Conditioned medium of E17 rat brain cells induced differentiation of primary colony of mice blastocyst into neuron-like cells

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ABSTRACT

Background: Conditioned medium is the medium obtained from certain cultured cells and contained secretome from the cells. The secretome, which can be in the form of growth factors, cytokines, exosomes, or other proteins secreted by the cells, can induce the differentiation of cells that still have pluripotent or multipotent properties.

Objectives: This study examined the effects of conditioned medium derived from E17 rat brain cells on cells with pluripotent properties.

Methods: The conditioned medium used in this study originated from E17 rat brain cells. The CM was used to induce the differentiation of primary colonies of mice blastocysts. Primary colonies were stained with alkaline phosphatase to analyze the pluripotency. The morphological changes in the colonies were examined, and the colonies were stained with GFAP and Neu-N markers on days two and seven after adding the conditioned medium.

Results: The conditioned medium could differentiate the primary colony, beginning with the formation of embryoid-body-like structure; round GFAP positive cells were identified. Finally, neuron-like cells testing positive for Neu-N were observed on the seventh day after adding the conditioned medium.

Conclusions: Conditioned medium from different species, in this case, E17 rat brain cells, induced and promoted the differentiation of the primary colony from mice blastocysts into neuron-like cells. The addition of CM mediated neurite growth in the differentiation process.

Keywords: Blastocyst; conditioned medium; neurons; growth factors; neurites

INTRODUCTION

Conditioned medium is the medium, where cells, particularly stem cells, are cultured. The medium contained factors secreted from the cells in the secretome, microvesicles, or...
Neuron differentiation by xenogeneic-CM

exosome [1]. The use of conditioned medium has attracted increasing attention because many studies reported that the injection of cytokines, chemokines, or growth factors could have superior effects to implanting cells directly to the organs. Kay et al. [2] reported that conditioned medium from a mesenchymal stem cells treatment reduced cartilage damage and suppressed the immune responses, and may provide effective cell therapy for inflammatory arthritis. Conditioned medium combined with a neural regeneration laboratory medium had a superior effect in inflammation reduction, neurite regeneration in vitro, and improved functional restoration in spinal cord injury rats in vivo [3]. Another research conducted by Sun et al. [4] reported that factors secreted from conditioned medium promoted the regeneration of sebaceous glands, angiogenesis, and wound healing.

Growth factors or secreted proteins found in conditioned medium have been attributed to a mechanism that delivers good results in both in vitro and in vivo experiments. Secretome in a paracrine mechanism can significantly repair the tissues while the differentiation of cells has a minor benefit [5]. The secretome in conditioned medium may also stimulate the host's endogenous cell survival mechanism [6]. The majority of the studies reported that the beneficial effect is most likely mediated by modulation of neurotrophic factor expression [7]. Moreover, the content of the conditioned medium that originated from the cells would resemble the ‘more physiological’ conditions of differentiation compared to the addition of chemical agents [8].

Related to the mechanism in which the conditioned medium had beneficial effects, some considerations are needed to choose the cells to produce conditioned medium because different types of cells will secrete different proteins or growth factors. There are many sources of the cells to produce or harvest conditioned medium, such as mesenchymal stem cells, neural stem cells, or adipocyte-derived stem cells [9-12].

One of the promising sources of secretome that is being extensively investigated is neuronal cell-derived conditioned medium. Lin et al. [13] reported that neuron-derived conditioned medium could protect microglia, astrocytes, and neurons from glucose, oxygen, and serum deprivation (GOSD)-induced cell death. Neural stem cell-conditioned medium has also been reported to inhibit cell apoptosis and have neuroprotective effects, making it a potential alternative and effective therapeutic intervention for ischemic stroke [10]. A previous study found that conditioned medium from a heterogeneous cell population of differentiated cells from E17 rat neural progenitor cells secreted proteins under starvation conditions that were used to maintain cell growth and stimulate differentiation [11]. The results of those studies were certainly very encouraging to open up future therapies for neurodegenerative diseases. On the other hand, research from a basic to clinical trial needs to be done and deepened so that the future application will truly be appropriate and safe to minimize the negative effects.

This study examined the effects of the conditioned medium derived from E17 rat brain cells on the cells with pluripotent properties. The primary colony of mice blastocyst was used as the first initial stage of embryonic stem cell propagation. This study evaluated whether the content of the conditioned medium that came from the different species could induce the differentiation of the primary colony.
MATERIALS AND METHODS

Production of conditioned medium of E17 rat brain cells
The conditioned medium used in this study originated from E17 rat brain cells. The E17 (days gestation) pregnant Wistar rat was euthanized by intraperitoneal injection of a ketamine-xylazine cocktail (91 mg/kg ketamine + 9.1 mg/kg xylazine) 0.2 mL/100 g body weight. The lower abdomen was sprayed with 70% alcohol then the uterus was then exposed by medial cutting under aseptic conditions to avoid contamination. All fetuses were removed and stored in a sterile, cold dissection solution (HBSS containing 0.3% glucose). The methods for isolating the neuronal cells and producing the conditioned medium were reported by Budiariati et al. [11]. The conditioned medium used in this study was collected 24 hours after serum deprivation. The experimental procedures of this research were approved by The Animal Care and Ethics Committee of the Faculty of Veterinary Medicine, IPB University (No: 123/KEH/SKE/IV/2019).

Culture of primary colony of mice blastocyst
Female mice were induced to superovulation by an intraperitoneal injection of 5 IU PMSG (Folligon®, Intervet, The Netherlands) and 5 IU hCG (Chorulon®, Intervet, Netherland) at 48 h intervals. The female mice were mated overnight with DDY males. On the following morning, the mice were examined for the presence of vaginal plugs. The blastocysts were harvested on day 3.5.

Harvested blastocysts were cultured for one hour before being exposed to 0.25% pronase for zona pellucida removal. Free-zona blastocysts were then cultured for one hour before being seeded on four-well gelatin-coated dishes for three–four days. The medium was knock-out DMEM (GibcoTM) supplemented with 15% knock-out serum replacement (GibcoTM), 1 mM GlutaMax® (GibcoTM), 0.1 mM β-mercaptoethanol, 0.1 mM nonessential amino acids (GibcoTM), 1% antibiotic-antimycotic (100x) (GibcoTM), and 1000IU/ml LIF (Sigma L-5158).

Alkaline phosphatase (ALP) Staining
ALP staining was performed after 3–4 days of culture, the medium was aspirated, and the primary colonies were fixed using 4% paraformaldehyde for 2 min. The fixative was discarded and washed 1X with TBST rinse buffer. The colonies were stained with 200 μg/mL Naphtol AS-MX phosphatase and 1 mg/mL fast red TR salt in 100 mM Tris buffer for 30 min at room temperature. The stained colonies were washed with TBST rinse buffer 1X and covered in 1X PBS to prevent drying. The colonies expressing ALP stained red.

Induction of differentiation using conditioned medium of E17 rat brain cells
The primary colony of mice blastocysts was induced for differentiation on day three–four after seeding by exchanging the medium with NRA medium consisting of neurobasal medium MACS®Neuro-Medium (Miltenyi Biotec) containing 2% MACS NeuroBrew-21 w/ retinoic acid (Miltenyi-Biotec), 1% antibiotic-antimycotic (100x) (GibcoTM), and 1% GlutaMax® (GibcoTM) for four days. The medium was changed into a combination of neurobasal medium: conditioned medium from E17 rat brain cells (1:1 (v/v)). The morphological changes of the colonies were examined, and the colonies were stained on days two and seven after adding the conditioned medium using GFAP or NeuN markers.
**Immunocytochemistry**

The differentiation after induction of the primary colony was assessed by immunocytochemistry. GFAP (Santa Cruz sc) and NeuN (Abcam ab104225) antibody markers were used in this study. The secondary antibody was a secondary HRP-conjugated antibody (Trekkie Universal Link, Starr Trek Universal HRP Detection Kit Biocare®). Staining was performed using the procedures reported by Rinendyaputri et al. [14].

**RESULTS**

**Culture of E17 rat brain cells and conditioned medium production**

The whole brain from E17 Wistar rat fetuses was isolated and cultured for four days. The cells were characterized by flow cytometry to analyze the heterogeneity of the cells population using two markers PSA-NCAM and A2B5. The results showed that the cell population consisted of immature neuron PSA-NCAM+, glia progenitor A2B5+, and double-positive markers (PSA-NCAM+/A2B5+), as shown in Fig. 1. The dominant cells of the population were the immature neuron (62.98%), whereas the glial cells with positive expression of A2B5 markers were 22.04%. The cells were then cultured under serum deprivation with minimum essential medium (MEM) without any supplement so that the collected conditioned medium will only contain proteins secreted from the cells. The conditioned medium was aspirated or collected 24 h after serum deprivation.

**Culture of mice blastocysts into primary colonies and pluripotency assay**

The fertilized blastocysts were seeded on four-well gelatin-coated dishes after being treated in 0.25% pronase for zona removal (Fig. 2A and B). On the third to fourth days after cultures, the primary colony formed as multilayer cells, as shown in Fig. 2D. Forty-one blastocysts were used in this study. The attachment rate or the capability of the blastocyst to attach to the dish was 86.63 ± 6.43, whereas the primary colony rate was 83.88 ± 8.64 (Fig. 3). Fig. 2C and D show the development of the blastocysts after attaching the dish to primary colony formation. ALP staining was performed to check the pluripotency of the colony. The results showed that the colony still had pluripotency indicated by the red color (Fig. 2E and F).

**Induction of differentiation of the primary colony of mice blastocyst**

The primary colonies of mice blastocysts were induced to differentiate using the conditioned medium from E17 rat brain cells. In the initial step of differentiation induction, the medium was exchanged with NRA medium, a neurobasal medium supplemented with retinoic acid...
contents, for four days. The morphology of the colony changed, and embryoid bodies-like structures were observed. They were embryoid bodies-like because they were not floating like common embryoid bodies features (Fig. 4A). The primary colony was divided into two groups. The first continued to be cultured in the NRA medium, while the second was cultured in the neurobasal medium: conditioned medium 1:1 (v/v).

The morphological changes were analyzed on the sixth day. Both groups showed morphological changes. The colony cultured in the conditioned medium formulation differentiated into round shape cells, as shown in Fig. 4C, whereas while the colony cultured in the NRA medium
did not show the same differentiated cells (Fig. 4B). The round shape cells were identified by GFAP staining to test the prediction that these cells might be neuroepithelial cells, which is the precursor for neuronal cells (Fig. 5A). The results showed that the round shape cells that originated from the primary colony were positive for GFAP. The results strengthen the hypothesis that the induction of a primary colony of blastocysts to neuronal cells occurred through a transformation to neuroepithelial cells. The trophectoderm cells were used as a negative control of the staining because the cells were also found when whole blastocysts were cultured but did not differentiate into neuronal cells (Fig. 5B).

The morphology of the primary colony cultured in the NRA medium for six days was compared with the primary colony cultured with the conditioned medium. The primary colony cultured in the NRA medium showed a larger undifferentiated area compared to another group (Fig. 6A and B). The primary colonies cultured with the supplementation of conditioned medium might differentiate faster and express positive results when stained with GFAP (Fig. 6C and D).

Neuron-like cells were identified after seven days of culturing the primary colony that had been cultured in conditioned medium formulation (neurobasal: conditioned medium 1:1 (v/v)), as shown in Fig. 6E. In contrast, the same neuron-like cells were not found in the control group. The neuron-like cells of the treatment group of the conditioned medium were then stained with the Neu-N marker. The results revealed cells positive for the Neu-N staining (Fig. 6F).
DISCUSSION

Over the last few decades, cell-based therapies have become a choice for degenerative diseases because of the limitations of current pharmacological drugs to solve those health issues. At least two major mechanisms have been proposed to explain how this therapy provides positive results. The first one is based on the engraftment of cells to damaged tissue. The second is the stimulation of the self-healing processes of endogenous tissue through the trophic effects mediated by cytokine and growth factor secretion [15]. Although a low count of the transplanted cells can have a beneficial effect, the homing of the cells and the mechanism in which the immune system does not reject them are still a mystery. In recent years, there has been a paradigm shift in that the cells act by secreting humoral factors that will improve the function of the tissues [16]. This leads to the development of secretome-based therapy instead of direct cell-based therapy.

As reviewed by Vizoso et al. [17], secretome as a cell-free therapy might resolve safety considerations and can be evaluated for the dosage and other pharmaceutical agents. Storage can be achieved without applying potential cryopreservative chemicals, making the process
more economical and allowing modification to desired cell-specific effects and possible for mass production. On the other hand, some considerations are needed to choose the cells that will produce the conditioned medium because different types of cells will secrete different proteins or growth factors.

This study used heterogeneous neuronal cells from the E17 rat whole brain instead of the purified type of cells, such as progenitor to immature neurons or astrocytes. Although previous studies used homogenous cells [7,8,10], based on their origin under in vivo conditions, the neurotrophic factor and growth factors that play a role in neurogenesis or brain repair originate from different regions of the adult and embryonic brain [18].

The cells were isolated from the whole brain of E17 rat embryos, and the cells were heterogeneous. They consisted of three types of cells, PSA-NCAM+, A2B5+, and those that express both markers. Based on the results, after four days of culture, the cells positive for the PSA-NCAM marker representing immature neurons were dominant (62.98%), while the glial cells with positive expressions of the A2B5 markers were 22.04%. Most PSA-NCAM+ cells that represent immature neurons are related to neurogenesis in the mammalian central nervous system. Neurons were found in the embryonic period, while most glial cells were produced after birth [19]. Neurogenesis of mouse or rat cerebral cortex commences around embryonic day 12 (E12), peaks at E15, and finishes around birth. The findings showed that the dominant cells from E17 rat brains after four days of culture were immature neurons; glial cell progenitors were obtained. Hence, gliogenesis had started at this time. In mice, gliogenesis starts at E16 and continues to postnatal life [20].

A previous study analyzed the conditioned medium obtained from a short and long period of serum deprivation culture of E17 rat brain cells. The proteins secreted from the cells treated with a short duration of starvation were dominated by immature neurons consisting of proteins used to maintain cell growth, stimulate differentiation, and produce energy. On the other hand, there were also proteins for the activation of autophagy and proteins secreted in response to microenvironmental stress. The cells cultured after long periods of starvation with the dominance of glial cells secreted proteins that act as neuroprotectors [11]. Hence, the conditioned medium from the cells cultured in serum-free conditions for 24 hours was used to test whether the conditioned medium would induce the differentiation of the primary colony of mice blastocyst.

This study used the conditioned medium from different species as an initial study to assess the potency of an inter-species differentiation mechanism mediated by proteins secreted from the cells. Lotfinia et al. [21] reported that secreted molecules from human embryonic stem cell-derived mesenchymal stem cells have therapeutic potentials on mouse models of acute hepatic failure. Another research conducted by Jonas et al. [22] reported that small extracellular vesicles from hypoxic mesenchymal stem cells from bone marrow samples of selected healthy donors could promote cerebral angiogenesis, brain remodeling, and neurological recovery after focal cerebral ischemia in mice. In veterinary practice and research, Lee et al. [23] reported that equine-induced pluripotent stem cells generated from adipose-derived stem cells could induce the regeneration of injured muscle of Rag1/mdx mice, highlighting the therapeutic effects.

This study assessed the ability of conditioned medium from rat brain cells to induce differentiation from mice blastocysts. The brain cells were selected to examine the specificity
of the cells that produced secreted proteins on the directed neuronal differentiation of the primary colonies of mice blastocyst. Timmusk et al. [24] reported that one of the main neurotrophic factors, brain-derived neurotrophic factor, is tissue-specific, where each brain region and peripheral tissue has a different level of expression of neurotrophic factors. Bach et al. [25] reported a cross-species effect of bioactive components and factors secreted by notochordal cells on improving the degeneration of intervertebral discs. Therefore, this initial study was conducted in the concept of the possibility of an inter-species manner.

The primary colonies from the mice blastocyst used were the initial cells of embryonic stem cells propagation [26]. Based on these results, the attachment rate was 86.63 ± 6.43, whereas the primary colony rate was 83.88 ± 8.64. The rate of the attachment and primary colony of the mice blastocyst can be varied, but the number showed an effectiveness of the culture of the blastocysts. The limitation of this study was the characterization of pluripotency. ALP staining was conducted as a common traditional marker of pluripotent embryonic stem (ES) cells, but the expression of pluripotency genes, such as Oct4, Nanog, and Sox2, are needed for the passage of the ES cell line [27]. The primary colonies tended to have pluripotency, as indicated by the positive expression visualized by the red color. The ALP can be detected in preimplantation embryos from two cells to blastocysts, in the inner cell mass and trophectoderm but later on the inner cell mass only.

The initial induction of the primary colony was achieved by adding a supplement containing retinoic acid. These findings revealed morphological changes in the primary colony to form embryoid bodies-like structures one day after the medium had been changed to an NRA medium. Retinoic acid is typically used at concentrations ranging from 5 μmol/L to 5 mmol/L to facilitate the differentiation of ESCs into neural progenitor cells. The retinoic acid will act through some reported mechanisms, such as cyclic AMP-binding protein (CREB), c-JunN-terminal kinase K, or among signaling pathways, such as the Wnt/β-catenin, FGF, and Erk pathways [28]. High concentrations of retinoic acid will increase the expression of neuronal genes and suppress the mesodermal genes resulting in the formation of embryoid bodies [29].

After the initial induction, the primary colonies were divided into two groups: the NRA groups and the neurobasal-conditioned medium groups. The results suggested that the conditioned medium promoted differentiation faster than the NRA groups. The primary colony cultured in the NRA medium showed a larger undifferentiated area than the other group. The colony cultured in the conditioned medium formulation differentiated into round shape cells that tested positive for GFAP. The cells were predicted to be neuroepithelial cells to radial glial cells with a radial morphology and glial characteristics [30]. Indeed, radial glial cells have cellular cells and molecular characteristics of astroglia, and also express the astrocyte-specific glutamate transporter GLAST, S100β, glutamine synthase (GS), vimentin, and tenascin-C (TN-C), and in certain species, GFAP. Nevertheless, further analysis using more specific markers will be needed.

On the seventh day after adding the conditioned medium, the cells developed into neuron-like cells. Neu-N staining was performed to confirm the morphological appearance; the cells were positive for the Neu-N marker. Based on the results, the conditioned medium might contain proteins secreted from E17 rat brain cells that promoted the differentiation of the primary colony of mice blastocysts into neuronal cells.
Tjalsma et al. [31] introduced the secretome terminology, which is defined as biomolecules secreted by the cells, tissue, or organisms through various secretory mechanisms. The biomolecules consisted of growth factors, cytokines, adhesion molecules, hormones, neurotransmitters, and proteases that reflected the function of the cells [32]. In the cell culture, the secretomes were secreted to the medium, which is called conditioned medium. The most common source of conditioned medium is stem cells. Secretome from mesenchymal stem cells can increase the proliferation and differentiation from progenitor neurons in the brain regions [33]. Ribeiro et al. [34] reported that conditioned medium from adipose-derived stem cells induced neuritogenesis mediated by nerve growth factor (NGF) stimulation. Proliferation and differentiation involve growth factors secreted by cells, such as brain-derived neurotrophic factor (BDNF), NGF, vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), and secretory vesicles that triggered neuronal differentiation in vitro and in vivo [35].

In addition to stem cells, it was also reported that other cells could be used to produce conditioned medium. The addition of conditioned medium from microglial cells could increase neuroblast production in the subventricular zone [36,37]. Srivastava et al. [38] reported that conditioned medium from PC-12 cells induced the neuronal differentiation of mesenchymal stem cells using a 50% conditioned medium concentration. Their results became the reason for the concentration determination of conditioned medium (neurobasal medium: conditioned medium (1:1) (v/v)).

Conditioned medium from different species, in this case, E17 rat brain cells, induced and promoted the differentiation of the primary colony from mice blastocyst into neuron-like cells. The differentiation process began with the initial induction to form embryoid bodies-like structures using a retinoic acid supplement, followed by the development of neuroepithelial to radial glial cells, then by neurite growth mediated by the addition of the conditioned medium. Staining using Neu-N marker showed positive results, but confirmation as to whether the cells are functional requires further characterization. Yang et al. [39] reported that definitive neuronal differentiation is determined by the neuronal morphology, expression of neuronal markers, polarization and action-potential of the cells, membrane stability, and functional receptor to neurotransmitter as well as the pre and post synapsis examination. This initial study, showed that conditioned medium from E17 rat brain cells has potency in neuronal differentiation of primary colonies from mice blastocysts in an inter-species manner.

Galvão et al. [40] reported that a single exogenous neurotrophic factor, which is the brain-derived neurotrophic factor that is given intravenously, could not increase neurogenesis in the subventricular zone and instead might decrease. On the other hand, the proliferation and neurogenesis of the cells in that zone responded well to the stimulation of combined extracellular factors, such as EGF, FGF2, PDGF, BMPs, noggin, prolactin, and erythropoietin [40-43]. The conditioned medium used in this study contained various proteins secreted from the cells that mediated the differentiation process because of its multiple growth factors and other secreted proteins [11].

The results showed that the conditioned medium from rat brain cells could differentiate the primary colonies of mice blastocysts. It showed that an interspecies approach might be used in further research or veterinary practices. On the other hand, rats and mice are both rodents with a close genetic distance. The genetic distance is closely related to the high similarity of
the neurotrophic factors secreted by the cells. For example, the rodents have BDNF genes that consist of nine exons with eight non-coding exons and single coding (exon IX). Nair and Wong-Riley [44] reported a high similarity between the BDNF genes of the rats and mice, especially on binding site nuclear respiratory factor 2 (NRF-2), which control the expression of the energy mediator metabolism (cytochrome oxidase) and neuronal activity. The study, which used the inter-species concept from longer genetic distances, might be conducted in a more specific and in-depth study. Nevertheless, these results are expected to open opportunities to substitute defined growth factors and strengthen the paracrine hypothesis in inter-species differentiation that may be a new direction for treatment in cell-based therapies in veterinary practice.

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