RESEARCH ARTICLE

Effect of Cell Culture Medium on the Proliferation and Stemness of CD24$^-$/CD44$^+$ Human Breast Cancer Stem Cells

Yalista Fatia Nadia[1], Resda Akhra Syahrani[2], Sekar Arumsari[2], Mohamad Sadikin[3], Septelia Inawati Wanandi[2,3],*

[1]Master's Programme in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya, Kenari, Jakarta, 10430, Indonesia
[2]Molecular Biology and Proteomics Core Facilities, Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya, Kenari, Jakarta, 10430, Indonesia
[3]Department of Biochemistry and Molecular Biology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya, Kenari, Jakarta, 10430 Indonesia

*Corresponding author. E-mail: septelia.inawati@ui.ac.id

Received date: Jul 29, 2021; Revised date: Sep 20, 2021; Accepted date: Sep 23, 2021

Abstract

BACKGROUND: Cancer stem cells (CSCs) is defined as tumor initiating cells within tumor that maintain stemness properties and tumorigenicity. Extracellular pH of CSCs in *in vitro* condition is important for supporting cell proliferation which may also regulate the expression of stemness markers such as OCT4. This work aimed to examine the effect of cell culture media on the proliferation and stemness of human breast cancer stem cells (BCSCs).

METHODS: Human CD24$^-$/CD44$^+$ BCSCs were grown in Dulbecco's Modified Eagle Medium/F-12 (DMEM/F-12) with 15mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), without HEPES and adjusted to pH 7.4, or without HEPES but pH was not adjusted. BCSCs were grown under standard conditions for various days. Viable cell number was measured using trypan blue exclusion, whereas proliferation rate using MTS assay. OCT4 mRNA and protein were analyzed using quantitative real time PCR (qRT-PCR) and Western Blot assay, respectively. *In vitro* tumorigenic activity was determined using mammosphere formation unit (MFU) assay.

RESULTS: Our results showed a higher viable cell number and proliferation of BCSCs in DMEM/F-12 HEPES (-) compared to HEPES (+) medium until 4 day incubation. OCT4 mRNA and protein level, as well as MFU of BCSCs were significantly higher in HEPES (-) compared to HEPES (+) medium on day 2.

CONCLUSION: DMEM/F-12 medium without HEPES facilitates CD24$^-$/CD44$^+$ BCSCs to have higher proliferation and stemness on day 2 incubation compared to those with HEPES.

KEYWORDS: breast cancer, cancer stem cell, OCT4, stemness, proliferation

Indones Biomed J. 2021; 13(4): 355-63

Introduction

Cancer is a complex disease that characterized by uncontrolled cell growth followed by metastasis acquisition.(1) In solid tumor including breast cancer, cancer stem cells (CSCs) also defined as tumor initiating cells, are a minor subpopulation within tumor that maintain stemness properties and tumorigenicity. The main properties of CSCs are self-renewal, unlimited potential for proliferation, and the ability to bring up daughter cells with the potential for differentiation through asymmetric cell division.(2,3) Moreover, it has been suggested that CSCs play pivotal roles in tumor formation, angiogenesis, metastasis, and drug-induced resistance.(4) Thus, understanding the underlying mechanisms that regulate the stemness and proliferation of CSCs is important to target CSCs for diagnosis and anti-cancer development.

Our previous studies have isolated and identified human breast cancer stem cells (BCSCs), named CD24$^-$/

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$CD44^+$ cell line (Patent from the Directorate General of Intellectual Property Rights, Ministry of Law and Human Right, Republic of Indonesia No. IDP 000056854), based on OCT4 expression and mammosphere formation indicating pluripotency and tumorigenic capacity, respectively.(5) Among major pluripotency marker, OCT4 is the most critical one due to its effect on BCSC activity, especially in maintaining the stemness of BCSCs.(6,7) OCT4 is thought to play essential roles in self-renewal, epithelial-mesenchymal transition, and drug resistance.(8) The tumorigenicity that is characterized by the ability of BCSCs to form new tumor can be analyzed in vitro by the mammosphere formation assay.(9)

CSCs grow in tumor microenvironment (TME) influenced by various physicochemical factors such as extracellular pH (pHe) that regulate their proliferation and stemness properties.(10) The pHe in normal tissue between 7.2 to 7.5 while pH range of TME is between 6.4 and 7.0.(11) It has been reported that pHe could change the glucose metabolism of CSCs to maintain its stemness.(5) In *in vitro* study, pHe is generated by cell culture medium and maintains cell viability and affects their properties during cell propagation. This depends on the buffer system in a cell culture medium such as sodium bicarbonate (HCO_3) that is stabilized by carbon dioxide (CO_2) in incubator atmosphere to balance the medium pH.(12) Furthermore, some non-volatile buffer compounds such as HEPES, MES, PIPES are supplemented into culture medium.(13) Yet, little is known about the appropriate buffer system that provide an optimal pH of BCSC culture medium which facilitates high proliferation and stemness properties simultaneously. Thus, this study is aimed to analyze the effect of various cell culture media on the proliferation and stemness of human breast cancer stem cells (BCSCs).

Methods

Human $CD24^-/CD44^+$ BCSCs Culture and pHe Measurement

Human $CD24^-/CD44^+$ BCSCs have been isolated and identified based on their pluripotent and tumorigenic activity, as described previously.(5,14) These cells retain high stemness properties when grow in serum-free medium, Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM/F-12).(15) In this study, we used various DMEM/F-12 media for growing BCSCs, namely 1) DMEM/F-12 with 15 mM HEPES (Cat. no. 12400-024, Gibco, Thermo Fisher Scientific, Inc. (Waltham, MA, USA)) supplemented with 15 mM $NaHCO_3$ (Cat. no. s5761-500G, Sigma-Aldrich, MA, USA); 2) DMEM/F12 without HEPES (Cat. no. 12500-039, Gibco, Thermo Fisher Scientific, Inc.) supplemented with 30 mM $NaHCO_3$ and adjusted to pH 7.4 with 1N HCl; and 3) DMEM/F-12 without HEPES but pH was not-adjusted. Media were supplemented with 1% antifungal Amphotericin B (Cat. no. 15290026, Gibco, Thermo Fisher Scientific, Inc.) and 1% antibacterial Penicillin/Streptomycin (Cat. no. 15140122, Gibco, Thermo Fisher Scientific, Inc.) to prevent bacterial or fungal contamination. After being adjusted to desired pH, media were filtered with 0.22 µM filter prior to used. BCSCs were grown in each medium and incubated in a CO_2 incubator under standard conditions (at 37°C, 5% CO_2 and 20% O_2).

Since the pHe could be affected by changes of CO_2 concentration and temperature, the pH of conditioned medium was immediately measured after being removed from the CO_2 incubator. After harvesting the cells by centrifugation for 10 minutes at 1000 rpm, supernatant was collected for pH analysis using pH electrode (Milwaukee MI150, NC, USA). pH of conditioned medium with or without cells were measured.

Cell Proliferation Assays

About 10^5 cells/well were grown in a 12-well plate containing 1000 µL medium. The medium was changed with fresh medium every two days. Cells were harvested on day 2, 4, or 6 and centrifuged at 1000 rpm for 10 minutes before being used for further analysis. Trypan Blue exclusion assay was used to determine viable cell number. Briefly, 0.4% trypan blue solution was used to stained cell suspensions with 1:1 mixture. Viable cells were counted using an automated cell counter (Luna®, Logos Biosystems Inc., Anyang, Gyeonggi, Korea). Viable cells defined as cells that did not absorb trypan blue intracellularly.(16)

To analyze the proliferation rate of BCSCs, we performed the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter-AQueous MTS assay; Cat. no. G5421, Promega, Madison, WI, USA). Briefly, about $5x10^3$ cells/well were grown in 100 µL medium in a 96-well plate. Cells were incubated under standard conditions for 1, 2, 3, 4, 5, or 6 days without changing the medium. Viable cell numbers were determined following 1 hour of incubation time with MTS according to the manufacturer's protocol. The formazan product was measured using spectrophotometer at 490 nm wavelength (Varioskan Flash®, Thermo Scientific, Finland).(17)

Quantitative Reverse Transcription-PCR

After cell culture, total RNA was extracted using Tripure® RNA Isolation Kit (Roche, Germany) according to the manufacturer's protocol and its concentration was counted using spectrophotometer at 260 nm wavelength (Varioskan Flash®, Thermo Scientific, Finland). To analyze the expression level of mRNA, we performed quantitative reverse transcription PCR (qRT-PCR) using KAPA SYBR Fast® qPCR (Cat. no. 07959389001, Kapa Biosystems, USA) and Exicycler™ 96 Real Time PCR machine (Bioneer Corporation, Daejeon, Korea). The PCR primers for 18S rRNA are 5'-AAA CGG CTA CCA CAT CCA AG-3' for forward and 5'-CCT CCA ATG GAT CCT CGT TA-3' for reverse primer with annealing temperature of 60°C. The PCR primers for OCT4 are 5'-GAGGAGTCCCAGGACATCAAA-3' for forward and 5'-AGCTTCCTCCACCCACTTCT-3' for reverse primer with annealing temperature of 57°C.(18) Livak method ($2^{-\Delta\Delta Ct}$) was used to calculate the mRNA expression level. (19) 18S rRNA was used as a reference gene, while $CD24^-/CD44^+$ cells cultured in DMEM/F12 HEPES (+) were used as a calibrator.

Western Blot Analysis

Western Blot analysis was performed as described previously.(16) Briefly, the blotted nitrocellulose membranes were incubated with rabbit anti-human polyclonal antibody against OCT4 (1:500 dilution; cat. no. ab18976; Abcam Cambridge, UK) or mouse anti-human monoclonal antibody against β-actin (1:1000 dilution; Cat. no. 8H10D10; Cell Signaling Technology, USA) at 4°C overnight. Then, the membranes were incubated in HRP-conjugated anti-rabbit (1:500 dilution; Cat. no. sc2357 Santa Cruz Biotechnology, Inc., Texas, USA) or anti-mouse (1:2000 dilution; Cat. no. sc-2302 Santa Cruz Biotechnology, Inc., Texas, USA) antibodies for 2 hours, respectively. Protein were visualized by ECL as the HRP substrate (Optiblot ECL Substrate Kit, Abcam, USA).

Mammosphere Forming Unit Assays

BCSCs were seeded in ultralow-attachment 96-well plate (Corning Incorporated, New York, NY, USA) with density of 100 cells/100 µL medium and incubated for 48 hours under standard culture conditions. Following the incubation, mammosphere formation was analyzed using an inverted microscope (OPTIKA Srl, model no. IM3, Ponteranicam, Italy) at 100x magnification. Mammosphere forming unit (MFU) was determined based on the mammosphere area of ≥700 μm^2 observed using OPTIKA Srl software (OPTIKA Srl, version 2.7) according to the Patent from the Directorate General of Intellectual Property Rights, Ministry of Law and Human Right, Republic of Indonesia No. IDP 000060309.

Statistics Analysis

All data were analyzed using SPSS software (IBM Corporation, Armonk, NY, USA). Data are presented as mean±SEM with at least 3 replicates for pH measurements, viability, proliferation, mRNA, and mammosphere forming unit. Data were tested for normality and homogeneity of variance. Differences between groups were analyzed using one-way ANOVA or t-test analysis. Differences were considered significant at $^*p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$ or $^{\#}p<0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$.

Results

Effect of HEPES Buffering in the Culture Medium on the Extracellular pH of BCSCs

To determine the effect of HEPES buffer on the pHe of BCSCs, we calculate the pH change (ΔpH) by substracting pH of medium with BCSCs (conditioned medium) from that of cell-free medium after incubation at indicated day. Table 1 demonstrates that the value of ΔpH after incubation with all used media was negative, indicating that pHe of BCSCs was significantly lower than that without cells (*p*-value range is from 0.000 to 0.006). After 2, 4, and 6 day-incubation, the HEPES (-) medium pH with or without cells was significantly higher than that of HEPES (+) medium (*p*-value range is from 0.000 to 0.022). Consequently, the ΔpH value of HEPES (-) either with or without adjusted pH was significantly more negative than that of HEPES (+) after all incubation days (*p*-value range is from 0.000 to 0.045). Although the medium has been changed with fresh medium every two days, all ΔpH was gradually decreased along with the length of cell culture. Moreover, all conditioned media became acidic (pH below 7) on day 6.

Effect of HEPES Buffering in the Culture Medium on the Viable Cell Number and Proliferation Rate of $CD24^-/CD44^+$ BCSCs

Viable cells number were counted using trypan blue exclusion assay to determine the proliferation of BCSCs in fresh culture medium every two day incubation. Since the conditioned medium was replaced with fresh medium every two days, there will be no effect of nutrient deprivation on the cell proliferation. Following 2 and 4 day-incubation time, viable BCSCs grown in DMEM/F-12 HEPES (-) medium,

Table 1. pH of cell-free medium and cell medium of BCSCs after incubation.

Day	Type of Medium	Medium pH After Incubation		ΔpH
		Without Cells	With Cells	
2	Hepes (+)	7.42±0.02**	7.35±0.01	-0.07
	Hepes (-) adjusted	8.22±0.05***	7.93±0.03###	-0.29###
	Hepes (-) not adjusted	8.27±0.03***	8.01±0.02###	-0.26###
4	Hepes (+)	7.42±0.02***	7.23±0.01	-0.19
	Hepes (-) adjusted	8.22±0.05***	7.75±0.02###	-0.47###
	Hepes (-) not adjusted	8.27±0.03***	8.02±0.02###	-0.25#
6	Hepes (+)	7.42±0.02***	6.45±0.00	-0.97
	Hepes (-) adjusted	8.22±0.05***	6.81±0.07##	-1.77###
	Hepes (-) not adjusted	8.27±0.03***	6.36±0.02#	-1.91 ###

Data are presented as mean±SEM. Statistical analysis was performed using Student's t-test to compare medium pH with and without cells after incubation. ANOVA analysis followed by LSD test were used to determine the difference between ΔpH of HEPES (+) and HEPES (-) medium either with or without adjusted pH. Differences were considered statistically significant at ***p*<0.01, ****p*<0.001 between medium pH without and with cells an after incubation at indicated days, whereas #*p*< 0.05, ##*p*< 0.01, ###*p*<0.001 for pH or ΔpH of HEPES (-) either with or without adjusted pH compared to HEPES (+) medium.

either with or without adjusted pH, were significantly higher (*p*-value range is from 0.000 to 0.015) than those in HEPES (+) medium as shown in Figure 1. Nevertheless, on day 6, viable BCSCs grown in both DMEM/F-12 HEPES (-) media decreased; in turn there was no significant difference of viable cell numbers between HEPES (+) and (-) media. In Figure 2, cell density and morphology were observed. Indeed, we showed that the density of BCSCs was higher when cultured in DMEM/F-12 medium without HEPES buffer until 4 days and tended to form more and larger mammospheres compared to that in medium with HEPES.

Using MTS assay, we further observed the proliferation rate of $CD24^-/CD44^+$ BCSCs every day from day 0 until 6 of incubation in HEPES (+), HEPES (-) with adjusted pH, or HEPES (-) without adjusted pH. Compared to the experiment in Figures 1 and 2 that applied 100 cells/µL medium, less cell density (50 cells/µL) was seeded and the culture medium in this experiment was not replaced until cells were harvested. Similar to the data of viable cell number presented in Figure 1, the proliferation rate of BCSCs in DMEM/F-12 HEPES (-) was significantly higher than that in HEPES (+) medium until day 4 (*p*-value range is from 0.000 to 0.035). When BCSCs were grown more than 4 days, there was no significant differences between BCSC proliferation rate in HEPES (+) and HEPES (-) media (Figure 3).

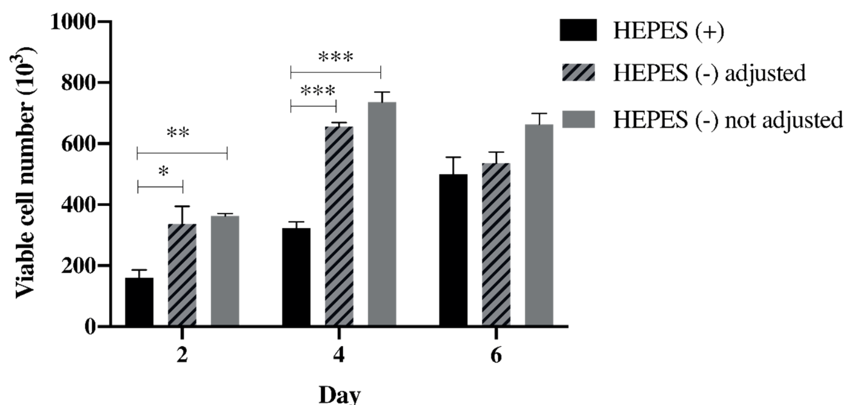

Figure 1. Viable cell numbers of $CD24^-/CD44^+$ BCSCs after incubation with various buffer system media. About 10^5 cells were grown in DMEM/F-12 medium with HEPES (+), HEPES (-) and adjusted pH to 7.4, or HEPES (-) but without adjusted pH to 7.4 under standard culture conditions for 2, 4, or 6 days. Medium was changed every two days. Cells were harvested and viable cells were counted using trypan blue exclusion assay and automatic cell counter. Data are presented as mean±SEM. One-way ANOVA analysis followed by LSD test were used to determine the differences of viable cell number among three buffer systems used in this experiment. Data with *p<0.05, **p<0.01, and ***p<0.001 were considered statistically significant compared to the indicated counterpart.

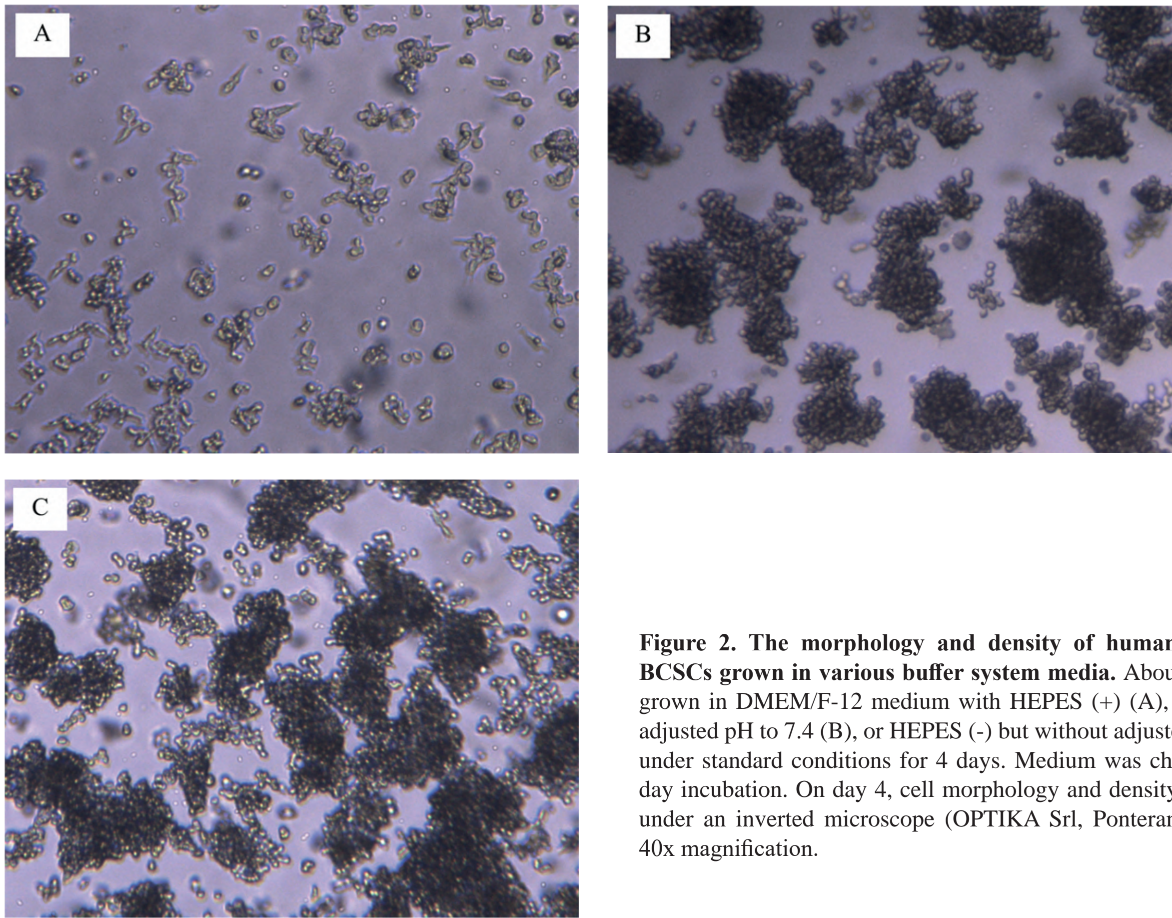

Figure 2. The morphology and density of human CD24-/CD44+ BCSCs grown in various buffer system media. About 10^5 cells were grown in DMEM/F-12 medium with HEPES (+) (A), HEPES (-) and adjusted pH to 7.4 (B), or HEPES (-) but without adjusted pH to 7.4 (C) under standard conditions for 4 days. Medium was changed after two day incubation. On day 4, cell morphology and density were observed under an inverted microscope (OPTIKA Srl, Ponteranica, Italy) with 40x magnification.

Effect of HEPES Buffering in the Culture Medium on the OCT4 Expression of CD24-/CD44+ BCSCs

The mRNA and protein OCT4 expression was to determine the effect of HEPES buffer on the expression of OCT4 in CD24-/CD44+ BCSCs. The result showed that BCSCs grown in DMEM/F-12 HEPES (-) either with (p=0.000) or without adjusted pH (p=0.000) had higher mRNA expression level on day 2 compared with HEPES (+). However, after day 4 and 6 incubation, there were no significant differences of OCT4 mRNA level among BCSCs grown in those culture media (Figure 4A).

Similar to mRNA expression, the OCT4 protein expression of BCSCs after day 2 incubation without HEPES medium was higher than that with HEPES (+).

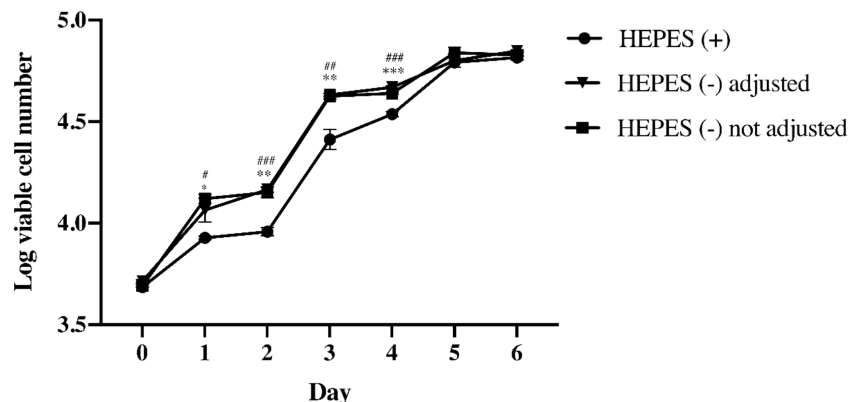

Figure 3. Proliferation of human CD24-/CD44+ BCSCs after incubation under standard condition. Data are presented as mean±SEM. Differences were analyzed using One-way ANOVA followed by LSD test. Data with *p<0.05, **p<0.01, and ***p<0.001 were considered statistically significant between HEPES (+) and HEPES (-) adjusted, whereas those with #p<0.05, ##p<0.01, and ###p<0.001 were considered statistically significant between HEPES (+) and HEPES (-) not adjusted.

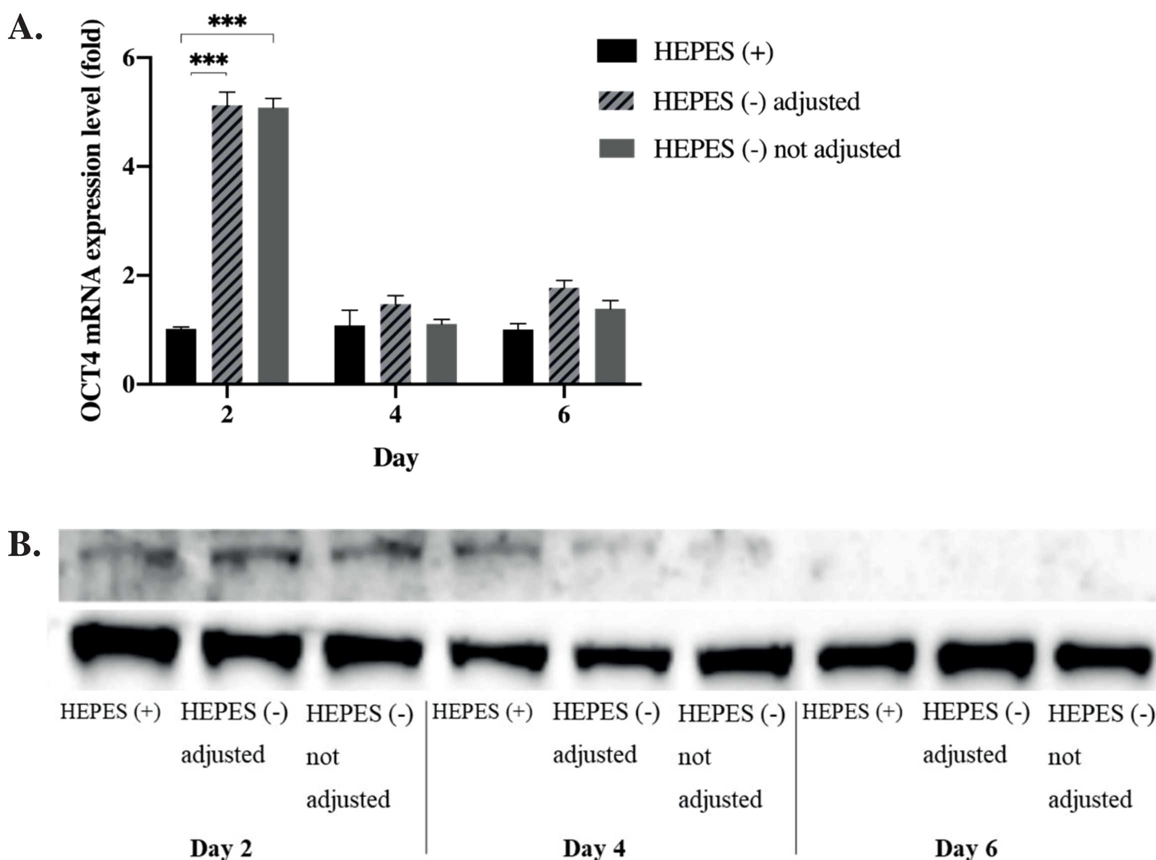

Figure 4. OCT4 expression in human CD24⁻/CD44⁺ BCSCs grown in various buffer system media. A: OCT4 mRNA expression level was determined using qRT-PCR and calculated using Livak method ($2^{-\Delta\Delta Ct}$). *18S* rRNA gene was used as a reference gene. $CD24^-/CD44^+$ cells cultured in DMEM/F12 HEPES (+) were used as a calibrator. B: OCT4 protein expression was determined using Western Blot assay. Data are presented as mean±SEM for OCT4 mRNA expression. Differences were analyzed using One-way ANOVA followed by LSD. Data with ***p<0.001 was considered statistically significant.

Nevertheless, our result showed that the OCT4 protein expression was decreased when BCSCs were prolonged grown in medium without HEPES for 4 days. On day 6, OCT4 protein expression could not be detected in BCSCs grown in all media (Figure 4B).

Effect of HEPES Buffering in the Culture Medium on the Mammosphere Formation of $CD24^-/CD44^+$ BCSCs

To investigate the capacity of $CD24^-/CD44^+$ BCSCs in tumor formation, the mammosphere forming unit was determined. Figure 5A demonstrates the morphology of a mammosphere with its area of 7476.54 μm^2. We also indicated that the MFU of BCSCs in HEPES (+), HEPES (-) adjusted, and HEPES (-) not-adjusted media were 20.00±1.73, 37.67±1.45, and 32.00±1.15, respectively (Figure 5B). Furthermore, mammosphere formation capacity of BCSCs is higher in HEPES (-) than HEPES (+) medium. There were significant differences (p-value range is from 0.000 to 0.033) between all type of medium.

Discussion

Cell culture media is a complex mixture of nutrients, growth factors, and physical environment required for *in vitro* cell growth.(20) In a cell culture system, the addition of buffering compounds in the culture medium, either volatile or non-volatile, is obligatory to maintain acid-base balance by controlling the suitable pH during cell growth. Ultimately, *in vitro* propagation of BCSCs needs further consideration particularly on the appropriate buffer system of culture medium that can accommodate a high cell proliferation whilst preserving their stemness properties. Nowadays, this issue becomes challenging in the research field of cancer stem cells such as drug development targeted to CSCs.

Human $CD24^-/CD44^+$ BCSCs that has been established in our laboratory were used to grow in DMEM/F-12 medium, neither with HEPES nor adjusted pH, and supplemented with 30 mM $NaHCO_3$ to establish

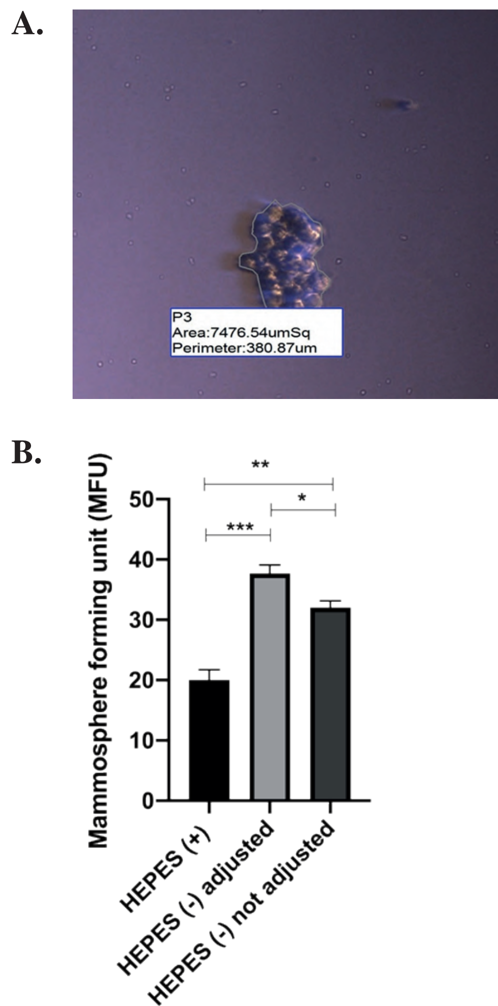

Figure 5. Morphology of CD24-/CD44+ BCSC mammosphere (A) and MFU of CD24-/CD44+ BCSCs grown in various culture media (B). Data are presented as mean±SEM. Statistical differences were considered at **p*<0.05, ***p*<0.01, and ****p*<0.001 using One-way ANOVA followed by LSD test.

a physiological CO_2/HCO_3^- buffering system. The CO_2/HCO_3^- buffer creates a natural transmembrane gradient of CO_2 and its HCO_3^- ions stimulate membrane transport processes that are essential for the homeostasis of cellular pH.(12) Interestingly, the bespoke medium without HEPES and pH adjustment has more alkaline pH range from 7.7 to 8, indicating that the medium needs to be adjusted with 1N HCl to reach pH 7.4. Furthermore, we noticed that the pH of medium without HEPES varied depending on storage time and temperature. In contrast to that, the respective medium containing 15 mM HEPES resulted in a lower and more stable pH compared to those without HEPES; it reached the physiological pH of 7.4 without any pH adjustment. It has been reported that HEPES as a non-volatile buffer can strictly stabilize medium pH without being affected by the shifting of CO_2 concentration when the medium is placed outside the CO_2 incubator. However, this non-volatile buffer capability cannot replace the CO_2/HCO_3^- buffering system involved during incubation in the CO2 incubator.

In general, our result revealed that pH of conditioned medium (after incubation with BCSCs) was lower than that of cell-free medium. Of note, pH decrease, as indicated by ΔpH, was lesser in the conditioned medium with HEPES compared to that without HEPES, either with or without adjusted pH. Cells that are metabolically active secrete lactic acid during aerobic glycolysis (Warburg effect) resulting in a decrease of pHe.(21) The decrease of medium pH caused by lactic acid production during cell culture can be avoided by the buffering capacity of medium.(12) Thus, we suggest that the presence of HEPES buffer in the culture medium can enhance the buffering capacity of the medium that has been supplemented with $NaHCO_3$.

The viable cell number of BCSCs in HEPES (-) was higher than that in HEPES (+) medium until 4 day incubation suggesting that BCSCs proliferate better in the medium without HEPES, albeit in a more alkaline condition. Following day 6 incubation, the number of viable BCSCs was not elevated anymore or yet slightly decreased. This might be due to the abundant upsurge in the density of BCSCs grown in HEPES (-) medium after 4 day incubation leading to cell death (Figure 2). Moreover, the lag-phase of BCSC proliferation rate was reached on day 5 incubation with all kind of media used in this study. This might be related to the acidic pH of all conditioned media measured on day 6 (Table 1) as a consequence of high active metabolizing cell density. It should be noteworthy that BCSCs grown in HEPES (+) medium exhibited lower proliferation rate than those in the medium without HEPES within day 1 until day 4. The supplementation of HEPES in the medium reduced endothelial cell growth by stimulating toxic oxygen metabolites production.(22) After being absorbed by cells, HEPES exerted various negative effects on various cell functions.(23) Likewise, HEPES has been shown to affect drug uptake and transport into cells.(24) Surprisingly, unexpected effects may be raised when combining several buffering systems for example HEPES and CO_2/HCO_3^-. These includes long-term toxicity,(25) glycolytic stimulation, and bonding to Ca^+.(12) Therefore, it has been suggested that the preparation of culture medium containing non-volatile buffers should take into account the total osmolarity and changes in free Ca^{2+} to evade the non-physiological effects.(12)

In an *in vitro* study, cancer cells with any alteration they have including CSCs needs exclusive extracellular milieu to maintain their aggressiveness. The present study

demonstrated that DMEM/F-12 medium without HEPES buffer could provide an optimal condition for BCSCs to boost the expression of OCT4, as one of the major pluripotent markers, at mRNA and protein levels. Nevertheless, this remarkable induction of OCT4 expression occurred only until day 2 and gradually disappeared when BCSCs grew more than two days in line with the increase of proliferation rate. As a key driver of tumor progression, CSCs, the minor sub-populations in tumor, is dormant (non-proliferative and in the quiescent state) until activated and able to survive and preserve their stemness for long-term period in extreme tumor microenvironmental conditions such as acidic pH.(26,27) We suggest that the induction of BCSC proliferation might stimulate cell differentiation which in turn suppress the stemness of BCSCs. Unlike normal stem cells, CSCs have the capability to self-renew and form a new colony, called tumorigenicity.(28) Our study showed that the higher tumorigenicity, signified by the MFU value, of BCSCs grown in HEPES (-) medium for two days was in line with their higher OCT4 expression on the same day compared to those in HEPES (-) medium. Notably, we highlight that BCSCs tumorigenicity was superior when grown in the medium without HEPES but with pH adjustment to 7.4.

Conclusion

Taken together, we conclude that human $CD24^-/CD44^+$ BCSCs exhibit high proliferation and stemness properties simultaneously when grown in DMEM/F-12 with a physiological CO_2/HCO_3^- buffering system, but without HEPES, for less than 4 days under standard conditions. The adjustment of medium pH to 7.4 optimizes buffering conditions of this medium which could enhance BCSC tumorigenicity. Combining HEPES with CO_2/HCO_3^- buffering system in the BCSC culture medium may be necessary for prolonged cell culture in an extreme pH, since HEPES buffering system could provide a stable physiological pH. Additional studies are required to elaborate cell death mechanism affected by these buffering systems.

Acknowledgements

The authors are thankful for the Research Grant (Penelitian Dasar Unggulan Perguruan Tinggi/PDUPT/ PDUPT NKB-2777/UN2.RST/HKP.05.00/2020) from Directorate General of Research and Development Strengthening, Ministry of Research, Technology and Higher Education of the Republic of Indonesia.

Authors Contribution

SIW was involved in concepting, planning the research, preparing, editing the manuscript. YFN implemented the research in general, including data collecting and analysis, and preparing the manuscript. RAS and SA were involved in supervising the technical work. MS was involved in preparing and reviewing the manuscript.

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