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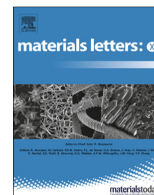

Influence of a melt derived bioactive glass (F18) over endothelial cells nitric oxide production

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ABSTRACT

Intense research efforts are being devoted to developing materials capable of releasing nitric oxide (NO) for numerous clinical applications, such as cardiovascular devices, wound healing and bone regeneration. However, the use of such technologies is still challenging due to their complexity, high cost, toxicity and lack of specificity. Here we investigated whether a novel biomaterial, F18 bioactive glass, can induce NO cell production *in situ*. Results indicated that F18 indeed increased the NO synthesis by a NOS activation-dependent mechanism, being this material a promising alternative for biomedical applications that aim rapid tissue regeneration.

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1. Introduction

Hypertension, atherosclerosis and diabetes mellitus are the main causes of death worldwide, and their common denominator is endothelial dysfunction (ED) [1]. The vascular endothelium is a monolayer of cells located between vascular smooth muscle cells and vessel lumen, being responsible for the release of endothelial factors. These factors play an essential role in the regulation of vascular tone, angiogenesis, wound healing, platelet activity and smooth muscle proliferation [2]. The nitric oxide (NO) is the main endothelial factor and its reduced release and bioavailability are always present in ED [1]. So, the integrity of the endothelial cells is crucial for vascular health and for an effective tissue healing process [3]. NO plays an important role in numerous physiological processes, such as in the regulation of bone metabolism, modulating inflammation, vasodilation, etc. [4,5]. Thus, developing materials that can induce NO production could greatly impact the healing process. However, the clinical application of these NO-donors technologies has been restricted due to problems, such as burst release, high toxicity, low payloads, lack of specificity, and untargeted delivery [4,6]. NO-donors are typically obtained via complex processes, being costly and generally non-effective [7]. Thus, simple strategies or easily processable biomaterials that increase NO cell production are highly desirable for tissue engineering applications, mainly when early vascularization is desired.

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A previous study has shown that bioactive glasses (BAG) can enhance the differentiation and survival of endothelial progenitor cells (EPC), inducing early vascularization [8]. Also, BAG have proven to present an angiogenic potential and to induce neovascularization throughout *in vitro* and *in vivo* studies [9–12]. Recently, the use of F18 (a new BAG) has increased the vascular endothelial growth factor (VEGF) production, leading to an accelerated wound healing process [12]. However, the mechanisms that led to this rapid healing are still under investigation. Hence, the aim of this study was to evaluate whether the F18 can enhance NO production in HUVEC cells.

2. Experimental

2.1. F18 bioactive glass preparation

F18 belongs to the $SiO_2.Na_2O.K_2O.MgO.CaO.P_2O_5$ system and its manufacture process has been described in detail elsewhere [13]. Briefly, the glass was melted in a platinum crucible at 1450 °C for three hours and then quenched by splat cooling. Subsequently, powdered samples were obtained with a particle size of 1–2 mm.

2.2. In vitro assays

HUVEC (Immortalized human umbilical vein endothelial cells) were cultured in DMEM (Dulbecco Modified Earle's medium) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 μg/mL) – (90% humidity and 5% CO_2 at 37 °C).

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Then, the HUVEC were seeded (24 well plate) in a concentration of 5×10^4 cells/well and a F18 was added to the cell culture (0.01 g/ml–500 µl/well). After 48 h, the detection of intracellular NO was determined with a selective fluorescent probe 4, 5-diaminofluorescein (DAF-2DA – 10 µM) for 30 min. Then, cells were treated with PBS (negative control), ionophore A23183 (A23 – positive control) or NG-nitro-L-arginine methyl ester (L-NAME) for 60 min. The detection of NO was held in SpectraMax GeminiXS fluorometer (Molecular Devices) at 485 nm excitation and 538 nm emission wavelength.

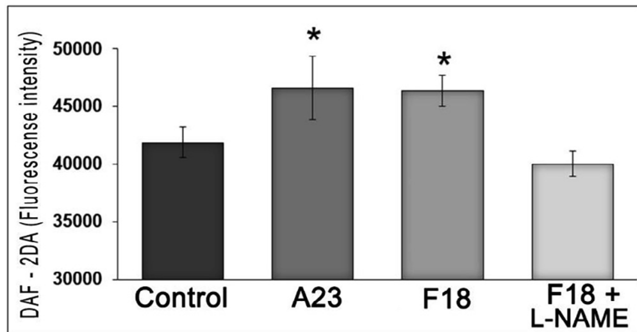


Fig. 1. Measurement of intracellular NO production in HUVEC by DAF-2DA. The fluorescence intensity (FI) after 60 min of treatment with A23, F18 and F18 + L-NAME $^*p < 0.05$, indicate significant difference to the CONTROL (n = 6).

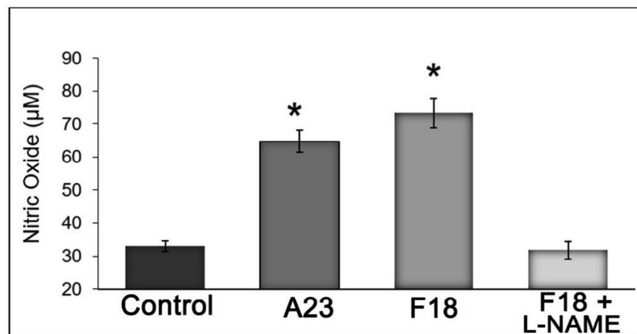
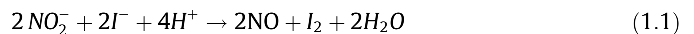


Fig. 2. Measurement of NO production in HUVEC supernatant by selective electrode. NO concentration obtained after 60 min of treatment with A23, F18 or F18 + L-NAME $^*p < 0.05$, indicate significant difference to the CONTROL (n = 6).

NO detection was done by a selective NO electrode (InNO-T-II, Nitric Oxide Measuring System, – Innovative Instruments, Inc.) by the indirect quantification of nitrite (NO_2^-) which is a stable metabolite of NO. For that, the supernatant from each well was placed in a shaker vessel along with 1 mL of a 1 M sulfuric acid and 90 mM potassium iodide solution. Therefore, reduction of nitrite in NO occurs (Eq. 1.1) and the compound is detected by the electrode.



$$2\,NO_2^- + 2I^- + 4H^+ \rightarrow 2\mathrm{NO} + I_2 + 2H_2O \tag{1.1}$$

Statistical analysis was performed using ANOVA one way (post hoc test: Newman–Keuls) in Statistica software 7.0 (StatSoft, Inc – USA 3.0). Data are expressed as mean ± SD. Values of $p < 0.05$ were considered significant.

3. Results and discussion

Fig. 1 shows the results obtained by the fluorescence method (DAF absorbance). The incubation of HUVECs with A23 significantly increased the NO production when compared to the negative control, as expected. Interestingly, F18 samples alone were able to induce an increase of the same order of magnitude. The inhibition of NOS by L-NAME abolished the F18 effect, indicating that the F18 action is mediated by NOS activation.

To endorse the results obtained by the DAF, the quantification of NO was performed by the selective NO electrode. The results indicate a similar effect (Fig. 2) obtained by two different quantification methods, confirming that F18 induced NO production in endothelial cells.

NO synthesis and release by endothelium is a critical regulator of cardiovascular homeostasis, it is a main signaling molecule of the vascular system and is produced by the constitutively

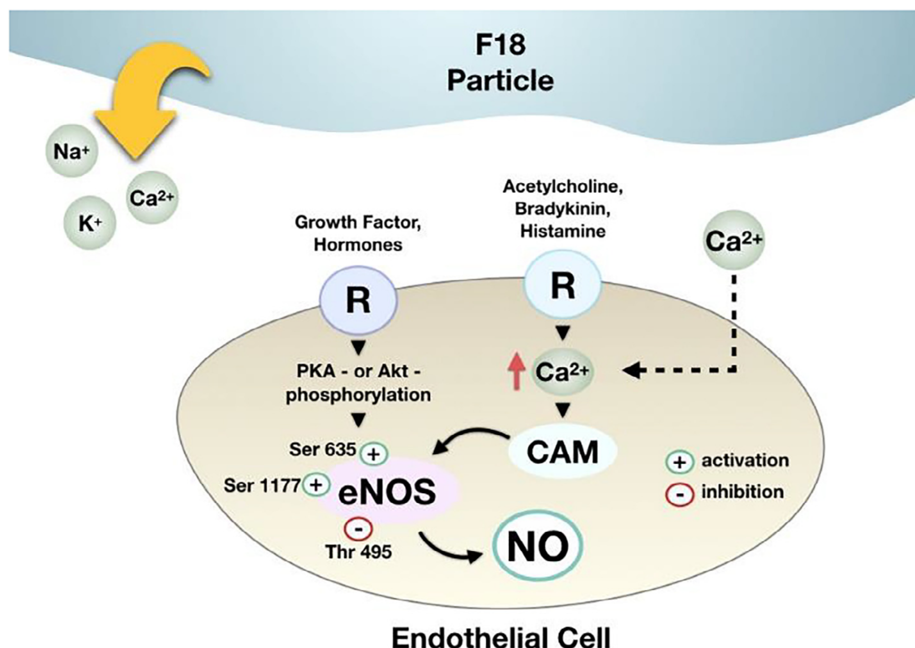


Fig. 3. eNOS activation process. Agonists act on specific receptors (R) on the endothelial cell membrane to increase the intracellular concentration of calcium, which binds to calmodulin (CaM) and leads to the activation of calmodulin-binding domain of eNOS to produce nitric oxide (NO). F18 bioactive glass leaches Ca^{2+} to the medium, promoting an increase in the intracellular Ca^{2+} concentration, hence stimulating NO production (dashed line).

expressed eNOS [14]. The eNOS synthesizes NO in a pulsatile Ca^{2+}/calmodulin-dependent manner with eNOS activity markedly increasing when intracellular Ca^{2+} increases. Ca^{2+} induces the binding of calmodulin to the enzyme thus increasing the rate of electron transfer from NADPH to heme center leading to efficient NO synthesis. Also, CaM can activate CaM kinase II, which may phosphorylate eNOS on S1179. [14,15]. In this sense, eNOS is classified as a constitutive and strictly Ca^{2+}/CaM-dependent enzyme [16].

The activity of eNOS, hence the production of NO, can be initiated/enhanced by several stimuli including shear stress, acetylcholine, bradykinin, histamine, and 17b-estradiol, in both calcium-dependent and -independent manners. Agonists, such as acetylcholine, bradykinin, and histamine, act on specific receptors on the endothelial cell membrane to increase the intracellular concentration of calcium, which binds to calmodulin and leads to the activation of calmodulin-binding domain of eNOS [17]. The increase in cytoplasmic calcium levels activates CaM that causes eNOS activation and NO production [15]. This study showed an increase in NO concentration when HUVEC cells were exposed to F18, as well as the inhibition of L-NAME, revealing that this effect was eNOS dependent (Fig. 3).

Many studies have shown that BAG leach soluble ions, such as Ca, Si, and P when in contact with fluids, which leads to favorable intracellular responses [18], one of them being the increase of VEGF, a potent mitogen for vascular endothelial cells [11,18–20], which also stimulates PI3K/AKT by phosphorylation and activation of eNOS and consequently NO production [15,21]. In HUVEC cells VEGF also induces a NO production by eNOS, via VEGF-induced Ca^{2+} mobilization, through tyrosine phosphorilation of phospholipase C (PLC) that results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate into D-myo-inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. The elevated IP3 levels cause the release of Ca^{2+}, which finally causes eNOS activation and NO production [15,22].

4. Conclusion

Our results indicate that the F18 induced NO production in endothelial cells by NOS activation-dependent mechanism. Thus, F18 can be a simple-manufactured, inexpensive and effective NO-inductor biomaterial and a promising alternative for several biomedical applications aiming a rapid and effective regenerative process.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mlblux.2019.100022.

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