Teratogenic Potential of Traditionally Formulated and Nano-Encapsulated Vitamin A in Two Vertebrate Models, *Rattus norvegicus* and *Xenopus Laevis*

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Abstract

Nano-encapsulation is applied for the preparation of functional food to preserve micronutrients degradation and to ameliorate their absorption. Being nano-encapsulation already related to increased vitamin A embryotoxicity, we aimed to evaluate the effect of traditionally formulated (BULK-A) and nano-encapsulated vitamin A (NANO-A) in two different vertebrate models: rat post implantation Whole Embryo Culture (WEC) and Frog Embryo Teratogenesis Assay-Xenopus (FETAX). After benchmark-dose modelling, WEC results showed that NANO-A was 7 times more effective than BULK-A, while FETAX results indicated that *X. laevis* development was affected only by NANO-A. The relative potency of WEC was 14 times the potency of FETAX, suggesting a minor role of preformed vitamin A in *X. laevis* development in respect to mammal embryogenesis. Results from this work prompt the necessity to monitor the use of food supplemented with NANO A, since even low doses can elicit teratogenic effects on vertebrate embryos due to its increased bioavailability.

Keywords: Functional food; Retinol; WEC; FETAX; Alternative models

Introduction

Over the past few decades, the evolution of a number of new technologies has revolutionized the food sector. Among these, nanotechnology is an advanced interdisciplinary scientific field that involves the manufacture, processing and application of materials with size in the nanometer range[1]. Nano-encapsulation is the coating of various substances within another material at sizes on the nano scale. The encapsulation material is known as the external phase, the shell, coating or membrane. The shell ensures higher availability than the core components itself, that usually are easily degraded or difficultly transferred across the biological membranes[2]. Applications for nano-encapsulation in food industry (nutraceutical production and human or veterinary food fortification) have been increasing because of the many advantages that these technologies can confer to the encapsulated material. It is generally used to deliver different nutraceutical products and bioactive molecules such as vitamins and antioxidants, allowing production of functional foods with enhanced functionality and stability by protecting the encapsulated materials from environmental, enzymatic and chemical changes, as well as ameliorate their organoleptic properties[3]. Lipid-based nano-encapsulation systems enhance the performance of lipophilic molecules by improving their solubility. In these systems, the lipophilic substance (core) is surrounded by an amphiphilic shell (coat) made of surface-active material that enhances solubility in aqueous media[4]. Nano-encapsulation provides significant savings to formulators, as it allows reducing the amount of active ingredients, increasing their bioavailability and shelf-life[5]. In addition, it has been proposed that the larger surface area of small lipid droplets...
facilitates the crossing of biological barriers and allow the encapsulated lipophilic substances to quickly and efficiently reach the biological targets enhancing their bioavailability[16,17]. On the other hand, encapsulation may modify the metabolism, distribution, and excretion processes, thereby altering its potential for promoting toxicity[9].

Because of the potential toxic effects on human health and environment, the increased use of nano-formulations in food and beverages has become matter of concern[12,13,14]. Since nano-formulations are essentially different from their corresponding bulk formulations, the European Food Safety Agency (ESFA) and the Food and Drug Administration (FDA) recommended to carefully evaluate and monitor nano-formulated molecules and promoted the search of valid and rapid methods for the identification, characterization and evaluation of the risks deriving from the use of these new substances[10].

Vitamin A is an essential nutrient which has received much attention in developed countries it has been supplemented into a variety of foods, leading to excessive vitamin A intake[15,16]. It has been established that the recommended daily dose of vitamin A (retinol) is 600 μg/day in adult women, 700 μg/day in pregnant women and 1000 μg/day during lactation, which corresponds to approximately 2000 IU, 2500 IU and 3500 IU respectively. Furthermore, the maximum tolerated dose of vitamin A is 3000 μg/day, which corresponds to about 10000 IU[12]. Plasma concentration of retinol is guaranteed constant in normal conditions and is close to 1-2 μM, corresponding to about 950-2000 IU/L[13,14]. Hypervitaminosis A is difficult to be obtained by excessive dietary intake, but it is usually a result of consuming an excess of preformed vitamin A from supplements[15]. Vitamin A values > 4μM (nearly 4000 IU/L) suggest hypervitaminosis A and associated toxicity. About 28-37% of general population uses supplements containing vitamin A[14], while a relatively small number of studies have reported plasma concentrations after ingestion of retinol supplements.

Vitamin A is obtained from the diet either as preformed vitamin A (mainly retinol and retinyl esters) in foods of animal origin or as provitamin A carotenoids in plant-derived foods. Once retinol has been taken up by a cell, it can be oxidized to retinaldehyde by retinol dehydrogenases and then retinaldehyde can be oxidized to retinoic acid (RA) by aldehyde dehydrogenases (ALDHs)[15]. A number of studies demonstrate that retinoic acid metabolites mediate most, if not all the toxic effect of retinol.

As far as embryo development is concerned, RA is a well-known morphogen and key determinant during embryonic development of both vertebrate and invertebrate chordates because of its implication in patterning the anterior-posterior axis by controlling the induction of HOX genes expression[18-20]. RA levels in embryos tissues are finely controlled by a precise balance between ALDH synthetic activity and the metabolizing activity of CYP26, a cytochrome P450 enzyme. Consequently, alterations of RA levels due to deficiency or excess of vitamin A intake cause embryonic defects in different organs including eyes, heart, lungs and genital tract, or defects in the anterior-posterior patterning of the neural tube[11,13,14]. Perturbation of RA levels in rodent embryos during neurulation is reported to alter branchial morphogenesis and branchial neural crest cell migration, with consequent craniofacial malformations[22-25]. In addition, also in the amphibian Xenopus laevis, larvae developed from embryos exposed to RA at the neurula stage showed anterior- or dysmorphogenesis, specifically at the level of the craniofacial cartilages of the 1st and 2nd branchial arch[26].

The rat, by the post implantation rodent Whole Embryo Culture (WEC) and the amphibian X. laevis by the Frog Embryo Teratogenesis Assay-Xenopus (FETAX) are here proposed to evaluate and compare the effects on embryo development of the exposure to bulk and nano-encapsulated vitamin A. The final aim is to evaluate alternative tests in order to reconsider vitamin hazard evaluation after nano-encapsulation.

Results

Rat whole embryo culture

At the end of 48 h of culture, the morphometric parameters showed the absence of a general toxicity in any group except for 20000 IU NANO-A, were a significant reduction of the total score was evident (Table 1). By contrast, the two forms of vitamin A were both able to induce specific abnormalities (Table 2). At the top concentrations (40000 IU BULK-A) and in NANO-A 10000 IU and 20000 IU groups a significant increase of embryos showing multiple abnormalities (plurimal formed embryos) was observed. Even if branchial arch abnormalities were recorded with a dose dependent trend both in BULK and NANO vitamin A groups, branchial defects were less severe (reductions) after BULK exposure than in NANO-A groups (when fusions were recorded) (Table 2). Different branchial arch phenotypes are shown in Figure 1. Encephalic abnormalities (open neural tube) were finally recorded only in NANO-A exposed groups (Table 2). BMD analysis showed that NANO-A was nearly 7 times more effective in inducing abnormalities than BULK-A.

Figure 1: Morphological appearance of rat embryos after 48 h of culture: phenotypes observed in different samples. A, A': Rat embryo after 48 h of culture showing anormal phenotype.
Note the dorsal region of the embryo (s), the heart (h), the cephalic region with the optic (>), the otic (°) vesicles and the branchial apparatus with the first (I), second (II), and third (III) separated branchial arches. Magnification: 20X (A), 40X (A’).
B: Plurimalformed embryo with complete fusion of the branchial arches (black rectangle). Magnification 20X.
C: Embryo with reduced branchial arches. Note the extreme reduction of the first branchial arch (I). Magnification: 40X.
D: Embryo with total fusion of the branchial arches forming a unique anterior structure (§). Magnification: 40X.
E: Embryo with fused branchial arches forming a continuous structure (*). Magnification: 40X. Note the open neural tube (>>).

**Table 1: Embryonic morphometric parameters recorded in rat embryos after 48 h of culture (MEAN ± SD)**

|                    | CON+ETH     | BULK A 20000 | BULK A 40000 |
|--------------------|-------------|--------------|--------------|
| Yolk sac diameter (mm) | 3.67±0.4    | 3.93±0.19    | 3.79±0.26    |
| Somite number      | 23.56±2.35  | 23.25±1.36   | 22.78±1.86   |
| Total score        | 38.67±1.22  | 37.67±1.15   | 22.78±1.86   |

**Table 2: Abnormalities observed in rat embryos after 48 h of culture (%)**

|                 | CON+ETH     | BULK A 20000 | BULK A 40000 |
|-----------------|-------------|--------------|--------------|
| Total examined  | 9           | 12           | 14           |
| Abnormal **     | 0           | 17           | 79           |
| Plurimalformed embryos ** | 0       | 0           | 36           |
| Embryos with BA abnormalities ** | 0 | 17       | 43           |
| Reduced BA **   | 0           | 17           | 43           |
| Fused BA        | 0           | 0            | 0            |
| Encephalon abnormalities | 0  | 0           | 0            |

**Table 2: Abnormalities observed in rat embryos after 48 h of culture (%)**

|                 | CON         | NANO A 2500 | NANO A 10000 | NANO A 20000 |
|-----------------|-------------|-------------|--------------|--------------|
| Total examined  | 13          | 9           | 10           | 13           |
| Abnormal **     | 0           | 11          | 100          | 100 #        |
| Plurimalformed embryos ** | 0 | 0       | 10           | 38 #         |
| Embryos with BA abnormalities ** | 0 | 11       | 90           | 62 #         |
| Reduced BA      | 0           | 11          | 0            | 0 #          |
| Fused BA        | 0           | 0           | 90           | 62 #         |
| Encephalon abnormalities ** | 8 | 0       | 90           | 100 #        |

** p<0.01 vs. CON (ANOVA followed by Tukey’s test)  ## p<0.01 vs. BULK A 20000 (Student’s T test)  ** p<0.01 vs. CON (ANOVA followed by Tukey’s test)  ## p<0.01 vs. BULK A 20000 (Student’s T test)
Figure 2: Evaluation of the relative potency factor (RPF) of the nano-formulation (dose-response curve with triangles) in respect to the bulk form (dose-response curve with circles) of vitamin A in WEC. RPF was calculated at the effective concentration at 50% (EC50). RPF of the nano-encapsulated vitamin A was 7.53 (BMD modelling by using PROAST software).

FETAX
Controls and *X. laevis* larvae exposed from NF stage 13 to NF stage 26 to the BULK and the NANO form of vitamin A at the end of the test reached NF stage 46. No abnormalities were recorded in larvae exposed to the BULK form of vitamin A (Table 3). On the contrary, the exposure to NANO-A resulted effective in inducing oral abnormalities in a concentration-dependent manner, with a significant linear trend (p<0.0001). Abnormal larvae showed a marked reduction of the oral opening (after cartilage staining this picture has been related to a general reduction of cartilaginous oral structures) or a funnel shaped mouth (due to a total fusion of the anterior cartilaginous elements) (Table 3, Figure 3). The BMD approach was not applicable due to the absence of abnormalities in the BULK-A groups.

Table 3: Abnormalities observed in *X. laevis* larvae (%).

|                | CON+ETH | BULK A 10000 | BULK A 20000 | BULK A 40000 |
|----------------|---------|--------------|--------------|--------------|
| Total examined | 28      | 19           | 25           | 27           |
| Abnormal       | 0       | 0            | 0            | 0            |
| Reduced oral opening | 0   | 0            | 0            | 0            |
| Fused oral cartilages | 0 | 0            | 0            | 0            |

|                | CON | NANO A 5000 | NANO A 10000 | NANO A 20000 | NANO A 40000 |
|----------------|----|-------------|--------------|--------------|--------------|
| Total examined | 55 | 18          | 18           | 29           | 39           |
| Abnormal **    | 0  | 0           | 0            | 6 #          | 18 §         |
| Reduced oral opening ** | 0 | 0           | 0            | 6 #          | 10 §         |
| Fused oral cartilages ** | 0 | 0           | 0            | 0            | 8 §          |

** p<0.01, Chi-squared test for trend  # p<0.05 vs. BULK A 20000 (Chi-square test)  § p<0.01 vs. BULK A 40000 (Chi-square test)
Neural crest cell migration evaluation (whole mount CRABPI immunostaining)

After CRABPI immune staining, normal rat embryos at the end of the culture showed the stained tissue distributed in the fronto-nasal region, in the welding edge of the neural tube, around the optic and otic vesicle and in the pharyngeal area. Three distinct migratory flows of migrating cells were well distinguishable from the rhombencephalon to the branchial arches, where the ectomesenchyme appeared condensed (Figure 4). Embryos exposed to BULK-A 40000 IU/L showed normal neural crest cell distribution of immunostained tissues, whereas embryos exposed to 20000 IU/L NANO-A showed a continuous immunostained mass migrating from the hindbrain to the fused branchial arches (Figure 4).

Similarly to what observed in rat embryos, after immunostaining of X. laevis embryos at stage 26, the immunostained tissues were distributed around the optic and otic vesicles, at the level of the frontal region and at the level of the branchial arches. Controls and embryos from 40000 IU/L BULK-A showed distinct migratory flows from the rhomboencephalon to the branchial arches, while in embryos exposed to NANO-A 40000 IU/L a disorganization of the CRABPI positive tissues into the branchial region was observed (partially fused migration streams and a continuous branchial immunostained mass) (Figure 4).

Benchmark approach to compare NANO-A results in the two methods

BMD approach revealed that in WEC method NANO-A potency in inducing abnormalities was 14 times higher than the potency observed in FETAX (Figure 5).

Figure 5: Evaluation of the relative potency factor of the effects of the nano-formulation of vitamin A in WEC (dose-response curve with triangles) in respect to FETAX (dose-response curve with circles). RPF of the effects nano-encapsulated vitamin A in WEC was 14 in respect to FETAX (BMD modelling by using PROAST software).

Discussion

The aim of the present work was to evaluate and compare the effects of bulk and nano-encapsulated vitamin A on embryo development by using two different alternative models: the rat WEC and the X. laevis FETAX. The final goal was the identification of a rapid alternative test applicable in order to reconsider vitamin hazard evaluation after nano-encapsulation. Nano-encapsulation has greatly increased in food industry (nutraceutical production and human or veterinary food fortification), due to the many advantages that this recent technology confers to the encapsulated material[3,7]. Nevertheless, nano-encapsulation also raised some questions about the potential toxic effects of nano-encapsulated nutrients in food and beverages on human health. For these reasons, EFSA and FDA promoted the search for valid alternative methods able to identify and evaluate the risks deriving from nanotechnologies[10]. Indeed, rat and Xenopus have already been proposed as animal models to assess the developmental toxicity of nano-molecules as well as the effects of nutrient excess or deficiency on embryos[27-33]. The reason stays in the interclasses similarities at the phylotypic stages (pharyngula, as named by Ballard[34], corresponding in vertebrates to the neurulation and branchial arch organization stages) at both morphological and molecular level. This consideration constitutes the basis for the use of simple culture systems, enabling the in vitro develop-
ment of low vertebrate whole embryos. In the present work, the nano-encapsulated vitamin A resulted effective in eliciting the specific developmental defects related to vitamin A exposure. NANO-A was effective in both models and induced quite similar neural crest cell-migration defects in embryos at the phylotypic stage (WEC at term of culture, FETAX at NF stage 26), WEC resulting more sensitive than FETAX. The teratogenic effect of hypervitaminosis A is not a novelty, and is known to be related to the increase of its active metabolite, RA.RA is a morphogen implicated in a wide range of biological processes during animal differentiation and morphogenesis[35]. This molecule is also implicated in cranio-facial morphogenesis, driving the correct differentiation of the branchial apparatus along the antero-posterior axis. Our results with WEC model showed vitamin A-related branchial malformations similar to those previously reported also by our group after RA exposure[23]. In rat embryos cultured in vitro, NANO-A resulted 7 times more effective than BULK-A, inducing more severe abnormalities as well. These results suggest a major bio-availability of the test molecule in the target sites. FETAX model results indicated that *X. laevis* embryo is insensitive, during the morphogenetic phylotypic stages, to BULK-A. On the contrary, the highest NANO-A concentrations caused specific cranio-facial abnormalities which are quite similar to the anterior dysmorphogenesis observed in *X. laevis* exposed to exogenous RA[26,36,37]. This suggested that, also in *X. laevis*, the teratogenic action of NANO-A could depend on a perturbation of the RA pathway even if the effective concentrations are in the amphibian odler than those effective in WEC. The hypothesis of a perturbation in RA homeostasis is in agreement with data by Pennati et al.[38], who exposed ascidian embryos to bulk and nano-vitamin A and reported alterations to ascidian anterior structures similar to those induced by Nagatomo et al.[39] by an excess of RA. In addition, the results obtained on neural crest cell migration in both models (CRABPI immunostaining) support the hypothesis that the observed alterations could be related to an increase of the endogenous RA levels. In fact, neural crest cell specification and migration from hindbrain to the branchial apparatus is driven by RA and exogenous RA exposure is known to induce similar migration defects both in rat and *X. laevis* models[40,41].

The observed different sensitivity to vitamin A in the two models needs a further evaluation. The most plausible explanation could be related to the fact that in nature preformed vitamin A itself (retinol) is only marginally accumulated in amphibian yolk egg deposits while the alternative carotenoid precursors are most abundant.

Results from this work prompt the necessity to monitor the use of food supplemented with nano-vitamin A, suggesting that even low doses can elicit teratogenic effects due to its increased bioavailability. Moreover, these data demonstrated that different animal models have different susceptibility to the action of nano-encapsulated vitamin A and recommend to routinely use more than one models when testing new formulations of molecules.

In conclusion, the collected data: 1) confirm the teratogenic activity of hypervitaminosis A; 2) show that nano-encapsulation increases vitamin A embryotoxicity, probably acting on the vitamin bioavailability at target structure; 3) indicate a common target apparatus (branchial region and its cranio-facial derivatives) and a common dysmorphogenic pathway (neural crest cell migration alterations) in both the tested species; 4) suggest minor role of preformed vitamin A (retinol) in *X. laevis* development if compared to mammal embryogenesis; 5) strongly suggest the need of hazard re-evaluation for nano-encapsulated vitamins devoted to human nutrition.

**Material and Method**

**Chemicals**

All analytical grade reagents, retinol palmitate (the esterified and biological active form of vitamin A- bulk A), Tyrode solution, 3-amino-benzoic acid ethylester (MS222) and salts for FETAX solution were purchased from Sigma-Aldrich S.r.l., Italy. For the rat WEC, NANO A formula was dissolved in Tyrode solution, while for the amphibian, all suspensions and stock solutions were prepared in FETAX medium whose composition in mg/L was: 625 NaCl, 96NaHCO₃, 30KCl, 15CaCl₂, 50CaSO₄·H₂O, and 70 MgSO₄. Nano-formulations with (NANO-A) or without (shell) encapsulated retinol palmitate were kindly provided by Aquanova* (Novasol® GmbH, Germany). For each experiment, solutions were freshly prepared and maintained in the dark.

**Experimental design**

**Rat whole embryo culture:** Virgin female Crl:CD rats (Charles River, Calco, Italy) housed in a thermostatically maintained room (T=22 ± 2°C, relative humidity = 55 ± 5%) with a 12-h light cycle (light from 6:00 AM to 6:00 PM), free access to food (4RF21, Charles River, Calco, Italy) and tap water, were caged overnight with males of proven fertility. The morning of positive vaginal smear was considered day 0 of gestation. 9.5 day old embryos (early neurula stage) were cultured according to the method proposed by New[42], partially modified by Giavini et al.[43]. Each culture flask contained heat inactivated rat serum (5 ml) and 5 embryos. Tested concentrations for BULK-A were dissolved in 100% ethanol and added (5 µl/bottle) to the culture medium of the treated group in order to obtain final concentrations of five and ten times as the plasma concentration considered indicative of hypervitaminosis A (20000 IU/L and 40000 IU/L). A control group (CON) and a control group containing the solvent alone (5 µl 100% ethanol/bottle, CON+ETH) were also performed. NANO-A has been supplied as a 10% emulsion of nanoliposomes, formed by a shell of lipids from soya lecithin and a core of retinol palmitate. NANO-A formula was dissolved in Tyrode solution and added to the culture medium in order to obtain final concentrations of 2500 IU/L, 10000 IU/L and 20000 IU/L (concentrations determined by a preliminary range-finding test). A control group exposed to the shell alone (dilutions equal to NANO-A 20000 group) was evaluated during the range finding test and was unable to affect embryo development (data not shown). After 48 h of culture, embryos were morphologically examined under a dissecting microscope in order to evaluate morphometrical parameters (yolk sac diameter, somite number, total score according to Brown and Fabro[44] and any embryonic abnormality and then fixed in Dent’s fixative overnight at -20°C and processed for immunostaining.

**FETAX methodology**

Adults of *X. laevis* (Harlan Italia, Bresso, Italy) were maintained

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in controlled conditions (T = 20 ± 2°C; pH = 7.5 ± 0.5; Conductivity = 1000 ± 100 μS; 12h light/dark cycle) in an automatic breeding system (TecnoPlus, Techniplast, Italy) and fed a semi synthetic diet three times a week (XE40 by Mucedola; Settimo Milanese, Italy). Embryos were obtained from natural mating of couples, which were housed in well-aerated mating tanks overnight. After mating, adults were removed and embryos collected in Petri dishes and processed according to Bacchetta et al.[43].

Embryos were exposed from NF stage 13 (early neurula stage) to NF stage 26 (phyloptystic stage, corresponding to rat embryos at the end of the culture period)[40] to BULK-A (10000 UI/L, 20000 UI/L and 40000 UI/L dissolved in 100% ethanol) or NANO-A (5000 UI/L, 10000 UI/L, 20000 UI/L and 40000 UI/L dissolved in FETAX), BULK- and NANO-A concentrations were selected based on a preliminary range finding test. A group exposed to the shell alone (dilutions equal to NANO-A 40000 group) was evaluated during the range finding test and was unable to affect embryo development (data not shown). A control group (CON) and a control group containing the solvent alone (1 µl 100% ethanol/mL FETAX, CON+ETH) were also prepared. At NF stage 26, 5 embryos/group were fixed in 10% buffered formalin overnight at 4°C and processed for immunostaining, while the remaining were removed from treatment solution, rinsed and incubated in FETAX solution till controls reached NF stage 46 (free swimming larva, end of the test). At this stage, all the surviving larvae were anaesthetized and then fixed for the subsequent morphological evaluation and cartilage staining. This assay was repeated three times under the same experimental conditions. To visualize the cartilage structures, fixed larvae were stained with Alcian Blue as previously described[40].

Immunostaining for CRABPI

In embryos exposed to the top concentrations, immunostaining of CRABPI was used to mark the migrating neural crest cells [40]. The whole mount immunostaining procedures on rat and X. laevis embryos have been previously described respectively in Menegola et al.[23] and Di Renzo et al.[47]. The monoclonal antibody was the anti-CRABPI (cellular retinoic acid binding protein), ABR, Italy, dilution 1:500. The anti-mouse-Ig-peroxidase (Fab fragment Boehringer, Italy) was diluted 1:40 in PBS. Immuno-reactivity was visualized with the substrate 4-Cl-1-naphthol (Sigma, Italy) and 0.006% H2O2. Stained cells appeared dark brown at light microscope. Negative control was performed incubating embryos only with secondary antibody.

Statistical analysis

For both rat and X. laevis tests, each assay was performed in triplicate. Data, expressed in percentage of malformed samples, were analyzed by Chi-square test. Data expressed as mean and standard deviation were analyzed by ANOVA followed by Tukey’s test or by Student’s T test. The level of significance was set at p < 0.05.

Benchmark-dose analysis

Benchmark-dose (BMD) approach was applied on abnormalities. Data were modelled by using PROAST 65.2 software in order to characterize the single dose response curves and obtain the relative potency factor (RPF) of nano-versus bulk- vitamin A and in order to compare the results obtained in the two models.

Conflict of Interest: The authors declare that they have no conflict of interest.

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