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First Estimation of *Drosophila* EPS Solution for Permeabilizing Lepidoptera *Galleria mellonella* Embryos

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Abstract

The increased importance of the *G. mellonella* for wide range of scientific research and commercial sides will need to create a germplasm resource banking by cryopreservation. Impermeability is a fundamental limiting factor for the successful cryopreservation of arthropods embryos. The successful permeability of *Drosophila* embryo by using an embryo permeabilization solvent (EPS) solution encouraged this trial on *G. mellonella* embryos (stage of 24 hours Post-oviposition (h PO)). Permeability assessment with Rhodamine B and crystal violet dyes showed that *G. mellonella* embryos can be permeabilized by EPS of D-limonene that has 3 mol ethoxylated alcohol. The permeabilization for 30 sec exposure time was resulted 61.5% ± 5.8% survival rate, 31.7% ± 3.1% uptakes dyes and 40.5% ± 0.3% was the survival rate post loading in 12% Ethylene glycol (EG). The low viability after immersion in liquid nitrogen (LN) (0.6% ± 0.08%) is due to the dual toxicity of EPS and cryoprotectant (CPA) solutions. However, fluorescence images showed sufficient permeability that confirms the possibility to increase the permeability of *G. mellonella* embryos with EPS solution, and to have the opportunity to improve the viability after LN by improving procedures of loading and dehydration with various CPAs and exposure times, which decrease the toxicity effect.

Keywords

Permeabilization, *G. mellonella*, *Drosophila*, D-Limonene, Eggshell

1. Introduction

Using *Galleria mellonella* (Lepidoptera, Pyralidae) has important benefits similar to *Drosophila melanogaster* Meigen (Diptera, Drosophilidae) as a host infec-

tion model in the entomopathogenic research. The pharmacological approaches of investigation developmental events in *G. mellonella* larvae have been largely expand [1]. In addition, *G. mellonella* has important role as parasite of honeybee hive feeding on the wax used by bees to build their honeycomb [2]. In addition, their larvae have been using as a live food for pets, birds and for fishing bait. These scientific and economic roles have needed to create and maintain wide groups of *G. mellonella* eggs. However, such maintenance would be expensive and time consuming as well as to the possibility of genetic draft from continue rearing. The cryopreservation of insect eggs has the potential of establishing the germplasm resource banking. The embryogenesis studies of *G. mellonella* [3] [4] supported the selection of a proper embryonic stage that tolerated a cryogenic temperature [5]. Insufficient permeability stayed as a main problem prevents the high viability post cryopreservation in *G. mellonella* embryos [6].

The *Drosophila melanogaster* Meigen (Diptera, Drosophilidae) was the first cryopreserved insect species [7] [8] [9]. This approach encouraged researches to cryopreserve other lepidopteran species like *G. mellonella* eggs [10]. For cryopreservation and assaying small molecules uses of *Galleria* embryos, the main obstacle was the impermeability of the eggshells (Chorion and wax layer). Permeability of cryopreservation is a critical for removing cell water and presenting CPAs (loading) subsequent removal of these compounds (unloading-thawing) from embryos immersed in LN. While in assaying toxicology research, the permeability requires to introduce drug or exposure to toxin for testing their effect on the development under imagine assessment.

The eggshells are important for exchange the oxygen and water flux across eggshells to prevent the desiccation of Lepidoptera embryos [11] [12]. The eggshell difference between *G. mellonella* and *D. melanogaster* eggs was known that Lepidopteran eggs have thickness more than dipteran eggs, which make difficulties for cryopreservation of lepidopteran eggs [6] [13] [14].

The non-polar organic solvent (alkane), such as heptane, octane and hexane are used usually for permeabilization of the dechorionated insect eggs [15] [16] [17] [18] [19]. Hexane was the preferred solvent per permeabilization of Dipterans as well as to Lepidoptera *Bombyx mori* eggs [20]. Heptane was preferred permeabilizer for Lepidopterans like *G. mellonella* eggs [10]. These solvents were so toxic and could decrease the viability of treated eggs. In particular, treatment of *G. mellonella* eggs is requires a transition out of and back into the aqueous phase making it technically challenging to avoid desiccation. For this reason, some attempts were conducted in order to improve the permeability of *Galleria* without using these solvents. Hence, the use of non-toxic surfactant Tween 80 (polyoxyethylene sorbitan monooleate) is effective in solubilizing hydrocarbons. Adding Tween 80 to organic solvents of dechorionation solution with different concentrations was succeeding. 0.04% Tween 80 was added to 1.25% sodium hypochlorite resulted in a hatching rate not significant different from that of the untreated eggs [10]. While adding 0.08% Tween 80 was significantly different and lower viability from the untreated eggs. However, solution

of 0.04% Tween 80 + 1.25% sodium hypochlorite has showed same shrinkage and reswelling percentage of eggs exposed to heptane for 10 seconds [6].

The objective of the present study was to evaluate the effect of EPS inversely related to hatching rate and demonstrated properties of Rhodamine B and Crystal violate as markers of ideally permeabilized embryos and their subsequent development.

Cosi and co-worker showed the low survival with organic solvent heptane that encouraged to permeabilizing the *G. mellonella* embryos by non-organic solvent (0.08% Tween 80 with sodium hypochlorite), which was less efficiency than organic solvents. The new non-organic solvent of EPS solution (90% D-limonene + 5% cocamide DEA + 5% ethoxylated alcohol or Alcohol Ethoxylates (AEs)) was found effective substitute for wax layer, and Limonene was enable permeabilized *Drosophila* embryos uptake of dyes have various molecular mass up to 995 Da (1Da = 1 g/mol) [21]. The encouraged motivation for testing EPS with *G. mellonella* embryos was the molecular mass of most permeable CPAs is less than 100 g/mol in used dyes (Ethylene glycol: 62.07 g/mol, Dimethyl sulfoxide: 78.13 g/mol, propylene glycol: 76.09 g/mol), with taking into consideration that *Drosophila* eggshell is a half size of that in *G. mellonella*. These facts generated the aim of this study to focus on the possibility for permeabilizing *G. mellonella* eggs with considered previous EPS results of *Drosophila* embryos.

2. Material and Methods

2.1. Embryo Collection and Culturing

The research was carried out in the Research Center for Agrobiology and Pedology (CREA-Florence, Italy). *Galleria mellonella* eggs were obtained from routinely rearing in room with temperature and relative humidity of 26˚C ± 2˚C and 70% ± 10%, respectively. The methods for maintaining the insect, collecting eggs and dechorionation the embryos were described by Roversi and co-workers [10]. The eggs were collected for period of 30 min after female deposited on a paper support, which was placed on the upper part of cages containing 100 - 150 adults with a sex ratio of about 1.0 (male/female). The eggs batches placed in aerated cages and incubated in a humidified chamber, with near-normal brood nest conditions: darkness, temperature 30˚C ± 0.5˚C and relative humidity 80% ± 2% [22]. In *Galleria mellonella*, all treatment procedures of synchronization of embryonic development, oviposition period, incubation conditions and selection of 24h PO stage were performed using the protocol of Cosi and co-workers [6].

2.2. Dechorionation-Permeabilization (D/P)

The embryos of *G. mellonella* were removed from the paper by agitating in double distilled water (DDW). Pre-treatment, eggs transferred into cell strainer basket 40 μm Nylon (BD Biosciences, NY, USA). As described in Berkebile and co-workers [22], to facilitate handling, the eggs mass is separate by washing in DDW with agitating by a glass medicine dropper and without using 0.5% NaOH

that caused toxicity and low survival rate. The primary D/P treatment was consists of 1.25% NaOCl + 0.08% Tween 80 for 2 min [6].

2.3. Drosophila EPS Solution

The exposure time in any permeabilization solution is an important factor affects the chemical toxicity and viability rate. The permeability treatment by EPS solution [21] consists of 90% D-limonene + 5% cocamide DEA + 5% AEs (3 mol) was compared under two exposure times (25 and 30 seconds) (Exposure times were chosen based on less toxicity). EPS was diluted with Phosphate Buffered Saline (PBS) to 1:5 (PH: 6.8 - 7) and used to permeabilizing *G. mellonella* eggs. Many successive washes in PBS (500 ml) followed the EPS treatment, and final washing per twice-in PBS has 0.05% Tween 20 (PBStw) (50 ml).

2.4. Alcohol Ethoxylates (AEs)

AEs powder was used to prepare three different molarity solutions (3, 6, and 10 mol) for selecting the best effect in EPS treatment. Post-treatment in EPS, the eggs loaded in 12% EG (in TMN-FH insect medium) for 55 - 57 min, and then recovered by warming step to remove EG from eggs. The warming procedure is consists of three sequence washing steps: 1) Solution of 0.5 M trehalose (in TMN-FH insect medium) per 2 min at room temperature. 2) Solution of 0.25 M trehalose per 10 min. 3) TMN-FH insect medium per 10 min, and 3 times washes in PBStw for removing the medium from treated eggs and to prevent bacterial and fungal contamination [23]. The eggs in this time are ready to transfer inside Petri dish (35 mm) filled agar and placed inside a bigger Petri dish (100 mm) covered by adhesive glue traps for the hatched larvae. Petri dishes were incubated into an aerated cage in a humidified chamber (Binder, Germany) (darkness, temperature, 30˚C ± 0.5˚C, and relative humidity 80% ± 2%) to hatching time. To calculate the hatching rate, the unhatched eggs and new hatched larvae were counted.

2.5. Cryopreservation

The results of AEs molarity showed the 3 mol as the best viability post treatment in EPS and confirmed by the assessment in dyes. The EPS-permeabilized embryos were continued vitrification procedures of cryopreservation as previous work of Roversi and co-workers [10]. After permeabilization process, embryos are placed in a loading solution of 12% EG per 45 min (Loading 1) and 60 min (Loading 2). Then higher viability eggs (Loading 1) were transferred by brush to a strips (15 × 25 × 0.2 mm) of chromatography paper (nylon membranes, Roche) for dehydrating in TMN-FH insect medium consists of 34 wt% EG + 4.5 wt% polyethylene glycol (PEG MW 6000-7500, AppliChem GmbH) + 15.5 wt% trehalose (dehydrate, AppliChem GmbH) per 7 min at 0˚C (on ice). Post-dehydration and concentrate the EG, the eggs left to dry on the Whatman paper before immersion in LN. The eggs on nylon membrane relaxed in LN vapor per

20 - 30 sec and dropped in LN per 5 - 10 min then finally recovered by previous warming and incubation steps.

2.6. Permeability Assessment

For evaluating the efficacy of different permeabilization procedures, 50 - 100 embryos per each treatment were prepared to uptake two dyes, Rhodamine B (MW = 479.0 g/mol, excitation wavelength = 520 - 550) and Crystal Violet (MW = 321.3 g/mol, excitation wavelength = 570 - 600) dyes. The treated eggs using a camel-hairbrush were transferred into 2 ml of 0.1% aqueous solution of dyes. Post staining exposure per 5 min with rocking or the drop-on step, the embryos rinsed twice in 2 ml of TMN-FH medium. Embryos washed four times in PBStw prior to visualizing by microscopes and then continue the incubation of development in the chamber (Binder, Germany). The assessment step was eliminate without counting all damaged eggs from that one have a good staining or uptakes of Rhodamine B and Crystal Violet dyes. Embryos colored with ruby red or dark pink considered to have been successfully permeabilized [6] [16] [24].

2.7. Microscopy

Images of stained embryos were captured by the following setups of microscope. The Nikon (SMZ 10A) stereomicroscope equipped with digital camera (SIGHT DS-Fi1) (Nikon, Japan) for vertical images with Rhodamine B. Differential interference contrast (DIC) is known also as Nomarski inverted microscope and images acquired by those microscopes using a 20 × 0.45 NA plan Fluor (ELWD; Nikon, Japan) objective. For fluorescent imaging, the same inverted microscope equipped with fiber Illuminator or a xenon arc lamp (C-HGFI, Nikon, Japan) that linked to filter has an excitation wavelength 540 - 525 nm (Channel 2). Images were captured by DIC and Fluorescent microscopes using internal digital camera and associated PC software (Nikon, Japan). Images processing was done with Adobe Photoshop.

2.8. Statistical Analysis

Three different egg batches represented 6 - 3 replications for each test. A minimum number of 200 eggs per replicate were tested. Percentages of hatched larvae for treated and control repetitions were calculated. For normality and homoscedasticity, data were tested, and if necessary, normalized by arcsine transformation. Effects of permeabilization treatments on cryopreservation steps were analyzed with two-way factorial analysis of variance (ANOVA) and differences among means were evaluated by the Tukey post-hoc test. When the p-value is < 0.05, and then accepted as statistically significant. The data were analyzed with SPSS 10.0 software (1999).

3. Results

Embryo Hatching Assessment

The separation of embryos mass was the main step that found has primary effect

on successful permeabilization treatment with high viability. Although it is help the permeability by remove the mucus layer, the exposure to 0.1% NaOH per 1 min was toxic and decreased the survival of embryos. Agitation of small groups of eggs is more preferred than aggregates of large groups that have difficulty to separate. Here, the short deposition time plays a vital role in preventing the oviposition to form the large gatherings of eggs. The oviposition for half an hour gives more than 50% homogeneous eggs in the embryonic characters stage [25].

The importance of separation the egg mass to uptakes dye should have the same role later to uptakes CPAs (**Figure 1(A)**, **Figure 1(B)**), and the hatchability was not affected by the toxicity of dye staining test (**Figure 1(C)**, **Figure 1(D)**). This result showed the positive side in *G. mellonella* embryo to establish in vitro conditions that permit drug delivery for toxicological studies.

The dechorionation procedure consists of 1.25% NaOCl + 0.08% Tween 80 had confirmed clearly the permeabilized eggs by fluorescence images that took a big molecular mass of Rhodamine B compared to the control eggs (**Figure 2**).

Statistical ANOVA analysis of hatching rates from two exposure periods of EPS permeabilized-embryos showed no significant difference between 25 and 30 sec resulted 61.5% ± 5.8% and 75.7% ± 9.3%, respectively ($P > 0.05$) (**Figure 3(A)**). The viability of *G. mellonella* eggs post-permeabilization in EPS revealed no significant difference between various molarity of AEs (3, 6, and 10 mol) of EPS solution 33.4% ± 4.8%, 34.1% ± 6.8%, and 44.0% ± 6.4%, respectively (**Figure 3(B)**). But the different molarity has a significant effect during loading of 12% EG as resulted 40.5% ± 0.3%, 22.8% ± 0.0%, and 30.5% ± 1.5%, respectively ($P > 0.05$). Tukey test showed the survival rate of EPS with 3 mol AEs has a

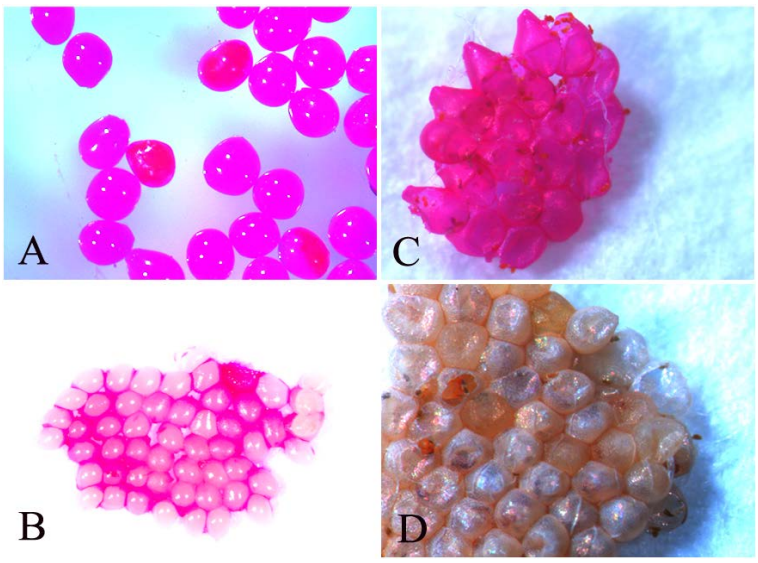

Figure 1. The effect of separation cluster mass eggs of *G. mellonella* on the staining uptake by Rhodamine B. The sufficient separation of the mass eggs showed better staining (A). Mass eggs stained by Rhodamine B showed less uptake of dye (B). Hatched mass eggs stained with Rhodamine B (C). Hatched control without any staining (D). Scale bars: 50 μm (A), (C), (D), 100 μm (B).

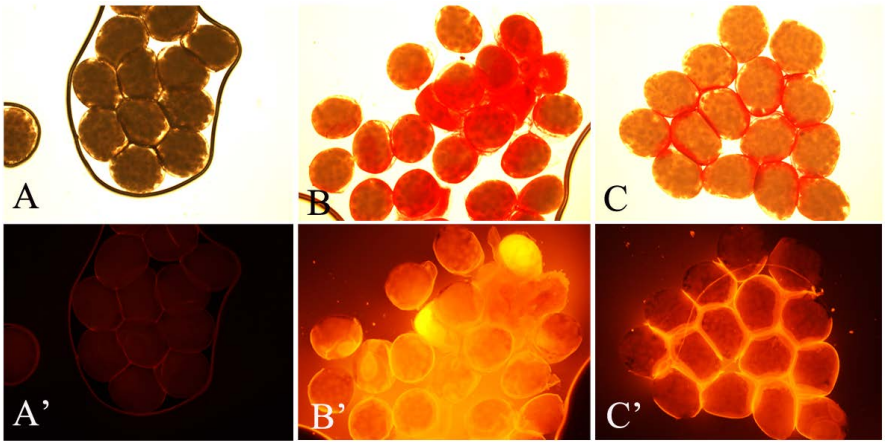

Figure 2. Synchronized images of DIC (A)-(C) and Fluorescent (A')-(C') microscopes. Control eggs without staining of Rhodamine B (A), (A'). Dechorionated eggs by solution of 1.25% NaOCl + 0.08% Tween 80 (B) (B') (C) (C'). The lighted eggs are uptakes the dye staining. Scale bars of all panels: 100 μm.

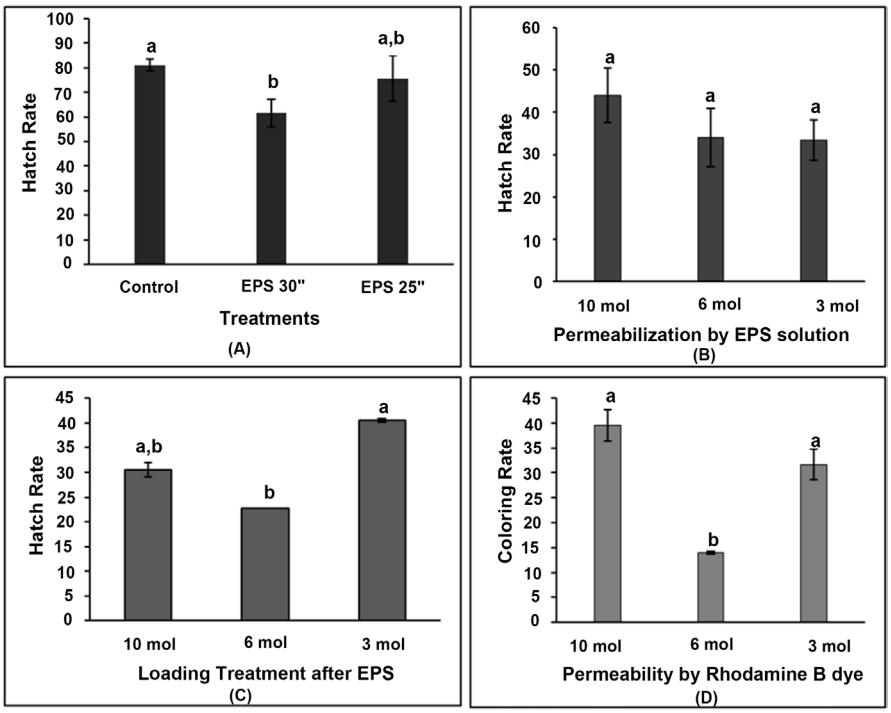

Figure 3. (A): The permeabilization of *G. mellonella* embryos (mean ± SE) with different exposure time of EPS. (B): The permeabilization treatment by different solution of EPS with different molarity of ethoxylated alcohol. (C): The assessment of permeability by uptake of Rhodamine B + EPS solution has different molarity of ethoxylated alcohol. (D): The effect of different molarity of ethoxylated alcohol of the permeabiliztion by EPS on loading solution of 12% EG. Mean values with different letters on the bar are significantly different from one another (Tukey post-hoc test; $P < 0.05$).

better survival rate than 10 mol and 6 mol respectively (**Figure 3(D)**). The permeability evaluation by staining with Rhodamine B was confirmed the significant difference as the staining uptake were 31.7 ± 3.1, 14.0 ± 0.2, and 39.6 ±

3.2 %, respectively (Figure 3(C)). Visual estimation with time between Rhodamine B and Crystal violet dyes confirmed the significant difference to uptakes dyes between various molarity of AEs (Figures 4-6).

The last test of cryopreservation steps showed a survival rate after permeabilization treatment (55.1% ± 3.9%) was not significantly different from control eggs (87.9% ± 5.3%) ($P > 0.05$). The effect of loading time was significantly different as the Tukey showed that 45 min loading time (Loading 1) was high viability than 60 min (Loading 2) due to the toxicity effect of EG (80.2 ± 8.6 and 40.6% ± 9.8%, respectively) ($P > 0.05$). The survival rate after dehydration treatment (50.6% ± 14.1%) has no significant difference from Loading 1 step (45 min exposure time) to 12% EG ($P > 0.05$) (Figure 7).

The low survival rate of *G. mellonella* eggs after immersion in LN (0.6% ± 0.1%) was confirm the sequenced stress of CPAs and cryogenic temperature of LN, which can be decrease with improving Loading/Dehydration steps. The sufficient permeability of EPS showed in microscopic observations referred to the improvement in the time permeability in *G. mellonella* eggs compared to previous results [6] [10].

4. Discussion

Successful cryopreservation of insect eggs must have a sufficient permeability of CPAs, which Cosi and co-workers [6] had tested their kinetic permeation and toxicity into *G. mellonella* eggs. Measurements of volume change by light microscopy [15] [21] [26] [27] and exposure times of permeability in Rhodamine B had been mostly used to study cell and embryo membrane permeability to CPAs [6] [16] [22].

As in *Drosophila* eggs, three-chorion layers of *G. mellonella* eggs were removed routinely with sodium hypochlorite (bleach), while the wax layer limiting the permeability of insect eggs was removed partially by adding the solvent 0.08% Tween 80 [6]. The wax layer is a waterproofing layer, able to exclude entry of even small solutes such as a sodium hypochlorite [21], but D-limonene as a monoterpene oil (as a major constituent of the oil in orange peels) was used to

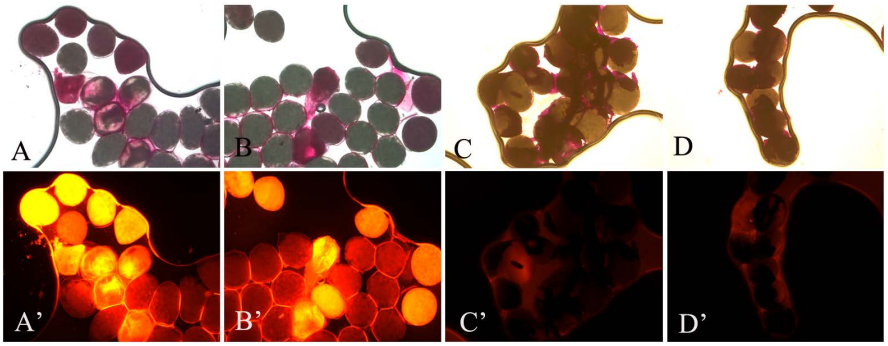

Figure 4. Synchronized images of DIC (A)-(D) and Flourscent (A')-(D') micropcopes. Permeabilization during 30 sec. in 3 mol EPS. Rhodamine B staining (A) (A') (B) (B'). Crystal Violet staining (C) (C') (D) (D'). Scale bars of all panels: 100 μm.

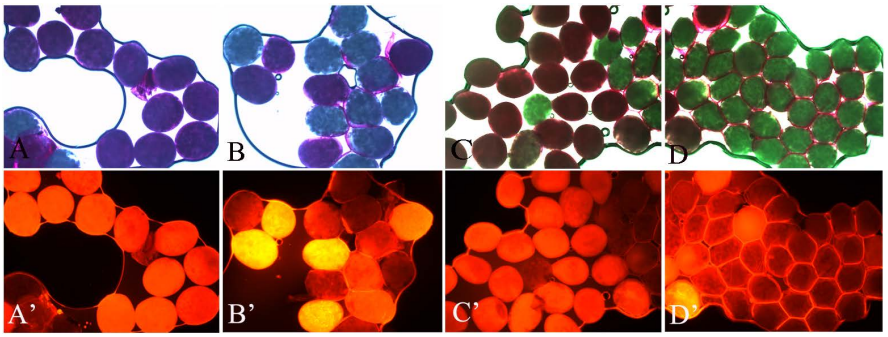

Figure 5. Synchronized images of DIC (A)-(D) and Flourscent (A')-(D') micropcopes. Permeabilization during 30 sec. in 10 mol EPS. Rhodamine B staining (A), (A'), (B), (B'). Crystal Violet staining (C), (C'), (D), (D'). Scale bars of all panels: 100 μm.

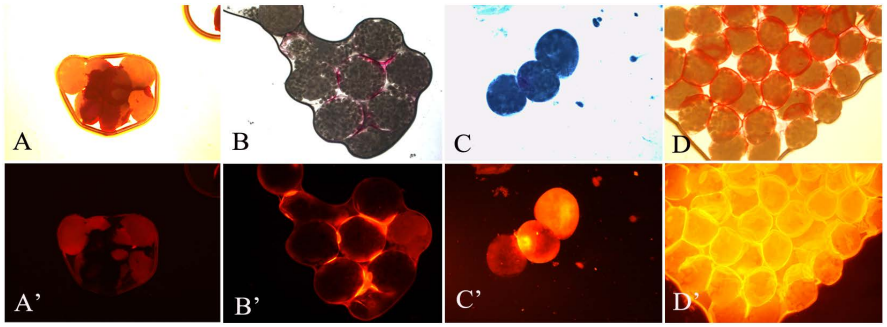

Figure 6. Synchronized images of DIC (A)-(D) and Flourscent (A')-(D') micropcopes. Permeabilization during 30 sec. in 6 mol EPS. Rhodamine B staining (B), (B'), (D), (D'). Crystal Violet staining (A), (A'), (C), (C'). Scale bars of all panels: 100 μm

increase the permeability of dechorionated eggs. The D-limonene need surfactant and oil to have composition for render limonene miscible with water for removal wax layer of dechorionated eggs.

As the eggshells in *G. mellonella* embryo is thicker than of *Drosophila* embryos [3], low permeability was led to the low loading of CPAs before quenching in LN and this could be one of reasons to the low survival rate.

One of the first unexpected results was increased the survival slightly after loading with 12% EG for EPS (3 mol AEs), while decreased with other two EPS have 10 and 6 mol of AEs (**Figure 3(D)**). Its look as eggs viability with low molarity of AEs in EPS will not affecting much by EG toxicity during loading exposure time.

The decreased viability of *G. mellonella* embryos after first experiment in **Figure 3(A)** than **Figure 3(B)** maybe due to store the EPS solution in lab for 3 weeks before use it again, which confirm that our composition was not stable due to unknown reason for more two months as with Rand and co-workers [21]. In addition, dye uptake occurs in higher level of EPS with 3 and 10 mol AEs than 6 mol gave another unknown reason for this significant difference as expected to be sequenced levels with their molarity and survival. The only accepted explanation for that is the unstable composition with EPS of AEs 6 mol during store time.

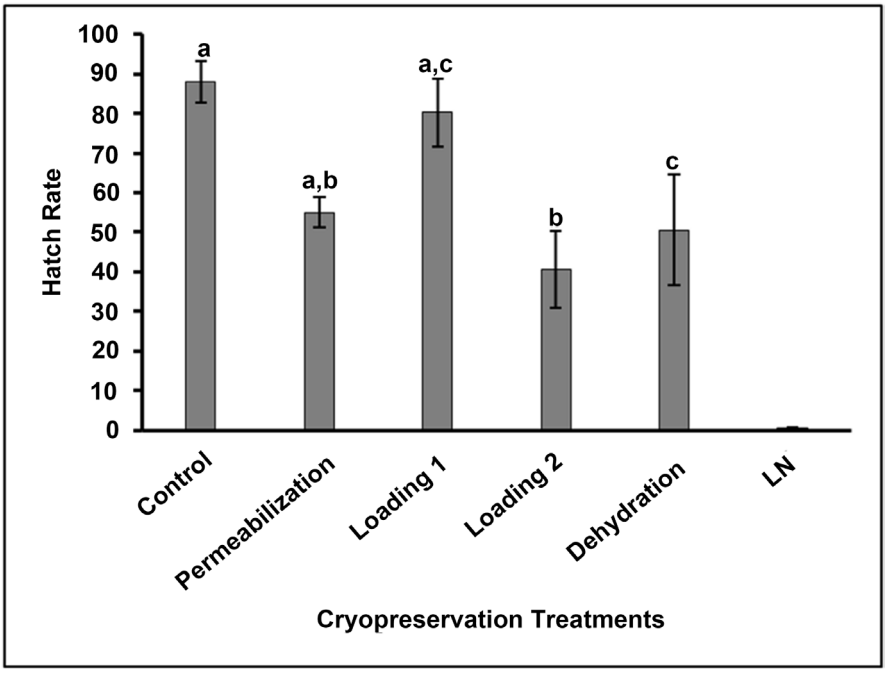

Figure 7. The survival of *G. mellonella* embryos (mean ± SE) after cryopreservation steps. Permeabilization step was in EPS solution have 3 mol of ethoxylated alcohol. Loading process was carried out with two times of exposure to EG for 45 and 60 min (Loading 1 and Loading 2, respectively). Dehydration in TMN-FH insect medium have (34 wt% EG + 4.5 wt% PEG + 15.5 wt% trehalose) for 7 min at 0˚C (on ice). The eggs were relaxed in LN vapor for 20 - 30 seconds and dropped in LN for 5 - 10 min, then washed in warming steps. Mean values with different letters on the bar are significantly different from one another (Tukey post-hoc test; $P < 0.05$).

For higher survival rate, chosen 45 min loading results to continue for dehydration step was not enough for testing the EPS effects on post-immersion in LN. The loading period of 45 min is not enough to present sufficient CPAs for preventing ice formation during cooling and waring steps.

5. Conclusion

In summary, our prediction showed that EPS composition for only two weeks store can give a better viability and permeability results. However, dye uptake traits have been an implicated in previous studies [6] [21], and here, it was not a big different of expected permeability that induced by EPS. Nevertheless, EPS time exposure used here was a very low compared to that used before (2 min).

The successful staining of *G. mellonella* embryos with big molecular weight dyes confirms the possibility to use this procedure in rendering the dechorionated eggshell permeable to small molecules, and use it for medical experimental drugs or loading small moleculae weight cryoprotectants for cryopreservation technology.

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Disclosure Statement

No potential conflict of interest was reported by the author.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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