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Article

Co-stimulation effect of fluid shear stress-material surface chemistry on the behavior of human umbilical vein endothelial cells

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Abstract: Objective: The improvement of bone repair scaffolds to enhance their bioactivity and in vivo vascularization is a current research hotspot. **Method:** HUVECs are subjected to both fluid shear stress (FSS) and chemical stimuli simultaneously. The release of ATP, NO, and the expression of eNOS were examined. The adhesion spots and cytoskeleton formed by HUVEC on the material surface were also observed. **Result:** LFSS (low fluid shear stress, 5 dyn/cm^2) did not trigger a response on Glass and -NH_2 HUVECs, but induced a strong response on -OH and -CH_3, while PFSS (physiological fluid shear stress, 15 dyn/cm^2) and HFSS (high fluid shear stress, 20 dyn/cm^2) generated responses of all groups of cells, among which the strongest response level was from the -NH_2 group, followed by Glass, and among which equal response levels of the -OH and -CH_3 groups existed at the lowest. **Conclusion:** The chemical functional groups changed the initial threshold of HUVECs response to FSS and the shear force stimulation threshold for optimal cellular response by influencing the quality of adhesion spots and cytoskeleton formed by HUVECs on the surface of the material, thereby altering the response state of endothelial cells to shear force stimulation.

Keywords: SAMs; material surface chemistry; FSS; HUVECs

1. Introduction

With dynamic reconstruction and rich blood vessels [1] , bone plays critical roles in support, protection, movement, and metabolism [2]. Full healing after severe trauma or large bone tissue loss is still a clinical challenge. In addition, treatment of large bone defects remains a complex task for surgeons. Existing treatment methods include bone grafting, vascularized graft and periosteum induction [3]. But these methods are technically demanding, with their own limitations and complexities [3]. Tissue engineering presents promising possibilities in achieving successful treatments of human body tissue loss that current methods fail to accomplish or secure satisfactory clinical outcomes. In scaffold-based bone tissue engineering, a high-performance scaffold lays a sturdy foundation for the success of a certain bone tissue engineering strategy. A major direction in the field is to produce bone tissue engineering scaffolds with desirable shape, structural, physical, chemical and biological features for enhanced biological performance and complex bone tissue regeneration [4]. The emergence of tissue-engineered bone can not only meet morphological and quantitative requirements for bone tissue repair, but also satisfy the needs of biocompatibility and osteogenesis induction after implantation in vivo. However, due to the lack of a perfect vascular system in the scaffolds, central ischemic necrosis often occurs in the repair of large bone defects [5,6]. Thus, at present, it is mainly applied to the repair of small bone tissue defects. The failure to improve the vascular system

of tissue-engineered bone poses an insurmountable hindrance to its wide application. Therefore, the fabrication of tissue-engineered bone with a vascular system has always been an imperative task to be worked out in the clinical repair of large bone defects.

As a crucial seed cell of tissue-engineered bone, human umbilical vein endothelial cells (HUVECs) participate in the formation of vascular network in tissue-engineered bones and play a pivotal role in this process [7]. Liu et al. planted smooth muscle cells on natural and artificial bone scaffolds, then inoculated HUVECs for co-culture 3 weeks later, and finally preformed the microvascular network in vitro [8]. Fabio et al. implanted umbilical vein endothelial cells and cardiomyocytes on hydrogel scaffolds and accurately distributed them in 3D space by 3D printing technology, inducing the formation of heart tissue with functional blood vessels and successfully integrating with the vascular production function in mice [9].

Endothelial cells are subjected to both biophysical and biochemical stimulation in vivo. Biophysical stimulation is mainly the shear force stimulation generated by blood flow in the lumen on endothelial cells in the vascular wall [10,11]. Biochemical stimulation refers to different chemical groups in the surrounding matrix during cell life [12]. In the field of tissue-engineered bone, scaffolds play a primary role in the study of tissue-engineered bone. Different scaffold materials can provide different biophysical and biochemical stimuli, and then affect the behavior and function of cells [13,14]. The biomaterials and medicine have been employed for the diagnosis or treatment of the human body. A large number of experiments in this field have confirmed the important role of the extracellular microenvironment in controlling the fate of cells. However, due to the complexity of the surface of biomaterials, the influence of a single key chemical group on the behavior of a particular cell remains unclear [15]. Chemical groups on the surface of materials can affect the conformation and number of adsorption proteins, and impact the extracellular matrix (ECM) and specific adhesion of cells, then influence the transmission of all levels of signaling pathways including the signaling pathways of the cytoskeleton system, and ultimately regulate cell proliferation, differentiation and apoptosis [16,17]. Appropriate physical stimulation is also essential for vascular endothelial cells to generate blood vessels and maintain the normal function of the vascular system itself [18]. Fluid shear stress (FSS) is the most important force that vascular endothelial cells are subjected to. However, shear stress is not a real force and is defined as a unit area amount of force acting on the fluid parallel to a very small element of the surface. The force stimulation is transmitted through the extracellular matrix, adhesive plaque and cytoskeleton, and eventually the stimulation signal is transmitted into the intracellular nucleus to regulate cell behavior. Though it is still unclear how vascular endothelial cells perceive mechanical stimulation and then transform physical signals into chemical signals to control cell behavior, there is much evidence supporting that adhesive plaques and the cytoskeleton indeed contribute greatly to this mechanical conduction system [19,20].

In conclusion, in order to fabricate vascularized tissue-engineered bone in vitro, HUVECs can form a normal structure and functional vascular network, which requires the combined action of appropriate chemical stimulation and mechanical stimulation on the surface of materials. So far, there have been many reports on the effects of

material surface chemistry alone and FSS stimulation alone on the adhesion and function of vascular endothelial cells both at home and abroad, but there are relatively few studies on the effects of material surface chemistry and FSS combined stimulation on endothelial cell behavior and cell-related behavior mechanisms [13–17]. Hence, it is of great significance for this paper to study the influence of fluid shear force stimulation and material surface chemical stimulation on the behavior of HUVECs, thereby providing a strong theoretical basis for the design of a bone tissue engineering repair scaffold and the exploration of the vascularization of tissue engineering bone.

Our research focuses on the stimulation of HUVECs by FSS integrated with chemical functional groups on the surface of materials, to observe the response of HUVECs on the surface of different materials to gradient FSS, and to explore the effect of material chemistry on cell response to shear force stimulation. Consequently, Self-assembled monolayers (SAMs) with terminal groups of -OH, $-CH_3$ and $-NH_2$ were prepared on the surface of Glass slides by SAMs technology, and blank Glass slides were used as controls to provide chemical stimulation on the surface of materials. HUVECs were inoculated on the surface of 4 groups of slides and loaded with 5 dyn/cm^2, 15 dyn/cm^2 and 20 dyn/cm^2 FSS, accordingly. The release of Adenosine triphosphate (ATP) and Nitric Oxide (NO) and the expression of endothelial nitric oxide synthase (eNOS) were detected. The total protein content was determined by the bicinchoninic acid method (BCA).

2. Materials and methods

2.1. Materials

High purity nitrogen (99.999% purity); Hydrogen peroxide (30%, Xi'an Sanpu Chemical Reagent Co., LTD.); Concentrated sulfuric acid (98%, Xi'an Sanpu Chemical Reagent Co., LTD.); Dimethyloctadecyl chlorosilane (Nanjing Chemical Reagent Co., LTD.); N-hexane (analytically pure, Beijing Baolaibo Technology Co., LTD.); 3-triethoxylmethylsilane-1-propylamine (Alfa Aesar, USA); Acetone (analytically pure, Nanjing Chemical Reagent Co., LTD.); Anhydrous ethanol (analytically pure, Nanjing Chemical Reagent Co., LTD.); DMEM/F12 medium (Hyclone corporation, USA); Fetal Bovine Serum (Hyclone corporation, USA); 0.25% trypsin (China, Beijing Solibor Technology Co., LTD.); ATP Detection Kit (Shanghai Biyuntian Biotechnology Co., LTD., China); BCA Protein Detection Kit (Beijing Solebo Technology Co., LTD., China); NO Detection Kit (Shanghai Biyuntian Biotechnology Co., LTD., China); RIPA Cracking Buffer (China, Shanghai Biyuntian Biotechnology Co., LTD.); PMSF Protease inhibitor (Beijing Solebo Technology Co., LTD., China); Sds-page Gel Preparation Kit (Beijing Solebo Technology Co., LTD., China); PVDF Membrane (China, Beijing Solebo Technology Co., LTD.); ECL Luminescent Reagent Detection Kit (Beijing Soleibao Technology Co., LTD., China); Rainbow 180 broad spectrum protein Marker (China, Beijing Solaibao Technology Co., LTD.); ENOS Rabbit pAb (Wuhan AgTECH Biotechnology Co., LTD., China);ACTB Rabbit mAb (Wuhan Agtech Biotechnology Co., LTD.); HRP Goat Anti-Rabbit IgG (China); HRP Goat Anti-Rabbit IgG (China); Paraformaldehyde (Shanghai Aladdin Reagent Co., LTD., China); Triton X-100 (China, Beijing Solebo Technology Co., LTD.); Bovine serum albumin BSA (Merck Germany);Anti-vinculin

Antibody (Wuhan AgTECH Biotechnology Co., LTD., China); FITC Polyclonal Goat Anti-rat IgG (Wuhan AbTECH Biotechnology Co., LTD., China);Rhodamine labeled Ghoupenopeptide (Wuhan AIbotec Biotechnology Co., LTD., China); DAPI (Shanghai Biyuntian Institute of Biotechnology, China); Glycerol (China, Nanjing Chemical Reagent Co., LTD.).

2.2. Preparation and characterization of SAMs on Glass slides

The experimental slides were immersed in acetone, anhydrous ethanol and deionized water in turn and thoroughly cleaned. After drying, the slides were obtained, and the cleaned slides were marked as Glass. The Glass slides were immersed in a beaker containing Pirahan solution (30% H_2O_2:98% H_2SO_4 = 15:7), then taken out in a constant temperature water bath at 80 ℃ for 1 h, next cleaned with deionized water, and finally blow-dried with high-purity nitrogen. The Glass slides with material surface chemistry of -OH were obtained, marked with -OH.

The obtained Glass slides with the material surface chemistry of -OH were immersed in the surface reaction solution containing -NH_2 (acetone: 3-triethoxylmethylsilane-1-propylamine = 20:1) and the surface reaction solution containing the -CH_3 monolayer (N-hexane: dimethyloctadecylchlorosilane = 30:1). Then we took them out after a water bath at 60 ℃ and 55 ℃ for 3 h and 1h, respectively. The Glass slides were washed with anhydrous ethanol and deionized water and then blow-dried with high purity nitrogen. Material surface chemistry of NH_2 and CH_3 were obtained and labeled. -NH_2 and -CH_3. 5 uL of deionized water was dropped on the surfaces of different prepared materials (Glass, -OH, -NH_2 and -CH_3), and the water contact Angle was determined by contact Angle meter, denoted by θ. The hydrophilicity and hydrophobicity of the material can be judged by measuring the water contact Angle of the material surface.

2.3. Fluid shear stress

The FSS platform was constructed based on Nauman's parallel plate flow chamber to provide 0 dyn/cm^2, 5 dyn/cm^2 (LFSS), 15 dyn/cm^2 (PFSS) and 20 dyn/cm^2 (HFSS). As shown in **Figure 1**, the length, width and height of the flow chamber are 7.5 cm, 2.5 cm and 0.3 cm respectively. The shear force stimulation can be calculated according to the formula $\tau = 6\eta Q/H^2W$, in which τ stands for FSS, η represents the viscosity of the liquid, W means the bottom width of the flow chamber and H expresses the height of the flow chamber.

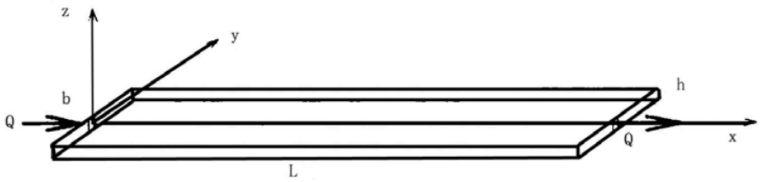

Figure 1. Parallel plate flow device.

2.4. Cell culture

HUVECs were purchased from the Kunming cell bank of the Chinese Academy of Sciences and cultured in DMEM, with the addition of 5% FBS, then placed in a 5%

CO_2 incubator at 37 ℃ [19]. Next, HUVECs were correspondingly inoculated on the surfaces of -OH, -NH_2, -CH_3 slides and Glass with an inoculation density of 200,000 cells per piece. After 48 h, when the degree of HUVECs fusion approximated 80%, the culture medium was discarded and washed with PBS 3 times. Eventually, the HUVECs in each group were set on a variable-strength FSS platform.

2.5. Determination of ATP, NO, and eNOS releases

Various types of cells release ATP in response to mechanical and biochemical stimuli, which functions as the energy source for nearly all living processes. Exposure of HUVECs to FSS stimulation generated by flowing blood can change their morphology, function and gene expression in accordance with the alteration of shear force stimulation, such as angiogenesis and vascular remodeling [21]. Plenty of studies have proven that as a signal factor, ATP is rather sensitive to shear stress mechanical stimulation in HUVECs; similarly ATP release from vascular endothelial cells is a critical indicator of its biological activity and function on the surface of materials [22,23].

After sensing FSS triggered by blood flow, vascular endothelial cells activate eNOS synthesis and release vasodilator factor NO to dynamically regulate blood pressure and vascular tension, thus achieving self-functional adjustment, generally deemed as the core ability of HUVECs [22]. Meanwhile, the eNOS-NO pathway is engaged in the regulation of angiogenesis, and also mediates the transmission of the angiogenic factor signaling pathway [23]. The eNOS and NO release on the surface of different materials can directly reflect the activity and functional status of HUVECs.

HUVECs after being seeded on SAMs for 48 h (reaching ~80% confluence) were employed for the determination of ATP, NO and eNOS releases. After HUVECs on slides were exposed to LFSS, PFSS and HFSS for a predetermined time.

2.6. ATP release

After HUVECs on SAMs were loaded with LFSS, PFSS and HFSS for 0, 1, 2, 3, 4, 5, 10 and 15 min, 2 mL of medium was withdrawn and replenished with an equal volume of fresh culture medium to maintain a constant circulating fluid volume. The extracted sample was labeled as ATP samples in groups and then settled into the refrigerator at −20 ℃ for storage. The same procedure was performed for samples without FSS exposure.

ATP concentration and total protein content were determined by ATP kit and BCA protein detection kit, while the ratio of ATP concentration to total protein was employed as the final index of ATP release in each group.

2.7. NO release

After HUVECs on SAMs were loaded with LFSS, PFSS and HFSS for 0, 5, 10, 15, 30, 45 and 60 min, we extracted 2 mL medium from each sample group, and in the meantime added 2 mL fresh medium. The extracted samples were tagged as NO samples in groups and relocated to the refrigerator at −20 ℃ for freezing storage. The same procedure was performed for samples without FSS exposure.

NO concentration and total protein content were measured by the NO kit and BCA protein detection kit, and the ratio of NO concentration to total protein was adopted as the final index of ATP release in each group.

2.8. eNOS release

After HUVECs on SAMs were loaded with LFSS, PFSS and HFSS for 1 h, RIPA lysis buffer was evenly dripped on the surface of each slide, then gently shaken to uniformly spread over the slide surface. Next, the slides were placed in an ice bath for cracking for 30 min, after which the cracking solution was uniformly spread over the slides.

The surface lysate was absorbed and displaced to a centrifuge tube already pre-cooled on ice, which would be marked and placed in a high-speed centrifuge for 20 min at 12,000 r/min at 4 ℃. After extraction and centrifugation, the supernatant in the tube was subtransferred into a 0.5 mL pre-cooling centrifugal tube, labeled, and stocked in a refrigerator at −80 ℃. The BCA kit was used to detect the protein concentration in the sample and complete the protein quantification.

2.9. The morphology and adhesion spots of HUVECs before FSS exposure

This part of the experiment has been done in the previous research [24].

2.10. Statistical analysis

Data were expressed as means ± SD ($n \geq 6$ for all experiments). Quantitative data were analyzed by one-way analysis of variance and expressed as mean ± standard deviation ($\overline{x}$ ± S). $P < 0.05$ indicated a significant difference and was represented by "*".

3. Results

3.1. Characterization of SAMs: Water contact angle measurements

The detection results describing the water contact Angle on the surface of the prepared material are displayed in **Table 1**. There are prominent diversities in the hydrophilicity and hydrophobicity of materials with different chemical groups. The hydrophilicity of -OH is the strongest with the surface water contact Angle as 9.8°. Glass water contact Angle is 29.4°. -NH_2 has moderate hydrophilicity and hydrophobicity with its surface water contact Angle as 64.5°, close to 66°. CH_3 is a hydrophobic surface with a water contact Angle of 100.7°. This result is verified by previous studies, indicating that materials with different chemical functional groups on the surface have been successfully prepared [25,26].

Table 1. Water contact angles of various SAMs.

SAMs	Contact angle
Glass	29.4 ± 1.1°
-NH_2	64.5 ± 2.1°
-CH_3	100.7 ± 2.8°
-OH	9.8 ± 1.4°

3.2. ATP, NO and eNOS releases on various surface chemistries with no FSS exposure

For better observation of the influence of material surface chemistry on the release of factors related to HUVECs, the release of ATP, NO and eNOS in each group without FSS was integrated into **Figure 2A,B** and **Figure 3**. As we can see, there was no remarkable variation in ATP, NO and eNOS release of HUVECs when chemical stimulation of -OH, -NH_2 and -CH_3 was given alone, and also no distinct effect was made aware of on ATP, NO and eNOS release of HUVECs by chemical stimulation alone.

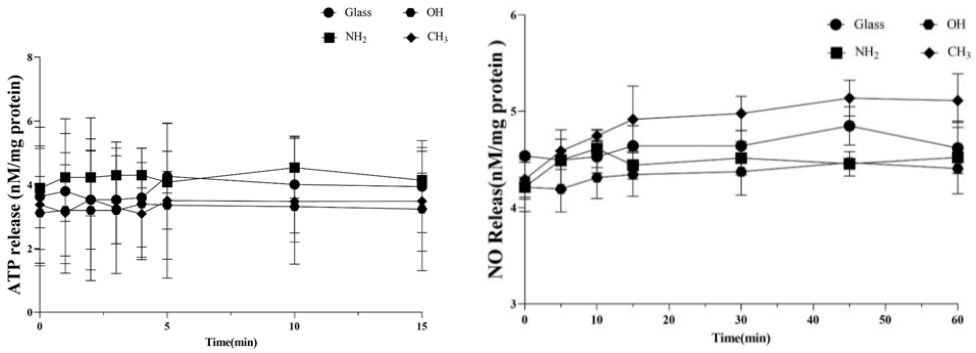

Figure 2. (A) Effect of surface chemical stimulation on ATP release of HUVECs; **(B)** Effect of surface chemical stimulation on NO release from HUVECs.

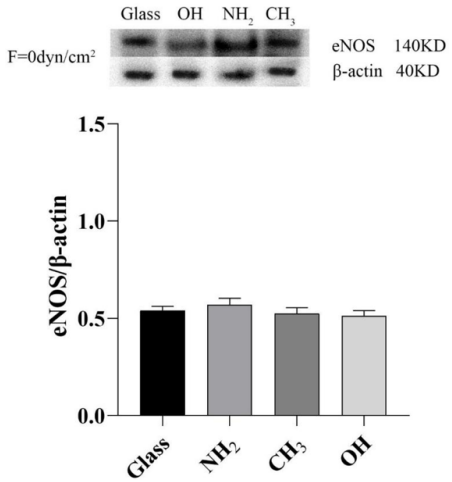

Figure 3. eNOS content in HUVECs on various surface chemistries with no FSS exposure.

3.3. The effects on HUVECs adhesion plaque and cytoskeleton

Figure 4 showed images of HUVECs cultured on each material for 48 h, using blue (DAPI), green (FITC) and red (Rhodamine) to represent the nucleus, Vinculin and F-actin correspondingly. Vinculin and F-actin in the images were statistically analyzed by Image J, and the results were shown in **Table 2**. After 48 h of culture, the HUVECs on -NH_2 were observed to contain a large amount of plaque binding protein and a clear cytoskeletal protein F-actin, and in contrast, only a small amount of HUVECs on -OH and -CH_3 were monitored. HUVECs on Glass had fewer adhesion

spots, while F-actin differed greatly from -NH_2. As a result, the formation of adhesive spots and F-actin in HUVECs on the chemical functional groups on the surfaces of different materials follows the pattern of -NH_2 > Glass > -CH_3 = -OH, which means HUVECs on -NH_2 formed the best adhesion spots and F-actin.

Table 2. The vinculin number (vinculin/cell) and the spreading area of F-actin (μm^2/cell).

SAMs	Vinculin	F-actin
Glass	185.47 ± 18	121.23 ± 14
-NH_2	271.61 ± 24	147.17 ± 20
-CH_3	142.75 ± 16	82.65 ± 17
-OH	139.34 ± 11	81.19 ± 19

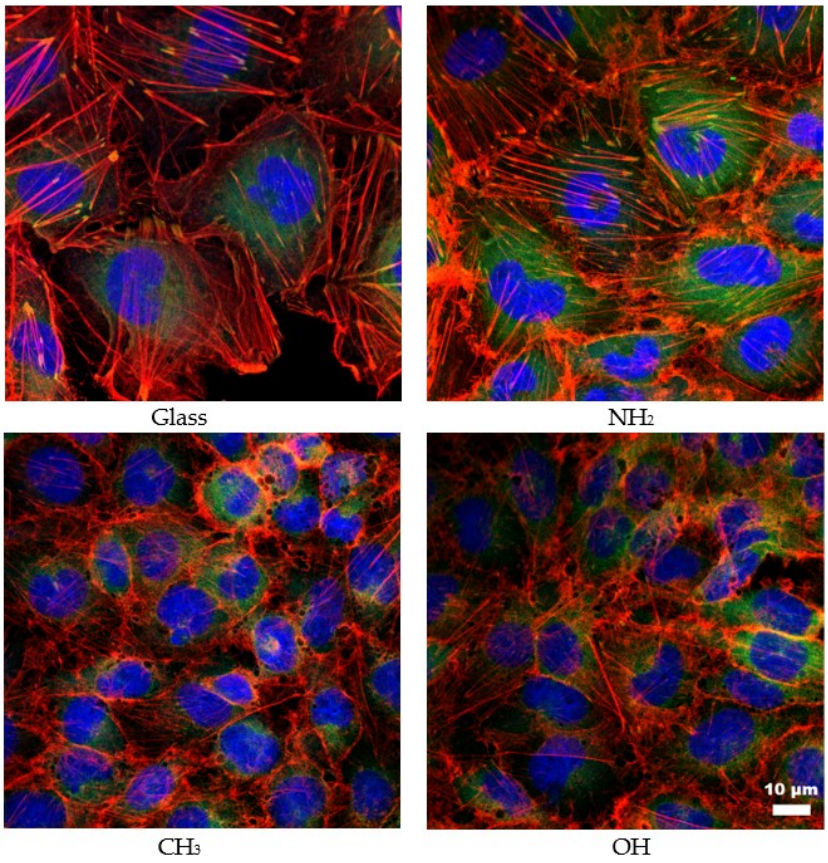

Figure 4. Effects of surface chemical stimulation on adhesion plaque and cytoskeleton of HUVECs.

3.4. ATP, NO and eNOS releases on various surface chemistries with FSS exposure

3.4.1. ATP, NO and eNOS releases with LFSS exposure

Under the action of FSS, the release of ATP in CH_3-LFSS and OH-LFSS significantly increased with that in -CH_3 and -OH, peaked at 1 min of shear force stimulation loading, and then decreased slowly but still remained higher than that at static condition. The release of ATP in -CH_3-LFSS was equal to that in OH-LFSS. NH_2-LFSS and Glass-LFSS did not change dramatically compared with -NH_2 and

Glass where the chemical stimulation was applied alone. This indicated that HUVECs growing on the surfaces of -NH_2 and Glass materials exhibited no ATP release response after loading F = 5 dyn/cm^2 stimulation, whereas HUVECs growing on the surfaces of -OH and -CH_3 materials presented obvious ATP release response. ATP release in each group demonstrated the phenomenon of OH-LFSS = CH_3-LFSS > -NH_2-LFSS = Glass-LFSS, as is shown in **Figure 5a–e**. The release of NO in each group also showed a similar phenomenon, which is revealed in **Figure 6a–e**. As NO is the end product of eNOS synthesis, the same trend is expected between the content of eNOS and the amount of NO released in each group, as is shown in **Figure 7**.

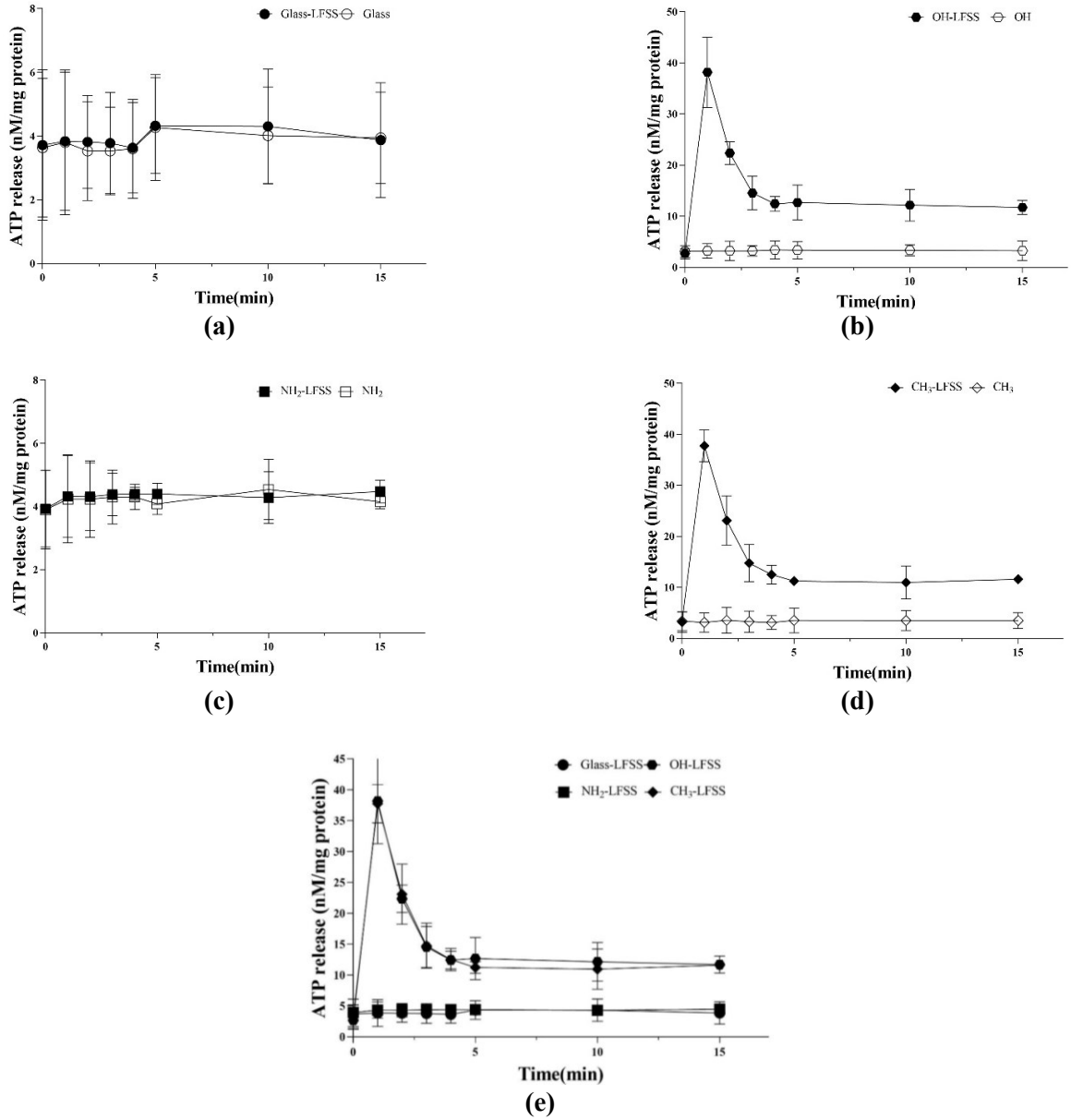

Figure 5. ATP release from HUVECs under material chemical and 5 dyn/cm^2 LFSS co-stimulation: (**a–d**) ATP release of HUVECs receiving both material chemistry and LFSS stimuli (X-LFSS) compared to those receiving material chemistry stimuli alone (*X*) and LFSS stimuli alone; (**e**) The differences of ATP releases of HUVECs was the comparison result among the LFSS groups. *X* represents material chemistry stimuli alone of NH_2, OH, or CH_3. Blank Glass slides (Glass) were used as controls of various material chemistries. Glass-LFSS was considered to provide LFSS stimuli alone.

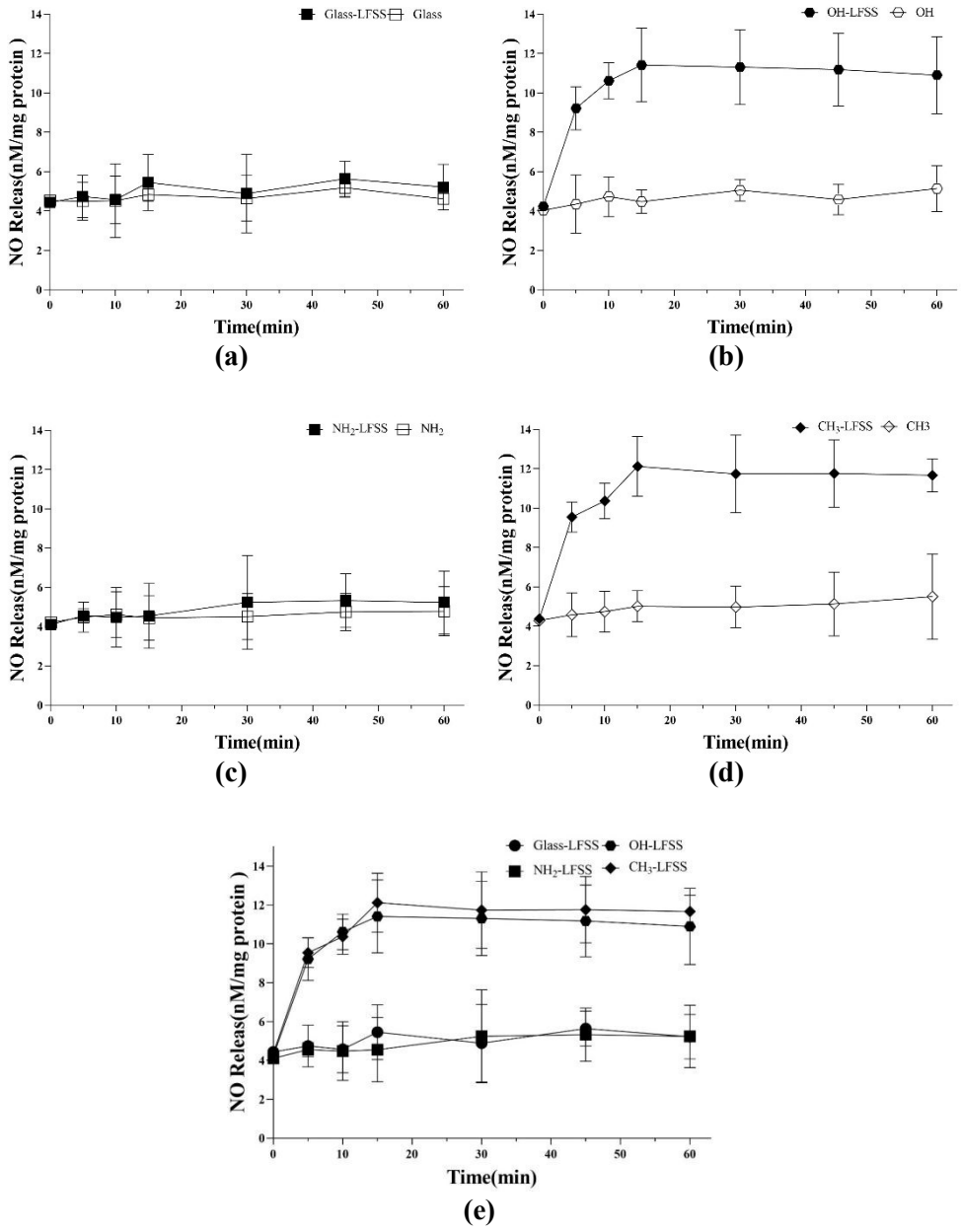

Figure 6. NO release from HUVECs under material chemical and 5 dyn/cm^2 LFSS co-stimulation: **(a–d)** NO release of HUVECs receiving both material chemistry and LFSS stimuli (*X*-LFSS) compared to those receiving material chemistry stimuli alone (*X*) and LFSS stimuli alone; **(e)** The differences of NO releases of HUVECs was the comparison result among the LFSS groups. *X* represents material chemistry stimuli alone of NH_2, OH, or CH_3. Blank Glass slides (Glass) were used as controls of various material chemistries. Glass-LFSS was considered to provide LFSS stimuli alone.

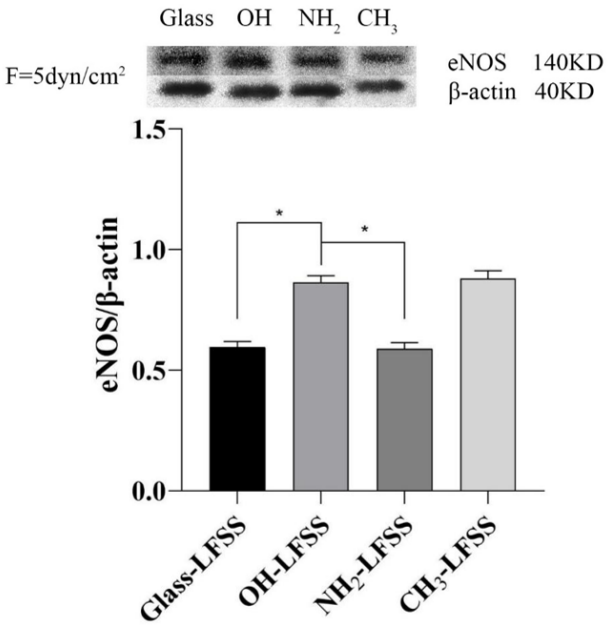

Figure 7. eNOS content in HUVECs under material chemical and 5 dyn/cm^2 LFSS co-stimulation.

3.4.2. ATP, NO and eNOS releases with PFSS exposure

This part of the work was reported in our previous research, and here is just a brief introduction [26]. The experimental results of material surface chemistry combined with PFSS on HUVECs illustrated that ATP, NO and eNOS measurements indicated all groups responded to PFSS, but the response degrees varied drastically, with -NH_2-PFSS group as the highest response to FSS stimulation, followed by the Glass-PFSS group. In comparison, the -OH PFSS group and CH_3-PFSS group had the same response to FSS stimulation and were the lowest among the four groups. The release level of chemical factors in each group represented the biological activity of cells. Under this circumstance, the biological activity of HUVECs in each group was ordered as NH_2-PFSS > Glass-PFSS > OH-PFSS = CH_3-PFSS.

3.4.3. ATP, NO and eNOS releases with HFSS exposure

After HFSS loading, ATP, NO and eNOS release responses were generated in all groups, but differences were detected in the response levels of each group. -NH_2 was the strongest, followed by Glass, and -CH_3 and OH were equivalent but weakest. Only -NH_2 had a similar response level to PFSS stimulation. The other groups exhibited a decrease in PFSS. On the whole, the NH2-HFSS group had the highest response to FSS stimulation, followed by the Glass-HFSS group, whereas the OH-HFSS group and the CH_3-HFSS group had the same response to FSS stimulation and were the lowest among the four groups. This trend was the same as the release response of each group under PFSS stimulation, as is shown in **Figures 8–10**.

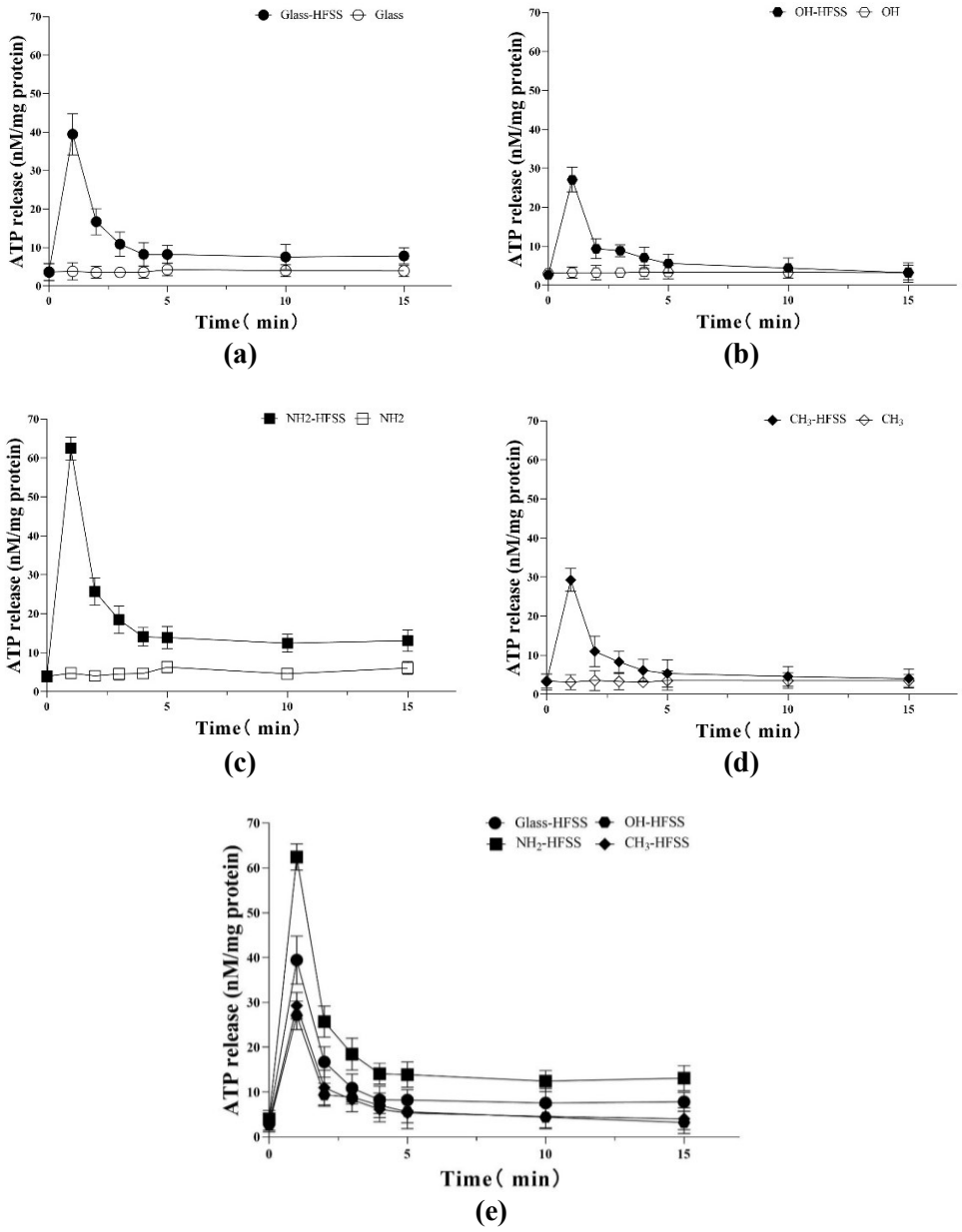

Figure 8. ATP release from HUVECs under material chemical and 20 dyn/cm^2 HFSS co-stimulation: **(a–d)** ATP release of HUVECs receiving both material chemistry and HFSS stimuli (*X*-HFSS) compared to those receiving material chemistry stimuli alone (*X*) and HFSS stimuli alone; **(e)** the differences of ATP releases of HUVECs was the comparison result among the HFSS groups. *X* represents material chemistry stimuli alone of NH_2, OH, or CH_3. Blank Glass slides (Glass) were used as controls of various material chemistries. Glass-HFSS was considered to provide HFSS stimuli alone.

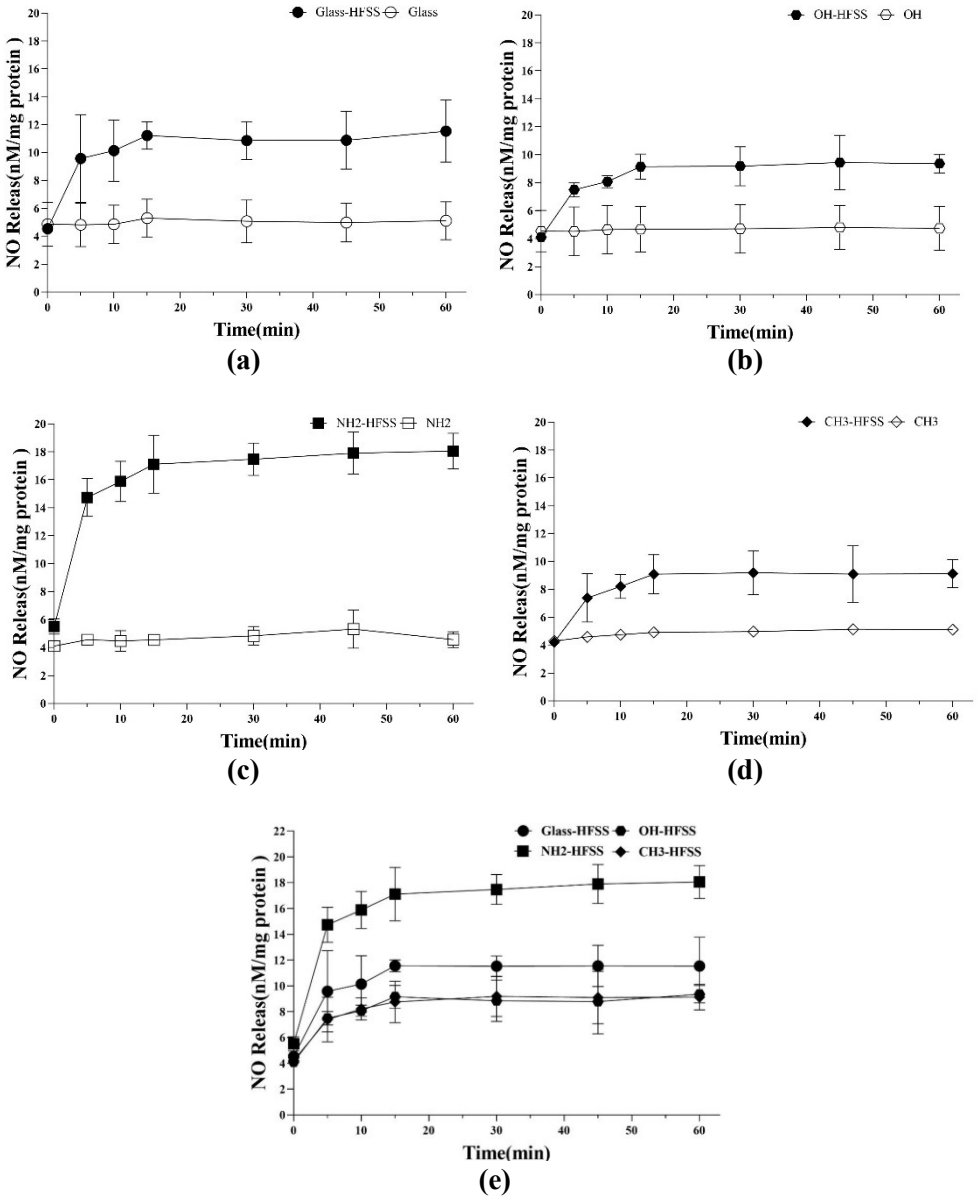

Figure 9. NO release from HUVECs under material chemical and 20dyn/cm^2 HFSS co-stimulation: **(a–d)** NO release of HUVECs receiving both material chemistry and HFSS stimuli (*X*-HFSS) compared to those receiving material chemistry stimuli alone (*X*) and HFSS stimuli alone; **(e)** The differences of NO releases of HUVECs was the comparison result among the HFSS groups. *X* represents material chemistry stimuli alone of NH_2, OH, or CH_3. Blank Glass slides (Glass) were used as controls of various material chemistries. Glass-HFSS was considered to provide HFSS stimuli alone.

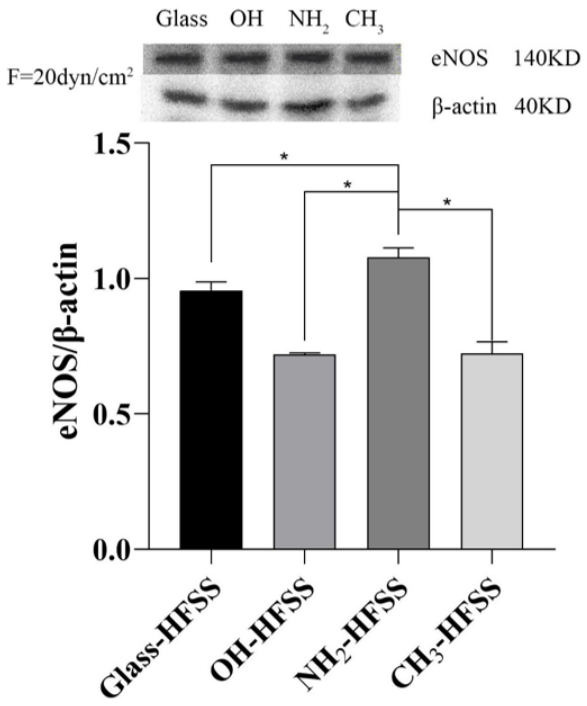

Figure 10. eNOS content in HUVECs under material chemical and 20 dyn/cm^2 HFSS co-stimulation.

In order to better observe the influence of different FSS on HUVECs by material surface chemistry combination, data were integrated, as is shown in **Figures 11–13**.

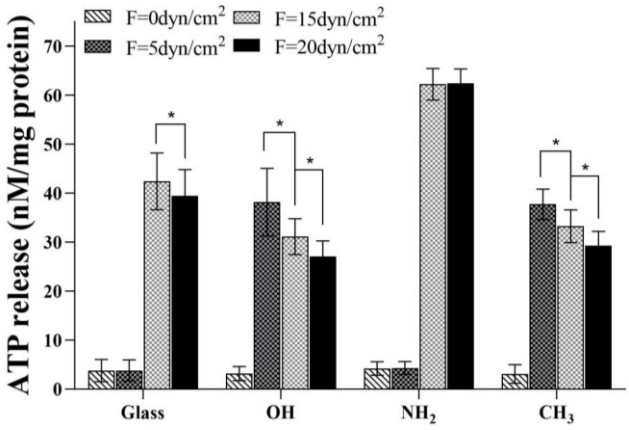

Figure 11. ATP release from HUVECs under material chemical and different FSS co-stimulation.

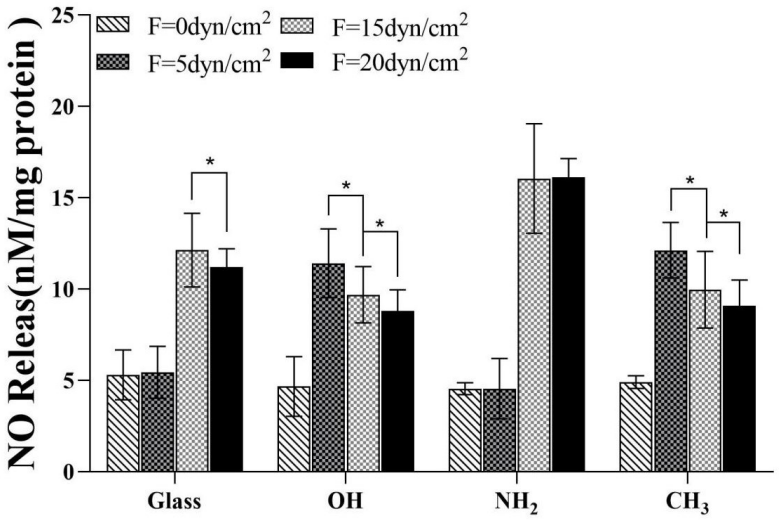

Figure 12. NO release from HUVECs under material chemical and different FSS co-stimulation.

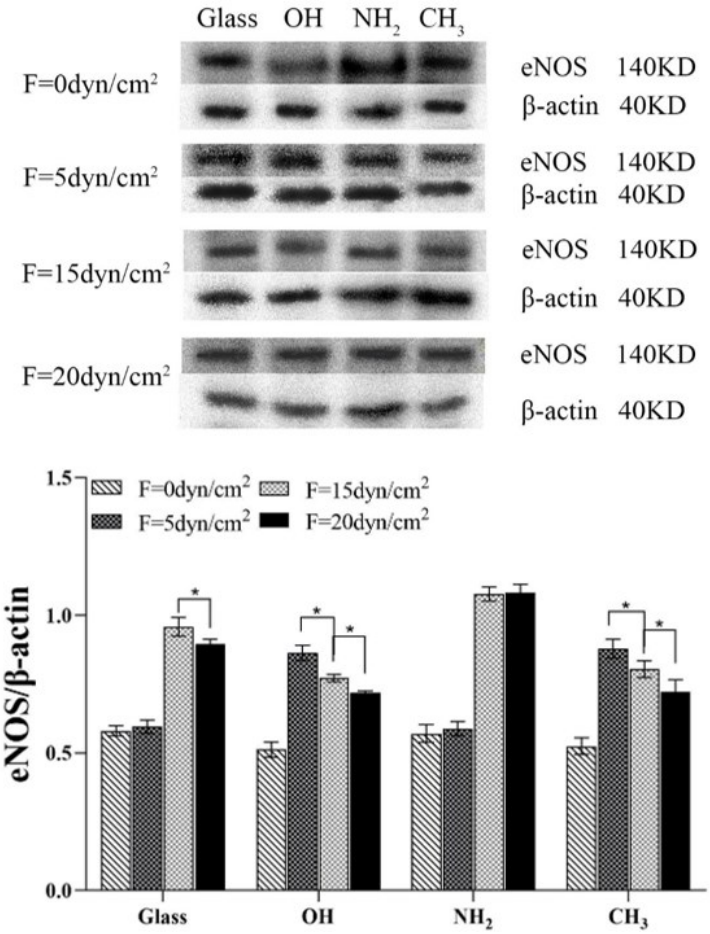

Figure 13. eNOS release from HUVECs under material chemical and different FSS.

4. Discussion

The results demonstrated that since LFSS did not reach the response threshold, HUVECs, therefore, did not produce a release response. Contrastingly, PFSS was the optimal shear force stimulation of HUVECs, so the release response exhibited the

strongest, whereas HFSS exceeded the ideal shear force stimulation of HUVECs, meaning, that the intensity of HUVECs release response was smaller than that of PFSS. All the experiments were conducted simultaneously.

Stained before FSS exposure, HUVECs were found to have adhesion spots and cytoskeletons in different states and of varying qualities in different functional groups. Adhesion spots and the cytoskeleton were not only the key factors determining the cell adhesion state, but also primary receptors for cells to sense external mechanical force stimulation and to shift mechanical force stimulation into cells, which in turn would control the cellular response to FSS. The adhesion spots and cytoskeleton quality of the $-NH_2$ were in optimal condition in each group. Hence, the adhesion state of the HUVECs living on the $-NH_2$ surface slide was at its best and most stable state, quite difficult to produce morphological changes. The threshold value of the initial FSS causing the stimulation response of the cells also increased accordingly. Optimal FSS falls into the range where HUVECs produce the strongest stimulus response. Stable adhesion also widens the optimal FSS interval correspondingly, allowing cells to have a good state and produce a strong stimulus response even when motivated by larger FSS. HUVECs living on -OH and $-CH_3$ surface slides are in a poor adhesion state, which is unstable and prone to morphological changes. Consequently, the threshold of response will drop, along with the decreased response level generated by FSS enhancement.

$-NH_2$ prompted HUVECs to form a cytoskeleton with better adhesion quality and better adhesion spots, so the initial response threshold of FSS increased likewise. LFSS did not cause a stimulus response in the Glass, nor did it produce one in the $-NH_2$. The range of optimal shear force stimulation was also enlarged, and the response level of HFSS stimulation resembled PFSS.

HUVECs on -OH and CH_3 can down-regulate the initial response threshold of FSS and produce a strong response at LFSS due to the formation of poor adhesion spots and cytoskeleton. At the same time, the optimal FSS threshold was down-regulated to narrow the range of the optimal FSS threshold. Under the gradient FSS stimulation, it was noted that the release response of the -OH group and the $-CH_3$ group manifested the strongest, while the response level gradually lowered against the rise of FSS. Therefore, PFSS is not optimal for the -OH and CH_3 groups, but LFSS is.

By integrating HUVECs response data to different FSS gradients and comparing the combined effects of material surface chemistry, we found that different chemical functional groups on the surface of materials resulted in HUVECs being attached to producing adhesive spots and cytoskeletons of distinct qualities, thus affecting the response threshold and response level of HUVECs to loaded FSS. Adhesion plaques and cytoskeletons on HUVECs may be the coordination site of physical stimulation and material chemical stimulation.

5. Conclusions

In general, -OH, $-NH_2$ and $-CH_3$ material surface chemistry were successfully prepared by SAMs in this study.

By means of influencing the quality of HUVECs adhesion spots and cytoskeleton, the surface chemical functional groups of materials changed the initial threshold of

HUVECs response to FSS stimulation and the FSS threshold of HUVECs response to FSS stimulation, thereby changing the state of HUVECs response to FSS stimulation. HUVECs adhesion plaque and cytoskeleton may be the joint action sites of physical stimulation FSS and material chemical stimulation. These findings provide a possible mechanism by which material chemistry influences HUVECs responses to FSS and may provide guidance for the design of scaffold chemistry and selection of mechanical loading in order to in vitro produce functional tissues.

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