Review Article

ผลกระทบของฟีนอลเรดต่อการเพาะเลี้ยงเซลล์

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บทคัดย่อ

ผลกระทบของฟีนอลเรดต่อการเพาะเลี้ยงเซลล์

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การเพาะเลี้ยงเซลล์เป็นโมเดลระดับหลอดทดลองในการศึกษาศักยภาพเบื้องต้นทางเภสัชวิทยาและพิษวิทยาของสารก่อนการศึกษาในสัตว์ทดลองและการศึกษาทางคลินิกต่อไป ฟีนอลเรดเป็นตัวบ่งชี้ระดับ pH ในอาหารเลี้ยงเซลล์ที่บอกเวลาที่เหมาะสมสำหรับการเปลี่ยนอาหารเลี้ยงเซลล์ ฟีนอลเรดมีสมรรถนะของเอสโตรเจนเนื่องจากมีโครงสร้างเอสโตรเจนที่ไม่ใช่สเตียรอยด์ที่คล้ายคลึงกับ 17 บีต้าเอสตราไดออลและบิสฟีนอล บางกรณีอาหารเลี้ยงเซลล์ปราศจากฟีนอลเรดจึงถูกใช้ทั้งระหว่างการเลี้ยงเซลล์และการทดสอบสารเพื่อหลีกเลี่ยงผลไม่พึงประสงค์ต่างๆ เช่น การรบกวนสมรรถนะของเอนไซม์เฟส 1 และ เฟส 2 การแสดงสมรรถนะของเอสโตรเจน การกระตุ้นการเจริญเติบโตและการเปลี่ยนแปลงรูปร่างของเซลล์ การลดฤทธิ์ทางเภสัชบำบัดของยาต้านมะเร็ง และการเพิ่มสมรรถนะเสริมสร้างร่างกายของสารบางชนิด โดยเฉพาะอย่างยิ่งเซลล์ที่ไวต่อเอสโตรเจน เช่น เซลล์มะเร็งเต้านมและเซลล์รังไข่ ฟีนอลเรดอาจแสดงผลลัพธ์ที่แตกต่างกันได้ตามความเข้มข้นและชนิดของเซลล์และหลักฐานเชิงประจักษ์นี้ยังมีอย่างจำกัดในปัจจุบัน ดังนั้นการใช้อาหารเลี้ยงเซลล์ที่มีหรือปราศจากฟีนอลเรดระหว่างการเลี้ยงเซลล์และการทดสอบสารจึงเป็นปัจจัยสำคัญหนึ่งที่จำเป็นต้องพิจารณาในการออกแบบการทดลองเพื่อป้องกันผลลัพธ์ที่ไม่น่าเชื่อถือในการศึกษาทางการแพทย์และเภสัชกรรม

คำสำคัญ: ฟีนอลเรด, การเพาะเลี้ยงเซลล์, ผลของเอสโตรเจน, การเจริญเติบโตของเซลล์, โมเดลระดับหลอดทดลอง

Effect of phenol red on cell cultures

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Abstract

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Cell culture is an *in vitro* model for primary screening of the pharmacological and toxicological potential of a substance before *in vivo* and clinical studies. Phenol red is the pH indicator in culture medium indicating a suitable time for medium change. Phenol red has estrogenic activity due to its non-steroidal estrogenic hormone-structure like 17β-estradiol and bisphenol. Phenol red-free medium is sometimes employed for culturing and treatment periods to avoid undesirable effects, e.g. interference with phase I and phase II enzyme activities, estrogenic activity, stimulating cell proliferation and differentiation, decreasing pharmacotherapeutic activity of anti-cancer drugs, and enhancing activity of anabolic compounds, particularly in estrogen-sensitive cells such as breast cancer cells and ovarian cells. Phenol red shows variable effects that can depend on its concentration and the type of cell, although relevant evidence is limited. Therefore, using phenol red-contained or -free medium during cell culture or treatment is a crucial choice for experimental design in order to prevent unreliable outcomes.

Keywords: phenol red, cell culture, estrogenic effect, cell proliferation, *in vitro* model

1. Introduction

The cell culturing method was discovered in 1870 by Ross Granville Harrison, the American embryologist who employed a blood, agar, and saline solution to culture live tissue (Taylor, 2014). Harrison identified major limitations of the method using primary nerve cells, which were restricted growing times and microbial contamination (Harrison *et al.*, 1907; Jedrzejczak-Silicka, 2017). Aseptic technique and immortalized cell lines were established by Montrose Burrows and Alexis Carrel in 1910 and 1912, respectively, to solve contamination problems and prolong growing times (Carrel and Burrows, 1911; Taylor, 2014). These discoveries formed a basis for the application of cell culture in embryology, histology, and biotechnology (Jedrzejczak-Silicka, 2017; Taylor, 2014). At present, cell culture is widely used as a primary study tool to understand the pharmacological and toxicological mechanisms of

compounds or to unravel the pathological mechanism of diseases prior to *in vivo* and clinical studies (Allen *et al.*, 2005; Kapałczyńska *et al.*, 2016; Mirabelli *et al.*, 2019; Monks *et al.*, 1991). Due to the artificial physiological conditions of cell culture, results can usually not be directly extrapolated to *in vivo* conditions (Antoni *et al.*, 2015; Duval *et al*, 2017). Hence, it is of interest to understand how the influencing factors in cell culture mimic environmental and physiological conditions (Niu and Wang, 2015). One important factor affecting cell culture is the culture medium, which is normally supplemented with additives such as serum, glucose, antibiotics, and phenol red (Eagle, 1955; Yao and Asayama, 2017) to ensure suitable conditions to maintain cell viability and enhance proliferation (Ackermann and Tardito, 2019; Eagle, 1955; Yao and Asayama, 2017).

Phenol red is a pH indicator additive in commercial culture media (Santos *et al.*, 2018; Welshons *et al.*, 1988). At the optimal pH of 7.4, phenol red is a bright red color that turns yellow in acidic culture medium containing waste products generated during the culturing period (Santos *et al.*, 2018). Phenol red possesses a weak estrogenic effect in accordance with its non-steroidal estrogenic like structure, which stimulates cell proliferation and can interfere with anti-cancer drugs in cell culture models (Berthois *et al.*, 1986; Welshons *et al.*, 1988). Hence, phenol red-free medium is often recommended for the treatment period to avoid these interfering effects, such as affecting the activity of phase I and/or phase II metabolizing enzymes (Baxter and Minet, 2017). Nevertheless, studies into the effects of phenol red on gene expression in different cell types, especially human cell lines, are limited and controversial (Welshons *et al.*, 1988). Therefore, this review focuses on the use of phenol red in cell culture models, and evidence of its effect in cell culture and treatment.

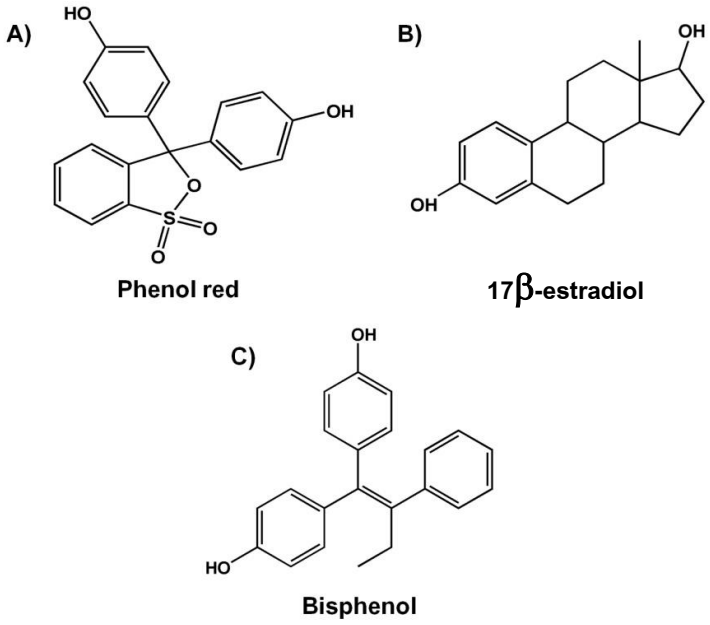

Figure 1 Phenol red and non-steroidal estrogen-related structures.
A) phenol red, B) 17β-estradiol, and C) bisphenol.
(Adapted from Sengupta *et al.*, 2013; Welshons *et al.*, 1988)

2. Significance of phenol red

Phenol red (3H-2,1-benzoxathiole 1,1-dioxide, $C_{19}H_{14}O_5S$) or phenolsulfonphthalein (Figure 1A) was invented by Rowntree and Geraghty in 1912 (Goldring *et al.*, 1936). In the past, phenol red was marketed as a non-absorbable indicator that was excreted via tubular secretion in the kidney (Clarke and Williams, 1971, Goldring *et al.*,

1936). Then, it was used as a universal test of renal function in humans by determining the relative concentration of phenol red in urine and plasma (Goldring *et al.*, 1936). Moreover, phenol red was used as a gastric emptying marker in humans due to its low mucus adsorption property (Clarke and Williams, 1971). However, these roles of phenol red in renal function and gastric emptying are not currently used. Currently, phenol red is applied in the “phenol red thread test” as an alternative clinical dry eye diagnosis due to its color change (from yellow to red) in weakly alkaline tears (Sakamoto *et al.*, 1993; Senchyna and Wax, 2008). Phenol red is also applied in “phenol red chromo-endoscopy”, a non-invasive gastric test for *Helicobacter pylori* infection in patients with contraindications for gastric biopsy. The urease enzyme produced by this bacterium can turn infected areas alkaline, with phenol red subsequently staining the gastric mucosa red (Ibrahim *et al.*, 2015).

Nowadays, phenol red is widely used as a pH indicator due to its obvious color visibility (Michl *et al.*, 2019). Phenol red changes color upon pH; acidic conditions induce protonation of its tautomeric form (Figure 2A) and phenol red appears an orange to yellow color detected at wavelength 466 nm while under basic conditions it is in equilibrium between phenol (Figure 2B) or phenate (Figure 2C) forms, appearing as a bright red color detected at wavelength 481 nm (Thomas and Brogat, 2017). Therefore, at the physiological pH of 7.4, phenol red is in bright red and turns yellow during the culturing period when cells eliminate waste products, which signals a suitable time for medium change (Santos *et al.*, 2018).

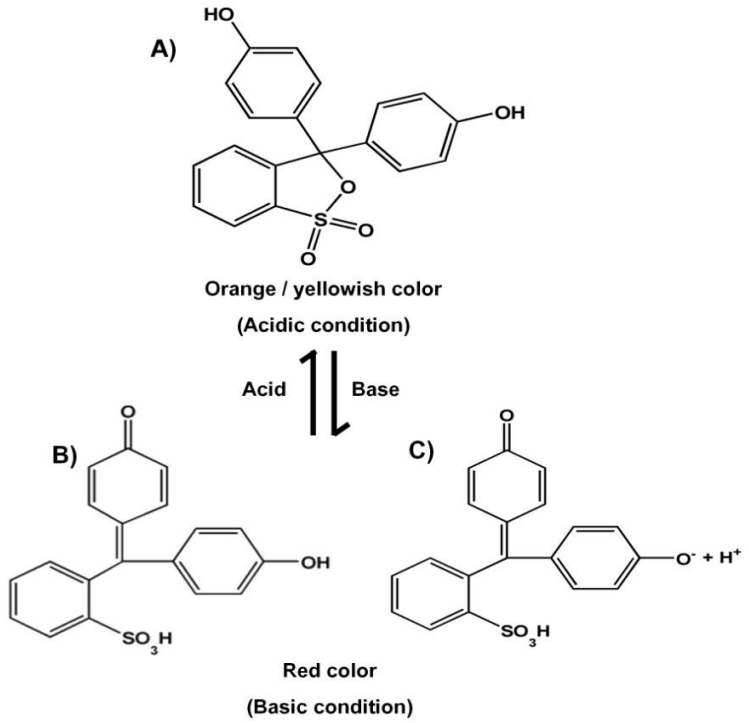

Figure 2 Chemical structural of phenol red in different pH conditions.
A) tautomeric form, B) phenol form, and C) phenate form.
(Adapted from Thomas and Brogat, 2017)

3. Phenol red in commercial culture medium

Phenol red causes endocrine-disruption and subsequently interferes with production of endogenous compounds and metabolism of hormones (Santos *et al.*, 2018; Wesierska-Gadek *et al.*, 2007). Moreover, phenol red exhibits weak estrogenic activity, which can be considered an interfering factor in cell culture (Baxter and Minet, 2017).

Low dose of phenol red is added in culture medium to prevent toxic effects to cells (Berthois *et al.*, 1986). There are several commercial culture mediums that contain phenol red (Ackermann and Tardito, 2019; Grady *et al.*, 1991), e.g. from high to low concentrations of phenol red, Dulbecco's modified Eagle's medium (DMEM; phenol red 39.90 - 45.00 μM), Iscove's modified Dulbecco's medium (IMDM; phenol red 39.85 - 45.15 μM), Eagle's minimal essential medium (MEM; phenol red 26.57 - 31.04 μM), Plasmax™ (phenol red 25.00 μM), Dulbecco's modified Eagle's medium with Ham's F-12 nutrient mix (DMEM/F-12; phenol red 21.50 - 24.35 μM), Roswell Park Memorial Institute 1640 medium (RPMI 1640; phenol red 13.30 - 15.00 μM), human plasma-like medium (HPLM; phenol red 14.00 μM), and Ham's F-12 nutrient mix medium (F-12; phenol red 3.20 μM) (Ackermann and Tardito, 2019; Wesierska-Gadek *et al.*, 2007; Santos *et al.*, 2018) (Table 1).

Table 1 Concentration of phenol red in commercial culture medium

Commercial culture medium	Concentration of phenol red (μM)	References
DMEM	39.90 - 45.00	Wesierska-Gadek *et al.*, 2007; Santos *et al.*,2018; Ackermann and Tardito, 2019
IMDM	39.85 - 45.15	Santos *et al.*, 2018; Ackermann and Tardito, 2019
MEM	26.57 - 31.04	Santos *et al.*, 2018; Ackermann and Tardito, 2019
Plasmax™	25.00	Ackermann and Tardito, 2019
DMEM/F-12	21.50 - 24.35	Santos *et al.*, 2018; Ackermann and Tardito, 2019
RPMI 1640	13.30 - 15.00	Santos *et al.*, 2018; Ackermann and Tardito, 2019
HPLM	14.00	Ackermann and Tardito, 2019
F-12	3.20	Ackermann and Tardito, 2019

4. Effects of phenol red on cell cultures

Phenol red (Figure 1A) is a chemical with partial estrogenic effect due to its non-steroidal estrogenic hormone-structure like 17β-estradiol (Figure 1B) and bisphenol (Figure 1C) (Liu *et al.*, 2013; Welshons *et al.*, 1988). Phenol red has a phenol hydroxyl group, the important part for estrogenic structure-related activity, located in a similar position as 17β-estradiol (Liu *et al.*, 2013; Welshons *et al.*, 1988). In addition, phenol red has a structure that resembles bisphenol, which is triphenylethylene, that partially activates the estrogen receptor, which subsequently induces cell proliferation (Obiorah *et al.*, 2014; Sengupta *et al.*, 2013; Welshons *et al.*, 1988). Therefore, phenol red-free medium is sometimes recommended during treatment (Baxter and Minet, 2017). Studies on cytochrome P450s (CYPs) activity in Caucasian primary hepatocytes, commercial viral infected hepatic cells, and primary airway epithelial cells suggest the use of phenol red-free medium for treatment to avoid interference with enzymatic activity (Baxter and Minet, 2017; Kim *et al.*, 2015). Some study designs employ phenol red-containing medium for both culture and treatment periods to reveal the impact of phenol red on cell culture.

4.1 Hormonal effects of phenol red on cell cultures

Phenol red (15 - 45 μM) in culture medium was reported to have estrogenic activity (Berthois *et al.*, 1986; Welshons *et al.*, 1988). Human breast cancer MCF-7 cells were cultured in a 25 cm^2-flask (1.5×10^5 cells/flask) with MEM phenol red-free medium supplemented dextran-coated charcoal-treated calf serum (5%) for 1 week and the medium was collected to incubate with [^{3}H] estradiol (2.5 nM) and estradiol (0.1 nM - 1 mM) or an estradiol competitor, phenol red (0.1 nM - 1 mM), for 17 hours. Competitive binding estrogen receptor analysis was indicated by hydroxyl apatite radioactivity bound [^{3}H] estradiol. Estradiol presented 100%

relative binding to the estrogen receptor. Phenol red had 0.001% binding affinity to the estrogen receptor, with an equilibrium dissociation constant of 0.2 μM while estradiol had an equilibrium dissociation constant of 0.2 nM. These findings indicate that phenol red demonstrated a partial estrogenic activity in MCF-7 cells (Berthois *et al.*, 1986).

The progesterone receptor is stimulated by estrogen (Berthois *et al.*, 1986). MCF-7 cells were cultured in 24 well-plates (1×10^4 cells/well) that contained phenol red-free MEM or MEM with phenol red (28.21 μM) for 7 days before determining the response of the progesterone receptor. The results showed that phenol red stimulated the response by 50% (Welshons *et al.*, 1988). Correspondingly, MCF-7 cells cultured in a 25 cm^2-flask (1.5×10^5 cells/flask) MEM with phenol red (30 μM) supplemented with dextran-coated charcoal-treated calf serum (5%) for 5 days showed a basal level of progesterone receptor 3 times higher (2.0 - 2.2 pmol/mg DNA) than in phenol red-free MEM (0.70 - 0.71 pmol/ mg DNA) (Berthois *et al.*, 1986) (Table 2).

Table 2 Effects of phenol red contained medium in cells

Cells	Concentration of phenol red (μM)	Effects	References
MCF-7	28.21	Stimulate progesterone receptor 50%	Welshons *et al.*, 1988
	15 – 60	↑ Cell proliferation	Wesierska-Gadek *et al.*, 2007
	30	↑ Cell proliferation ↑ Progesterone receptor level	Welshons *et al.*, 1988; Berthois *et al.*, 1986
	300	↓ Cell proliferation	Welshons *et al.*, 1988
	45	↓ Anti-cancer drug activity (e.g. roscovitine)	Santos *et al.*, 2018; Wesierska-Gadek *et al.*, 2007
Primary mouse endometrial cells	21.57	↑ Cystic fibrosis transmembrane conductance regulator	Tsang *et al.*, 2001
Primary rat osteoblast-like cells	1.5 – 300	↑ Cell proliferation	Ernst *et al.*, 1989
Primary human ovarian surface epithelial cells	45	↑ Differentiation of ovarian cell to large oocyte phenotype cell	Bukovsky *et al.*, 2005
HL-60	15-200	↔ Cell proliferation	Morgan *et al.*, 2019
ZR-75-1	42.32	↔ Anti-cancer drug activity (e.g. tamoxifen, *trans*-hydroxytamoxifen)	Glover *et al.*, 1988
T-45-D	42.32	↔ Anti-cancer drug activity (e.g. tamoxifen, *trans*-hydroxytamoxifen)	Glover *et al.*, 1988
Bone marrow stromal cells	42.32	↑ Anabolic stimulation (i.e. prostaglandin E2, prostaglandin A1, basic fibroblast growth factor)	Still *et al.*, 2003

Note. (↑) increase; (↓) decrease; (↔) unchanged.

Phenol red affects cystic fibrosis transmembrane conductance regulator (CFTR) related ovarian hormones in mice (Tsang *et al.*, 2001). CFTR is the chloride channel normally expressed in the epithelium of the reproductive tract (Ajonuma *et al.*, 2005). Binding of estrogen to the estrogen receptor increases both expression and activity of CFTR, which can be applied as a biomarker of ovarian hyperstimulation syndromes (Tsang *et al.*, 2001; Ajonuma *et al.*, 2005). Primary mouse endometrial cells obtained from uteri of immature ICR mice (4-week-old) were cultured

on a Matrigel® coated nitrocellulose Millipore® filter with DMEM/F12 containing phenol red (21.57 μM) or phenol red-free DMEMF/12 for 3 days before determination of ion channel activity via the short circuit current technique (ISC). Forskolin-stimulated ISC is a parameter to predict expression and chloride exchange activity of CFTR. Mouse endometrial cells in phenol red-free DMEMF/12 showed a decrease in forskolin-stimulated ISC from 16.95±1.53 μA/cm^2 to 9.72±0.89 μA/cm^2, while cells in DMEM/F12 containing phenol red showed an increase in forskolin-stimulated ISC compared to the basal ISC value. These findings imply that phenol red induced expression of CFTR in mouse endometrial cells via estrogen receptor binding (Tsang *et al.*, 2001) (Table 2).

According to the impact of phenol red on the expression and activity of estrogen and progesterone receptors, phenol red-free medium is recommended for all hormone-associated studies that involve these receptors, particularly in MCF-7 cells and primary mouse endometrial cells.

4. 2 Effects of phenol red on cell proliferation and differentiation

Phenol red is known as a cell proliferation agent due to its estrogenic like structure, particularly for estrogen receptor positive MCF-7 cells (Santos *et al.*, 2018; Welshons *et al.*, 1988; Wesierska-Gadek *et al.*, 2007). MCF-7 cells were cultured in microtiter plates (5 × 10^3 cells/well) with DMEM contained phenol red (45 μM) or phenol red-free DMEM supplemented with fetal calf serum (FCS) (10%) for 53 hours. A CellTiterGlo™ luminescent cell viability assay was performed at 0, 29, and 53 hours. MCF-7 cells in the DMEM with phenol red showed a 3-fold increase in cell proliferation, while MCF-7 cells in phenol red-free DMEM showed a 2-fold increase compared to the cell viability at 0 hour. Likewise, MCF-7 cells cultured in phenol red-free medium for 6 months could increase their cell viability by 50% after culture in medium supplemented with phenol red (15 - 60 μM) and FCS (20%) for 1 day (Wesierska-Gadek *et al.*, 2007) (Table 2). Moreover, phenol red (30 μM) stimulated cell proliferation in MCF-7 cells cultured in a 24 well-plate (4 × 10^4 cells/well) with MEM for 3 days, by increasing DNA level per well 2-fold compared to baseline. In contrast, phenol red at a higher concentration (300 μM) showed cytotoxicity (Welshons *et al.*, 1988) (Table 2). MCF-7 cells were cultured in a 25 cm^2-flask (1.5 × 10^5 cells/flask) with 3 types of media including 1) regular MEM with phenol red, 2) phenol red-free MEM, and 3) phenol red-free MEM plus phenol red (30 μM), supplemented with dextran-coated charcoal-treated calf serum (2 - 20%) for 8 days and cell proliferation was determined by cell counter method. Regular MEM with phenol red and phenol red-free MEM plus phenol red (30 μM) showed cell proliferation around 2 times more than those of phenol red-free MEM (Berthois *et al.*, 1986) (Table 2).

Primary rat osteoblast-like cells from calvariae of newborn Sprague-Dawley rats were cultured in a 35 mm-culture dish (1.3 × 10^4 cells/cm^2) with viscous phenol red-free alpha-Eagle's modified minimal essential medium with Ham's F-12 nutrient mix (α-MEM/F-12) supplemented with delipidated bovine serum albumin (0.25%) and methylcellulose (0.8%). Concentration of phenol red was varied (1.5, 15, 50, 150, and 300 μM). Cell proliferation was determined by counting the number of cells per colony 3 times per week for 20 days. Osteoblast-like cells with α-MEM/F-12 contained phenol red showed significantly increased numbers of cells per colony, from 132 to 175% of the control (phenol red-free α-MEM/F-12 as 100%) (Ernst *et al.*, 1989) (Table 2).

Ovarian surface epithelial cells from women aged 39 - 52 years old were cultured in a 24 well-plate (and cell density was dependent on cell-size) with phenol-free DMEM/F-12 or DMEM contained phenol red (45 μM) supplemented with 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (25 mM), glucose (4,500 mg/L), and FCS (20%) for 5 days. The size and phenotype of the cells were monitored under a phase-contrast microscope to indicate cell differentiation. Ovarian surface epithelial cells cultured in DMEM with phenol red for 5 days demonstrated cell differentiation to large oocyte phenotype cells. By contrast the cells in phenol red-free DMEM/F-12 appeared as small sized granulosa phenotype cells. Therefore, phenol red contained in culture medium was involved with cell differentiation (Bukovsky *et al.*, 2005) (Table 2).

On the other hand, human promyelocytic leukemia HL-60 cells were cultured in phenol red-free RPMI1640 supplemented with FCS (10%), glucose (5 mM), glucose oxidase (25 mU/ml), and antibiotics (1%) in a 96 well-plate (5×10^4 cells/well) and then treated with phenol red (15, 50, 100, and 200 µM) for 3 hours. CellTiter-Glo® luminescent assay was performed at 1 and 3 hours. The results showed that incubation of HL-60 cells with phenol red did not change cell viability (Morgan *et al.*, 2019) (Table 2).

Phenol red has been reported to enhance cell proliferation in MCF-7, HL-60, and primary rat osteoblast-like cells and promote differentiation in primary human ovarian surface epithelial cells. Hence, the concentration of phenol red in standard commercial media (3 – 45 µM) is suitable for activating cell growth to achieve cell confluence with a desirable phenotype before starting treatment.

4. 3 Effects of phenol red on pharmacotherapeutic activity of anti-cancer drugs

Phenol red can interfere with the pharmacotherapeutic effects of anti-cancer drugs (Santos *et al.*, 2018). MCF-7 cells were cultured in phenol red (45 µM) and phenol red-free DMEM supplemented with FCS (10%), and then treated with a cyclin-dependent kinase inhibitor, roscovitine (20 µM), for 24 hours. The number of G_2 stage-arrested cells was 30% in the phenol red-contained medium while the number of G_2 stage arrested cells in the phenol red-free medium was 40% (Santos *et al.*, 2018; Wesierska-Gadek *et al.*, 2007) (Table 2).

Human breast cancer ZR-75-1 and T-47-D cells were cultured at low (2 to 8 × 10^4 cells/dish) and high density (2 to 5 × 10^5 cells/dish) with RPMI1640 contained phenol red (42.32 µM) or phenol red-free RPMI1640 supplemented with FCS (1%) for 7 days. Both ZR-75-1 and T-47-D cells were treated with tamoxifen (1 µM) or *trans*-hydroxytamoxifen (10 nM) for 12 and 14 days, respectively. For the high density ZR-75-1 cells, tamoxifen and *trans*-hydroxytamoxifen inhibited cell growth only in the phenol red contained medium. Conversely, for the low density of ZR-75-1 cells in the phenol red-free medium, tamoxifen weakly stimulated cell growth after 7 days of incubation, which turned to an inhibitory effect after 12 days of incubation, while *trans*-hydroxytamoxifen inhibited cell growth in either phenol red-contained or free medium. For T-47-D cells, tamoxifen and *trans*-hydroxytamoxifen showed cell inhibitory effects after 14 days of incubation in cell density- and phenol red concentration-independent patterns. Other than phenol red, cell type and density, including culturing time, were factors influencing pharmacotherapeutic activity of the anti-cancer drugs, tamoxifen and *trans*-hydroxytamoxifen (Glover *et al.*, 1988) (Table 2).

Phenol red was shown to decrease the efficacy of roscovitine in MCF-7 cells and interfere with the pharmacotherapeutic activity of tamoxifen and its derivatives in ZR-75-1 and T-47-D cells. Therefore, the presence of phenol red in the media, together with the selection of a suitable cell type, are key concerns for studies of pharmacotherapeutic activity, and for studies of anti-cancer drugs in estrogen-dependent MCF-7 cells.

4.4 Effect of phenol red on enhancing anabolic activity in cell culture

Expression of alkaline phosphatase, collagen, and calcium in osteoblastic cells are the parameters indicating stages of osteoblast differentiation (Still *et al.*, 2003). The colony-forming unit-fibroblastic assay is used to determine osteoblastic induction by positive colony-staining of alkaline phosphatase, collagen, and calcium. Bone marrow stromal cells were cultured in a 55 cm^2-petri dishes (1×10^6 cells/dish) with phenol red-free DMEM supplemented with FCS (12%), dexamethasone (10 nM), and ascorbic acid (283.89 µM) for 5 days. The cells were treated with prostaglandin E2 (PGE2; 0.1 µM), prostaglandin A1 (PGA1; 1 µM), and basic fibroblast growth factor (bFGF; 0.02 nM) in phenol red (42.32 µM) and phenol red-free DMEM for 15 days. In the presence of phenol red, PGE2, PGA1, and bFGF increased alkaline phosphatase positive colony-staining to 208%, 233%, and 245%, respectively, compared with control. For collagen positive staining, PGE2, PGA1, and bFGF elevated the number of colonies to 179%, 206%, and 206% of the control level, respectively. Correspondingly, PGE2, PGA1, and bFGF increased the number of calcium positive colonies to 257%, 301%, and 261% of the control, respectively. In contrast, in the phenol red-free DMEM, PGE2, PGA1, and bFGF did not alter alkaline phosphatase and the number of collagen and

calcium positive colonies compared to control. Therefore, phenol red was required in culture medium to activate anabolic compounds, such as PGE2, PGA1, and bFGF, in osteoblastic differentiation (Still *et al.*, 2003) (Table 2).

Phenol red can influence the stage of osteoblastic differentiation in primary osteoblastic cells due to its ability to activate anabolic compounds. Hence, phenol red-free medium is recommended for studies of the effects of anabolic drugs on bone forming processes.

5. Conclusion

Cell culture is a useful tool for screening cell/tissue characteristics before stepping forward to *in vivo* and clinical studies. Nonetheless, an optimal culture model that mimics physiological conditions is required. Phenol red is the pH indicator in culture media indicating an appropriate time for medium change. Phenol red is recommended at low concentration, ranging from 3 to 45 µM, to avoid cell toxicity and interference effects. In addition, in studies on metabolic enzymes such as CYPs, phenol red-free medium is preferred during treatment periods to avoid interference with probe and enzyme activities. Phenol red exhibited estrogenic and anabolic enhancing effects due to its non-steroidal estrogenic like structure. Phenol red partially binds with the estrogen receptor to increase expression of proteins related to estrogenic hormones including the progesterone receptor and CFTR, and to stimulate cell proliferation and differentiation, especially in estrogenic hormone-related cells, e.g. MCF-7 cells and primary ovarian stem cells. Phenol red also reduced the pharmacotherapeutic activity of the anti-cancer drug roscovitine by antagonizing the estrogen receptor, and increased the anabolic activity of PGE2, PGA1, and bFGF (Figure 3). Nevertheless, the impact of phenol red on cell culture models remains variable and controversial at present. Therefore, it is worth studying how phenol red effects other aspects, such as gene expression profiles, in several cell-types and culture mediums to reveal a suitable cell culture model and provide reliable outcomes. In addition, a review of the impact of phenol in studies using compounds possessing estrogenic like structures and/or in estrogen-dependent cells is required.

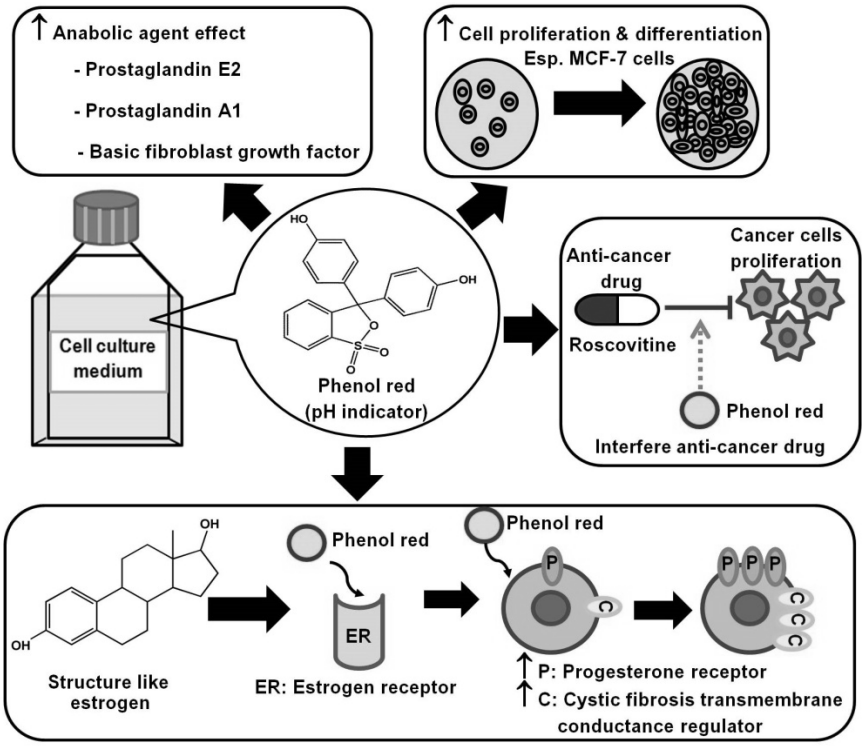

Figure 3 Summary on effects of phenol red in cell cultures.

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