Rapid Communication

Cardiac side effects of RNA-based SARS-CoV-2 vaccines: Hidden cardiotoxic effects of mRNA-1273 and BNT162b2 on ventricular myocyte function and structure

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Short Title: Cardiomyocyte effects of mRNA-1273 and BNT162b2

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Abstract

Background and Purpose

To protect against SARS-CoV-2 infection, the first mRNA-based vaccines, Spikevax (mRNA-1273, Moderna) and Comirnaty (BNT162b2, Pfizer/Biontech), were approved in 2020. The structure and assembly of the immunogen – in both cases, the SARS-CoV-2 spike (S) glycoprotein – are determined by a messenger RNA sequence that is translated by endogenous ribosomes. Cardiac side effects, which for the most part can be classified by their clinical symptoms as myo- and/or pericarditis, can be caused by both mRNA-1273 and BNT162b2.

Experimental Approach

As persuasive theories for the underlying pathomechanisms have yet to be developed, this study investigated the effect of mRNA-1273 and BNT162b2 on the function, structure, and viability of isolated adult rat cardiomyocytes over a 72 h period.

Key Results

In the first 24 h after application, both mRNA-1273 and BNT162b2 caused neither functional disturbances nor morphological abnormalities. After 48 h, expression of the encoded spike protein was detected in ventricular cardiomyocytes for both mRNAs. At this point in time, mRNA-1273 induced arrhythmic as well as completely irregular contractions associated with irregular as well as localized calcium transients, which provide indications of significant dysfunction of the cardiac ryanodine receptor (RyR2). In contrast, BNT162b2 increased cardiomyocyte contraction via significantly increased protein kinase A (PKA) activity at the cellular level.

Conclusions and Implications

Here we demonstrated for the first time, that in isolated cardiomyocytes, both mRNA-1273 and BNT162b2 induce specific dysfunctions that correlate pathophysiologically to

cardiomyopathy. Both RyR2 impairment and sustained PKA activation may significantly increase the risk of acute cardiac events.

Keywords: mRNA-1273; BNT162b2; SARS-CoV-2 spike (S) glycoprotein; ryanodine receptor (RyR2); protein kinase A (PKA); cardiac side effects; cardiac dysfunction

Rationale for rapid communication:

- The present preclinical cardiac safety data point to the need for a reassessment of the risk–benefit ratio of RNA-based SARS-Cov-2 vaccines, given indication of their cardiotoxicity.

What is already known:

- While mRNA-based SARS-Cov-2 vaccines can induce cardiac side effects, the underlying mechanisms are not understood.

What this study adds:

- Both mRNA-1273 and BNT162b2 can induce disturbances to regular contractile function.
- However, the associated mechanisms are functionally as well as pathophysiologically distinct.

Clinical significance:

- The findings support both the diagnosis and treatment of cardiac events following mRNA-based COVID vaccination.
- The results may provide an explanation for persistent cardiac symptoms in connection with post-COVID syndrome.

Introduction

With the approval of the first mRNA-based vaccines, an effective preventive measure against symptomatic infection with SARS-Cov-2 was available just a few months after the start of the pandemic. mRNA technology is based on the concept of relocating the production of specific (foreign) antigens in the body cells of vaccinated persons, which promises to significantly

reduce development and manufacturing times. The structure of the immunogen is determined by the sequence of messenger RNA (mRNA) that is protected from degradation by means of lipid nanoparticles (LNPs) and absorbed by the cells as an LNP-mRNA complex by simple endocytosis (Teo, 2021).

Development, preclinical and clinical safety and efficacy testing, and emergency approval were realized in under 12 months for mRNA-1273 (Spikevax, Moderna) and BNT162b2 (Comirnaty, Pfizer/Biontech), both of which encode the identical SARS-CoV-2 spike (S) glycoprotein. While basic immunization with mRNA-1273 and BNT162b2 efficiently reduced hospitalization and mortality rates associated with SARS-CoV-2, approximately four months after the start of the vaccination campaigns, the incomplete cardiac safety testing and exploration of the adverse event profile resulted in the diagnosis of cardiac side effects, which based on their clinical symptoms have been predominantly classified as myocarditis, pericarditis, or a combination thereof (Butt et al., 2021; Nyberg et al., 2022; Ferdinandy et al., 2019; D'Angelo et al., 2021; Heymans, Cooper, 2022; Sun et al., 2022). It should be noted that cardiac safety issues of many therapeutics appears only in the late phase of clinical developments or during their clinical application, termed hidden cardiotoxicity (Ferdinandy et al., 2019).

Numerous studies have demonstrated a statistical association between mRNA-based vaccines and adverse cardiac events and have analyzed risk as a function of age and sex (Sharff et al., 2022; Chua et al., 2022; Karlstad et al., 2022; Le Vu et al., 2022; Buchan et al., 2022). In the Kaiser Permanente Northwest study, the risk of perimyocarditis within 30 days of two mRNA injections was reported to be 1/2650 in male adolescents aged 12 to 17, and 1/1862 in the 18-to-24 age group (Sharff et al., 2022). These data are consistent with findings from the Hong Kong study by Chua et al., who calculated an incidence of 37.32 (95% CI, 26.98-51.25) per 100,000 male adolescents (Chua et al., 2022). In the “Nordic Cohort” study, the incidence rate was also determined separately for both vaccines, which was 3.3 times higher after the second vaccination dose for mRNA-1273 (excess events: 5.55 [95% CI, 3.70-7.39] vs. 18.39 [9.05-27.72]) (Karlstad et al., 2022). Data from France confirmed this risk ratio (8.1 [95% CI, 6.7-9.9] vs. 30 [21-43]); the authors also cautioned that both myocarditis and pericarditis posed a substantial risk in all age groups and for both sexes (Le Vu et al., 2022).

Compared with BNT162b2, a vaccine dose of mRNA-1273 contains 3.3 times the amount of mRNA (100 µg vs. 30 µg), so based on empirical data, there may be a correlation between the incidence of myocardial alterations and the amount of mRNA used. Due to the large number of components involved, the number of mRNA copies used cannot be extrapolated one-to-one to the synthesis rate of the spike protein, but the significantly higher geometric mean titers (GMTs) of neutralizing antibodies after basic immunization with mRNA-1273 also indicate significantly higher concentrations of the spike protein (Khoury et al., 2021; Tyner et al., 2022).

A currently favored theory about the underlying pathomechanisms of myocardial alterations is based on the possibility of immunological cross-reactions. Antibodies directed against epitopes of the spike protein as a result of vaccination could also react with epitopes of the alpha-myosin heavy chain (alpha-MHC) (Vojdania and Kharrazian, 2020). However, it must be considered that alpha-MHC is a sarcomeric protein expressed almost exclusively in atria and cannot interact directly with circulating antibodies because of its purely intracellular localization (Racanelli et al., 2011).

Autoantibodies against alpha-MHC can be detected in the course of various diseases of the myocardium; however, etiology and pathogenetic mechanisms are still poorly understood, so that a causal contribution to cardiac symptoms cannot be confirmed at present (Kaya et al., 2012).

The application of mRNA is carried out by means of LNPs, the distribution of which in the organism was studied by the use of radiolabeled tracers over a period of two days in Wistar rats (SARS-CoV-2 mRNA Vaccine, report number: 185350; Li et al., 2022). Because lipid particles can reach the myocardium from the injection site after only a few minutes, in this study we examined the direct effect of mRNA-1273 and BNT162b2 on the function, structure, and viability of isolated rat cardiomyocytes over a 72 h period. After isolation, cellular and molecular effects directly attributable to the intrinsic effect of the two mRNA vaccines can be studied on the serum-free incubated ventricular muscle cells; the absence of immunocompetent cells allows indirect influences due to possible antigen-antibody reactions to be excluded. Additionally, the cardiomyocyte cell line AC16 was used to demonstrate the uptake of LNP-mRNA complexes and the translation of the encoded spike protein in human cells of ventricular origin (see Fig. 1 "*Study design*").

Our present findings show for the first time in the literature that left ventricular myocytes both from rat and human origin express the encoded spike protein after incubation with both mRNA-1273 and BNT162b2; functionally, both mRNAs induce disturbances to normal contractile function within 24 to 48 h of application, which, however, differ fundamentally with respect to their symptomatology as well as their respective pathomechanisms.

Methods

This study was performed in conformance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Isolation and cultivation of ventricular cardiomyocytes

Left ventricular cardiomyocytes were isolated from the hearts of 3-month-old male Wistar rats (RjHan:WI). Anesthesia was administered on one experimental animal at a time in the desiccator (vol: 6 l) under 4-5% isoflurane (IsoFlo 100% w/w, Zoetis Deutschland GmbH, Germany). After the eyelid reflex was extinguished, killing was performed by cervical dislocation.

Subsequently, hearts were perfused using the Langendorff technique at 37°C, first blood-free and then recirculating for 25 min with 50 ml of Powell medium (PM), 0.06% (w/v) type 2 collagenase (Worthington Biochemical Corp., USA), and 25 µmol/l $CaCl_2$ (Composition of PM in mmol/l: NaCl, 110; KH_2PO_4, 1.2; KCl, 2.6; $MgSO_4$, 1.2; Hepes, 25; and glucose, 10; adjusted to pH 7.4 and gassed with 5% CO_2–95% O_2). Mechanical comminution was followed by further enzymatic digestion for 5 min in 12 ml PM. The cell suspension was filtered through a 200-µm nylon mesh and separated from non-myocytic cells; purification and increase of calcium concentration from 0.2 to 0.5 mmol/l were performed in three successive centrifugation steps. Subsequently, cells were taken up in 20 ml CCT cell culture medium (medium 199 with Earle's salts supplemented with 5 mmol/l creatine, 5 mmol/l L-carnitine, 5 mmol/l taurine, 100 IU/ml penicillin, and 100 µg/ml streptomycin, pH 7.4), resuspended, and plated out on 35 mm culture dishes (Falcon, type 3001, Corning Inc., USA) that had previously been pre-incubated overnight with CCT medium plus 4% (v/v) FCS. Non-adherent cells were removed by changing the medium after one hour (Schreckenberg et al., 2015).

Transferring a complex *in vivo* situation to an *in vitro* model is often a major challenge, especially with regard to the concentrations of the applied active ingredients. In this study, the amount of mRNA used was calculated from the lipid concentration detected in the rat heart 2 h after injection of 50 µg [^{3}H]-labeled LNP mRNA. The lipid equivalent of 1.4 µg per gram of cardiac tissue corresponds to approximately 3% at the applied LNP amount of 50 µg. Consequently, 10 µl (≙ 1.0 µg RNA/ml) or 3.3% of a regular inoculation dose of BNT162b2 (300 µl per dose) or 16.6 µl (≙ 3.3 µg RNA/ml) of an inoculation dose of mRNA-1273 (500 µl per dose) was applied to the culture dishes, each of which contained 1 ml of cell culture medium (≙ approximately 1 gram) (SARS-CoV-2 mRNA Vaccine, report number: 185350). The culture dishes of the respective control groups were not treated.

All cell culture work, including the application of mRNA-1273 and BNT162b2, was performed separately from the subsequent analysis, using different lab space and personnel. Accordingly, all experiments were performed in a blinded manner, without knowledge of the group affiliation of each culture dish.

Determination of cell contraction

Absolute and relative cell shortening as well as parameters of contraction dynamics were determined on isolated cardiomyocytes using the "Cell-Edge Detection System". In the electric field set up by two AgCl electrodes, the cells were made to contract by successive square-wave pulses (50 V) of opposite polarity (Schreckenberg et al., 2015).

A total of n=1161 cells from n=6 or n=8 independent preparations were measured and analyzed. At a stimulation frequency of 2 Hz, a contraction was recorded every 15 s for one minute using a 500 Hz line scan camera. The average of these four individual measurements represents the contraction of the cell. The cardiomyocytes of a further n=32 culture dishes from n=8 independent experiments could not be measured or evaluated at 48 h after application of mRNA-1273 in the two concentrations 1.0 as well as 3.3 µg/ml of mRNA, because the cell pattern was predominantly characterized by arrhythmic as well as irregularly contracting myocytes.

Valid measurements can only be obtained from regularly beating cardiomyocytes. The cell boundaries of irregularly contracting myocardial cells are not properly detected by our

system. Arrhythmically beating cells do not provide consistent values within the four contractions recorded per cell (and these values are in some cases nonphysiological).

Visualization of calcium transients

For the analysis of calcium transients, myocytes were plated out on 35 mm glass-bottomed cell culture dishes (CELLview #627861, Greiner Bio-One International GmbH, Germany) following isolation. 50 µg of Fluo-4, AM (membrane-permeable, excitation at 488 nm, emission at 520 nm, Invitrogen™, USA) were first dissolved in 200 µl DMSO and then diluted 1:200 in a CCT cell culture medium. Immediately before measurement, cells were loaded with Fluo-4 via medium exchange for 10 min at 37°C, washed, and allowed to contract at a stimulation frequency of 2 Hz. Calcium transients were visualized using ION Optix imaging systems (IonOptix LLC, USA).

Phalloidin staining protocol

Imaging of the sarcomere structure of isolated cardiomyocytes was performed by confocal laser scanning microscopy (CLSM) detection of filamentous actin (F-actin). Phalloidine tetramethylrhodamine B isothiocyanate (phalloidine-TRITC, sc-301530, Santa Cruz Biotechnology, Inc., USA) was used, which exhibits an emission maximum of 570–573 nm after excitation at wavelengths of 540–545 nm, giving the labeled F-actin an orange-red appearance. Cell fixation with 4% paraformaldehyde was followed by incubation with 0.2% Triton-X-100 and then staining with 100 µmol/l Phalloidin-TRITC. For morphological assessment, images of three independent cell preparations and three culture dishes per experimental condition were obtained using the Axiovert 200 confocal laser microscope (Carl Zeiss AG, Germany).

Measurement of PKA activity

PKA activity in the cell lysate of isolated cardiomyocytes was performed using the "PKA (Protein Kinase A) Colorimetric Activity Kit" (#EIAPKA, Invitrogen™, USA). The cells were lysed and harvested exactly according to the manufacturer's instructions; the sample material was then prepared for analysis using an ELISA reader. Set to the recommended parameters, a Tecan Infinite M200 (Tecan Group Ltd., Switzerland) and the associated Tecan i-control software were used to quantify PKA activity.

Langendorff Perfusion

The clearance of mRNA-1273 and BNT162b2 from coronary vessels into the myocardium and into myocytic and non-myocytic cells was studied *ex vivo* in isolated perfused hearts of 3-month-old male Wistar rats. Flow-constant perfusion was performed retrogradely via 16-gauge aortic cannula using a Krebs-Henseleit solution, gassed with carbogen (95% O_2, 5% CO_2) and warmed to 37°C. Heart rate, left ventricular pressure, and aortic pressure were recorded continuously. After functional stabilization, the hearts were first perfused with either mRNA-1273 or BNT162b2 for 2 h in a recirculating fashion and then flushed with an open circuit for 15 min. However, the hearts of the respective control groups were perfused exclusively with Krebs-Henseleit buffer according to an identical protocol.

PCR-based detection of the respective mRNA was performed either directly on left or right ventricular tissue, septum, and atria, or on cardiomyocytes and non-myocytic cells isolated according to the above protocol (see above "*Isolation and cultivation of ventricular cardiomyocytes"*) directly following Langendorff perfusion (Schreckenberg et al., 2015).

A total of n=16 Langendorff hearts were perfused. Fig. 6 A): n=2 controls, n=2 mRNA-1273 and n=2 BNT162b2: For each heart, PCR-based mRNA detection was performed at n=8 different regions. All PCRs were performed in duplicate. Fig. 6 B): n=2 controls, n=2 mRNA-1273 (1:2000), n=2 mRNA-1273 (1:4000), n=2 BNT162b2 (1:2000) and n=2 BNT162b2 (1:4000): Myocytes and nonmyocyte cells were isolated/separated from each heart. Thus, mRNA uptake was examined in duplicate in n=4 myocyte fractions and n=4 nonmyocyte fractions from mRNA-1273 and BNT162b2 perfused hearts, respectively. All treatment groups provided consistent results, so in accordance with the 3Rs Guidelines, we saw no need to add more hearts to the group size.

PCR-based detection of mRNAs

Complete RNA was isolated from cardiac tissue, isolated cells, and AC16 cardiomyocytes using TRItidy G™ (A4051, AppliChem GmbH, Germany) following the manufacturer's protocol. After incubation with 1 U DNase/µg RNA for 15 min at 37°C, cDNA synthesis was performed using SuperScript™ III reverse transcriptase (#18080093, Invitrogen™, USA). All PCRs were performed with the CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Germany) using iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc., Germany).

The primers used were constructed based on the sequences published by Jeong et al. (Jeong et al.). The following primer pair was used to detect mRNA-1273: Forward: GCCTACAGCAACAACAGCAT, Reverse: TTGAACAGCAGGTCCTCGAT, Product Size: 356 bp; for the detection of BNT162b2: Forward: GGATCCTCTGAGCGAGACAA, Reverse: ACAGGTCGTTCAGCTTGGTA, Product Size: 293 bp.

SARS-CoV-2 Rapid Test

Qualitative detection of the spike protein encoded by mRNA-1273 and BNT162b2 was performed using the Koch Antigen Rapid Test NCV10 (Koch Biotechnology Co., Ltd., China), which specifically and exclusively detects the SARS-CoV-2 spike (S) glycoprotein. With the exception of sample preparation, the test was performed according to the prescribed protocol. For the detection of the spike protein in the cell culture medium, 30 µl supernatant was pipetted onto the corresponding field of the test strip. For intracellular detection, cells were washed with PBS after complete removal of the CCT medium and harvested in 100 µl of lysis buffer (Cell Lysis Buffer #9803, Cell Signaling Technology, Inc., USA). Immediately afterwards, the test was performed with 30 µl of native cell lysate.

AC16 human cardiomyocyte cell line

Human AC16 cardiomyocytes were cultured on 100 mm dishes (Falcon, type 3003, Corning Inc., USA) in Dulbecco's Modified Eagle's Medium (D6429, EMD Millipore Corp., USA) supplemented with 12.5% heat-inactivated FBS (35-079-CV, Corning Inc., USA) and under 1% antibiotic-antifungal cover (30-004-CI, Corning Inc., USA) according to protocol (#SCC109, EMD Millipore Corp., USA). All AC16-based experiments in this study were performed on cells previously transferred to 60 mm culture dishes (Falcon, type 3002, Corning Inc., USA). Independent confirmation of all test results for spike protein was performed using the Vazyme antigen detection kit (#C8602C, spike protein recognition regions: aa345-410 and aa420-450, Nanjing Vazyme Medical Technology Co., Ltd., China).

Using the cardiomyocyte cell line AC16, we demonstrated both uptake of the LNP-mRNA complexes and translation of the encoded spike protein by AC16 cells. Specific detection of the respective mRNA was performed 15, 30, 60, and 90 min after application of mRNA-1273 or BNT162b2 in the three series of experiments listed. Subsequently, spike protein was detected every other day in the cell fraction and supernatant on a total of n=5 control dishes, n=5 mRNA-1273, and n=5 BNT162b2 dishes, respectively, resulting in 90 samples analyzed in the three series.

Data and Statistical Analysis

All data are expressed as boxplots. First, data were tested using the non-parametric Kruskal-Wallis-Test with subsequent pairwise Wilcoxon-Tests for independent data and Bonferroni-Holm p-value adjustment for multiple testing. A p-value less than 0.05 is considered to be statistically significant.

Because of the exploratory study design in combination with an hypothesis-free study approach, an *a priori* case number calculation was not performed. The estimation of the required number of independent cell preparations (≙ the number of required experimental animals) was based on our long-term experience regarding the cultivation and functional characterization of isolated adult rat cardiomyocytes (Schreckenberg et al., 2004).

Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

Results

The effects of mRNA-1273 and BNT162b2 on the function of left ventricular myocytes 24 and 48 hours after application

The function of cardiomyocytes was characterized at a beating frequency of 2 Hz by determining its relative cell shortening (dL/L in %), contraction velocity, and relaxation velocity (µm/s).

After 24 h, cells treated with mRNA-1273 or BNT162b2 did not differ functionally (see Fig. 2 A-C) or morphologically (see Fig. 3 A) from untreated control cells.

However, 48 h after application of mRNA-1273, quantification of contraction parameters was no longer possible, since the cell pattern was predominantly characterized by arrhythmic as well as irregular, partially "peristaltic" contracting myocytes (see Vid. 1 A-F). Regular contractile activity could be recorded in a sufficient number of cardiomyocytes only when the mRNA concentration was reduced to 0.3 µg per ml of culture medium. Although relative cell shortening was not affected, there was significant deterioration in both dynamic functional parameters (see Fig. 2 D-F).

In contrast, BNT162b2-treated cells contracted rhythmically and uniformly but, compared with control myocytes, exhibited both a significant increase in relative cell shortening (+22.6%) as well as contraction (+31.9%) and relaxation velocity (+32.1%). With application of 3.3 times the dose of BNT162b2 (BNT162b2 (3.3 µg)), the mRNA concentration per dish was approximately the same as x1 dose of mRNA-1273; while this enhanced the aforementioned effects (cell shortening: +34.6%, contraction velocity: +51.4%, and relaxation velocity: +54.8% vs. control), the characteristic mRNA-1273 symptomatology was not observed (see Fig. 2 D-F).

The contraction behavior that BNT162b2-treated cells develop in the period 24 to 48 h after application is largely consistent with functional changes that cardiomyocytes (or myocardium) exhibit as a result of catecholamine stimulation. In our model, nearly identical functional parameters could be obtained in untreated cardiomyocytes that received acute beta-adrenergic stimulation with isoprenaline (10 nmol/l final concentration) after 48 h (see Fig. 2 D-F).

The "functional state" of isolated cardiomyocytes 24, 48, and 72 hours after mRNA application

Figure 3 illustrates the (functional) state of the cardiac muscle cells over time and in relation to the particular experimental condition. At 24 h (see Fig. 3 A), the untreated controls and the mRNA-1273 / BNT162b2-incubated culture dishes showed regularly contracting cells (percentages of 75% and 77%, respectively). After 48 h (see Fig. 3 B), the number of contracting myocytes decreased, and the proportion of non-beating but morphologically intact myocytes and the proportion of rounded or hypercontracted cells increased. In all of the above categories, controls and BNT162b2-treated cells differed only insignificantly. By contrast, the proportion of regularly contracting myocardial cells was reduced to 10% by the application of mRNA-1273, and arrhythmic as well as irregularly beating cells together accounted for approximately 52% of total cells.

Our model of isolated rat cardiomyocytes allows accurate quantification of myocyte contraction parameters up to 48 h after isolation. After 72 h (see Fig. 3 C), the "functional state" was therefore assessed solely in a qualitative fashion. Compared to the control dishes, which still had a 41% share of contracting cardiomyocytes, the cells almost completely ceased to function after mRNA-1273 incubation. After BNT162b2 application, the proportion of beating myocytes was reduced to 27%; both mRNAs also resulted in a significantly increased proportion of rounded or hypercontracted cells.

Reducing the mRNA-1273 dose to 1.0 µg RNA per ml did not improve the previously described symptomatology over time. By contrast, a further decrease to 0.3 µg/ml significantly increased the proportion of regularly contracting myocardial cells, but was only transiently effective within the first 48 h (see Fig. 3 D).

The effects of mRNA-1273 and BNT162b2 on the calcium transients of isolated cardiomyocytes

In order to visualize calcium transients, cells from all three treatment groups were loaded with Fluo-4 AM for 10 min after 48 h of incubation and then electrically stimulated at 2 Hz.

In untreated control myocytes and in BNT162b2-treated cells, both the systolic release and diastolic decrease of calcium were rhythmically and uniformly detectable over the entire

cross-sectional area of the respective cell. The calcium cycles of mRNA-1273-treated cardiomyocytes showed arrhythmic as well as localized and irregular transients, which must be interpreted as correlates of the previously described dysfunctions (see Vid. 2 A-G).

The BNT162b2-induced increase in all contraction parameters causally underlies a significant increase in protein kinase A (PKA) activity (see below "*The effects of mRNA-1273 and BNT162b2 on PKA activity after 48 hours of incubation*"). Thus, the intensity and dynamics of calcium transients in BNT162b2-treated myocytes increased.

Detection of an intact sarcomere structure using phalloidin staining

Especially with regard to the irregular contractions induced by mRNA-1273, structural damage within the sarcomere structure could not be ruled out. After 48 h of incubation, cells from three preparations were examined for the regular arrangement of their transverse striations by phalloidin staining (see Fig. 4).

Characteristic, clustered irregularities in the structure of the parallel myofibrils could not be observed in any treatment group; thus, an assignment to the three experimental conditions was not possible via the assessment of the histological preparations, which was performed in a blinded fashion.

The effects of mRNA-1273 and BNT162b2 on PKA activity after 48 hours of incubation

The maintenance as well as the demand-responsive adaptation of cardiac function are primarily under the control of the beta-adrenergic receptor, which regulates the activity of adenylyl cyclase and thus the level of cAMP by means of Gs protein. Both positive inotropic and positive lusitropic effects are mediated by cAMP-dependent activation of PKA.

Functionally, BNT162b2 application after 48 h in the absence of extracellular ligands of the beta-adrenergic receptor caused both a significant increase in relative cell shortening, as well as in contraction and relaxation rates. Consequently, ELISA-based myocyte PKA activity was determined (see Fig. 5), which increased after 48 h in BNT162b2-treated cells to levels comparable to those in untreated cells that received acute beta-adrenergic stimulation with isoprenaline (10 nmol/l final concentration) after 48 h. While mRNA-1273 had no effect on

PKA activity, BNT162b2 (3.3 µg) led to a further increase – which, however, comparable to the functional effects, was not proportional to the dose increase.

Uptake of mRNA and production of spike protein in rat cardiomyocytes

PCR-based detection of mRNA was conducted to investigate the incorporation of LNP-mRNA complexes in cardiac tissues and ventricular cells. After 2 hours of Langendorff perfusion with mRNA-1273 and BNT162b2, respectively, and a 15-minute washout period, mRNA could be detected in all sections of the left and right ventricular myocardium, in the septum, and in both atria (see Fig. 6 A). Following cell isolation, it was also possible to demonstrate cell-specific uptake in cardiomyocytes as well as in non-myocytic cells (endothelial cells, fibroblasts). In a direct comparison, the cell fraction of non-myocytic cells showed consistently higher levels of mRNA compared to the myocyte fraction after perfusion in the case of both mRNA-1273 and BNT162b2, for both concentrations used (1:2000 and 1:4000) (see Fig. 6 B).

Under duplicate measurement, all Langendorff hearts gave concordant results with respect to mRNA uptake. Neither mRNA-1273 nor BNT162b2 had any effect on cardiac function or hemodynamics during 2 hours of perfusion (data not shown).

The Koch NCV10 rapid antigen assay, which is designed for specific detection of the SARS-CoV-2 spike (S) glycoprotein, was used to monitor the translation of the vaccine-encoded spike protein. 24 h after incubation of isolated cardiomyocytes with mRNA-1273 and BNT162b2, respectively, spike protein was not detectable in the cell lysate or cell culture medium. After 48 h, positive test results could be obtained exclusively for the intracellular fraction of mRNA-1273 and BNT162b2 treated cells; the supernatant always remained “negative” (see Fig. 6 C). Because of the rapid and simple assay procedure, successful translation after application of the two mRNAs was verified by random sampling in all previously described experiments; the rate for positive protein detection was 100% for both mRNA-1273 and BNT162b2 after 48 h of incubation.

Uptake of mRNA and spike protein production in human AC16 cardiomyocytes

Using the cardiomyocyte cell line AC16, we were able to demonstrate the efficient uptake of LNP-mRNA complexes and the translation of the encoded spike protein in human cells of ventricular origin. Specific detection of the respective mRNA was performed 15, 30, 60, and 90 min after application of mRNA-1273 and BNT162b2, respectively (see Fig. 7 A). In contrast to the isolated cardiomyocytes of adult rat hearts, the spike protein could be detected in AC16 cultures in both the cell fraction and supernatant. By reducing the RNA concentration used to 0.66 µg/ml (mRNA-1273) and 0.2 µg/ml (BNT162b2) in experiment no. 3, the number of positive test results could be increased, due to reduced cell damage in comparison to the first two experiments (see images). In addition, the results regarding the production time of the spike protein must take into account the two cell passages in a ratio of 1:5, so that the positive findings of the last four days are mathematically based on only 4% of the originally incubated cells (see Fig. 7 B).

Discussion

Functionally, in ventricular cardiomyocytes, both mRNA-1273 and BNT162b2 cause characteristic symptoms, each of which is based on independent as well as highly specific pathomechanisms. Whereas mRNA-1273 induces both arrhythmic and irregular contractions via massive disruption of sarcoplasmic calcium release, BNT162b2 leads to a disproportionate increase in cardiomyocyte functional parameters via chronic activation of PKA.

Within the first 24 h after application of the mRNAs, neither functional disturbances nor morphological abnormalities were observed. By contrast, after 48 h, specific symptomatology could be clearly diagnosed for both mRNA-1273 and BNT162b2. As a causal agent, the time kinetics in both cases point to the spike protein, which must first be translated and cytoplasmically enriched in sufficient concentration. Immunological cross-reactions can be excluded by the cell culture model used; direct damage to the cell membrane by the LNP-mRNA complexes would have already become symptomatic after 24 h (Tsilingiris et al., 2022).

The rapid and efficient uptake of LNP-mRNA complexes could be reliably demonstrated in both *ex vivo* perfused Langendorff hearts as well as human AC16 cardiomyocytes. While our two cell culture systems do not permit any concrete conclusions regarding the duration or extent of spike protein production, all results for AC16 cells indicate a highly efficient and possibly long-lasting translation.

The spike protein, encoded by both mRNA-1273 and BNT162b2, is stabilized in its prefusion form (S-2P) by double proline substitution at positions 986 and 987. Translation results in proteins with identical amino acid sequences in both cases, but BNT162b2 has 4,284 nucleotides, 280 nucleotides more than mRNA-1273; other differences include the design and modifications of the 5'- and 3'-UTRs, codon optimization, and the chemical structure of the LNPs (Xia, 2021; Granados-Riverona and Aquino-Jarquin, 2021). Consequently, post-translational modifications could be responsible for the different pathomechanisms in cardiomyocytes; NMR analyses could be used to identify both structural differences and potential interaction partners.

Regulation of myocardial contraction occurs at the cellular level primarily by influencing intracellular calcium homeostasis. Voltage-gated L-type calcium channels are responsible for the influx of so-called “trigger calcium” that activates intracellularly located ryanodine receptors (RyR2), which subsequently release large amounts of calcium from the sarcoplasmic reticulum (SR). The increase in cytosolic calcium from 10^{-7} mol/l to approximately 10^{-5} mol/l is almost entirely due to sarcoplasmic calcium and can be viewed as an initial step in the contraction. The magnitude and dynamics of calcium transients are mainly under the control of the PKA, which, after beta-adrenergic stimulation, regulates the release via phosphorylation of the L-type calcium channel as well as RyR2 and, via phospholamban, the activity of SERCA2a and, thus, the return of calcium to the SR (Schreckenberg, 2016).

The arrhythmic as well as completely irregular contractions, together with the irregular as well as localized calcium transients, indicate a significant dysfunction of RyR2, which was induced exclusively after incubation with mRNA-1273. Direct impairment of SR-dependent calcium release also explained the significantly reduced contractile velocity after application of the reduced mRNA-1273 dose.

As a result of transfection with recombinant SARS-CoV-2 spike protein, Clemens et al. found comparable effects on rhythm and calcium transients in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) (Clemens et al., 2023).

Numerous papers have identified mutation-related RyR2 dysfunction as a causal agent of arrhythmias, ventricular tachycardia, and sudden cardiac death (Thomas et al., 2005; Jóna, Nánási, 2006; Seidlmayer et al., 2018; Sleiman et al., 2021). RyR2-induced cardiomyopathies may further be characterized by a structurally remodeled myocardium; our histological findings do not provide evidence of disruption of sarcomeric transverse striation, so that the described symptomatology 48 h after application is in all likelihood purely functional in causation (Benitah et al., 2021). However, severe dysfunction of isolated cardiomyocytes, regardless of the underlying cause, can cause immediate structural damage in the myocardial cellular complex by mechanical forces on directly adjacent cardiomyocytes (Schauer et al., 2022).

Our results on the effects of BNT162b2 are fully consistent with the pathological findings of Gill et al., who disagree with the clinically diagnosis of “classic myocarditis” as a cause of death in both cases examined; they describe the cardiac changes as a consequence of catecholamine-induced toxic cardiomyopathy (Gill et al., 2022). Without being able to identify the exact molecular mechanism, incubation with BNT162b2 causes functionally comparable effects to acute stimulation of cardiomyocytes with isoprenaline via an increase in PKA activity. BNT162b2-induced sustained PKA activation could thus correspond histopathologically to catecholamine-induced cardiomyopathy. The permanent stimulation of beta-adrenergic signaling mechanisms not only increases the energy demand of the myocardium; the increase in heart rate simultaneously reduces the diastolic filling time of the coronary arteries, thus also lowering energy supply (Lefkowitz et al., 2000; Kassim et al., 2008; Casey et al., 2017).

Moreover, our present findings contradict the assumption that the high mRNA concentration of an mRNA-1273 vaccine dose is alone responsible for the increased rate of cardiac side effects. The 3.3-fold dose of BNT162b2 potentiates the previously induced effects of the 1-fold dose, but the symptomatology triggered by mRNA-1273 cannot be observed, even after application of identical mRNA concentrations. If there is no other common pathomechanism contributing to the cardiac side effects by differential diagnosis *in vivo*, the expression or

severity of clinical symptoms alone could explain the unequal risk ratio. This assumption is supported by our findings regarding the "functional state" of the cells 72 h after application: the proportion of regularly contracting cells was significantly reduced on BNT162b2-incubated culture dishes compared to untreated controls, but in the presence of mRNA-1273, cardiomyocytes almost completely ceased to function.

The main experiments in this study were performed on cardiac muscle cells from 3-month-old male Wistar rats. In further studies, we will investigate the influence of age and gender on the described pathomechanisms as well as on the evolution and severity of dysfunction (Bugiardini et al., 2023; Ferdinandy et al., 2019; Ferdinandy et al., 2023). In addition, it would be important to determine the influence of mRNA-1273 and BNT162b2 on the function of muscle cells specific to the cardiac conduction system, which are histologically differentiated from working myocardial cells. In these cells heart rate and conduction velocity are also under the control of PKA, which is able to regulate the level and rate of calcium cycling via its influence on RyR2 and SERCA2a. In the study published by Mansanguan et al., approx. 18% of 301 adolescents aged 13 to 18 years studied developed ECG abnormalities after two doses of BNT162b2 vaccine (Mansanguan et al., 2022).

Contrary to the clinically diagnosed side effects, which are predominantly classified as myo- and/or pericarditis, both mRNA-based SARS-Cov-2 vaccines induce functional disturbances on isolated cardiomyocytes that correspond pathophysiologically to cardiomyopathy. RyR2 impairment and sustained PKA activation – both of which are attributable to the intracellular interactions of the spike protein – are risk factors for sudden cardiac death, ventricular tachyarrhythmias, and contractile dysfunction. Both mechanisms also provide a possible explanation for persistent cardiac symptoms observable in the context of long COVID / post-COVID syndrome (Gyöngyösi et al., 2023). Because rodents became susceptible to SARS-CoV-2 with the emergence of the spike N501Y mutation, the rat model offers the advantage of directly comparing the effects of mRNA-based COVID vaccines and the consequences of infection with SARS-CoV-2 at the level of the isolated cardiomyocyte (Shuai et al., 2021).

Our present study has some limitations. Although all of our present findings obtained from isolated perfused hearts and isolated cardiomyocytes indicate the LNPs of both vaccines on their own do not negatively affect cardiomyocyte function or structure, neither acutely nor within the first 24 hours, the effect of control LNPs were not studied here. Unfortunately,

LNPs loaded with suitable control mRNA or “unloaded” LNPs are not currently available for the research community. Accordingly, it is not possible to conclusively assess the effects that may be specifically attributable to the nanoparticles themselves at this time.

In conclusion, currently approved SARS-CoV-2 vaccines have been shown to be effective in preventing COVID-19 and its sequelae, but our present data suggest that the risk–benefit ratio of mRNA-based vaccines should be reevaluated taking into consideration of current preclinical cardiac safety data that revealed a hidden cardiotoxic effect of the vaccines. Furthermore, the present findings should be taken into account in future diagnosis and therapy of cardiac symptoms temporally coincident with mRNA-based COVID vaccination.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design and Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

Author Contributions

Participated in research design: Rolf Schreckenberg, Nadine Woitasky, Nadja Itani, Péter Ferdinandy, Rainer Schulz. Conducted experiments: Rolf Schreckenberg, Nadine Woitasky, Nadja Itani, Laureen Czech. Performed data analysis: Rolf Schreckenberg, Nadja Itani, Rainer Schulz. Contributed to the writing of the manuscript: all authors.

List of Abbreviations

alpha-MHC	alpha-myosin heavy chain
CLSM	confocal laser scanning microscopy
dL/L in %	relative cell shortening
F-actin	filamentous actin
GMT	geometric mean titer
ISO	isoprenaline
LNP	lipid nanoparticle
mRNA	messenger RNA
phalloidine-TRITC	phalloidine tetramethylrhodamine B isothiocyanate
PKA	protein kinase A
PM	Powell medium
RyR2	cardiac ryanodine receptor
SR	sarcoplasmic reticulum

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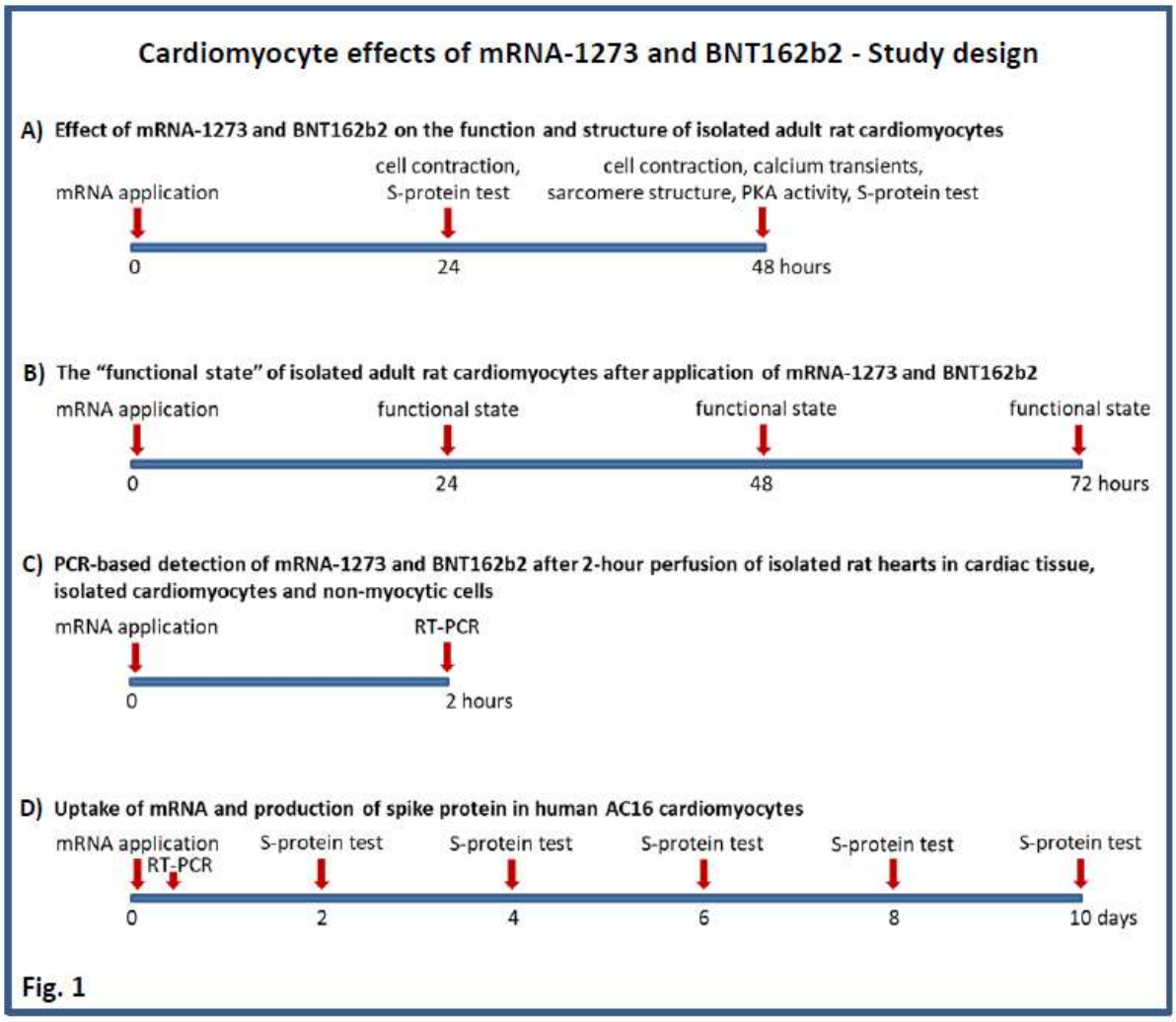


Fig. 1: A) The effect of mRNA-1273 and BNT162b2 on the function and structure of isolated rat cardiomyocytes was examined over a period of 48 h; both the cell fraction and supernatant were assayed for spike protein (S-protein) after 24 h and 48 h, respectively. B) 24 h, 48 h, and 72 h after application of mRNA-1273 and BNT162b2, isolated cardiomyocytes were stimulated in a blinded fashion using a 2 Hz electric field and assigned to one of the following categories: regular contraction, arrhythmic contraction, irregular contraction, no contraction, and rounded/hypercontracted. C) mRNA uptake was assessed after 2 hours of Langendorff perfusion in all sections of the left and right ventricular myocardium, septum, and both atria. Following the additional step of cell isolation, it was also possible to detect cell-specific uptake in cardiomyocytes and non-myocytic cells. D) Using the human cardiomyocyte cell line AC16, it was possible to demonstrate both the efficient uptake of LNP-mRNA complexes and translation of the coded spike protein in human cells of ventricular origin.

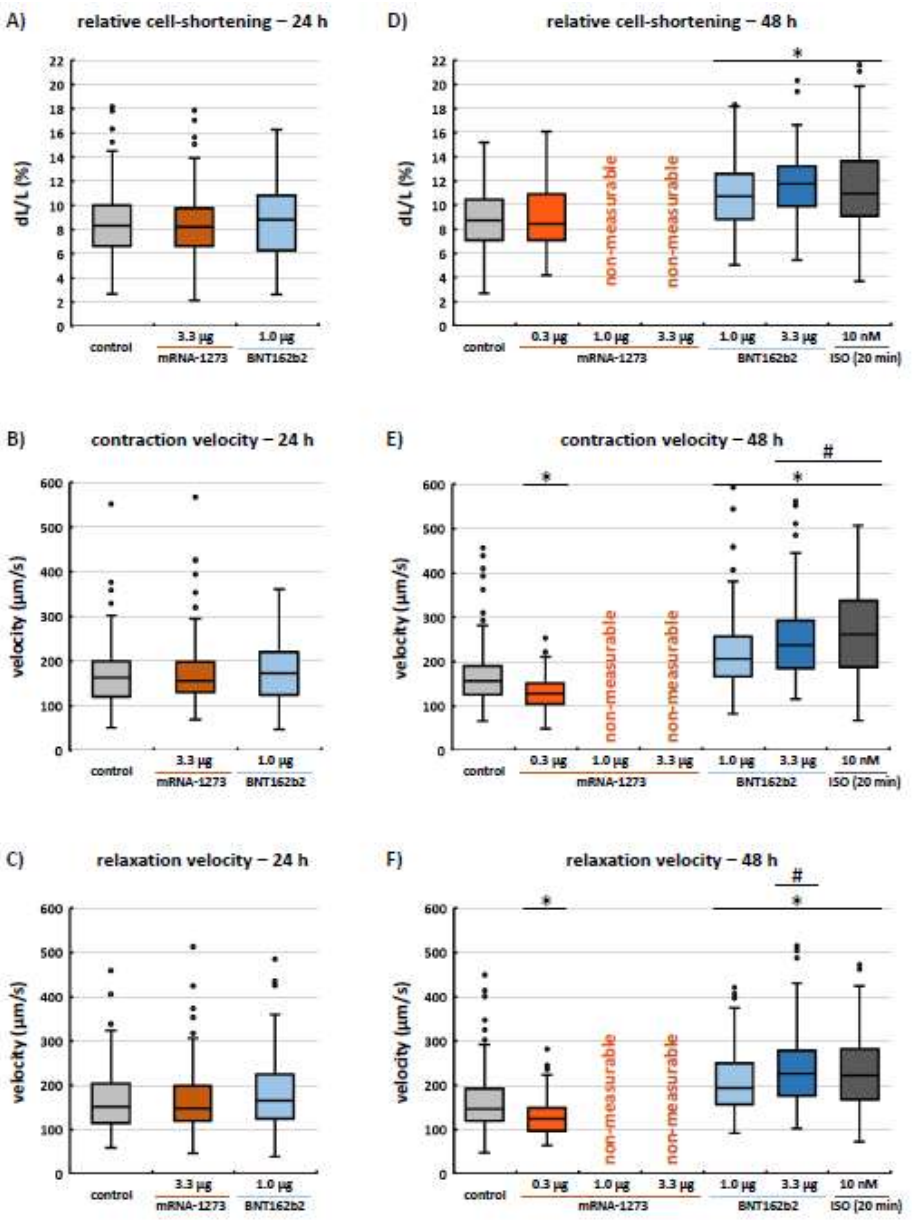


Fig. 2: Functional analysis of isolated cardiomyocytes: After 24 (A-C) and 48 h (D-F), the effect of mRNA-1273 and BNT162b2 on relative cell shortening (dL/L in %), contraction velocity, and relaxation velocity (µm/s) was examined. mRNA-1273: At 48 h, we could only obtain valid functional data after reducing applied RNA concentration to 0.3 µg per ml; for technical reasons, regular contraction parameters could no longer be recorded on the predominantly arrhythmic and irregularly contracting myocytes after application of 1.0 and 3.3 µg of mRNA per ml. BNT162b2 (3.3 µg): 3.3-fold dose of BNT162b2 (equivalent to x1 dose of mRNA-1273) was used to investigate the possible relationship between applied mRNA concentration and cardiac symptoms. ISO (10 nM): The 20-minute application of the nonselective beta-sympathomimetic isoprenaline causes functionally comparable effects to the 48-hour incubation with BNT162b2. Number of measured cells: A-C: n=171 cells from n=8 independent preparations; D-F: n=144 cells from n=8 (control, mRNA-1273 (0.3 µg-3.3 µg), BNT162b2 (1.0 µg)) and n=108 cells from n=6 (BNT162b2 (3.3 µg), ISO (10 nM)) independent preparations. *, p< 0.05 vs. control, #, p< 0.05 vs. BNT162b2 (1.0 µg).

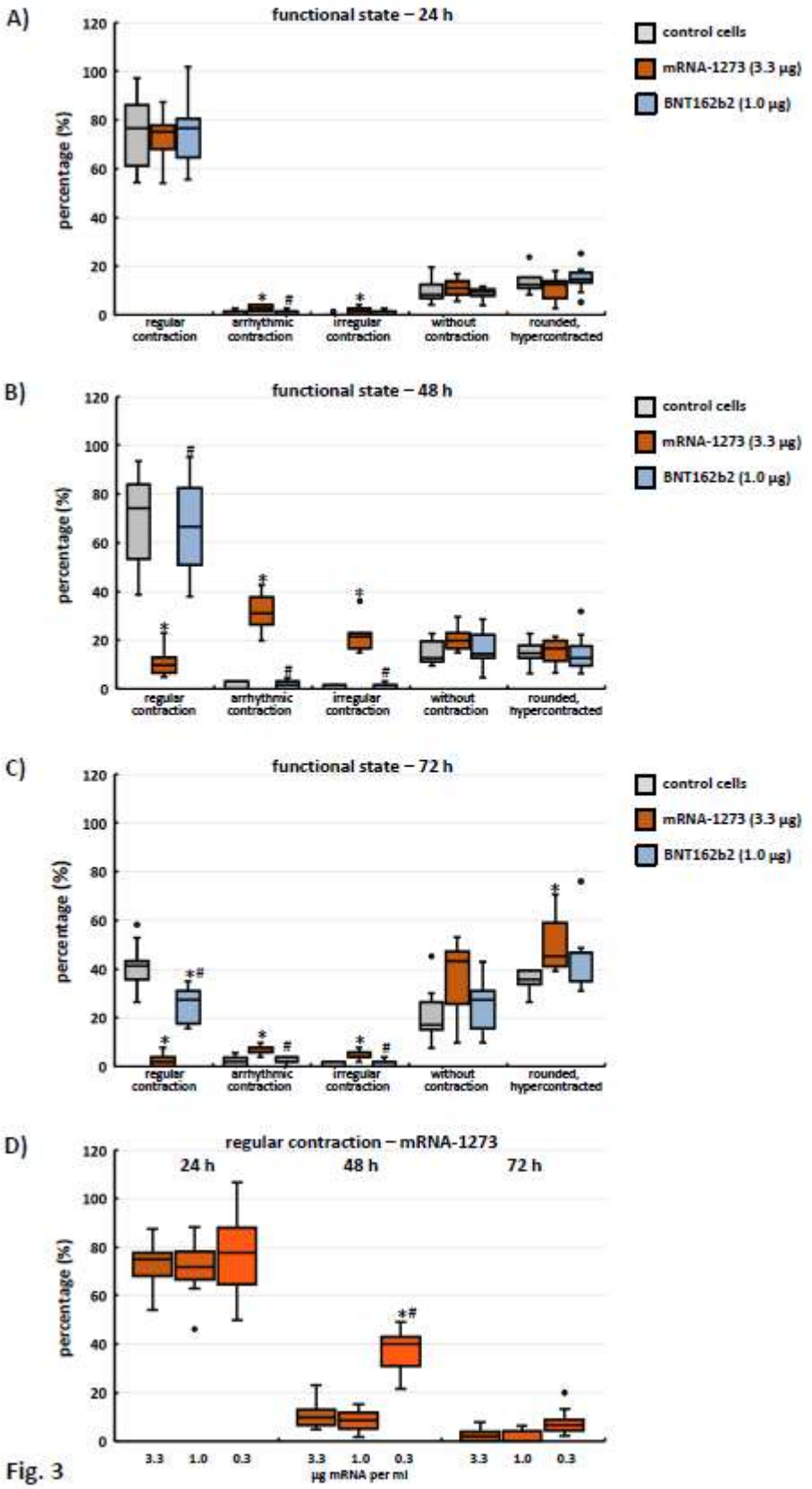


Fig. 3

Fig. 3: The functional state of isolated cardiomyocytes after A) 24, B) 48, and C) 72 hour cultivation: The evaluation is based on three independent preparations. Cardiomyocytes from nine culture dishes per experimental condition and time point were stimulated by electric field at 2 Hz and assigned to the five criteria in a blinded fashion. Identical visual fields were counted; number of cells evaluated: control/mRNA-1273/BNT162b2; 24 h: 647/648/681; 48 h: 558/548/567; 72 h: 478/458/462. *, p< 0.05 vs. control cells, #, p< 0.05 vs. mRNA-1273 (3.3 µg). Figure D) plots over time the percentage of regularly contracting cardiomyocytes considering the three mRNA-1273 concentrations used. Number of cells evaluated after incubation with mRNA-1273: 3.3 µg/1.0 µg/0.3 µg; 24 h: 648/698/686; 48 h: 548/527/581; 72 h: 458/432/403. *, p< 0.05 vs. mRNA-1273 (3.3 µg), #, p< 0.05 vs. mRNA-1273 (1.0 µg).

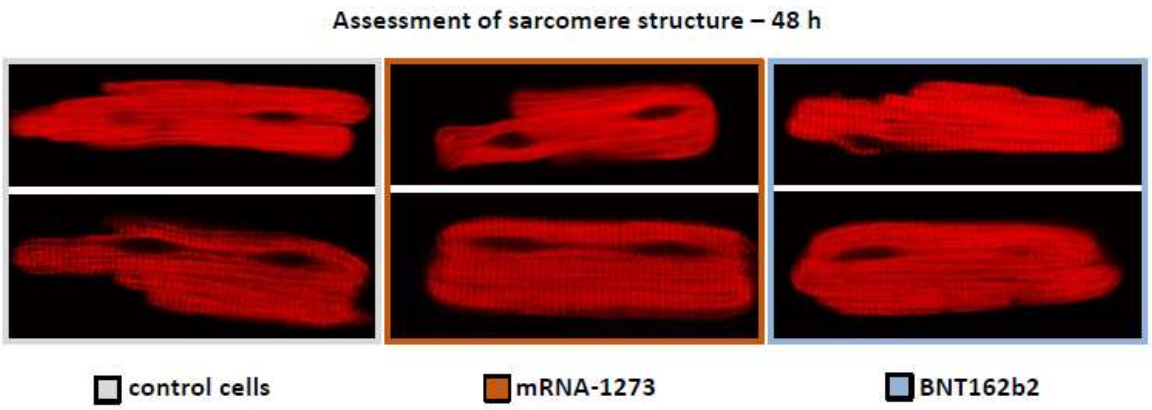


Fig. 4: Assessment of sarcomere structure using phalloidin staining. After 48 hours of cultivation, isolated cardiomyocytes from three culture dishes per experimental condition from three independent preparations were examined for regular transverse striation. Microscopically visible structural damage was not caused by either mRNA-1273 or BNT162b2.

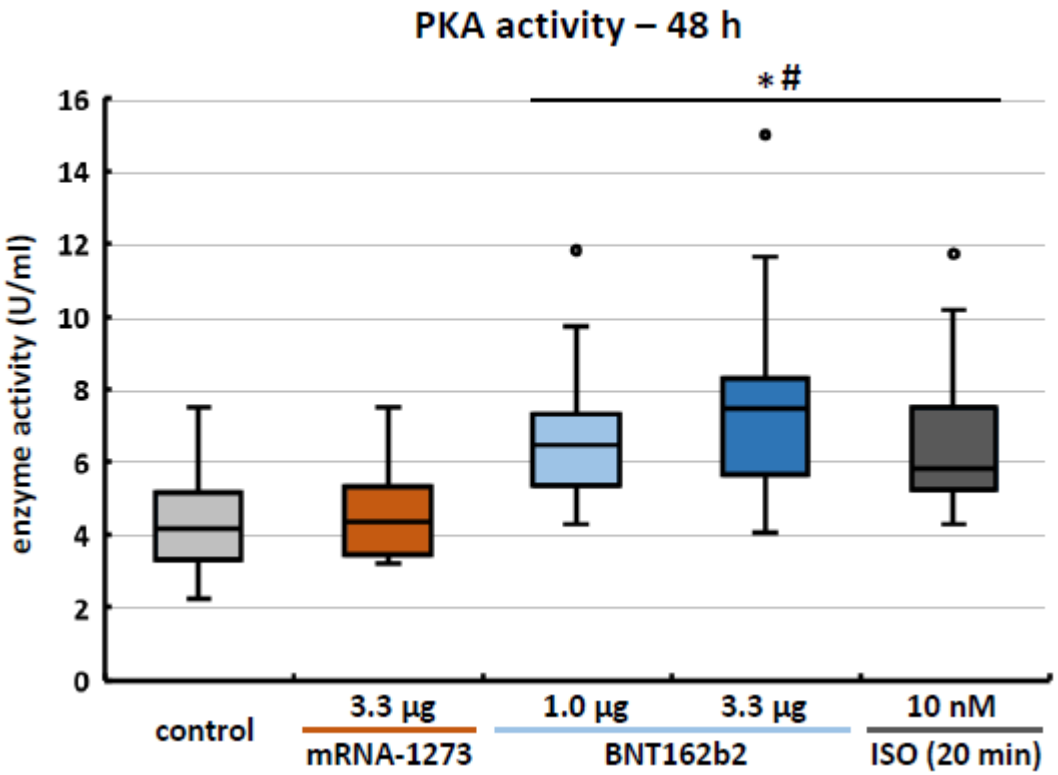


Fig. 5: ELISA-based determination of myocyte PKA activity. In contrast to mRNA-1273, BNT162b2 causes a significant increase in PKA activity after 48 hours of incubation. Comparable enzyme activity could be obtained by stimulating cardiomyocytes with isoprenaline (10 nmol/l final concentration) for 20 minutes. Data are based on n=18 (control, ISO (10 nM)) and n=16 (mRNA-1273 (3.3 µg), BNT162b2 (1.0 µg, 3.3 µg)) culture dishes from n=8 independent preparations. *, $p < 0.05$ vs. control, #, $p < 0.05$ vs. mRNA-1273 (3.3 µg).

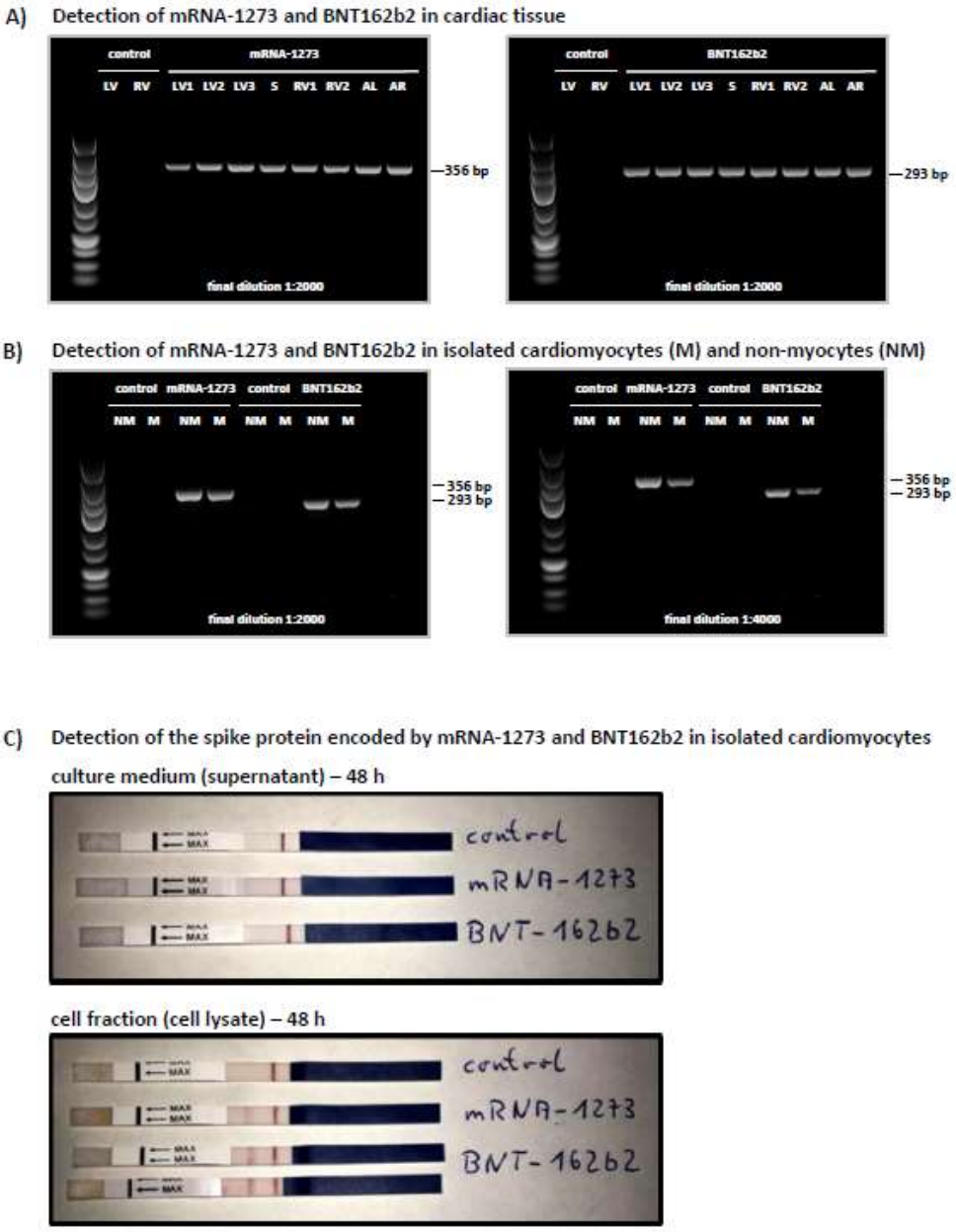
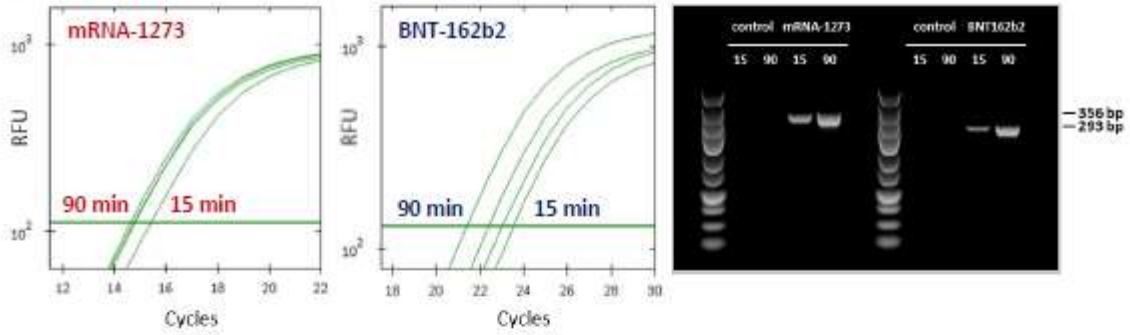


Fig. 6: Cellular uptake of mRNA and production of spike protein in rat cardiomyocytes. PCR-based detection of mRNA-1273 and BNT162b2 after 2-hour Langendorff perfusion was performed in A) cardiac tissue and B) cardiomyocytes (M) and non-myocytic cells (NM) isolated immediately after perfusion (n=2 hearts per experimental condition; mRNA-1273 and BNT162b2 were added at 1:2000 and 1:4000-fold dilution, respectively). Qualitative detection of SARS-CoV-2 spike (S) glycoprotein was performed using the NCV10 rapid antigen assay from Koch Biotechnology Co. Positive test results could be obtained exclusively in the cell fraction after 48-hour incubation with mRNA-1273 or BNT162b2. C) negative test result of the culture medium and positive result of the cell fraction (BNT162b2 – top: 1-fold dose; bottom: 3.3-fold dose).

A) Detection of mRNA-1273 and BNT162b2 in AC16 cardiomyocytes



B) Detection of the spike protein encoded by mRNA-1273 and BNT162b2 in AC16 cardiomyocytes

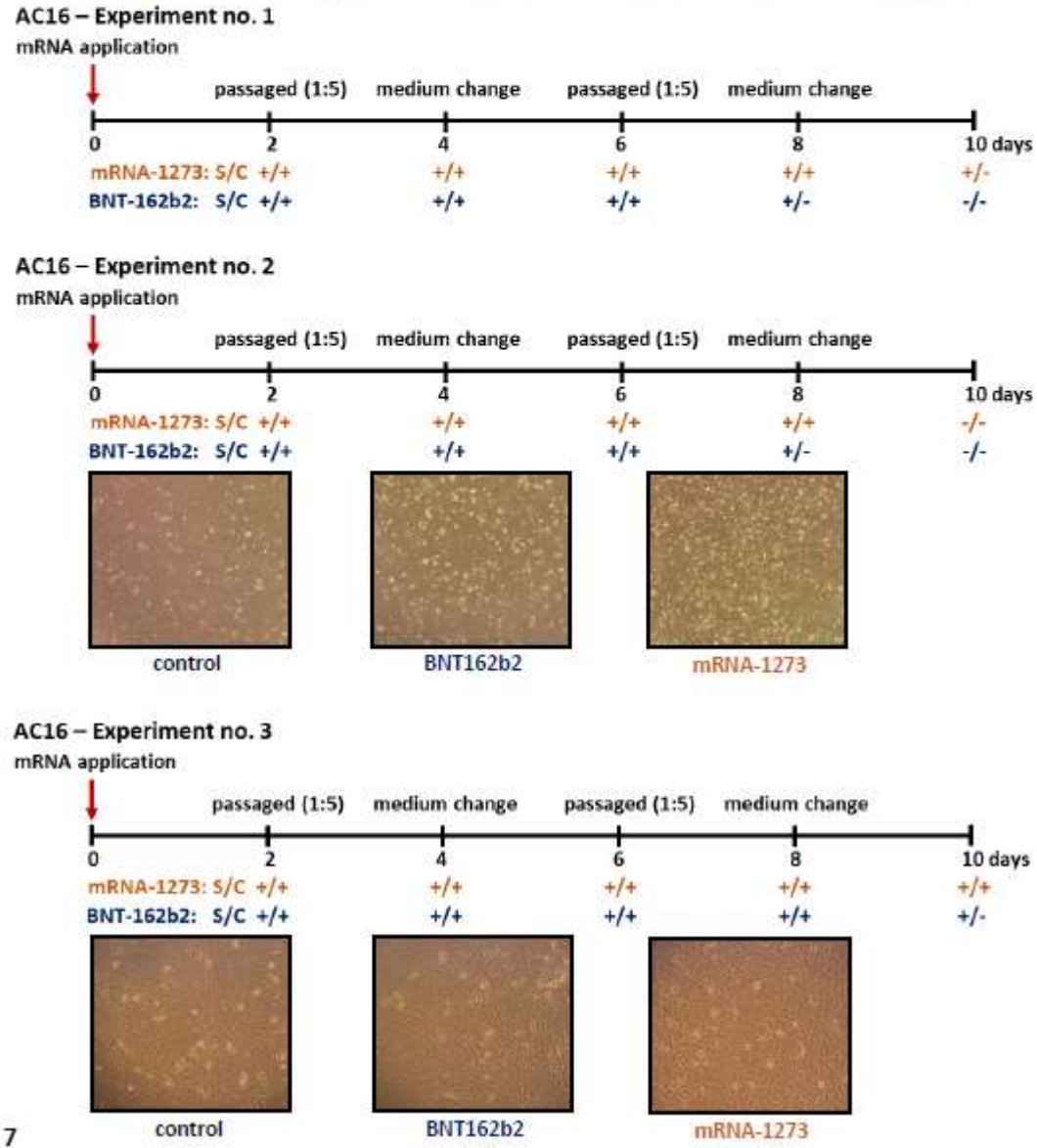


Fig. 7

Fig. 7: Uptake of mRNA and production of spike protein in human AC16 cardiomyocytes. The amount of mRNA taken up was determined 15, 30, 60, and 90 min after application of mRNA-1273 and BNT162b2, respectively, in the cell fraction. Detection and visualization were performed A) by real-time PCR and agarose gel. Spike protein could be detected over a period of B) 6 to 10 days in both the supernatant (S) and the cell fraction (C); release of the spike protein into the cell culture medium was not demonstrated for the isolated rat cardiomyocytes. Reducing the amount of mRNA applied in experiment no. 3 resulted in a prolongation of positive test results due to less cellular damage (see images). mRNA concentration used in experiment no. 1 and no. 2: mRNA-1273 (3.3 µg/ml), BNT162b2 (1.0 µg/ml); Experiment no. 3: mRNA-1273 (0.66 µg/ml), BNT162b2 (0.2 µg/ml).