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ORIGINAL RESEARCH

The acute toxic effects of platinum nanoparticles on ion channels, transmembrane potentials of cardiomyocytes in vitro and heart rhythm in vivo in mice

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Background: Platinum nanoparticles (PtNPs) have been considered a nontoxic nanomaterial and been clinically used in cancer chemotherapy. PtNPs can also be vehicle exhausts and environmental pollutants. These situations increase the possibility of human exposure to PtNPs. However, the potential biotoxicities of PtNPs including that on cardiac electrophysiology have been poorly understood.

Methods: Ion channel currents of cardiomyocytes were recorded by patch clamp. Heart rhythm was monitored by electrocardiogram recording. Morphology and characteristics of PtNPs were examined by transmission electron microscopy, dynamic light scattering and electrophoretic light scattering analyses.

Results: In cultured neonatal mice ventricular cardiomyocytes, PtNPs with diameters 5 nm (PtNP-5) and 70 nm (PtNP-70) concentration-dependently (10^{-9} – 10^{-5} g/mL) depolarized the resting potentials, suppressed the depolarization of action potentials and delayed the repolarization of action potentials. At the ion channel level, PtNPs decreased the current densities of I_{Na}, I_{K1} and I_{to} channels, but did not affect the channel activity kinetics. In vivo, PtNP-5 and PtNP-70 dose-dependently (3–10 mg/kg, i.v.) decreased the heart rate and induced complete atrioventricular conduction block (AVB) at higher doses. Both PtNP-5 and PtNP-70 (10^{-9} – 10^{-5} g/mL) did not significantly increase the generation of ROS and leak of lactate dehydrogenase (LDH) from cardiomyocytes within 5 mins after exposure except that only very high PtNP-5 (10^{-5} g/mL) slightly increased LDH leak. The internalization of PtNP-5 and PtNP-70 did not occur within 5 mins but occurred 1 hr after exposure.

Conclusion: PtNP-5 and PtNP-70 have similar acute toxic effects on cardiac electrophysiology and can induce threatening cardiac conduction block. These acute electrophysiological toxicities of PtNPs are most likely caused by a nanoscale interference of PtNPs on ion channels at the extracellular side, rather than by oxidative damage or other slower biological processes.

Keywords: platinum nanoparticle, transmembrane potential, ion channel, arrhythmia, nanotoxicity, heart

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Background

Platinum-based preparations/drugs, including platinum nanoparticles (PtNPs) and special PtNPs-delivering systems, have been regularly used in cancer chemotherapy.[1–9] PtNPs are considered a nontoxic anticancer nanomaterial relative to other chemotherapeutics.[1] In vitro, PtNPs may kill cancer cells via biological and physical ways. For example,

PtNPs inhibit the proliferation and induce apoptosis of cancer cells by means of DNA fragmentation[10] or via photothermal ablation.[8,11,12]

Although PtNPs are generally considered nontoxic in cancer chemotherapy, substantial in vitro studies have reported the toxic effects of PtNPs on genes and a variety of cells. For example, PtNPs induce apoptosis on Raw264.7 macrophages.[13] PtNPs size-dependently induce DNA strand breaks in human bronchial epithelial cells.[14] PtNPs inhibit matrix metalloproteinases and decrease cell viability in L929 fibroblast cells.[15] PtNPs induce DNA damage and p53-mediated growth arrest in human lung fibroblasts.[16] A few studies also revealed biotoxicity of PtNPs in vivo. In zebrafish embryos, PtNPs induce hatching delay, heart rate (HR) reduction, touch response and axis curvatures.[17] PtNPs (1 nm) are hepatotoxic and nephrotoxic and can also exacerbate drug (carbon tetrachloride or cisplatin) toxicity in mice.[18]

Now that platinum- and PtNP-based therapeutic agents are widely used in cancer therapy, patients with tumors may have more chance to be exposed to these agents. In addition, PtNPs and platinum-group elements are vehicle-exhaust catalysts and are new kinds of environmental pollutants which are hazardous to organisms including humans.[19] Environmental PtNP pollution increases the chance of PtNP exposure not only to tumor patients but also to healthy people and all other organisms. Therefore, a thorough understanding of PtNP biotoxicity is warranted. PtNPs may exert acute and chronic toxic effects on organisms including humans. We noticed that PtNPs decrease the HR in zebrafish.[17] This report encourages us to investigate the potential toxic effects of PtNPs on cardiac electrophysiology which are seldom investigated till now, also taking into account that cancers usually occur in elder people who have more incidence of cardiovascular diseases and are susceptible to abnormal cardiac electrophysiology and heart rhythm.

The present study aimed to investigate the potential acute toxic effects of PtNPs on cardiac electrophysiology at the molecular, cellular and integrative levels in mice both in vitro and in vivo and the potential underlying mechanisms. As the biological effects of PtNPs may be size-dependent, we selected two sizes of PtNPs to do the experiments: diameters 5 nm (PtNP-5) and 70 nm (PtNP-70). We first observed the acute hazardous effects of PtNPs on the transmembrane potential (TMP) including the resting potential (RP) and the action potential (AP). As ion channel activity is the basis of TMP, we then examined the acute effects of PtNPs on several major ion channels in the cardiomyocytes, including the inwardly rectifying potassium channel (I_{K1} channel) which takes a major role in the maintenance of RP and late-phase repolarization of AP, the voltage-gated sodium channel (I_{Na} channel) which is responsible for the depolarization of AP and the transient outward potassium channel (I_{to} channel) which is the key channel for the early repolarization of AP and takes a major role in AP repolarization especially in mice cardiomyocytes.[20] At the integrative level, we recorded the surface electrocardiogram (ECG) and observed whether these PtNPs would affect HR and rhythm. Results showed that PtNPs of both sizes similarly inhibited the activities of above ion channels, and thus depolarized the RP, suppressed the depolarization and decelerated the repolarization of AP. To our expectation, PtNPs decreased the sinus HR at lower doses and induced complete atrioventricular conduction block (AVB) at higher doses in vivo.

To explore the potential mechanisms of the above electrophysiological toxicities of PtNPs, we observed the time-dependent endocytosis of PtNPs in cardiomyocytes and measured the generation of ROS and leak of lactate dehydrogenase (LDH) in cardiomyocytes after acute exposure to PtNPs. Results revealed that endocytosis, oxidative stress and membrane injury are not responsible for the acute toxicities of PtNPs on cardiac electrophysiology. We suggest that patients with cardiac diseases especially with high susceptibility to AVB should be cautious when using PtNPs for cancer therapy or when encountering serious air pollution with PtNP-containing vehicle exhausts.

Materials and methods
Ethical approval
The cell and animal use protocols were approved by the Life Ethics Committee of Shanxi Medical University and were in compliance with the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publication 85–23, revised 1985).

Preparation and characterization of PtNPs
Citrate-coated PtNPs with diameters 5 nm (PtNP-5) and 70 nm (PtNP-70) were purchased from nanoComposix (San Diego, CA, USA). Both PtNP-5 and PtNP-70 had a purity of 99.99% according to the manufacturer. The PtNP concentration of the stock solution was 1 mg/mL (Pt) in sodium citrate. Prior to experiments, PtNPs were filter-

sterilized, and then lipopolysaccharide (LPS) was examined with limulus reagent (Genscript Biotech. Co., Hong Kong, China). Ultrasonic vibration was performed for 20 mins to disperse the PtNP stock solution before use. A certain amount of stock PtNPs was diluted with 5% isotonic glucose solution for in vivo experiments, or diluted with water or respective extracellular solutions (culture medium, Tyrode's solution or external solutions for recordings of TMP and channel currents for in vitro experiments).

The morphology of PtNPs was observed using a JEM-1400 plus transmission electron microscope (JEOL Ltd., Tokyo, Japan). A small amount of PtNP stock solution was diluted to 10^{-5} g/mL with distilled water, and then the diluted PtNP solution was dropped onto a copper grid and TEM was performed. Images of PtNPs were taken with a CCD camera (Veleta, Olympus Soft Imaging Solutions GmbH, Münster, Germany) attached to the electron microscope.

The hydrodynamic diameters and zeta potentials of PtNP suspensions were, respectively, determined with the methods of dynamic light scattering (DLS) and electrophoretic light scattering (ELS) analyses, using a ZetasizerNano ZS90 analyzer (Malvern Instruments Ltd, Malvern, UK). Briefly, stock solutions of both PtNP-5 and PtNP-70 were dispersed in 1 mL of various solutions including distilled water, serum-containing culture medium, Tyrode's solution and bath solutions for recording TMP and channel currents, incubated for 5 mins, and then DLS and ELS were performed at room temperature. Three independent experiments were performed for each treatment.

Isolation and culture of neonatal ventricular myocytes

Cardiomyocytes were isolated from the ventricles of neonatal (1–2 days old) C57BL6/J mice using an enzymatic method as we reported previously.[21] Briefly, mice were decapitated, and hearts were harvested and ventricular tissues were trimmed; then they were put into cold PBS and digested with 0.25% trypsin at 37°C for 8 mins. The digestion was stopped with FBS and cell pellets were harvested. The residual tissues were again digested for another 8 mins. These steps were repeated several times until most ventricular tissues were well digested. The collected cell suspension was filtered with a cell strainer (aperture 74 μm) and then centrifuged at 1,200 rpm for 5 mins. All collected cell pellets were resuspended in high-glucose DMEM added with 100 U/mL penicillin, 100 μg/mL streptomycin and 15% FBS, and primarily cultured in 3.5-cm culture dishes. After culture for 48 hrs, cardiomyocytes were used for patch clamp study.

Patch clamp

Whole-cell recording configuration of the patch clamp technique was used to record channel currents or TMPs of the cultured cardiomyocytes using an EPC-10 amplifier and PatchMaster software (Heka Elektronik, Lambrecht, Germany). Current clamp mode was used to record the TMP, and voltage clamp mode was applied to record channel currents. The pipettes (resistance 5–7 MΩ) were pulled with the P-97 horizontal puller (Sutter Instruments, Novato, CA, USA) using standard-wall borosilicate glass (inner diameter 0.86 mm, outer diameter 1.5 mm). All patch clamp experiments were conducted at room temperature (23±2 °C), and the series resistance was mostly compensated by about 70%.

To record TMP including RP and AP using current clamp mode, the bath solution contained (mmol/L): NaCl 140, KCl 4, CaCl$_2$ 2, MgCl$_2$ 2, HEPES 5, glucose 5, pH 7.4 adjusted with NaOH. Pipette solution contained (mmol/L): NaCl 10, KCl 140, ethylene glycol diethyl ether diamine tetraacetic acid (EGTA) 2, MgCl$_2$ 1, Mg-ATP 5, HEPES 10, pH 7.3 adjusted with KOH. APs were elicited by rectangular current pulses of 1 Hz and 1 ms duration at 125% threshold level. TMPs at baseline and after exposure to PtNP-5 or PtNP-70 (both $10^{-9} - 10^{-5}$ g/mL) for 5 mins were recorded and compared.

Voltage clamp experiments were performed to record the I_{Na}, I_{K1} and I_{to} of cultured cardiomyocytes at baseline and after PtNP-5 or PtNP-70 exposure at different concentrations (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} g/mL).

To record the I_{Na} and to establish the I–V curves and activation curves of I_{Na} channels, the bath solution contained (in mM): NaCl 15, N-methyl-D-glucamine (NMDG) 125, KCl 2.5, CaCl$_2$ 1, MgCl$_2$ 1, HEPES 10, pH 7.4 adjusted with HCl. Pipette solution contained (in mmol/L): CsF 105, NaCl 10, EGTA 10, HEPES 10, pH 7.3 adjusted with CsOH. The recording protocols were the same as we reported previously.[21] Briefly, the holding potential was set to −90 mV followed by 50-ms step depolarization pulses from −90 mV to +20 mV in 10-mV steps. The measured I_{Na} was divided by cell capacitance to obtain current densities. The I–V curves and the activation, inactivation and recovery curves of I_{Na} channels were
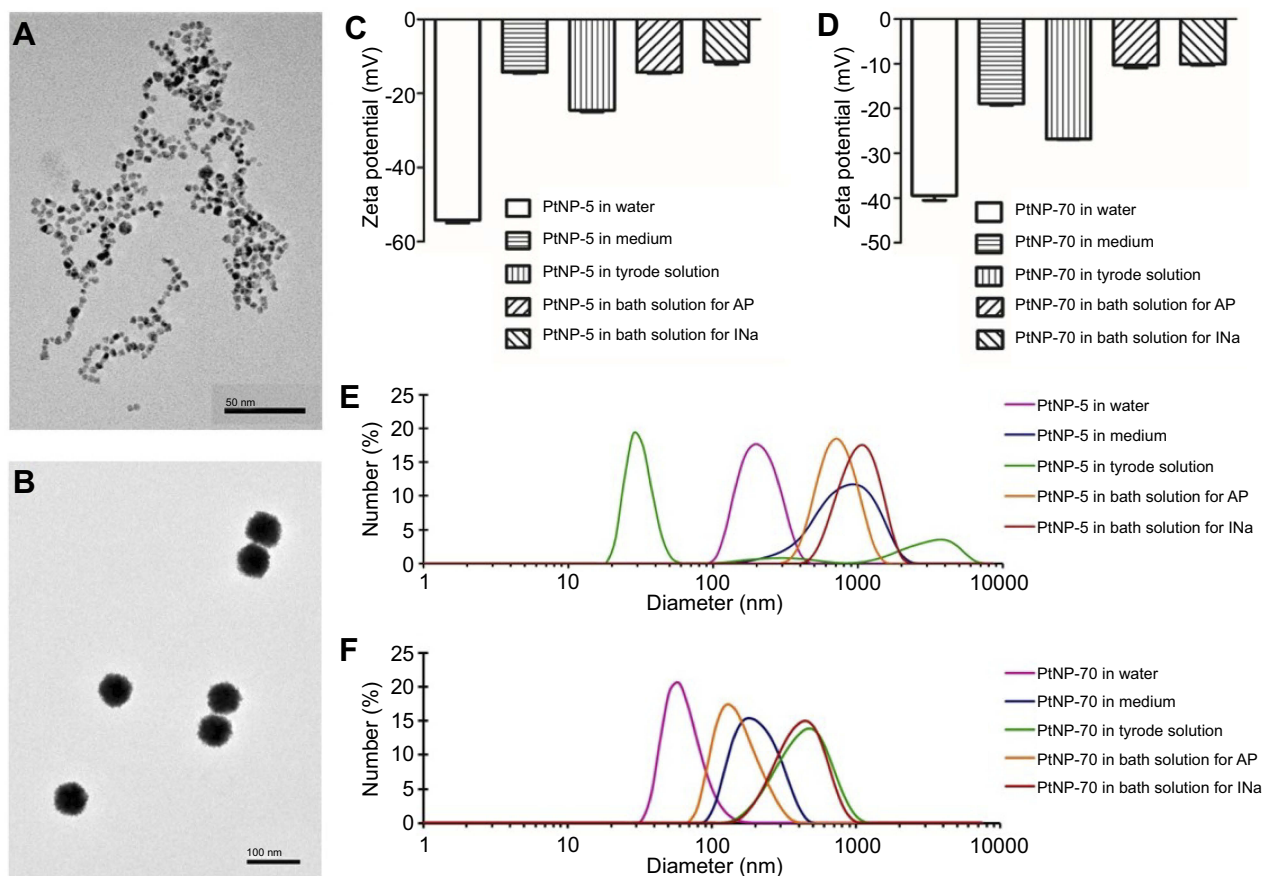
Figure 1 The morphology and characterization of PtNPs. (**A** and **B**) Transmission electron microscopy (TEM) images of PtNP-5 and PtNP-70 in the culture medium, respectively. (**C** and **D**) Zeta potentials, respectively, for PtNP-5 and PtNP-70 in different solutions 5 mins after incubation. (**E** and **F**) Hydrodynamic diameters, respectively, for PtNP-5 and PtNP-70 in different solutions 5 mins after incubation.
Abbreviations: PtNP, platinum nanoparticle; PtNP-5 and PtNP-70, platinum nanoparticles with diameters of 5 and 70 nanometers.

Both PtNP-5 and PtNP-70 were well-dispersed in various solutions including water, cell culture medium containing 15% FBS and external solutions for recording AP and I_{Na} currents after incubation for 5 mins. PtNP-5 showed zeta potentials (mV) of –54.20±0.44, –14.27 ±0.16, –24.53±0.22, –14.30±0.15 and –11.40±0.40, respectively, in water, culture medium, Tyrode's solution, external solution for recording of AP and external solution for I_{Na} recording (Figure 1C). PtNP-70 showed zeta potentials (mV) of –39.43±0.62, –18.90±0.18, –26.80±0.07, –10.28±0.33 and –10.04±0.17, respectively, in water, culture medium, Tyrode's solution, external solution for AP recording and external solution for I_{Na} recording (Figure 1D). The zeta potentials of both PtNP-5 and PtNP-70 were more negative in water than in other solutions.

The hydrodynamic diameters for PtNP-5 (Figure 1E) and PtNP-70 (Figure 1F) in different solutions were basically consistent with Gaussian distribution. The variations of zeta potentials and sizes of PtNPs in different solutions might be contributed to adsorption of medium components onto the PtNP surface or mild degree of aggregation of PtNPs in different solutions.

PtNPs depolarize the RPs, suppress the depolarization and delay the repolarization of APs in cultured neonatal mice cardiomyocytes

The TMPs were recorded using the current clamp mode of patch clamp technique in cultured neonatal mice cardiomyocytes. The baseline RP was about –70 mV, the action potential amplitude (APA) was about 110 mV and the APD90 was about 90 ms (Figure 2). PtNP-5 (Figure 2A) concentration (10^{-9}–10^{-5} g/mL)-dependently depolarized the RP, suppressed the depolarization and delayed the repolarization of AP after exposure for 5 mins. PtNP-5 at
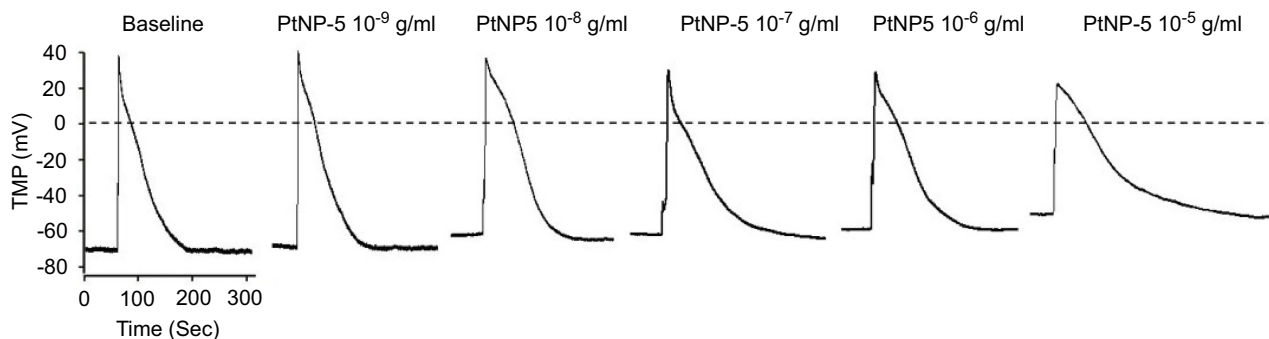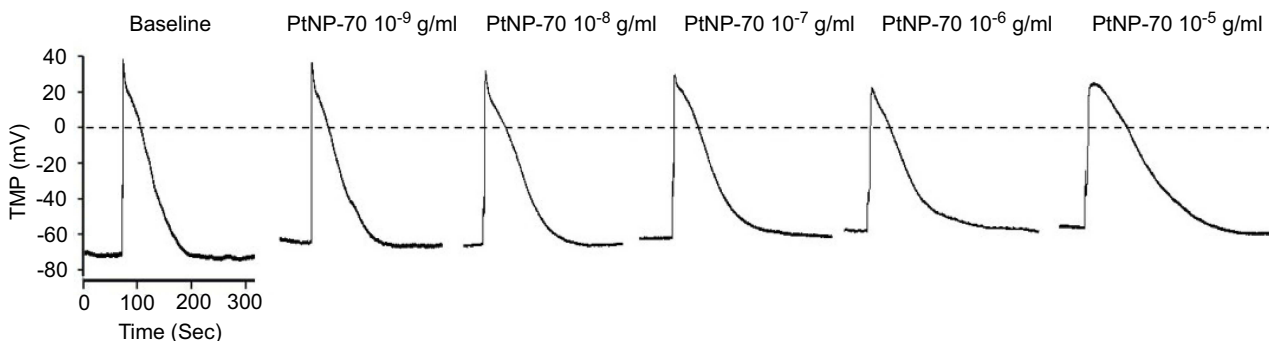
A PtNP-5, 5 min

B PtNP-70, 5 min

Figure 2 The hazardous effects of PtNP-5 and PtNP-70 on the transmembrane potentials (TMPs) of cultured neonatal cardiomyocytes. (**A**) PtNP-5. (**B**) PtNP-70. Note that both PtNP-5 and PtNP-70 concentration-dependently depolarized the RP, decreased the APA and prolonged the APD within 5 mins after exposure.
Abbreviations: RP, resting potential; AP, action potential; APA, action potential amplitude; APD, action potential duration.

10^{-9} g/mL only slightly depolarized the RP, while it did not significantly affect the shape of AP. Higher concentrations of PtNP-5 induced more obvious changes of AP shape, including more obvious RP depolarization, APA reduction and APD prolongation (Figure 2A). PtNP-70 (Figure 2B) exerted similar effects on the RP and AP as that induced by PtNP-5 after exposure for 5 mins. Statistical results of TMP parameters showed the quantitative changes of TMPs of cardiomyocytes after exposure to PtNP-5 or PtNP-70 for 5 mins (Figure S1).

PtNPs decrease the current densities of I_{Na} channels but do not significantly alter the channel activation, inactivation and recovery kinetics in cultured neonatal cardiomyocytes

Voltage clamp mode was used to record the I_{Na} currents. PtNP-5 concentration (10^{-9}–10^{-5} g/mL)-dependently decreased the I_{Na} currents compared with baseline (control) I_{Na} currents after exposure for 5 mins (Figure 3A). PtNP-70 exerted similar

inhibitory effects on the I_{Na} currents as that did by PtNP-5 (Figure 3B). The I–V curves of I_{Na} channels showed that both PtNP-5 and PtNP-70 significantly decreased the I_{Na} current densities after exposure for 5 mins (Figure 3C and D).

Either PtNP-5 or PtNP-70 at 10^{-7} g/mL did not significantly affect the activation (Figure S2), inactivation (Figure S3) and recovery (Figure S4) kinetics of I_{Na} channels.

PtNPs decrease the current densities but do not significantly alter the activation kinetics of I_{K1} channels in cultured neonatal mice cardiomyocytes

As PtNPs depolarized the RP of cardiomyocytes (shown in Figure 2), we estimated that PtNPs may suppress the I_{K1} channels which are known to be responsible for the maintenance of RP. Results confirmed our estimation that both PtNP-5 and PtNP-70 at 10^{-7} g/mL moderately but significantly decreased the I_{K1} (Figure 4A and B). However, PtNP-5 and PtNP-70 at 10^{-7} g/mL did not
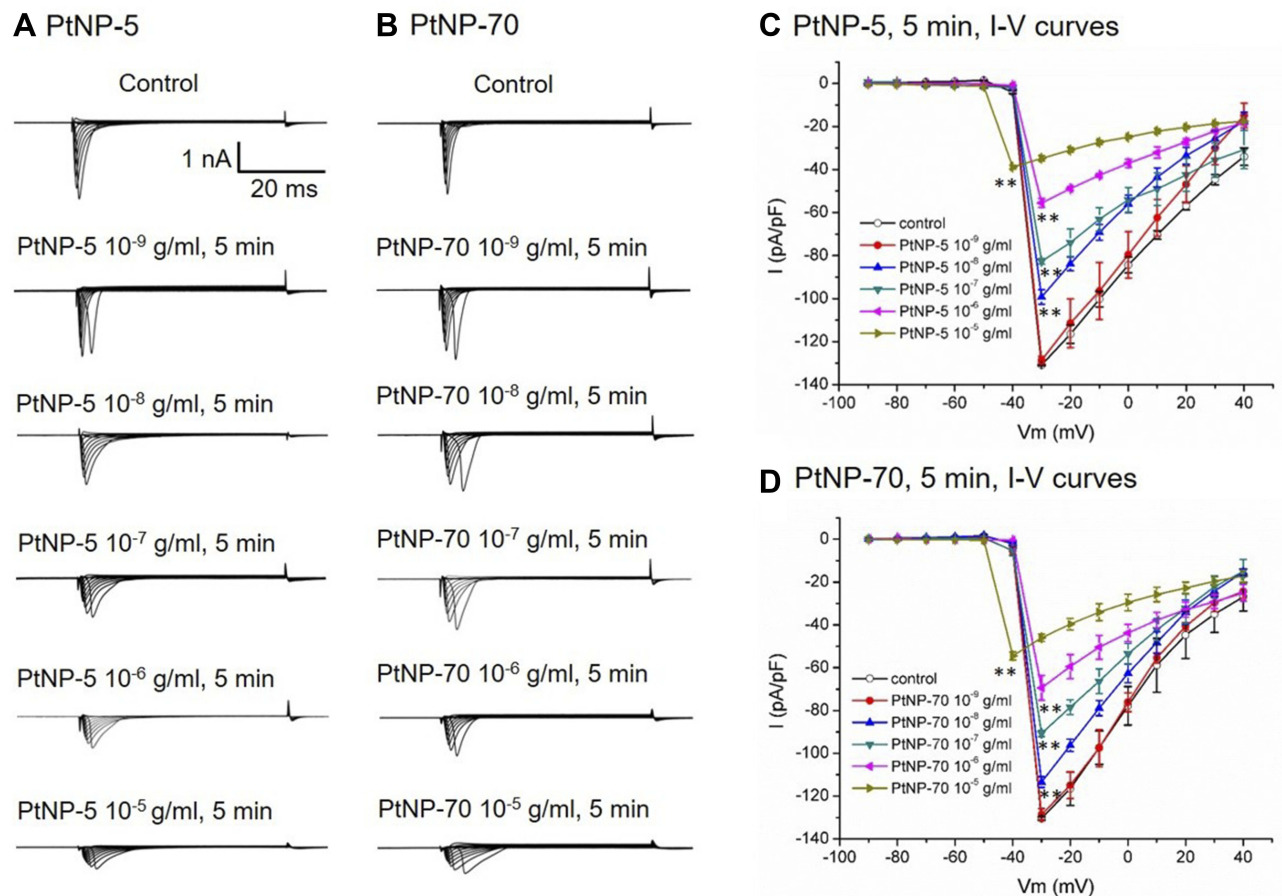
Figure 3 The concentration-dependent inhibitory effects of PtNPs on I_{Na} channels in cultured neonatal mice cardiomyocytes. (**A** and **B**) Typical I_{Na} current tracings at baseline and after exposure to PtNP-5 and PtNP-70 ($10^{-9} - 10^{-5}$ g/mL) for 5 mins, respectively. (**C** and **D**) The I–V curves of I_{Na} channels for PtNP-5 and PtNP-70, respectively. **P<0.01 vs the corresponding value of the control.

significantly affect the activation curves of I_{K1} channels in the cardiomyocytes (Figure 4C).

PtNPs decrease the current densities but do not significantly alter the activation kinetics of I_{to} channels in cultured neonatal cardiomyocytes

As we observed that PtNPs blunted the early repolarization of AP (shown in Figure 2), and I_{to} channels are known responsible for the early repolarization of AP, we thus examined the effects of PtNPs on I_{to} channels. Results showed that both PtNP-5 and PtNP-70 at 10^{-7} g/mL moderately but significantly suppressed the I_{to} currents and current densities (Figure 5A and B) but did not significantly affect the activation kinetics of I_{to} channels in the cardiomyocytes (Figure 5C).

PtNPs dose-dependently decrease the HR, prolong the P-R intervals and finally lead to complete A-V conduction block in adult mice in vivo

The suppressing effects of PtNPs on RP and AP (shown in Figure 2) suggest that PtNPs may induce cardiac conduction block and bradyarrhythmias. We, therefore, observed the effects of PtNPs (i.v.) on the ECGs of mice in vivo. PtNP-5 i.v. at 3 mg/kg did not affect the ECG (Figure S5). PtNP-5 i.v. at 4 mg/kg slightly decreased the sinus HR and prolonged the P-R intervals (degree I AVB) but did not induce other types of arrhythmias within the observed 90 mins (Figure S6). PtNP-5 i.v. at 6 mg/kg decreased the HR and significantly prolonged the P-R intervals but did not induce complete AVB within 90 mins (Figure S7). PtNP-5 i.v. at 8 mg/kg decreased
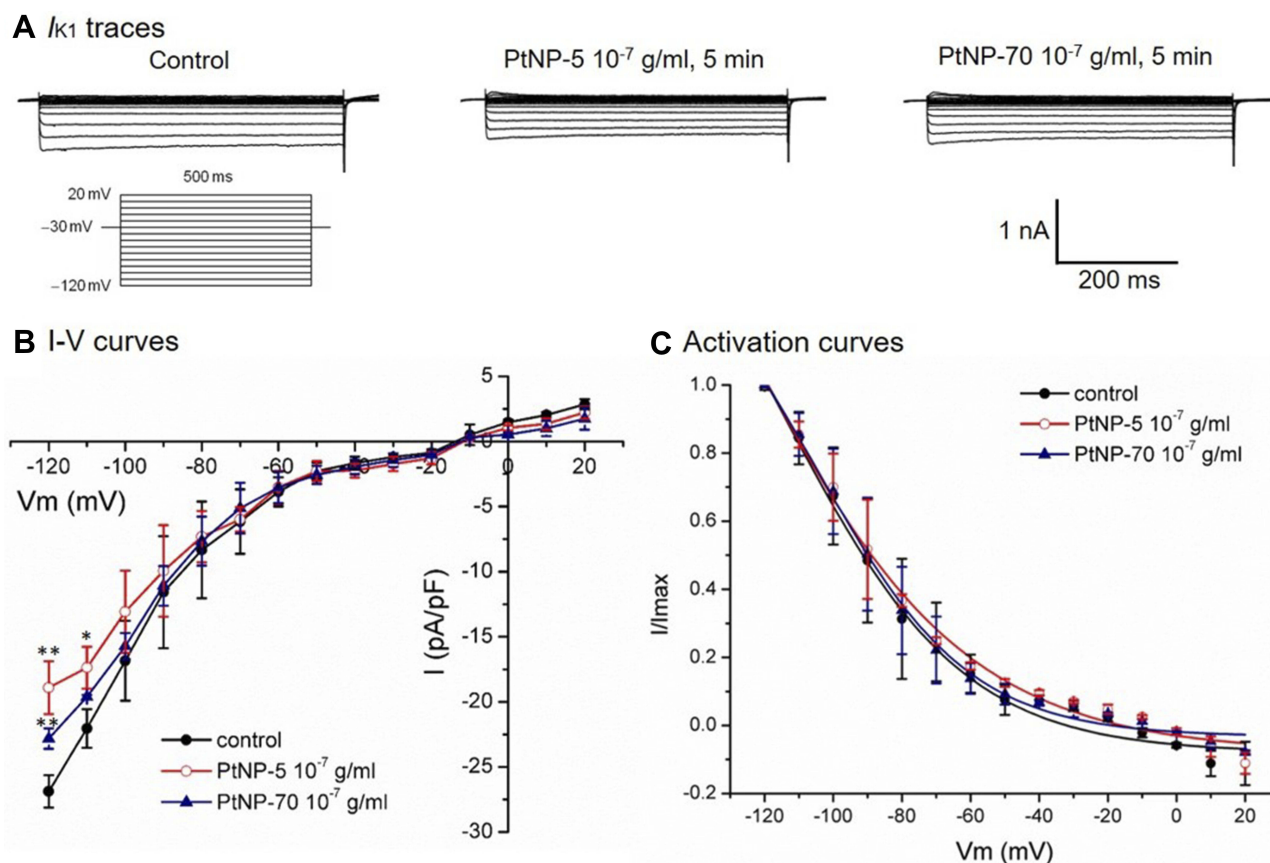
A I_{K1} traces

B I-V curves

C Activation curves

Figure 4 The suppressing effects of PtNP-5 and PtNP-70 (10^{-7} g/mL) on the I_{K1} channel currents in cultured neonatal cardiomyocytes. (**A**) Typical I_{K1} current tracings at baseline (control) and after exposure, respectively, to PtNP-5 and PtNP-70 for 5 mins. (**B**) The I–V curves of I_{K1} channels at baseline and after exposure to PtNP-5 and PtNP-70 for 5 mins. (**C**) the activation curves of I_{K1} channels at baseline and after exposure to PtNP-5 and PtNP-70 for 5 mins. Note that both PtNP-5 and PtNP-70 decreased the I_{K1} current densities but did not significantly affect the channel activation kinetics. * $P<0.05$, ** $P<0.01$ vs the corresponding value of control.
Abbreviations: Vm, membrane potential; I-V curve, current/voltage curve.

the HR and severely prolonged the P-R intervals, but also did not induce complete AVB within 90 mins (Figure S8). PtNP-5 i.v. at 10 mg/kg significantly decreased the HR and further induced complete AVB but did not lead to cardiac asystole and animal death within 90 mins (Figure 6). PtNP-70 i.v. at doses of 3–10 mg/kg exerted similar effects on the ECGs including the induction of complete AVB at 8–10 mg/kg, and also did not induce cardiac asystole and animal death within the observed 90 mins after intravenous injection (Figure 7; Figures S9–S12). No tachyarrhythmias (such as ventricular tachycardia and ventricular fibrillation) were observed after PtNP-5 or PtNP-70 intravenous injection.

Note that both PtNP-5 and PtNP-70 also slowed down the intraventricular conduction. For example, PtNP-5 widened QRS complex at higher doses (shown in Figures 6 and 7).

PtNPs do not significantly induce oxidative stress and membrane injury after short-time exposure in cultured neonatal cardiomyocytes

To identify whether the above acute toxic effects of PtNPs on cardiac electrophysiology were caused by oxidative stress and cell membrane injury, we measured the acute effects of PtNPs (5 mins after exposure) on the generation of intracellular ROS and LDH leak from the cardiomyocytes which represents surface membrane injury. Results showed that both PtNP-5 and PtNP-70 at a concentration range of $10^{-9} - 10^{-5}$ g/mL did not significantly increase the intracellular ROS levels (Figure S13A). PtNP-5 and PtNP-70 at a concentration range of $10^{-9} - 10^{-6}$ g/mL also
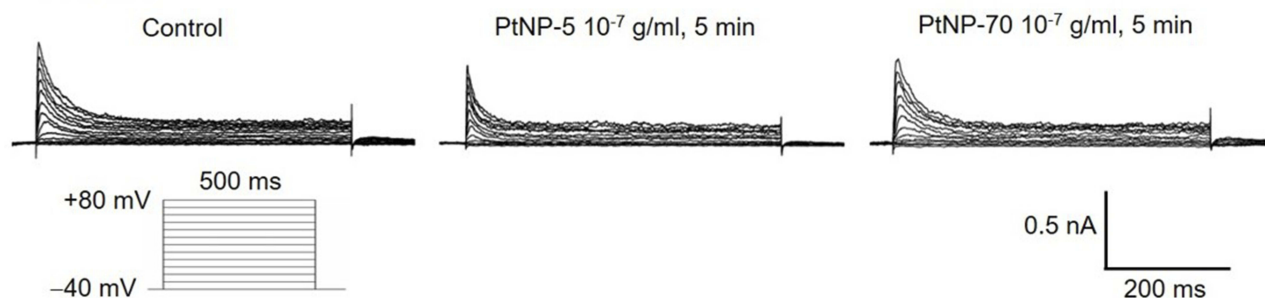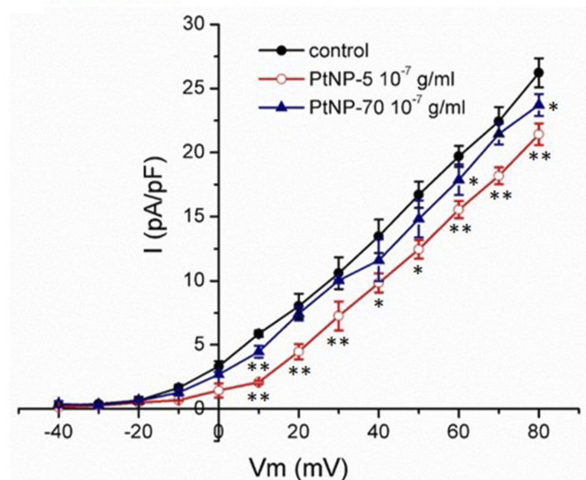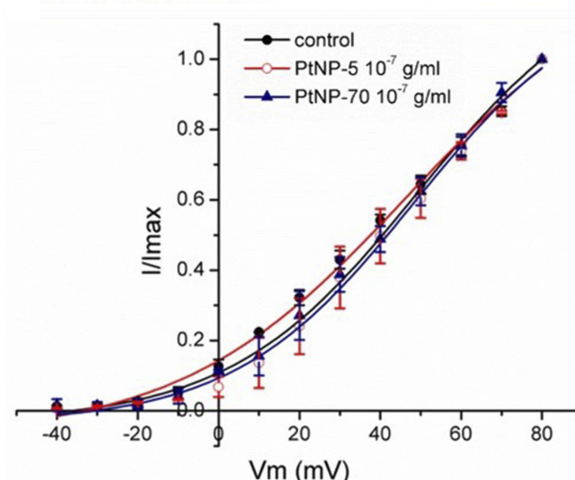
A I_{to} traces

Control

PtNP-5 10^{-7} g/ml, 5 min

PtNP-70 10^{-7} g/ml, 5 min

+80 mV

500 ms

–40 mV

0.5 nA

200 ms

B I-V curves

- control
- PtNP-5 10^{-7} g/ml
- PtNP-70 10^{-7} g/ml

I (pA/pF)

Vm (mV)

C Activation curves

- control
- PtNP-5 10^{-7} g/ml
- PtNP-70 10^{-7} g/ml

I/Imax

Vm (mV)

Figure 5 The suppressing effects of PtNP-5 and PtNP-70 (10^{-7} g/mL) on the I_{to} channels in cultured neonatal cardiomyocytes. (**A**) Typical I_{to} current tracings at baseline and after exposure to PtNP-5 and PtNP-70 for 5 mins. (**B**) I–V curves of I_{to} channels at baseline and after exposure to PtNP-5 and PtNP-70 for 5 mins. (**C**) The activation curves of I_{to} channels at baseline and after exposure to PtNP-5 and PtNP-70 for 5 mins. Note that both PtNP-5 and PtNP-70 decreased the I_{to} current densities but did not significantly affect the channel activation kinetics. *$P<0.05$, **$P<0.01$ vs the corresponding value of control.

both did not significantly increase LDH leaking, except that PtNP-5 at 10^{-5} g/mL slightly but significantly increased LDH leak (Figure S13B). These results suggest that oxidative stress and membrane injury was not a leading mechanism underlying the acute toxic effects of PtNPs on cardiac electrophysiology.s

The rapid hazardous effects of PtNPs on cardiac electrophysiology are internalization independent

Nanoparticles may impair the cell function through an extracellular way or an intracellular way. Some studies have demonstrated that nanoparticles can be internalized by cells after certain time of exposure. To determine whether the rapid hazardous effects of PtNPs on cardiac electrophysiology were actually caused by extracellular or intracellular PtNPs, we used TEM to examine the internalization of PtNPs in cultured neonatal cardiomyocytes after

cell exposure to PtNP-5 or PtNP-70 (both 10^{-5} g/mL) for 5 mins and 1 hr. Figure 8A and B showed the appearance of PtNP-5 or PtNP-70, respectively, in the culture medium (extracellular space); note that the sizes of these two types of PtNPs just matched the scale. Figure 8C shows a control cardiomyocyte without exposure to PtNPs. Both PtNP-5 (Figure 8D) and PtNP-70 (Figure 8E) could not be internalized within 5 mins after exposure at 37ºC, and neither PtNP-5 nor PtNP-70 was found inside the cell. After exposure to PtNP-5 or PtNP-70 for 1 hr, both PtNP-5 (Figure 8F) and PtNP-70 (Figure 8G) were found internalized by the cardiomyocytes.

Discussion
Along with the increased clinical use of PtNP-based cancer therapeutic agents and the worsened air pollution with vehicle PtNP exhausts, attention ought to be paid to the potential acute and chronic biotoxicities of PtNPs. However, investigations into the toxicities of PtNPs are
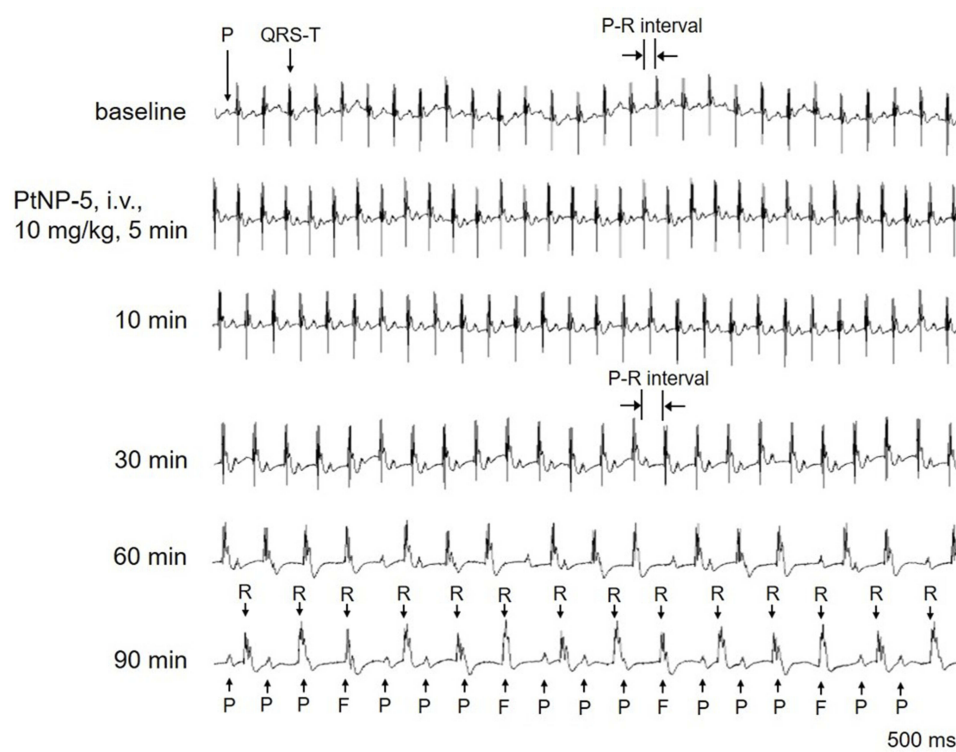
Figure 6 Representative ECGs showing the effects of intravenous PtNP-5 (10 mg/kg) on the heart rate and rhythm of a mouse in vivo. PtNP-5 at this dose successively decreased the heart rate, prolonged the P-R intervals (degree I AVB) and induced complete atrio-ventricular conduction block (AVB) (degree III AVB) within the observed 90 mins. PtNP-5 did not induce ventricular tachyarrhythmias or cardiac asystole within 90 mins.
Abbreviations: ECG, electrocardiogram; P, P wave; QRS-T, QRS complex and T wave; F, fusion of P wave with QRS complex; R, R wave.

insufficient at present. Most of the studies on PtNP toxicities are conducted on the cellular level, seldom studies have been done at the integrative level, and no report is available to show the potential toxic effects of PtNPs on cardiac electrophysiology. One of the reasons may be that Pt/PtNP-based therapeutic agents are basically not very toxic and are even considered nontoxic.[1] Even so, there are still some reports showing the toxicities of PtNPs at the cellular and integrative levels as described above.[13–18] The present study focused on the acute toxic effects of PtNPs on the cardiac electrophysiology in a mouse model both in vitro and in vivo. We did find substantial toxicities of PtNPs on the cardiac electrophysiology at different levels. PtNPs exerted these toxic effects in a concentration/dose-dependent manner both in vitro and in vivo, and the lowest toxic concentration/dose was 10^{-9} g/mL in vitro and 4 mg/kg i.v. in mice in vivo. Generally, PtNPs suppressed the activities of several ion channels, affected the depolarization and repolarization of AP and thus changed the shape of TMP and decreased the HR and induced complete AVB at higher doses in vivo. Cardiac ion channel activities endow the maintenance of RP and the formation of AP

in the cardiomyocytes. AP conduction in the myocardium triggers the excitation–contraction coupling and thus accomplish the pumping function of the heart. The effects of PtNPs on ion channels better explain the effects of PtNPs on TMP and heart rhythm in the present study.

We first observed that PtNPs of both sizes depolarized the RP in a concentration-dependent manner. It is known that mild-to-moderate RP depolarization increases the excitability and the susceptibility to delayed after-depolarization and triggered activity, and severe RP depolarization would lead to decrease and even loss of excitability in the cardiomyocytes. Both PtNP-5 and PtNP-70 at the highest tested concentration (10^{-5} g/mL) depolarized the RP by about 20 mV (roughly from −70 mV to −50 mV), and this intensity of RP depolarization did not reach the membrane potential threshold for Na^+ channel inactivation (usually at −40 mV). In other words, the RP-depolarizing effect of PtNPs was not as severe as silver nanoparticles (AgNPs), AgNPs could severely depolarize the RP and lead to total loss of cardiomyocyte excitability at higher concentration (10^{-6} g/mL).[21] This may be one reason why PtNPs could not lead to loss of cardiomyocyte excitability and cardiac
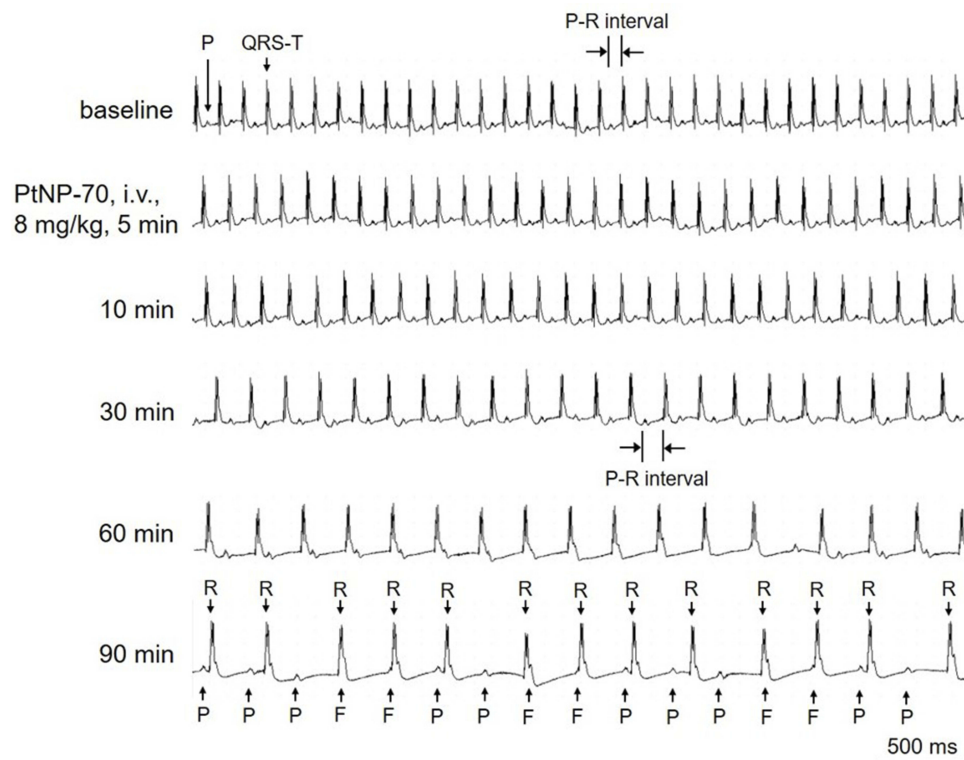
Figure 7 Representative ECGs showing the effects of intravenous PtNP-70 (8 mg/kg) on the heart rate and rhythm of a mouse in vivo. PtNP-70 at this dose had similar effects on the heart rate and rhythm as that did by PtNP-5, ie, the ECGs successively showed heart rate slowing, P-R interval prolongation and complete atrio-ventricular conduction block (AVB), but the animals did not die within the observed 90 mins.
Abbreviations: P, P wave; QRS-T, QRS complex and T wave; F, fusion of P wave with QRS complex; R, R wave.

asystole even at higher concentration in vitro or higher dosage in vivo. The RP-depolarizing effect of PtNPs was mainly contributed to the inhibitory effect of PtNPs on I_{K1} channels, as we observed the suppressing effect of PtNPs on the I_{K1} currents, and I_{K1} currents are considered the primary conductance controlling the RP.[22]

We then observed that both PtNP-5 and PtNP-70 significantly suppressed the depolarization of AP with similar efficacies, as shown by the reduction of APA (shown in Figure 2 and Figure S1). These effects of PtNPs contributed to the repressing effects of PtNPs on voltage-gated Na^+ channels as we present here. It is known that normal conduction of myocardial excitation depends on a normal depolarization which is reflected by APA. Fall of APA to a certain extent would harm the excitation conduction and lead to conduction block as we observed here.

Except for suppressing the depolarization, PtNPs also delayed the repolarization of AP, as shown by the prolongations of APD50 and APD90 (shown in Figure 2 and Figure S1). Generally, APD decides the effective refractory period (ERP). Prolongation of APD means longer ERP and increases the severity of functional conduction

block. Therefore, suppressions of AP depolarization and repolarization may both contribute to cardiac conduction block. We observed the inhibitory effects of PtNPs on I_{to} and I_{K1} channels which can explain why PtNPs prolonged the APD, because these two channels provide the main outward currents which accelerate the AP repolarization in mice cardiomyoctes. We did not check the effect of PtNPs on the delayed rectifier K^+ (I_K) channels (including I_{Kr} and I_{Ks}), because the expressions of I_K channels are rapidly declined in mouse myocardium after birth, and both I_{Kr} and I_{Ks} could not be detected in adult cardiomyocytes.[23] In addition, we observed that PtNPs did not affect the activation, inactivation and recovery kinetics of I_{Na} channels, and also had no effect on the activation kinetics of I_{K1} and I_{to} channels. These properties of PtNPs on channel kinetics were different from that of AgNPs; AgNPs not only decreased the channel current densities but also changed the channel kinetics as we previously reported in cardiomyoctes.[21] This may be one reason why PtNPs were less toxic than AgNPs.

The inhibitions of I_{Na}, I_{to} and I_{K1} by PtNPs and subsequent changes of RP and AP suggest that PtNPs
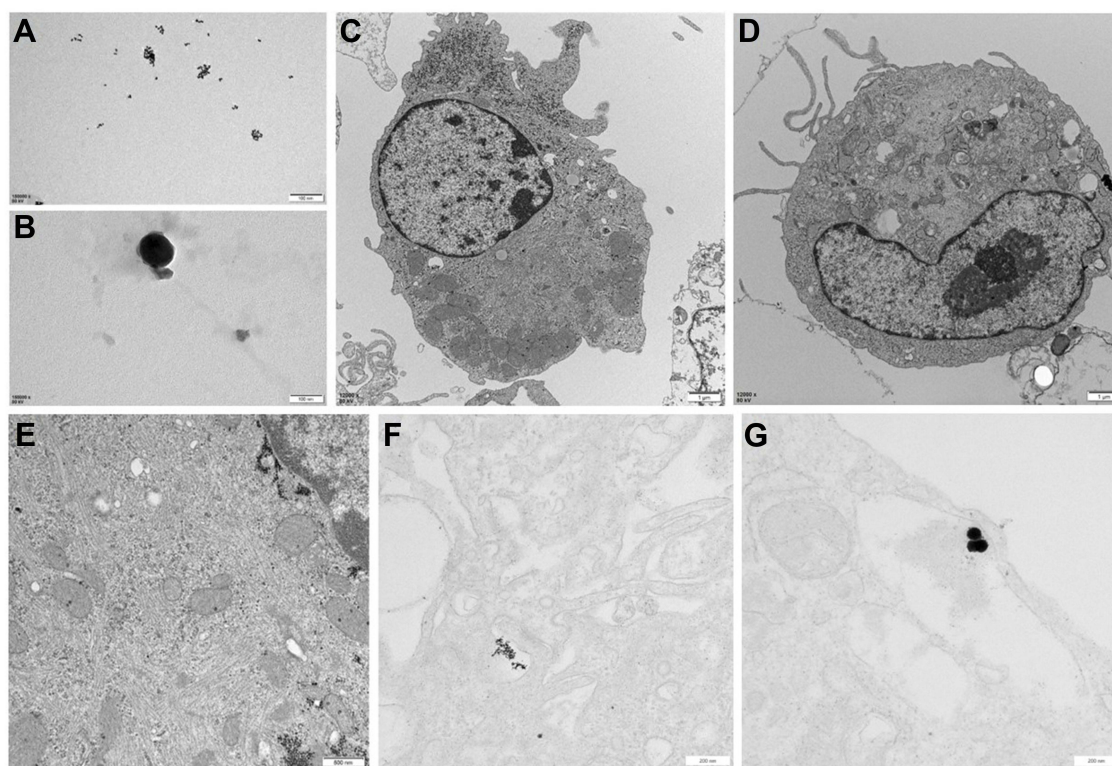
Figure 8 Transmission electron microscopy (TEM) images showing the internalization of PtNP-5 and PtNP-70 (both 10^{-5} g/mL) in cultured neonatal mice cardiomyocytes before and after exposure for 5 mins and 1 hr. (**A**) Extracellular PtNP-5. (**B**) Extracellular PtNP-70. (**C**) A control cardiomyocyte without exposure to PtNPs. (**D**) A cardiomyocyte exposed to PtNP-5 for 5 mins, no PtNP-5 was observed inside the cell. (**E**) A cardiomyocyte exposed to PtNP-70 for 5 mins, no PtNP-70 was observed inside the cell. (**F**) A cardiomyocyte exposed to PtNP-5 for 1 hr, PtNP-5 was observed inside the cytoplasmic vesicles, indicating PtNP-5 internalization. (**G**) A cardiomyocyte exposed to PtNP-70 for 1 hr, PtNP-70 was observed inside the cell. Scale bars in panels A through G were, respectively, 100 nm, 100 nm, 1 μm, 1 μm, 200 nm, 500 nm and 200 nm.
Abbreviation: PtNP, platinum nanoparticle.

may induce cardiac conduction block and bradyarrhythmias. Results proved our speculation that PtNPs prolonged the P-R intervals (degree I AVB) at lower doses and caused complete (degree III) AVB at higher doses. In addition to causing AVB, PtNPs also slowed down the intraventricular conduction, as indicated by the widened QRS complex at higher doses (shown in Figures 6 and 7). Repression of Na$^+$ channels by PtNPs could underlie this effect and partially explained why AVB and hampering of ventricular conduction happened. In addition, direct inhibition of I_{K1} and I_{Na} channels by PtNPs may also disturb the voltage-dependent characters of other channels and indirectly affect the activities of these channels. These direct and indirect effects of PtNPs on ion channels might together cause bradyarrhythmias as we observed.

We noticed that PtNP-induced arrhythmias were dominantly bradyarrhyrthmias which mainly exhibited decelerated sinus HR and degrees I and III AVB. No tachyarrhythmia was observed after PtNP exposure. The inhibitory effect of PtNPs on I_{Na} channels may be one reason, because tachyarrhythmias require rapid excitation conduction, while inhibition of I_{Na} channels would prohibit the high-efficacy AP conduction in the myocardium. In addition, APD prolongation also hampers the high-frequency AP initiation which is necessary for tachyarrhythmias.

It is known that the toxicity of nanoparticles is size dependent. For example, Isoda et al.[18] reported that PtNP-1 (diameter 1 nm, i.v.), but not PtNP-8, showed acute hepato-renal toxicity in mice; Konieczny et al.[19] showed that smaller PtNPs (5.8 nm) exhibited a more deleterious effect on DNA stability than bigger PtNPs (57 nm) in primary keratinocytes. In the present study, it is likely that PtNP-5 showed slightly higher inhibitory effects on I_{Na}, I_{to} and I_{K1} than PtNP-70 in cardiomyocytes in vitro, while PtNP-5 and PtNP-70 exerted similar effects on the TMP in vitro and heart rhythm in vivo. Overall, we only find mild, but not striking, difference in the ion channel toxicity between PtNP-5 and PtNP-70.

Upon the mechanisms underlying the acute toxic effects of PtNPs on cardiac electrophysiology, we noticed that the toxic effects appeared rapidly (within 5 mins) either at the cellular AP level (shown in Figure 2) or at the ion channel level (see Figures 3–5). Even at the integrative level, HR showed rapid (within 5–10 mins) and a significant decrease after PtNP exposure (see Figures 6 and 7). The in vivo effects of nanoparticles usually appear slower than their in vitro effects, as nanoparticles need time to travel and diffuse to the target cells under in vivo condition. These phenomena suggest that PtNPs exerted the acute toxic effects in an endocytosis-independent manner. The TEM results confirmed this viewpoint: PtNP endocytosis could not occur within 5 mins but occurred 1 hr after exposure. Usually, the completion of endocytosis process needs at least 10 mins even in cells with high phagocytic capability such as macrophages as we previously observed,[21,24] and cells with lower phagocytic capability need longer time to complete the internalization of nanoparticles such as 3T3 L1 cells[25] and cardiomyocytes. Furthermore, potential changes of gene expression and other relatively slower biological processes also unlikely underlie the acute toxic effects of PtNPs, as these processes need more than 5 mins. This quick action pattern of PtNPs on cells is similar to AgNPs[21] but is different from multiwalled carbon nanotubes, the latter affect cells on the intracellular side and thus is endocytosis dependent as we have reported previously.[26] Another potential mechanism might be oxidative stress caused by PtNPs.[27,28] However, we observed here that short-time exposure (5 mins) of cardiomyocytes to PtNPs did not significantly increase the production of ROS and membrane injury as indicated by LDH leak. A third potential mechanism might be that Pt ions released from PtNPs might exert the acute toxicity. However, Pt is a well-known inert metal. It is extremely stable and cannot be oxidized or dissolved in most regular mediums such as water and physiological solutions, while it can only be dissociated to release Pt ions at extreme conditions such as in aqua regia.[29,30] Therefore, Pt ions could not be a contributor to the acute toxicity of PtNPs. Taken together, in terms of mechanism, we suggest that PtNPs exerted the acute toxic effects on cardiac electrophysiology via a nanoscale-level interference with ion channels at the extracellular side, and not likely via changes of protein expression or other slower biological processes, oxidative stress and membrane injury.

The present study may have a translational potential. By scanning the concentration/dosage ranges and respective cardiomyocyte/heart electrophysiological responses both in vitro and in vivo, we found that the lowest toxic concentration of PtNPs was 10^{-9} g/mL in vitro and the lowest toxic dose of PtNPs was 4 mg/kg (*i.v.*) in mice in vivo. In real life, people may encounter risk of PtNP exposure in situations of medical PtNP administration, occupational exposure, severe environmental pollution and accidentally, poisoning. The above measures in mice shown here might be used as references when evaluating PtNP toxicities and exposure level–toxicity relations in human. In addition, blood Pt levels after exposure are also valuable in the assessment of exposure-toxicity associations and thus need to be measured both in animal and human in the future.

Conclusion

PtNPs (PtNP-5 and PtNP-70) rapidly (within 5 mins) and concentration-dependently reduced the current densities of I_{K1}, I_{Na} and I_{to} channels with similar efficacies but did not significantly affect the kinetics of these channels in cultured neonatal mice cardiomyocytes. These channel effects of PtNPs could better explain the hazardous effects of PtNPs on the TMP, including depolarization of RP and repressions of AP depolarization and repolarization. In vivo, PtNPs dose-dependently decreased the sinus HR and led to degrees I and III AVB, but could not cause cardiac asystole and animal death at the highest tested dose (10 mg/kg, i.v.) in adult mice. Overall, the cardiac electrophysiological toxicity of PtNPs is lower than AgNPs as we previously reported.[21] PtNPs exerted these rapid toxicities on cardiac electrophysiology directly via a nanoscale-level interference on ion channels at the extracellular side, but not via ROS generation, membrane injury and other slower biological processes. The study warns that PtNPs are toxic to cardiac electrophysiology and can lead to threatening cardiac conduction block. Attention should be paid to patients especially those with heart diseases and those encountering PtNP exposure.

Limitations

The first limitation of this study was that we could not demonstrate the exact mechanism by which PtNPs affect the cardiac electrophysiology, although results suggest that extracellular PtNPs directly interfere with ion channels at the nanoscale level. The second limitation was that we did not observe the effects of PtNPs on other ion channels such as I_{Ca-L}, I_{K} and I_{Kur} channels in the cardiomyocytes. A third limitation was that we did not measure the blood Pt level after intravenous PtNP injection. Investigations into the association between blood Pt level and exposure

dosage are warranted in future studies both in animal and human.

Author contributions

CXL and JMC designed the study; CXL performed major parts of the experiments and analyzed the data and JLG performed the transmission electron microscopy (TEM) experiments. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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