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Original Research

Effect of Agarose on Viability and Proliferation of BRIN-BD11 Cell Lines

Mozan Hassan[1,2], Siti Pauliena Mohd Bohari[1,3*]

[1] Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, Skudai, Johor Bahru, 81300, Malaysia
[2] Department of Anatomy, College of Medicine and Health Sciences, United Arab Emirates University, Al Ain, 15551, United Arab Emirates
[3] Institute Bioproduct Development, Universiti Teknologi Malaysia, Skudai, Johor Bahru, 81300, Malaysia

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ABSTRACT

Nowadays, there is an increasing effort to shift from using two-dimensional culture (2D) to three-dimensional culture (3D). This shift is led by the tissue engineering research study that needs to use the 3D culture system to form tissue graft. Agarose hydrogel has been widely used to provide a solid 3D environment for the cells due to its ability to support the cells and to mimic the extracellular matrix (ECM). In this study, 3D culture system was used to evaluate the agarose biodegradability and cell viability of BRIN-BD11 insulin-producing cells in culture. Results revealed the degradation rate of agarose was relatively slow for all concentrations. This could be due to the mechanical stiffness of agarose crosslinkers which gives support for the cells to grow and proliferate. MTT assay showed that cells viability decreased at higher agarose concentration (3%) and low seeding density (5×10^4 cell/mL), could be due to the smaller pore size which limits the space for the cells to grow. Meanwhile, 1% agarose concentration enhanced cell viability the best (≈50%). Higher seeding densities, 1×10^6 cell/mL was found to be more suitable for seeding on agarose at low concentration, could be due to the large pore size which allows movement of nutrients and accommodates large number of metabolizing cells. In conclusion, this study demonstrated that agarose hydrogel is a suitable and compatible material for 3D culture of D11 cells. Precisely, by altering the agarose concentration and the seeding density, cell proliferation in agarose can be controlled.

INTRODUCTION

Tissue engineering is a noteworthy technique to regenerate a three-dimensional (3D) tissue. It involves a combination of cells, biomaterials, biochemical and physiochemical factors. The anticipation of this technique is biomaterial can mimic the native physiological structure of tissues (Guven et al., 2015). The basic approach to tissue engineering is 3D culture system which requires the uses of cells and scaffold, to engineer the tissue ex-vivo (Rezwan et al., 2006). Growth in conventional two-dimensional (2D) surfaces can cause cells to flatten and affects nuclear shape, which may lead to altered gene expression and protein synthesis (Knight & Przyborski, 2015). Furthermore, 2D cultures are not capable of mimicking the nature of cells environment, which may lead to loss of signals that govern major cellular processes like proliferation and differentiation (Zanoni et al., 2016). Cells need to relay on a scaffold material that can support the cells and mimic the ECM of native tissue temporarily, till the cells are capable in constructing their own ECM (Chan & Leong, 2008). The ideal scaffold should provide mechanical and structural integrities to the cells. Simultaneously, scaffold should be biodegradable, biocompatible and allow the movement of nutrients to cells (Naahidi et al., 2017; Orive et al., 2003). Hydrogels are promising scaffold due to their structural similarity to ECM of many tissues. They are hydrophilic polymers that can swell in the presence of water (Huang et al., 2006; Kong et al., 2003), and their high-water content allows diffusion of nutrients and

* Siti Pauliena Mohd Bohari (pauliena@utm.my)

Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, Skudai, Johor Bahru, 81300, Malaysia

cells infiltration which will enhance the cells viability (Nisbet et al., 2008; Zhang & Khademhosseini, 2017). Hydrogels are also can easily be manipulated and fabricated to resemble cells native physiological environment (Zhang & Khademhosseini, 2017).

Agarose is a type of hydrogel with a linear polysaccharide structure that can be extracted from marine red algae (Zarrintaj et al., 2018). This thermosetting hydrogel has a gelation process based on the response to temperature reduction (Buckley et al., 2009; Zarrintaj et al., 2018). Agarose hydrogel has been used in many studies to provide 3D environment due to its mechanical stiffness and its ability to absorb water (Goldman & Barabino, 2016; Lahooti & Sefton, 2000; Lee & Yun, 2018; López-Marcial et al., 2018; Pollot et al., 2018; Zarrintaj et al., 2018). These characteristics ensure nutrients availability for the growing cells and allow them to continue differentiation (Balgude et al., 2001; Larson et al., 2012). Agarose is also not cytotoxic and has non-immunological property which makes it suitable for long term culture (Xiao et al., 2019; Zarrintaj et al., 2018). Agarose biocompatibility and non-cytotoxic properties have made it the right candidate for this study. Agarose has been widely used in tissue engineering applications. A previous study revealed that agarose used to encapsulate islets β-cells to enhance cells proliferation and improved the insulin production in diabetic mice (Holdcraft et al., 2014). Another study also showed that long term culture of agarose encapsulated islets cells enhances the expression of insulin producing genes when compared to free islets cells cultured overnight (Dumpala et al., 2016). In addition, agarose was used to support and provide the 3D-construct for C2C12 myogenic cells and allowed cell proliferation and maturation into myofibrils which can be used to study skeletal muscles disorders (Larson et al., 2012). Agarose was used to study neurodegenerative disorders and successfully supported the survival, and the differentiation of human induced pluripotent stem cells aggregates into dopamine-producing neurons. Meanwhile, the study found that the free cells aggregates were unable to survive due to limited nutrient supply and formed necrosis, leading to less dopamine production (Konagaya & Iwata, 2015).

Scaffold degradation is very critical in tissue engineering since it gives space for the newly growing cells to proliferate and differentiate (Zhang et al., 2012). Scaffold degradation rate should be tailored to the new tissue formation, one of the major drawbacks of agarose is that its degradation properties cannot be easily controlled (Rahman et al., 2015). Yet, a study found that agarose scaffold encourages chondrocytes growth and enhances the ECM production in vitro (Cigan et al., 2016). Another attempt was done to overcome the agarose uncontrollable degradation by using different ratios of agarose/hyaluronic acid (HA) in composites. The study revealed that composites with higher HA and lower agarose content degraded faster compared to the composites with lower HA and higher agarose ratio (Zhang et al., 2012).

Thus, the aim of this study was to observe the degradation of different agarose gel concentrations in the medium to ensure if the degradation rate of agarose can be controlled and optimized for seeding the insulin secreting cells (BRIN-D11). Additionally, this study also aimed to determine the effect of different agarose concentrations and seeding densities on D11 cells viability. Consequently, the potential applications of agarose hydrogel for the insulin secreting cells, tissue engineering and transplantation of islet secreting insulin cells on diabetic patients can be evaluated.

MATERIALS AND METHOD

Chemicals and Reagents

Roswell Park Memorial Institute (RPMI-1640) medium and fetal bovine serum (FBS) were bought from Biowest SAS (France). Gibco™ Trypsin was purchased from Bio-Diagnostic Sdn Bhd (Selangor, Malaysia). Trypan blue solution, phosphate buffer saline (PBS) tablets, sodium azide powder, agarose powder and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) powder were purchased from Sigma-Aldrich® Group.

Preparation of Agarose Solutions and Discs

Agarose was prepared in three different concentrations [1%, 2% and 3% (w/v)] by dissolving agarose powder in PBS. Agarose solutions were then autoclaved at 121°C for 30 min. Then the liquid agarose was kept in oven (37°C) to prevent gelling. To mould the agarose solutions into a disc shape; a gas pipe with 9 mm diameter was used and cut into 1 cm thickness (Figure 1a). The agarose solutions were poured into the gas pipes using pasture pipette (Figure 1b) and allowed to gel at a room temperature for 30 min to form agarose gels (AU - Strobel et al., 2018; Buckley et al., 2009). Agarose gels were then washed with PBS and incubated at room temperature in RPMI medium supplemented with 10% FBS and 0.1% sodium azide (Ji et al., 2019).

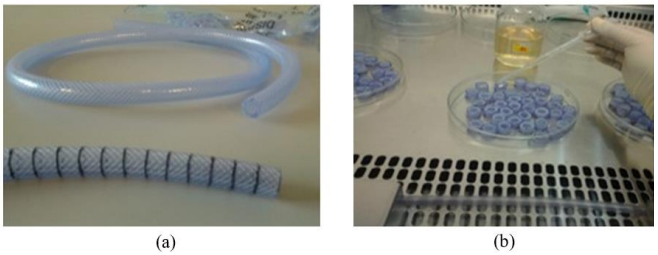


Fig 1. Preparation of agarose gels (a) gas pipe ready to be cut into 1 cm thickness (b) pouring of agarose solution into mould.

Agarose Gels Swelling and Degradation Studies

The swelling and the degradation of different concentrations of agarose gels was observed for 14 days (Day 0, 2, 4, 6, 8, 10, 12 and 14). Three gels from each agarose concentrations were removed every 2 days from the medium and the agarose wet weight was measured to determine the swelling of different agarose concentrations in the RPMI medium. Then the gels were kept at -80°C for 24 hours and freeze-dried for another 24 hours before measuring the dry weight of lyophilized agarose (Fereshteh et al., 2016; García-Astrain & Avérous, 2018). The percentage of weight loss of agarose gel due to incubation in RPMI medium was calculated using the formula: Weight loss (%) = (initial dry weight – weight of dried disc) / Initial dry weight × 100 (Zeng et al., 2015).

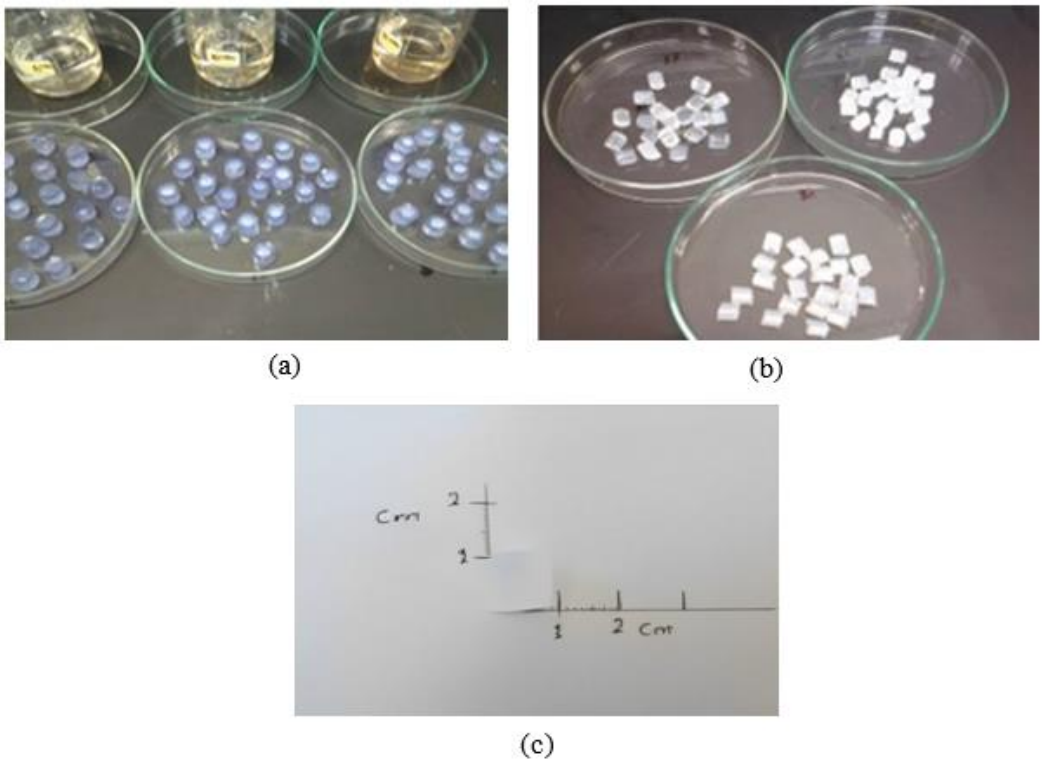


Fig 2. Moulding of agarose gels (a) from left to right: Agarose 1%, 2% and 3% moulded in gas pipe and allowed to gel. (b) Agarose gels 1%, 2% and 3% after gelling. (c) Size and diameter of agarose gels.

Cell Culture

BRIN BD11 cell line is a hybrid cell line derived from clonal B cell-RIN which developed by electrofusion of NEDH rat normal pancreatic B cells with RINm5F cell line (Green et al., 2018). D11 cells were maintained in RPMI medium supplemented with 10% FBS, 1% pen-strep antibiotic and incubated at 37°C in CO2 incubator. Cells were monitored and passaged when reached 70%–80% confluency and medium was changed every 2–3 days.

Cells Seeding on Agarose Gels

All materials including gas pipes, petri dishes, pipettes and pipette tips were sterilized with UV light before used. Agarose solutions were prepared as previously mentioned. Then, the agarose solutions were poured under sterile condition into 1 cm gas pipe mould (see Figure 2a). Gas pipes were removed after agarose gelling (30 min), resulting in 1 cm disc shape agarose gel (see Figure 2b and Figure 2c). Various concentrations of D11 cells (5×10^4, 2.5×10^5, 5×10^5, and 1×10^6 cell/mL) were optimized to be used in this study. For cell seeding purpose, agarose gels were placed in 6 well plates and the cell suspension was loaded dropwise on the top of each agarose gel via micropipette. Then, the plates were incubated at 37°C, 5% CO_2 for one hour for the cells to immerse into the agarose gel. The cells construct (cells + agarose) was supplemented with the RPMI medium and the plates were returned to the CO2 incubator. Medium was changed every 2 days.

MTT Assay

MTT assay was used to measure the cell viability, in which the water-soluble yellow dye MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was converted into insoluble purple formazan by cleavage of the tetrazolium ring from active mitochondrial dehydrogenases of living cells. Formazan was then solubilized and the colour intensity was directly proportional to the number of living cells (Ghasemi et al., 2021). The constructs (with and without cells) were removed from 6 well plates and placed in a centrifuge tube with 1 ml of RPMI medium and 10% of MTT solution. The tested samples were incubated in CO_2 incubator for 4 hours to allow formazan production. MTT solution was removed, and 2 ml of solubilizing agent 0.01 M acidic isopropyl alcohol was added into the construct and incubated for 15 min. The solubilized solution was measured spectrophotometrically at 570 nm wavelength and the survival percentage was calculated using formula: Survival rate (%) = (absorbance of sample – absorbance of blank) / (absorbance of control – absorbance of blank) × 100 (Kamiloglu et al., 2020).

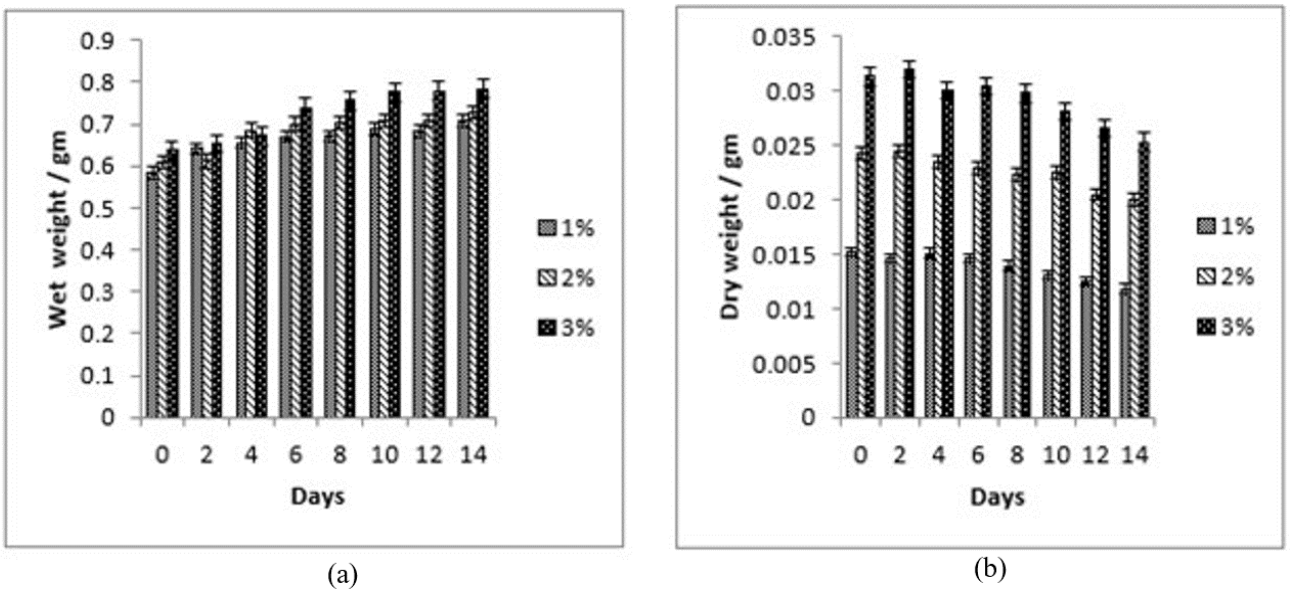
Statistical Analysis

Data obtained were analysed by using SPSS (version 16.0). The Normality data was checked using the Shapiro-Wilk test. Normal data was tested using the independent t-test and the asterisk indicates the significance of differences between the groups. P-values were marked with an asterisk < 0.05 (*), < 0.01 (**) and < 0.001 (***).

RESULTS

Agarose Swelling

The swelling behaviour of agarose gel in the media can lead to increase in the pore size, leading to the determination of the agarose abilities to accommodate cells, to absorb the culture media and to ensure nutrient availability for the growing cells. Moreover, it can eventually facilitate agarose disintegration in long term culture (Felfel et al., 2019). The swelling activity of different concentrations of agarose in medium for 14 days is shown in Figure 3a. The figure shows that all the agarose concentrations were able to swell and absorb the medium till the last day of experiment (day 14) and the wet weight slightly differed with different agarose concentrations.



(a) (b)

Fig. 3 (a) Wet weight of different agarose gels concentrations for 14 days. (b) Dry weight of different agarose gels concentrations for 14 days.

percentage of weight loss was greater in 1% agarose that reaching 22.36% by day 14, followed by 2% and 3% agarose in which the weight loss percentage was 19.16% and 17.3%, respectively. This result indicates that 1% agarose can accommodate more cells while degrading, which allows for cell growth and proliferation.

Table 1 Percentage of dry weight loss in different agarose gels concentrations compare to the first day (day 0)

Days	1% agarose	2% agarose	3% agarose
Day 2	3.65%	1.20%	2.20%
Day 4	3.95%	2.89%	2.87%
Day 6	3.95%	5.37%	4.15%

Degradation Studies

Previous studies stated that when cells start to proliferate, agarose scaffold should be degraded and breakdown into polymer fragments to be easily excreted from the body (Naahidi et al., 2017). Degradation of agarose gels (1%, 2%, and 3%) in this study was observed for 14 days. This was done to determine the agarose concentration that can be degraded at a controllable rate. Hydrogels that are able to degrade with controlled manner is important. After the seeded cells start to form a tissue, this temporary scaffold will be degraded and replaced with the cells ECM by time in culture. When this happens, the cells are forming 3D tissues (El-Sherbiny & Yacoub, 2013). The dry weight of 1%, 2% and 3% agarose (Figure 3b) were 0.015, 0.02 and 0.03 g, respectively at day 0. The graph implied that longer incubation time for the gels in the medium decreases the dry weight for all the agarose concentrations. The results represented that the agarose gels were degrading but the degradation process prolonged. The degradation result is directly proportional with the swelling study. When the agarose gels increase its swelling rate, the dry weight of the agarose gel decreases by time in culture. As shown in Table 1, the

MTT Assay

The effect of different agarose concentrations and seeding densities on cell viability was assessed by using MTT assay for 7 days interval (Day 2, 4, 7). Generally, the percentage of viable cells in different agarose concentrations increased over time. This indicates that all cells were able to grow, but the number of proliferating cells differed with different agarose concentrations and different seeding densities. 1% agarose showed the best percentage of cells viability (≈50%) when gels seeded in high density 1×10^6 cell/mL. Meanwhile, the percentage of viable cells by day 7 was 15%, 18% and 23% in seeding densities of 5×10^4, 2.5×10^5, 5×10^5 cell/mL. respectively. Both 2% and 3% agarose showed lower cell viability percentage in all used seeding densities. Generally, increasing seeding density increased cell viability percentage, while increasing agarose concentration resulted in a decrease of cell viability percentage. This was shown with the 1% agarose gel enhanced the cell viability the best when compared to 2% and 3%. Figure 4 represents the effect of different agarose concentrations and different seeding densities based on cell viability for 7 days.

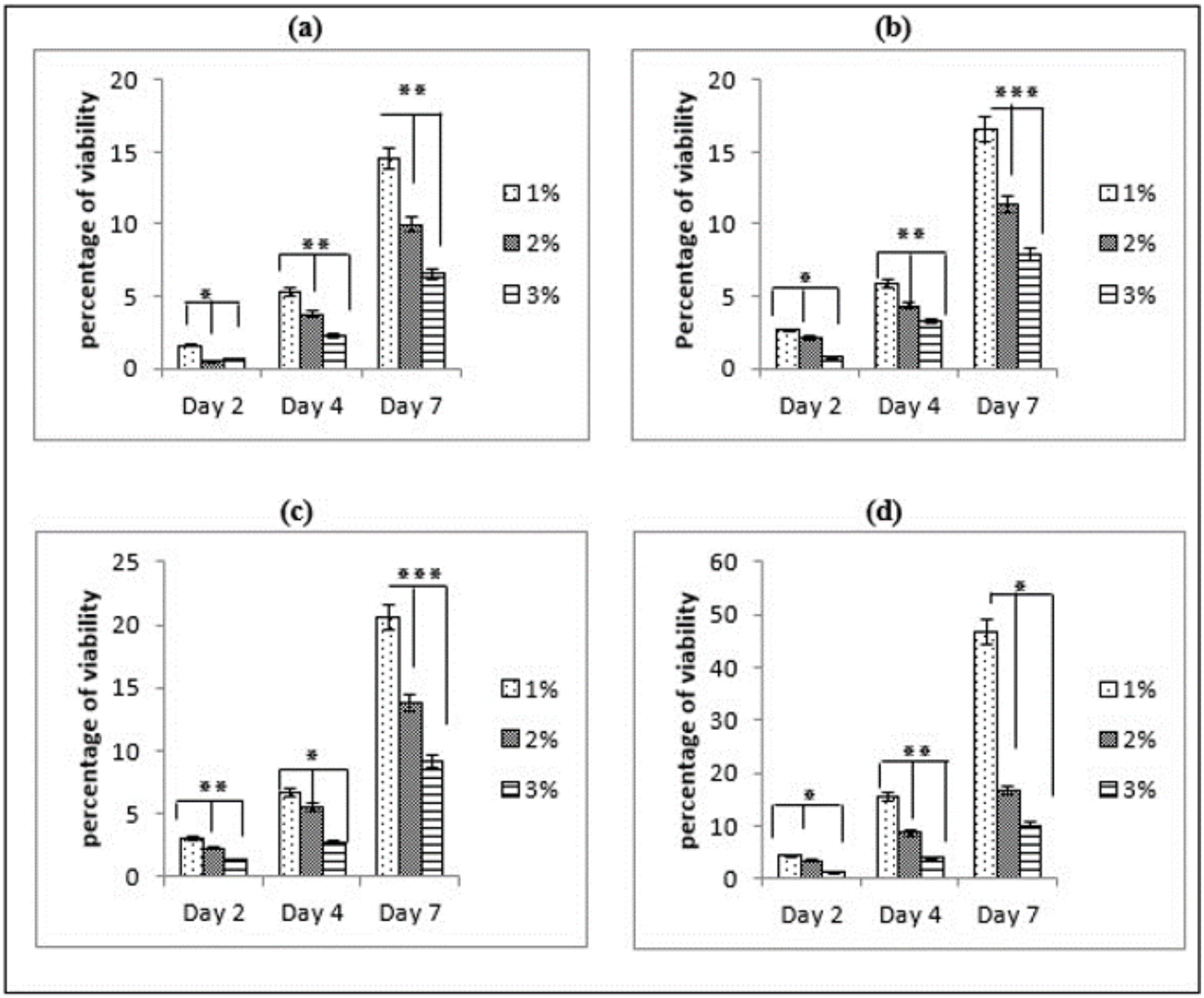


Fig. 4 MTT percentage of viable cells in agarose for (a) low seeding density 5×10⁴ cell/mL, (b) intermediate seeding density 2.5×10⁵ cell /mL, (c) intermediate seeding density 5×10⁵ cell/mL and (d) high seeding density 1×10⁶ cell/mL. The results showed that higher seeding density exhibited good proliferation/cell viability on 1% agarose concentration. P-values < 0.05, all measurements were in triplicates. Error bars represent standard deviation and asterisk represents the significance difference.

DISCUSSION

Agarose hydrogel has been widely used in tissue engineering applications due to its resemblance to ECM, biocompatibility property and also the ability to support cell growth and proliferation (Zarrintaj et al., 2018). Agarose gel contains crosslinked hydrophilic polymers that are capable of swelling and entraps water (Ahmed, 2015; Gulrez, 2011). Results of agarose wet weight showed that different agarose concentrations were able to swell till 14 days. This result indicated that agarose could absorb the medium and ensure the nutrient availability for the seeded cells.

Biodegradation is a required property for an ideal scaffold and the degradation rate should be tailored to match cell growth (Zarrintaj et al., 2018). Agarose hydrogel is known to degrade following incubation in aqueous medium. This characteristic is beneficial in certain tissue engineering applications such as drug delivery (Kim et al., 2016), bone tissue engineering (Sánchez-Salcedo et al., 2008), neural generation (Rossi et al., 2012), and brain tissue engineering (Pettikiriarachchi et al., 2010). Disintegration of agarose gels can occur due to hydrolysis of hydrogen bonds that formed during the agarose gelation process. This process eventually leads to loss in crosslinking and degradation of gels which later accelerates the degradation process (Sigala et al., 2015; Zarrintaj et al., 2018). The difference in degradation rate (dry weight) in different agarose concentrations could be explained by the number of crosslinkers. The number of crosslinkers in the scaffold can increase significantly by increasing the agarose concentration, affecting the stiffness of agarose gel (Kazi et al., 2019; Rossi et al., 2012).

The present study found that all concentrations (1%, 2% and 3%) of agarose hydrogel were degrading after incubation in culture media with lower agarose concentration (1%) degraded first followed by 2% and 3%. This study also observed the degradation rate was slow and the agarose scaffold lost 17%–22% of its weight by day 14 as in table (1). This indicates the stability of the hydrogel and its ability to support new formed tissue. The stability and the minimal degradation exhibited by agarose was proved previously (Roberts et al., 2011). Another

in vivo study showed that when agarose tissue engineered scaffolds implanted into the hind legs of mice, there was no considerable change in agarose scaffold till 23 weeks, suggesting its ability to support cells for longer period (Forton et al., 2016).

The cell viability on agarose scaffold was dependent on agarose concentration and density at which the cells seeded. The lowest agarose concentration (1%) enhanced cell viability the best compared to 2% and 3% agarose concentrations. This result can be linked to that 1% agarose degraded earlier than 2% and 3% agarose, but in a controllable manner that also supported the cell growth and proliferation. While in higher agarose concentrations, the stiffness of the scaffold hinders the cell growth resulting in low number of viable cells and reduces the viability percentage (Roberts et al., 2011). A study done also showed that low agarose concentrations enhanced chondrocytes clusters 1.7 folds in 7 days while in 2% and 4% agarose showed no changes in clusters number (Karim & Hall, 2017). Another study suggested that nutrient viability can be hindered by higher agarose concentrations such as 3% (Ng et al., 2006). Also, the number of crosslinkers in different agarose concentrations can affect the dynamic stiffness and the pore size of agarose gels. In the highest agarose concentrations, cells cannot penetrate further in agarose gel due to the stiffness and the smaller pore size of the gel (Kock et al., 2013). Moreover, nutrients and oxygen will not diffuse properly inside the hydrogel, reducing the cell viability (Mori et al., 2013).

Several studies have demonstrated the effect of agarose on cell viability and observed that there is inverse correlation between gel concentration and proliferation rate, and that low stiffness is associated with increase proliferation rate (Benning et al., 2018). When MC3T3-E1 cell line cultured in different agarose concentrations, lower agarose concentration (0.5%, 2%) showed elevated proliferation compared to stiffer gels (3%, 5%) (Kazi et al., 2019). In addition, the elasticity in low agarose concentration makes it more resembling the natural ECM of normal tissues which consequently will improve cell viability. In a study done also revealed that tissues cultured in low agarose concentrations are more similar to healthy tissues due to their elasticity, while tissues cultured in high agarose concentrations are similar to diseased tissues (Fuente et al., 2019).

Agarose gels were seeded with different cell concentrations in this study to optimize the suitable cell seeding density that can be used for agarose without causing cells to overgrow or die before the end of the experiment. The highest cell seeding density was able to proliferate the best in 1% agarose, might be due to its flexibility and suitable degradation rate that allowed more cells to grow. Many studies have shown that higher seeding densities are the best for seeding the cells and that higher seeding density will enhance the synthesis and assembly of ECM (Sun et al., 2017). Another study also showed that when chondrocytes cultured at lower densities experienced less growth while chondrocytes cultured at higher seeding densities approached properties of human knee cartilage (Cigan et al., 2016).

CONCLUSION

The purpose of this study was to investigate the effect of agarose concentrations on the viability of insulin-secreting cell line (BRIN BD11). The results showed that cells viability and proliferation was enhanced at lower agarose concentration (1%). This could be due to the lower density of agarose crosslinkers in lower agarose concentrations. Hence, high porosity allows more cells to proliferate and eases the nutrients penetration which consequently increases cells viability. The seeding density also was optimized in this study and higher seeding densities at 1×10^6 cell/mL showed to be more suitable for seeding. This can be due to higher cell number which resulted in better cell colonization and more cells can penetrate deeper into the scaffold region, resulting in high viability with high seeding densities.

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