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Using the balance between proliferation and apoptosis to assess the cryopreservation and thawing protocol in mouse 4-cell embryos

Mostafa M. El-Naggar, Hassan Nasrat, Hassan Jamal, Samar Al-Saggaf, Mohamed H. Badawod

ABSTRACT

Aims: The criteria used to assess the optimal conditions for cryopreservation of the embryos in the in vitro fertilization (IVF) protocols are still a matter of discussion. This study aimed at evaluating the use of cell proliferation and apoptosis to assess the optimal conditions for cryopreservation/thawing of the 4-cell embryos. Methods: Fertilized ova were collected from mated female MF1 mice 24 hours after hCG injection. They were cultured in KSOM medium and kept in CO2 incubator at 37°C and 5% CO2 up to the stage of the 4-cells. Two methods of cryopreservation were used; the step-rate and the ultra-rapid vitrification. Slow and fast thawing was done. Slides were prepared from samples of the embryos, and stained immunohistochemically for proliferative and apoptotic cells. The proliferative capacity was measured by labeling with bromodeoxyuridine (BrdU) and the apoptotic ability was measured with TUNEL technique. Results: Vitrification with fast thawing of the 4-cell embryos gave better morphology, higher proliferative capacity, and lower apoptotic ability. Following step-rate cryopreservation with slow or fast thawing, cell labeling index for BrdU was 0% and 17%, respectively and was 66% and 83%, respectively following vitrification. The incidence of apoptosis following step rate cryopreservation with slow or fast thawing was 96% and 89%, respectively and was 42% and 13%, respectively following vitrification. Conclusion: It is concluded that cell proliferation and apoptosis could be used to assess the cryopreservation/thawing protocol for early embryos.

Keywords: Apoptosis, Cryopreservation, Embryos, Mouse, Proliferation, Thawing

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INTRODUCTION

In vitro fertilization (IVF) has become an established procedure to treat infertility. Cryopreservation of the extra embryos at –196°C would allow repeating the IVF procedure with the advantage of avoiding the

inconvenient induction of ovulation and the invasive procedure of oocyte retrieval. It would also give flexibility to the IVF program and allow selecting the proper time for the recipient mother for receiving the embryos. The specimens are stored at –196°C, where at this low temperature, storage for long periods theoretically have minimal impact on viability [1].

The optimal conditions for cryopreservation/thawing protocol for the early embryos are still a matter for discussion. Various criteria were used to assess the optimal conditions for cryopreservation of the embryos; such as in vitro light and electron microscopic morphological changes [2–5], metabolic activity [6], in vitro and in vivo embryo development [2, 3, 7, 8].

Since growth of the embryo is supported by increased cell proliferation and low apoptosis, these two factors are critical for further development of the embryo. However, the effect of cryopreservation on the proliferation/ apoptosis equilibrium of the embryonic cells was not investigated. The present work aimed at using proliferative capacity and the apoptotic ability of the embryonic cells to evaluate different cryopreservation/ thawing protocols of the 4-cell embryos. The proliferative capacity was measured by labeling the proliferating cells with BrdU. The apoptotic ability was measured by labeling the apoptotic cells with the TUNEL technique. Morphological changes were also used for comparison. Two cryopreservation methods (step-rate and ultra-rapid vitrification), and two thawing rates (slow and fast) were used.

MATERIALS AND METHODS

Collection of fertilized ova

Adult female outbred MF1 mice, (20–40 g) at age 5–6 weeks were obtained from the Animal House, King Fahd Medical Research Center, King Abdulaziz University, Jeddah. They were maintained on a standard diet [commercial cubes containing (w/w) approximately 18% protein, 3% fat, 77% carbohydrate and 2% of an inorganic-salt mixture with a vitamin supplement (Grain Silos and Flour Mills Organization, Jeddah, Saudi Arabia)] and water *ad libitum*. The mice were kept in a controlled environment (constant temperature 24°C, and a light cycle of 14 h on/ 10 h off). Superovulation was induced by subcutaneous injection of 5 IU of pregnant mare's serum (PMS) gonadotropin and, 48 hours later, with intraperitoneal injection of 5 IU of human chorionic gonadotropin (hCG). Each female was placed with a proven male in a breeding room and then examined for sperm plugs next morning (day 1). Fertilized ova were collected 24 hours after hCG injection. The ova were examined for signs of fertilization; extrusion of second polar body, formation of pronuclei or division of the zygote. The fertilized ova were incubated in potassium simplex optimized culture medium (KSOM, from Specialty Media, Sigma-Aldrich, St Louis, MO, USA) in 15 mm culture dish (Nunc, Fisher Scientific, Pittsburgh, PA, USA) to the 4-cell stage, before cryopreservation. The incubation was carried out in 20 µl droplets of the KSOM culture medium covered with paraffin oil, and kept in the CO2 incubator at 37°C and 5% CO2.

Cryopreservation/thawing

Two methods of cryopreservation were used; the step-rate and the ultra-rapid vitrification methods. The step-rate cryopreservation procedure is a modification of the method of Rajotte et al. (1983) and Taylor and Benton (1987) [9, 10]. The procedure consisted of equilibrating the embryos with the cryoprotectant dimethyl sulfoxide (DMSO). This was followed by cooling the ova to -8°C. Ice nucleation (seeding) is induced within the fluid, to permit the release of the latent heat of fusion by getting the tubes, which contain the embryos, in brief contact with liquid nitrogen. The tubes were then gradually cooled to -40°C and quenched in liquid nitrogen at –196°C for long storage.

The cryopreserved embryos were thawed by the slow or fast warming rates. For slow thaw the cryotubes were kept standing on the bench at room temperature for approximately 10 min. For fast-thaw the cryotubes were agitated in a water bath at 37°C. In both cases the tubes were transferred to an ice bath at 0°C, just before lyses of the last ice crystal. DMSO was drawn from the intracellular compartment by a hyperosmolar sucrose solution, which was then gradually diluted and replaced by culture medium.

The ultra-rapid (vitrification) procedure is the method described by Kasai et al. (1990) [1]. The procedure consisted of washing the embryos in Dulbeco's phosphate buffered saline (D-PBS). Embryos were equilibrated in the vitrification solution for two minutes to dehydrate the cytoplasm. The vitrification solution (EFS40) consisted of 40% ethylene glycol in solution of 30% Ficoll, 0.5 M sucrose and BSA dissolved in D-PBS. The embryos were then transferred to 13 mm EFS40 column in the straw. The straw was allowed to cool slowly in liquid nitrogen vapor for at least three minutes before immersing in liquid nitrogen (-196°C) for storage.

The vitrified embryos were thawed by the slow or fast warming rates. For slow thaw, the straws were kept standing on air at room temperature for 15 seconds, and then immersed into a 20°C water bath. For fast thaw, the straws were agitated in a water bath at 37°C. When the sucrose solution began to melt, the straws were removed from the water bath and slowly perfused with 1 ml sucrose solution, the embryos recovered and transferred to drops of hyperosmolar sucrose in culture dish. Glucose was then gradually diluted and replaced by culture medium.

Morphological examination

Embryos were examined by phase contrast microscope immediately after thawing. Changes in the zona pellucida and blastomeres were recorded. The survival rate was calculated as the number of intact post-thawed embryos and expressed as a percentage of the total number of embryos. The in vitro development rate was calculated as the percentage of the number of the intact post-thawed embryos followed from day-3 (4-cells), through day-4 (8-cell mass/ morula), day-5 (morula) and day-6 (blastocyst).

Immunohistochemical examination

Slides were prepared from samples of the cyopreserved/ thawed 4-cell embryos, fixed with a drop of paraformaldehyde and stained immunohistochemically for bromodeoxyuridine (BrdU) to label the proliferating cells and for TUNEL to label the apoptotic cells. Slides were also prepared from specimens incubated without cryopreservation/thawing, and used as control. Controls for the specificity of the immunohistochemical methods were also used.

BrdU labeling for proliferation:

BrdU was added to the culture medium 12 hours, at least, before processing the specimens for fixation and staining. BrdU is incorporated into DNA of the nuclei of the dividing cells in place of thymidine. The slides were incubated with mouse anti-BrdU, then with anti-mouse Ig conjugated with alkaline phosphatase, for 30 min each at 37°C in a humid atmosphere. Nitroblue tetrazolium (NTB) was used as a color substrate to visualize the sites of the positive reaction. The cell labeling index (percentage of cells labeled) was measured by counting the number of BrdU labeled nuclei and express it as a percentage of the total number of nuclei scored.

TUNEL technique for apoptosis

TUNEL Technique was used to measure programmed cell death (apoptosis) by detecting DNA strand breaks in individual cells. It uses terminal deoxy-transferase (TdT) to label free 3'OH ends in genomic DNA with fluorescein-dUTP. The slides were incubated with TUNEL reaction mixture containing TdT and fluorescein-dUTP. During this incubation step, TdT catalyzes the attachment of fluorescein-dUTP to the free 3'OH ends in the DNA. The incorporated fluorescein is detected with an anti-fluorescein antibody peroxidase conjugate. The immune complex peroxidase is then visualized by a substrate reaction and detected by light microscope. The negatively stained nuclei were counter-stained with hematoxylin. The cell labeling index (percentage of cells labeled) was measured by counting the number of TUNEL labeled nuclei and express it as a percentage of the total number of nuclei scored.

Statistical Analysis

The significance of difference between the cryopreserved and control specimens was evaluated by students' t-test at $p < 0.05$.

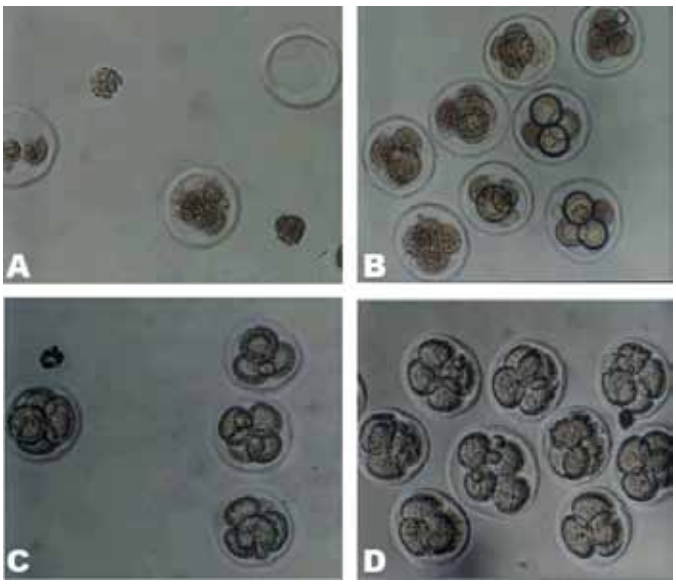
RESULTS

Morphological findings

Embryos were examined by phase contrast microscope immediately after thawing. Step-rate cryopreserved embryos with slow-thawing showed fractured zona and loss of part or all of the blastomeres which were seen outside the zona (Figure 1A). Fast-thawed embryos showed few embryos with well-defined blastomeres; whereas most of the embryos were having blastomeres with ill-defined borders (Figure 1B)

Vitrified 4-cell embryos with slow-thawing appeared in good condition with spherical well-defined borders of the blastomeres surrounded with thick zonae (Figure 1C). Similar results were found in the fast-thawed embryos which showed spherical thick zona pellucida with four well defined blastomeres inside (Figure 1D).

The step-rate cryopreserved embryos showed survival rates of 35% and 60%, and in vitro development rates of 6% and 46% with slow and fast thawing, respectively (Table 1). The best results were obtained with vitrification of the embryos both with slow and fast thawing, which gave survival rates of 86% and 94%, and in vitro development rates of 74% and 80%, respectively (Table 1).



Figures 1 (A-D): Step-rate cryopreserved 4-cell embryos, day-3, (A) 1 h after slow-thawing, (B) fast-thawing and vitrified 4-cell embryos, (C) 1 h after slow-thawing (D) fast-thawing. Inverted phase contrast microscope, X 100.

The in vitro differential developmental progress of the cryopreserved/ thawed embryos was much slower than that of the corresponding control embryos, especially at day-4 (stage of 8-cell mass/ morula). During day-5 (stage of morula) and day-6 (stage of blastocyst), their developmental progress was slightly slower than that of the control (Figure 2).

Bromodeoxyuridine (BrdU) labeling for proliferation

The nuclei of all the blastomeres of the control embryos appeared positively stained for BrdU (Figure 3A) indicating that they have undergone cell division. The nuclei of the step-rate cryopreserved slow-thawed embryos did not show any positive staining for BrdU (Table 2) indicating that these embryos did not undergo proliferation. Most of the step-rate cryopreserved fast thawed embryos showed blastomeres with nuclei that did not show any positive staining for BrdU (Figure 3B). Few embryos showed blastomeres with nuclei that appeared with positive reaction to BrdU, indicating that few embryos have undergone proliferation (Table 2). The nuclei of the blastomeres of most of the vitrified slow-thawed embryos showed positive staining for BrdU (Table 2). Similarly, the nuclei of the blastomeres of most of the vitrified fast thawed embryos appeared with positive reaction to BrdU (Figure 3C). This indicates that most of the vitrified embryos have undergone proliferation (Table 2).

The cell labeling index (percentage of cells labeled) for BrdU was nearly 100% in the control group. Following step-rate cryopreservation and slow or fast thawing, cell labeling index was 0% and 17%, respectively. Following vitrification and slow or fast thawing, cell labeling index was 66% and 83%, respectively (Table 2).

TUNEL technique for apoptosis

The nuclei of the blastomeres of most of the control embryos in the morula stage (day-4) appeared negatively stained for TUNEL (no brown stain) but counterstained with hematoxylin (blue stain) (Figure 4A) indicating that they are devoid of apoptosis. The nuclei of the blastomeres of nearly most of the step-rate cryopreserved slow or fast-thawed embryos were positively stained with TUNEL (Table 2, Figure 4B), indicating that they have undergone apoptosis. Few embryos, however, showed blastomeres with nuclei that did not show any positive staining (Table 2). The nuclei of some of the vitrified slow (Figure 4C) or fast-thawed (Figure 4D) embryos showed positive staining, whereas the nuclei of most embryos did not show any positive staining for TUNEL, but were counterstained with hematoxylin.

The cell labeling index (percentage of cells labeled) for TUNEL showed that the incidence of apoptosis in the

Table 1: Survival rate and in vitro development of the cryopreserved 4-cell embryos following slow and fast thawing. Significant results were determined at ($p < 0.05$)

	Control	Step-rate cryopreservation		Vitrification	
		Slow-thaw	Fast-thaw	Slow-thaw	Fast-thaw
No. of Cryopreserved embryos	149	70	70	70	70
No. of intact post-thaw (Day-3)	---	21	42	60	66
Survival rate (Day-3)	100%	35%	60%	86%	94%
		($p < 0.05$)	($p < 0.05$)	($p < 0.05$)	($p < 0.05$)
No. of in vitro development (Day-4)	120	4	32	52	56
In vitro development rate (Day-4)	80.6%	5.7%	45.7%	74.3%	80%
		($p < 0.05$)	($p < 0.05$)	($p < 0.05$)	($p < 0.05$)

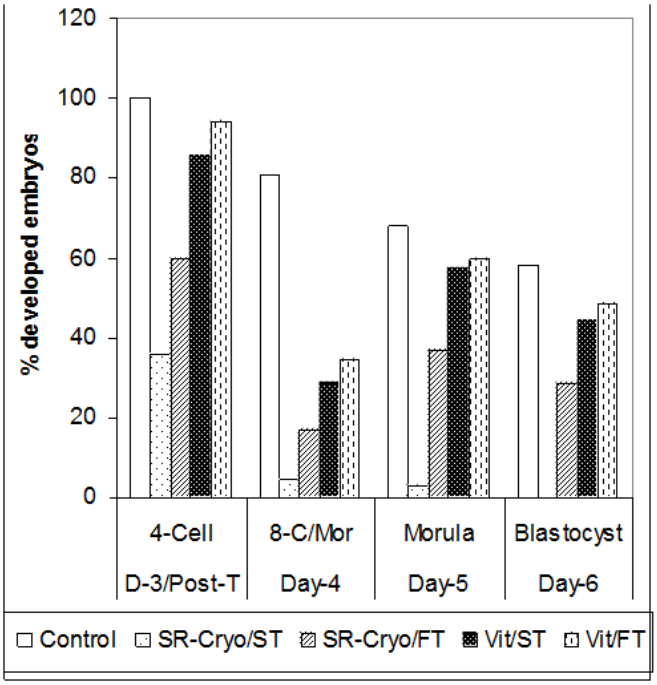
Table 2: The cell labeling index of the cryopreserved thawed 4-cell embryos after marking the nuclei of their proliferating blastomeres with BrdU and of the apoptotic blastomeres with the TUNEL technique. Each experimental group was compared with the control. Significant results were determined at ($p < 0.05$)

Stain	Control	Step-rate cryopreservation		Vitrification	
		Slow-thaw	Fast-thaw	Slow-thaw	Fast-thaw
BrdU	100%	0%	17%	66%	83%
		($p < 0.05$)	($p < 0.05$)	($p < 0.05$)	($p < 0.05$)
TUNEL	4%	96%	89%	42%	13%
		($p < 0.05$)	($p < 0.05$)	($p < 0.05$)	($p < 0.05$)

control embryos is about 4%. The incidence of apoptosis has increased following step rate cryopreservation and vitrification to 96% and 42%, respectively after slow thawing and to 89% and 13 %, respectively after fast thawing (Table 2).

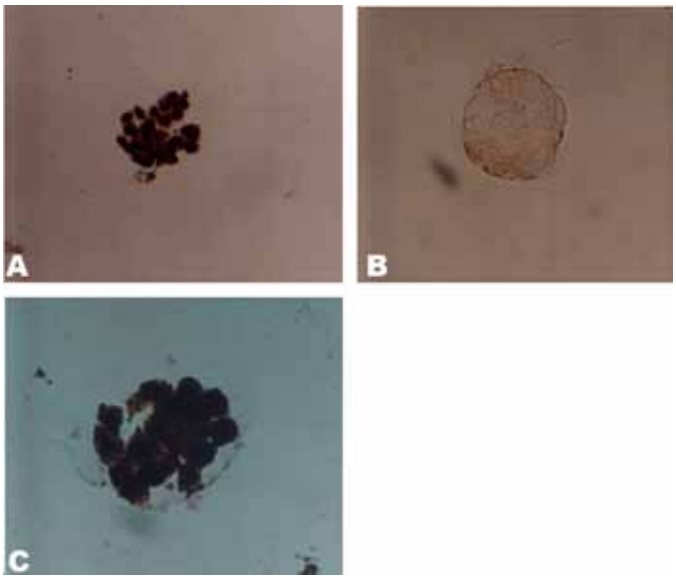
DISCUSSION

The results of this study showed that vitrification with fast thawing gave the best proliferation cell labeling index (83%) and the least incidence of apoptosis (13%). These proliferation/apoptosis results were more or less supportive to the morphological evaluation; survival rate of 94% and in vitro development rate of 80% following vitrification and fast thawing. The results of the morphological study obtained in this report are comparable to those obtained by other authors. The recorded survival rate following vitrification methods was 83–94% [11] in mouse, 63% [12], and 79.2% [13] in human embryos. High rates of in vitro development following vitrification was reported to be 84–98 % [1] in rat, 75–97% [14], 80–83% [15], 74–92% [16], 94–95% [17] in mouse, and 80% [18] in human embryos.

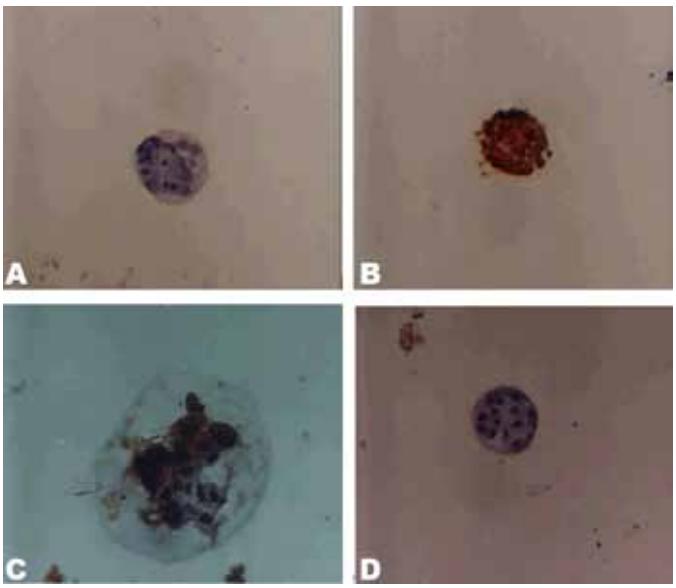


SR-Cryo: Step-rate cryopreservation
Vit: Vitrification
ST: Slow thaw
FT: Fast thaw

Figure 2: Diagram showing the in vitro differential developmental progress of the cryopreserved/ thawed 4-cell embryos. The embryos were followed from day-3 (4-cells), through day-4 (8-cell mass/ morula), day-5 (morula) and day-6 (blastocyst).



Figures 3 (A-C): Zona-free embryos in the stage of morula at day-4 after (A) incubation (control), (B) Step-rate cryopreservation, (C) Vitrification, and (D) The embryos were fast-thawed and cultured with BrdU for overnight. The dividing blastomeres showed nuclei positively stained for BrdU (brown color) (Immunoperoxidase stain for BrdU, X200).



Figures 4 (A-D): Zona-free embryos in the stage of morula at day-4 after (A) Incubation (control), (B) Embryos were step-rate cryopreserved and fast-thawed, (C) Vitrified and slow-thawed, and (D) Fast-thawed at 4-cell stage (day-3), then cultured with KSOM medium to day-4. The positively reacted nuclei (brown color) indicate blastomeres that have undergone apoptosis. The negatively reacted nuclei are counterstained with hematoxylin (blue color). (TUNEL technique, X100).

Although we could not find in literature any report critically investigating the effect of cryopreservation on both proliferation and apoptosis of the early embryonic cells, however, few reports could be located investigating the effect of either proliferation or apoptosis. Few investigations used proliferation ratio to provide

information about the developmental potential of embryos after cryopreservation [19, 20]. Takagi et al. (1996) [21] investigated proliferation of the inner cell mass (ICM) by examining the rate of DNA synthesis in frozen-thawed bovine blastocysts, by immunocytochemical staining. They found that the numbers of bromodeoxyuridine-immunoreactive ICM cells of frozen-thawed embryos were significantly lower than those of unfrozen embryos, suggesting that the rates of proliferation of ICM of frozen-thawed bovine embryos tend to be lower than those of unfrozen embryos. Few reports used the dUTP nick end-labeling (TUNEL) technique to describe the effect of cryopreservation on the apoptotic cell death [5, 22–26].

The mechanisms underlying the optimum control of the proliferation/apoptosis balance for embryonic development following in vitro fertilization and culture are unknown. Several factors have been investigated and claimed responsible for increasing or decreasing proliferation of early embryos. It has been suggested that lipid peroxides derived from polyunsaturated fatty acids (PUFAs) inhibit proliferation of various cells, a process that could be reversed by antioxidants [27]. Several factors could be involved in early embryonic cellular proliferation and development; such as Brca1 [28], tumor suppressor gene Brca2 [29], radiation-inducible gene muREC2/RAD51L1 [30], insulin [31], cyclin proteins [32], embryo-derived platelet-activating factor (EPAF) [33], polyamines [34], and all-trans retinoic acid [35].

Apoptosis, on the other hand, was found to be affected by several factors [36-38]. Levy et al. (1998) [39] has shown that apoptosis occurs in mammalian embryos as early as the cleavage stage, in order to regulate the inner cell mass. Current evidence indicates that hydrogen peroxide causes apoptosis of inner cell mass cells destined to develop into trophectoderm, the first apoptotic event during mammalian development [37, 40]. Yang and Rajamahendran (2002) [38] showed that apoptosis appears to be due to an interactions between the Bcl-2 family of proteins in pre-implantation embryos development.

CONCLUSION

It is concluded that proliferative capacity and apoptotic ability of the embryonic cells could be used as criteria for assessing cryopreservation/thawing protocols. Disturbance of this balance, in the form of inhibited cellular proliferation or stimulated apoptosis, may compromise the developmental process of the growing embryos. It is possible that these two processes were minimally disturbed with vitrification and fast thawing of the 4-cell embryos, since this procedure gave the best development rate in vitro. These results may have an impact on the human IVF protocol and developing a bank of embryos that could be cryopreserved for future use.

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Author Contributions
Mostafa M. El-Naggar – Substantial contributions to conception and design, Acquisition of data, Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Hassan Nasrat – Substantial contributions to conception and design, Acquisition of data, Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Hassan Jamal – Substantial contributions to conception and design, Acquisition of data, Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Samar Al-Saggaf – Substantial contributions to conception and design, Acquisition of data, Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published
Mohamed H. Badawod – Substantial contributions to conception and design, Acquisition of data, Analysis and interpretation of data, Drafting the article, Revising it critically for important intellectual content, Final approval of the version to be published

Guarantor
The corresponding author is the guarantor of submission.

Conflict of Interest
Authors declare no conflict of interest.

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