ORIGINAL ARTICLE

Effects of eluted components from 4-META/MMA-TBB adhesive resin sealer on osteoblastic cell proliferation

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Running title: Effects of resin sealer elution on osteoblasts

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Background/purpose: Adhesive resin sealer systems have been applied to seal the root canal system more effectively through the formation of a resin impregnation layer. The purpose of this study was to investigate effects of the eluted components of adhesive Super-Bond sealer (SBS) on the proliferation of osteoblastic cells *in vitro*.

Materials and methods: The standard powder-liquid ratio according to the manufacturer's instructions was used to produce a cylindrical block of SBS (5 mm in diameter, 10 mm in length) used in this elution study. The resin block was placed on a 100-mm culture dish. Osteoblastic cells were seeded at a density of 4×10^6 in α-MEM containing 10% fetal bovine serum, and were cultured in a humidified incubator. After 3 days of culture with or without SBS, the cells were retrieved and lysed according to the manufacturer's instructions. The cellular events induced by the eluted components from SBS were analyzed using an antibody assay for MAPKs and the MTT assay. Furthermore, the concentration of boron, a component of the catalyst, tri-*n*-butyl borane, was analyzed using an inductively coupled plasma optical emission spectrometer

Results: The expression of MAPKs was increased after SBS application. The MTT assay indicated that TBB, one of components of SBS, accelerated the proliferation of osteoblastic cells. The values of boron were found to be 1.66 ± 0.37 ppm and 1.74 ± 0.30 ppm in the cells cultured with and without FBS, respectively.

Conclusion: This study demonstrates that the eluted components from SBS could increase the expression of some MAPKs related to osteoblastic cell

proliferation and differentiation *in vitro*. Both the elution experiment and the treatment of cell culture with SBS components indicated that the boron originated from TBB was a probable candidate for activating the proliferation of osteoblastic cells.

KEY WORDS: adhesive resin sealer; osteoblastic cell proliferation; expression of MAPKs; MTT assay; boron concentration

INTRODUCTION

The purpose of root canal treatment is to prevent and/or to treat pulp and periapical pathoses.[1] Irrigation and medication usually yield sufficient infection control of the root canal system. Secondary infection is generally prevented by the final sealing of the root canal with various types of filling materials. Therefore, root canal filling to seal the root canal system from the outside environment is the most important step for ensuring a good long-term prognosis.

Traditional root canal filling material is composed of a gutta-percha core. However, gutta-percha characteristically demonstrates a high coefficient of thermal expansion and shrinkage. It also does not adhere to and seal root dentine. This means that a sealer cement consisting of zinc oxide and eugenol, calcium hydroxide, or epoxy resin is required to provide resistance to bacterial invasion after root canal filling.[2-5]

Resin systems that adhere to dentine have also been applied for endodontics. These adhesion technologies were originally borrowed from restorative dentistry. A 4-methacryloxyethyl trimellitate anhydride/ methacrylate- tri-*n*-butyl borane (4-META/MMA-TBB) adhesive resin (SB) developed in Japan (SUNMEDICA Co., Japan) is widely used in dentistry as a bonding agent.[6] Adhesive resins have been used to seal dentine through the creation of resin impregnated layer[7] that protects the pulp from the actions of oral fluids and their contaminants. Recently, a SB sealer (SBS) containing radiopaque zirconium oxide has been developed as a root canal sealer.[8] This modified type of SB adheres to root canal dentine and is

expected to seal the root canal system more effectively through the formation of a resin impregnated layer[7].

There have been several studies concerning the cytotoxicity and tissue responses after SB application *in vitro* and *in vivo*.[9-13] Its effect on the expression of phosphoinositol-3-kinase has been recently detected using a cDNA microarray and RT-PCR.[14] However, little is known about how SBS affects the mitogen-activated protein kinases (MAPKs) related to cell proliferation and differentiation, and which components of SBS directly influence osteoblastic cell growth. In the present study, the effects of SBS were examined using an antibody assay for the phosphorylation of MAPKs, and an MTT assay was used to analyze cell proliferation in osteoblastic cells cultured with each component of SBS. The first analysis of boron released from SBS *in vitro* was also carried out to investigate the effects of boron on the cellular activity.

MATERIALS AND METHODS

Antibody assay for the phosphorylation of MAPKs

The standard powder-liquid ratio {sealer powder 0.13 g, monomer 4 drops (0.08 g), and catalyst 1 drop (0.006 g)} was used according to the manufacturer's instructions to produce the cylindrical block of SBS (5 mm in diameter, 10 mm in length) used in this study. The resin block was placed on a 100-mm culture dish. Osteoblastic (NOS-1[15]) cells derived from human osteosarcoma were seeded at a density of 4×10^6 in α-MEM containing 10% fetal bovine serum (FBS), and were cultured in a humidified incubator at

37°C in an atmosphere of 5% CO_2 and air. After 3 days of culture with or without SBS, the cells were retrieved and lysed according to the manufacturer's instructions.

The relative level of phosphorylation of various signal transduction kinases was analyzed using a human phospho-MAPK assay kit (R&D Systems, Inc., Minneapolis, MN) that examines 21 kinds of MAPKs.[16] A phospho-site specific biotinylated antibody was used to detect twenty kinds of phosphorylated kinases via streptavidin-HRP, and then the membranes were exposed to X-ray film and the detected signals were developed on the film. Thereafter, each signal was quantitatively measured using the densitometric mode of a ChemiImager™ (Alpha Innotec, Switzerland).

MTT assay

NOS-1 cells were seeded at a density of 1×10^4 cells in 96-well flat-bottomed plates at 100μL per well. The culture was stimulated with varying concentrations of 4-META (0.125~10 *mM*), MMA (0.125~10 *mM*), and TBB (0.125~10 *mM*) dissolved in culture medium supplemented with or without FBS. Unstimulated cells were used as controls. The cell proliferation in response to the different treatments was detected using an MTT Cell Growth Assay Kit (Millipore, Billercia, MA). After incubation for 3 days (at 37°C with 5% CO_2), 10 μL of MTT reagent (3- (4, 5- dimethylthyazol-2-yl) - 2, 5- diphenyltetrazolium bromide) was added to the wells, and the cells were incubated for an additional 4 h at 37°C. After incubation, the resulting formazan crystals were dissolved by adding an MTT solubilizing solution. Within 1 h, the absorbance at 570/630 nm was

recorded using an ELISA plate reader (Model550, BIO-RAD, Tokyo, Japan). The data from control samples were considered to be 100% and those obtained for cells treated with the various concentrations were converted to percentages compared to the control. The differences between the control and TBB stimulated cells were compared using Student's *t*-test. A *P* value of <0.05 was considered to be significant.

Analyses of the boron concentration

A method similar to the above-mentioned methods for the MAPKs assay was used for the analyses of the boron concentration in culture medium containing components released from the cylindrical block of the SBS. The boron concentration was analyzed using an inductively coupled plasma optical emission spectrometer (limit of detection, < 10 ppb) (ICP SPS7700; Seiko Instruments Inc., Chiba, Japan). Standard boron levels were obtained by means of a standard curve constructed using 0, 1, 5, 10, and 20 ppm boron. All measurements were performed in duplicate.

RESULTS

Figure 1 shows the density of the reaction products using a human phospho-MAPK array. Five kinases out of 21 MAPKs exhibited a difference in their expression ratio between the experimental and control groups of $\geq$ 1.2. The ratios of ERK1, ERK2, and Akt1/2/pan were 3.0, 1.2, 1.6, 1.9 and 1.2, respectively (duplicate samples).

MTT assays showed that the supplementation of 4-META or MMA into the culture medium with and without FBS did not affect the cell proliferation at

any of the concentrations examined (Figs. 2A and B). There was a more prominent inhibitory tendency in the cells cultured with FBS than in those cultured without FBS. This tendency was also confirmed in the TBB group (Fig. 2C). The highest (percentage) of proliferation was after stimulation with 0.125 *mM* of TBB ($p<0.01$). The values at concentrations below 0.125 *mM* were lower than that at 0.125 *mM* of TBB (data not shown).

The amount of boron could be calculated from the medium sample cultured with SBS. The values were found to be 1.66 ± 0.37 ppm and 1.74 ± 0.30 ppm in the cells cultured with and without FBS, respectively.

DISCUSSION

This study was carried out to demonstrate the effects of the components dissolved from SBS on osteoblastic cells in culture. The PI3Ks, which represent a family of lipid kinases, are key mediators of intracellular signaling in many cell types.[17] PI3Ks are upstream regulators of a number of signaling cascades that control cell proliferation, growth, death, migration, metabolism, and a host of other biological responses.[18] Furthermore, the PI3K/Akt pathway is one of the most critical signaling pathways involved in the regulation of cell survival.[19,20] For example, the TGF-β2-induced epithelial-mesenchymal transition in postoperative remnants of lens epithelial cells is mediated by the downregulation of connexin 43, which is regulated through the PI3K/Akt pathway.[21] The data from real-time PCR[14] and the present phosphorylation antibody assay of MAPKs indicate that the PI3K/Akt/ERK pathway is activated by components from the SBS.

Our laboratory has previously reported that the application of SBS to the alveolar bone in rats using a canal model results in a mild postoperative inflammatory reaction, and that new bone formation occurs in the periapical space near SBS on day 5 (unpublished data). Thereafter, the osteoblatic cells were used for MTT assay to examine the effect of SBS. In an MTT assay using human pulp cells to examine the effects of SB on cell viability, the eluates from SB were found to be the least cytotoxic agents compared to other resin cements.[22] The cytotoxicity of 4-META/MMA-TBB was comparable to that of MMA-TBB. However, TBB induced a higher cytotoxicity than 4-META/MMA.[23] The cytotoxicity induced by 4-META resin may be primarily associated with TBB.[23] The concentration of TBB (over 1000ppm) found in this study was extremely high, and it was unlikely to be reached in the clinical setting. In fact, our present data from culture medium clearly demonstrated that the released boron from TBB was only a few ppm. Therefore, it is highly unlikely that a concentration of over 1000ppm of TBB release would ever occur under clinical conditions.

The present data showed that cells cultured without FBS showed higher MTT values than did those cultured with FBS. This means that FBS could interact with the components of the SBS block and mask their effects. The TBB-initiated polymerization has unique and different characteristics compared to those initiated by the other common initiators: the decrease in residual MMA is fast, sustained for a long time, and results in a very low value of MMA.[24] This TBB effect was likely responsible for the promotion of cell proliferation.

Boron is a vital micronutrient in plants[25] and may be essential for animal growth and development. The role of boron in plants has been extensively investigated,[26] including its importance in pollination,[27] in the stability of the membrane,[28] as well as for increasing crop size.[29] However, little is known about boron homeostasis and function in animal cells.

NaBC1, the mammalian homolog of AtBor1, is a borate transporter. In the absence of borate, NaBC1 conducts Na^+ and OH^- (H^+), while in the presence of borate, NaBC1 functions as an electrogenic, voltage-regulated, Na^+-coupled $B(OH)_4^-$ transporter.[30] At low concentrations (0.1 to 0.5 *mM*), borate activates the MAPK pathways to stimulate the cell growth and proliferation in rat submandibular glad acini and ducts, although it is toxic at high concentrations (above 1 *mM*).[30] Furthermore, in a mineralization assay of MC3T3-E1 cells, increased mineralized nodules were observed in the cells treated with boron (1 and 10 ng/ml concentrations) after 30 days of culture.[31] In the short term (24 h), boron decreased the cell survival rate beginning at concentrations of 1 ppm and above. After long term (72 h) culture no statistically significant difference was detected in cells treated with different boron concentrations (2-10 ppm).[31] A relatively low concentration (1.6-1.7 ppm) of boron was demonstrated in the present study. Furthermore, the present analyzed values were closely consistent with the concentration (about 1.35 ppm) of boron showing the biggest increases in cell proliferation observed in the MTT assay, as the 1 *mM* concentration of TBB contains about 10.8 ppm of boron. Thereafter, the boron released from the TBB as a catalyst in SBS was thus confirmed to be potentially useful for

accelerating osteoblastic cell proliferation.

In conclusion, the present study demonstrated that the eluted components from SBS could increase the expression of some MAPKs related to osteoblastic cell proliferation and differentiation *in vitro*. Both the elution experiment and the treatment of cells with each SBS component in culture indicated that the boron originated from TBB was a probable candidate for activating the proliferation of osteoblastic cells.

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Figures Legends

Fig. 1 Signals detected on a membrane spotted with various antibodies against phosphorylated MAPKs following incubation with cell lysates from untreated cultures (A) or SBS-treated cultures (B). The signals indicated by the squares are as follows; a- ERK1, b- ERK2, c- JNK2, d- p38 α, e- ribosomal S6 kinase (RSK)1, f- glycogen synthase kinase (GSK)-3α/β, g- Akt 1, h- Akt 2, i- Akt pan.

Fig. 2 The viability of NOS-1 cells was determined by MTT assay following incubation with 4-META (A), MMA (B), and TBB (C). The data represent the means ± standard error of values taken from three wells.

Fig.1

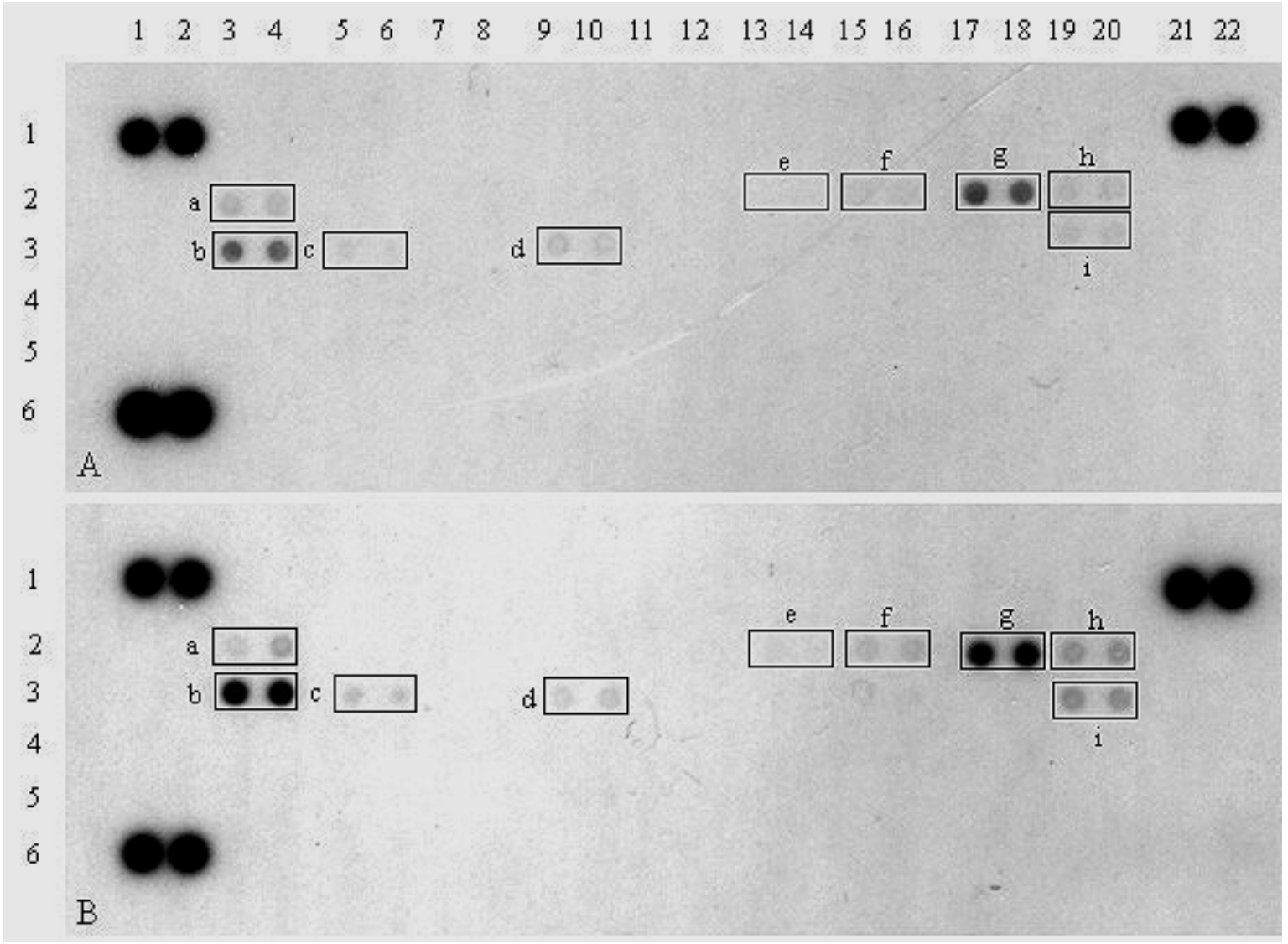
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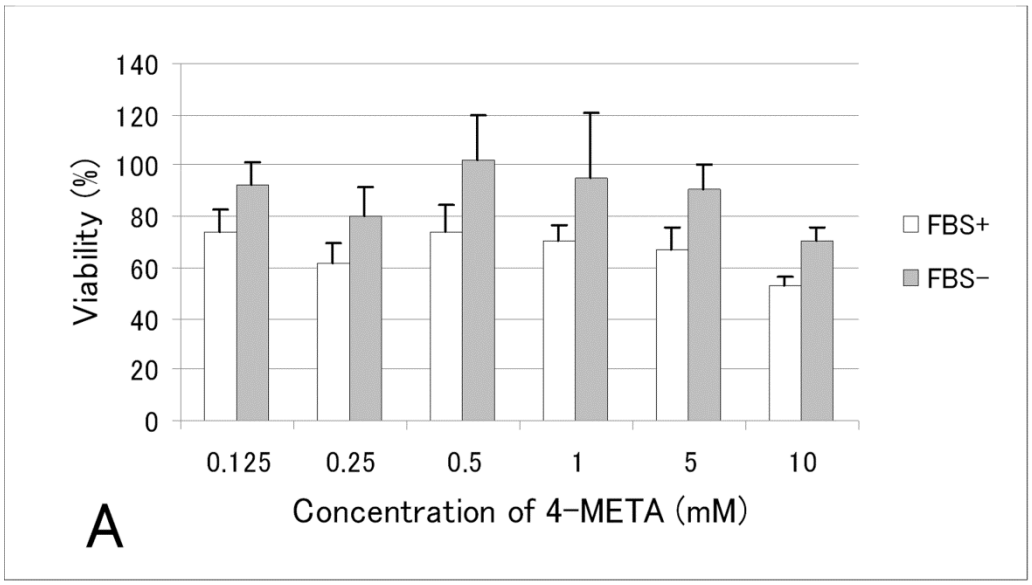
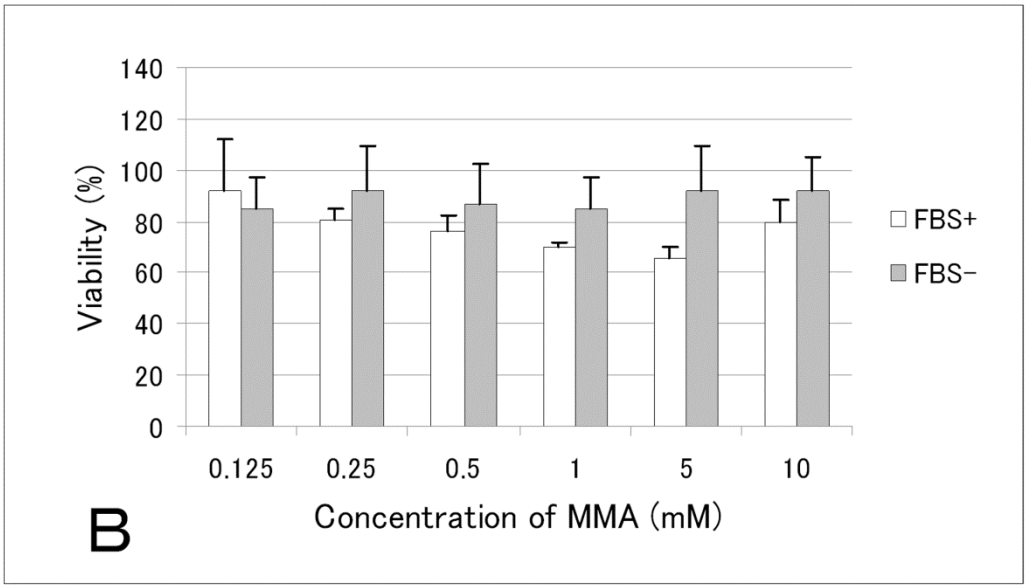
Fig.2


140
120
100
80
60
40
20
0
Viability (%)
0.125
0.25
0.5
1
5
10
Concentration of 4-META (mM)
FBS+
FBS-
A


140
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Viability (%)
0.125
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1
5
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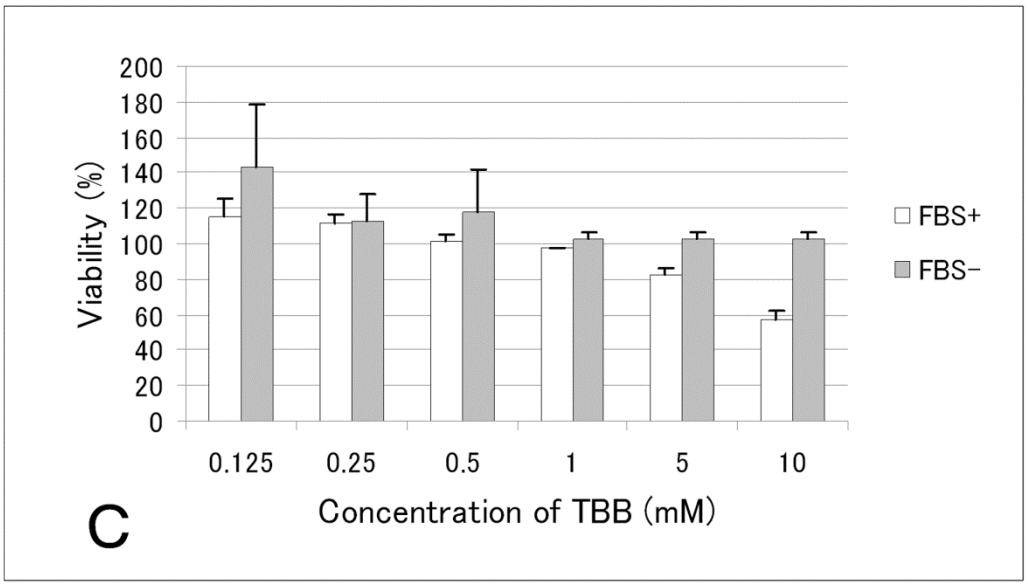
Concentration of MMA (mM)
FBS+
FBS-
B


200
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Viability (%)
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Concentration of TBB (mM)
FBS+
FBS-
C