RESEARCH ARTICLE

Teratogenic potential of nanoencapsulated vitamin A evaluated on an alternative model organism, the tunicate Ciona intestinalis

Roberta Pennati, Raoul Manenti, Antonella Stillitano, Gentile Francesco Ficetola, Giorgio Scarib, Silvia Mercurio and Elena Menegola

Department of Environmental Science and Policy, University of Milan, Milan, Italy; Department of Biosciences, University of Milan, Milan, Italy

ABSTRACT

Nano-encapsulation is a technology used to pack substances in order to enhance their stability and bioavailability, but this packing may interact with living systems, causing unexpected toxicity. Vitamin A (vit A) is a substance that has received attention, because in developed countries, the increasing availability of supplements is leading to its excessive intake. This study aims to compare teratogenic effects caused by exposure to the traditional formulation of vit A versus nano-encapsulated vit A. We used ascidian embryos as an alternative model. Ascidians are marine organisms closely related to vertebrates that share with them a body plan and developmental programme, including the morphogenetic role of retinoic acid (RA). Our data showed that the adverse effects of exposure to the same concentration of the two formulations were different, suggesting that the nano-encapsulation increased the bioavailability of the molecule, which could be better absorbed and metabolised to RA, the effective teratogenic substance.

INTRODUCTION

Nano-encapsulation is a novel technology used to pack substances in miniature. This technology is generally used to deliver various nutraceutical products and bioactive molecules, such as vitamins and antioxidants, enabling the production of functional foods with enhanced functionality and stability.

Lipid-based nano-encapsulation systems enhance the performance of lipophilic molecules by improving their solubility. In these systems, a lipophilic core is surrounded by an amphiphilic shell made of a surface-active material that enhances solubility in aqueous media. Thus, nano-encapsulation can provide significant savings to formulators, as it enables the amount of active ingredients to be reduced, thereby increasing their bioavailability (Astete et al. 2009).

Moreover, several studies showed that the bioavailability of highly lipophilic substances encapsulated within lipid droplets increases as the droplet size decreases (Acosta 2009). It has been proposed that the larger surface area of small droplets could enable quicker digestion, leading to easier content release and absorption. In addition, small droplets have longer residence time in the small intestine, and they can be directly transported across the epithelium by paracellular or transcellular mechanisms (McClements and Rao 2011).

In addition, it is well-known that nanoparticles furnish new chemical and physical properties, different from normal bulk formulation, and they may interact with living systems, causing unexpected toxicity (Das et al. 2009). Indeed, there has been growing concern regarding the increased utilisation of nanoparticles in foods and beverages because of potential toxic effects (Hagens et al. 2007; Chaudhry et al. 2008; Bouwmeester et al. 2009; Souto et al. 2009). Reducing the dimensions of a material to the nanometre scale may modify its biological fate within human body, including its absorption, distribution, metabolism and excretion, thereby altering its potential for promoting toxicity (Hagens et al. 2007; Bouwmeester et al. 2009).

Since nanomaterials are essentially different from their corresponding bulk formulations, the European Food Safety Agency (EFSA) and the Food and Drug Administration (FDA) have recommended careful evaluation and monitoring of nano-formulated molecules, since they can potentially cause risks to human health and the environment (EFSA 2011).
In this regard, vitamin A (vit A) has received considerable attention, because in developed countries, the increasing availability of supplements is leading to excessive vit A intake (Penniston and Tanumihardjo 2006), a growing problem when considering the diffusion of nano-formulations.

Vitamin A (retinol) is primarily obtained from animal food. Once retinol has been taken up by a cell, it can be oxidised to retinal (retinaldehyde) by retinol dehydrogenases, and retinaldehyde can later be oxidised to retinoic acid (RA) by aldehyde dehydrogenases (ALDHs).

RA is a morphogen known to play key roles during the embryonic development of both vertebrate and invertebrate chordates (Canestro et al. 2006). RA levels are finely tuned in embryos by a precise balance between ALDH synthetic activity and the catalytic activity of CYP26, a cytochrome P450 enzyme. Embryos deprived of RA showed defects in multiple organs, including eyes, heart, lungs and genital tract, whereas early exposure to exogenous RA primarily caused defects in the anterior–posterior (AP) patterning of their neural tubes (Shimeld 1996). Indeed, it has been demonstrated that in chordates, RA regulates the patterning of the antero-posterior embryo body axis by controlling the expression of several HOX genes (Shimeld 1996).

Ascidians are chordates belonging to the Tunicate taxon, which is considered the sister group of vertebrates (Delsuc et al. 2006). Adult ascidians are filter feeding sessile marine organisms; they develop through a swimming larva that retains the ancestral chordate body plan formed by a trunk and a locomotory tail, where the notochord is located. A dorsal hollow neural tube constitutes the central nervous system; on the basis of gene expression patterns, it can be subdivided into different regions homologous to the forebrain, hindbrain and posterior neural tube of vertebrates (Passamaneck and Di Gregorio 2005). Thus, ascidians, conjugating the handiness of an invertebrate with the body plan of a vertebrate, could represent an alternative model for investigating the toxicological potential of nanomolecules.

Exposure of ascidian gastrulae to exogenous RA causes a typical and highly reproducible phenotype characterised by round trunk (De Bernardi et al. 1994; Nagatomo et al. 2003). In the ascidian Ciona intestinalis, it has been demonstrated that exogenous RA upregulates Ci-Cyp26 expression and slightly downregulates Ci-Aldh2 expression in the embryo (Nagatomo and Fujiwara 2003). Moreover, RA strongly enhances Hox-1 expression in both C. intestinalis and Halocynthia roretzi (Katsuyama et al. 1995; Nagatomo and Fujiwara 2003).

In this work, we exposed ascidian embryos to nanoencapsulated and bulk vit A in order to compare the effects induced by the two formulations. We assumed that differences in the effects observed after treatment at the same concentration could be attributed to the augmented bioavailability of the nanoformulation, thereby enabling an indirect assessment of its toxicity to be obtained.

**Materials and methods**

**Animals**

Adults of Ciona intestinalis were obtained by the fishery service of the Station Zoologique de Roscoff (Roscoff, France) and were maintained in aquaria filled with artificial seawater (Instant Ocean; salinity approximately 32\%o) and provided with an internal circulation system, as well as mechanical, chemical and biological filters. Constant illumination was preferred to prevent spontaneous release of gametes. Sperm and eggs were obtained surgically from animal gon ducts and used for *in vitro* fertilisation in artificial seawater (ASWH: 122.75 g NaCl, 3.35 g KCl; 7.35 g CaCl2 2H2O; 22.40 g MgCl2 6H2O; 31.45 g MgSO4 7H2O; 0.90 g NaHCO3; 25 ml 1 M Hepes in 1 l of H2O) in order to obtain synchronously dividing embryos.

**Treatments**

Embryos at gastrula stage (4.9 hours post-fertilisation (hpf); Hotta et al. 2007) were exposed to different concentrations of retinol palmitate, the esterified and biologically active form of vit A (Sigma, Igea Marina, Italy), bulk vit A, nanoencapsulated retinol palmitate (Aquanova VR Novasol GmbH, Darmstadt, Germany) or nano vit A.

The tested concentrations for both formulations were 1.1 μg/ml (2 IU/ml), 5.5 μg/ml (10 IU/ml) and 11 μg/ml (20 IU/ml). Concentrations were chosen on the basis of previously reported effective concentrations of retinol on ascidian embryos (Groppelli et al. 2001).

Nano vit A is supplied as a 10% emulsion of nanoliposomes, formed by a shell of lipids from soya lecithin and a core of retinol palmitate. A stock solution of nano vit A was prepared by diluting 11 μl of emulsion in 20 ml of ASWH and heating at 37°C for 15 min with gentle rocking. Working solutions were
prepared by diluting either 0.8, 4 or 8 ml of stock solution to a final volume of 40 ml in ASWH.

A 1M stock solution of bulk vit A corresponds to a concentration of 10.876 IU/ml. Working solutions were prepared by diluting either 736, 368 or 73.6 μl of stock solution to a final volume of 40 ml in ASWH.

_Ciona intestinalis_ embryos at gastrula stage were treated in glass Petri dishes (4-cm diameter) containing 40 ml of working solution or 40 ml of ASWH as control. An independent experiment was performed to test the effects of the nanoliposomes by exposing embryos to empty shells of soya lecithin (Aquanova® Novasol® GmbH, Darmstadt, Germany). Each treatment was repeated three times on different batches. At least 50 embryos were exposed for each treatment. All treatments were performed at 18°C in a thermostatic room. Embryos were enabled to develop until they reached the hatching larva stage (17.5 hpf; Hotta et al. 2007) and later fixed in 4% paraformaldehyde for 1 h for subsequent analysis.

**Morphological and statistical analysis**

Control and treated larvae were mounted on glass for microscopic observations. Dead embryos and severely affected ones were counted. For the residual samples, the incidence of malformations at adhesive papillae, pigmented organs, trunk and tail was scored. We used generalised linear models to assess the impact of treatment and dose on larval mortality and on the frequency of malformed individuals. Subsequently, we repeated the analysis of the frequency of malformations on different portions of the body: adhesive papillae, pigmented organs, tail and trunk.

In all models, we used an quasi-binomial error distribution to take into account over-dispersion, and we calculated significance using an F test (Maindonald and Braun 2010). All analyses were performed using the R statistical environment (Maindonald and Braun 2010).

A chi-square test was performed to assess differences between controls and embryos exposed to empty nanoliposomes. A p value ≤ 0.05 was considered to be significant.

**Immunostaining**

Larvae exposed to 10 or 20 IU/l of bulk or nano vit A were processed for immunostaining of nervous fibres with a monoclonal anti-β tubulin antibody (Sigma, Igea Marina, Italy, clone 2-28-33).

Control and treated larvae were fixed in 4% PFA in 0.1 M phosphate buffer saline (PBS) at room temperature for 2 h. All of the following steps were performed with gentle rocking. Specimens were permeabilised with 0.1% Tween-20 and 0.25% Triton X-100 in PBS for 1 h, washed three times in PBS for 10 min each, and incubated for 2 h in 50% PBS/50% normal goat serum. Next, the samples were incubated overnight at 4°C with primary antibody diluted 1:400. After several washes in PBS, the samples were incubated in 1% bovine serum albumin (BSA) in PBS for 2 h at room temperature and incubated at 4°C overnight in goat anti-mouse AlexaFluor 488 diluted 1:800 in PBS. After several washes in PBS, the samples were mounted in 1,4-diazabicyclo[2,2,2]octane (DABCO, Sigma, Igea Marina, Italy) on microscope slides and examined using a Leica TCS NT confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany) equipped with an argon/krypton laser, 75 mW multiline. Series of “optical sections” attained by scanning whole-mount specimens were projected into one image with greater focal depth.

**In situ hybridisation**

Control and treated larvae were fixed in fresh 4% paraformaldehyde with 0.5 M NaCl and 0.1 M MOPS at pH 7.5 and room temperature for 90 min. Next, the samples were washed twice with PBT (0.1% Tween–10% in PBS) and digested with 2 μg/ml Proteinase K for 30 min at 37°C. Next, the samples were post-fixed in 4% paraformaldehyde in PBS for 1 h, washed twice in PBT and then three times, 10 min each, with 0.25% acetic anhydride in 0.1 M triethanolamine. The samples were then incubated in hybridisation solution (50% formamide, 5 x SSC, 50 μg/ml tRNA, 5 x Denhardt’s solution, 0.1% Tween–10%, 50 μg/ml heparin) for 1 h. The hybridisation was carried out overnight at 50°C with 0.3–0.6 ng/μl Dig-labelled probe. _Ci-Otx_ and _Ci-Hox-1_ dig-labelled antisense RNA was obtained from linearised plasmids using the dig-labelling kit (Roche, Segrate, Italy) according to the indications. On the second day, samples were washed with a descending series of SSC buffers in 50% formamide and 0.1% Tween–10% then incubated overnight at 4°C in a dilution (1:2000) of alkaline phosphatase-conjugated anti-DIG-antibody in blocking buffer containing 0.5% blocking reagent and 5% normal sheep serum. On the third day, the samples were washed several times in PBT and were rinsed with AP buffer containing NBT/BCIP substrates. When the colour reaction developed, larvae were washed in PBT, mounted on glass slides with
80% glycerol and observed using an optical microscope.

Results

Morphological analysis

Control larvae of Ciona intestinalis showed a normal phenotype characterised by an elongated trunk, pigmented organs correctly located in the sensory vesicle, three anterior palps well differentiated (Figure 1(A,B)). Larvae exposed to low concentrations (2 IU/ml) of nano and bulk vit A showed a conserved morphology even if in some rare cases their palps were not elongated (Figure 1(C,F)). Larvae exposed to the higher concentrations of nano or bulk vit A (10 IU/ml) showed recurrent malformations: the palps were not correctly developed or were absent; the pigmented organs were fused and dislocated on the upper side of the sensory vesicle; and the trunk was roundish (Figure 1(D,G)). Larvae exposed to the highest tested concentration (20 IU/ml) of bulk vit A showed the same altered phenotypes (Figure 1(E)). However, exposure to 20 IU/ml of nano vit A caused a prevalent more severe phenotype characterised by a short trunk, short and curled tail, and absence of palps (Figure 1(H)). Larvae exposed to the empty shell of soya lecithin were similar to control ones reared in ASWH (data not shown).

Incidence of malformations

Larvae developed from embryos exposed to nano or bulk vit A were scored to obtain the proportion of undeveloped, healthy and malformed specimens. The resulting data (see Supplementary materials A and B) were statistically analysed to evaluate the effects of the treatment, the dose and the interaction of dose and treatment. The percentage of undeveloped larvae was not influenced by the treatment ($F_{1,14} = 0.52; p = .48$), the dose ($F_{2,14} = 1.01; p = .38$) or the interaction between dose and treatment ($F_{2,14} = 1.26; p = .31$). These results demonstrate that the two tested formulations of vit A have no toxic effects on ascidian embryos and larvae.

However, statistical analysis showed a significant effect of dose ($F_{2,14} = 15.05; p = .002$) and of treatment ($F_{1,14} = 10.77; p = .005$) on the incidence of malformed specimens but not of the interaction between dose and treatment ($F_{2,14} = 1.26; p = .31$). The dose-dependent incidence of malformations with both the formulations confirms that the teratogenic properties of vit A are maintained following nano-encapsulation. The most striking result is the significant difference between the two different formulations in impacting the incidence of malformations, indicating that at the same dose, the number of malformed specimens is higher after exposure to the nano-encapsulated vit A than to bulk vit A. Figure 2(A) shows that exposure to 20 IU/ml nano vit A causes more than 50% malformed larvae (53.4%), whereas the proportion of malformed larvae caused by the same dose of bulk vit A is 11%.

Larvae exposed to the empty shell of soya lecithin showed an incidence of malformation that was very low and not significantly different from that of controls reared in ASWH (chi-square test: $p = .3105$; see Supplementary materials C). To better characterise the malformations that were induced, we identified four target organs and scored the exposed larvae according to the presence of anomalies in these structures. From

![Figure 1](image-url)
anterior to posterior, these targets are adhesive papillae, pigmented organs, trunk and tail. The data are reported in Table 1 and graphs are presented in Figure 2(B–E).

Table 1. Statistical analysis to evaluate the effects of the dose, the treatment and the interaction of dose and treatment on the frequency of malformations in different body portions.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Treatment</th>
<th>Concentration × treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>d.f.</td>
</tr>
<tr>
<td>Adhesive papillae</td>
<td>11.08</td>
<td>2,14</td>
</tr>
<tr>
<td>Pigmented organs</td>
<td>8.43</td>
<td>2,14</td>
</tr>
<tr>
<td>Trunk</td>
<td>9.12</td>
<td>2,14</td>
</tr>
<tr>
<td>Tail</td>
<td>1.14</td>
<td>2,14</td>
</tr>
</tbody>
</table>

F: degree of freedom (d.f.) and p value are reported. Significant effects are in bold.

For the incidence of malformation to the adhesive papillae, there was a significant effect of the dose but not of the treatment, which means that both the formulations of vit A have a similar dose-dependent effect on the papillae, the anterior-most organs. The incidence of malformations to the pigmented organs was significantly affected by the dose and by the interaction between dose and treatment; in other words, the differences between the two formulations are evident only at the highest tested doses. For trunk malformations, there were significant effects of concentration and treatment. The interaction between concentration and treatment suggests that the differences between the two formulations are wider at the highest concentrations. Finally, there were no significant differences in the incidence of malformations of the tail, the more posterior organ of the larva.

**Immunostaining**

To better characterise the induced malformations, we performed immunostaining of the nervous system fibres of larvae treated with the highest concentrations of nano and bulk vit A using an anti-β-tubulin monoclonal antibody. In control larvae, the fibres of the central and peripheral nervous system were well-marked; in particular, the papillary neurons and papillary nerves that run from the adhesive papillae to the posterior sensory vesicle were clearly recognisable (Figure 3(A)). In the sensory vesicle, several fibres occurred in correspondence with the pigmented sensory organs, the otolith and the ocellus, as clearly visible in an image obtained by the superimposition of confocal and transmission microscopy images (Figure 3(B)). From the posterior part of the sensory vesicle, the neural tube fibres run posteriorly into the dorsal tail (Figure 3(A)). The larvae exposed to 20IU/ml bulk vit A showed papillary nerves and neural fibres around the pigmented organs that were displaced more dorsally (Figure 3(C)) compared to control larvae (Figure 3(A)); moreover, the point of insertion of

Figure 2. Graphs indicating the incidence of severely malformed larvae (A) and the incidence of target organ malformations (B–E) after exposure to different concentrations of bulk or nano vit A. The values are in percentage and the standard deviation is indicated.
the sensory papillary nerves into the sensory vesicle was not clearly detectable. Fibres of the posterior neural tube were normally developed (Figure 3(C,D)). Nervous fibres in the trunk of larvae exposed to 20 IU/ml nano vit A were compromised severely. In particular, the papillary nerves showed an abnormal pathway and were shorter than those of control larvae. Moreover, the fibres around the pigmented organs were not detectable, and the sensory vesicle was shifted anteriorly (Figure 3(E,F)).

**In situ hybridisation**

Defects to the sensory vesicle in exposed larvae were further evidenced by in situ hybridisation experiments with a probe for *Ci-Otx*. In *Ciona intestinalis* larvae, *Ci-Otx* is expressed exclusively in the sensory vesicle (Wada and Satoh 2001) (Figure 4(A,B)). In 20 IU/ml bulk vit A larvae, the region of expression of *Ci-Otx* was reduced indicating a reduction of the overall dimensions of the sensory vesicle (Figure 4(C,D)). In larvae exposed to 20 IU/ml nano vit A, the sensory vesicle was slightly reduced compared to larvae exposed to the bulk formulation, and it was displaced dorsally (Figure 4(E,F)).

To verify that the observed malformations were due to a specific effect of vit A, we analysed the expression of *Ci-Hox-1* in control and exposed larvae. *Ci-Hox-1* expression is greatly enhanced in *Ciona intestinalis* larvae by exposure to RA, the active metabolite of vit A (Nagatomo and Fujiwara 2003). In control larvae, *Ci-Hox-1* was expressed in the endoderm of the trunk, in the neural tube at the visceral ganglion level and in the first tract of the posterior nerve cord (Figure 4(G)). In larvae exposed to 20 IU/ml bulk vit A, the limit of expression of *Ci-Hox-1* in the neural tube was shifted posteriorly (Figure 4(H)). This shift was more marked in larvae exposed to the nano-encapsulated
formulation of vit A (Figure 4(I)). These results suggested that the observed effects were attributable to the action of vit A which, upon its absorption, is metabolised to RA.

**Discussion**

In this paper, we analysed the effects of vit A exposure on the development of *C. intestinalis* embryos. Moreover, we compared the effects induced by two different formulations of this molecule, bulk and nano-encapsulated, to evaluate if the latter can increase vitamin bio-availability.

Morphological analysis of exposed larvae indicated that vit A caused malformations similar to those observed after RA treatment principally affecting the adhesive papillae, trunk and pigmented organs (Katsuyama et al. 1995; Nagatomo et al. 2003). Retinoic acid is the active metabolite of vit A and is known to be a morphogen implicated in a wide range of biological processes during differentiation and morphogenesis, including patterning of the antero-posterior body axis (Maden and Holder 1992).

Generally, exposure of ascidian embryos to RA results in a typical larval phenotype characterised by round trunk, reduced adhesive papillae, fusion of pigmented organs and extrusion of the sensory vesicle (Nagatomo et al. 2003).

In our experiments, we obtained the same phenotype, and it was particularly accentuated in the samples exposed to the nanoformulation. Moreover, the incidence of malformations caused by exposure to vit

---

**Figure 4. In situ hybridisation. (A–F) *Ci-Otx* expression. (A, B) Control larva. (C, D) Larva exposed to 20 IU/ml bulk vitamin A. (E, F) Larva exposed to 20 IU/ml nano vitamin A. (G–I) Expression of *Ci-Hox-1*. The arrow indicates the posterior limit of expression. (G) Control larva. (H) Larva exposed to 20 IU/ml bulk vitamin A. (I) Larva exposed to 20 IU/ml nano vitamin A. Scale bar: 100 μm.**
A was dose- and formulation dependent. At low concentrations, neither formulation caused a significant increase in malformations compared to controls. The no observed effect level (NOEL) is 2 IU/ml for both bulk and nanoformulations of vit A for C. intestinalis embryos. Whereas higher concentrations of nano-encapsulated vit A induced a significant increment of severely malformed larvae compared to the same concentration of the bulk form. We excluded the possibility that nanoliposomes are responsible for the observed malformations, since treatment with empty shells did not cause any significant effect.

In addition, the severity of the induced malformations was dependent on the formulation. Immunolabelling of nervous fibres revealed that the anterior papillary nerves and the nervous fibres around the pigmented organs were more severely affected after exposure to the highest concentration of nano vit A than after exposure to the same concentration of the bulk form. The sensory vesicle, as shown by in situ hybridisation with a Ci-Otx probe, was reduced and displaced dorsally following the exposure to nano vit A. This is a typical alteration caused by RA. The effects of exposure to the same concentration of the bulk form were less evident.

Considering all these aspects, our results suggested that the nano-encapsulation increased the bioavailability of the molecule, which could be better absorbed and metabolised to RA. This observation is strengthened by the analysis of expression of Ci-Hox-1 in control and exposed larvae. In Ciona intestinalis larvae, Ci-Hox-1 is expressed with a sharp posterior limit in the nerve cord (Ikuta and Saiga 2005) and is inducible by exogenous RA (Nagatomo and Fujiwara 2003). The increased expression of this gene in vit A exposed larvae indicated that in these larvae the levels of RA were augmented following an increment of the supply of its precursor. These data suggested that high concentrations of vit A are teratogenic for ascidian larvae, probably by inducing an increment of endogenous RA synthesis. Even though this phenomenon seems to be the more plausible action of vit A, it cannot be excluded that other mechanisms could be present, and the exact pathway of the teratogenicity of this molecule needs to be confirmed by further studies.

In any case, our data confirm that nano-encapsulated vit A differs from the bulk formulation in its effects at the same dose and can potentially be a risk to human health. In the last decade, considerable attention has been paid to developing delivery systems that increase the bioavailability of approved food grade nutrients and bioactive substances in order to produce functional foods or fortified foods requested by consumers. Food products are normally fortified with health-promoting and disease-preventing molecules, such as phytochemicals, vitamins, minerals and oils (omega 3 fatty acids) (Aditya et al. 2017).

In developed nations, due to fortified food, observational studies suggest that more than 75% of people may be routinely ingesting more than the recommended dietary allowance (RDA) of vit A (Allen and Haskell 2002). The use of nanoencapsulated vit A can increase the risk of hyper-vitaminosis in these situations. Thus, our results suggest that it is necessary to carefully monitor beverage and food supplementation with nano vit A and reconsider the RDA in light of its augmented bioavailability in accordance with recommendations of the EFSA and the FDA.

Acknowledgements

The authors want to thank the people of “IBIS: imaging bioscience support” for the use of the confocal microscope.

Disclosure statement

The authors report no conflicts of interest.

References


