DEVELOPMENT OF PVA/PVP BASED CRYO-CONTAINERS FOR VITRIFICATION OF BIOLOGICAL SAMPLES

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BY

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CERTIFICATE

This is to certify that the work in the thesis entitled "**DEVELOPMENT OF PVA/PVP BASED CRYOCONTAINERS FOR VITRIFICATION OF BIOLOGICAL SAMPLES"** submitted by **Ms. Jyoti Bala (212BM2009)**, in partial fulfilment of the requirements for the award of M. Tech (Biotechnology) at the National Institute of Technology Rourkela, is an authentic work performed by her under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any University/Institute for the award of any Degree or Diploma.

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ABBREVIATIONS

PVA	Polyvinyl Alcohol
PVP	Polyvinyl Pyrrolidone
OPS	Open pulled straw
CPA	Cryo-protective agent
VS	Vitrification solution
ES	Equilibration solution
DMEM	Dulbecco's Modified Eagle Medium
PBS	Phosphate Buffered Saline
DMSO	Dimethyl Sulfoxide
PROH	1, 2, Propendiol
EG	Ethylene Glycol
PEG	Polyethylene Glycol
SF	Solubility Factor
OD	Optical Density
GA	Glutaraldehyde

ABSTRACT

Vitrification is a physical phenomenon used for cryopreservation of biological samples such that they are retained in vitreous state. In this study, polyvinyl alcohol (PVA) and polyvinyl pyrrolidone (PVP) were the polymers, used in the form of blend, for development of Cryocontainers for vitrification of biological samples. Films with 5%, 3% PVA and 5%, 2% PVP were prepared using solvent evaporation method and were characterized for mechanical strength, swelling, solubility in water, stability in liquid nitrogen (LN_2), degradability, crystallinity, morphology and biocompatibility. Film prepared from 5% PVA and 5% PVP showed the best results with maximum folding endurance (380±5) , swelling (225%), solubility in water (33%±5) and minimum thickness (0.06±0.02 mm). These films were also stable in LN_2 for at least 7 days, showed maximum haemolysis of 3.52% and 97% viability of cells was seen in Trypan blue dye exclusion test which suggest their biocompatibility. All the results turned out to be promising and hence make PVA/PVP blend films good candidates for vitrification of biological samples.

KEYWORDS: Vitrification, Cryocontainers, PVA (polyvinyl alcohol), PVP (polyvinyl pyrrolidone)

1. INTRODUCTION

Cryopreservation of living specimens is critical for cost-effective, long term preservation of biological samples. The ultimate aim is to cool them to a point at which intracellular functions stop and therefore cells donot require additional energy input. Cells must also maintain their chemical and physical integrity which will allow them to function upon warming. Among distinctive sorts of strategies, vitrification is viewed as an important strategy for cryopreservation, owing to the glass-like vitreous state without the formation of ice crystals. Since first reported preservation in mouse embryos, tremendous improvements have been made to enhance the post-warming survival of cells (1). Vitrification is just a vitreous, transparent, ice-free solidification of water-based solutions at subzero temperatures, which is potentially applied faster, and relatively inexpensive, and it is becoming clinically established, and is seemingly more reliable and consistent than conventional cryopreservation when carried out appropriately. Chances of damage due to ice or other cryoinjuries are less likely which is foremost advantage over conventional freezing and benefits by minimising time and cost required for cryopreservation. It has wide application in cryogenics for preserving biological samples like embryos, sperm, somatic and stem cells and the banking of different organs to be used for transplantation. All developmental phases of embryos studied in vitro have been effectively vitrified and warmed. Vitrification is a standout amongst the most stimulating enhancements to avoid ice formation throughout the process of vitrification by making vitreous state instead of an ice solid state. To accomplish this glass-like state of live cells for cryostorage with high rates of cooling combined with usage of large amassing of cryoprotective agents, an essential system for vitrifying cells and tissue is to build the velocity of warm conductivity, while diminishing the amassing of the vitrificants to lessen their potential toxic effect [2].

Polymers have gotten to be progressively appealing on account of substantial number of provisions including biotechnology, biomedical, photonics, and optoelectronics. Recently they have been used for forming blends of different polymers or an inorganic particle with polymers, representing a strategic route for improving the performance of a biomaterial, enhancing the performance of parent polymers. This tailoring of the macroscopic properties by the process of polymer blending is the result of modifying the composite structure at macroscopic scale. Polymer blending is a useful framework for arranging materials with a wide combination of properties. Polymer blends offers an approach to process new materials by using successfully existing polymers, which are also cost-effective. Polyvinyl pyrrolidone (PVP) has excellent mechanical strength with simple processing, good electrical and thermal properties. Therefore composite materials formed from PVP in the final polymer system gives satisfactory mechanical and electrical properties as well. Solution blending, along with formation of new material also have variety of properties which are mainly dependant on characteristic of the parent homo polymers and the blend composition. Many studies on polymer blends are being carried out in recent years. Interest in novel structures and properties of blends is because of need of biomaterial with different properties than those observed in homopolymers and copolymers [4]. An important aspect of the properties of a blend is the miscibility of its components. Specific interactions between polymeric components contribute to the miscibility in polymer blends which usually give rise to a negative free energy of mixing in spite of the high molecular weight of polymers. These interactions include hydrogen bonding, ionic and dipole, π-electrons and charge transfer complexes. Polymer blends are physical mixtures of structurally different polymers interacting through secondary forces and are miscible at molecular level; the advantage being that the properties of the final product can be tailored to the application requirement. In this study, the components of blend are polyvinyl alcohol (PVA) and polyvinyl pyrrolidone

(PVP) [4].Among the polymers PVA has excellent physical properties such as mechanical strength, electrochemical stability, non-toxicity, good film-forming capability and biocompatibility. PVA is semi-crystalline, water-soluble, better film forming capacity, biocompatibility, mechanical and electrochemical properties and biodegradability which is comprehensively used in biomedical field. PVP is a vinyl polymer which possesses planar and highly polar side groups due to peptide bond in lactum ring. It also has wide applications in biomedical field because of its properties like conductivity, mechanical strength and good solubility in water. Right when these two polymers are mixed, the participation between PVA and PVP is required to take place through intermolecular hydrogen holding between the hydroxyl social event of PVA and carbonyl moiety of PVP [5].These blends are stable within the physiological environment because of this physical crosslink consisting of intermolecular hydrogen bonds, and the intramolecular hydrogen bonds within PVA which are responsible for solubility of PVA and PVP in water. Different types of cryocontainers are used for preservation purpose for example cryoloops, open pulled straw, EM grids or plastic straw. Here films made from PVA/PVP serve as one type of cryocontainers. This study has been designed with an objective to form films at various compositions of PVA and PVP with 5%, 3% PVA and 5%, 2% PVP were prepared using solvent evaporation method and were characterized for mechanical strength, swelling, solubility in water, stability in liquid nitrogen (LN_2), degradability, crystallinity, morphology and biocompatibility.

1. LITERATURE REVIEW

A definitive objective of cryopreservation of cells is to cool them to a point at which when intracellular function stop, and in this manner cells don't progress or require extra energy inputs. Cells should likewise keep up their physical and chemical integrity, which will permit them to function properly after warming. Both thermal shock and ice formation are hindrances to effective cryopreservation; however these impediments could be killed by utilizing cryoprotective agents, controlling cooling rates, and presenting cells to a high salt medium,

Table 1. Summary of Cryopreservation Protocols generally used

S. NO	PARAMETERS	SLOW FREEZING	CONVENTIONAL FREEZING	ULTR-RAPID VITRIFICATION
1	CONTAINERS	STRAW	STRAW	MINIMUM VOLUME CRYOCONTAINERS
2	TEMPERATURE OF TREATMENT	ROOM TEMPERATURE	ROOM TEMPERATURE	BODY TEMPERATURE
3	CONCENTRATION OF PERMEATING CPA	1.4 M	7.2 M	4.7 M
4	DURATION OF CPA PRE-TREATMENT	0 MIN	1-3 MIN	1-3 MIN
5	DURATION OF CPA TREATMENT(FINAL)	15-20 MIN	1 MIN	25-45 SEC
6	TIME REQUIRED FOR COOLING	90 MIN	3 MIN	<0.1 SEC

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at last dehydrating them [1,2]. Exorbitant drying out of cells has its set of issues, including permanent harm to cell structures, ph changes prompting protein damage, and amassing of ion particles bringing about toxicity. Indeed, all conventions connected with freezing and vitrification relies on upon various factors, huge numbers of which conflict with each other and are not entirely understood. Of course, about all parts of cryopreservation conventions temperature, timing, volumes, compartments, cryoprotectants, diluents, phase of foetuses, and so forth - were established by trial and error trying to adjust the vast number of restricting components that influence vitrification and freezing. Thermal shock and ice formation are the main causes of cryoinjuries. When cells are cooled too quickly thermal shock occurs. Ice formation occurs if cells are not allowed to dehydrate properly as freezing occurs. Damage to plasma membrane is initial step of thermal shock and is due to mechanical shearing and membrane elements shrinking in diverse manners resulting in conformational changes to the exterior of the cell [1, 3].

2.1 FUNDAMENTAL TECHNIQUES OF CRYOPRESERVATION

Presently there are two basic techniques used for cryopreservation: freezing and vitrification both differ in cooling rate and the CPA addition procedure.

2.1.1 SLOW FREEZING

This is the very first effective strategy sought for the cryopreservation of biological sample. The principle of this strategy is the impact of crystallization of extracellular water into ice in a moderate and well-controlled plan such that intracellular water is excluded out of the cell to bring about the great rise of viscosity within the cells or solidification without ice formation within the cell. As required, this procedure is also known as slow freezing or conventional slow freezing. The method includes two main steps: equilibration and freezing. In the first

step cells are exchanged from isotonic medium to a hyper osmotic solution holding one or additional penetrating CPA and duration of exposure to equilibration is very short to consume CPA. Throughout this exposure time, because of difference in intracellular and extracellular osmotic weight, cells shrivel instantly however as CPA saturates, water re-enters the cells to keep up the intracellular osmotic equilibrium and shrinkage stops at the point when the equilibrium is arrived between the water permeating outside and the CPA being in fluxed. This continues until at the point when no osmotic and synthetic slopes in contrast to water or CPA remain. After the equilibration is completed, cryo-containers are used to keep the cells for storage (usually plastic straws) and then cooling rate of 0.5- 2°C/min from normal temperature to a temperature marginally beneath the liquefying purpose of the solution, which is pretty nearly -5 to -7°C.maunal seeding is then performed at this temperature by manually touching the cryo-containers with a forceps (prechilled with fluid nitrogen),or by bringing it in contact with LN_2 in a programmable freezer, to initiate extracellular ice creation and to avoid super cooling.

2.1.2 VITRIFICATION

Vitrification alludes to the physical phenomena delineating the crystallization of water or water-based solutions into a glass-like indistinct liquid state (called vitreous state), due to great rise in consistency all through cooling, without the establishment of ice. Accomplishment of vitrification technique depends on two elements: lifted convergence of CPA to block the crystallization water into ice and a to a great degree high cooling and warming rates (up to 20,000°C/min) to pass through the risky temperature zone quickly (+15 and -5°C) to refuse cryo-damage. As most CPAs are significantly hazardous, presentation time ought to be decreased before utilization. On the off chance that the presentation is exorbitantly short, CPA may not penetrate to the cells and intracellular ice may structure even

without ice development outer surface of the cells. In this manner, the fundamental assembly for vitrification is to begin by equilibrating them in a solution (called equilibration solution, ES) which have a low convergence of one or all the more penetrating CPA before they are exchanged to a last solution (called vitrification solution, VS) holding a solid porous CPA and a non-penetrable CPA like sucrose. The ES solution comprises of 25% and half of the last pervading CPA fixation which might be included expanding focuses for instance 25% lastly half of the last CPA focus to be pervaded. Time for equilibration is generally 1-3 min while time of incubation in VS solution is bound to 25-45 seconds. Regardless, equilibration time (5-15 min) with porous CPA in low focus in the ES solution has also been used. Such control in CPA fixation and equilibration time in the ES permits vitrification investigations to be performed parallel. On the inverse, there is no development for controlling the CPA fixation and time of hatching in VS solution unless control over cooling rates is carried out. A indirect relationship exists between these two components: bring down the cooling rate, the higher the obliged amassing of CPA and the other way around. Despite the fact that it is troublesome to make cooling rate with present open techniques and extending the convergences of CPA additionally raises the poisonous quality and also the effect of osmosis. On the other hand, extending the time of incubation in VS solution extends the shots of danger by CPA yet useful as it gives an incredibly enhanced assurance to cell. 25 s hatching with VS solutions has realized splendid rates of survival after vitrification, yet pregnancies were gotten exactly when period of incubation in VS solution was amplified 60 s. Throughout cooling, specimen bearer gadget is stacked with a little volume of the VS solution holding the cells is stacked, which is then kept into a coolant (normally LN_2), to proceed with vitrification. This gadget is then totally fixed and kept in LN_2 tank for capacity. The warming and rehydration or CPA evacuation is to a degree like moderate solidifying strategy. In this step normally cells are warmed and emptied from the transporter gadget to a

plan of solutions with least osmolarities for rehydrating the cells and clearing CPA which is toxic [6].

2.2 CRYOCONTAINERS

Various types of containers (carrier or vessels) are widely used to hold cells during vitrification, storage and warming

2.2.1 STRAW TYPE CONTAINERS:

Initially compartments normally utilized for moderate solidifying, standard 0.25 ml plastic insemination straw are tried for vitrification notwithstanding constrained achievable cooling (100-1540°c/min) and warming (2000-2500°c/min) rates due to thick divider (0.15mm),large distance across (1.7mm) and moderately huge measure of solution (25-200 µl) needed for safe loading of the sample. A part of the straw could be stacked with ~30 µl of VS solution holding the cells and other small samples are stacked with vitrification solution and 150 µl of sucrose solution, with every one piece partitioned by an air bubble. The stacked straw is then kept into LN_2 for vitrification. For recuperation, the straw is warmed in water shower before the substance is casted out from the straw into sucrose respond in due order regarding rehydrating. One issue with the use of straw is the chances of its fold or impact on account of incredible weight change throughout immediate drenching of into LN_2 all through vitrification or into water shower all through warming [7].

TABLE: 2. CHARACTERISTICS OF COMMONLY USED CRYOCONTAINERS

S.NO	CONTAINERS	VOLUME OF SOLUTION (µL)	COOLING RATE (°C/min)	WARMING RATE (°C/min)
1	PLASTIC STRAW	25-200	<2500	2000-2500
2	EM GRID	<0.1	<20000	NA
3	OPS	0.5	16340-7700	13000-13900
4	CRYOTOP	<0.1	22800-2000	18000-42100
5	HEMI-STRAW	0.3	1600	3000
6	CUT STANDARD STRAW	0.5	600	30000-90000
7	CRYOLOPP	NA	15000	>48000
8	PAPER CONTAINER	2-5	100-1540	2000-2500

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2.2.2. CRYOVIALS AND CRYOTUBES

Cryovials and cryotubes, ordinarily used for the cryopreservation of physical and undeveloped cells, have similarly been used for the vitrification of biological samples. Regardless of the way that they have thicker divider with less demanding high temperature conductance than that of plastic straw. The examples are equilibrated in ES solution and moved into the cryotubes/cryovials (precooled on ice) using a pipette. The VS solution is by then added to the compartment, brought forth for looked for period (regularly 1 min), close and thereafter, plunged into LN_2 for limit. Recovery of cells is completed by holding the Cryotubes/cryovials at room temperature for 30 to 60s, including a far reaching volume (~1 ml) of sucrose solution into it for debilitating of CPA and rebuilding of water substance [8,9].

2.2.3 CONTAINER-LESS VITRIFICATION IN MICRO DROPS

Other than minimizing the volume of VS solution incorporating the phones, securing a prompt contact (without any thermo-ensuring layer) between the VS solution and the LN_2 is a substitute most perfect methodology to construct the rate of cooling. This could be accomplished by particularly dropping the transporter free VS solution, holding the cells, as meagre micro drops (5 to 20 μl) into the LN_2 and securing the vitrified micro drops in cryotubes on the other hand cryovials. Warming and debilitating is conceivable by clearly dousing the vitrified micro drops into the sucrose solution [10, 11].

2.2.4 METAL-MESH VITRIFICATION IN EM GRID

One issue with the holder less micro drop system was the advancement of a vapour blanket around the micro drop as it falls on the LN_2. The vapour spread structures due to gurgling and vanishing of LN_2 and not simply functions as a securing layer furthermore keeps the brief sinking of micro drop into the LN_2 and in this way, hampers the speedy cooling. To abstain from this issue, stacking of micro drop on a staggering material, for instance, metal cross area, was connected [13]. The EM grid was the in any case compartment in which both little sample volume and quick contact with LN_2 was achievable to get a particularly high cooling rates required for vitrification. The EM systems are copper organizes, 3 mm in estimation and 25 μm thick, open in different cross segment sizes [14]. After the cells are brought forth in VS solution, they could be stacked on the schema with minimum VS solution and vitrified by clearly plunging the network into LN_2. The skeletons, holding the vitrified examples, can then be set in cryotubes/cryovials and set away in LN_2 stockpiling tanks. Warming can achieved by particularly soaking the grid into warm sucrose solution whereupon cells separate from the network. The vitrified-warmed cells can now be rehydrated in decreasing center of sucrose respond in due order regarding remove the soaking CPA [2, 9, 15].

2.2.5 CRYOLOOP

In year 1999, Lane et al. balanced the possibility of using a little round that is consistently used inside X-shaft crystallography to hold the protein diamonds inside a film of CPA respond in due order regarding data gathering at cryogenic temperatures. The solution film traversing the crevice of the round is strong enough to hold the cells and with this immaterial result volume, the achievable cooling rate may be extraordinarily high, up to a normal 700,000°C/min [16]. Utilizing this device, safe vitrification may be achieved even in the Ln2 vapour. The round, called cryoloops, is right now financially available. It embodies a little nylon ring (20 µm thick and 0.5~0.7 mm in separation over) that is mounted on a stainless steel tube inserted into the spread of the cryovials. Cells could be stacked on the film of VS solution brings about the nylon ring, secured into the cryotubes and set away in LN_2. Warming and example recuperation is done by fundamentally plunging the round into sucrose solution [17].

2.2.6 OPS

The OPS is a change of standard plastic straw that is high temperature decreased and pulled physically, in the same route as a glass thin, to get an inside separation crosswise over of ~0.8 mm which gives a divider thickness of ~0.07 mm [19]. The pulled straw is then cut at the most slim point and used as OPS. Right when the tight end of OPS is dunked into a little droplet of VS solution holding the oocytes/foetus, the case is characteristically stacked into the OPS, which is then plunged into Ln2 for vitrification. Warming is conceivable by removing the cells direct into the sucrose solution [20, 21, and 22].

2.2.7 CRYOTOP

Cryotop was handled especially for the vitrification of human cells however has also been used viably in private creatures [23]. It contains an exceptionally created fine polypropylene strip attached to a plastic handle. The VS solution, holding the phones , could be stacked on the strip and wealth solution could be emptied essentially by and large by craving. The case is then soaked into Ln2 for vitrification likewise stockpiling. Cryotop grants higher cooling and warming rates than those achievable with OPS, easy to learn and perform and oblige essential control which decreases the peril of clash [24, 25].

2.2.8 CRYOTIP

Cryotip is a subsidiary of OPS that was imagined by to keep the likelihood of sickness transmission due to quick contact of the vitrified cases with unintentionally corrupted LN_2 [25]. It includes a feeble plastic straw (250 µm internal estimation, 20 µm divider thickness, and 3 cm length) connected with a thicker part (200 µm inward estimation, 150 µm divider thickness, and 4.5 cm length, and outfitted with a flexible protective metal sleeve). The cells may be stacked in the dainty plastic straw for vitrification and close by the compact guarding metal sleeve before limit in LN_2 limit tank. Then again, Cryotip obliges a long taking in twist to load and handle the device rightly and to keep up a vital separation from the breakage of a stacked Cryotip all through dealing with and stockpiling. Moreover, with respect to survival and pregnant rate, it didn't change from other cryo-compartments, for instance, cryotop.

2.2.9 HEMI-STRAW SYSTEM

Hamawaki et al. vitrified the cows creating lives by stacking them on the outside or inside surface of a plastic straw with irrelevant volume (<1 µl) of VS solution and clearly plunged it

in LN_2 for vitrification and capacity [27]. This procedure was called MVC framework yet was later changed by Vanderzwalmen et al. as Hemi-straw schema (HSS) wherein the straw was changed over to a hemi-straw by making an inclined cut to one side of the straw for example stacking. Cells could be stacked on the cut end of hemi-straw with slightest volume (~0.3 μl) of VS come about and plunged in LN_2 for vitrification. Under LN_2, the examples may be mechanically secured by embeddings the hemi-straw into a greater straw brought after by halting with plastic fittings at the two completions of the bigger straw for sealing [28].

2.2.10 VITRIFICATION SPATULA AND PLASTIC BLADE

The vitrification spatula and plastic honed steel are subsidiaries of cryotop. The complexity is the structure of example stacking region. In vitrification spatula, the example is stacked on a pedal (~1 mm2 district) made of a beat pipette tip, in to the extent that the plastic edge is a telephthalate strip (5 mm width). The pedal of spatula or the bit of front line may be stacked with example in <0.5 μl of VS result and plunged in LN_2 for vitrification and capacity. Both bearers have been represented to give something like 98~100% survival [29,30].

2.2.11 NYLON MESH

Right when example must be vitrified in generous numbers, which is customarily the case with private animals, a nylon cross area may similarly be viably used as holder to hold the sample[31.]The nylon grid can have a greater surface, in centimetres (cross section size of 60 μm), and has been used to pass on up to 65 dairy steers oocyte for vitrification with clearly higher survival rates. In an exchange study, using human creating lives at the cleavage organize, a 98% post-warming survival rate was obtained [32].

2.2.12 SOLID SURFACE VITRIFICATION USING PRE-COOLED METAL SURFACE

Solid surface vitrification (SSV) is an amazingly clear framework that incorporates setting the VS solution, holding the oocyte/hatchling, as a little droplet (<1.0 μl) particularly on the surface a metal square kept half-submerged in the LN_2. Precooled metal surface is said to have a temperature less demanding than LN_2 itself and thusly, a high cooling rate could be accomplished achieving high rates of survival and progression in a couple of creature mixtures tallying cows, goats, monkeys, and pigs [33]. SSV technique does not require any excellent device and has additional inclination of stacking a couple of oocyte/early life form in a lone droplet. We have further shown that metal piece may be supplanted with a commonplace aluminium foil, which may be physically caved in to make a vessel like structure and floated on the surface of LN_2. In any case, for achievement of SSV strategy, it is key to certification that the surface of the metal is kept dry going before setting the VS solution with oocyte/baby. Using this balanced SSV assembly, we procured up to 80% survival rate for pig oocytes which are the most astonishing recorded achievement for vitrified-warmed pig oocytes. In addition, all things considered, SSV is the primary method that allowed common, lipid-holding, pig oocytes vitrified at either the GV or MII stage to make up to blastocysts following in vitro medication. A contraption that uses precooled metal surfaces in spot of LN_2 for cooling is in a matter of seconds similarly open in a business form[34,35].

2.2.13 VITMASTER

Vitmaster is not a holder rather is a device that makes a fragmented vacuum over the LN_2 to decrease its temperature by a further 10-15°C to 208°C coming to fruition in the game plan of a slush. Utilization of LN_2 slush may keep the securing pocket of gas organizing around the

investigate as it does with liquid LN_2. The inventors of the Vitmaster reported that the rate of cooling of result in altered pulled straws procured with their device when the transporter was placed in LN_2 slush was 32,200°C/min between 25 and -140°C, underneath which there is no spontaneous ice nucleation, and only 8,100°C/min when the same transporter instrument was plunged into LN_2.[36,37]

2.2.14 **PAPER CONTAINERS:**

Great, effectively accessible and shabby option which satisfies all necessities could be done by utilizing paper holders that might be effortlessly ready from any non-elusive, retentive written work paper. Since paper holders could be effortlessly ready and effectively disinfected, they could conceivably be helpful in vitrification of incipient organisms. The plausibility of these compartments was completed alongside other accessible cryocontainers. The paper pieces were cut slanting to make two triangular pieces, a corner of which was then bended, using a forceps, to empower straightforward holding all through diverse steps of the vitrification-warming system. The ensuing paper compartments were open fibrillar structure of triangular shapes (fig. 1).The post warming survival of blastocytes on these holders ends up being practically identical with different cryocontainers (fig. 1) and brought about effective pregnancy prompting conception of full-term offspring [1].

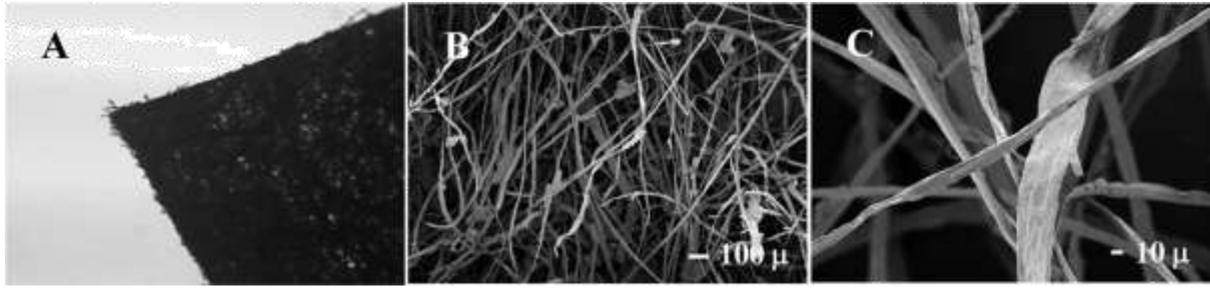

FIG. 1.- Paper Containers as observed by Kim et al (A) under inverted microscope/SEM, magnification (B) 150x, (C) 1000x

TABLE.3: CRYO-CONTAINERS AND DEVICES USED FOR VITRIFICATION

S.NO	CONTAINERS/DEVICES	
	ORIGINAL	DERIVATIVE
1	PLASTIC STRAW	STRAW-IN-STRAW
2	CRYOTUBES/CRYOVIALS	
3	DIRECT DROPPING INTO LN2	
4	ELECTRON MICROSCOPE (EM) GRID	STEEL GRID NYLON MESH MINIMUM DROP SIZE
5	CRYOLOOPS	
6	OPEN PULLED STRAW	SUPERFINE OPS CLOSED PULLED STRAW FLEXIPET DENUDING PIPETTE CRYOTIP
7	CRYOTOP	HEMI STRAW SYSTEM CRYOLOCK CRYOLEAF VITRIFICATION SPATULA PLASTIC BLADE
8	SOLID SURFACE VITRIFICATION	ALUMINIUM FOIL
9	VITMASTER	
10	HIGH HYDROSTATIC PRESSURE	

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2.3 .COMPONENTS OF VITRIFICATION SOLUTION

Answers for vitrification and warming hold one on the other hand more CPA in a base medium. Most VS solution by and large hold pervading CPA besides a non-penetrating CPA in base medium while ES solution hold simply the saturating CPA the focus used for making the VS solution. Of course, warming solution hold a non saturating CPA in decreasing fixations as analyzed previously.

2.3.1 BASIC MEDIUM

The base media to which the CPAs are incorporated have extended from PBS or DMEM media. Astounding results have been represented using both sorts of base media, there has been some stress over the buffering furthest reaches of different pad media all through cooling and warming steps. At low temperature, phosphate-padded media, for instance, PBS tends to get acidic. This reasonable ph development may further be influenced by the kind of protein and CPA utilized. Be that as it may, an adequate prevalence of natural cradled media over phosphate-underpinned media has not been made. An exchange decision is to use a temperature free upheld result which, nevertheless, has not been striven for the oocyte/fetus [38].

2.3.2 CPAs

Dimethyl sulfoxide (DMSO) was the first CPA that engaged the first productive cryopreservation of mouse developing lives in 1972, and had achieved the first compelling pregnancy and conception from human cryopreserved incipient organisms in 1983. Notwithstanding, a couple of entering (CPA that can enter the phones) and non-infiltrating (CPA that can't enter the phone) soon climbed and have been used successfully [39, 40].

2.3.2.1 PERMEABLE CPAs

Ethylene glycol (EG), DMSO, 1, 2 propendiol (PROH), and glycerol are the four huge infiltrating CPAs used for the cryopreservation .Although no specific entering CPA is on a very basic level dominating in assessments of achievement rates, the unified with higher vulnerability is basically supported subsequent to its quick saturation curtails the introduction time, reduces the deadly harm, and minimizes the osmotic swelling all through its clearing. Thus, EG has been for the most part used for vitrification owing to its low sub-atomic weight, high immersion, and low lethality. The usage of EG alone obliges higher centralizations of the CPA (≥ 5.5 M) and thus, is customarily joined with DMSO in 1:1 degree to reducing the storing up of either CPA fundamentally. DMSO and PROH have higher film penetrability than glycerol. DMSO has been showed to cause axle polymerization in oocyte, achieving a stretched potential for polyploidy. PROH is a less unsafe and after that some penetrable than DMSO. Furthermore, cryopreservation using PROH did not bring the aneuploidy rates up in oocyte and developing lives. All CPAs have negative effects, including harmfulness and osmotic harms, on oocyte and babies the level of which is relative to the amassing of CPA and the term of introduction. Acquaintance of specimen with regularly used CPAs (EG, PROH, DMSO, and glycerol) at amassing of 2.0 M alone realized spasmodic meiotic shaft morphology, expanded recurrence of aneuploidy and decreased treatment and embryonic change in mouse [39]. Although it is troublesome to completely forgo the CPA lethality, different philosophies can reduce the level of mischief realized by the CPA danger: Using an extraordinarily penetrable CPA at most insignificant obliged focus. Keeping the introduction time of CPA to perfect. Keeping up ideal temperature of the result. The penetration of CPAs into the specimen is profoundly influenced by the temperature. Raised temperature quickens the penetration of CPAs and along these lines, could increase the CPA danger. In this way, it is important to select suitable CPA with low lethality and treat the example at a suitable

temperature [41]. Joining two or more penetrable CPAs. Measuring the glass-framing viability of the CPAs that make the VS result has uncovered that a marvelous VS result could be framed by a mixture of an adjusted convergance of a solid glass previous , for instance, DMSO and a fragile glass previous, for instance, EG, acetamide, then again formamide that backings cell reasonability. The mixture of EG and DMSO in 1:1 extent has all the reserves of being a standout amongst the most well known combinations for vitrification. The combination of DMSO and EG gave pervasive results for human oocyte vitrification contrasted and DMSO and PROH. A mixture of EG and PROH has also been accounted for. Regardless, others have found agreeable survival, medication, cleavage rates, coming about pregnancy and implantation rates using it is conceivable that EG notwithstanding DMSO, EG notwithstanding PROH, or EG alone [42]. Expanding the consistency of the result. A few makers have prescribed growing the thickness of VS result may help drying out of example and may help diminishing the CPA harmfulness. Notwithstanding, apparently there gives off an impression of being no quick relationship between the thickness of CPA and their viability at supporting the vitrification. Case in point, EG or PROH are unquestionably not gooey and don't bring about a build in thickness of water anyway they are among the most gainful and by and large used CPA inside vitrification, instead of the outstandingly thick glycerol that was extensively used for conventional solidifying, however surrendered a couple of years again for vitrification purposes [43]. Applying a high hydrostatic weight. Subjecting the example to high hydrostatic weight (~200 times more stupendous than ecological weight) for 60 min, with a recuperation time of 1~2 hour going before vitrification has been represented to expand their cryotolerance and consequently, manufacture their improvement fitness. Regardless, this requires a broad machine and incorporates extra time for total time required for the vitrification process. Furthermore, its utility has not yet been represented unequivocally from

other free labs. Including a non-penetrating CPA. The attention of parts, for instance, Ficoll or Polyvinylpyrrolidone may enhance the ampleness of the saturating CPAs the reason behind is obscure [42, 44]**.**

3.2.2 NON-PERMEABLE CPA

supplanting the piece of porous CPA with non-penetrable polymers, for instance, mono- or disaccharides, Polyvinylpyrrolidone, polyethylene glycol (PEG), Ficoll, dextran and PVA offers an exchange likelihood for minimizing the CPA lethality. Typically, sucrose has been used as a piece of cryopreservation result at convergences of ~0.5 M. [45, 46] in the weakened results, afresh, sucrose has been the saccharide of decision. Then again, distinctive sugars, for instance, trehalose [49, 50] and galactose [51] have been just about as viable. In assurance, trehalose was represented to be superior to sucrose in various studies notwithstanding the way that in any case it stays less well known than sucrose. A late study in like manner reported the mixture of trehalose into the cytoplasm of oocyte to upgrade their cryosurvival. The trehalose is immediately discarded creating incipient organism and does not seem to frustrate further developmental fitness. Ficoll is yet an interchange non-immersing CPA that has been used pervasively as a piece of combination with EG and sucrose in VS solution [47, 48, 49].

2.4 VITRIFICATION-INDUCED DAMAGES

Some biotic specimens experience huge uneasiness (chilly daze and osmotic tension) all through vitrification warming besides may persist far reaching morphological moreover valuable damage. As demonstrated by the different temperature reaches out through which the cells pass, three sorts of damage may be recognized all through the cooling step. Between +15 and -5℃, oocyte/fetuses may cause chilling wounds that dominatingly hurts the

cytoplasmic lipid droplets and microtubules including the meiotic axles [48, 49]. Much of the time, the late mischief may be reversible however the past is always irreversible and all things considered helps the downfall of lipid-rich oocyte/ creating lives of particular species. Temperature underneath -150℃ is undoubtedly the smallest dangerous time of the vitrification framework. All through limit (ordinarily in LN_2 at -196℃), if not true blue done, accidental warming is in all likelihood the most consistent explanation behind harm which can result in devitrification (occasion of ice jewels), especially right when the CPA level is kept at the base level. All through warming, the same sorts of harms may happen as at cooling yet in inverse solicitation [46]. Separated from these damages, oocytes/fetus may moreover obtain mechanical mischief to the plasma lemma, cytoplasmic organelles, cytoskeleton and cell-to-cell contacts, the part of which is not completely seen yet is acknowledged to be associated with intracellular and extracellular ice encircling, absence of hydration, gas air pocket molding, extended thickness and the stretched in intracellular solute and ionic centering. The method for cryodamage, its examination and proposals vary between developmental periods of oocyte/ baby. In oocyte, setting of the zona pellucida, troublesome landing of cortical granules, and depolymerization of the microtubules and misalignment of the chromosomes are a significant part of the time viewed that achieves poor sperm invasion and pronuclear creation. As an aftereffect of CPA destructiveness and/or chilling damage, the poles of oocyte can't hold the chromosomes faultlessly at the metaphase plate going before polar body expulsion, provoking chromosomal diffusing, stretched rate of aneuploidy or polyploidy, and end of embryonic development. However, break of zona pellucida is not all that constant if there ought to be an event of vitrified-warmed oocyte. In the event that there ought to be an event of juvenile oocyte, resumption of meiosis upon in vitro improvement may be influenced. Of course, by virtue of hatchlings, the parameters used for the examination of achievement in vitrification are transcendently survival and coming about

headway despite the way that zona pellucida mischief and aggravation of assimilation framework have moreover been accounted for. In pronuclear in addition early-organize embryos, resumption of mitosis are much of the time used as a marker of fitting cryosurvival and have been shown to relate with the implantation potential [45, 51]. However, evaluation of cryodamage is more mind boggling in late-compose creating lives and blastocytes. The comprehensively recognized criteria for creating life survival and capability for trade is that at any rate 50% of the special blastomeres survive. In any case, not completely set up cryopreserved nascent living beings have a reduced proficience to make to the blastocyst mastermind in vitro and achieve blastocytes with diminished total cell numbers and decreased implantation rates. Evaluation of resumption of progression in vitrified-warmed blastocytes is, yet again, more limited since any augmentation in the measure of cells is troublesome to center without staining framework and further in vitro public opinion is at present not possible [1, 39, 50]. Incredibly incredible results have been represented with moderate cementing and likewise with vitrification in both neighborhood animals and human. On the other hand, a concurrence on which method to use for cryopreservation does not seem to exist. In composing, an extensive number of incredible review that almost as help either decrease setting or vitrification. One distinctive inclination of vitrification over moderate cementing is that, vitrification is less mind boggling and afterward a few worthwhile and does not oblige the purchase and upkeep of unreasonable supplies, for instance, controlled rate programmable cooler. Also, there is no demonstrate that vitrification is more unsafe, if any, than moderate solidifying. In an audit study, Al-Hasani et al. furthermore reported that the pregnancy rate got with vitrification of pronuclear stage hatchlings was 3 times higher than that got with the moderate rate solidifying. Examination of handling examples reveals that there is dynamic form in the exploratory energy to vitrification in both animal and human ART [52, 53].

2.5 BIOMATERIAL

Any matter, surface or construct that interacts with biological systems and encompasses elements of medicine, biology, chemistry, tissue engineering and material science. They can be derived from either naturally or synthesized manually using polymers, composite materials etc. main application being in medical field for treatment some ailments like joint replacements, bone plates, bone cement, artificial tissue, heart valves, skin damage and repair, drug delivery etc. It can be in form of autograft, allograft or xenograft as transplant material. Some important properties possessed by a biomaterial are biocompatibility, biodegradability, minimum concentration, non-immunogenicity, transparency, injectibility, resorption ability. They should have ability of remaining intact in a biological environment without damaging the surroundings and without getting damaged in that process, therefore require both biological and materials properties to suit a specific application. The fundamental requirement of any biomaterial concerns the ability of material to perform effectively with an appropriate host response for desired application. That is material and the tissue environment effect on each other and should be gradually resorbed before they finally disappear and are totally replaced by new tissues in vivo [53, 54].

2.6 POLYMER BLENDS

Polymers are a large class of materials consisting of many small molecules called monomers that can be linked together to form long chains thus also known as macromolecules. The two polymers used in this study are polyvinyl alcohol and polyvinyl pyrrolidone. These were selected because of excellent physical properties, mechanical strength, electrochemical stability non-toxicity, biocompatibility and good film forming capability [4, 5].

2.7 PVA

Polyvinyl alcohol is derived from vinyl acetate as monomer. Polyvinyl alcohol (C_2H_4O) contains hydroxyl group attached to methane carbon. It is an atactic polymer exhibiting crystallinity and hydrophilic nature. It is well-known biological friendly polymer due to its compatibility and appropriate mechanical properties and was one of the first synthetic polymers to be tested as an artificial cartilage.

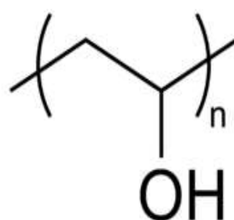

FIG. 2. STRUCTURE OF PVA

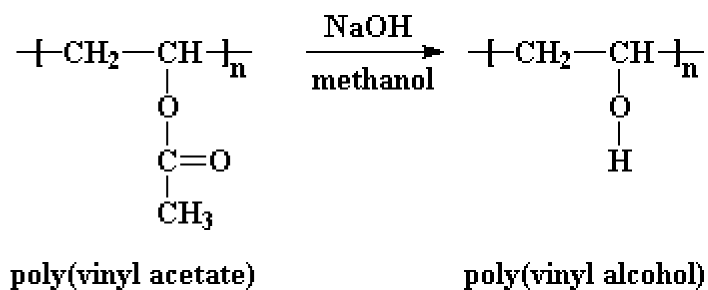

FIG. 3: PREPARATION OF PVA FROM POLYVINYL ACETATE

2.8 PVP

Polyvinylpyrrolidone $(C_6H_9NO)_n$ contains vinyl polymer possessing planar and highly polar side groups due to peptide bond in lactum ring. It is amorphous polymer with amphiphilic

behaviour and possess high glass transition temperature, because of the presence of pyrrolidone group which is drawing polar group and is known to form complexes with other polymers.pvp is one of the most commonly used polymer in medicine because of its solubility in water and its extremely low cytotoxicity.other pharmaceutical applications of PVP include its use as a matrix or as a additive for the controlled release of drugs for co-precipitation of other drugs and a solid dispersion for controlling drug diffusion. A recent work applies its application in skin for transdermal delivery of drugs [55]. Advantage in case of using these two polymers is that proton-accepting carbonyl moiety and hydroxyl group as side group. Therefore hydrogen bonding occurs between the two.

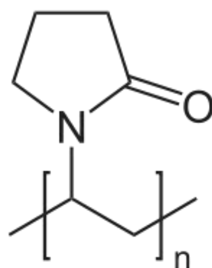

FIG. 4. STRUCTURE OF PVP

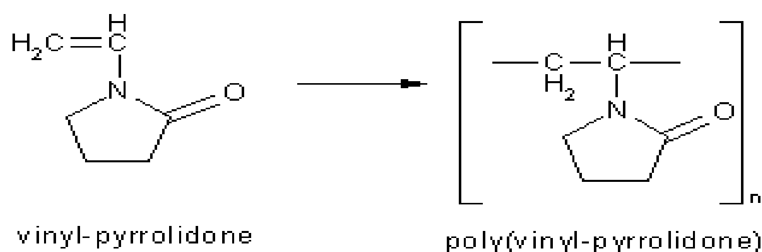

FIG. 5. POLYMERIZATION OF PVP FROM VINYL-PYRROLIDONE

Miscibility of components of a blend affects its final properties and assigned to specific interactions between polymeric components, like hydrogen bond, ionic interaction. Thus combination of these two polymers in blend has emerged as a new approach for developing biomaterials [2].

3. MATERIAL AND METHODS

HIMEDIA chemicals were used in this study PVA (60-125 KDa), PVP-(30 KDa) , PBS buffer (100ml,pH-7.4)- (, NaCl- 0.8g, KCl- 0.02g Na_2HPO_4- 0.144g,)KH_2PO_4- 0.024g, Glutaraldehyde was used as a cross linker, Base medium [DMEM with 10 % FBS], Vitrification solution [0.3M sucrose,5% PVP, 35% EG/ DMSO in base medium], Equilibration solution [4 % EG/DMSO in base medium], Warming solution [(0.4 M, 0.2 M, 0.1 M, 0.05 M) in base medium]. Four different samples were prepared with varying concentrations of PVA and PVP. Sample 1 (S 1) consisted of 8% PVA, Sample 2 (S 2) having 5 % each of PVA and PVP, Sample 3 (S 3) with 3% PVA and 2% PVP, and sample 4 (S 4) is sample 2 with 0.1 ml of Glutaraldehyde.

Table 4: Sample compositions

S.NO.	SAMPLE	COMPOSITION
1	S 1	8 % PVA
2	S 2	5 % PVA and 5 % PVP
3	S 3	3 % PVA and 2 % PVP
4	S 4	5 % PVA and 5 5 PVP with 0.1 ml GA

3.1 EXPERIMENTAL PROCEDURE

3.1.1. POLYMER SOLUTION PREPARATION

Two solutions of PVA were prepared with, 5% PVA (5 gm in 100ml) and 3% PVA .100 ml of distilled water in a beaker was kept on magnetic stirrer at 80° C. Slowly weighed PVA is dropped in the beaker. Once the homogenous transparent solution is prepared, magnetic stirrer is switched off and the solution is ready for the test. Similarly 3 solutions of PVP 5%, 2% were also made but at room temperature. Similarly 100 ml of distilled water in a beaker was kept on magnetic stirrer at room temperature and PVP is dropped in the beaker. Once the homogenous transparent solution is prepared, magnetic stirrer is switched off and the solution is ready for the test .Then these solutions were mixed in desired proportions and then stirred for 15 minutes at room temperature to make sample solutions.

3.2.2 FILM CASTING

Films were cast from aqueous solution in Petri dishes by solvent evaporation method at 60-70° C overnight in incubator. Films thus formed are cut in desired shape. After the removal of film from Petri dish, they are cut into strips with dimensions of 1 cm^2 area. These strips were then kept in PBS buffer for degradation, swelling, weight analysis.

3.2 CHARACTERISATION:

Samples were further characterised by following techniques:

3.3.1 FOLDING ENDURANCE VALUE:

This value is characterised as logarithm of the two fold overlap that are obliged to make a test piece break, Where F is folding endurance and D is number of double folds. It is expressed as a number of folds (number of times the insert is folded at same place, either to break the specimen or to develop visible cracks.) this test is important to check the ability to withstand

folding. This also gives an indication of brittleness. The specimen was folded in the centre, between the fingers and the thumb and then opened. This was termed one folding**.** This test measures a combo of flexibility, extend, and weariness properties. This test is moreover useful for measuring the decay of polymer after maturing as it is sensitive to switches which seem much sooner than there is a change in malleable, impact, or tearing safety. Slight extends in relative dampness cause a checked build in collapsing perseverance. This, coupled with the amazingly little region attempted, achieves wide assortments for special examples. In light of this variability, collapsing continuance is not normally used as a specific unless a tolerance of no short of what 20% is took into consideration high-audit polymers and 30% for standard polymers.

3.3.2 SWELLING TEST AND SOLUBILITY TEST

Swelling capacity of any biomaterial is based on its ability to hold water or buffer. Calculation is done by this equation, water uptake is expressed as:

$W = M_2 - M_1 / M_1$

M_2 = weight of water soaked film

M_1 = weight of dry film (initial)

Films were cut in strips of 1 cm2 and their initial weight is checked, then they are immersed in 5 ml of PBS buffer. After every few hours weight was checked and noted. Surface moisture was removed using tissue paper. Finally samples were allowed to dry until constant weight is achieved at 60°C and weighed (M_3)

Solubility factor (SF) % = M_1-M_3 (100) /M_1

Here M_3 is the weight of film after drying

3.3.3 DEGRADATION TESTS

There are several methods to assess biodegradability of films. But the ones used here are weight analysis and pH analysis. Films are kept in PBS buffer and weighed daily to see any changes and number of days in which it is completely degraded. These films were checked for 3 weeks and readings were taken. Before recording the readings films should be soaked with a tissue paper/napkin.

3.3.4 STABILITY TEST

Stability of the film in liquid nitrogen is a paramount angle as the capability to withstand such a solidifying temperature is important for vitrification. In this technique the specimen is kept in LN2 for a week or two and examined for time when it is totally dissolved.

3.3.5 THICKNESS OF THE CAST

Thickness of the cast film is measured using Digital Vernier Calliper. This device measures more precisely than could be done by reading or any other scale. It is scale that demonstrates where the estimation lies in the middle of two of the imprints on the principle scale. Final thickness of the film formed depends upon the volume used to fill the Petridish.

3.3.6 X-RAY DIFFRACTION

XRD measurements of samples were recorded with x-ray difractor operated at 45kV and 40Ma.the diffractometers were measured in the range of 2ɵ from 5-50 at a speed of 3 degree/min.

3.3.7 HEMOCOMPATIBILITY TEST

About 10 ml of 0.9% saline was added to 8 ml of blood (Dilution ratio was 10:8). Then in 9ml of saline and 0.5 ml of diluted blood was added to it, for test sample 0.5 ml of sample were added in the solution. 0.5 ml of 0.1 M HCL and 0.5 ml of saline were added instead of test sample in the mixture of blood and saline (9 ml saline + 0.5 ml diluted blood) to know the positive and negative control. The samples were incubated along with positive and negative control for 60 min and the centrifuged at 1000 rpm for 5 min. The supernatant is taken and OD was measured by using spectrophotometer at an absorbance of 545nm.5 haemolysis is calculates by below equation.

$$\% \text{ Hemolysis} = 100\times (OD_{sample} - OD_{-ve\ control}) / (OD_{+ve\ control} - OD_{-ve\ control})$$

3.3.8 TRYPAN BLUE EXCLUSION TEST

This exclusion test is used to determine the number of viable cells present in a cell suspension. The principle based on the live cells those posses' intact cell membranes that exclude certain dyes like Trypan blue. A cell sample is mixed with dye and hen visually examined to determine whether cell take up or exclude dye. The visualization is done by using microscope and dye mixtures visualized on haemocytometer .Presence of stained cell indicates that the cell was dead and the unstained cells indicate that the cells were live respectively. The sample is mixed with 0.4% Trypan blue solution in 1:4 ratio and filled in haemocytometer. Incubation of cells on haemocytometer is done for 1-2min at room temperature and cells are counted under the microscope in 4 1×1 square of one chamber.non-viable cells appear blue while viable remain unstained.

4. RESULTS AND DISCUSSIONS

4.1 FOLDING ENDURANCE VALUE

Folding endurance was performed to evaluate elasticity and brittleness of film. The films containing PVA /PVP showed maximum endurance 380, with increase in PVP content endurance increased. This may be attributed to their continuous polymeric structure which cannot be broken easily. Although folding endurance was high when both PVA and PVP are present in the film but there was a slight difference when both were present in equal amount. This suggest that with increase in PVP content, mechanical strength increases and the film was able to show more flexibility.

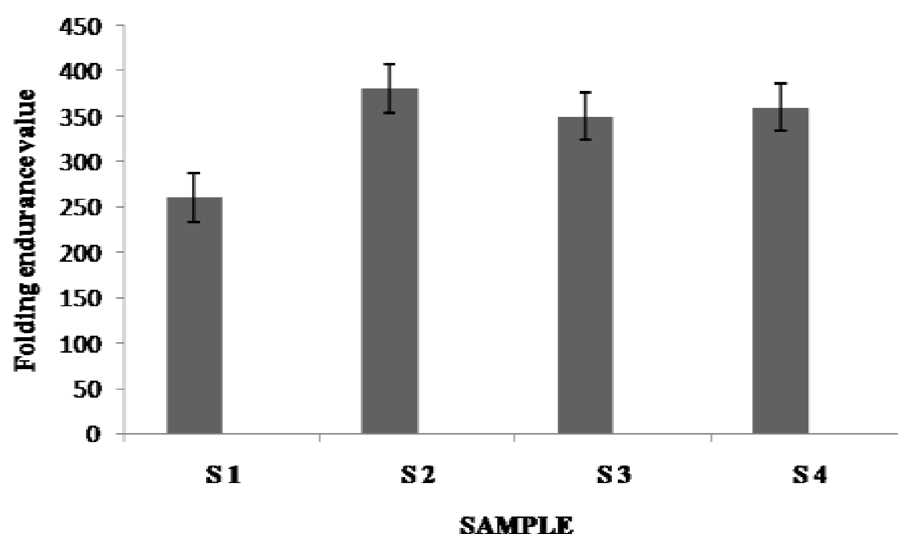

Fig.6. Folding endurance value of PVA/PVP film

4.3 DEGRADATION TEST: Samples were kept in PBS buffer for 20 days; pH and weight changes occurring in course of 20 days were observed and recorded

4.3.1 **pH Analysis**- As observed there was no change in pH in the duration of 20 days. These films showed minimum degradation with time as change in pH is one such criteria for degradation study. As seen from the graph below changes were insignificant. It was always between 7-8 which concludes that these films were not easily degradable.

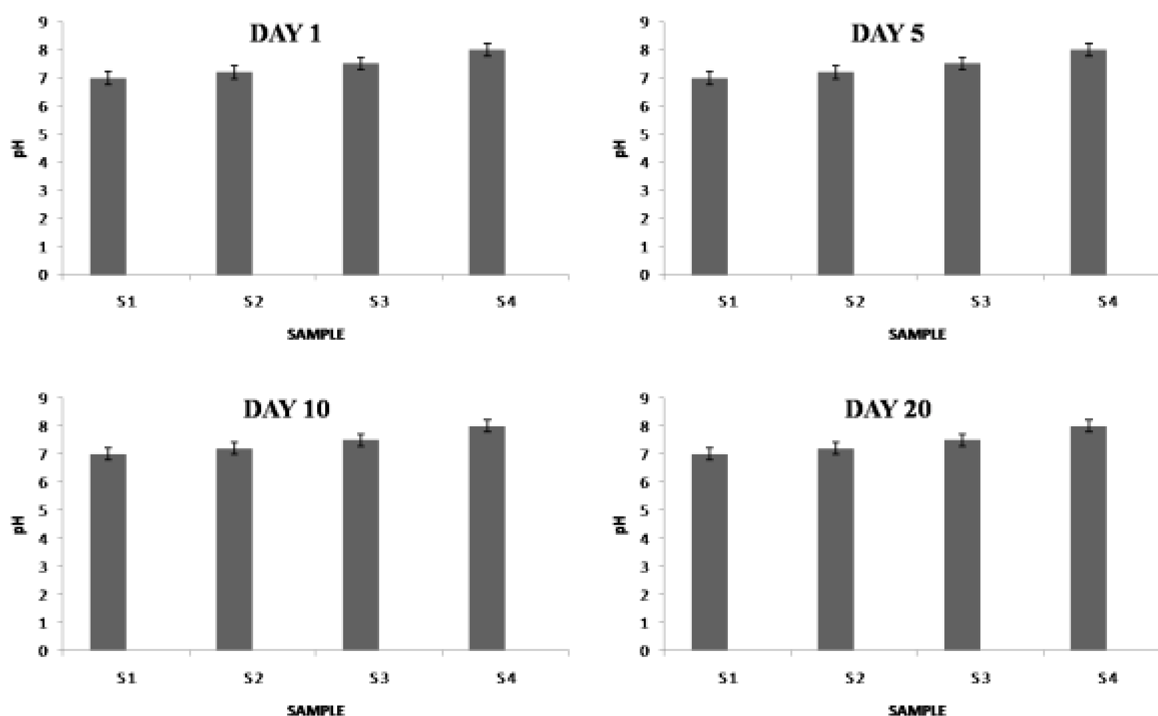

Fig.7.pH observed for PVA/PVP film

4.3.2. Weight analysis: Weight was checked for 20 days and following changes were observed. As shown in the data below, Weight of the sample increased with time but became constant after 48 hrs. In S1 weight of the film increased from 0.02 to 0.08 g as PVA has crystalline structure the ability to swell been comparatively less as compared to PVA/PVP film. In S2 with addition of PVP, weight increased from 0.04 to 0.13 g. S3 also has the

similar effect. Although S4 showed less water uptake with the addition of cross linker as compared to other samples constituting PVP.

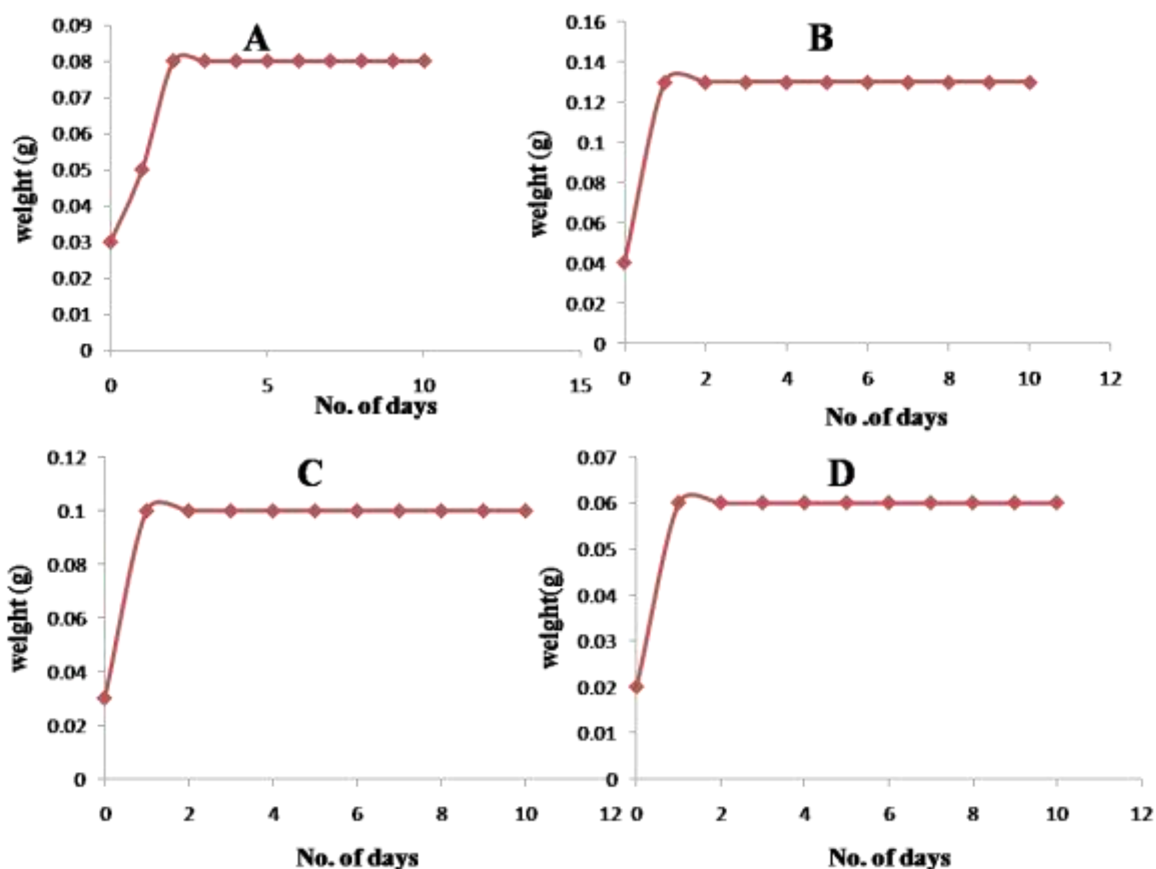

FIG. 8: Weight Analysis of PVA/PVP films (A) 8% PVA (B) 5% PVA-5% PVP (C) 3% PVA-2% PVP (D) 5% PVA-5% PVP WITH GA

4.4 SWELLING TEST: Swelling percentage was calculated once the final weight in each sample is obtained. PVA crystallites in the membranes play a double role as it reduce the absorbed solvent and restrict the swelling of the membrane by a physical cross linking effect. The degree of swelling is higher in case of PVA/PVP blend than just for PVA. It could be explained because PVP reduces the crystallinity of the blend. In addition PVP has higher

affinity for water and it makes that the film with PVP swell to a greater extent than PVA sample.

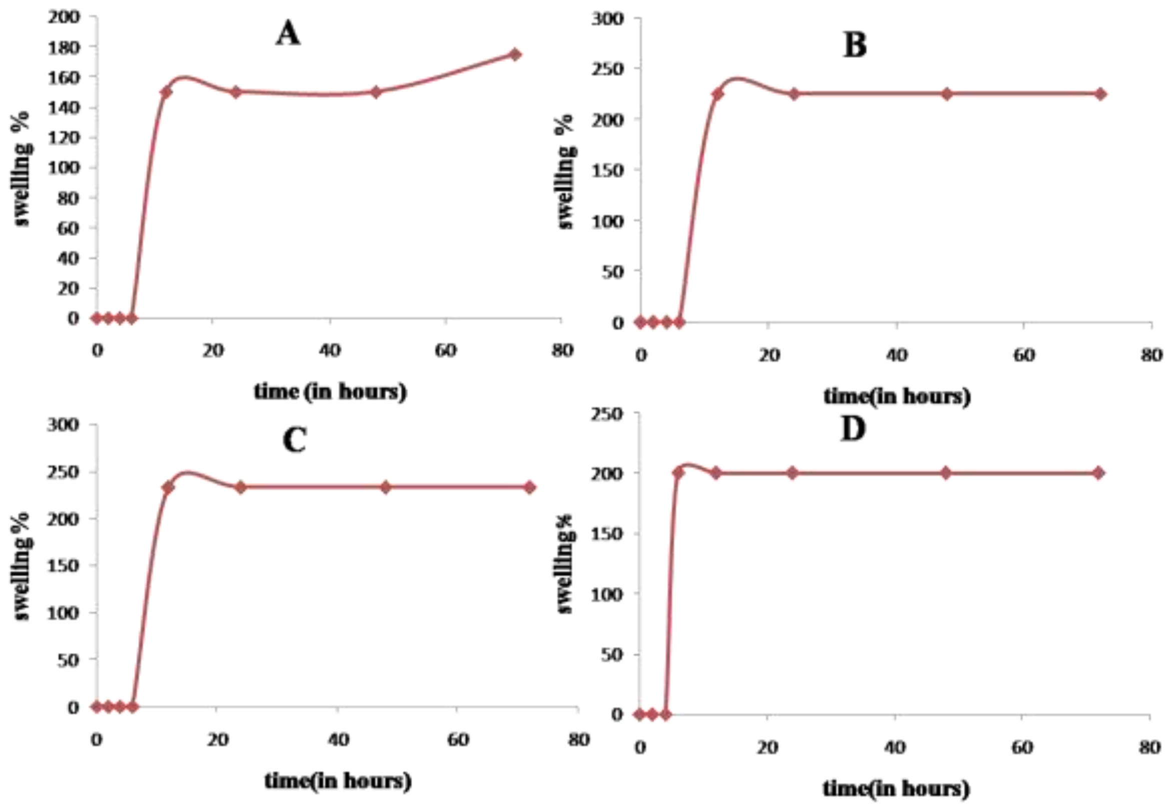

FIG. 9: Swelling % (A) 8% PVA (B) 5% PVA-5% PVP (C) 3% PVA-2% PVP (D) 5% PVA-5% PVP with GA

4.5. STABILITY TEST

Stability of the film in liquid nitrogen is a paramount angle as the capability to withstand such a solidifying temperature is important for vitrification. In this technique the specimen is kept in LN2 for a week or two and examined for time when it is totally dissolved. These films were stable for at least 7 days.

4.6. THICKNESS OF THE FILM

Thickness of the cast film is measured using Digital Vernier Calliper. This device measures more precisely than could be done by reading or any other scale. It is scale that demonstrates where the estimation lies in the middle of two of the imprints on the principle scale. Thickness of films are a important factor as it effects other properties of the film like swelling properties, the more thick film is, more water it will be able to hold, therefore thickness of the film can be calculated from the volume of the solution taken in the Petridish.

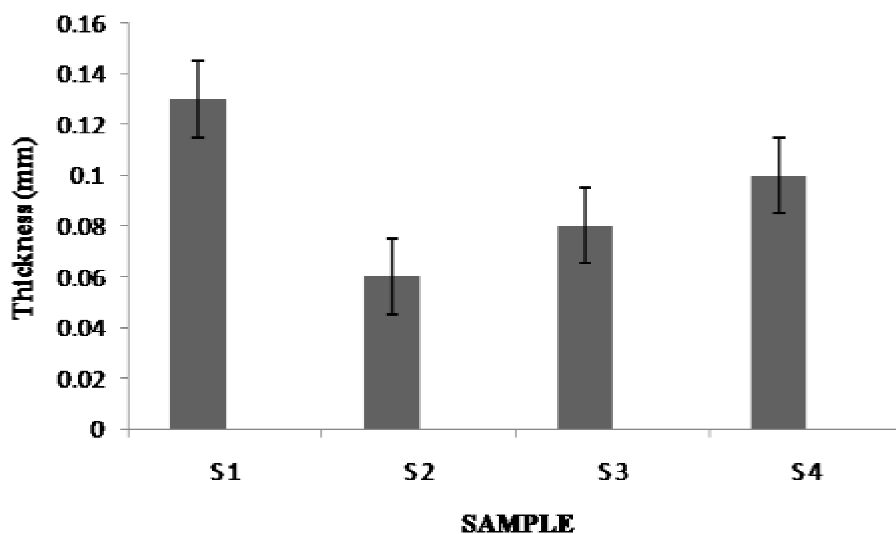

FIG.10 Thickness of the film

4.7 SOLUBILITY IN WATER

Solubility of PVA in water is attributed to the interaction of the hydroxyl groups with water molecules through hydrogen bonds. Solubility increases with the PVP content in the film because of the availability of space in the lattice of PVA/PVP blend .Once the sample is cross linked, the number of chains which are not joined to the matrix is reduced and therefore the solubility is reduced as well.The presence of GA crosslink PVA and the solubility is reduced. With addition of Glutaraldehyde swelling decreases because of cross linking the structure becomes more compact and rigid.

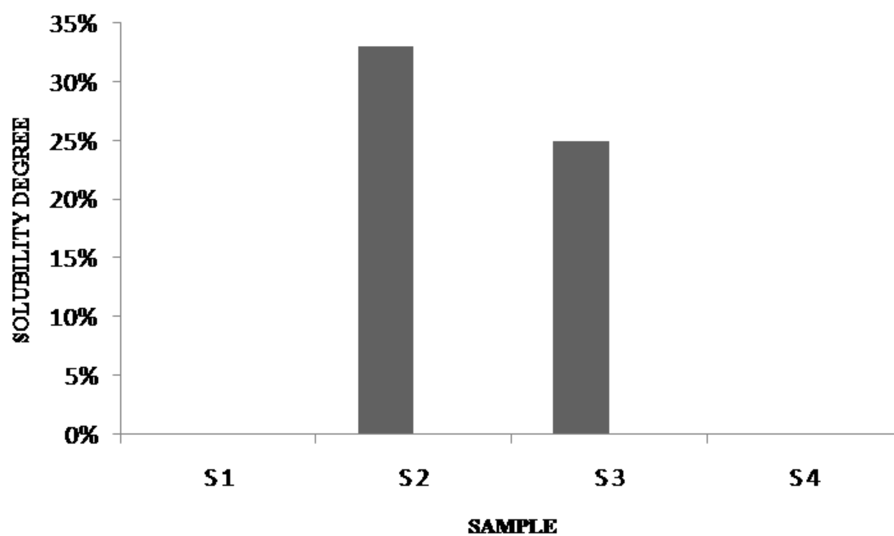

FIG.11 Solubility of films in water

4.8 XRD ANALYSIS

XRD measurements of samples are taken to measure crystallinity in the sample. A sharp with high intensity peak shows that the sample is crystalline in nature whereas a broader with less intensity peaks concludes that the sample taken is amorphous in nature. For these samples x-ray difrractor was operated at 45kV and 40Ma, the diffractometers were measured in the range of 2ɵ from 5-50 at a speed of 3 degree/min.PVA is crystalline in nature and peak comes at 20°, as PVP concentration is increased, the peak reduces and gets more broader in each case .This concludes that with addition of PVP the crystalline nature of PVA reduces and blend formed is less crystalline in nature.

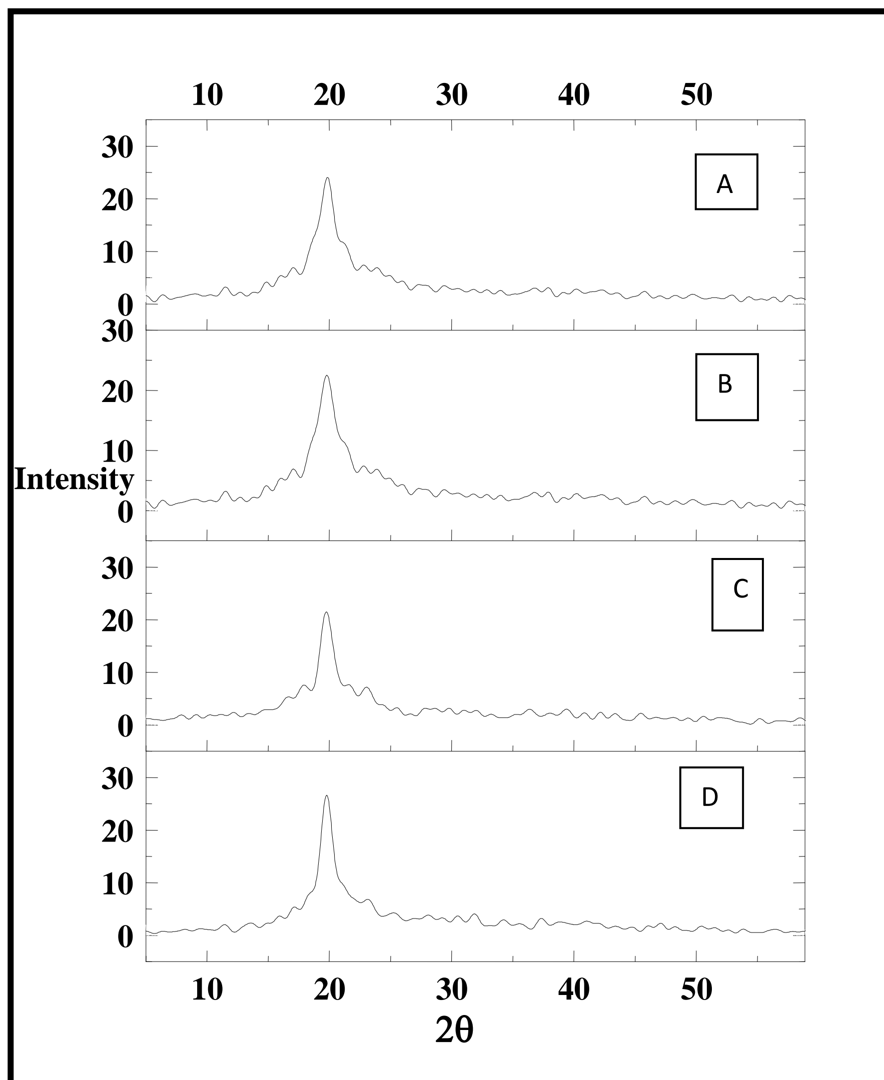

FIG. 12: XRD analysis of PVA/PVP films prepared from: (A) 8% PVA (B) 5% PVA-5% PVP (C) 3% PVA-2% PVP (D) 5% PVA-5% PVP WITH GA

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4.9 HEMOCOMPATIBILITY TEST

This test refers to ability of RBCs to rupture under stress.its the degree of hemolysis that occurs when a sample of blood cells are subjected to osmotic stress.a hypertonic solution with 9 % NACl is used to stress the cells.when the cells are kept in a hypertonic solution,due to high concentration of salt outside the cell allows the haemoglobin to expel out of the cell.the measure of this haemoglobin gives the measure of hemolysis percentage. The results indicate that these samples are highly hemocompatible in nature indicating its biocompatibility maximum shown by Sample 2 consisting 5 % PVA and 5 % PVP.

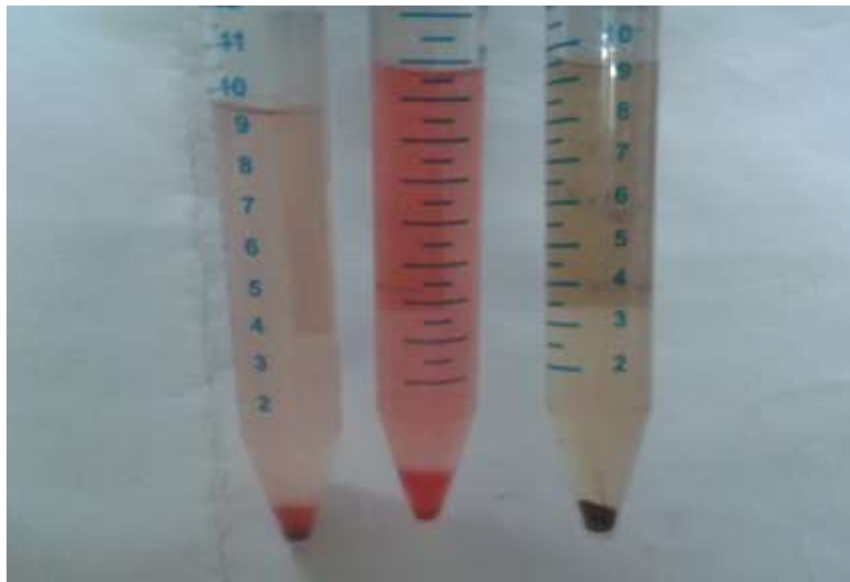

FIG. 13.Hemocompatibility test

Table 5: Hemocompatibility test: Respective OD and % Hemolysis

S.NO	SAMPLE	OD	% HEMOLYSIS
1	+VE CONTROL	0.838	
2	-VE CONTROL	0.094	
3	8% PVA	0.101	0.95
4	5% PVA-5% PVP	0.192	3.52
5	3% PVA-2% PVP	0.172	0.92
6	5% PVA-5% PVP with GA	0.103	1.2

4.10. TRYPAN BLUE EXCLUSION TEST

The principle based on the live cells those posses intact cell membranes that exclude certain dyes like Trypan blue. A cell sample is mixed with dye and then visually examined to determine whether cell take up or exclude dye. After the cell suspension was mixed Trypan blue dye and visualized under microscope on haemocytometer, we see that the cell which are not dead remain unstained as their cell wall is intact.[5] The cells whose cell wall has been compromised were stained blue. Presence of stained cell indicates that the cell was dead and the unstained cells indicate that the cells were live respectively. The sample is mixed with 0.4% Trypan blue solution in 1:4 ratio and filled in haemocytometer. Incubation of cells on haemocytometer is done for 1-2min at room temperature and cells are counted under the microscope in 4 1×1 square of one chamber. Non-viable cells appear blue while viable remain unstained. sample B found to have maximum viability of around 97%.

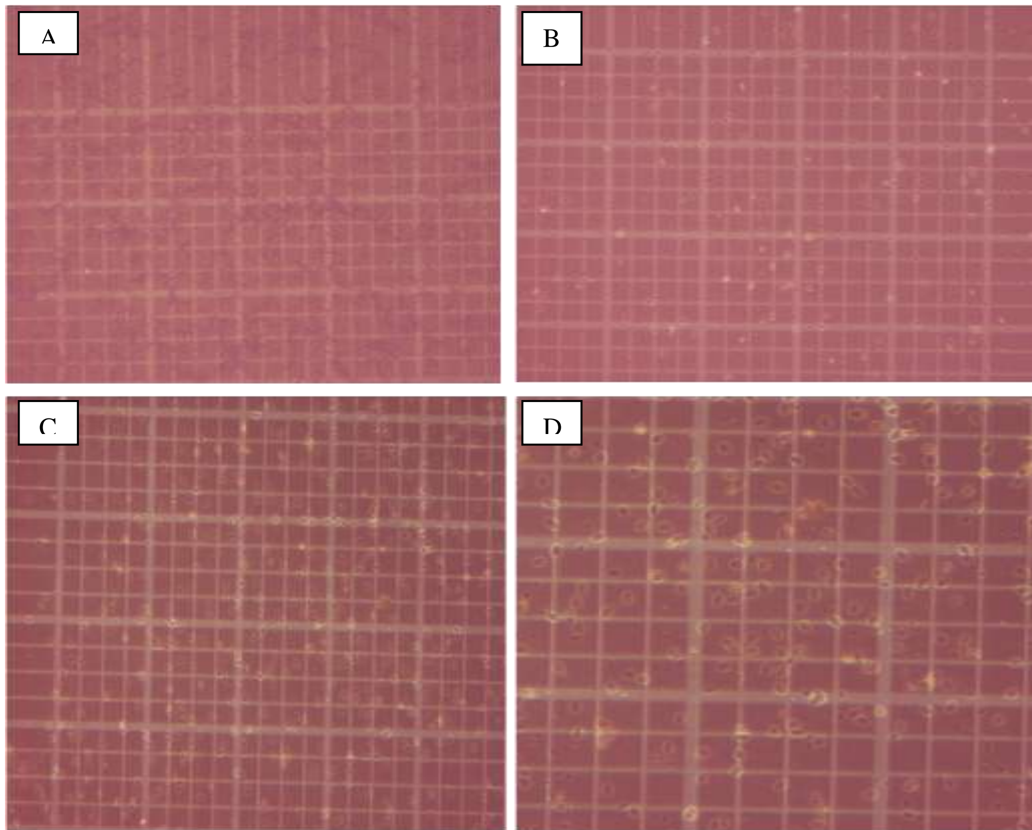

Fig. 14. Trypan Blue Dye Exclusion Test: (A) 8% PVA (B) 5% PVA-5% PVP (C) 3% PVA-2% PVP (D) 5% PVA-5% PVP with GA

Table 5: Viability assessment using trypan blue dye exclusion test

S.NO	SAMPLE	VIABILITY
1	S 1	80 ± 1 %
2	S 2	97 ± 1%
3	S 3	95 ± 1%
4	S 4	94 ± 1%

5. CONCLUSION

PVA/PVP Blends are versatile candidate for medical applications and films have been obtained by solvent evaporation method. Addition of Glutaraldehyde affected the structure of blend making it more rigid and compact, although difference was slight as physical cross linking already exists between the two polymers. These films have mechanical strength, swelling and solubility in water and stability in liquid nitrogen. On the basis of all the results it is concluded that films consisting of 5% PVA and 5% PVP were found to have best results so far with maximum folding endurance, swelling and solubility in water and biocompatibility. They can prove to be promising candidate for vitrification of the biological sample.

6. REFERENCES

1. Kim, Y. M., Uhm, S. J., Gupta, M. K., Yang, J. S., Lim, J. G., Das, Z. C., ... & Ko, D. H. (2012). Successful vitrification of bovine blastocysts on paper container. Theriogenology, 78(5), 1085-109
2. Fahy GM, MacFarlane DR, Angell CA, Meryman HT. Vitrification as an approach to cryopreservation. Cryobiology 1984; 21: 407-26.
3. Vajta G, Nagy ZP, Cobo A, Conceicao J, Yovich J. Vitrification in assisted reproduction: myths, mistakes, disbeliefs and confusion. Reprod Biomed Online 2009; 19 Suppl 3:1-7.
4. Cassu, S. N., & Felisberti, M. I. (1997). Poly (vinyl alcohol) and poly (vinyl pyrrolidone) blends: miscibility, microheterogeneity and free volume change.Polymer, 38(15), 3907-3911.
5. Bhajantri, R. F., Ravindrachary, V., Poojary, B., Harisha, A., & Crasta, V. (2009). Studies on fluorescent PVA+ PVP+ MPDMAPP composite films.Polymer Engineering & Science, 49(5), 903-909.
6. Berthelot F, Martinat-Botte F, Locatelli A, Perreau C, Terqui M. Piglets born after vitrification of embryos using the open pulled straw method. Cryobiology 2000; 41: 116-24.
7. Kuleshova LL, Shaw JM. A strategy for rapid cooling of mouse embryos within a double straw to eliminate the risk of contamination during storage in liquid nitrogen. Hum Reprod 2000; 15: 2604-9.
8. Nakao K, Nakagata N, Katsuki M. Simple and efficient vitrification procedure for cryopreservation of mouse embryos.Exp Anim 1997; 46: 231-4.

9. Kim DH, Park HS, Kim SW, Hwang IS, Yang BC, Im GS, et al. Vitrification of immature bovine oocytes by the microdrop method. J Reprod Dev 2007; 53: 843-51.
10. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod 1996; 54: 1059-69.
11. Fujino Y, Kojima T, Nakamura Y, Kobayashi H, Kikuchi K, Funahashi H. Metal mesh vitrification (MMV) method for cryopreservation of porcine embryos. Theriogenology 2008;70: 809-17.
12. Arav A, Zeron Y. Vitrification of bovine oocytes using modified minimum drop size technique (MDS) is effected by the composition and the concentration of the vitrification solution and by teh cooling conditions. Theriogenology 1997; 47: 341
13. Larman MG, Sheehan CB, Gardner DK. Vitrification of mouse pronuclear oocytes with no direct liquid nitrogen contact. Reprod Biomed Online 2006; 12: 66-9.
14. Liebermann J, Tucker MJ. Effect of carrier system on the yield of human oocytes and embryos as assessed by survival and developmental potential after vitrification. Reproduction 2002; 124: 483-9.
15. Vajta G, Lewis IM, Kuwayama M, Greve T, Callesen H. Sterile application of the Open Pulled Straw (OPS) vitrification method. Cryo-Letters 1998; 19: 389-92
16. Chen SU, Lien YR, Cheng YY, Chen HF, Ho HN, Yang YS. Vitrification of mouse oocytes using closed pulled straws (CPS) achieves a high survival and preserves good patterns of meiotic spindles, compared with conventional straws, open pulled straws (OPS) and grids. Hum Reprod 2001; 16: 2350-6.

17. Liebermann J, Tucker MJ, Graham JR, Han T, Davis A, Levy MJ. Blastocyst development after vitrification of multipronuclear zygotes using the Flexipet denuding pipette. Reprod Biomed Online 2002; 4: 146-50.
18. Kuwayama M, Vajta G, Ieda S, Kato O. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. Reprod Biomed Online 2005; 11: 608-14.
19. Ebrahimi B, Valojerdi MR, Eftekhari-Yazdi P, Baharvand H. In vitro maturation, apoptotic gene expression and incidence of numerical chromosomal abnormalities following cryotop vitrification of sheep cumulus-oocyte complexes. J Assist Reprod Genet 2010; 27: 239-46.
20. Zhou XL, Al Naib A, Sun DW, Lonergan P. Bovine oocyte vitrification using the Cryotop method: effect of cumulus cells and vitrification protocol on survival and subsequent development. Cryobiology 2010; 61: 66-72.
21. Hamawaki A, Kuwayama M, Hamano S. Minimum volume cooling method for bovine blastocyst vitrification. Theriogenology 1999; 51: 165.
22. Vanderzwalmen P, Bertin G, Debauche C, Standaert V, Schoysman R. In vitro survival of metaphase II oocytes and blastocysts after vitrificationi in an Hemi Straw (HS) system. Fertil Steril 2000; 74 (Suppl): 215.
23. Sugiyama R, Nakagawa K, Shirai A, Nishi Y, Kuribayashi Y, Inoue M. Clinical outcomes resulting from the transfer of vitrified human embryos using a new device for cryopreservation (plastic blade). J Assist Reprod Genet 2010; 27: 161-7.
24. Tsang WH, Chow KL. Mouse embryo cryopreservation utilizing a novel high-capacity vitrification spatula. Biotechniques 2009; 46: 550-2.

25. Nagashima H, Kashiwazaki N, Ashman RJ, Grupen CG, Nottle MB. Cryopreservation of porcine embryos. Nature 1995; 374: 416.
26. Matsumoto H, Jiang JY, Tanaka T, Sasada H, Sato E. Vitrification of large quantities of immature bovine oocytes using nylon mesh. Cryobiology 2001; 42: 139-44.
27. Dinnyes A, Dai Y, Jiang S, Yang X. High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. Biol Reprod 2000; 63: 513-8
28. Gupta MK, Uhm SJ, Lee HT. Effect of vitrification and betamercaptoethanol on reactive oxygen species activity and in vitro development of oocytes vitrified before or after in vitro fertilization. Fertil Steril 2010; 93: 2602-7.
29. Gupta MK, Uhm SJ, Lee HT. Cryopreservation of immature and in vitro matured porcine oocytes by solid surface vitrification. Theriogenology 2007; 67: 238-48.
30. Martino A, Songsasen N, Leibo SP. Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. Biol Reprod 1996; 54: 1059-69.
31. Arav A, Yavin S, Zeron Y, Natan D, Dekel I, Gacitua H. New trends in gamete's cryopreservation. Mol Cell Endocrinol 2002; 187: 77-81
32. Sieracki NA, Hwang HJ, Lee MK, Garner DK, Lu Y. A temperature independent pH (TIP) buffer for biomedical biophysical applications at low temperatures. Chem Commun (Camb) 2008: 823-5.
33. Kola I, Kirby C, Shaw J, Davey A, Trounson A. Vitrification of mouse oocytes results in aneuploid zygotes and malformed fetuses. Teratology 1988; 38: 467-74.

34. Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. Nature 1983; 305: 707-9.
35. Gook DA, Osborn SM, Bourne H, Johnston WI. Fertilization of human oocytes following cryopreservation; normal karyotypes and absence of stray chromosomes. Hum Reprod 1994; 9: 684-91
36. Huang JYJ CH, Tan SL, Chian RC. Effects of osmotic stress and cryoprotectant toxicity on mouse oocyte fertilization and subsequent embryonic development in vitro. Cell Preserv Technol 2006;4:149-60.
37. Du Y, Pribenszky CS, Molnar M, Zhang X, Yang H, Kuwayama M, et al. High hydrostatic pressure: a new way to improve in vitro developmental competence of porcine matured oocytes after vitrification. Reproduction 2008; 135:13-7.
38. Pribenszky C, Molnar M, Cseh S, Solti L. Improving postthaw survival of cryopreserved mouse blastocysts by hydrostatic pressure challenge. Anim Reprod Sci 2005; 87: 143-50.
39. Kim EY. Effect of vitrification method on the in vitro/in vivo development of bovine follicular oocytes [dissertation]. Seoul (Korea): Konkuk Univ.; 2003.
40. Kim YM. Development of a new vitrification container of bovine embryo freezing [dissertation]. Seoul (Korea): Konkuk Univ.; 2004.
41. Ryu CS. Cryopreservation of bovine mature and immature oocytes by ultra-rapid cooling [dissertation]. Seoul (Korea): Konkuk Univ.; 2000.
42. Lee YJ. Development of in vitro produced bovine embryos after vitrification with various containers [dissertation]. Seoul (Korea): Konkuk Univ.; 2002.
43. Wright DL, Eroglu A, Toner M, Toth TL. Use of sugars in cryopreserving human oocytes. Reprod Biomed Online 2004; 9: 179-86.

44. Eroglu A, Toner M, Toth TL. Beneficial effect of microinjected trehalose on the cryosurvival of human oocytes. Fertil Steril 2002; 77: 152-8.
45. Eroglu A, Elliott G, Wright DL, Toner M, Toth TL. Progressive elimination of microinjected trehalose during mouse embryonic development. Reprod Biomed Online 2005; 10: 503-10.
46. Chian RC, Huang JY, Tan SL, Lucena E, Saa A, Rojas A, et al. Obstetric and perinatal outcome in 200 infants conceived from vitrified oocytes. Reprod Biomed Online 2008; 16: 608-10.
47. Langer R, Vacanti JP. Tissue Engineering. Science 1993;260:920-926.
48. Rezwan K, Chen QZ, Blaker JJ, Boccaccini AR. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. Biomaterials 2006;27(18):3413-3431.
49. Hassan, C.M., Peppas, N.A., 2000. Structure and applications of poly (vinyl alcohol) hydrogels produced by conventional crosslinking or by freezing/thawing methods. Adv. Polym. Sci.153, 37–65.