrifugation machine. DGC is an important technique used in the ART laboratory to prepare sperm samples for intrauterine insemination (IUI) and intracytoplasmic sperm injection (ICSI). During the centrifugation process, the sperm reaches a point where their density matches that of the gradient and they will be distributed accordingly. The denser the sperm is, the further does it pass through the different gradients and reach the bottom of the test tube to form the pellet. In this way, denser sperm are seen in the pellet, and sperm that are less dense, round cells, and debris are seen in "rafts" in the supernatant. Interestingly, it is observed that sperm with progressive motility is commonly seen in the pellet, whereas immotile sperms remain in the supernatant. It should be stressed that even though this is the case, sperm cells are separated based on their densities and not motility. The result is a

highly concentrated semen sample in the pellet that contains the normal spermatozoa. For two-layer DGC, one can use commercially available gradients like Puresperm (Nidacon International AB, Gothenburg, Sweden) which are silane-coated silica particles. During DGC for sperm, the 80% gradient (lower layer) is first layered slowly along the sides of the test tube, followed by the 40% (upper layer) overlaying the 80%. Care should be taken that both gradient layers do not mix. Finally, the semen is overlaid above the 40% gradient. This tube is then centrifuged at high speeds like 300g for 20 minutes. After centrifugation, the pellet will contain the gradient-washed denser sperm [1,2]. Normal spermatozoa have a density of 1.12 g/mL, whereas abnormal sperm have densities between 1.06- 1.09 g/mL. Hence it is observed that only "normal" sperm cells can penetrate through both layers of gradients to reach the bottom of the test tube that is prepared for DGC. One of the major drawbacks seen by using DGC is that if the sperm cells are centrifuged for longer periods, it could cause the formation of reactive oxygen species (ROS) and can cause DNA fragmentations [2].

### Types of DGC

There are two kinds of DGC: rate zonal centrifugation and isopycnic centrifugation. Rate zonal centrifugation is a type of DGC that separates particles based on their sedimentation coefficient, sizes, and shapes. The sample to be separated is slowly and carefully layered on top of previously layered gradients and is then allowed to centrifugate. All the particles will move to different parts of the gradient depending on their size. Larger and spherically symmetrical particles will move faster and sediment at the bottom of the tube because their large size allows them to move through the viscous layers. The smaller particles remain on the top as they cannot penetrate the viscous gradients. This method has a few limitations such as the sample is less concentrated during separation and takes up a small area of the tube at the start of fractionation. Rate zonal centrifugation is used to separate protein molecules that differ in their molecular weights but have the same densities [3,4]. The other kind of DGC is the isopycnic or equilibrium density separation that is based on the particle density instead of the size. This kind of centrifugation is independent of time and occurs at very high speeds. Isopycnic centrifugation is used for the separation of nucleic acids [3,4].

### Step density gradient centrifugation

Discontinuous or step DGC is confined to isopycnic separations that form sharp bands. They can be either used as an overlaying of an underlying method. The overlaying method involves adding the gradients from the bottom of the test tube, to the top. This means the denser gradients are at the bottom followed by layering with the less dense gradients and finally the sample. The underlying method utilizes the opposite. The lightest solution is first layered followed by the denser solutions [5].

### Continuous density gradient centrifugation

Continuous DGC (CDGC) differs continuously and smoothly. The shapes of CDGC can either be linear, concave, or convex curves. This method can either be prepared in the diffusion or gradiometer method. The diffusion method involves the formation of a linear gradient kept for some amount of time. The starting point is a steep gradient that is 1 - 2 cm in thickness and a linear gradient should be formed through free diffusion in a stationary environment. The gradiometer method utilizes an apparatus to form a linear gradient. The apparatus consists of 2 chambers with an outlet, the mixing chamber has a stirrer and the third chamber is a reservoir. The gradient comprises two different concentrations of the solution; one will be the light solution having a low density and the other will be the heavy solution having a higher density [5].

### Which is better; two-layer DGC or three-layer DGC?

Two-layer DGC involves the use of two gradients (80% and 40%) whereas three layers DGC involves the use of three gradients (90%, 70%, and 40%). A study was conducted by Astarte and colleagues to determine which of the two methods of DGC was beneficial. It was observed that using three layers of gradients significantly reduced the concentration of sperm seen in the pellet, however, this also yielded

a large population of high motile sperm with normal morphology [6]. A reduction in concentration could be because while using three gradients, sperm has to penetrate through three viscous solutions which could be difficult, but this also allows us to select the best sperm for ART. Studies conducted by other groups [7] further showed that using four layers of gradients further reduced the concentration of spermatozoa in the pellet. This enhances the theory that sperm find it tedious to migrate to the bottom of the tube. Using two layers of DGC is suitable for IUI as we require a large concentration of sperm for successful fertilization, whereas in the case of ICSI since only one sperm is required for direct injection into the oocyte, we could go for the three layers DGC technique [6].

### **Role in virus samples**

DGC followed by swim-up (SU) is a technique that is adopted by many fertility clinics worldwide. This allows highly motile sperms with normal morphology and concentration to be chosen for IUI or in vitro fertilization (IVF). DGC followed by SU is a good measure to reduce the viral load (such as human immunodeficiency virus type 1, (HIV-1)) in any given sperm sample. This is because sperm cells and other viruses or bacteria are separated based on their densities. A study comprising of a total of 129 sub-fertile couples conducted between January 2020 and April 2012, at the Keio University Hospital, was conducted. The female was HIV-1 negative and the male partner was HIV-1 positive. The semen samples were prepared by Percoll DGC followed by SU to remove HIV-I from the samples. It was observed that almost all the samples had no viral load in them. Furthermore, horizontal and vertical transmission of the disease was not observed. DGC followed by swim-up can hence be used as a treatment option for patients with viruses like HIV-I [8]. DGC followed by SU can also be used for other viral samples like COVID-19 positive patients as the size of both HIV and SARS-CoV-2 are similar (0.1 [9] microns and 0.125 [10] microns respectively). This would allow for a safer measure to perform IUI and IVF/ICSI as the virus would be expelled in the supernatant [11].

### **What are the advantages and disadvantages of density gradient centrifugation?**

#### **Advantages of DGC and secured ICSI**

- It is a gentle separation method used to produce a very clean fraction of highly progressively motile sperm is obtained.
- The amount of reactive oxygen species (ROS) production is less.

#### **Disadvantages of DGC**

- It may not be the best method for severely oligozoospermic sperm samples.
- This procedure is relatively more expensive than the conventional swim-up method.
- Medical grade equipment such as test tubes is required which adds to cost.

### **Conclusion**

Men with Oligoasthenoteratozoospermia and bacteriospermia had a higher pregnancy rate when ICSI samples were prepared using modified density gradient methods. Embryo development and implantation rates in Oligoasthenospermia and Bacteriospermia have been shown to be quite high. Overall, the laboratory results were good, and no embryos were arrested at any stage. Before starting any ART treatment, patients with bacteriospermia should be provided thorough information so that they may make an informed decision regarding ICSI as soon as possible.

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