Effects of nitric oxide on ammonia decomposition by hepatocytes under shear stress

Tateki Sumii1,*, Yohei Nakano1, Takuma Abe1, Kazuhiro Nakashima2, Toshihiro Sera2, Susumu Kudo2

1Department of Mechanical Engineering, Graduate School of Mechanical Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan
2Department of Mechanical Engineering, Faculty of Engineering, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395, Japan

Received: 1 December 2015 / Accepted: 3 December 2015
© Japanese Society of Biorheology 2016

Abstract  Nitric oxide (NO) and shear stress modulates hepatocyte functions, including ammonia metabolism. This study investigated the simultaneous effects of NO and shear stress on hepatocyte functions. We developed a cell culture device to simultaneously apply NO and shear stress to hepatocytes, and measured changes in ammonia decomposition by hepatocytes in response to changes in NO concentration and shear stress. NO was supplied directly to cells at a constant rate at 0, 0.5, 5, and 25 ppm, and shear stress was either applied at 0.6 Pa or not (static culture). Ammonia decomposition in static culture was higher under all NO loads compared with 0 ppm NO, and was highest under 0.5 ppm NO and decreased under higher NO loads. In the absence of NO load, ammonia decomposition under shear stress was approximately double that in static culture. Under the simultaneous application of NO and shear stress load, ammonia decomposition under 0.5 ppm NO was approximately twice as high as under 0 ppm NO, but was almost the same under 25 ppm NO as under 0 ppm NO. These results indicate that both NO and shear stress enhance ammonia decomposition although the enhancement depends on the NO concentration in their immediate surroundings.

Keywords  nitric oxide, hepatocytes, shear stress, PDMS, ammonia decomposition

Abbreviations  NO, nitric oxide; L-NAME, L-N-omega-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; PDMS, dimethylpolysiloxane

*E-mail: sumii.t.377@s.kyushu-u.ac.jp, +81-92-802-3080

Introduction

A bioartificial liver acts as a supportive device that either allows a patient’s own liver time to regenerate or bridges the patient’s liver functions until a liver transplant is possible [1]. Bioartificial livers must exhibit good viability and functionality but hepatocytes by themselves do not satisfy these requirements [1]. In order to meet these requirements, important design considerations in liver bioreactor technology include fluidic shear stress (shear stress load), chemical cues, and the number of cell types and ratios (co-culture of hepatocytes with supporting cell types) [2]. Our previous work has shown both ammonia decomposition and nitric oxide (NO) concentration increase under medium flow load in a cultured hepatocyte model [3]. Moreover, ammonia decomposition decreased upon addition of 1-N-omega-nitro-L-arginine methyl ester (L-NAME), which inhibits NO synthesis in co-culture models employing hepatocytes and non-parenchymal cells [3]. These results indicate that ammonia decomposition may be influenced by NO.

NO has many important roles in the liver, including apoptosis inhibition [4, 5], liver regeneration [6], and injury prevention [7]. Sumii et al. have also shown that NO can increase hepatic function [3]. The amino-acid intermediates of the of urea cycle, namely ornithine, aspartate, and arginine, stimulate urea synthesis [8]. Arginine, in particular, is an intermediate metabolite in the NO synthetic pathway that is involved in urea cycle disorder. In contrast, Sesti et al. indicated that exposing hepatocytes to 0.1 mM bradykinin, which stimulates NO synthase (NOS), for 2 h results in a significant decrease in urea production [9]. Furthermore, Sesti et al. showed that L-NAME addition caused urea production to recover to control levels [9]. Based on these
results, NO could have either positive or negative effects on hepatic function. Aharoni-Simon et al. also reported that NO causes apoptotic cell death by different mechanisms, such as by activating kinases, affecting mitochondrial function, and inducing DNA damage. Paradoxically, NO inhibits hepatocyte apoptosis by inhibiting caspase-3 [4, 10–12]. The detailed effects, however, are unclear because the amount of NO generated using arginine [8], bradykinin [9], L-NAME [9], and DETA-NONOate as NO donors [12] may depend on the condition of the hepatocytes. Hence, the NO load applied to hepatocytes using such donor compounds cannot be accurately estimated. We therefore hypothesize that the effects of NO on hepatocytes may be dependent on the NO concentration in their surroundings. We subsequently developed a method to control the NO load on hepatocytes independently of their condition, which may alter the NO concentration in their surroundings.

It is also important to consider the effects of shear stress induced by the flow of culture medium [13]. Miyoshi et al. reported that fetal liver cells are sensitive to medium flow [13]. Moreover, ammonia decomposition by hepatocytes increased under flow load compared with in static culture [3]. Peak albumin production was observed in the culture incubated at 30 rpm (from 0.5 Pa to 3 Pa) between 0 and 120 rpm mechanical stress loading for 6 h [14].

In this study, we hypothesized that NO effects on hepatic functions are dependent on the concentration of NO that hepatocytes are subject to, and that the simultaneous application of NO and shear stress induces further effects on the improvement of hepatic functions. We investigated the effects of varying NO concentration while cells are under shear stress and in static culture using a new device to control the NO concentration and shear stress applied to cells. Shear stress is generally induced by medium flow; however, NO is oxidized immediately in the culture medium before reaching the cells. Therefore, our device can apply NO and shear stress on cells independently before NO is oxidized in the culture medium.

Materials and Methods

NO-loading device design

We developed a new cell culture device for simultaneously loading specific NO concentration and shear stresses. Figure 1 shows a schematic and images of the experimental system and the cell culture device. The medium flow through the device was induced by a pump, and the total volume of medium was 17 mL. The device was placed between the pulse damper and the flow reservoir, which were used to reduce pulsation and inspire O2 into the medium, respectively. Both the medium flow reservoir and pulse damper were warmed to 37°C using a water bath covered with a plastic cover. The device consists of three parts: a ring, a dimethylpolysiloxane (PDMS) disk, and a retainer plate (Fig. 1b and 1c). The eight cylindrical NO gas channels were placed in the PDMS (Dow Corning Toray, Japan) disk, on which cells were cultured and exposed by shear stress caused by the flow of culture medium (Fig. 1d). Thus, NO gas was expected to be transported through the PDMS membrane and delivered to the cells directly without oxidation of NO due to the presence of oxygen in the medium (Fig. 1e). The disk was sandwiched between a ring and retainer plate.

Flow load and NO load application

Cells were cultured under applied flow load for 24 h. The flow channel was restricted to 0.3 mm (height) × 12 mm (width) × 30 mm (length) with a silicon gasket (Fig. 1d and 1e). Three-dimensional simulation of hepatic portal vein has calculated shear stress to be ~0.6 Pa [15, 16], while ~0.5 Pa shear stress improved hepatic functions in a reactor of bioartificial liver [17]. In this study, therefore, shear stress was applied at either 0.6 Pa to mimic blood flow in the liver, or at 0.008 Pa as under static culture conditions. NO gas was supplied from a NO gas bottle (Air Liquide, Japan) containing a mixture of NO and N2. NO was applied at either 0 ppm which is 100% N2 gas, 0.5, 5, or 25 ppm concentration in addition to N2; the gas bottle pressure was 0.3 × 106 Pa.

NO concentration measurement

To validate the NO concentration transported through the PDMS, we measured the NO concentration in phosphate buffered saline (PBS; Nissui Pharmaceutical, Japan) after 24 h exposure of 25 ppm NO gas. This measurement was performed under no cell-culture. After each experiment, all the PBS (17 mL in volume) was collected from the device, including the tubes, flow reservoir, and pulse dumper. The NO concentration in a 0.1 mL sample of the collected PBS was measured using a NO2/NO3 Assay Kit-FX (Dojindo, Japan), so the measured NO concentration was the average value in the PBS circulating throughout the device. This kit can measure NO2 concentrations ranging from 1 μM to 10 μM. The NO2 concentration was determined with a microplate reader (Bio-Rad, USA) because NO3 molecules in the culture medium were reduced or oxidized to NO2.

Cell culture

Rat hepatocytes (RHT33; RIKEN, Japan) were seeded on PDMS dishes at a density of 1.5 × 107 cells/dish and grown to confluence. Our device was coated with collagen type I (BD Falcon, USA) for culturing. Hepatocytes were cultured in Dulbecco’s modified Eagle medium (DMEM; Invitrogen, USA) containing 10% fetal bovine serum (FBS; Biological Industries, Israel) and 1% penicillin/streptomycin (Wako,
Japan). All cultures were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

**Observation of cell condition and determination of cell viability**

Cells were imaged with a light microscope (Nikon, Japan) before and after application of the appropriate treatment, to assess their condition, and cell viability was measured after treatments. Hepatocytes were removed from PDMS-device after each experiment using 0.05% trypsin EDTA (Invitrogen). Dead hepatocytes were stained with Trypan Blue test (Sigma-Aldrich, USA), and the number of live hepatocytes was counted. We defined cell viability as the ratio of the number of viable cells after each experiment to the number before the same experiment.
Measuring ammonia decomposition by cultured hepatocytes

Ammonia decomposition was used to measure hepatic function because hepatocytes convert harmful ammonia into relatively harmless urea. Cells were cultured in DMEM containing serum, antibiotics, and 1.0 mM ammonium chloride (KOSO Chemical Company, Japan). The absorbance of ammonia in 0.5 mL medium was calibrated using the Ammonia Test Wako kit (Wako) and a microplate reader (Bio-Rad). The amount of ammonia decomposed was defined as the difference in ammonium concentration in DMEM measured before and after each experiment. This kit can measure ammonia concentrations ranging from 4.1 μM to 470 μM.

**Statistical analysis**

Data are presented as means ± standard deviations. The statistical significance of our experimental observations was determined by one-way analysis of variance (ANOVA) followed by the Scheffe test, with the significance set at $P < 0.05$. Descriptive statistical analyses were performed using Office Excel 2010 (Microsoft, USA).

**Results & Discussions**

**NO device development**

In this study, hepatocytes were simultaneously exposed to NO gas and shear stress. The hepatocytes were cultured on a PDMS membrane in which NO channels were placed, and the cells were loaded directly by shear stress of the medium flow (Fig. 1e). In the past, arginine, bradykinin, and NO donors were commonly used to study the role of NO in hepatic metabolism [8, 9, 12]. However, these methods cannot accurately estimate the amount of NO loaded on cells directly, and NO gas produced by these compounds may be oxidized before NO gas reached to cells. In contrast, in this study, NO was diffused through the PDMS and loaded on to cells on PDMS directly before oxidation by medium. Moreover, NO gas bottles were used as the NO source. Therefore, in this study, the hepatocytes were exposed constantly and directly for 24 h to both NO and shear stress.

Since the NO gas was transported through the PDMS membrane, the final concentration supplied to hepatocytes differed from the concentration on the bottle. Thus, we validated the NO concentration supplied to hepatocytes under each condition numerically. We assumed the temperature to be 37°C and calculated the NO concentration in the medium [18]. Briefly, NO passed through the PDMS membrane was oxidized to NO$ _2$ by O$_2$ in the culture medium, and the oxidation can be simplified to

\[
4\text{NO} + \text{O}_2 + \text{H}_2\text{O} \rightarrow 4\text{NO}_2 + 4\text{H}^+ \tag{1}
\]

The concentrations of NO and NO$_2$ in the bulk medium are denoted as $C_j(t)$ for gas species $j$, and calculated as shown in Eq. (2) and (3).

\[
\frac{dC_{\text{NO}}}{dt} = \frac{k_{\text{NO}} \cdot A}{V} \left( (a \cdot P_{\text{NO}} - C_{\text{NO}}) - 4 \cdot k_1 \cdot C_{\text{NO}}^2 \cdot C_{\text{O}_2} \right) \tag{2}
\]

\[
\frac{dC_{\text{NO}_2}}{dt} = 4 \cdot k_1 \cdot C_{\text{NO}}^2 \cdot C_{\text{O}_2} \tag{3}
\]

where the relevant rate constant ($k_1$) is $2.4 \times 10^6$ mol$^{-2}$·L$^{-1}$·s$^{-1}$, the liquid-phase mass transfer coefficient for NO ($k_{\text{NO}}$) is $1.11 \times 10^{-5}$ m$^2$/s, and the aqueous solubility for NO ($a$) is $1.5 \times 10^6$ mol·L$^{-1}$·Pa$^{-1}$ [18]. In this study, the partial pressure for NO ($P_{\text{NO}}$) is $0.3 \times 10^5$ (0, 0.5, 5, or 25 ppm). Pa, the surface area of the PDMS membrane ($A$) is $360 \times 10^{-6}$ m$^2$, the total liquid volume ($V$) is 17 mL, and $C_{\text{O}_2}$ was $210 \times 10^{-6}$ mol/L constantly, because O$_2$ was constantly discharged into the culture medium to the point of saturation. These equations were solved numerically by using Scilab (Digiteo, France), and $C_{\text{NO}}$ and $C_{\text{NO}_2}$ at $t = 24 \times 3600$ sec under 25 ppm NO were $0.09 \times 10^{-6}$ mol/L and $0.85 \times 10^{-6}$ mol/L, respectively, and the total NO concentration was $0.92 \times 10^{-6}$ mol/L. We measured the NO concentration in medium with a NO$_2$/NO$_3$ Assay Kit-FX (Dojindo, Japan) after 25 ppm NO gas was loaded to hepatocytes for 24 h experiment, as described in Materials and Method. The measured value was $(1.32 \pm 0.18) \times 10^{-6}$ mol/L (n = 9), close to the calculated value. With regard to other NO concentrations (0.5 and 5 ppm), however, the NO concentrations were undetectable experimentally because initial NO concentrations were too small. Theoretically, stable NO concentration in the bulk medium is determined by the liquid-phase mass transfer coefficient for NO, the aqueous solubility for NO, the partial pressure for NO, and the device geometry. Our result suggested that we could estimate and control the NO concentration delivered directly to hepatocytes with our device, when the concentration in the gas bottle was 0.5 ppm, 5 ppm, as well as 25 ppm.

**Effects of NO and shear stress on cell condition and viability**

We observed no significant difference from the control in cell condition and cell detachment under 0.6 Pa shear stress and 5 ppm NO load (Fig. 2a and 2b). We also observed no significant differences in cell viability under all experimental conditions (Fig. 2a). In particular, we found that cell viability was similar to the control, even under 25 ppm NO and 0.6 Pa shear stress load applied on hepatocytes. A previous study [9] found that the viability of hepatocytes following 2 h of incubation in 0.1 mM bradykinin is similar to that of untreated control cells; however, viability was significantly reduced compared to that of control cells by cell viability test after 24 h despite medium refreshment.
following 2 h of treatment with 0.1 mM bradykinin. This reported decrease in viability was significantly reduced by simultaneous treatment with L-NANE even when viability was still lower than that of the control cells. The authors of that previous study suggested that the observed cell toxicity following bradykinin treatment depended on exposure to increased levels of NO and that these cytotoxic effects of bradykinin were opposed by L-NAME [9]. To effectively determine if NO exerts positive or negative effects on hepatocytes, the direct control of NO levels is important to maintain high cell viability. In our device, NO is supplied from a NO bottle and fed to cells directly through PDMS. Hence, our device can control the NO concentration loaded both constantly and quantitatively, making it suitable for experiments involving long-term exposure of cells to NO.

Effects of NO on ammonia decomposition by cultured hepatocytes

Our results showed that NO loading affects ammonia decomposition (Fig. 3). The magnitude of ammonia decomposition was higher than the control at all NO concentrations tested. In particular, ammonia decomposition was greatest under 0.5 ppm NO. Ammonia decomposition under 0.5 ppm NO was approximately 2-fold greater than that under 0 ppm NO. Under this NO load, $C_{NO}(t)$, the concentration of NO that the hepatocytes were exposed to, was about $2 \times 10^{-9}$ mol/L, which is close to the levels of free nitric oxide ($3 \times 10^{-9}$ mol/L) in blood plasma [19]. Furthermore, the amount of ammonia decomposition decreased as the NO concentration exceeded 0.5 ppm NO, and there was a significant difference in ammonia decomposition measured under 0.5 ppm and under 5 ppm.

Nagao et al. have previously reported that the rate of urea
Fig. 3 Effects of NO load on ammonia decomposition by hepatocytes in static culture after 24 h. *, † P < 0.05 compared with decomposition measured under 0 and 5 ppm NO.

synthesis by hepatocytes cultured with 0.2 mM arginine for 12 h was higher than the control [8]. They proposed that this increase might be induced by NO since NO is produced by the oxidation of L-arginine. NO therefore has positive effects on hepatic function. Conversely, in comparison to the control, treatment with 0.1 mM bradykinin significantly decreased urea production while treatment with 0.1 mM bradykinin and L-NAME did not [9]. These results indicate that NO produced by hepatocytes reduces urea production. In particular, in comparison to the control, only the cell viability measured after 24 h of incubation in 0.1 mM bradykinin indicated any significant difference; cell viability measured after 2 h incubation did not differ significantly from the control [9]. These studies suggested that the decrease in hepatic functions is related to cell function but not to cell viability. Although these previous studies reported that there are positive and negative effects on hepatic functions, our results showed that NO increased hepatic function compared to the control within the NO concentration range tested and that NO effects are dependent on the NO load that cultured hepatocytes are subject to.

Another study reported that a significant increase in urea synthesis was observed only after a 2 h treatment with 10 μM of the NO donor ((±)-(E)-4-ethyl-2-{[(E)-hydroxyimino]-5-nitro-3-hexenamide; NOR-3). A significant decrease in urea synthesis was also observed after 2 h of treatment with 100 or 150 μM NOR-3 [20]. Moreover, they also reported a significant decrease in cell viability 2 h after treatment with 50, 100, or 150 μM of NOR-3, and that this decrease was more marked 24 h after treatment [20]. Hence, the reduction in urea synthesis might be related to a reduction in cell viability.

In this study, NO was supplied to hepatocytes constantly for 24 h and there are no significant differences in cell viability. Nevertheless, ammonia decomposition was greatest at 0.5 ppm NO. The amount of decomposition was still higher for cells cultured under 25 ppm NO than the control even though decomposition under 25 ppm was significantly less than under 0.5 ppm. We speculated that NO might have positive effects on hepatocytes if the cell viability is sufficiently high, and that such positive effects might be enhanced by low NO concentrations. In a previous study, treatment with 0.01 mM bradykinin did not result in an increase in NO production compared to the control [9]. Our device can supply any low NO concentration to hepatocyte directly through PDMS before oxidization in the culture medium. Moreover, our device is useful for determining the effects that NO has on hepatic function.

Based on our findings, we propose the following mechanism to explain how low NO concentrations increase the rate of urea synthesis. We believe that NO synthase (NOS) activity during the urea cycle is influenced by NO concentration [20]. When NO is synthesized from arginine by the NO synthase (NOS) reaction, citrulline is formed; however, urea is also synthesized from arginine. Thus, the NOS reaction allows for the urea cycle to be bypassed [21]. In another study [20], when hepatocytes were exposed to a low concentration of NO (10 μM NO-donor) for 2 h, cell damage was not induced and the level of urea synthesis increased. When the hepatocytes were subjected to high NO concentrations (100 and 150 μM NO-donors), however, cell damage was observed and the level of urea synthesis decreased. Therefore, they hypothesized that a low concentration of NO inhibits cell damage and that NOS activity allows for the urea cycle to be bypassed. Furthermore, this process induces an increase in urea levels. In contrast, a high concentration of NO induces cell toxicity, reduces cell viability, and induces the bypassing of the urea cycle due to NOS activity. Therefore, a low concentration of NO may inhibit bypassing of the urea cycle from arginine to citrulline, consequently leading to an increase in urea levels.

Effects of NO in the presence of shear stress on ammonia decomposition by cultured hepatocytes

The variation in the amount of ammonia decomposition with NO load under shear stress is shown in Fig. 4. In the absence of NO loading (0 ppm NO), applying a shear stress of 0.6 Pa to hepatocytes caused an approximately 2-fold increase in ammonia decomposition compared with a shear stress of 0.008 Pa. Previous studies have also investigated the effect of applying shear stress on hepatocytes. Kan et al. observed enhanced rates of ammonium removal in a coculture system for hepatocytes with non-parenchymal cells under 0.5 Pa shear stress [17]. Additionally, they found that aggregates of hepatocytes formed abundantly in the perfusion system and that the hepatocytes developed a cuboidal shape [17]. Based on these observations, they hypothesized that medium perfusion affects the function and morphology of hepatocytes in the co-culture system [17].

In a separate study, Torii et al. reported that albumin production by liver tissue was significantly higher under shear stress of 30 rpm (0.05 to 2 Pa) than under 120 rpm
and 0 rpm (static culture) after 6 h and 12 h treatments of shear stress load [22]. They also reported that the liver structure when either not exposed to shear stress at all or when exposed to high shear stress (120 rpm) was destroyed during early stages of incubation [22]. These results indicate that there may be a relationship between hepatocyte morphology induced by shear stress and the levels of hepatic functions. Moreover, Miyazawa et al. observed that albumin production reached a peak in a culture incubated under a mechanical stress of 30 rpm (0.5 to 3 Pa) applied for 6 h. They also found that albumin production by hepatocytes was greater under mechanical stress at 30 rpm than in stationary culture even up to 36 h of stress application [14]. Additionally, they reported that actin filaments polymerized around the periphery of hepatocytes placed under mechanical stress, enabling the hepatocytes to adhere to one another and aggregate into large spheroids [14]. Miyazawa et al. therefore proposed that mechanical stress induces the polymerization of actin filaments, thereby stimulating hepatocyte aggregation and improving hepatocyte specific function [14].

McCarty et al. also reported that albumin secretion by cultured hepatocytes increases steadily if an ultrathin, pure collagen nanolayer is deposited on top of the cells, reaching a plateau on day 10 of culture; without this top matrix, albumin secretion by hepatocytes apparently remains low [23]. Moreover, the actin filaments in seeded hepatocytes are diffuse or radially oriented around the nucleus in the absence of a top matrix layer; however, actin fibers are reorganized along the cell-to-cell borders in hepatocytes covered with the ultrathin collagen layer [23].

Based on these previously reported results, we hypothesized that the alteration of hepatocyte morphology and actin polymerization by shear stress may be accompanied by an increase in ammonia decomposition. We therefore measured the effects of simultaneous shear stress and NO loads on ammonia decomposition by cultured hepatocytes. We found that ammonia decomposition was higher under all NO concentrations tested when hepatocytes were cultured under 0.6 Pa shear stress compared with hepatocytes cultured in the absence of a NO load under the same shear stress (P < 0.05) (Fig. 4). This result suggests that simultaneously applying a NO load and 0.6 Pa shear stress on hepatocytes had a compounding effect that enhanced ammonia decomposition. The amount of decomposition, however, decreased as the NO load was increased, meaning that it was greatest under 0.5 ppm NO and lowest under 25 ppm NO. In fact, in the presence of 0.6 Pa shear stress, the amount of ammonia decomposition measured under 25 ppm NO was similar to that in the absence of NO load (0 ppm NO) (Fig. 4). This result indicates that NO concentrations above 25 ppm may not increase hepatic metabolism when 0.6 Pa shear stress is applied. A possible explanation is excessive NO concentration resulting from NOS. Takagi et al. suggested that hepatocytes generate NOS [24], and our previous study found that 0.6 Pa shear stress increased NO production by hepatocytes [3]. Therefore, in our experimental system, hepatocytes may produce NO in response to the 0.6 Pa shear stress applied, so that 25 ppm NO may exceed the NO concentration limit for increasing hepatic metabolism in the presence of 0.6 Pa shear stress.

Endothelial cells, not hepatocytes, are under shear stress in vivo. However, hepatocytes are exposed to NO produced by endothelial cells, and their function could be improved [3]. Previous studies have investigated the relationship between hepatic function and shear stress because hepatocytes are directly exposed to shear stress of medium flow in a bioartificial liver (BAL), and reported that the hepatic function was improved in BAL [13, 17]. Furthermore, using hepatocytes in a culture dish, some studies have investigated the relationship between shear stress and improvement of hepatic function [14, 22]. On the other hand, based on the previous studies about relationship between NO and the hepatic function, improvement of hepatic function may depend on NO concentration [9, 20]. However, it is difficult to control the NO concentration from endothelial cells under shear stress only. In this study, therefore, we hypothesized that NO effects on hepatic functions are dependent on the concentration of NO that hepatocytes are subject to, and that the simultaneous application of NO and shear stress can induce further improvement of hepatic function. We investigated the effects of varying NO concentration while cells are under shear stress and in static culture using a new device to control the NO concentration and shear stress applied to cells. Shear stress is generally induced by medium flow; however, NO is generally oxidized immediately in the culture medium before reaching the cells. Our device can apply NO and shear stress on cells independently before NO is oxidized in the culture medium. Our results show that both NO and shear stress enhance ammonia decomposition, and the enhancement depends on the
NO concentration in their immediate surroundings, indicating potential to improve hepatic function in bioartificial liver.

In summary, we have developed a new device to simultaneously apply specific NO concentration and shear stress to hepatocytes. We have also found that NO increased hepatic function, as measured by ammonia decomposition, both in the presence and absence of shear stress; however, the magnitude of the increase is dependent on NO concentration. Our data highlights the importance of controlling the NO concentration quantitatively when studying the relationship between NO concentration and hepatic function. Moreover, the effects of NO may depend on the culture environments such as the presence or absence of shear flow.

Acknowledgements This study was supported in part by a Grant-in-Aid for JSPS Fellows (255172). We would like to thank Hiroshi Sakamoto and Shinichiro Doi for their technical assistance.

References