Final Report (October 1, 2016 – September 30, 2019)

Examination of the possible mechanisms involved in the delayed antiarrhythmic effect of sodium nitrite

Introduction

It is established that organic nitrites and nitrates protect against the ischemia and reperfusion (I/R) injuries, including the suppression of the generation of severe ventricular arrhythmias [1]. Our previous results showed that sodium nitrite ($NaNO_2$), in a concentration of 0.2 μmol kg^{-1} min^{-1}, markedly suppressed the incidence and severity ventricular arrhythmias when it was administered either just prior to and during ischemia [2] or 24h before the I/R insult [3].

There are some other *in vivo* and *in vitro* studies, which showed the late cardioprotective effect of $NaNO_2$ [4, 5]. For example, it has been found that $NaNO_2$ administered in rats, 24h prior to I/R, reduced myocardial infarct size and hepatic reperfusion injury [4]. This study pointed out that the nitrite mediated delayed protection involved similar mechanisms to the acutely administered nitrite therapy; i.e. S-nitrosylation of mitochondrial proteins and preservation of mitochondrial function during reperfusion injury play a mandatory role in the protection [4]. This hypothesis was supported by our previous studies, showing that following the acute administration of nitrite elevated the SNO level of certain mitochondrial proteins [2]. However, we have not had any information which mechanisms are involved in the delayed phase of the nitrite-induced protection, particularly against arrhythmias; i.e. how nitrite induces protection 24h after its administration?

Therefore, the aim of the grant during the last three years period (2016-2019) was to examine the mechanisms involved in the delayed cardioprotective effect of sodium nitrite. In this regard, the following mechanisms were examined; (1) the role mitochondrial structural and functional changes, (2) changes in the mitochondrial and calcium homeostasis (3) whether nitrite itself, or after converting to NO, would act as a trigger for the protection, and (4) the possible modulator role of nitrite on cardiac gap junctions. All these mechanisms may influence the generation of arrhythmias resulting from acute ischaemia and reperfusion. The examination of changes in calcium homeostasis was not part of the research proposal originally, but it was included later because of the potential contribution of calcium overload to arrhythmia generation.

I. Examination of the role of mitochondria in the delayed antiarrhythmic effect of nitrite administration.

The idea to examine the delayed phase and the involved mechanisms of the protective effect of nitrite comes from studies of Shiva and colleagues [4]. They suggested that the administration of $NaNO_2$ induces delayed protection, and this effect occurs at the mitochondrial level. It is well known that the

damage of the mitochondrial electron transport chain during ischaemia contributes to the reperfusion injury by generating the burst of reactive oxygen species [6]. They have proposed that the stable S-nitrosylation of complexI at specific cysteine residues attributes to the late protection of nitrite by decreasing the ROS producing activity of the mitochondrial electron transport chain. This protection occurs shortly after the nitrite treatment and it remains stable for 24h [4].

Starting from the assumptions that the target of the delayed cardioprotective effect of nitrite might be a mitochondria mediated process, in the first year of the project we designed studies in which changes in mitochondrial morphology and respiratory function were examined in dogs undergoing an I/R insult, 24h after nitrite administration.

Methods: To examine this, four experimental groups were established from thirty dogs. On day one, 15 dogs received saline, and another 15 dogs an intravenous injection of $NaNO_2$ (0.2 μmol/kg/min) for 20 min. Twenty-four hours later, 10 control (IC) and 10 nitrite ($NaNO_2$+I/R) treated dogs underwent a 25 min period of occlusion of the left anterior descending coronary artery (LAD) followed by a rapid, 2 min reperfusion. Five nitrite ($NaNO_2$) and five saline (SC) treated dogs, underwent the same surgical interventions, but no I/R was applied. At the end of the experiments, myocardial tissue samples were taken for in vitro analyses; TEM (transmission electron microscopy) analysis was performed to assess the mitochondrial morphology and the size (area, perimeter) or the shape (roundness and circularity) of the mitochondria were calculated.

To examine the functional changes of the mitochondria, the CI (ComplexI) and CII (ComplexII)-dependent respiration was examined by Clarke-type O_2 electrode. For the respiratory parameters, the basal respiration (State2), the active respiration (OXPHOS; State3) rate, the capacity of the inhibition of OXPHOS (State4) and the electron transport system (ETS) were measured. From these measured parameters, the respiratory control ratio (RCR= OXPHOS/State4) and the P/E coupling control ratio (OXPHOS/ETS) were calculated. RCR indicates the coupling between oxygen consumption and oxidative phosphorylation and the P/E control coupling ratio is a measure of the limitation of OXPHOS capacity by the phosphorylation system. The ATP production rate was also assessed by bioluminescence assay.

The level of tissue superoxide was determined by the DHE (dihydroethidium) staining of 20μm thick myocardial tissue sections and the peroxynitrite production was assessed by measuring the 3-NT (3-nitrotyrosine) level with Western Blot.

Results: The *in vivo* results showed that $NaNO_2$, administered 24h before I/R, significantly attenuated the severity of ischaemia, and markedly reduced the number and incidence of ventricular arrhythmias [7]. From the *in vitro* experiments, the results of the TEM measurements showed that nitrite significantly attenuated the I/R-induced structural alterations of the mitochondria, such as

reductions in the mitochondrial area, perimeter and Feret diameter were all improved, whereas the mitochondrial roundness reverted to its original shape [7].

In our dog model, compared to the SC group, a 25 min ischemia and 2 min reperfusion resulted in a significant reduction in the CI (24%), and also in the CII-supported OXPHOS, a decrease in ATP and an increase in superoxide productions. Furthermore, the P/E coupling ratio was similar to the SC controls, suggesting that such a period of I/R inhibits the respiratory complexes of the ETS, and consequently, increases the generation of ROS [7].

In contrast, nitrite itself, and following an I/R insult, substantially reduced mitochondrial respiration. There was a marked decrease in the CI-dependent OXPHOS (48% vs. 24% in the IC group), in RCR and in the P/E coupling ratio [7]. The decrease in P/E indicates that nitrite affects the phosphorylation system, and that the reduction in the CI-dependent OXPHOS would result from the modification of the phosphorylation system rather than of the other ETS members. We assume that nitrite might act on one of the components of the phosphorylation system, for example, the ATP synthase, the phosphate transporter or the ADP/ATP translocator ANT [7].

In summary, the results of this study confirm that the administration of $NaNO_2$ provides protection against the ischaemia and reperfusion-induced severe ventricular arrhythmias, 24h later. We have now shown that this protective effect may involve, among a number of other NO-dependent effects, changes in mitochondrial morphology and function. Nitrite prevents the I/R-induced structural alterations of the mitochondria, and most probably by interfering with the phosphorylation system, inhibits the ROS producing components of the ETS thereby reducing the ROS formation during the early phase of reperfusion [7]. These results were published in Frontiers in Pharmacology in 2018 [7].

II. Changes in calcium homeostasis in the delayed antiarrhythmic effect of sodium nitrite

It has been well known that, apart from the oxidative stress products, changes in the intracellular calcium homeostasis are also implicated in the generation of severe ventricular arrhythmias resulting from I/R [8]. There is increasing evidence for the interplay between calcium and oxidative stress products [9]. ROS can modulate calcium release/uptake by modifying proteins of calcium channels and transporters, whereas calcium can regulate mechanisms associated with oxidative stress [9]. Since calcium channels and transporters are also redox sensitive, accumulation of ROS is directly responsible for the I/R-induced calcium overload [10].

Starting from these abovementioned facts, for the second and third year of the research period we planned to examine whether the changes in calcium homeostasis may play a role in the $NaNO_2$-induced delayed cardioprotective effect of sodium nitrite.

Methods: To examine the role of calcium movements in the late occurring effect of $NaNO_2$, 30 dogs of both sexes were used and divided in four experimental groups. In two groups, either saline (I/R=4) or $NaNO_2$ (0.2 μmol/kg/min) was administered intravenously for 20 min (NaNO2+I/R=6) and 24h later, these dogs were subjected to a 25 min ischaemia followed by a 2 min reperfusion. In the other two groups, either saline (SC=10) or $NaNO_2$ (NaNO2=10) was infused, and the experiments were terminated without I/R, 24h later. At the end of the experiments, tissue samples were taken for *in vitro* analyses. The cellular and mitochondrial calcium content were measured by TEM analysis.

To measure the tolerance of the mitochondria to Ca^{2+}-overload, mitochondria were isolated from sham and nitrite treated animals (without I/R), and Ca^{2+}-overload was induced by consecutive injections of 30 μM $CaCl_2$. The respiratory response was assessed by Clark-type O_2 electrode. The susceptibility of mitochondrial permeability transition pore (MPTP) opening, also called calcium retention capacity or CRC, were measured using a calcium sensitive fluorescent dye, Calcium-Green 5N.

Cardiomyocytes were also isolated from the SC and $NaNO_2$ groups to assess cellular calcium transients in response to stimulated ischaemia. For these experiments, an 18 min of stimulated ischaemia was applied followed by a 10 min reperfusion period. The cells were loaded with the Ca^{2+}-sensitive fluorescent dye, Fluo-4-AM, and were stimulated at a constant frequency of 1 Hz. Changes in parameters of Ca^{2+} transient were characterized by the emitted fluorescence intensity at 535 nm wavelength. Diastolic $[Ca^{2+}]_i$ levels were determined immediately before the trigger stimulus. Systolic $[Ca^{2+}]_i$ was determined at the peak of the corresponding transient. The rise to peak parameter determines the elapsed time between the end-diastolic (or trigger) and maximal peak value. In parallel, the half relaxation time measures the time starting from the maximum peak value until the fluorescence intensity is halved.

To determine the action potential (AP) changes 24h after nitrite administration, ventricular papillary muscles were isolated from the SC and $NaNO_2$ groups. Stimulated ischaemia was induced for 25 min, followed by 10 min reperfusion. The preparations were stimulated with constant current pulses of 2 ms duration at a rate of 1 Hz and various AP parameters, were recorded such as APD25, 50, 90 (APD: action potential duration); AMP: amplitude, Vmax: maximum upstroke velocity; RMP: resting membrane potential; CT: conduction time; ERP: effective refractory period.

Results: The Ca^{2+}-transients showed that, compared to the SC, stimulated ischaemia significantly reduced the amplitude of the calcium transient, increased the diastolic calcium level and the time to the peak value, without substantially modifying the half relaxation time. In contrast, the amplitude of the calcium transient in the nitrite-treated dogs was significantly less than in ischemic controls and

the time to the peak value was also prolonged in samples of the nitrite treated dogs. Following reperfusion, the cardiomyocytes isolated from the nitrite treated dogs showed a better return to the baseline values than the ischaemic, untreated controls. Furthermore, the diastolic calcium level was significantly less in the nitrite treated cells than in the ischaemic control ones. These results suggest that nitrite is able to attenuate the I/R-induced calcium overload, even 24h after its administration.

The attenuation effects of nitrite on the cellular calcium transients are also supported by the TEM measurements, which showed a reduced the number of calcium deposits both in the intracellular space and within the mitochondria in the nitrite treated samples compared to the sham controls.

The AP recordings showed that $NaNO_2$, administered 24h prior to simulated ischaemia, significantly prolonged the ERP/APD ratio compared to the ischaemic controls without affecting the other AP parameters. This indicates that nitrite prolongs the ERP duration which might also contribute to the antiarrhythmic effect of nitrite.

Furthermore, our current results indicate that nitrite treated mitochondria, when exposed to calcium overload, showed an increase in CRC, and the mitochondrial respiration stopped at higher calcium concentrations than the control mitochondria. These results suggest that $NaNO_2$ delays the deterioration of mitochondrial function under conditions of calcium overload, most probably by delaying the opening of MPTP [11] and therefore, attenuates the harmful consequences of calcium overload. This is particularly important during reperfusion, when calcium overload occurs and have substantial role in the generation of fatal reperfusion-induced ventricular arrhythmias.

In summary, we propose that nitrite influences the calcium homeostasis by reducing the harmful consequences of calcium overload under pathologic conditions. This effect may come from direct regulatory effects of nitric oxide on ion channels and transporters that are responsible for calcium transport, but, as the results suggest, nitrite can modulate calcium homeostasis indirectly, by reducing the mitochondrial ROS formation.

The results of this study were presented at conferences [12, 13] and a manuscript was submitted to the International Journal of Molecular Sciences.

III. Examination of the conversion of nitrite to NO following sodium nitrite infusion

This part of the experiments has attempted to test the hypothesis as to whether nitrite can convert to NO under physiological conditions, and then, in turn, would be able to induce S-nitrosylation of those mitochondrial proteins which are known to be important in the nitrite induced cardioprotection [4]. The conversion of nitrite to NO can be induced by the reaction of nitrite with deoxy-Hb to form met-Hb and NO, which explains the hypoxic generation of NO from nitrite [14]. However, there is also evidence that in the absence of hypoxia, hemoglobin may act as a nitrite reductase [14] making possible the nitrite-NO conversion even in the absence of ischemia. Taking into consideration the abovementioned facts, we designed studies in which the time course changes of nitrate/nitrite (NOx) levels and S-nitrosothiol formation of proteins (RSNO) in blood samples were examined.

Methods: For these experiments, blood samples were taken from those groups which had been used at the calcium homeostasis studies (Paragraph II, $NaNO_2$ group). Blood samples were taken before and at the end of nitrite administration (at 0 and 20 min), and then 15, and 30 min, as well as 1, 3, 6, 12 and 24h after the injection of nitrite (0.2 μmol/kg/min, i.v.). For the NOx measurements, rat samples were also used with the same sampling protocol. Using two species for the NOx measurements, we were able to compare the kinetic changes of nitrite conversion between two species with two different metabolism rates.

The changes in nitrite/nitrate (NOx) levels were determined by the Griess reaction and the RSNO levels of the plasma proteins were assessed by the Saville reaction.

Results: The infusion of $NaNO_2$ significantly increased the plasma nitrite, nitrate and the NOx levels, immediately after the administration of $NaNO_2$ (Fig1). Both in the rat and dog samples the level of nitrite reached the maximum values at the end of the infusion period, but it has started to decrease already 15 min after stopping the infusion (Fig1). In contrast, there was a significant increase in the NOx levels from 15 min after the cessation of the nitrite infusion. This increase in NOx resulted primarily from the marked elevation in the nitrate levels. For the rat samples, we have seen the same changes for the nitrite-nitrate conversion during the examined 24h period (Fig1B).

We concluded from these results that under oxygenated conditions nitrite converts to nitrate rather quickly, (within 15 min) in both species. This is probably due to the quick oxidation process of nitrite to nitrate under normal physiological conditions. Since, the nitrate levels remained significantly high compared to the baseline values even 24h after of its administration, this might indicate that the nitrite is available in nitrate form for a longer time period both in dogs and rats and nitrate will be the primary source for nitrite and NO when the animals are subjected to ischaemia. These results are in accordance with our previous findings where we obtained elevated nitrate level 24h after the

administration of $NaNO_2$ in dogs [3]. The result of this study clearly revealed that dogs treated with nitrite, might use nitrate as a primary source for nitrite and NO during ischemia [3].

In order to determine, whether nitrite is able to convey protein-SNO during such a short period of time and before its conversion to nitrate, we measured SNO levels of the blood plasma proteins from dog samples. These results showed however, that there was no significant change in the plasma protein-SNO content compared to the baseline values. However, it should be noted that although the administration of nitrite in such a low concentration might induce SNO of the plasma proteins, the Saville reaction might be not sensitive enough to measure these minor changes. Therefore, it would be worth to apply a more sensitive technique, such as the biotin switch method, in order to determine the potential ability of nitrite to induce protein-SNO even under physiological conditions. This warrants further examination.

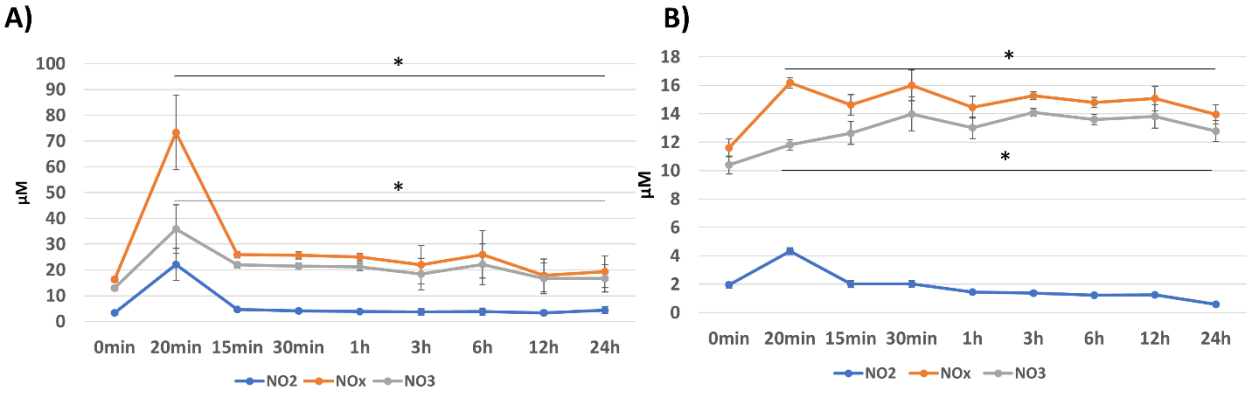

Figure 1.Changes in nitrite (NO2), nitrate (NO3) and NOx plasma levels determined in the venous blood during the administration of sodium nitrite (at 0 and 20 min), and also at 15 and 30 min, as well as 1, 3, 6, 12h and 24h after the infusion of nitrite in blood samples taken from dogs (A) and rats (B).*P < 0.05 compared with baseline.

IV. Examination of the possible modulator role of nitrite on gap junction structure and function

Gap junction-mediated intercellular coupling (GJIC) is essential for the rapid electrical and metabolic communication between adjacent cells. There is strong evidence that ischemia impairs GJIC and leads to GJ uncoupling. For example, it is known that the uncoupling of GJs contribute to the generation of phase 1B arrhythmias that occur after 15 min of a coronary artery occlusion [15]. Although it is well known that GJIC maintains the electro-metabolic communication between adjacent cells, the significance of the metabolic coupling during myocardial ischemia and reperfusion is much less known. During reperfusion, GJIC has been shown to mediate cell-to-cell propagation of

cell death, and there is evidence that this phenomenon explains the continuity of necrotic areas and significantly contributes to the final size of the infarct [15].

We have previous evidence that NO may modulate GJ function [16]. For example, sodium nitroprusside resulted in marked antiarrhythmic effect, which was attributed to the preservation of gap junctional function [17]. Furthermore, a NO donor, SNAP, partially inhibited the hypoxia-induced reduction in Cx43 content and resulted in functional activation of metabolic GJIC [18].

Methods: To examine the coupling of GJs in the delayed cardioprotective effect of sodium nitrite, we examined the phosphorylation level and the content of the main GJ forming protein, Cx43 (connexin43) from those experimental groups which have been used for the calcium homeostasis studies (Paragraph II). In order to compare changes in the phosphorylation status of Cx43 during ischaemia and after reperfusion respectively, two more groups were included. In these groups, the protocol was stopped at the end of the ischaemic period without reperfusion both in the control (I; n=3) and in the nitrite treated dogs ($NaNO_2$+I, n=3).

To examine the possible modulator role of sodium nitrite on metabolic GJIC, the cell scrape dye load method was used on cardiomyoblast (H9c2) cells with LY/RD (LuciferYellow/RhodamineDextrane) fluorescent dyes. Western blot was used to assess the changes in the phosphorylation and total protein content of Cx43.

Results: In the normal, non-ischaemic myocardium, Cx43 is mostly present in phosphorylated form [19-20]. Our results showed that this ratio was shifted towards dephosphorylation (35/65 ± 3%) in the ischaemic dogs and it further but it was slightly increased at the end of the reperfusion (44/56 ± 4%). This result confirms our previous findings that the Cx43 protein is substantially dephosphorylated after an I/R insult [19-20].

The administration of nitrite, however, administered 24h before the occlusion, shifted this ratio toward the phosphorylated form of Cx43 both after occlusion and at the end of reperfusion (51/49 ± 6%) (Fig2A).

The total protein content of Cx43 was not changed in these experimental groups (Fig2B).

There is substantial evidence that GJ function and the normal phospho/dephospho ratio is preserved in various cardioprotective circumstances, such as in the cardiac pacing-induced preconditioning [19]. This protection was associated by the prevention of the dephosphorylation of Cx43, resulting from myocardial ischaemia. In such a case the phospho/dephospho ratio shifts towards the normal, phosphorylated form of Cx43, which might also contribute to an antiarrhythmic effect under ischaemic conditions [19].

The current experiments, however, failed to unambiguously show, whether nitrite directly or indirectly modifies the phosphorylation pattern of Cx43, since the observed phospho/dephospho alterations may also result from the nitrite-induced reduction in ROS levels. The nitrite-induced attenuation of the ROS mediated processes might have an indirect effect on the phosphprylation staus of Cx43. A more direct evidence for the role of GJ in the nitrite-induced late cardioprotective effect would be the S-nitrosylation of Cx43 protein. There is evidence that this posttranslational modification also has a functional effect on the GJ function [21]. It has been reported that the SNO of the cysteine residue 271 on Cx43 increases and denitrosation decreases the permeability of myoendothelial gap junctions [21].

Therefore, in our experiments the SNO level of Cx43 was assessed by the biotin-switch method. The preliminary data in Fig2C shows that, 24h after the nitrite treatment, the SNO level for Cx43 is elevated compared to the untreated controls. This preliminary result shows that nitrite has a direct effect on Cx43, and it is able to induce S-nitrosylation of this protein. Whether this increased SNO level of the Cx43 affects the GJ channel metabolic conductance warrants further experiments where the permeability of the GJ channels induced by S-nitrosylation agents should be monitored.

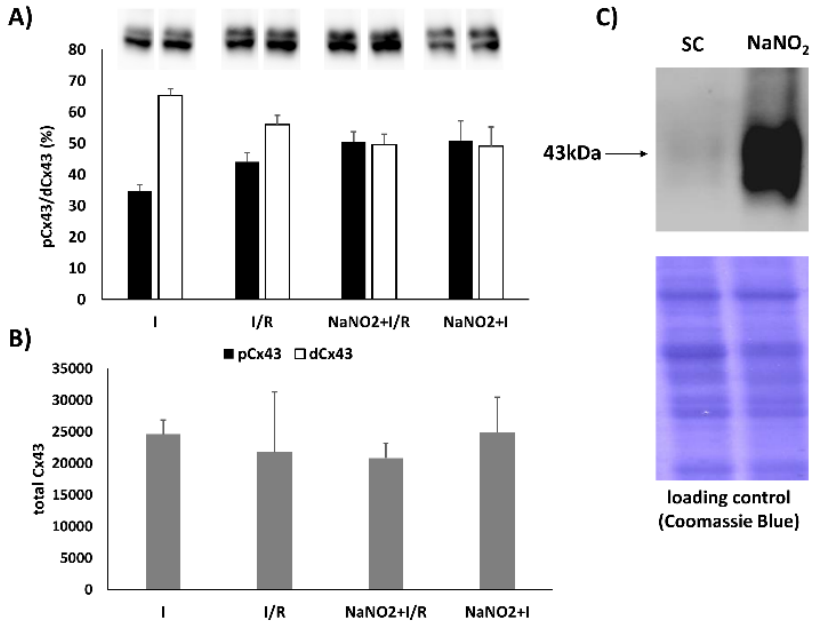

Figure 2. The effect of nitrite on the phospho/dephospho ratio (A) and total protein content of Cx43 (B) ischaemic controls and in the ischaemia and reperfusion dogs (I and IR) as well as the sodium nitrite treated groups with ($NaNO_2$+I/R) or without I/R ($NaNO_2$). P-Cx43/DP-Cx43: phosphorylated and dephosphorylated form of Cx43. The level of Cx43-SNO is shown in (C) 24h after nitrite administration without I/R.

In order to assess the metabolic GJIC in response to nitrite treatment, a cellular model of the popular scrape load/dye transfer technique has been attempted to establish for the rat cardiomyoblast cell line, H9C2. However, the fluorescent dye, LuciferYellow from the FL/RD mixture, failed to spread to the cells from the penetration site created by a scrape wound, indicating no communication between the neighboring cells (Fig3A). In order to explain this phenomenon, the cells were stained for Cx43. Fig3B clearly shows that the cells are able to express Cx43 but only surrounding of the nucleus, and no GJs are formed on the surfaces of the adjacent cells (Fig3B). The absence of mature GJs at the adjacent sites of the H9C2 cells might explain their inability for cell-to-cell communication and this also explains why the LY/RD experiment failed. In order to induce the GJ forming characteristics of these cells, H9c2 was initiated to differentiate towards a cardiac phenotype, when the media serum is reduced in the presence of retinoic acid, creating multinucleated cells with low proliferative capacity but it did not change the results for the LY/RD experiments and these cells also failed to establish GJs at their adjacent surfaces. To establish a cellular model to accurately monitor the metabolic GJIC, perhaps another cell line with pronounced GJs, such as astrocytes should be used in the future.

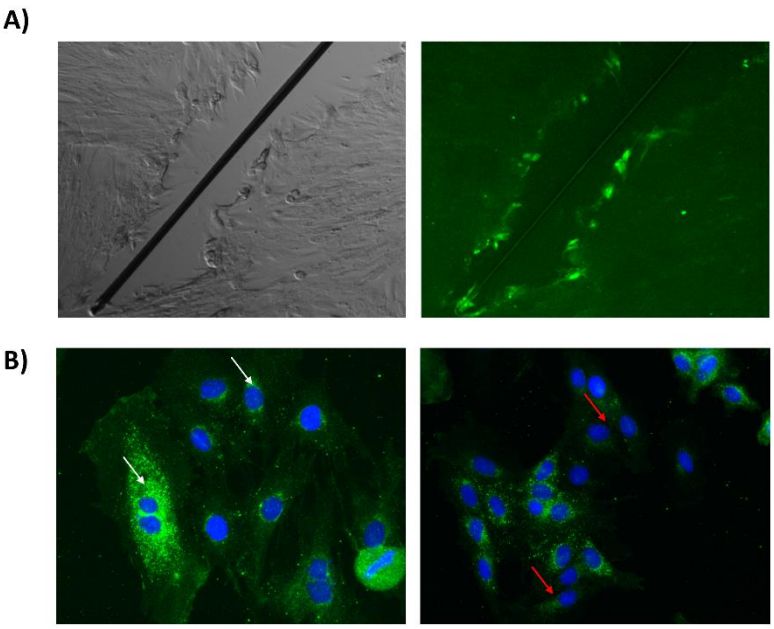

Figure 3. Scrape load/dye transfer experiment on H9C2 cells (A) and Cx43 staining on H9C2 cells (B). The white arrows indicate the hemi-channels surrounding the nucleus while no visible GJs are formed at the adjacent sites of the cells (red arrows).

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