**TiO$_2$ nanoparticles induce omphalocoele in chicken embryo by disrupting Wnt signaling pathway**

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Titanium dioxide nanoparticles (TiO$_2$ NPs) are among abundantly used metal oxide NPs but their interactions with biomolecules and subsequent embryonic toxicity in higher vertebrates is not extensively reported. Physicochemical interactions of TiO$_2$ NPs with egg albumen reveals that lower doses of TiO$_2$ NPs (10 and 25 µg/ml) accounted for higher friccohesity and activation energy but an increment in molecular radii was recorded at higher doses (50 and 100 µg/ml). FTIR analysis revealed conformational changes in secondary structure of egg albumen as a result of electrostratic interactions between egg albumen and TiO$_2$ NPs. The morphometric data of chicken embryo recorded a reduction at all the doses of TiO$_2$ NPs, but toxicity and developmental deformity (omphalocoele and flexed limbs) were recorded at lower doses only. Inductively coupled plasma optical emission spectrometry (ICP-OES) confirmed presence of Ti in chicken embryos. mRNA levels of genes involved in canonical and non-canonical Wnt signaling were lowered following TiO$_2$ NPs treatment resulting in free radical mediated disruption of lateral plate mesoderm and somite myogenesis. Conformational changes in egg albumen and subsequent developmental deformity in chicken embryo following TiO$_2$ NPs treatment warrants detailed studies of NP toxicity at lower doses prior to their biomedical applications.

Nanotechnology is a rapidly expanding field, with a wide range of applications in communications, robotics, medicine, clothes, sporting goods, etc$^{12}$. According to a recent survey, the number of nanotechnology-based consumer products available in the world market is more than 1800$^3$. The increased use of nanomaterials is also under scrutiny due to their adverse effects on the environment, physiology and overall survival of organisms. Titanium dioxide nanoparticles (TiO$_2$ NPs) are the most abundantly used nano metal oxides with their documented industrial uses in pigments and additives for paints, paper, ceramics, plastics, foods, and other products. The estimated worldwide production of TiO$_2$ NPs is 10000 tons/year for 2011–2014 and 2.5 million metric tons/year by 2025$^4$. Therefore, risk assessment studies have predicted that TiO$_2$ NPs will be the most prevalent nanomaterials in environment$^4$.

Cytotoxic potential of TiO$_2$ NPs is well documented in a variety of cell lines. Oxidative DNA damage and apoptosis in HepG2 cells and in human epidermal cells$^6$, apoptosis and/or necrosis in human astrocytoma (astrocytes-like) U87 cells$^7$ and mitochondrial dysfunction in BRL 3A cells$^8$ are some of the recent reports on cytotoxicity of TiO$_2$ NPs. Toxicity of TiO$_2$ NPs based on difference in their size has been documented in nematodes$^9$ and earthworms$^{10}$. The ability of TiO$_2$ NPs to produce reactive oxygen species and surface charge are the reasons accredited for their toxicity$^{6,11}$. Several engineered nanometals including TiO$_2$ NPs have been known to persist in the food chain and move across trophic levels resulting in various forms of toxic manifestations$^{11}$. Hence, their effect on reproductive performance and embryonic development cannot be ignored. Accelerated hatching of larvae and deformed embryos in zebrafish$^{12}$ and histopathological changes in juvenile carp$^{13}$ are few evidences on TiO$_2$ NPs induced toxicity on embryonic and post-hatch development. Hatching inhibition and malformation of embryos of Abalone have been reported following TiO$_2$ NPs exposure$^{14}$. Also, prenatal exposure of TiO$_2$ NPs in female rats impacts genes controlling brain development in offspring$^{15}$ providing compelling evidences on systemic and developmental toxicity.

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Chicken embryo is a sensitive and popularly used model for assessing developmental toxicity and teratogeny of various nanoparticles. Hence, chicken embryo was chosen as an experimental model in our study to assess the impact of TiO₂ NPs on embryonic development. Other studies had reported developmental toxicity of graphite¹⁶, copper¹⁷, carbon¹⁸, platinum¹⁹, pristine graphene²⁰ and silver²¹ nanoparticles on chicken embryo, but their in ovo physicochemical interactions with biomolecules such as egg albumen have not been taken into account. In the present study, we assess the interaction of TiO₂ NPs with egg albumen and its subsequent impact on chicken embryonic development.

Results

In DLS analysis, TiO₂ NPs presented a single distribution with peak centered at 88.6 nm. The plot showed that the nanoparticles have a narrow size distribution with an average diameter of about 88.6 nm (Supplementary Figure S1).

Physicochemical analysis. Results shown herein are quantification of interaction of peptide bonds with TiO₂ NPs and alteration in the Lennard-Jones potential that varies spontaneity and strength of interactive force. There was a decrement in density (1.031064 kg.m⁻³) of TiO₂ NPs + albumen at 10 µg/ml, whereas, 25, 50 and 100 µg/ml recorded steadily ascending values (1.031567, 1.031979 and 1.032092 kg.m⁻³ respectively) (Fig. 1a and Supplementary Table S2). Lower concentrations of TiO₂ NPs (1 and 5 µg/ml) recorded higher viscosity indices (2.57 and 2.60 mPa.s). The viscosity indices of 10, 25 and 50 µg/ml doses were comparable to each other (2.47, 2.49 and 2.45 mPa.s) but, 100 µg/ml dose accounted for a decline in viscosity (2.35 mPa.s) (Fig. 1b and Supplementary Table S2). Indices of surface tension showed an increase at 10 µg/ml concentration of TiO₂ NPs (65.98 mN.m⁻¹) as compared to 1 and 5 µg/ml concentrations (65.45 and 65.44 mN.m⁻¹ respectively). However, 25, 50 and 100 µg/ml doses recorded a steady increment in surface tension (66.01, 66.64 and 66.65 mN.m⁻¹) (Fig. 1c and Supplementary Table S2). Friccohesity indices showed a decline at 10 µg/ml TiO₂ NPs (0.002009 s.m⁻¹) as compared to 1 and 5 µg/ml concentrations (0.002113 and 0.002136 s.m⁻¹ respectively). A steady decline in friccohesity (0.002027, 0.001974 and 0.001899 s.m⁻¹ at 25, 50 and 100 µg/ml doses respectively) was also observed in this study (Fig. 1d and Supplementary Table S2). A dose dependent decline in activation energy was recorded from 1–100 µg/ml doses with −57.81 KJ.mole⁻¹ as the highest value and −61.26 KJ.mole⁻¹ as the lowest value respectively (Fig. 1e and Supplementary Table S2). An increase in molecular radii (5–11.53 nm) was observed at 1–10 µg/ml TiO₂ NPs. Further, a steady increase in molecular radii (15.69, 19.66 and 24.45 nm) was observed at 25, 50 and 100 µg/ml doses respectively (Fig. 1f and Supplementary Table S2).

Spectroscopic characterization. Comparative FTIR spectra (400–4000 cm⁻¹) of native albumen and TiO₂ NPs + albumen depicting amide A (around 3400 cm⁻¹), amide B (about 3090 cm⁻¹), amide I and II (region between 1600–1700 cm⁻¹) domains are shown in Fig. 2a. The broad peak at 3591 and 3434 cm⁻¹ in the amide A region of native albumen corresponds to the H-O-H asymmetric and symmetric stretching respectively whereas TiO₂ NPs + albumen recorded a shift in H-O-H stretching peak to 3478 cm⁻¹. Also, a shift in peak from 2071 cm⁻¹ (in albumen) to 2083 cm⁻¹ (TiO₂ NPs + albumen) was recorded. The amide I and II secondary

Figure 1. Physicochemical analysis of titanium dioxide nanoparticles and their interaction with egg albumen. Density, Viscosity, Surface tension, Friccohesity, Activation energy and Molecular radii of TiO₂ NPs in albumen (a–f) respectively.
fingerprint regions (in albumen) recorded two peaks at 1651 cm\(^{-1}\) and 1642 cm\(^{-1}\) but in TiO\(_2\) NPs + albumen a peak was recorded at 1641 cm\(^{-1}\). A peak in the amide III region was recorded at 1243 cm\(^{-1}\) in albumen whereas, TiO\(_2\) NPs + albumen recorded a shift to peak 1551 cm\(^{-1}\). Also, peaks at 1457 and 1451 cm\(^{-1}\) in albumen and TiO\(_2\) NPs + albumen respectively are due to –CH\(_2\) scissoring vibration whereas, a peak at 675 cm\(^{-1}\) in TiO\(_2\) NPs + albumen corresponds to Ti-O vibrational mode of TiO\(_2\) NPs. Results obtained in deconvoluted Gaussian fitted spectra (Fig. 2b,c) and integrated peak areas of secondary-derivative structure element (Fig. 2d and Supplementary Table S3) and in albumen and TiO\(_2\) NPs + albumen showed that albumen was mainly composed of side chain (1610 cm\(^{-1}\), 20.93%) inter or intramolecular \(\beta\) sheet (1621–1629 cm\(^{-1}\), 39.11%) closely followed by \(\alpha\) helices (1664 cm\(^{-1}\), 13.94%) with minor proportions of \(\beta\) turns (1681 cm\(^{-1}\), 11.95%) and \(\beta\) sheet (1695 cm\(^{-1}\), 10.76%). After interaction with TiO\(_2\) NPs, a decrease in side chain (1607 cm\(^{-1}\), 4.11%), \(\beta\) turns (1681 cm\(^{-1}\), 10.31%) and \(\beta\) sheet (1695 cm\(^{-1}\), 8.81%) and an increase in inter or intramolecular \(\beta\) sheet (1621 cm\(^{-1}\), 53.39%) and \(\alpha\) helices (1661 cm\(^{-1}\), 17.46%) was recorded.

**Natality and Morphometry of Chick embryos.** Lower doses (10 and 25 \(\mu\)g/ml) of TiO\(_2\) NPs treatment accounted for 12.5% and 25% viable embryos respectively on 19th day of incubation. Also, 56.25% and 43.75% embryos were found to be malformed at 10 and 25 \(\mu\)g/ml doses. However, higher doses (50 and 100 \(\mu\)g/ml) recorded viable embryos ranging between 75–87.5% (Fig. 3a). Morphometry of the embryos (whole weight and length) recorded significant decrement at all the doses (10–100 \(\mu\)g/ml) (Fig. 3b). Whole weights of liver, brain and heart showed non-significant decrement at all the said doses (Supplementary Table S4).

**ICP-OES analysis of embryos.** After 4-days of TiO\(_2\) NPs treatment, the contents of Ti in the chick embryo were measured by ICP-OES. Significantly high levels of Ti was detected (3 times increase) in embryos of eggs treated with 10 \(\mu\)g/ml TiO\(_2\). But, higher dose (100 \(\mu\)g/ml) accounted for a moderate non-significant content of Ti in embryos (Fig. 3c).

**Deformity.** Control and TiO\(_2\) NPs treated chick embryos were examined as per Hamburger-Hamilton standard that revealed presence of flexed limbs at 10 and 25 \(\mu\)g/ml doses of TiO\(_2\) NPs (Fig. 3d). Also, omphalocele (ventral body wall defect) was observed at 10 \(\mu\)g/ml dose. These deformities were not seen at any of the higher doses (50 and 100 \(\mu\)g/ml). Further confirmation of flexed limbs of 10 and 25 \(\mu\)g/ml TiO\(_2\) NPs treated embryos was obtained by alcian blue- alizarine red staining (Fig. 3e).

**Expression of Wnt signaling genes.** RT-PCR analysis was performed to assess the effect of TiO\(_2\) NPs on expression of key genes of canonical (CTNNB1, PITX2 and LEF1), non-canonical Wnt/Ca\(^{2+}\) (WNT11, PRKCA and CAMK2D) and Planar Cell Polarity (ROCK1 and ROCK2) pathways associated with Wnt signaling. Expression levels of genes of canonical pathway (CTNNB1, PITX2 and LEF1) were downregulated significantly
in embryos treated with TiO₂ NPs (10 µg/ml). A similar trend of significant decrement was also observed in cadmium treated embryos, whereas, TiO₂ bulk treatment could not manifest any significant change (Fig. 4a–c). Expression levels of key genes of non-canonical Wnt/ Ca²⁺ signaling (WNT11, PRKCA and CAMK2D) showed significantly lowered expression levels following TiO₂ NPs or cadmium treatment. However, the TiO₂ bulk treatment showed non-significant changes in the expression levels of the said genes (Fig. 4d–f). mRNA expression of key genes of Planar Cell Polarity pathway (ROCK1 and ROCK2) accounted for non-significant decrement following TiO₂ NPs or cadmium treatment. TiO₂ bulk treatment accounted for moderately significant increment in ROCK1 expression and non-significant increment in expression of ROCK2 (Fig. 4g and h). Expression levels of HOXD13 showed significantly lowered expression levels following TiO₂ NPs or cadmium treatment, whereas, the TiO₂ bulk treatment showed non-significant changes (Fig. 4i).

**Somite development.** It was observed that 10 µg/ml dose of TiO₂ NPs accounted for 20% decrement in the number of somites after 24 h which was comparable to that of the cadmium treated group, whereas, TiO₂ bulk treatment could not manifest any significant change (Fig. 4j).

**Discussion**

Nanomaterials have been reported to interact with protein molecules in unique ways and form a ‘protein corona’ that alters its physicochemical identity and affect its bio-distribution, kinetics and subsequent toxicity²². A previous study in our lab had shown that TiO₂ NPs interact with protein components of RPMI-1640 and result in higher indices of intermolecular interaction²³. Egg albumen is reservoir of protein in an avian egg that meets the nutritional requirements of an embryo. Besides egg shell, shell membrane and chorio-allantoic membrane; egg albumen also regulates the trafficking of exogenous elements by acting as a natural biological barrier²⁴. In the present study, a dose dependent increase in density of albumen was observed following addition of TiO₂ NPs. But, relatively lowest density observed at 10 µg/ml hints at effective dispersion of TiO₂ NPs in egg albumen. Higher
intermolecular forces and cohesion are the key factors that determine viscosity and surface tension of liquids. In our study, a dose dependent decrement in viscosity and a reciprocal increment in surface tension are in support of our claim that higher intermolecular forces are as a result of higher concentration of TiO$_2$ NPs. Friccohesity is a product of frictional and cohesive forces within similar (protein-protein) and dissimilar (protein-nanoparticle) molecules$^{25}$. A dose dependent decrement in friccohesity suggests weaker inter conversion between cohesive and

**Figure 4.** Expression of Wnt signaling pathway-related genes in TiO$_2$ NPs-treated chicken embryos. The expression of Wnt signaling pathway-related genes (a–h) including CTNNB1, PITX2, LEF1, WNT11, PRKCA, CAMK2D, ROCK1 and ROCK2 and (i) limb development gene HOXD13 was analyzed using reverse transcription polymerase chain reaction (RT-PCR) in control and TiO$_2$ NPs-treated embryos ($n = 3$), 4 h after treatment in shell-less culture at 60 h. All the Wnt signaling pathway related genes and limb development gene were downregulated in TiO$_2$ NPs-treated embryos compared to those of control embryos. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = not significant. (j) Somite numbers in control and TiO$_2$ NPs-treated embryos 24 h after treatment in shell-less culture (HH-23). There is a reduction in the number of somites in TiO$_2$ NPs-treated embryos as compared to the control embryos. The data are expressed as Mean ± SD. Statistical analysis was done by one way ANOVA followed by Dunnett’s test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns = not significant.
frictional forces implying towards a stronger nanoparticles-egg albumen interaction. Also, a decrement in activation energy is an indicator of increased proportion of collision/chemical reaction between the test compounds. Relatively higher indices of activation energy recorded in TiO$_2$ NPs + albumen (10 µg/ml) is suggestive of more quantum of interaction between TiO$_2$ NPs and egg albumen. Molecular radii play an important role in dispersion of nanomaterials and its impact on biological systems. A dose dependent increment in molecular radii resulting due to TiO$_2$ NPs-egg albumen interactions implies towards formation of nanoparticle agglomerates at higher doses. TiO$_2$ NPs + albumen recorded a shift in H-O-H stretching peak to 3478 cm$^{-1}$ confirming interaction between TiO$_2$ NPs and albumen. Further a shift in peak from 2071 cm$^{-1}$ (in albumen) to 2083 cm$^{-1}$ (TiO$_2$ NPs + albumen) is attributable to interaction between C-O and amide groups of amino acids present in albumen. Peaks observed in the amide I and II secondary fingerprint region (in albumen) at 1651 cm$^{-1}$ and 1642 cm$^{-1}$ are attributable to C=C stretching and H-O-H bending respectively. However, a minor shift in C=O stretching and depletion of H-O-H bending (at 1641 cm$^{-1}$) was possibly on account of electrostatic interaction due to Vander Waal forces taking place between albumen and TiO$_2$ NPs. Peak in amide III region (at 1243 cm$^{-1}$) in albumen occurs due to N-H bending and C-N stretching of amino groups but, TiO$_2$ NPs + albumen recorded a shift (at 1551 cm$^{-1}$) from amide III to amide II region. This shift also portrays major conformational changes in secondary components (α helices and β sheet) of proteins possibly due to their interaction with TiO$_2$ NPs. Fourier-self deconvolution approach was employed to assess secondary conformational changes in amide I and II region. Venyaminov and Kalnin had reported that amide peak at 1610 ± 4 corresponds to NH bending of CO-NH$_2$ bond in glutamine. In our study, amide peak at 1610 cm$^{-1}$ in TiO$_2$ NPs + albumen indicates possible deformity of glutamate in egg albumen. Role of glutamate in nutrition and metabolism is well reported and hence impact of structurally altered egg albumen are possibly due to TiO$_2$ NPs mediated conformational changes, formation of aggregates or amyloids with protein moieties in egg albumen. These findings are the first to showcase interaction of TiO$_2$ NPs with egg albumen and the said physicochemical alterations.

Interaction of TiO$_2$ NPs with egg albumen prompted us to assess its impact on embryonic development using chicken egg as a model. Significant reduction in morphometric indices (body weight and length) and higher percentage mortality was recorded in developing chicken embryos at lower doses of TiO$_2$ NPs (10 µg/ml). But, the higher doses of TiO$_2$ NPs (50 or 100 µg/ml) failed to elicit a dose-dependent toxicological response possibly because TiO$_2$ NPs underwent physicochemical alterations as evidenced by relatively higher indices of density, viscosity and friccohesity coupled with lower activation energy hinting at formation of NP agglomerates. Whereas, lower extent of NP-egg albumen interactions observed at 10 µg/ml of TiO$_2$ NPs was instrumental in its effective bio-distribution and manifested said toxicity. Percentage mortality of bulk TiO$_2$ treated chicken embryos was comparable to that of higher doses (50 or 100 µg/ml) of TiO$_2$ NPs thus providing conclusive evidence that an altered physicochemical identity of NPs failed to induce a dose dependent toxicity in chicken embryos. Nanoparticles are known to cross biological barriers like the blood brain barrier and blood placenta barrier. The results obtained herein indicate that the TiO$_2$ NPs could cross biological barriers within an avian egg and reach the embryo. The same was confirmed by ICP-OES studies that revealed presence of higher levels of TiO$_2$ NPs in the embryonic tissue at the lower dose (10 µg/ml).

Omphalocele is a ventral body wall defect and is accompanied by herniation of midgut into the abdominal cavity, failure in fusion of the anterior abdominal wall with 1/3000 frequency of occurrence in human population. Teratogenic agents such as cadmium, specific radiations, fungal toxins, etc. are known to induce omphalocele in various animal models. However, no known nanomaterials have been reported to induce omphalocele. Wnt signaling pathway has been implicated in various events of embryonic development such as cell differentiation, survival, migration, proliferation, adhesion and somite formation. Canonical Wnts relay their signal via β-Catenin pathway that control cell fate determination. Whereas, the non-canonical Wnt signaling either through Wnt/Ca$^{2+}$ pathway or planar cell polarity pathway that controls cell adhesion and movement. Results obtained herein were compared with cadmium induced omphalocele chicken embryo model. PITX2, a bicoid-type homeodomain transcription factor, has known to be regulated by β-Catenin dependent Wnt pathway. In the Wnt/β-Catenin pathway, the accumulation of β-Catenin in the nucleus converts DNA-binding factor, lymphoid enhancing factor-1 (LEF1), to a transcriptional activator and is regulated through direct physical interaction with PITX2 and β-Catenin. In this study, downregulation in expression levels of CTNNB1, PITX2 and LEF1 following TiO$_2$ NPs treatment (10 µg/ml) could be a key factor in the disruption of somite myogenesis by inhibiting Wnt/β-Catenin pathway. It has been postulated that cells from somites migrate into the parietal layer of lateral plate mesoderm (LPM) to assist in forming the lateral body folds. PITX2 is known to regulate cell survival and its downregulation may induce abnormal apoptosis in the somite and LPM that could further interfere with the movement of the lateral body wall folds. These results justify the decrement in somite count obtained in our study following TiO$_2$ NPs treatment (10 µg/ml). WNT11, a member of the noncanonical Wnts, is an important epithelialization factor acting on the dermomyotome whereas, PRKCA and CaMK2D control actin-cytoskeleton organization and cell contractility. Previous studies had implicated PRKCA and CaMK2D (activated by WNT11) in the regulation of cell-cell adhesion molecules (CAMs) such as cadherins. The resultant linkages between E-cadherin and actin filaments reinforce the cell-cell junctional connection. In our study, downregulation of WNT11, PRKCA and CaMK2D genes after TiO$_2$ NPs treatment (10 µg/ml) possibly interfered with actin-cytoskeleton organization, cell movement and cell adhesion, thus disrupting noncanonical Wnt/Ca$^{2+}$ signaling that resulted in omphalocele. Rho kinases (ROCK) are involved in the regulation of various cellular functions (contraction, adhesion, migration, proliferation and apoptosis) including tissue closure during embryonic development. ROCK1 and ROCK2 mediate signaling from Rho to the actin cytoskeleton in the Wnt non-canonical pathway. ROCK1 knockout (KO), ROCK2 KO, and ROCK1/2 double heterozygous mice has been reported to exhibit omphalocele phenotype due to disorganization of actin filament in the epithelial cells of umbilical ring. Downregulation of ROCK genes following TiO$_2$ NPs treatment possibly disrupted actomyosin.
assembly, resulting in the failure of ventral body wall closure resulting in omphalocele. Defects in ventral body wall closure and omphalocele has also been reported with accompanying limb deformities in genetically modified experimental models42. Hox genes are important regulators of limb pattern in vertebrate development, HOXD13 misexpression in the hindlimb results in shortening of the long bones, including the femur, the tibia, the fibula and the tarsometatarsals. In our study, significantly lowered expression of HOXD13 in TiO2 NPs treated embryos corroborate with the observed omphalocele. Cadmium is known to use Ca2+ ion channels and membrane transporters to enter into the cells of a developing embryo. Further, it disrupts lateral plate mesodermal cells and induces omphalocele. Therefore, cadmium treated chicken embryos were used as a disease control in our study wherein; expression levels of key genes of the Wnt signaling pathways were comparable to TiO2 NP treated embryos. TiO2 NPs are also known to cause free radicals induced cellular damage. Free radical induced disruption of lateral plate mesoderm and somite myogenesis culminating in omphalocele in TiO2 NPs treated chicken embryos is hypothesized in our study.

Besides their widespread industrial use, TiO2 NPs have gained prominence in biomedical applications due to their long term photostability, superior biocompatibility, catalytic efficiency and a strong oxidizing power. Photodynamic therapy for cancer, cell imaging, genetic engineering, drug delivery and biosensors are some of the reported biomedical applications of TiO2 NPs. Also, their use in diagnosis of cardiovascular diseases, diabetes mellitus, cancer and orthopaedic disorders underlines their prominence. But, omphalocele formation only at sub lethal (lower) concentrations reported herein raises concerns of toxicity benchmarks impacting foetal development. Hence, it raises an urge to study interactions of nanoparticles with biomolecules vis-à-vis particle size or surface modifications prior to their use in diagnostics or biomedical applications.

Conclusion
Nanometal oxides witness a wide range of biomolecules in a physiological environment that can alter their behavior and responses. In the present study, TiO2 NPs were found to interact with egg albumen as evidenced by changes in their proteinc secondary structure. These interactions could possibly allow TiO2 NPs to traverse the biological barriers (shell membrane and CAM) within chicken egg and affect the growth and development of embryos and cause malformations like omphalocele and flexed limbs. Also, the observed mortality and significant decrement in morphometry (whole weight and length) are attributable to TiO2 NPs-albumen interactions. Omphalocele formation in TiO2 NPs treated groups is possibly due to the disruption of somite myogenesis as evidenced by alterations in expression of key genes of Wnt signaling pathway. Hence, use of TiO2 NPs in diagnostics and therapy warrants a detailed research in embryos by taking into account its particle size, surface modifications and interaction with biomolecules.

Materials and Methods
Availability of Data and Materials. The datasets supporting the conclusions of this article are included within the article.

Nanoparticles. Titanium (IV) oxide nanopowder (TiO2 NPs, mixture of Anatase and rutile, Cat. no. 634662, particle size <100 nm, 99.5% purity) was procured from Aldrich (St. Louis, MO, USA). TiO2 NPs (1 mg/ml) were suspended in water and probe sonicated (LMUC-4, Labman scientific instruments Pvt. Ltd., Kolkata, India) for 30 min. After sonication, the particle size distribution was measured using a 90 plus DLS (Dynamic light scattering) unit from Brookhaven (Holtsville, USA).

Physicochemical analysis. The physicochemical properties such as density, viscosity, surface tension, activation energy, fracturehessity and molecular radii were assessed in absence or presence of TiO2 NPs in egg albumen (freshly collected). TiO2 NPs were suspended in egg albumen at 1, 5, 10, 25, 50 and 100 μg/ml concentrations. Density of TiO2 NPs in water and egg albumen were determined with Anton Paar Density and Sound velocity Meter (DSA 5000 M). Density was calculated using equation 1:

$$\rho = \rho^° + S^m \rho + S^m \rho^2$$  
(1)

($\rho^°$ at m → 0 is limiting density, $S^m$ is the 1st slope)

Viscosity was measured as viscous flow times (VFT) using Borosil Mansingh Survismeter at physiological temperature of 37 °C (LAUDA ALPHA RA 8 thermostat) and calculated by equation 2:

$$\eta = \left( \frac{1}{t} \right) \left( \frac{\rho}{\rho^°} \right) \eta^°$$  
(2)

($\eta^°$, $t$ are viscosity of water and t, $t$ are flow times of solvent and mixtures respectively)

The $\eta$ data were regressed with following equation 3:

$$\eta = \eta^° + S^m \rho$$  
(3)

($\eta^°$, $t$ are limiting viscosity; $S^m$ is the 1st degree slope).

Surface tension was measured by counting pendent drop numbers (PDN) using Borosil Mansingh Survismeter and calculated by equation 4:

$$\gamma = \left( \frac{\eta}{\eta^°} \right) \left( \frac{\rho}{\rho^°} \right)$$  
(4)
(γ° is surface tension of water, η° and η are pendent drop numbers of medium and solutions respectively)
The γ data were regressed for limiting values γ° at m → 0 with following equation 5:

\[ \gamma = \gamma^° + S_m \]  

(γ° is limiting surface tension, and S_m is the 1st degree slope)
Friccohesity was calculated using Mansingh equation 6:

\[ \sigma = \alpha \left( \frac{t}{t_0} + \frac{B}{r} \left( \frac{\eta}{\eta_0} \pm 0.0012(1 - p) \right) \right) \]  

(σ is friccohesity, t and t° are the sample and solution viscous flow times respectively, η° and η are the pendent
drop numbers of medium and solutions respectively)
Reference friccohesity was calculated by equation 7

\[ \sigma = \frac{\eta}{\gamma^°} \]  

where, η° and γ° are the viscosity and surface tension of references respectively.
For activation energy, the partial molar volume V_2 was calculated with following equation 8:

\[ V_2 = \frac{1000(\rho^° - \rho)}{m_\rho^° \rho} + \frac{M}{\rho} \]  

( M is molar mass, ρ^° is density of water and ρ is density of solution)
The V_1 for water or albumen at 37 °C is calculated with equation 9:

\[ V_1 = \frac{M}{\rho} \]  

V_1 and V_2 are used for calculating activation energy by using equation 10:

\[ \Delta \mu^*_1 = R \ln \left( \frac{\eta V_1}{hN} \right) \]  

(Δμ^*_1 is activation energy of water or albumen, R is gas constant, h is Planck constant and N is Avogadro number
(6.023 \times 10^{23}). Activation energy (Δμ^*_2 J/mol) was calculated by using equation 11:

\[ \Delta \mu^*_2 = \Delta \mu^*_1 - \left( \frac{RT}{V_2} \right) (1000\eta) - (V_1 - V_2) \]  

Molecular radii r (nm) is calculated by using equation 12:

\[ r = \sqrt{\frac{3p}{4\pi Nc}} \]  

(φ is volume fraction of water or albumen entangled with NPs, N is Avogadro number, c is concentration and π
is constant).
Each parameter was measured in triplicates.

**Spectroscopic characterization.** Protein-nanoparticle interaction was identified by Fourier transform infrared (FTIR) spectra (PerkinElmer spectrum 65 series, PerkinElmer, Inc., MA, USA). Sample was prepared
by making pellet of 1.5 to 2 mg of sample mixed with 200 mg KBr (AR, Sigma, USA) in the KBr press machine
(model Mp-15) at 5 kg/cm² pressure for 2 min. After taking background scan, samples were analyzed at 400–
4000 cm⁻¹. FTIR direct-transmittance spectroscopy (KBr) was used to indicate the degree to which oxygen
groups were removed and the IR absorption of water from the air was mostly eliminated. Each measurement
was repeated in triplicates to minimize the error.

**Chicken Embryo model and experimental groups.** The experimental protocol (MSU-Z/IAEC/03–2017) was approved by the Institutional Animal Ethical Committee (IAEC) and the Committee for the Purpose
of Control and Supervision of Experiments on Animals (827/GO/Re/S/04/CPCSEA). Fertilized eggs (55 ± 2.1 g)
of White leghorn (Gallus gallus domesticus) were obtained from Shakti hatcheries, Sarsa, Gujarat, Gujarat, stored for 2
days at 12 °C and then incubated under standard conditions (37.5 °C, humidity 60%) for 48 hours and guidelines
of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) were hereby
followed for all the experiments conducted on the chicken embryo. The procedures for in ovo experimentation
were as per the standard operating protocols of our laboratory. Candling was done to confirm the fertility of
eggs and unfertile eggs were discarded. Eggs were randomly divided into six groups of 16 eggs/group viz. control
(untreated), placebo (treated with PBS) and TiO₂ treated (10, 25, 50 and 100 µg/ml) groups. TiO₂ NPs powder was
suspended in saline (1 mg/ml) and sonicated (LMUC-4, Labman scientific instruments Pvt. Ltd. Kolkata, India)
for 30 min. Further, TiO₂ NPs were diluted to 10, 25, 50 and 100 µg/ml doses. Test samples were injected in ovo
(0.3 ml/egg) in airspace using sterile 1 ml tuberculin syringe and incubated for 18 days.
erol/0.8% KOH, 50% glycerol/0.5% KOH and 80% glycerol/0.2% KOH respectively) and stored in 100% glycerol.

De-staining of embryos was done in a graded series of glycerol/potassium hydroxide (20% glycerol, 10% glycerol/0.25% KOH, 10% glycerol/0.5% KOH, 5% glycerol/1% KOH, 1% glycerol/5% KOH, 0.5% glycerol/10% KOH and 0.25% glycerol/20% KOH) and dehydrated to 100% ethanol. Embryos were then fixed in 100% ethanol for 2 h, rinsed in a graded series of ethanol (100%, 80%, 60%, 40%) and dehydrated to 100% ethanol. Embryos were transferred to 100% acetone for 2 h and then embedded in paraffin. Eight micrometer sections were cut, placed on glass slides and stained with hematoxylin-eosin for light microscopic examination.

### Table 1. Primers used for RT-PCR.

| Gene Name | Accession number | Primer Sequence (5′-3′) |
|-----------|------------------|-------------------------|
| GAPDH     | NM_204305.1      | F: ACTGTCAAGGCTGAGACGG  |
|           |                  | R: AATGTCCGATCCTGGACATGA |
| HOXD13    | NM_205434.1      | F: TCTGCTAATGCTGAGACGG  |
|           |                  | R: AATGTCCGATCCTGGACATGA |
| CTNNB1    | NM_205081.1      | F: GTGCGTATGAGGGAGCAAGCC |
|           |                  | R: GATTTGGGGGAAATACAGGAA |
| PITX2     | NM_205010.1      | F: CGATGAGTTGCATAGAGACCG |
|           |                  | R: AGGAGGAACTGGAGGAGGG |
| LEF1      | XM_015276137.1   | F: TCACCTACAGGAGAGGACGAC |
|           |                  | R: TATCAGGAGGGAGGAGGACG |
| WNT11     | XM_01528085.1    | F: TTACATCTTGCCCTGACGCTC |
|           |                  | R: AGCTCGATGAGGGAGGAGGAG |
| PRKCA     | XM_004946229.2   | F: ACAACCGAGACTTCTTGAG |
|           |                  | R: CCTGCTAGAGGACGGCTCCA |
| CAMK2D    | XM_015276279.1   | F: GCCAATCCACACCATATTACCC |
|           |                  | R: CCATCGATGACTGGGAGGAG |
| ROCK1     | XM_015277931.1   | F: TGACTCGTGTTGCTAGTGGAGG |
|           |                  | R: TAGGAATACGCTGCTGTCAGG |
| ROCK2     | XM_015276085.1   | F: GACTGTTGTCCTCGAGGAGAGT |
|           |                  | R: GCAGTCTCTGGAAGGAGGAGG |

### Autopsy, morphometry and staining.

On 19th day, the experiment was terminated by opening the eggs and the viable embryos were weighed and decapsitated. The morphology of the embryos was examined according to the Hamburger and Hamilton standards and ratios of live vs. dead/malformed were recorded. Deformities of the head, limbs, body and tail were observed under a dissecting microscope and photographed with a digital (Nikon coolpix p900) camera. Two embryos per group were processed for Alizarine-alcian blue staining. Briefly, skin and viscera was removed and embryos were fixed in 96% ethanol for 3 days followed by acetone for 2 days. Later, embryos were then rinsed in ethanol for 1–2 h and stained with Alizarine-alcian blue stain (0.015% Alcian blue, 0.005% Alizarin red in 70% ethanol, 20% acetic acid and 10% dH2O) at 37 °C for 4 h. Embryos were rinsed in ethanol and running tap water for one hour each and muscles were cleared in an aqueous solution of 1% potassium hydroxide. De-staining of embryos was done in a graded series of glycerol/potassium hydroxide (20% glycerol/0.8% KOH, 50% glycerol/0.5% KOH and 80% glycerol/0.2% KOH respectively) and stored in 100% glycerol.

### Inductively coupled plasma optical emission spectroscopy (ICP-OES).

Four days old control and treated eggs (three per group) were opened and embryos were digested overnight at 40 °C in 6 ml of conc. nitric acid and 3 ml of hydrogen peroxide. Contents were heated in an oven (110 °C) for 2 h, cooled at room temperature and diluted with 11 ml of dH2O. The concentrations of nanosized particles in the embryo were quantified by ICP-OES.

### Shell-less culture and dosing.

In a separate set of experiment, procured eggs were incubated for 60 h in standard conditions and later were explanted into shell-less culture as per Dugan, et al. The embryos were divided into four groups of six eggs each viz control (50 µl PBS), positive control (50 µl of 50 µM CdCl2), TiO2 NPs treated (50 µl of 10 µg/ml) and bulk TiO2 (50 µl of 10 µg/ml). Dosing was done directly on blastodisc using a micropipette and embryos were incubated for 4 h or 24 h.

### Autopsy, RNA isolation and qPCR study.

Developing embryonal discs (three per group, HH 17; whole embryo) were transferred in RNA later solution (Invitrogen, California, USA). Total RNA was isolated using TRIzol reagent (Invitrogen, California, USA) and cDNA was synthesized by reverse transcription of 1 µg of total RNA using iScript cDNA Synthesis kit (BIORAD, California, USA). For HOXD13, total RNA was isolated from limb bud of 4 day old control and treated embryos. Quantitative RT-PCR was performed using SYBR Select Master Mix (Applied Biosystems) in QuantStudio12K (Life Technologies) real-time PCR machine with primers (Table 1) to detect selected messenger RNA (mRNA) targets. The relative mRNA expression levels were normalized against expression levels of GAPDH for each sample and analyzed using 2−∆∆CT method.

### Somite development.

The embryos (three per group) were dissected from their membranes 24 hours after treatment (HH 23) and inspected using the dissecting microscope to count somite numbers.

### Statistical analysis.

Data analysis was carried out by unpaired Student’s t-test or one way analysis of variance (ANOVA) using Graph Pad Prism 6.0 (CA, USA). Differences between control and treatment groups were deemed to be significant when P < 0.05.

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Author Contributions
R.D. conceptualized the idea and with S.P. designed the study. S.P. and S.J. conducted experiments on chick embryo toxicity whereas, R.C. performed physicochemical studies and later, M.S., and R.C. did data analysis. R.D., S.P. and M.S. drafted the manuscript with inputs from S.T., S.J. and R.C. All authors gave intellectual input to the study and approved the final version of the manuscript.

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