Titanium dioxide nanoparticles promote arrhythmias via a direct interaction with rat cardiac tissue

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Titanium dioxide nanoparticles promote arrhythmias via a direct interaction with rat cardiac tissue

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Abstract

Background

In light of recent developments in nanotechnologies, interest is growing to better comprehend the interaction of nanoparticles with body tissues, in particular within the cardiovascular system. Attention has recently focused on the link between environmental pollution and cardiovascular diseases. Nanoparticles <50 nm in size are known to pass the alveolar–pulmonary barrier, enter into bloodstream and induce inflammation, but the direct pathogenic mechanisms still need to be evaluated. We thus focused our attention on titanium dioxide (TiO$_2$) nanoparticles, the most diffuse nanomaterial in polluted environments and one generally considered inert for the human body.

Methods

We conducted functional studies on isolated adult rat cardiomyocytes exposed acutely in vitro to TiO$_2$ and on healthy rats administered a single dose of 2 mg/Kg TiO$_2$ NPs via the trachea. Transmission electron microscopy was used to verify the actual presence of TiO$_2$ nanoparticles within cardiac tissue, toxicological assays were used to assess lipid peroxidation and DNA tissue damage, and an in silico method was used to model the effect on action potential.

Results

Ventricular myocytes exposed in vitro to TiO$_2$ had significantly reduced action potential duration, impairment of sarcomere shortening and decreased stability of resting membrane potential. In vivo, a single intra-tracheal administration of saline solution containing TiO$_2$ nanoparticles increased cardiac conduction velocity and tissue excitability, resulting in an enhanced propensity for inducible arrhythmias. Computational modeling of ventricular action potential indicated that a membrane leakage could account for the nanoparticle-induced effects measured on real cardiomyocytes.

Conclusions

Acute exposure to TiO$_2$ nanoparticles acutely alters cardiac excitability and increases the likelihood of arrhythmic events.
Keywords

Pollution, Cardiac arrhythmia, Experimental model, Titanium dioxide nanoparticles, Supernormal conduction, Membrane leakage

Background

The explosive growth in nanotechnology – i.e., the design and development of systems at the atomic or nano scale – and bioengineering nanotechniques has led to the development of a large number of new nanomaterials with novel biological, physical and chemical properties. Nanoparticles (NPs) have been manufactured for several decades on an industrial scale. Several metal oxide NPs possess photo-catalytic ability, high electrical conductivity, ultraviolet absorption and photo-oxidizing capacity against chemical and biological species [1]. Some commercial products, such as cosmetics, are also likely source of NPs that could become uncontrollably delivered to the human environment [1]. Because of the widespread presence of potential sources of NPs, environmental release of manufactured NPs is expected to increase for the near future. Thus, understanding the molecular and cellular basis of nanotoxicity is an essential challenge today.

We focused the present study on one of the most-produced NPs species, namely titanium dioxide (TiO$_2$), 1.45 million metric tons of which were produced in the United States in 2007 alone [2], and which was considered biologically inert up to recently [3]. Most studies concerning TiO$_2$-NP toxicology have focused to date on pulmonary inflammation [4-7] and have elucidated its behavior inside cells. Unfortunately, it has been very difficult to detect, track and precisely quantify TiO$_2$-NPs at the cellular level. Thus, the effects on cellular and nuclear membrane protein domains, as well as on the interference with metabolic pathways, remain poorly investigated.

The National Institute of Occupation Safety and Health (NIOSH) recommends airborne exposure limits of 2.4 mg/m$^3$ for fine TiO$_2$ and 0.3 mg/m$^3$ for ultrafine TiO$_2$, as time-weighted average concentrations for up to 10 hours per day during a 40-hour work week, but NIOSH also admits that there is insufficient data to classify TiO$_2$ as a hazard for human health [8,9].

Recently, attention has moved to the study of how TiO$_2$ translocates from lungs to other systemic organs [1,10], including the gastrointestinal tract, kidney and heart [11]. Indeed, the heart is the first organ that inhaled TiO$_2$-NPs may reach, after passing the alveolar epithelial fenestrated barrier and entering the pulmonary circulation. Thus, we hypothesized that acute exposure to TiO$_2$-NPs might cause detrimental effects on cardiac electromechanical function. In fact, we have previously described in cultured engineered neonatal cardiac tissue that TiO$_2$-NPs may influence cardiac action potential (AP) by enhancing conduction and upstroke velocities and disrupting myofibrils via production of reactive oxygen species (ROS) [12]. However, comprehensive in vivo assessment of cardiac risk is lacking.

Here, we demonstrate with conventional electrophysiological techniques – i.e., patch-clamp, Epicardial Potential Mapping (EPM) and cellular motion detection – that acute exposure (<5 hours) to TiO$_2$-NPs (diameter range: 30–100 nm) is detrimental for cardiac performance and increases the propensity for arrhythmia. Biophysical characterization of the NPs was conducted with a number of techniques – i.e., Atomic Force Microscopy (AFM), Dynamic
Light Scattering (DLS), Raman spectroscopy, and Transmission Electron Microscopy (TEM). TiO$_2$ toxicology was also characterized with ROS and ThioBarbituric Acid Reactive Substance (TBARS) analyses.

Results

Particles size, type and aggregation

AFM imaging revealed that a relevant fraction of TiO$_2$-NPs had a diameter <100 nm (Figure 1A): specifically, single NPs had a diameter in the 25–35 nm range (Figure 1B); the overall size distribution frequency of the NPs is given in Figure 1C. In addition, NP aggregates of variable size and morphology were also present, composed of up to tens of single particles (Figure 1D). By measuring volume, we estimated that ~40% of NPs had a diameter <100 nm, with the remaining particulates made up of aggregates.

The Raman spectrum of the TiO$_2$-NPs (Additional file 1: Figure S1) had peaks corresponding to a mixture of anatase (tetragonal polymorph, space group I4$_1$/amd, characterized by Raman peaks at ~143, 196, 396, 516 and 638 cm$^{-1}$) and rutile (tetragonal polymorph, P4$_2$/mmm, with characteristic Raman frequencies at ~143, 238, 445 and 609 cm$^{-1}$) TiO$_2$ minerals. All peaks for TiO$_2$-NPs were larger than those of the pure polymorphs, confirming the presence of nanosized (<100 nm) TiO$_2$ particles [13,14]. The amount of anatase was determined with a calibration procedure using the intensities of the Raman peaks of the two polymorphs present in the mixture (see Additional file 1: Methods section). The results of this procedure on different Raman peaks coherently indicated 93 wt% anatase in the TiO$_2$-NP powder, with an estimated uncertainty of about ±1%. Finally, in order to better characterized charge and size of the adopted NPs, DLS was employed: Z-potential and hydrodynamic diameter values are reported in Table 1.

Table 1 Biophysical properties on TiO$_2$ NPs in different solutions

<table>
<thead>
<tr>
<th>TiO$_2$-NPs</th>
<th>Water</th>
<th>Saline</th>
<th>Tyrode</th>
<th>PBS-BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Potential (mV)</td>
<td>$-31.74 \pm 1.02$</td>
<td>$-18.36 \pm 2.30$</td>
<td>$-24.60 \pm 1.61$</td>
<td>$-13.05 \pm 2.13$</td>
</tr>
<tr>
<td>Hydrodynamic Diameter (nm)</td>
<td>$154 \pm 3.00$</td>
<td>$498 \pm 0.55$</td>
<td>$538 \pm 0.37$</td>
<td>$449 \pm 0.85$</td>
</tr>
</tbody>
</table>

TiO$_2$ nanoparticles impair cardiomyocyte contractility

The effect of TiO$_2$ on contractility was investigated on cardiomyocytes isolated from adult rats: $n = 104$ non-exposed control cardiomyocytes (CTRL), and $n = 102$ TiO$_2$-NP-exposed cells (NP$_C$). Figure 2A–C give representative recordings of shortening under different pacing frequencies (0.5 Hz, 1 Hz and 2 Hz) in CTRL (black line) and NP$_C$ (red line). The average diastolic sarcomere length (Figure 2D) was nearly identical in CTRL and NP$_C$ at all pacing rates ($1.73 \pm 0.003 \, \mu m$ and $1.74 \pm 0.003 \, \mu m$, respectively). In contrast, fractional shortening (FS) and maximal rates of shortening and re-lengthening were significantly reduced in NP$_C$ at all frequencies (~ −50%, $p < 0.01$; Figure 2E–G).
Spontaneous contractions (SCs) were then assessed in cardiomyocytes that had been conditionally trained for 60 s with field stimulation set at 0.5 Hz. The percentage of cells exhibiting SCs in the 60 s after stopping electrical pacing was significantly higher in NP<sub>C</sub> than in CTRL (Figure 2H). Furthermore, in cardiomyocytes exhibiting SCs, the number of events per cell within the 60 s period was two-fold higher in NP<sub>C</sub> (Figure 2I, Additional file 1: Figure S2).

TiO<sub>2</sub> nanoparticles slightly depolarize resting membrane potential but dramatically alter other action potential parameters

In order to assess whether SCs were associated with sub- or supra-threshold fluctuations of resting membrane potential (V<sub>r</sub>), we analyzed variability (ΔV<sub>r</sub>) over 60 s recording periods in the absence of electrical stimulation; similarly to motion measurements, cells were conditionally trained (40 beats at 5 Hz) before V<sub>r</sub> recording. We found three types of V<sub>r</sub> fluctuation (Figure 3A): in one group of cardiomyocytes (type 1), V<sub>r</sub> was stable over time – considering the expected signal-to-noise ratio for this type of voltage recording – with a sharp frequency distribution (Figure 3B, left panel); in a second group (type 2), V<sub>r</sub> underwent small (~1 mV), continuous fluctuations, resulting in a continuously distributed frequency around a peak value of −70 mV (Figure 3B, middle panel); finally, type 3 cardiomyocytes displayed dispersed transitions to discrete V<sub>r</sub> levels (ΔV<sub>r</sub> up to 3–4 mV), resulting in well-separated peaks within the frequency distribution (Figure 3B, right panel). The fraction of type 2 and 3 cardiomyocytes increased after exposure to TiO<sub>2</sub>-NPs (64% in NP<sub>C</sub> vs. 36% in CTRL). Overall, ΔV<sub>r</sub> and the coefficient of variability of V<sub>r</sub> (CV<sub>Vr</sub>) were significantly higher in NP<sub>C</sub> (ΔV<sub>r</sub>: 2.8 ± 0.24 mV in CTRL vs. 4.0 ± 0.35 mV in NP<sub>C</sub>; CV<sub>Vr</sub>: 0.53 ± 0.04 in CTRL vs. 0.70 ± 0.07 in NP<sub>C</sub>). TiO<sub>2</sub>-NP-exposed cardiomyocytes also had, on average, a remarkable reduction in AP duration (APD) with respect to both the early (APD<sub>20</sub>) and late (APD<sub>60</sub>) phases of repolarization (Figure 4A,B). A dose-dependent effect was found at concentrations from 5 to 50 µg/ml (Additional file 1: Figure S3). Of note, despite APD shortening – which is expected to decrease beat-to-beat variability of APD in this cell type [15] – CV<sub>APD60</sub> increased significantly by 28% (Figure 4C).

Figure 3 Variability in resting membrane potential in cardiomyocytes exposed to TiO<sub>2</sub> NPs. A. Traces representative of the three types of V<sub>r</sub> behavior found over a 60 s recording period subsequent to conditioning training at 5 Hz for 40 beats. B. Frequency distribution of V<sub>r</sub> for the three ΔV<sub>r</sub> types.

Figure 4 TiO<sub>2</sub> NPs-induced changes in cellular electrophysiology. A. Representative action potential (AP) waveforms recorded from control (CTRL, black line) and TiO<sub>2</sub>-NP (NP<sub>C</sub>, red line) cardiomyocytes at the physiological driving rate of rat heart (5 Hz). B–E. Graphs of action potential duration (APD) measured at −20 mV (APD<sub>20</sub>) and −60 mV (APD<sub>60</sub>), beat-to-beat variability of APD<sub>60</sub> (CV<sub>APD60</sub>), AP upstroke (UPS) and membrane
capacitance ($C_m$). In all graphs, CTRL is given by white columns, and NP$_C$ by the red columns ($n = 37$ NP$_C$ and $n = 49$ CTRL). * $p < 0.05$ vs. CTRL. F, APs simulated with the Pandit model, without (black trace, CTRL) and with (red trace, NP$_C$) a simulated 1.5 nS constant potassium leakage.

AP upstroke (UPS) and electrical membrane capacitance ($C_m$) values were also significantly reduced after acute exposure to TiO$_2$-NPs (Figure 4D–E). Mean values of $V_r$, resting membrane resistance ($R_m$), AP amplitude (APA), and rheobase and chronaxie values were comparable in NP$_C$ and CTRL (Additional file 1: Table S1).

In order to provide a mechanistic explanation for the cellular findings, we ran simulations with a modified version of the Pandit et al. rat ventricle AP model [16]. The above-presented in vitro findings could be reproduced in silico by introducing into the Pandit model’s pool of ion currents a 1.5 nS leakage conductance selectively permeable to potassium ions (for the sake of comparison, amounting only to ~5% of maximum $I_{K1}$ conductance). Of note, the experimental and simulated APs were similar, with a quasi-superimposable reduction of APD without any significant changes in $V_r$ (compare Figures 4A and F; Additional file 1: Figure S4). Moreover, we ran simulations of Pandit-modelled APs with and without the addition of a $K^+$ leakage current, and setting extracellular [K+] at values ranging from 3.0 to 23.2 mmol/l. We found that the simulated leakage current led to an increase in $dV/dt_{\text{max}}$ from 180 to 183 V/s (Additional file 1: Figure S5, left panel), with a maximum peak corresponding to a $[K]_o$ of about 6 mmol/l, which is known to characterize supernormal conduction of sodium current in engineered neonatal rat cardiac tissue [17] (Additional file 1: Figure S5, right panel).

**ECG and epicardial electrograms indicate faster electrical activation after exposure to TiO$_2$**

Rats were anesthetized as described below and instilled tracheally with either physiological solution (Vehicle) or 2 mg/Kg TiO$_2$-NPs (NP$_R$). Rats were left to recover for 4 hours before undergoing anesthesia (see Figure 5A). NP$_R$ had a normal QRS pattern of activation, as evidenced by 3-lead ECG (data not shown). Electrograms (EGs) derived from the electrode array positioned on the epicardial surface (Additional file 1: Figure S6) did not indicate any qualitative difference in the two experimental groups. Indeed, EGs (Figure 5B) had the expected biphasic form, with positive electrocardiographic R and negative S waves whose amplitudes were correlated to the area explored by the electrode array. In detail, EGs had an Rs shape (see Additional file 1: Methods section) when positioned near the heel of the pulmonary artery, and an rS shape near breakthrough points.

**Figure 5 Instillation of TiO$_2$-NPs and in vivo recordings of cardiac electrical performance.** A. Time-scale (hours) of the experimental protocol. B. Representative EGs recorded from an 8x8 epicardial electrode array. Each waveform of the grid represents the time-course of extracellular potential at the corresponding position. The scheme on the right hand side explains the EG parameters, as measured from their root mean square (RMS)-derived signals. Magenta thin trace represents the first time derivative, whose minimum value is taken as a marker of the end of the QT interval C. Representative activation time maps (isochrones, ms) from Vehicle (left) and NP$_R$ (right), showing differences in longitudinal (red arrows) and transverse (blue arrows) propagation.

Since EG waves were not qualitatively different, we focused our attention on their duration (right panel of Figure 5B, Table 2). We found that P wave duration and the PQ segment were...
significantly reduced (−12%), as was QRS complex duration (−5%), in NP<sub>R</sub> compared with Vehicle. There was a non-significant increase in heart rate (R-R reduction), while QT duration was reduced 22% in NP<sub>R</sub> (Table 2).

Table 2 In-vivo electrophysiological parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>NP&lt;sub&gt;R&lt;/sub&gt;</th>
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<tbody>
<tr>
<td>P wave (rns)</td>
<td>33.0 ± 0.22</td>
<td>28.9 ± 0.21**</td>
</tr>
<tr>
<td>PQ segment (rns)</td>
<td>24.2 ± 0.18</td>
<td>21.3 ± 0.21**</td>
</tr>
<tr>
<td>QRS complex (rns)</td>
<td>16.0 ± 0.09</td>
<td>15.2 ± 0.09**</td>
</tr>
<tr>
<td>QT interval (rns)</td>
<td>40.0 ± 0.28</td>
<td>31.1 ± 0.54**</td>
</tr>
<tr>
<td>RR interval (rns)</td>
<td>237.6 ± 0.90</td>
<td>231.3 ± 2.47</td>
</tr>
<tr>
<td>Rheobase (µA)</td>
<td>21.4 ± 1.68</td>
<td>20.6 ± 2.73</td>
</tr>
<tr>
<td>Chronaxie (ms)</td>
<td>0.75 ± 0.04</td>
<td>0.64 ± 0.04*</td>
</tr>
<tr>
<td>CVI (mis)</td>
<td>0.63 ± 0.004</td>
<td>0.70 ± 0.004</td>
</tr>
<tr>
<td>CVt (m/s)</td>
<td>0.33 ± 0.002</td>
<td>0.32 ± 0.002</td>
</tr>
<tr>
<td>Anisotropy ratio</td>
<td>1.96 ± 0.01</td>
<td>2.33 ± 0.02**</td>
</tr>
</tbody>
</table>

* p < 0.05 vs Vehicle **p < 0.005 vs Vehicle.

Exposure to TiO<sub>2</sub> nanoparticles increases cardiac excitability and conduction velocity

_in vivo_, we observed a non-significant decrement of rheobase (Vehicle: 21.4 ± 1.68 µA; NP<sub>R</sub>: 20.6 ± 2.73 µA) and significantly decreased chronaxie (Vehicle: 0.75 ± 0.04 ms; NP<sub>R</sub>: 0.64 ± 0.04 ms; p < 0.05) in NP<sub>R</sub>, pointing to an overall increase in cardiac excitability (Table 2).

From the isochrone maps, we evaluated the average transverse and longitudinal conduction velocity (CVt and CVl, respectively) (Figure 5C). While CVt was unchanged, CVl increased significantly by 11% in NP<sub>R</sub> (Table 2, **, p < 0.005). The anisotropy ratio CVl/CVt – the augmentation of which is recognized as a key arrhythmogenic factor [18] – was 19% higher in NP<sub>R</sub> (**, p < 0.005).

Tracheal instillation of TiO<sub>2</sub> nanoparticles exacerbates arrhythmogenesis

Refractoriness was measured as the effective refractory period (ERP) using a dedicated protocol [19,20] (Figure 6) – see Additional file 1: Methods section - for parameters definition. An increase in ERP duration and ERP spatial dispersion – commonly recognized as an index of vulnerability to arrhythmia – was also found in NP<sub>R</sub> (Figure 6B–C).

Figure 6 Susceptibility to arrhythmias in Vehicle and NP<sub>R</sub> rats. A. Ventricular ectopic couplet (top) and ventricular fibrillation (bottom) recorded during evaluation of the effective refractory period (ERP). Scale bar =500 ms. B. Evaluation of ERP in Vehicle and NP<sub>R</sub>. **, p < 0.005. C. Percentage of inducible arrhythmic events.

Similarly, the likelihood of establishing arrhythmic events during the ERP was assessed from 60 electrode positions in NP<sub>R</sub> and 44 in Vehicle. We observed a roughly two-fold increase in induced ventricular ectopic activity, mainly extra-systole couplets (Figure 6A, top) and ventricular fibrillation (Figure 6A, bottom), when pacing near the ERP (33% of the total electrodes for NP<sub>R</sub> vs. 18% Vehicle; Figure 6C).
**TiO$_2$ nanoparticles acutely reach lungs and ventricles, and are internalized within cells**

A key aspect for the interpretation of the above findings was to identify the presence of TiO$_2$-NPs not only in the lungs but also in exposed cardiomyocytes. We found that TiO$_2$-NPs entered not only directly into cultured cardiomyocytes (Additional file 1: Figure S7A,B), but also *in vivo* into left and right ventricular cardiomyocytes of TiO$_2$-instilled rats, suggesting that contamination of cardiac tissue can occur via the lungs. In particular, morphologic evidence provided by TEM indicates that NPs leave the capillary lumen, cross the endothelial layer, penetrate the sarcolemma and reach the myoplasm by establishing intimate contact with myofibrils and mitochondria (Figure 7A–C). Ultrastructural analysis also showed the presence of TiO$_2$ -NPs in lung tissue, with a tendency to agglomerate in the cytoplasm of alveolar cells and macrophages (Figure 7D–H).

**Figure 7 Presence of titanium dioxide (TiO$_2$) nanoparticles (NPs) in the rat ventricular myocardium after tracheal instillation: TEM analysis.** A. Right Ventricle. Electron-dense NPs in two longitudinally oriented cardiomyocytes and in the wall of a vascular structure. B. Left Ventricle. NPs accumulating at the edge of longitudinally oriented cardiomyocytes, as well as in the sarcolemma. NPs are also present in the interstitial space, in endothelial cells and within the capillary lumen (L). C. Left ventricle. The lumen of a capillary neighboring a cardiomyocyte containing TiO$_2$ NPs, which also appear to be connected to and engulfed by endothelial cells. GJ marks a gap junction location. Blue rectangles include areas shown at higher magnification in the lower panels (A1, B1 and C1). Scale Bars: A and B =5 µm; A1 and B1 = 2 µm; C =1 µm; C1 = 200 nm. Bottom. Ultrathin sections of lung samples from NP-exposed treated rats. **D.** The bronchial epithelium is apparent by the presence of ciliated cells (*). Electron-dense NPs are best seen in cytoplasm at high magnification (**D**1). Clusters of NPs were found within the lung parenchyma (**E**) and in macrophages (**F**). N, nucleus. **G,H.** The typical shape of titanium NPs is apparent at higher magnification. Scale Bars: D =5 µm; D1 = 2 µm; E =2 µm; F =1 µm; G =200 nm; H =100 nm.

**TiO$_2$ Nanoparticles are cardiotoxic and genotoxic**

The interplay between TiO$_2$-NPs and cardiomyocytes denoted signs of intracellular damage after acute administration. Indeed, we observed a 25% increase in damaged nuclear DNA already after one hour of exposure, accompanied by a 16% increase in ROS formation (Figure 8A, B). We also analyzed TBARS *ex vivo*, from trachea, lungs and heart. While only tracheal tissue showed positive TBARS values in Vehicle –probably owing to the instillation maneuver – NP$_R$ had lipid peroxidation in the lungs as well as in the heart (Figure 8C), indicating membrane damage of cardiomyocytes due to ROS production.

**Figure 8 TiO$_2$ NPs-induced toxicological effects.** A. DNA damage detected in single isolated cardiomyocytes by Comet assay (pH >13) in CTRL (white columns) and NP$_C$ (red columns) after 1 h and 5 h of exposure. DNA damage is expressed as tail intensity (TI%; *p < 0.05). B. Percent increase of ROS in single isolated cardiomyocytes, NP$_C$ (red column) after 1 h. C. Evaluation of TBARS in trachea, lungs and heart tissue after tracheal instillation of saline solution (Vehicle) or saline solution containing TiO$_2$-NPs (2 mg/Kg, NP$_R$). *, p < 0.05 vs. Vehicle.
**Discussion**

Our findings indicate that TiO$_2$-NPs can: i) reach the heart via the respiratory system; ii) enter ventricular cardiomyocytes; and iii) enhance the susceptibility to cardiac arrhythmias via shortening of repolarization time, and by increasing cardiac excitability. To the best of our knowledge, this is the first time that a direct effect of TiO$_2$ on cardiac electrical performance has been reported and explained *in vivo*.

It was described recently that, depending on size and bioavailability, NPs may reach the bloodstream after passing the pulmonary barrier [10]. Furthermore, we previously reported that electrically charged NPs modulate excitability by producing membrane disruption (positively charged NPs) or life-compatible nanopores (negatively charged NPs) [21]. We also reported that negatively charged nanoparticles can be internalized via clathrin-mediated membrane protrusions [22]. Such findings are in line with our leakage hypothesis and explain our observation of a significant decrease in membrane capacitance due to membrane loss caused by the internalization process.

TiO$_2$ is widely used as a nanomaterial because it is considered inert [23,24]: its toxicity, documented in cellular and animal models, remains controversial [25,26] since surface charge may differ depending on the vehicle [26]. Z-potential characterization demonstrated that NPs were negatively charged in our experimental setting and, therefore, able to transiently produce nanopores that, in turn, resulted in the development of leakage currents, as reported previously [21]. It is known that, upon tracheal instillation, TiO$_2$ generates a detrimental sequence of dysfunctions, which might ultimately lead to atherosclerosis and, thus, affect the function of the heart pump [27]. Here, we show how quickly TiO$_2$-NPs may directly affect cardiac electrical activity via the reduction of repolarization velocity, enhancing temporal dispersion and increasing excitability.

In order to test the hypothesis that an NP-induced leakage current might provide, *per se*, a mechanistic explanation of our electrical findings, we introduced a 1.5 nS potassium leakage into the membrane equations of the Pandit rat ventricular AP model [16]. Of note, simulations were consistent with the results obtained in patch-clamped cardiomyocytes, i.e., reduction of APD and UPS, and slight depolarization of $V_r$. In order to provide a mechanistic explanation of the increment of CVI found *in vivo*, we simulated electrically paced AP trains by means of the Pandit model endowed or not with the leakage current. Simulated APs exhibited the highest $\Delta dV/dt_{\text{max}}$ for $[K]_o = 5.8$ mmol/l, which corresponds to the point of supernormal sodium conduction [17] in cardiac tissue, suggesting that the small TiO$_2$-induced depolarization may: i) speed up the kinetics for reaching the activating threshold, and ii) lead to the observed increment in CVI, which can thus be ascribed to supernormal conduction [12]. Notably, conduction block and increased likelihood of alternans [28] consequent to supernormal conduction are known to make cardiac tissue prone to structural and functional reentrant arrhythmias [17,29]. Although arrhythmogenesis is frequently associated with prolongation of repolarization, measured at the cellular and organ level (APD and QT), the role of QT and APD shortening in favoring transition to arrhythmic events has been also extensively documented [30]. This is particularly relevant for our findings, where TiO$_2$-NPs – in contrast with diesel-exhaust particulates, for instance [31] – induced APD and QT shortening.
Also, physiological sarcomere contraction is undoubtedly affected by the presence of TiO\textsubscript{2}. In fact, not only were NPs detected among the myofibrils by TEM, but dynamic sarcomere measurements on the same cells indicated that their excitation–contraction coupling machinery was significantly impaired. Besides the reported arrhythmogenic behavior due to dyssynchronous sarcomere contractions mediated by the mechano-electric feedback [32,33], we also found an increased incidence of spontaneous contractions (cf. Figure 3) following high-frequency conditioning pacing, which may be synergic with an increment of inducible arrhythmic events.

Although our experiments do not reveal any evidence for NP-induced changes in rate-dependent shortening of the sarcomere, their lengths, FS and maximum rates of shortening and re-lengthening (±dl/dt\textsubscript{max}) were all affected by the presence of NPs. Our multi-level approach generated parallel \textit{in vitro} and \textit{in vivo} results (reduction in the APD, increase in the APD temporal dispersion, reduction in the QT interval and increase in its dispersion), which substantially rule out cardiac inflammatory process caused by TiO\textsubscript{2}-NPs as the main mechanism responsible for excitation–contraction coupling unbalance. Nonetheless, chronic effects, including inflammatory progression, certainly need further evaluation.

A recent study on Langendorff-perfused isolated heart elegantly demonstrated that TiO\textsubscript{2}-NPs produced abnormal electrical activity accompanied by a dose-dependent increase in heart rate [34]. Our study confirms those findings \textit{in vivo} anesthetized rats (ECGs and EGs) and focuses for the first time on the underlying mechanism. Indeed, the evaluation of excitability parameters \textit{in vivo} indicated that cardiac tissue becomes more excitable, in line with previously described results [12]. Moreover, we adopted a single NP dose (2 mg/Kg) in solution in order to be aligned not only with NIOSH recommendations on the time-evaluation of human exposure to TiO\textsubscript{2}-emitting sources, but also because such a concentration in ~50 µl of instilled solution is a good compromise between the volume of vehicle needed and the optimal disaggregation of NPs (cf. Figure 1). A half-dose instillation of TiO\textsubscript{2}-NPs (1 mg/Kg) evaluated in three rats produced the same effects observed with the full dose (data not shown).

Although the pathological effects on the cardiovascular system are gaining more attention than they have had in the past [35], the effect of NPs on cardiovascular tissue is usually viewed as secondary to inflammatory processes initiated at the lungs [36]. Despite the fact that the latter mechanism can be present chronically, we observed in the acute setting that ROS production and an increase in inducible arrhythmias occurred after perfusion with TiO\textsubscript{2}-NPs, which were rapidly internalized within cardiomyocytes. ROS-induced membrane damage and NP-induced leakages are expected to locally depolarize cardiac tissue and promote arrhythmogenesis [37] via source-sink electrical mismatch. Occurrence of arrhythmias was estimated in healthy rat hearts \textit{in vivo} by the well-known S1-S2 protocol (see Additional file 1: Methods section). Through this protocol, we found a significant increase in the incidence of ectopic events (duplicates or triplets) and of ventricular fibrillation in rats administered TiO\textsubscript{2}. Therefore, these malignant events may be exaggerated by acute and sub-acute exposure to an environment polluted with TiO\textsubscript{2}.

\textbf{Study limitations}

While this study proposes supernormal sodium-based conduction at the single-cell level as a physiological mechanism underlying the arrhythmia \textit{in vivo}, it only partially clarifies the difference in anisotropy ratio observed in rats exposed to NPs. Further investigation is
necessary to evaluate: i) the effect of TiO$_2$-NPs on cell-to-cell coupling, e.g., intercalated
discs; ii) the possible re-localization of connexins from the end to the side of sarcolemmal
microdomains as responsible for ensuring longitudinal and lateral connection among
cardiomyocytes; and iii) the possible pro-arrhythmic role of inflammatory processes in lungs,
vasculature and myocardium. Moreover, chronic exposure to TiO$_2$-NPs needs to be
investigated in order to confirm not only the molecular mechanisms underlying the
propensity to arrhythmias, but also to identify a time limit for workers who are exposed daily
to TiO$_2$.

**Conclusion**

Our results clearly describe the short-term direct effect of TiO$_2$-NPs on myocardial tissue.
Although additional epidemiological and toxicological studies will be required to further
establish a link, TiO$_2$ exposure is very likely to increase propensity to arrhythmias, and thus
needs to be monitored in terms of ultra-fine particle emissions and length of exposure (in the
range of a few hours). Notably, our study highlights a rapid effect that is not based on
cumulative NP absorption and that could possibly lead to a fast-developing pro-arrhythmic
scenario.

**Methods**

**Experimental animals**

The study population consisted of male Wistar rats bred in our departmental animal facility,
aged 12–14 weeks and weighing 300–350 g. The animals were kept in single-sex groups of
four individuals from weaning (4 weeks after birth) until the onset of the experiments, in a
temperature-controlled room at 20–24 °C, with the light on between 7.00 AM and 7.00 PM.
The bedding of the cages consisted of wood shavings; food and water were freely available.
Rats were anesthetized with a mixture of 40 mg/kg ip ketamine chloride (Imalgene, Merial,
Milano, Italy) and 0.15 mg/kg ip medetomidine hydrochloride (Domitor, Pfizer Italia S.r.l.,
Latina, Italy), both for the *in vivo* and *ex vivo* experiments. This study was carried out in
accordance with the recommendations in the Guide for the Care and Use of Laboratory
Animals of the National Institute of Health. The protocol was approved by the Veterinary
Animal Care and Use Committee of the University of Parma and conforms to the National
Ethical Guidelines of the Italian Ministry of Health (Permit number: 41/2009-B). All effort
was made to minimize suffering.

**Particle suspension**

TiO$_2$-NPs (Titanium (IV) oxide, Sigma, code: 677469, Milan, Italy) were suspended in
sterilized high-purity water at a concentration of 2.5 mg/ml for the *in vitro* studies, and in
physiological saline solution (10 mg/ml, stock solution) for *in vivo* experiments. Immediately
before the experiments, the suspensions were vortexed and directly sonicated (Branson
Ultrasoundics, Danbury, CT, USA) through five cycles of 20 s at 65% of the maximum power
at room temperature in order to minimize particle aggregation.
TiO₂ nanoparticle characterization

After sonication, the TiO₂-NP suspension at 2.5 mg/ml was diluted in low-calcium solution ([Ca²⁺] =0.1 mmol/l) to a final concentration of 50 µg/ml and analyzed by AFM. A drop of TiO₂-NP suspension was deposed onto freshly cleaved mica and onto mica treated with poly-ornithine prepared as follows: 10 µl poly-ornithine solution (10 mg/ml) was deposed onto freshly cleaved mica for 1 minute, the disk was then rinsed with milliQ water and dried with a gentle nitrogen flow. Afterward, the NP suspension (50 µg/ml) was deposed and incubated for 5 minutes at room temperature. The mica disk was then rinsed with milliQ water and dried with nitrogen. AFM imaging was performed on the dried sample with a Nanoscope IIIa microscope equipped with scanner J and operating in tapping mode. Commercial diving board silicon cantilevers (MikroMasch, Tallinn, Estonia) were used. Images were analyzed with ImageJ software. The percentage of NPs <100 nm, with respect to the total number of NPs present in the topographical image was determined using the “Laplacian volume” routine of Gwyddion software (ver. 2.32, see Additional file 1: Methods section).

The anatase and rutile proportions of the TiO₂ preparation used were determined with a Jobin-Yvon Labram micro-Raman apparatus equipped with an Olympus BH-4 confocal microscope with 4x, 10x, 50x and 100x objectives (lateral spatial resolution of approximately 25, 10, 2 and 1 µm, respectively). The spectrometer employs a 20 mW He-Ne Laser emitting at 632.8 nm, an edge filter, a 256x1024 pixel CCD detector, an 1800 grooves/mm grating and a density filter wheel. The spectral resolution is about 1 cm⁻¹. The calibration of the spectrometer was controlled on the silicon Raman peak at 520.6 cm⁻¹. Raman spectra were acquired with 4x and 10x objectives for 2–5 seconds and 5–8 repetitions, and recorded at 10 different points for each sample. The baseline subtraction with a 2nd degree polynomial curve, the normalization and the peak fitting were made with LABSPEC 5.78.24, Jobin Yvon/Horiba software package. Since the behavior and the aggregation state of NPs strongly depend on their surface charge and the ionic strength of the medium, we carried out further characterizations using DLS and Z-potential techniques. The measurements were performed using the 90Plus Phase Analysis Light Scattering (PALS) instrument by Brookhaven Corp. Particle size measurements were made with a 658 nm laser, collecting data at a scattering angle of 90°. In order to estimate the Stokes-Einstein hydrodynamic radius of the NP agglomerates in suspension, we performed a measurement of the autocorrelation function fitted, assuming a lognormal distribution of relaxation times. The measurement of the Z-potential was carried out by PALS, which determines the electrophoretic mobility of charged colloidal suspensions [38].

Single-cell studies

Cardiomyocyte isolation

Individual myocytes were enzymatically isolated from the left ventricle (LV) with collagenase perfusion in accordance with a procedure previously described [15] (see Additional file 1: Methods section).

Acute exposure of adult cardiomyocytes to TiO₂ nanoparticles

Half of the freshly isolated cells were incubated with buffered maintenance solution (see Additional file 1: Methods section) supplemented with TiO₂-NPs (stock solution of 2.5
mg/ml in high purity water) at final concentrations of 50, 25 and 5 µg/ml. Patch-clamp and contractility recordings were started 1 hour after exposure to TiO$_2$-NPs, and lasted 2–5 hours. Oxidative stress and genotoxicity in single cells were assessed in the same experimental conditions.

**Contractility properties**

Mechanical properties of CTRL and NP$_C$ were assessed by using the IonOptix fluorescence and contractility systems (IonOptix, Milton, MA) as previously described [39]. Briefly, cells were field-stimulated at frequencies of 0.5, 1 or 2 Hz by constant depolarizing pulses (2 ms in duration, and twice diastolic threshold in intensity) by platinum electrodes placed on opposite sides of the cell chamber connected to a MyoPacer Field Stimulator (IonOptix). The stimulated cardiomyocytes were displayed on a computer monitor by means of the IonOptix MyoCam camera connected to the side port of the inverted microscope (Nikon TE2000, Nikon Instruments, Japan). The following parameters were computed: mean diastolic sarcomere length, FS (%) and ± dl/dt$_{max}$. See Additional file 1: Methods section.

**Patch-clamp recordings**

CTRL and NP$_C$ in the whole-cell patch-clamp configuration were used for transmembrane potential (V$_m$) measurements in current-clamp mode using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). Data recordings and analysis were performed with Clampfit9 software (Molecular Devices, Sunnyvale, CA, USA). Stability of V$_m$ during resting conditions was assessed as ΔV$_r$ (maximum–minimum V$_r$ values, mV) and CV$_Vr$ (%) over 60 s recordings. C$_m$ and R$_m$ were derived according to a protocol previously described [40]. Sequences of APs were elicited by means of brief (3 ms) depolarizing current pulses with amplitude 1.5 times the current threshold, and sampled at 10 kHz. From the average of 10 consecutive beats, AP parameters, i.e., UPS, APA and APD at −20 mV (APD$_{20}$) and at −60 mV (APD$_{60}$) were measured as described in the supplementary section.

**Action potential simulations**

All simulations reported in this study were performed by means of the Pandit et al. rat ventricular AP model [16]. The model was recompiled in Matlab language using the COR facility at http://cor.physiol.ox.ac.uk. The ‘ode15s’ Solver built into the R2010b version of Matlab (MathWorks Inc., Navick, MA, USA) was used to integrate model equations. Simulations were run on a PC with an Intel Core 2.24 GHz CPU. Initial conditions for all simulations were set after a simulated train of 50 conditioning beats elicited at a frequency of 2 Hz. A leakage current (I$_{leak}$) was simulated by adding the following equation to the set of ion currents included into the Pandit model:

\[ I_{leak} = G_{leak} \cdot (V_m - E_K) \]

where G$_{leak}$ is the hypothesized membrane leakage conductance and E$_K$ is the potassium reversal potential.

**Genotoxicity assay**

DNA damage was measured using single-cell gel electrophoresis (SCGE, Comet assay). The alkaline version (pH >13) of the assay was performed to detect single-strand breaks and alkali-labile sites, such as apyrimidinic and apurinic sites that are formed when bases are lost
and oxidized. SCGE was performed basically according to [41], with some minor modifications applied to adapt the procedure to cardiomyocytes (see Additional file 1: Methods section).

**Measurement of ROS formation in single cells exposed to TiO$_2$-NPs**

ROS production was measured by fluorescence using 5’-(and 6)-carboxy-2’-7’-dihydrodichlorofluorescein diacetate (DCFDA) as described by [42]. See Additional file 1: Methods section.

**Multicellular studies**

*Intratracheal instillation*

After anesthesia and in order to avoid forcing out of NPs into the lung, a 16-gauge catheter was gently inserted into the trachea of rats in order to deliver ~50 µl of saline solution (Vehicle, n = 6) or saline solution +2 mg/kg TiO$_2$ (NP$_R$, n = 8) by means of a laboratory bench P200 pipette (Gibson, UK). The volume was determined on a body weight basis in order to ensure good NP dispersion (after sonification). This amount of saline solution minimizes the dose of fluid contacting the lungs. Furthermore, an empty sterile syringe (1 ml) was used to gradually inflate the lung with air twice prior to administration of 0.15 mg/kg atipamezole hydrochloride (Antisedan, Pfizer, Italy); finally, the cannula port was connected to a ventilator (Rodent ventilator UB 7025, Ugo Basile, Comerio, Italy). We chose a single concentration of 2 mg/kg as the LD$_{50}$ for this nanomaterial is 59.2 mg/Kg, and we wanted to operate safely (~4%) in line with recently described studies [43]. After waking, the rats were left conscious for four hours before performing *in vivo* electrophysiological recordings.

Before exposing the rat heart, pre-cordial ECGs (3 unipolar and 3 bipolar leads) were recorded in order to screen possible anoxic effects on cardiac electrical activity due to the tracheal instillation (data not shown). Two animals, one per group, were excluded from the final analysis because of the presence of anoxic signs, possibly related to mechanical injury due to the instillation procedure (atrioventricular block).

*Epicardial multiple leads recording*

Four hours after the instillation, the rats were re-anesthetized. Under artificial respiration, the heart was exposed through a longitudinal sternotomy and suspended in a pericardial cradle. Body temperature was maintained with infrared lamp radiation. In the present study, an 8 × 8 row electrode matrix with 1-mm inter-nodal resolution was fabricated from surgical cotton gauze. The electrode array was positioned in order to cover part of the anterior surface of the right (RV) and left (LV) ventricles [44], and the following parameters acquired: ECG waves, excitability parameters (Rheobase and Chronaxie), longitudinal and transversal conduction velocities (CVl, CVt) and ERP. See Additional file 1: Methods section.

**Detection of TiO$_2$ nanoparticles in heart and lungs by TEM**

Heart (RV and LV) and lung samples from Vehicle and NP$_R$ groups were analyzed by TEM in order to document the presence of NPs within the tissues. Moreover, freshly isolated rat
cardiomyocytes were incubated with 50 µg/ml TiO$_2$ and processed for ultrastructural analysis (see Additional file 1: Methods section).

**Thiobarbituric acid reactive substance detection**

Tissue samples were extracted in the dark, washed in PBS and included in cryovials prior to freezing at −80 °C. Frozen tissue samples (trachea, lungs and heart) were homogenized and sonicated in phosphate-buffered saline supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Insoluble debris was pelleted and lipid peroxidation products detected in the supernatants by the TBARS method, based on the condensation of malondialdehyde derived from polyunsaturated fatty acids, with two equivalents in order to give a fluorescent red derivative. More details in the Additional file 1: Methods section.

**Statistical analysis**

The SPSS statistical package was used (SPSS 17th version, Chicago, IL, USA). Normal distribution of variables was checked by means of the Kolmogorov-Smirnov test. Statistics of normally distributed variables included mean ± standard error (SE), paired and unpaired Student’s t test. Statistical significance was set at p < 0.05.

**Abbreviations**

(TiO$_2$), Titanium dioxide; (NPs), Nanoparticles; (NIOSH), National Institute of Occupation Safety and Health; (AP), Action potential; (APD), Action potential duration; (APA), Action potential amplitude; (UPS), Action potential upstroke; (ROS), Reactive oxygen species; (AFM), Atomic force microscopy; (V$_m$), Transmembrane potential; (V$_r$), Resting potential; (C$_m$), Membrane capacitance; (R$_m$), Membrane resistance; (CTRL), Control non-exposed cells; (NP$_C$), TiO$_2$-exposed cells; (NP$_R$), TiO$_2$-exposed rats; (TBARS), Thiobarbituric acid reactive substance; (CV), Coefficient of variability; (RV), Right ventricle; (LV), Left ventricle; (SCs), Spontaneous contractions; (CVt), Transverse conduction velocity; (CVl), Longitudinal conduction velocity; (ERP), Effective refractory period; (SCGE), Single-cell gel electrophoresis; (TEM), Transmission electron microscopy; (FS%), Fraction of shortening.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

M.S, S.R, L.B; designed, performed electrophysiological and mechanical experiments and analysis; L.G and F.C performed patch-clamp and computational model experiments; A.P, A.B performed comet assay experiments; D.A, C.R conceived and performed AFM experiments; R.A, M.G, S.P performed T-Bars experiments; M.C. performed DLS analysis, I.A, PP.L and D.B conceived and performed Raman experiments; C.F, A.G, F.Q, K.U. designed and performed TEM experiments; M.P, D.S, E.M, A.M conceptual and technical advises, M.Z and M.M conceptual design, experimental design, data analysis, preparation of the manuscript. All authors critically read and approved the final manuscript.
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**A**

Type 1

Type 2

Type 3

**B**

Type 1

Type 2

Type 3

Frequency

$V_r$ (mV)
A

B

C

D

E

F

G = 5% I_K leakage

CTRL

K leak

Vm (mV)
Additional files provided with this submission:

Additional file 1. Supplementary Material (981k)
http://www.particleandfibretoxicology.com/content/supplementary/s12989-014-0063-3-s1.zip