POSTER PRESENTATION

Health Science

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EFFECT OF RESVERATROL ON COBALT CHLORIDE INDUCED STEM CELL MARKER EXPRESSION IN HEAD AND NECK CANCER CELL CARCINOMA

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ABSTRACT

Resveratrol is known to possess various anti-cancer activities, including anti-cancer stem cell. As hypoxia has been shown to favor the growth of cancer stem cell, this research aims to investigate whether resveratrol can inhibit the expression of cancer stem cell under hypoxia. $CoCl_2$ was used to mimic hypoxic condition. Using RT-PCR, we found that $CoCl_2$ dose-dependently induced the expression of cancer stem cell marker including *Oct-4*, *Nanog*, *CD-44*, *CD-105* and *CD-133*. Performing Real-time PCR, we showed that the induction of these cancer stem cells markers were inhibited by resveratrol. These results indicate that resveratrol might be able to target cancer stem cells in hypoxia-associated tumor.

Keywords: Resveratrol, Head and neck cancer cell carcinoma, Cancer stem cells, Cobalt chloride

Introduction

Cancer therapy remains a huge challenge in cancer research. One of the major causes of ineffective treatment is cancer recurrence. It has been shown that cancer stem cells (CSCs) play an important role in the recurrence of cancer (Li et al., 2013). Though cancer stem cells are constituted only in a very small percentage of tumor cells, they are capable of self-renewal and tumorigenesis (Clarke et al., 2006). The isolation of cancer stem cells from extrahepatic cholangio carcinoma xenografts revealed that *CD-24*$^{+}$ *CD-44*$^{+}$ *EpCAM*^{high} cells exhibited self-renewal and high tumorigenic potential (Wang et al., 2011). In addition, properties of CSCs such as DNA repair ability, overexpression of anti-apoptotic proteins and drug efflux transporters are associated with drug resistance and anti-apoptotic resulting in cancer cell survival (Baumann et al., 2008). Thus, targeting cancer stem cells is an important approach for the development of new compounds for anti-cancer therapy.

Resveratrol is polyphenol compounds found in many plants. Various pharmalogical properties of resveratrol have been studied including cancer prevention, anti-virus, and anti-oxidants (Aggarwal et al., 2004; Sun et al., 2010). Moreover, it has been reported that resveratrol inhibited the self-renewal and increased apoptosis in pancreatic CSCs (Pandey et al., 2011; Shankar et al., 2011). In addition, resveratrol impeded CSC properties, epithelial-mesenchymal transition (EMT) and metabolic reprogramming of nasopharyngeal CSCs (Shen et al., 2013). *In*

vitro and *in vivo* study in breast cancer showed that resveratrol inhibited the proliferation of breast cancer stem cells as well as the growth of xenograft tumors in NOD/SCID mice (Fu et al., 2014).

Hypoxia, a condition of low oxygen tensions, has been shown to play an important role in the growth of cancer stem cells (Heddleston et al., 2009). Hypoxia enhances stemness of cancer cells by up-regulating cancer stem cell markers such as *CD-44, CD-105, Oct-4, Rex-1* and *Nanog* (Ketkaew, 2014). The inhibition of hypoxia inducible factors (HIFs), a key transcription factors in hypoxic cells, resulted in inhibition of cells proliferation, self-renewal and cells survival (Heddleston et al., 2009).

Our previous study in head and neck squamous cell carcinoma showed that resveratrol inhibited the proliferation of cancer cells and reduced the expression of *vascular endothelial growth factor (VEGF)*, a key regulator in angiogenesis and cancer cell growth (Sintuyanon, 2013). In this study, we test whether resveratrol could target head and neck cancer stem cell by determining the expression of cancer stem cell marker under hypoxia. Cobalt chloride ($CoCl_2$), a hypoxia-mimicking agent, is used to simulate the hypoxia conditions.

Objective

The aim of this study was to explore the effect of resveratrol on the expression of cancer stem cell induced by $CoCl_2$.

Materials and methods

Cell culture: The head and neck cancer cell lines HN-30 was esophagus squamous cell carcinomas. HN-30 cells were grown in 10%DMEM containing 10% fetal bovine serum (FBS), 1%L-glu and 1%Ab/Am. Cells were maintained in 95% humidity at 37°C and 5%CO_2. Resveratrol (Sigma, Italy) was dissolved in DMSO to the desired concentration, and was added to the medium to make a final concentrations of 5, 10, 20 µM.

MTT assay: HN-30 cells (3.0×10^5 cells) were seeded in each well of 12-well plates and treated with various concentration of $CoCl_2$ (Sigma, Switzerland) for 24 hours. After that, 10%DMEM was replaced by MTT solution (USB, USA). During the assay, MTT was reduced to violet colored formazan dye by dehydrogenases enzymes in active cells, which was detected at 570 nm using Microplate Reader (Biorad, USA).

Reverse transcription polymerase reaction (RT-PCR): HN-30 cells (5.0×10^5 cells) were seeded in each well of 6-well plates and treated with either normoxia or $CoCl_2$ (50 and 100 µM) for 6 and 24h. RNA was extracted from cells using trizol (Invitrogen). RNA was transcribed to cDNA using the ImProm-IITM Reverse Transcription system (Promega). PCR was performed using Hot start taq DNA Polymerase (Sigma). Products were separated on 1.5% agarose gels and measured by gel imaging machine (VALBER LOURMAT, Germany). According to previous studies, *Oct-4, Nanog, CD-44, CD-105, CD-133* expression was found to be correlated to head and neck cancer stem cells.(Chiou et al., 2008; Damek-Poprawa et al., 2011; Joshua et al., 2012; Ketkaew, 2014; Sintuyanon, 2013). In this study, we used these CSC markers for test.

Quantitative real-time RT-PCR: HN-30 cells (5.0×10^5 cells) were seeded in each well of 6-well plates. Cells were pretreated with 5, 10, and 20 µM resveratrol and incubated with 100µM $CoCl_2$ for 6 h. SYBR Green I qRT-PCR was performed.

Statistical Analysis: Each experimental group were calculated and expressed by mean±SD. Significant differences between groups were analyzed by one way ANOVA, followed by Bonferoni's post hoc tests. A p-value <0.05 was considered statistically significant.

Results

Cobalt chloride reduces cell viability in a dose-dependent manner: The concentration of $CoCl_2$ that is not toxic to HN-30 cells was first determined. Cells were cultured with various concentrations of $CoCl_2$ (0, 10, 25, 50, 100, 200 and 400 µM) for 24 hours. The viability of HN-30 cells was studied using MTT assay. As shown in Figure1, cell viability was not significantly changed when cells were treated with 0-100 µM $CoCl_2$. However, 200-400 µM $CoCl_2$ markedly decreased cell viability. As $CoCl_2$ at the concentrations of 50 and 100 µM was not toxic to the cells, we use these concentrations for the next experiments.

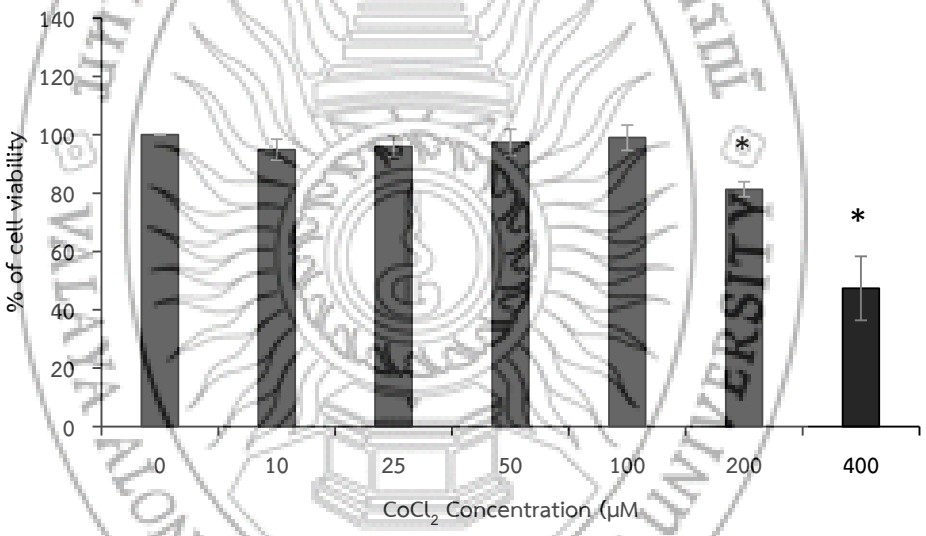

Figure 1 Effect of the $CoCl_2$ on cell viability was determined by MTT assay. The HN-30 cells were treated with 0, 10, 25, 50, 100, 200 or 400 µM $CoCl_2$ and incubated for 24 hours. *$p < 0.05$ compared with $CoCl_2$ at 0 concentration.

Cobalt chloride dose-dependently induced cancer stem cell markers expression: Next, we study whether $CoCl_2$ can induce stem cell marker expression. Cells were treated with 50 and 100 µM $CoCl_2$ and incubated for 6 and 24 h. *vascular endothelial growth factor (VEGF),* a HIF-α-targeted gene, was studied as positive control. The mRNA expression was examined by semi-quantitative RT-PCR. As shown in figure 2, the mRNA expression of *Oct-4, Nanog, CD-44, CD-105, CD-133* and *VEGF* was clearly increased after 6 h treatment with 100 µM $CoCl_2$. With the exception of *Nanog*, the induction of cancer stem cell marker at 24 h was less prominent than those of 6 h. Bio-1D software used to compare the intensity of amplification bands.

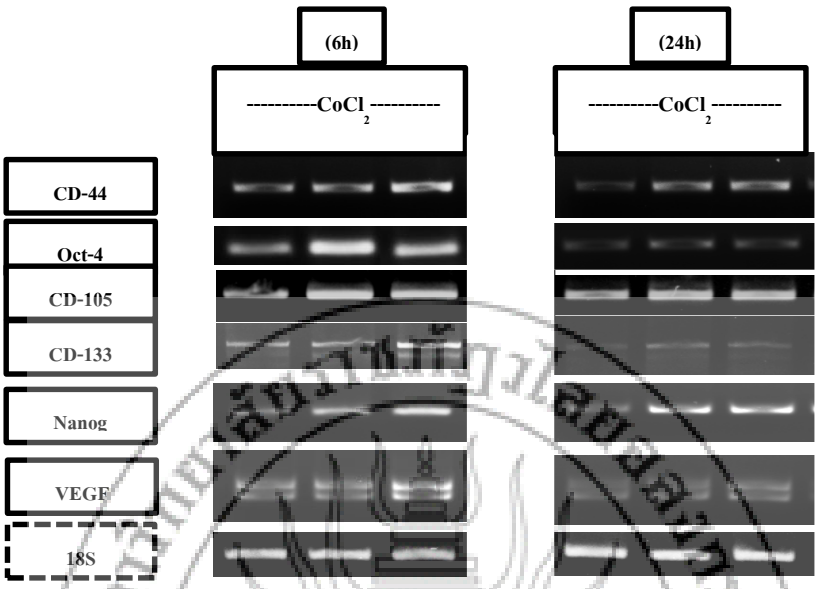

Figure 2 Effect of $CoCl_2$ on cancer stem cell markers expression. HN-30 were incubated with 0, 50, 100 µM $CoCl_2$ for 6 and 24 h. The mRNA expression was analyzed by RT-PCR. *18s* gene as housekeeping gene.

Resveratrol inhibits the expression of Oct-4, Nanog, CD-44, CD-105, CD-133 and VEGF in HN-30 cells: Next, the inhibitory effect of resveratrol on cancer stem cell marker and *VEGF* expression was studied by Real-time PCR. Cells were pretreated with various concentrations of resveratrol for 30 min. The hypoxic condition was mimicked by treatment with 100 $CoCl_2$ for 6 h. As shown in figure 3, resveratrol significantly inhibit mRNA expression of *Oct-4, Nanog, CD-44, CD-105, CD-133* and *VEGF*. Interestingly, low dose of resveratrol (5 µM) was found to be more potent than higher concentrations (10 and 20 µM).

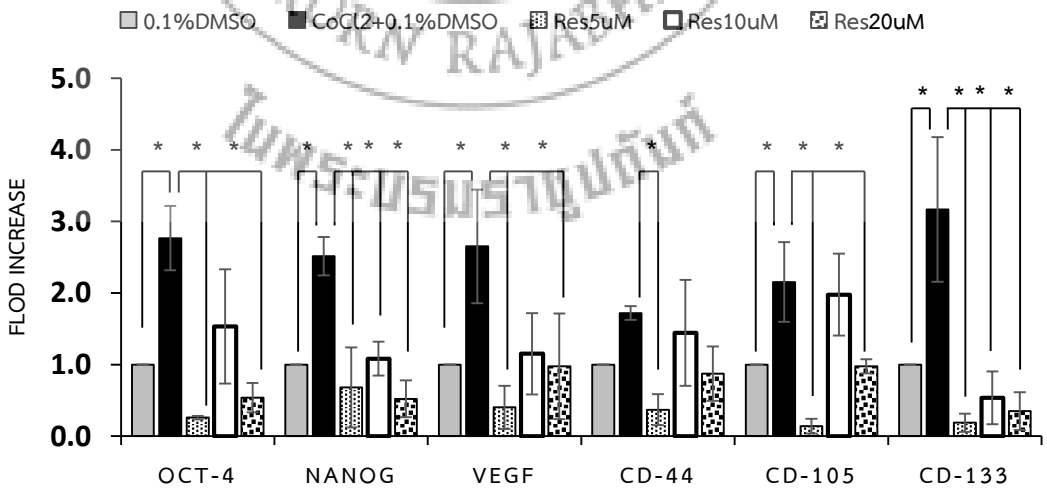

Figure 3 Effect of resveratrol on cancer stem cell markers expression under hypoxic-like condition. HN-30 were pre-treated with 5, 10, 20

resveratrol and incubated with 100 µM $CoCl_2$ and for 6 h. The mRNA expression was analyzed by Real-time PCR. : Res; resresvertrol. *p < 0.05 compared with 0.1% DMSO (control).

Discussion and Conclusion

Resveratrol a group of polyphenol compounds play many roles in cancer stem cells. For example, resveratrol inhibited self-renewal, and increased apoptosis in pancreatic (Shankar et al., 2011) and breast cancer stem cells (Pandey et al., 2011). It has been shown that hypoxia increased self-renewal, proliferation and tumorigenesis capacity of cancer stem cells by up-regulation of cancer stem cell factors, such as *Oct-4* and *Nanog* (Heddleston et al., 2009). In addition, Li et al. found that self-renewal capacity, decreased apoptosis and increased tumorigenesis in cancer stem cells was induced by HIF-α (Li et al., 2009). In this study, we treated cells with $CoCl_2$ which is known to create hypoxic-like condition by stabilizing HIF-α. $CoCl_2$ binds to Fe^{2+}-binding site of prolyl hydroxylase domain (PHD) enzyme and thereby inhibiting HIF-α degradation (Yuan et al., 2003). In similar to our previous study in conventional hypoxia (Ketkaew, 2014), we found that $CoCl_2$ markedly increased cancer stem cell marker expression. These results imply that the induction of cancer stem cell markers occur through HIF-dependent pathway. Most impostantly, we found that resveratrol could abolish the effect of $CoCl_2$ on the expression of *Oct-4*, *Nanog*, *CD-44*, *CD-105*, and *CD-133*. These markers are important for cancer stem cell survival. For example, *Oct-4* plays a key role in controlling the self-renewal and pluripotent (Bourguignon et al., 2012). *Nanog* plays a role in embryonic stem cells self-renewal and pluripotent (Bourguignon et al., 2012). Taken together, our findings suggest that resveratrol might be able to inhibit the proliferation and self-renewal of cancer stem cells. Future study using flow cytometry is required to confirm that resveratrol can reduce the number of cancer stem cells.

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SHEAR BOND STRENGTH OF RESIN COMPOSITE TO BLOOD AND HEMOSTATIC AGENT CONTAMINATED REINFORCED GLASS IONOMER CEMENT

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ABSTRACT

The aim of this study was to evaluate the effect of blood and hemostatic agent contaminated on bonding of reinforced glass ionomer (RMGIC) (GC Fuji II LC®) to resin composite (Filtrek™ Z350XT) with total-etch and self-etch adhesive systems. Sixty samples of cylinder were used as mold, 2 mm high and 4 mm diameter hole was made in the middle for RMGIC application. Sample surfaces were grounded by 600 grit SiC paper for 1 minute. Samples were divided into 2 groups based on adhesive system types as total-etch (TE) and self-etch (SE). In each group, subgroups were divided as control (CTE, CSE), hemostatic agent (ATE, ASE) (ViscoStat® Clear) and blood contamination (BTE, BSE). Contamination was done on RMGIC surface for 1 minute and rinsing process was followed before bonding procedure. There was no contamination used in control group. Total-etch (Adper™ Single Bond 2) and self-etch (Clearfil™ SE Bond) were used following the instruction prior to resin composite restoration. After resin composite was polymerized, samples were stored in 95% humidity for 24 hours. Universal testing machine and stereomicroscope were used to record shear strength and mode of failure data, respectively. Independent T-Test, Two-way Anova and Turkey's HDS test were conducted for statistical analysis. The results showed that both type of contamination and adhesive system had significantly effect on shear bond strength. Hemostatic agent contamination showed significantly lower shear bond strength than blood contamination ($p=0.04$) and control group ($p=0.01$), with no significant different between blood contamination and control group ($p>0.05$). Self-etch system provided significantly higher shear bond strength than total-etch system in

hemostatic agent contamination (p=0.00) and control group (p=0.02), but not for blood contamination (p=0.09). Failure mode observation on ATE revealed adhesive failure, whereas, others groups showed mostly mix failure mode. In conclusion, hemostatic agent contamination reduced shear strength in total-etch adhesive while both hemostatic agent and blood contamination had no effect on self-etch adhesive.

Keywords: Shear strength, blood contamination, hemostatic agent contamination, sandwich technique, RMGIC.

Introduciton

The use of sandwich technique for restorative dentistry has been done tremendously in class II, V and deep restoration to gingival tissue. Sandwich restoration is termed when glass ionomer (GIC) is used as an intermediate layer between tooth structure and a resin based composite. Some advantages can be related to the intermediate layer such as reducing polymerization shrinkage of resin composite, providing chemical bond to tooth structure, reducing post-operative sensitivity and anti-cariogenic action of fluoride. It was reported earlier that fracture was commonly seen between composite and GIC due to the poor bonding(Smith & Soderholm, 1988). However, Resin Modified Glass Ionomer Cement (RMGIC) has been reported to have better bonding strength in previous studies according to the chemically bond of resinous component and surface mechanically bond of RMGIC (Chadwick & Woolford, 1993; Pamir, SEN, & EVCIN, 2012).

In clinical situation, bleeding is frequently occurred due to traumatic procedure. Blood contains substances such as nutrients, oxygen and cells in composition. Blood protein has potential to improve the cascade of clotting reaction. This clotting process interferes the penetration of adhesive system and reduce boding strength to dentine (Dietrich, Kraemer, Losche, Wernecke, & Roulet, 2000; Eiriksson, Pereira, Swift, Heymann, & Sigurdsson, 2004). Moreover, good adhesion of adhesive system can not be achieved due to present of blood contamination (Yoo & Pereira, 2006). Hemostatic agent is used commonly for tissue management in dentistry. Aluminum Chloride used as hemostatic agent contains highly acidic environment which pH of these solutions are

mostly ranged from 0.7-3.0 (Tarighi & Khoroushi, 2014; Woody, Miller, & Staffanou, 1993). It was reported earlier of the effect of homeostatic agent on adhesive system. Contamination of these solution exhibits completely removal of smear layer on tooth structure, reduce polymerization and bonding mechanism of self-etch adhesive to dentine (Land, Couri, & Johnston, 1996). In contrast, it was reported to improve bonding ability of total-etch system to dentine (Kuphasuk, Harnirattisai, Senawongse, & Tagami, 2007). Up to date, there is no report regarding to the bonding strength of RMGIC to resin composite with present of contaminations in sandwich technique.

Research objective

The aim of this study was to evaluate the effect of blood and hemostatic agent contamination to RMGIC-resin composite bonding by total-etch and self-etch adhesive systems in term of shear strength.

Materials and methods

Sample preparation

Sixty cylinders filled with acrylic resin were made for the study. On the flat surface of acrylic resin, 2 mm depth and 4 diameter hole was made on the center by straight handpiece tapper carbide bur. RMGIC (GC Fuji II LC®) was mixed for 10 seconds on amalgamator and placed into prepared hole. Then, the excess of RMGIC was removed by using flat plastic instrument prior to complete polymerization by 20 seconds light curing (Kerr Demi™ Plus LED 400-470 nm wavelength, intensity 1100 mw/cm^2 to 1330 mW/cm^2). In order to standardize the surface of RMGIC, samples were handed ground by 600 grits SiC paper for 60 seconds under running water. Samples were randomly divided into two groups for adhesive types, total-etch (TE) and self-etch (SE). Then, each group was randomly further divided into three subgroups (n=10) for contamination type.

Contamination preparation

Blood was taken from blood bank of Srinagrarind Hospital, Khon Kaen Univerisity with approval of ethical committee, Khon Kaen University (Ref: HE591326) and stored in vacutainer containing heparin. The surface contamination was done by coating 1 ml of blood on the surface of RMGIC. Contamination was left for 60 seconds without disturbance. Then blood was rinsed and air dry for 60 seconds. Hemostatic agent (ViscoStat® Clear, 25% Aluminum Chloride) was dispended and directly applied on the RMGIC surface by microbrush tip. The contamination was done for 60 seconds and rinsing - drying for 60 seconds.

Group TE

Control group (CTE): no contamination was used in this group. Total-etch system (Adper™ Single Bond 2) was followed the instruction. Application of etching was done for 15 seconds, cleaned and dried for 10 seconds, application of bonding for 15 seconds and light cure for 10 seconds.

Group ATE: Hemostatic agent contamination was used in this group. Application of contamination was done as previously described. Bonding procedure was done as same as control group.

Group BTE: Blood contamination was done on surface of RMGIC as previously described. The bonding procedure was performed similarly as the other groups.

Group SE

Control group (CSE): No contamination was used in this group. Self-etch adhesive system was applied on the surface of RMGIC by primer solution for 20 seconds, air for 20 seconds, bonding solution application, air blow to have uniform appearance, and light cure for 10 seconds.

Group ASE: Hemostatic agent was used to contaminate on RMGIC surface following the procedure as described above. Bonding procedure was done as same as control group.

Group BSE: Blood contamination was used according to previously procedure. Self-etch adhesive system was applied based on the same procedure.

All samples were ready for resin composite restoration (CR). To restore the CR, special design metal mold was used to have 2 mm high and 2 diameter of CR. Excessive

of resin composite was removed by flat plastic and light cure was done for 20 seconds to complete the polymerization. Then samples were stored for 24 hours in 95% humidity. Shear strength was recorded by using universal testing machine (LLOYD instruments LR 30K). 100 N force was applied for 0.5 mm/ 1 minutes to the sample. Stereomicroscope (Nikon MEASURESCOPE 20) was used for failure mode under 20X magnifications. Two-way Anova and Turkey's HDS tests were used for statistical analysis. Moreover, Independent T-Test was used to compare between adhesive systems in each contamination group. List of materials are showed in table 1 and summary process of the study are showed on figue1.

Table 1: Materials used in the study

Material	Composition
GC Fuji II LC®	**Powder**: Alumino silicate glass **Liquid**: Distilled water (20-30%), Polyacrylic acid (20-30%), 2-hydroxyethylmethacrylate (30-35%), Urethanedimethacrylate (<10%), Camphorquinone (<1%)
Filtek™ Z350XT	**Resin matrix**: Bis- GMA, UDMA, TEGDMA and Bis-EMA **Filler***:* Nano-agglomerated/non-aggregated 20 nm silica filler, 4-11nm zirconia particles. **Filler load**: 72.5% by weight (55.6% by volume)
Adper™ Single Bond 2	• 35% phosphoric acid • Primer: HEMA, polyalkenoic acid polymer, water Adhesive: Bis-GMA, HEMA, tertiary amines (both for light-cure and self-cure initiators), photo- initiator.
Clearfil™ SE Bond	• Primer: MDP, HEMA, hydrophilic dimethacrylate, photo-initiator, water • Bond: MDP, HEMA, Bis-GMA, hydrophobic dimethacrylate, photo-initiators, silanated colloidal silica.
ViscoStat® Clear	• 25% Aluminum Chloride

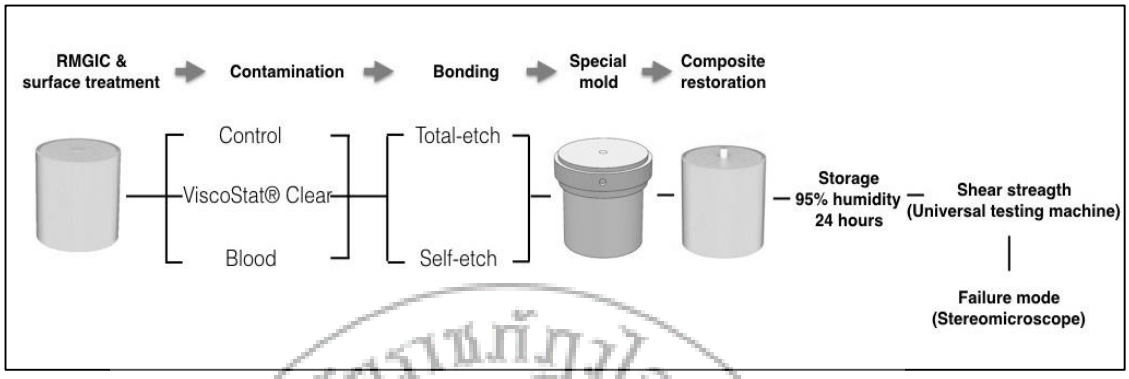

Figure 1: Summary process of the experiment.

Results

Shear bond strength of both adhesive systems to contaminated RMGIC were shown in table 2. Test of normality was done to ensure the normality distribution of data. According to Two-way anova, both contamination type ($p=0.009$) and adhesive system ($p<0.001$) showed highly significant effect on shear bond strength. Control group provided highest mean value of shear bond strength (17.84 ± 2.79) followed by blood (17.41 ± 2.93) and hemostatic agent (15.11 ± 3.98). Within three groups, hemostatic agent contamination showed significantly lower shear bond strength than blood contamination ($p=0.04$) and control group ($p=0.01$), with no significant between blood contamination and control group ($p>0.05$). Regarding to adhesive system, self-etch system showed higher significant than total-etch system ($p<0.001$). The Independent T-Test showed self-etch (ASE, CSE) provided significantly higher shear bond strength than total-etch (ATE, CTE) in both hemostatic agent ($p=0.006$) and control group ($p=0.026$), but not for blood contamination group (BTE, BSE) ($p=0.093$). The observation failure mode revealed mix failure mode among those groups except adhesive failure was prominently in ATE group. Detail of failure mode was shown in table 3 and figure 2.

Table 2: Mean value of shear strength (mean ±SD) in MPa

Group	Control (C)	Hemostatic agent (A)	Blood (B)	Total
Total-etch (TE)	16.49 ± 2.53[a]	12.82 ± 3.43[b]	16.30 ± 2.20	15.20 ± 3.18[c]
Self-etch (SE)	19.20 ± 2.44[a]	17.39 ± 3.19[b]	18.52 ± 3.24	18.37 ± 2.97[c]
Total	17.84 ± 279[d]	15.11 ± 3.98 [d, e]	17.41 ± 2.93[e]	

The same superscripts applied to the columns present significantly different.

Table 3: The observation of failure mode

Group	Cohesive	Adhesive	Mix
CTE	3	0	7
ATE	0	6	4
BTE	3	3	4
CSE	2	2	6
ASE	0	3	7
BSE	3	1	6
Total	11	15	34

Types of failure mode

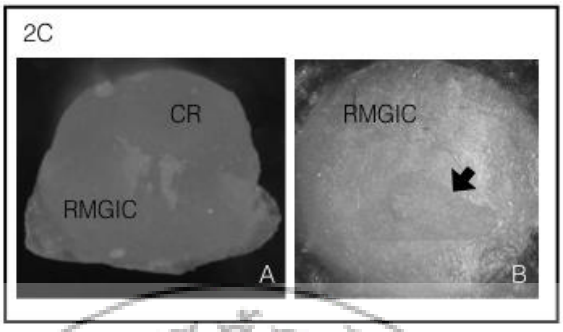

Figure 2: 2A: cohesive failure (A) RMGIC fracture part on CR, (B) RMGIC fracture surface, 2B: Adhesive failure (A) CR surface was flat, (B) RMGIC surface was flat, 2C: Mix failure (A) Partial of RMGIC fracture part on CR, (B) RMGIC fracture part on surface.

Discussion

Based on this result, hemostatic agent containing 27% aluminum chloride showed significantly lower shear strength than control and blood contamination group. It had been reported of the displacement of calcium by aluminum in hydroxyapatite and resulted in an insoluble compound on dentine (Martin, 1986). As RMGIC contains calcium particles, the displacement of aluminum and calcium may be occurred. Etching and cleaning procedure might not completely remove the contamination. With the agreement of previous studies (Navimipour et al., 2012; Zanata, Navarro, Ishikiriama, Souza Junior, & Delazari, 1997), this phenomenon was considered to affect the wettability of adhesive system and reduce chemically bonding ability of materials.

This study found that blood contamination had no effect on both adhesive systems. Blood protein and macromolecules were reported earlier as it still remained on dentine surface after decontaminated by distill water (Oztoprak, Isik, Sayinsu, Arun, & Aydemir, 2007). In this current study, the process of blood contamination was done by using hepatized blood which coagulation was less likely to occur. This condition might enhance effectiveness of decontamination. Most of the blood concentration might be removed during surface decontamination by both one-minute water and air dry. This was confirmed with the study of Dietrich et al. 2002, on effectiveness of fresh blood contamination over anticoagulation blood on microleakage on dentine (Dietrich, Kraemer, & Roulet, 2002). However, there might be different in effectiveness if using

totally fresh blood which coagulation is vulnerable to happen faster than the contamination.

Application of total-etch showed less significant shear bond strength than self-etch in hemostatic contamination and control group. Using total-etch in bonding RMGIC to resin composite was depending on both chemically bond of methacrylate group and microretention of both materials (Kerby & Knobloch, 1992; Zanata et al., 1997). Moreover, the highly acidic solution of hemostatic agent seemed to enhance etching effect of total-etch solution (Kuphasuk et al., 2007). According to the result of this study, hemostatic agent might have adverse effect and reduced bonding strength by damaging oxygen inhibition layer of RMGIC. Copolymerization between resin composite and RMGIC may not completely exist and reduce bonding strength. On the other hands, Clearfil™ SE Bond is self-etch system containing weak acidity approximately pH 2. It was reported earlier of low acidity self-etch bond better than strong and medium acidity self-etch (Kandaswamy, Rajan, Venkateshbabu, & Porkodi, 2012). However, present of the aluminum chloride on surface was reported to lower the etching effect of primer (Tuncer et al., 2014). Even though primer function was reduced, shear strength in this current group was retained appropriate. Primer was dilute well on RMGIC surface (Hinoura, Suzuki, & Onose, 1991). Microretension seemed not to be important effect in this group (Jaberi Ansari, Panahandeh, Tabatabaei Shafiei, & Akbarzadeh Baghban, 2014). Remarkable component presenting in current self-etch system is 10-methacryloyloxi-decyl-dihydrogen-phosphate (MDP). The current study indicated that the MDP function might remain as stable as in surface without contamination. This means that MDP has ability to provide strong ionized bond to the calcium and resin monomer of the RMGIC even though surface is contaminated. Moreover, one minute cleaning and drying process were done in the study. Indeed, it was reported that MDP provide immediate superior bond strength and reduction in long-term bond durability (Matsui et al., 2015). However, this study was not involved the thermocycling process and long term bonding ability evaluation.

Mode of failure in the present study showed different according to the group. CTE, BTE groups presented in mix failure within agreement of previous study (Deepa, Dhamaraju, Bollu, & Balaji, 2016). In contrast, cohesive failure was predominantly

shown in other studies for RMGIC-composite bond without contamination (Chadwick & Woolford, 1993). There are two explanations related to such particular mode of failure. First, it might be due to different of composition of RMGIC using in the studies. Different materials lead to a deviation of physiochemical properties, nature of bonding between adhesive and materials (Deepa et al., 2016). Second, RMGIC was weak in early stage after light curing (Hashem, Foxton, Manoharan, Watson, & Banerjee, 2014).

According to the condition of the study, hemostatic agent contamination reduced shear bond strength in bonding of RMGIC to resin composite. Application of total-etch system in hemostatic agent contamination was not recommended and self-etch system could be used to restore shear bond strength in contaminated RMGIC of sandwich technique.

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