‘’Indirect toxic effects of nanoparticles in *in vit*ro models of the human placenta’’

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September/October 2022
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Contents

Abstract

With the ever-increasing use of nanotechnology, humans may be incidentally exposed to nanoparticles, which raises concerns about their potential toxic effects, particularly in pregnant women and developing foetuses. Advanced in vitro placental models should complement in vivo animal studies to gain insights into placenta-mediated toxicity. Using such approaches, it has been shown that CoCr nanoparticles can induce DNA-damaging signaling across BeWo cellular barriers, an in vitro model of the human placenta, and cause neuronal toxicity without crossing the barriers. In these studies, human fibroblasts were exposed to CoCr nanoparticles indirectly through monolayered and bilayered/multi-layered BeWo cellular barriers grown on transwell inserts. Media was conditioned under the BeWo barrier during nanoparticle exposure and transferred onto differentiating neural progenitor cells (NPCs). In vivo experiments included intravenous injection of CoCr nanoparticles in pregnant mice in early pregnancy and when the placenta is fully established. They found that CoCr nanoparticles caused DNA damage and chromosome aberrations on human fibroblasts without crossing the BeWo barrier. The indirect damage was caused by intercellular signaling within the barrier through pannexin and connexin channels and the secretion of factors such as ATP. This was only evident in bilayered/multi-layered barriers and not monolayered ones. The initiation of the signaling cascade was mediated by autophagy and involved IL-6 release, resulting in DNA damage in differentiating NPCs, dependent on astrocytes' presence. In pregnant mice, nanoparticle exposure caused DNA damage in the neonatal blood, liver, and hippocampus and reactive astrogliosis in the foetuses. These indirect effects of nanoparticles might be significant in human pregnancy since it is in early pregnancy that the placental villi are bilayered and the embryo is most vulnerable. Exposure of the human placenta to nanoparticles may have detrimental effects on the embryo during neurodevelopment. New advanced in vitro placental models with high predictive value are necessary to understand the indirect developmental toxicity of nanoparticles.

Introduction

Nanoparticles are nanoscale particles ranging between 1 and 100 nm in size. Nanoparticles can occur naturally by chemical, photochemical, mechanical, thermal, and biological processes [1], or be formed incidentally or intentionally from anthropogenic resources [2]. Nanomaterials are widely used in many fields, such as cosmetics, pharmaceuticals, food additives, and biomedicine. They have an increased surface-to-mass ratio; therefore, their chemical/catalytic reactivity is higher than normal-sized forms above 100 nm of the same substance [3].

Their unique characteristics are driving the development of advanced technologies in promising directions but also raise questions about their safety. With the rapidly growing field of nanotechnology, novel engineered nanomaterials can be industrially produced on large scales in many industries [4]. Humans are being exposed to airborne nanoparticles throughout evolution, but exposure has increased extensively due to anthropogenic factors [3]. Large-scale production and extensive use of novel nanomaterials will further increase human exposure and raise safety concerns regarding human health risks.

This is particularly important for vulnerable populations, including pregnant women and the developing fetus [5]. Despite growing concern about the potential risks of nanomaterials to human health, information on their potential toxicity is still scarce. Maternal exposure to nanomaterials during pregnancy may lead to developmental toxicity in offspring [6]. Exposure of pregnant women and their unborn children to engineered nanoparticles is not yet a primary public concern [7]; however, with the ever-increasing use of novel nanoparticles in many consumer products, it is essential to gain knowledge about their potential developmental toxicity and to understand the underlying mechanisms regarding their toxicity [8].

Direct and indirect developmental toxicity of NPs

The first indications of the developmental toxicity of nanoparticles came from an epidemiological study by Dadvand *et al*. in 2013, showing an association between maternal exposure to particulate matter with low birth weight [9]. Indications that nanomaterials can cross the placental barrier and induce developmental toxicity came from animal studies, and transfer was later confirmed in human *in vitro* models [5]. In principle, the developmental toxicity of nanomaterials can arise from two different routes: a direct and an indirect route [8].

Direct developmental toxicity

Direct developmental toxicity may arise from maternal blood particles crossing the placental barrier and reaching the fetus [8]. After maternal exposure to nanoparticles via inhalation, ingestion, dermal exposure, and injection, nanoparticles can reach the fetal tissues and cause damage due to their high surface reactivity. They can induce inflammation and production of reactive oxygen species (ROS) resulting in oxidative stress [10], effects that are responsible for developmental toxicity of the fetus, such as growth restriction, preterm birth, and cardiovascular and behavioural defects [8]. There is evidence that nanoparticles can cross the placental barrier from animal studies and human *in vitro* studies.

Indirect developmental toxicity

Direct toxicity from nanomaterials crossing the placental barrier is often considered the key pathway of developmental toxicity and has been the primary focus of developmental toxicity studies [5], [8]. However, besides direct toxicity from translocated particles, developmental toxicity can even occur in the absence of placental transfer, as nanomaterials can indirectly interfere with fetal development via

the release of maternal or placental mediators [5]. Figure 1 shows a schematic representation of the direct and indirect effects of nanomaterials on fetal tissues.

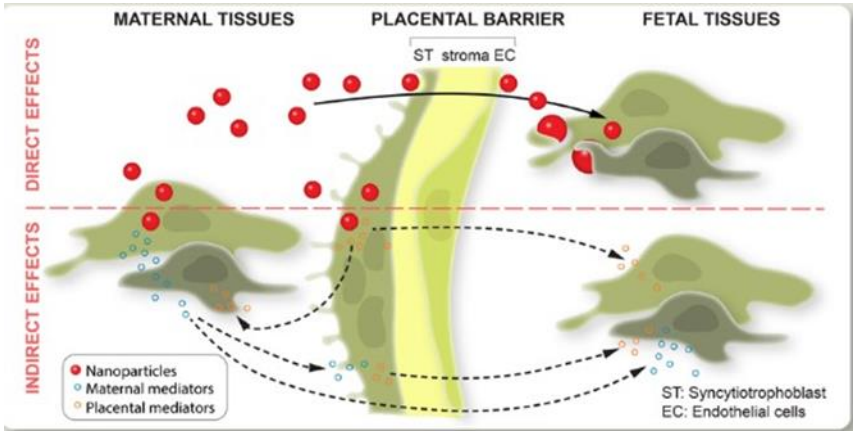

Figure 1. Nanoparticles can affect fetal development directly via crossing of the placental barrier or indirectly by inducing the release of maternal and placental mediators. Figure reproduced from Buerki-Thurnherr et al.

However, only small percentages of the applied doses of nanomaterials can reach fetal circulation, raising questions about whether these small amounts of transferred particles can account for adverse effects on the fetus [5]. The indirect effects of nanomaterials on fetal development through the release of maternal and placental mediators are not yet fully understood and are only slowly gathering some attention [8].

Following maternal exposure to nanomaterials, maternal or placental mediators, can cause toxic effects to the fetus, known as maternal-mediated developmental toxicity or placental-mediated developmental toxicity, respectively. In maternal mediated developmental toxicity, nanomaterials deposited in primary maternal tissue barriers at the point of entry may cause maternal organ dysfunction. Maternal injury might induce oxidative stress and inflammation, leading to the release of inflammatory mediators and soluble signaling factors (e.g., hormones, cytokines) than can reach the placenta and the fetus to induce potential toxic effects [8]. Alternatively, in placental-mediated developmental toxicity, nanoparticles reaching the placenta may cause placental dysfunction resulting in oxidative stress followed by inflammation and induction of placental signaling factors release, that may affect fetal development [8].

Most available studies in the literature, have focused on the direct toxicity of nanoparticles. However, the indirect effects of nanoparticles have only recently gained interest and there is a substantial need to understand the mechanistic pathways behind indirect mediated toxicity. Dugershaw et al. aimed to review the potential indirect pathways in developmental toxicity of nanoparticles. They included studies that reported adverse effects of nanomaterials on gestation and fetal development, with no detectable transfer of particles from mother to fetus [8]. They identified ten studies reporting developmental toxicity without nanomaterial translocation across the placental barrier.

Of all the studies identified in the review of Dugershaw et al., most used pregnant mice as the experimental model, and only a few used *in vitro* cell culture systems for more mechanistic studies [8]. However, mechanistic support for the indirect effects of nanoparticles is difficult to obtain from *in vivo* studies [5]. Moreover, the placenta is the most species-specific organ among mammals [5], [8], [11]. The rodent and human placenta belong to the hemochorial type, where the maternal blood directly faces the trophoblast barrier, which separates the embryonic from the maternal compartment [12]. Although placentas from different species fulfill the same function, supporting the embryo, there are significant differences in placental development, architecture, function and pathology between

rodents and humans [11]. Therefore, extrapolating animal data to humans is challenging and should be done with caution [8], [11].

One of the major challenges of human gynecologic and toxicological research is to find a suitable model for the human placenta [13]. There are a variety of promising models to examine indirect mechanisms of nanoparticles in the human placenta such as the *ex vivo* placental perfusion system, placental explants, primary trophoblast cultures, cell line based *in vitro* human placental model (e.g. BeWo cell lines) [5]. To achieve the most comprehensive knowledge on placental-mediated toxicity, especially to address indirect mechanisms, advanced human *in vitro* and *ex vivo* placental models should complement *in vivo* studies in pregnant rodents [5]. Using such approaches, some recent studies investigated the indirect developmental toxicity of CoCr nanoparticles [14]–[16]. They showed that CoCr nanoparticles can induce DNA-damaging signalling across BeWo cellular barriers, and cause neuronal toxicity without crossing the barriers. [5], [8].

This report aims to describe in detail the three literature studies by Bhabra et al., Sood et al., and Hawkins et al., on the indirect developmental toxicity of CoCr nanoparticles, with emphasis on the models that they used and the conclusions we can draw about human pregnancy from their findings.

Indirect DNA damage of nanoparticles across cellular barriers

In this section, the three studies on the indirect developmental toxicity of CoCr nanoparticles will be discussed in detail. All three studies used the BeWo cell line as an in vitro model of the placental barrier. First, some important information about the anatomy and function of the human placenta, and on the BeWo cell line, will be provided to better understand the content of these studies.

Human placenta

The human placenta is a multifunctional transient organ serving as a barrier between the mother and the fetus [11]. The placenta should be a key focus in any mechanistic study on nanomaterial-mediated developmental toxicity due to its position at the interface between maternal and fetal tissues and its numerous essential functions during pregnancy [8]. It mediates the transfer of nutrients and metabolic waste products while also secreting hormones that maintain pregnancy [17]. As a transient organ, the placenta starts forming after implantation of the conceptus in the uterine wall [8] and consists of tissues of maternal and fetal origin.

The trophoblasts are cells forming the outer layer of the blastocyst, a cluster of dividing cells made by a fertilized egg, the earliest stage of an embryo [18]. As soon as the blastocyst has attached to the uterine epithelium, the polar trophoblast undergoes differentiation to generate the syncytiotrophoblast [18]. The remaining trophoblasts, now referred to as cytotrophoblast, act as stem cells, which rapidly divide and subsequently fuse with the syncytiotrophoblast, resulting in a continuous expansion of the latter [18]. In the first trimester of pregnancy, the villous cytotrophoblast is present as a complete cell layer below the syncytial layer. Thus, at that stage, the placental villi are covered by a two-layered trophoblast epithelium [18]. As pregnancy progresses, the cytotrophoblast becomes progressively sparser and these cells will soon fuse with the syncytiotrophoblast and become an integral part of the polarized multinucleated syncytial layer [18]. Figure 2 is a schematic representation of the human placental barrier in early and late pregnancy.

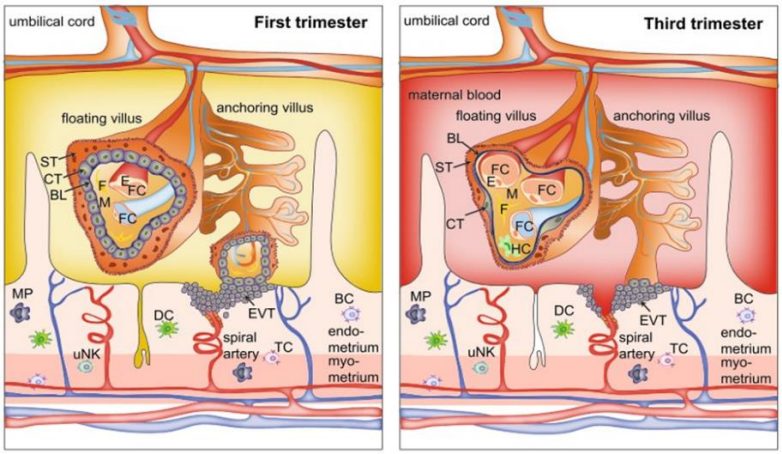

Figure 2. In the first trimester of human pregnancy, the villous trophoblast comprises two layers, a superficial layer of syncytiotrophoblast (ST) that rests in a second layer of cytotrophoblast (CT). Later in pregnancy, the cytotrophoblast becomes progressively sparser and the syncytiotrophoblast is mostly monolayered. Figure reproduced from Dugershaw et al.

In other words, throughout pregnancy, the placenta undergoes significant structural and functional changes to meet the growing needs of the developing fetus [8]. During early pregnancy, the placental barrier is relatively thick (20-30 µm) and bilayered, whereas towards the end of pregnancy the placental barrier is thinner (2-4 µm) and becomes predominantly monolayered [8].

The future of cytotrophoblastic cells in the differentiation pathways depends on several factors including the surrounding environment and regulators such as cytokines and hormones [19]. Furthermore, gap junctional intercellular communication also appears important in each differentiation pathway [19]. Gap junctions allow direct communication between adjacent cells [20]. In the animal kingdom, there are three families of gap junction proteins: the innexins, expressed only in protostomes, and the connexins and pannexins, expressed in deuterostomes [21]. Connexin gap junctions are formed when six connexin (Cx) subunits form a hemichannel (connexon) in the plasma membrane which can dock to another hemichannel in the plasma membrane of an adjacent cell, to assemble a complete gap junctional channel [20]. Gap junctions mediate cell-to-cell communication via the passive diffusion of small signaling molecules such as cAMP, cGMP, inositol triphosphate (IP3), and Ca^{2+}. In the first trimester of human pregnancy, the superficial layer of the syncytiotrophoblast rests on a second layer of cytotrophoblast. These two layers are interconnected by gap junctions formed by gap junction alpha-1-protein, also known as connexin 43 (Cx43) [19]. In humans, it appears that Cx43 allows gap junctional intercellular communication required for the fusion process of cytotrophoblastic cells leading to the formation of syncytiotrophoblast, the site of numerous placental functions [19].

BeWo cell line

BeWo b30 is a human trophoblast choriocarcinoma-derived cell line, which has been widely used to create a well-established *in vitro* model barrier [14]. This cell line retains cell properties and hormonal profiles of mononucleated cytotrophoblasts [22]. The b30 BeWo clone forms a confluent, polarized monolayer that provides a good *in vitro* model system which has been successfully used to study the transcellular distribution of many substances across the placental trophoblast, such as amino acids, immunoglobulins, hormones, fatty acids, transferrin and viruses [14], [17], [22]. Figure 3 shows a schematic representation of BeWo cells grown on a transwell insert.

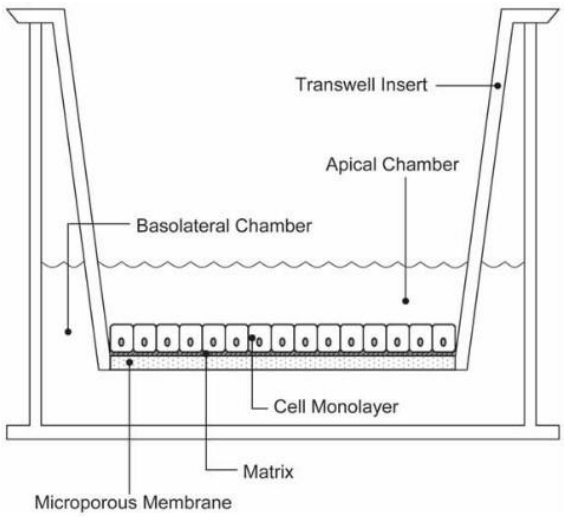

Figure 3. The BeWo cell line forms a confluent, monolayer trophoblast cell line. When grown on a transwell insert, the monolayer is polarized in the expression of apical and basolateral marker enzymes and transporters in a manner consistent with normal trophoblasts. Figure reproduced from Bode et al.

BeWo b30 cells are grown on a transwell insert to form a cell layer, separating an apical chamber, representing the maternal compartment, from a basolateral chamber, representing the fetal compartment [22]. When grown on transwell microporous inserts, the cells get polarized with respect to the expression of functional transporters and enzymes specific to the apical and basal membranes in a manner consistent with primary trophoblasts [17], [22].

The BeWo cell line is particularly attractive because it is stable, easy to maintain by passage and can grow to a confluent monolayer in a relatively short period of time [17], [23]. More importantly, BeWo cells demonstrate morphological and hormonal secretion properties common to typical trophoblasts [17], [23]. Human cytotrophoblasts have been successfully purified and cultured [23]. However, in primary culture, these cells spontaneously differentiate to a syncytiotrophoblast [23], whereas, BeWo cells are not capable of differentiating and they consist predominantly of undifferentiated cytotrophoblasts with a few syncytialized cells [17]. Undifferentiated BeWo cells are morphologically similar to primary cultures of trophoblasts, exhibiting close cell apposition and microvillar projections on the apical side of the monolayer [17].

In the studies to be discussed below, BeWo cells are used to form a confluent cell barrier on a transwell insert above a layer of human fibroblasts, to assess the cellular toxicity of nanoparticles when a fully confluent cellular barrier separates the nanoparticles from the fibroblasts, as an attempt to test the indirect effects of nanoparticle exposure.

CoCr nanoparticles can damage human fibroblast cells across an intact cellular barrier [14]

Bhabra *et al*. aimed to investigate whether a cellular barrier in the form of a confluent layer of BeWo cells can protect human fibroblasts from damage when indirectly exposed to surgical cobalt-chromium (CoCr) alloy particles [14].

Humans are exposed internally to CoCr nanoparticles by wear mechanisms associated with metal-on-metal orthopaedic joint replacements [14]. Total hip replacement has been proven to be cost-effective and highly efficient in relieving pain and disability, and its success has resulted in an increased use for the treatment of hip arthritis among young adults [24]. Metal-on-metal implants can generate 10^{12}-10^{14} CoCr nanoparticles sized between 20 and 60 nm per year [24]. Despite the use of metal

implants since the 1930s, there is still lack of knowledge of the biological responses to orthopaedic biomaterials [24]. When CoCr nanoparticles were applied directly to human fibroblasts in tissue culture above a certain concentration threshold, they had genotoxic and cytotoxic effects, causing DNA damage, chromosome aberrations and cell death [25]. However, there were no previous studies to evaluate the cellular toxicity of nanoparticles when a fully confluent cellular barrier separates them from human fibroblasts [14]. The authors used a multi-layered barrier, 3-to-4-cells in thickness, to ensure that they were studying the indirect effects of particle exposure, rather than the direct effects of particles that may have leaked through the barrier [14].

DNA damage of human fibroblasts

Human fibroblasts were placed below a confluent layer of BeWo cells grown on a transwell insert and were exposed to CoCr nanoparticles for 24 hours. Fibroblasts were exposed either through the BeWo cell barrier (indirect), through the insert without the BeWo barrier (insert) or directly without a BeWo barrier or insert (direct). A schematic of the indirect exposure setup is presented in Figure 4.

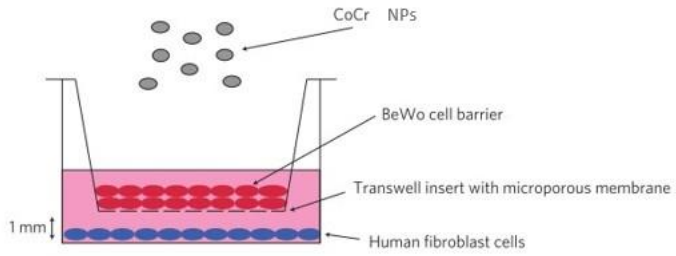

Figure 4. Human fibroblasts were placed below a confluent multi-layered BeWo barrier grown on a transwell insert with microporous membrane. Fibroblasts were exposed to CoCr nanoparticles indirectly through the BeWo barrier. Figure reproduced from Bhabra et al.

The level of damage on the human fibroblasts after indirect exposure was measured with the alkaline comet assay, which detects alkaline labile sites, single-strand and double-strand DNA breaks, and with γ-H2AX staining of the fibroblasts for measurement of double-strand DNA breaks. The results are presented in figure 5.

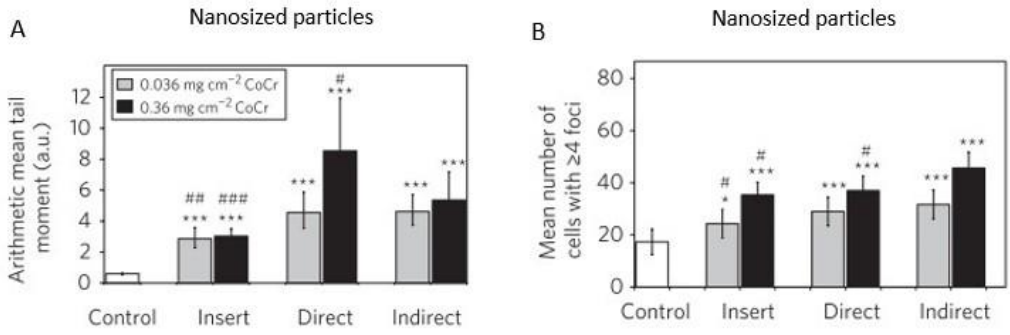

*Figure 5. Fibroblast DNA damage as recorded with the alkaline comet assay (A) and γ-H2AX staining (B). Indirect exposure of nanoparticles caused significant damage when compared with the control. Damage after indirect exposure is greater than that after exposure through insert. All values are means ± 95% CI. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 when compared with control. #P ≤ 0.05, ##P ≤ 0.01, ###P ≤ 0.001 when compared with indirect exposure. Figure reproduced from Bhabra et al.*

Measurement with the alkaline comet assay showed that the level of damage after indirect exposure was greater than that of fibroblasts with no CoCr exposure (control), and was similar to or less than that observed after direct exposure (Figure 5A). Measurement with γ-H2AX staining showed a similar or greater DNA damage of the fibroblasts after indirect exposure compared with direct exposure (Figure 5B). Moreover, fibroblast DNA damage in the presence of the BeWo layer was greater than that after exposure of the cells to CoCr nanoparticles placed behind the transwell insert in the absence of the cellular barrier (insert). This indicates that the BeWo barrier cells contributed to the process of DNA damage.

Behaviour of particles in the BeWo barrier

As mentioned earlier, when CoCr nanoparticles were added directly to cultured cells above certain concentrations, they had genotoxic and cytotoxic effects [25]. Here, the BeWo barriers are exposed to a low concentration of CoCr nanoparticles (0.04 mg/ml). The integrity of the barrier was checked by transepithelial electrical resistance (TEER) measurement and it was confirmed that in these concentrations of nanoparticles, the barrier remained intact.

Transmission electron microscopy (TEM) imaging revealed that the nanoparticles were located within the cells of the superficial layer of the 3-to-4 cell-thick barrier after 24 hours of exposure, as shown in Figure 6. This means that CoCr nanoparticles were internalized by the BeWo cells and did not cross the barrier.

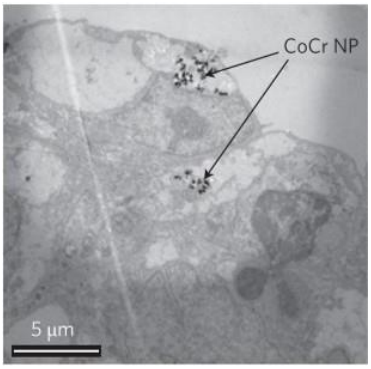

Figure 6. TEM image of BeWo cell barrier showing aggregates of CoCr nanoparticles internalized in the superficial layers of the barrier.

DNA damage was mediated by a novel proposed mechanism

It appears that the BeWo barrier plays an important role in the DNA damaging process. For this reason, the authors investigated the possibility that intercellular signalling pathways associated with the intact BeWo barrier may mediate the DNA damage. As mentioned earlier, gap junctions mediate intercellular communication that support the bidirectional transport of ions, small molecules and metabolites across cells. Hexameric unpaired hemichannels made of connexins or pannexins are further sources of intercellular signalling.

To test the role of connexin gap junctions or hemichannels on the intercellular signalling process, the authors first confirmed that BeWo cell expressed connexin 43 (Cx43). Then they used specific blockers of gap junctions/hemichannels, the connexin mimetic peptides, Gap 26 and Gap 27, to see the effects on the observed DNA damage. Applying gap junction blockers above or below the BeWo barrier during a low-dose nanoparticle exposure above the barrier prevented DNA damage in the fibroblasts, including double-strand DNA breaks. When they applied AAP10, an antiarrhythmic peptide which increases gap junction communication as well as Cx43 expression and ATP release, there was an increased DNA damage in the fibroblasts.

Then, they used similar experiments to determine if additional or complementary signalling pathways involving pannexin channels were operational in the BeWo cellular barrier. First, they confirmed that BeWo cells expressed pannexin receptors (P2X) and pannexin channel 1 (PNX1). Addition of the pannexin channel blocker, Panx1 or a pannexin receptor antagonist to the fibroblast-containing medium during indirect exposure to CoCr nanoparticles, resulted in a decrease in DNA damage.

In order to further elucidate the mechanism of the nanoparticle-induced DNA damage, they investigated the role of ATP in the damaging signalling. Because ATP is known to pass through gap junctions and hemichannels, they aimed to see if ATP acts as a signalling molecule from any connexin or pannexin channels in the BeWo barrier and contributes to DNA damage. By placing ATP below the BeWo cell barrier, there was induction of the DNA damage in the fibroblasts. To confirm the role of ATP in the DNA damage, they added molecules which inhibit the purinergic transmission below the BeWo barrier. Addition of apyrase (an enzyme hydrolysing ATP), PPADS (which blocks the P2 purinergic receptor), NBMPR (inhibiting ATP uptake or release), or allopurinol (which inhibits xanthine oxidase), significantly decreased the DNA damage in the fibroblasts. Also, DNA damage induced by placing ATP below the barrier, was reduced by Panx1, or the P2X7 antagonist.

As ATP seemed to contribute to the DNA damage, and knowing that purinergic transmission can lead to calcium wave propagation, they performed calcium assays after ATP addition to BeWo cells, to further explore the signalling cascade. They found a strong ATP-dependent spike in calcium concentration. When ATP was added in the presence of a P2Y receptor blocker, Ca^{2+} flux was reduced, suggesting that the ATP induced Ca^{2+} flux was P2Y receptor dependent. Furthermore, DNA damage after indirect exposure to nanoparticles was reduced by the presence of cyclosporine A or FK506, which bind to cyclophilin A and inhibit calcineurin, a critical component for several calcium-dependent signalling pathways.

Based on these results, the authors suggested a novel mechanism for the indirect toxicity of CoCr nanoparticles to fibroblast cells. The top layer of BeWo cells is damaged perhaps through nanoparticle damage to mitochondria or from hypoxic mimicking actions of cobalt ions. Both conditions may result in ATP release from the top layer which activates P2Y receptors of the second non-metal damaged layer or pass through connexin gap junctions and into the second layer of cells. In the second layer of the barrier, ATP (either directly or via P2Y receptor activation) can cause rise in intracellular calcium and a subsequent ATP secretion, again via connexin and pannexin hemichannels. This ATP then causes DNA damage to human fibroblasts beneath the barrier via P2 receptors on the fibroblasts. Figure 7 is a schematic representation of the described mechanism.

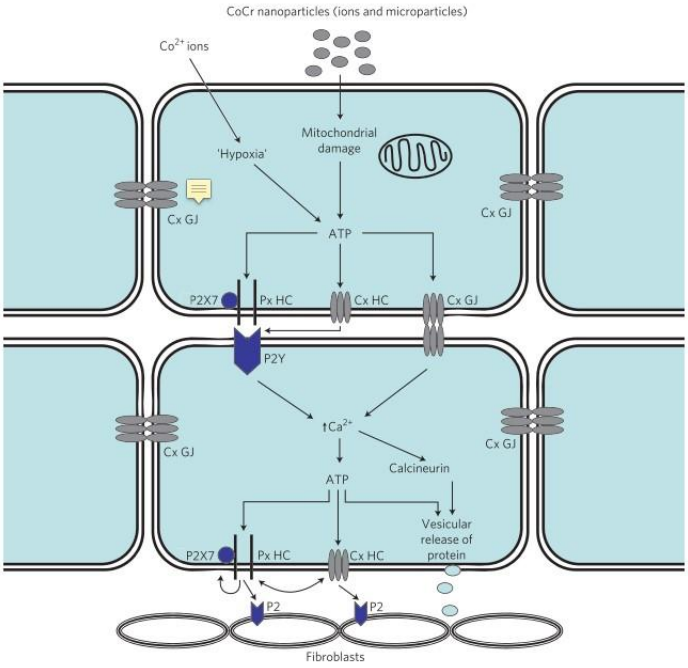

Figure 7. Schematic of the proposed mechanism of the indirect toxicity of CoCr nanoparticles to human fibroblasts across a BeWo cell barrier. Figure reproduced from Bhabra et al.

The indirect effects of nanoparticles depend on the barrier thickness [15]

So far, it has been shown that CoCr nanoparticles on one side of a multi-layered cellular barrier can cause DNA damage and chromosome aberrations on the other side, without crossing the barrier. Sood et al. aimed to investigate whether these indirect effects might vary for different types of barriers. By using *in vitro*, *ex vivo* and *in vivo* models, they showed that the indirect damage of nanoparticles depends on the barrier thickness.

Importance of barrier thickness

The importance of barrier thickness on the level of DNA damage in the underlying fibroblasts was tested by growing BeWo cells on transwell inserts for different periods. At 4 days growth, 90% of the barrier was monolayered, while at 7 days, more than 99% of the barrier was at least bilayered and 10-15% was multi-layered. The barriers were exposed to CoCr nanoparticles, the same way as described in Figure 4. The barriers are shown in Figure 8.

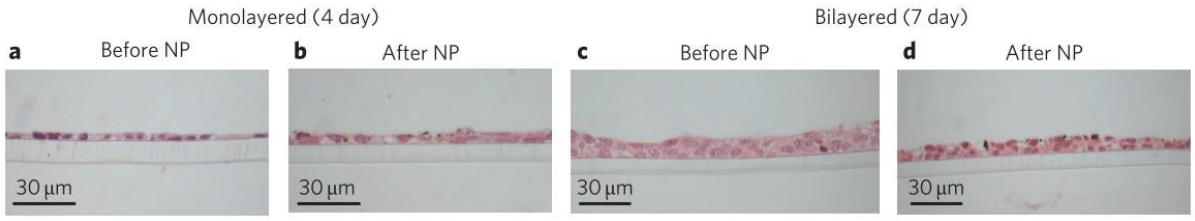

Figure 8. Light micrographs of predominantly monolayered (4-day growth) (a,b) and bilayered (7-day growth) (c,d) BeWo barrier before (a,b) and after (c,d) exposure to 0.04 mg/ml CoCr nanoparticles. Figure reproduced from Sood et al.

Despite the barrier being thinner, no DNA damage was observed in fibroblasts below 4-day BeWo barriers. However, in fibroblasts placed below 7-day BeWo barriers, DNA damage was evident. The integrity of the barrier was not affected by nanoparticle exposure.

The proposed mechanism by Bhabra *et al.*, where DNA damage results from signalling from the bilayered BeWo barrier to the fibroblasts, share some similarities with the radiation-induced

bystander effect [26]. Radiation or chemical exposure of an individual cell causes it to send a DNA-damaging signal to a neighbour cell via gap junctions [26]. In the radiation-induced bystander effect, mitochondria and free radical generation are important sources for the DNA-damaging signal. For that reason, Sood *et al*. wanted to investigate the role of mitochondria and free radicals in signalling across BeWo barrier. Nanoparticle exposure caused a short-term increase in free radicals within the BeWo cells. To test the role of free radicals in the DNA damage, they used the antioxidants vitamin C and MitoQ, and indeed these antioxidants were effective in preventing the DNA-damaging signalling to the fibroblasts. Thus, the authors suggested that nanoparticles can induce an increased barrier stress response characterized by impaired mitochondrial activity with ROS formation.

Complementary *ex vivo* and *in vivo* experiments

To test whether indirect signalling can occur across a placenta *in vivo*, pregnant mice were intravenously injected with CoCr nanoparticles at two stages of pregnancy, at 9.5 days and at 12.5 days, where the placenta is fully established with three layers. Observations were made 7 days after the exposure, to ensure that the outcomes were not a result of a direct damage to the placenta. No pathological changes were observed. However, after exposure at 12.5 days, there was an increase in DNA damage in neonatal blood and liver. The levels of Co and Cr had not increased in the neonates.

Signalling was also tested with *ex vivo* experiments, using human placental explants in the first trimester, when the trophoblast barrier is bilayered, and at term when the barrier is monolayered. The explants were maintained in culture medium and exposed to altered oxygen from 1% to 21% as this stimulus is found to cause the BeWo barriers to send DNA-damaging signals to fibroblasts. This method was chosen instead of exposing the explants to nanoparticles because in this case any transfer of culture medium could contain metal, which would then cause direct than indirect exposure by metal nanoparticles. When the media surrounding the first trimester explants was then transferred to fibroblasts, there was an increase in DNA damage in the fibroblasts. This was not the case for the third trimester explants.

Nanoparticle-induced neuronal toxicity across placental barriers [16]

Hawkins *et al*. attempted to further investigate the mechanism behind the indirect developmental toxicity of CoCr nanoparticles. The most common developmental abnormalities in humans are due to maternal exposure to toxins and involve neurodevelopment. It has been found that nanoparticles can cause neurodevelopmental toxicity to the fetus because of their transfer across the placenta. However, there is evidence that nanoparticles can cause intrauterine growth restriction without crossing the placenta [27]. In this study by Hawkins *et al*. they used *in vitro* and *in vivo* experiments and showed that nanoparticles induced neuronal toxicity across the BeWo placental barrier, which was mediated by autophagy and is dependent on astrocytes.

Nanoparticles can impair the autphagic flux in BeWo placental models

BeWo cells were grown on transwell inserts for 7 days to form a confluent barrier of 2-to-3 cells in thickness, at which point the CoCr nanoparticles were applied to the upper layer for 24 hours. TEER measurements showed that the barriers remained intact.

As shown in the previous experiments, exposure of the BeWo barrier to nanoparticles induces an increased barrier stress response characterized by impaired mitochondrial activity [15]. As mitochondrial inhibition due to oxidative stress can induce autophagy via p53, the authors proceeded to determine the levels of autophagic activity. Western blot analysis of stress-induced autophagy markers revealed increased protein expression for LC3-II and P62, which indicates formation of autophagosomes associated with a blockade of autophagosome clearance. Autophagosomes are membrane vesicles that enclose cellular components and fuse with lysosomes which digest these

components during autophagy. Immunocytochemistry analysis revealed that this phenomenon occurred predominantly in the upper layer of the barrier where nanoparticles accumulate after internalization. Therefore, it seemed that nanoparticle exposure results in an altered autophagic flux.

Astrocytic and neuronal DNA damage

To investigate the effect of DNA-damaging signalling on the differentiation of the neural cell lineage, media was conditioned under the BeWo barrier during nanoparticle exposure and then transferred onto differentiating neural progenitor cells (NPCs). These were derived from human fetal cortex and differentiated in culture to form neurons and astrocytes. Figure 9 shows a schematic representation of the experimental set up.

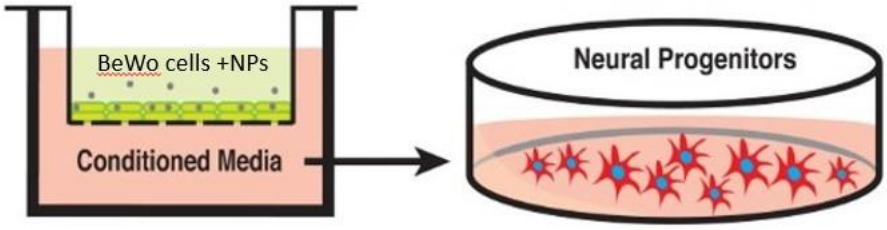

Figure 9. BeWo barrier-media transfer model with neural progenitor cells. Figure reproduced from Hawkins et al.

Serum was omitted from the nanoparticle media due to the possibility of its transfer to the basal chamber, as it is known to drive NPCs differentiation in a glial lineage. Results were compared to no barrier and no nanoparticle exposure controls, and barrier only without nanoparticle exposure controls.

Exposure of NPCs to conditioned media had no effect on cell survival. Immunostaining revealed glial cell differentiation and further immunostaining assays confirmed the astrocytic identity of these glial cells. Astrocytes underwent morphological changes following indirect exposure to nanoparticles, evidenced by increased nuclear and cytoplasmic areas. Neurons in culture with astrocytes showed no obvious morphological changes. MTS assay showed that the changes seen in astrocytes were not due to oxidative stress, so the authors looked for evidence of astrocytic DNA damage responses. γ-H2AX immunostaining revealed an increase in the numbers of γ-H2AX foci in the exposed astrocytes and neurons, with the effect being more pronounced in astrocytes than in neurons.

Neuronal DNA damage is mediated by astrocytes

The authors went on to determine whether the astrocytes might influence the observed toxic effects on neurons. Because astrocytes mediate neuroprotection by the production of glutathione (GSH), they measured glutathione levels in NPC culture following indirect exposure to nanoparticles. There was a large increase in GSH in NPCs differentiated in media underneath the BeWo barrier with nanoparticle exposure, compared to controls. However, when primary cultures that were characterized as > 99% neuronal were exposed, only low levels of γ-H2AX foci were detected in the neurons, with no difference compared to controls. When an additional stage of media conditioning was introduced, in which the media was first placed onto cultures of astrocytes derived from NPCs and then placed on primary neuronal cultures, increased levels of DNA damage were observed in the primary neurons. Therefore, the authors suggested that the formation of γ-H2AX foci in the primary neurons was dependent on the presence of the astrocytes in the culture.

IL-6 contributes to the neuronal toxicity

Further experiments were performed in order to establish the mechanism by which DNA-damaging signalling is communicated to astrocytes. From the previous studies, it was believed that the signalling was transmitted via ATP secretion from the barrier, followed by additional release of other factors [15]. The authors examined the release of several chemokines/cytokines by performing ELISA on media conditioned by the BeWo barrier and also from culture of astrocytes exposed to that media, before and after nanoparticle exposure. They found that IL-6 was secreted by the BeWo barrier, and its secretion was significantly increased after nanoparticle exposure, and remained elevated after culture of the astrocytes. In the nervous system, IL-6 is known to be a mediator of neuroinflammation with a possible role in astrocyte activation, but is also thought to have astrocyte neuroprotective effects in various central nervous system insults.

The role of IL-6 in the DNA damaging signalling was further investigated by shutting down IL-6 function in the BeWo barrier. They did that by transfecting the BeWo barrier with lentiviral vectors that expressed an IL-6 shRNA to knock out IL-6 gene expression, and by utilizing an IL-6 blocking antibody. γ-H2AX immunostaining revealed significantly reduced levels of DNA damage in astrocytes exposed to media from these barriers compared to barriers without IL-6 suppression, suggesting that IL-6 contributes to the neurotoxic effect.

The authors went on to further experiments to investigate the link between autophagy and DNA-damaging signalling, including the role of IL- 6. They transfected the BeWo barrier with a lentiviral vector that inhibits autophagy. After nanoparticle exposure, media was transferred onto differentiating NPCs and γ-H2AX immunostaining was performed on astrocytes in culture. They observed reduced DNA damage in astrocytes exposed to media from autophagy-impaired barriers, indicating that autophagy has a key role on the initiation of the DNA-damaging signalling. They also measured IL-6 levels in media derived from autophagy-impaired barriers exposed to nanoparticles. These barriers released less IL-6 than non-impaired autophagy barriers exposed to nanoparticles. These results suggested that nanoparticle exposure to BeWo barriers, causes impairment of autophagic flux and IL-6 release resulting in DNA damage in NPC-derived astrocytes.

Neurodevelopmental abnormalities after nanoparticle exposure

The authors also performed *in vivo* experiments, with nanoparticle exposure of pregnant mice, to evaluate the potential of nanoparticles to cause indirect developmental neurotoxicity. Mice were intravenously injected with CoCr nanoparticles at two stages of pregnancy, 9.5 days (E9.5) and 12.5 days (E12.5) when the placenta is fully established. In previous *in vivo* experiments by Sood *et al.*, it was shown that there was an increased DNA damage in the neonatal blood and liver, without increased levels of Co and Cr in the neonates. Here, Hawkins *et al*., analysed a number of astrocytic genes in whole-brain samples of the offspring that are upregulated in astrocytes in response to stress/injury.

GFAP (glial fibrillary acidic protein) expression, which is increased by astrocytes in response to neurological insults, was significantly increased in the E12.5 group, whereas no differences in GFAP expression were observed in the E9.5 group, highlighting the stage-specific effects of nanoparticle exposure during pregnancy. Immunohistochemical staining for GFAP showed a significant increase of this marker in the fetal hippocampus in the E12.5 group as well as elevated STEAP4 mRNA levels, a marker of astrocyte activation. Immunohistochemical staining of the hippocampus in the E12.5 neonates also revealed significantly increased γ-H2AX foci. In summary, the *in vivo* experiments revealed reactive astrogliosis and DNA damage in the neonatal hippocampus after maternal exposure to nanoparticles.

Discussion

In the study of Bhabra *et al*., the BeWo barrier, 3-to-4 cells in thickness was not intended as a replica of the human placenta. Instead, the authors aimed to create a multi-layered barrier to ensure that even tiny gaps in the barrier would be covered by the underlying cells. In this way, the indirect effects of nanoparticle exposure could be tested [14]. However, trans-barrier signalling resulting in DNA damage might be significant in human pregnancy. In the first trimester of human pregnancy, the villous trophoblast comprises two layers, a superficial layer of syncytiotrophoblast that rests on a second layer of cytotrophoblast, and these two layers are interconnected by Cx43 gap junctions. In this context, it is possible that indirect DNA damage mediated by Cx43 gap junctions in a multi-layered barrier might be relevant to the human placenta because it is in the first trimester the embryo is most vulnerable to teratogenic effects. This suggestion is further supported by the results of the study by Sood *et al*. where they found that indirect toxicity was only evident in bilayered/multi-layered barriers but not in monolayered barriers [15]. Since it is in the early pregnancy that the placental villi are bilayered, while later in pregnancy it becomes monolayered, we could suggest that the indirect toxicity observed in the multi-layered BeWo barrier may be relevant to human pregnancy, especially during the first trimester.

Hawkins *et al*. showed that CoCr nanoparticles can induce developmental neurotoxicity across BeWo cellular barriers via autophagic flux and astrocyte-mediated DNA damage. Considering the relevance to the placental barrier, this indirect neurotoxicity could have important implications for human pregnancy [16]. The placenta has an essential role in fetal brain development. During periods of stress, for example due to food deprivation, autophagy may be upregulated in the placenta to protect the fetal brain. However, dysfunction of the autophagic pathways is associated with developmental conditions such as intrauterine growth restriction and neonatal encephalopathy [28]. The role of astrocytes in disease is also of particular importance, as they can cause neurotoxicity and may be involved in neurodegenerative conditions. Therefore, exposure of the human placenta to nanoparticles may have detrimental effects on the embryo during neurodevelopment.

Although the results from these studies have significant implications for nanoparticle exposure during human pregnancy, they were based on the *in vitro* BeWo placental model, which raises questions about their predictive value. Studying the toxic effects of nanoparticles on the human placenta still remains a challenge. Although animal studies have provided some evidence of indirect developmental toxicity of nanoparticles, translation to human pregnancy should be done with caution, as the placenta is a species-specific organ. Advanced *in vitro* models are considered the cornerstone for mechanistic studies on the indirect toxicity pathways of nanoparticles. Interconnection of multiple *in vitro* models such as the placenta, maternal tissues and the embryo, either directly in co-cultures [14], [15] or indirectly via the transfer of conditioned media [16] seem to be very promising.

However, the combination of different models often requires compromises and modifications of the cultivation conditions (e.g. culture medium, cultivation time), and, therefore, their predictive value should be carefully validated [8]. For example, the use of serum-free media is required for cultivation of neural precursor cells to prevent differentiation, which could affect the protein corona of nanomaterials [8]. In a biological environment, nanoparticles will immediately acquire a corona of various biomolecules on their surface, which impacts their biological behaviour [29]. For a better approximation of the *in vivo* situation, future studies should use more complex, organotypic placental models with pre-treatment of nanomaterials with human plasma to obtain a relevant protein corona [11]. Also, the use of BeWo cells as an *in vitro* placental model has some limitations. Choriocarcinoma trophoblast cells continue to proliferate in tissue culture, unlike primary trophoblast cells [13]. Although these trophoblastic cells share similar characteristics with primary trophoblast cells, they

also differ in several aspects due to their malignant transformation [13]. Additionally, in the *in vivo* environment, trophoblasts exist in close proximity to various cell types, whereas this is not the case in the BeWo cells, so the potential influence of these other cell types is not taken into account.

The increasing use of nanotechnology raises concerns about the potential toxic effects of nanomaterials on humans, especially for vulnerable populations such as the unborn child. Research on the potential toxic effects of nanoparticles is a relevant and important issue, particularly with regard to indirect effects, for which information is still scarce. New advanced models of the human placenta are necessary to reveal the mechanisms behind the indirect developmental toxicity of nanoparticles. A new promising approach of a 3D-vascularized human placental barrier model was developed for the first time by Nishiguchi et al. This model can be conveniently divided into segments using several transwells, allowing the study of cross-talk between different cell types and can be used to elucidate the mechanisms of cellular signalling across barriers [13]. However, this approach is still in its early stages and the experimental setup is more complex and expensive than other *in vitro* models.

Conclusion

In bilayered BeWo *in vitro* models of the placental barrier it has been shown that CoCr nanoparticles can cause damage to DNA and chromosomes without crossing the barrier. The damage is mediated by a novel mechanism involving pannexin and connexin hemichannels and gap junctions and purinergic signalling. This indirect toxicity only occurs in bilayered/multi-layered barriers and not in single layered barriers. The signalling cascade triggered by nanoparticles causes DNA damage in differentiating neural progenitor cells. The initiation of this signal is mediated by autophagy, whereas the resulting DNA damage in neurons and astrocytes that may be present is dependent on astrocytes. Exposure of the human placenta to nanoparticles could have detrimental effects on the embryo, especially in early pregnancy where the placental villi are bilayered.

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