International Journal of Food and Nutritional Science

ISSN: 2377-0619

Research Article

OMMEGA Publishers

OPEN ACCESS

DOI: 10.15436/2377-0619.19.2493

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

Maria Battistoni[1*], Renato Bacchetta[2], Francesca Di Renzo[2], Francesca Metruccio[3], Roberta Pennati[2], Elena Menegola[2]

[1]Department of Biomedical and Clinical Sciences, Università degli Studi di Milano, Milan, Italy
[2]Department of Environmental Science and Policy, Università degli Studi di Milano, Milan, Italy
[3]ICPS, ASST Fatebenefratelli Sacco, Milan, Italy

***Corresponding author:** Maria Battistoni, Department of Biomedical and Clinical Sciences, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy. +39 02 50314753; E-mail: maria.battistoni@unimi.it

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

Received Date: April 30, 2019
Accepted Date: May 14, 2019
Published Date: May 17, 2019

Citation: Battistoni, M., et al. Teratogenic potential of traditionally formulated and nano-encapsulated vitamin A in two vertebrate models, Rattus norvegicus and Xenopus laevis. (2019) J Food Nutr Sci 6(1): 43-51.

facilitates the crossing of biological barriers and allow the encapsulated lipophilic substances to quickly and efficiently reach the biological targets enhancing their bioavailability[6,7]. On the other hand, encapsulation may modify absorption, distribution, metabolism, and excretion processes, thereby altering its potential for promoting toxicity[8].

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[1,8,9]. 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 careful 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 attentionas in developed countries it has been supplemented into a variety of foods, leading to excessive vitamin A intake[11]. 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 retinol is 3000 µg/day, which corresponds to about 10000 IU[12]. Plasma concentration of retinol is guaranteed constant in normal condition and is close to 1-2 µM, corresponding to about950-2000 IU/L[13,14]. Hypervitaminosis A is difficult to be obtained by excessive dietary intake, but 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 retinal (retinaldehyde) by retinol dehydrogenases and then retinaldehyde can be oxidized to retinoic acid (RA) by aldehyde dehydrogenases (ALDHs)[17]. 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 tubes[18,21]. 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 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.

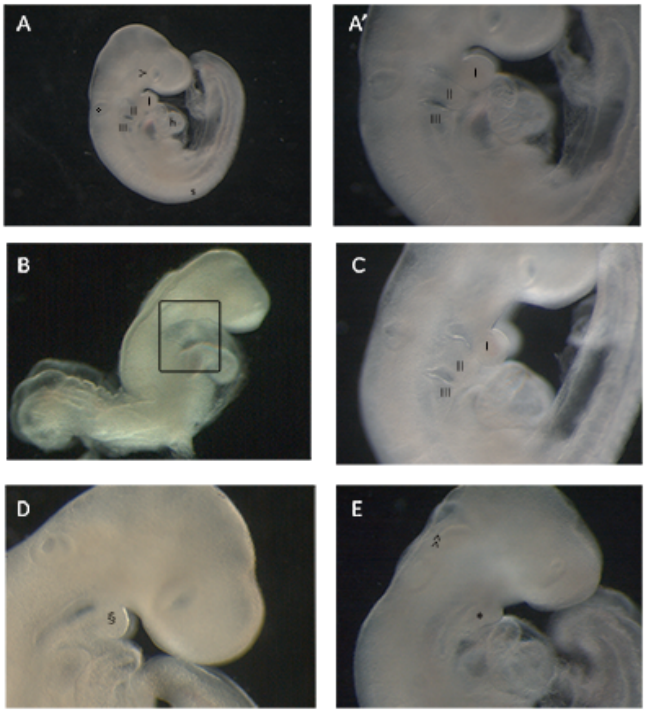

Figure 1: Morphological appearance of rat embryos after 48 h of culture: phenotypes observed in different samples.
A, A': Rat embryo after 48 h of culture showing anormal phenotype.

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
	9	12	9
Somite number	23.56±2.35	23.25±1.36	22.78±1.86
	9	12	9
Total score	38.67±1.22	37.67±1.15	22.78±1.86
	9	12	9

	CON	NANO A 2500	NANO A 10000	NANO A 20000
Yolk sac diameter (mm)	3.76 ± 0.21	3.86 ± 0.18	3.59 ± 0.2	3.68 ± 0.26
	13	9	8	12
Somite number	23.38 ± 2.02	22.44 ± 2.79	22 ± 2	21.2 ± 1.81
	13	9	9	10
Total score	37.92 ± 1.71	36.22 ± 1.3	37.11 ± 1.17	34.6 ± 4.14 ** ##
	13	9	9	10

** p<0.01 vs. CON (ANOVA followed by Tukey's test) ## p<0.01 vs. BULK A 20000 (Student's T test)

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
Abormal **	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

	CON	NANO A 2500	NANO A 10000	NANO A 20000
Total examined	13	9	10	13
Abormal **	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, Chi-squared test for trend
# p<0.01 vs. BULK A 20000 (Chi-square test)

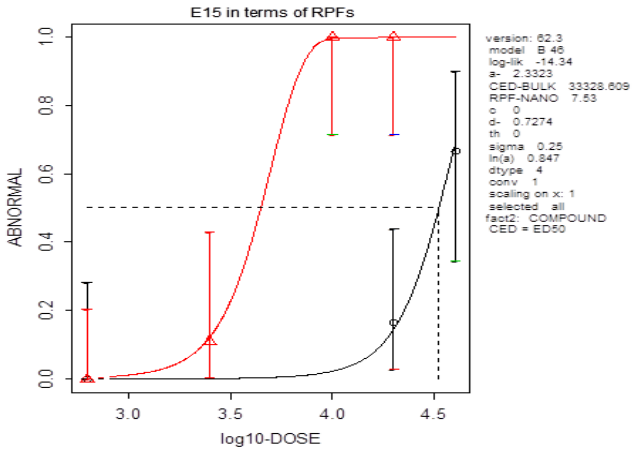

Figure 2: Evaluation of the relative potency factor (RPF) of the nano-formulation (dose-response curve with triangles) in respect to the bulk form (dose-response curve with circles) of vitamin A in WEC. RPF was calculated at the effective concentration at 50% (EC50). RPF of the nano-encapsulated vitamin 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.

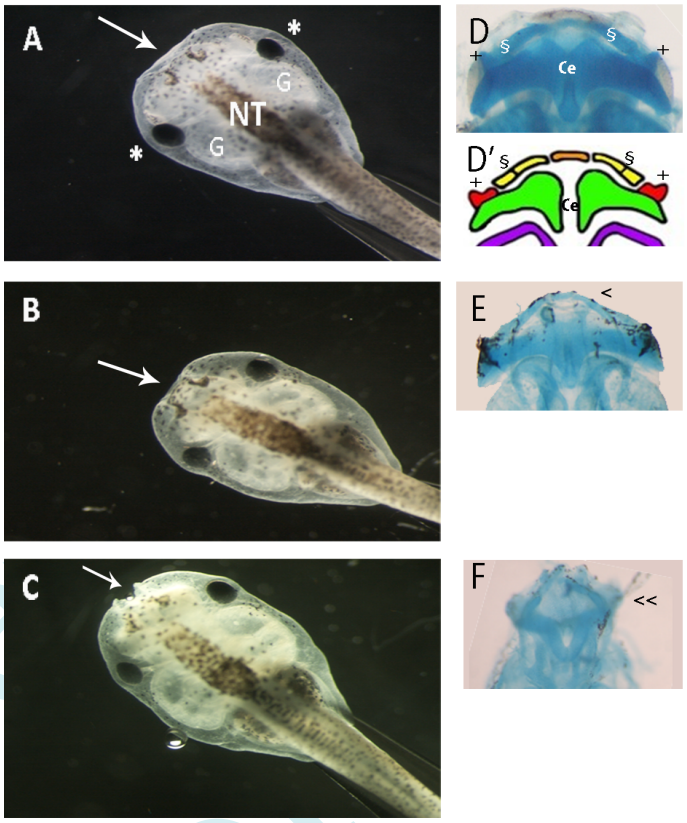

Figure 3: Morphological appearance of NF stage 46 larvae. Magnification: 16X (A-C), 20X (D-F).
A: dorsal view of a larva with a normal phenotype. Note the dorsalneural tube (NT), the well-expanded gill basket (G), eyes (*) and the oral opening (arrow).
B: dorsal view of a larva with reduced oral opening (arrow).
C: dorsal view of a larva with a funnel-shape mouth (arrow).
D, D': ventral view of head cartilagesisolated from a normal larva. D' shows the scheme of normal cartilaginous structures. Note the ventral cartilaginous structure is ceratohyal (Ce), maxilla (+) and mandibular arch (§).
E: ventral view of head cartilages isolated from a larva with reduced oral opening. Note the bent mandibular arch (>).
F: ventral view of head cartilages isolated from a larva with funnel-shaped oral opening. Note the complete fusion of the anterior cartilages (>>).

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 showeddistinct migratory flows from the rhomboencephalon to the branchial arches, while in embryos exposed to NANO-A40000 IU/L a disorganization of the CRABPI positive tissues into the branchial regionwas observed (partially fused migration streams anda continuousbranchial immunostained mass) (Figure 4).

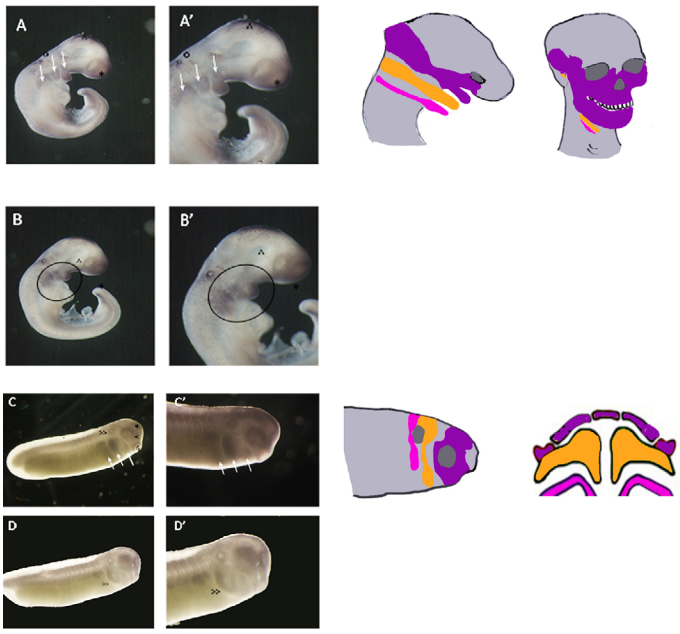

Figure 4: Rat embryos immunostained with antibody anti-CRABP after 48 h of culture (A-B') and *X. laevis* embryos at NF stage 26 immunostained with antibody anti-CRABP (C-D'). Magnification: 20X, 40X. On the right, schemes illustrating the skeletal elements derived from branchial neural crest cells in mammals and in amphibian larva.

A, A': Normal embryo. Note the immunostained areas at the level of the optic and frontonasal region (asterisk), at the welding edges of the neural tube (>), at the level of the otic vesicle (o) and at the level of the branchial arches with three separate streams (arrows), indicating the migration of neural crest cells.

B, B': rat embryo with fused branchial arches. Note the continuous mass (circle) at the level of the fusedbranchial arches.

C,C': X. *laevis* embryo (control). Note the immunostained areas at the level of the frontal region (*), of the otic vesicle (>>), of the optic vesicle (>),and at the level of the branchial arches with separate neural crest migratory flows (arrows).

D, D': X. *laevis* embryos exposed to NANO-A 40000. Note the altered migration of the neural crest cells:the migration streams appear fused forming a ventrally indistinct immunostained mass.

Benchmark approachto 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).

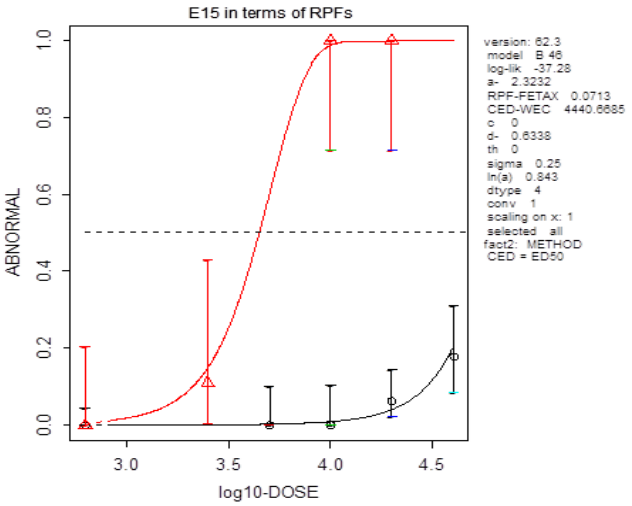

Figure 5: Evaluation of the relative potency factor of the effects of the nano-formulation of vitamin A in WEC (dose-response curve with triangles) in respect to FETAX (dose-response curve with circles). RPF of the effects nano-encapsulated vitamin A in WEC was 14 in respect to FETAX (BMD modelling by 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 severeabnormalities 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 abnormalitieswhich 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 odel higher 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 reportedalterations to ascidian anterior structures similar to those induced by Nagatomo et al.[39] by an excess of RA. In addition, the results obtained onneural crest cell migration in both models (CRABPI immunostaining) support the hypothesis that the observed alterations could be likely 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 using 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) suggesta 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, 96$NaHCO_3$, 30KCl, 15$CaCl_2$, 60$CaSO_4 \cdot {}_2H_2O$, and 70 $MgSO_4$). 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 6:00AM 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 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

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, Italia) 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.[45].

Embryos were exposed from NF stage 13 (early neurula stage) to NF stage 26 (phylotypic stage, corresponding to rat embryos at the end of the culture period)[46] 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[46].

Immunostaining for CRABPI

In embryos exposed to the top concentrations, immunostaining of CRABPI was used to mark the migrating neural crest cells [48]. 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-naphtol (Sigma, Italy) and 0.006% H_2O_2. 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.

References

1. Chaudhry, Q., Scotter, M., Blackburn, J., et al. Applications and implications of nanotechnologies for the food sector. (2008) Food Addit Contam Part A Chem Anal Control Expo Risk Assess 25(3): 241–258.
   Pubmed | Crossref | Others
2. Lamba, A., Garg, V. Nanotechnology approach in food science: A review. (2018) Int J Food Sci Nutr 3(2):183–186.
   Pubmed | Crossref | Others
3. Mozafari, M.R., Johnson, C., Hatziantoniou, S., et al. Nanoliposomes and Their Applications in Food Nanotechnology. (2008) J Liposome Res 18(4):309-327.
   Pubmed | Crossref | Others
4. Wilson, N., Shah, N.P. Microencapsulation of vitamins. (2007) ASEAN Food J 14(1): 1-14.
   Pubmed | Crossref | Others
5. Astete, C.E., Sabliov, C.M., Watanabe, F., et al. Ca^{2+} Cross-Linked Alginic Acid Nanoparticles for Solubilization of Lipophilic Natural Colorants. (2009) J Agric Food Chem 57(16): 7505–7512.
   Pubmed | Crossref | Others
6. Acosta, E. Bioavailability of nanoparticles in nutrient and nutraceutical delivery. (2009) Curr Opin Colloid Interface Sci 14(1): 3–15.
   Pubmed | Crossref | Others
7. McClements, D.J., Rao, J. Food-Grade Nanoemulsions: Formulation, Fabrication, Properties, Performance, Biological Fate, and Potential Toxicity. (2011) Crit Rev Food Sci Nutr 51(4): 285–330.
   Pubmed | Crossref | Others
8. Bouwmeester, H., Dekkers, S., Noordam, M.Y., et al. Review of health safety aspects of nanotechnologies in food production. (2009) Regul Toxicol Pharmacol 53(1): 52–62.
   Pubmed | Crossref | Others
9. Souto, E.B., Martins-Lopes, P., Lopes, C.M., et al. A Note on Regulatory Concerns and Toxicity Assessment in Lipid-Based Delivery Systems (LDS). (2009) J Biomed Nanotechnol 5(4): 317–322.
   Pubmed | Crossref | Others
10. EFSA. Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Guidance for risk assessment of engineered nanomaterials. (2011) EFSA J 9(5): 2140.
    Pubmed | Crossref | Others
11. Penniston, K.L., Tanumihardjo, S.A. The acute and chronic toxic effects of vitamin A. (2006) Am J Clin Nutr 83(2): 191–201.
    Pubmed | Crossref | Others
12. EFSA. Scientific Opinion on Dietary Reference Values for vitamin A: Dietary Reference Values for vitamin A. (2015) EFSA J 13(3): 4028.
    Pubmed | Crossref | Others
13. Blomhoff, R., Wake, K. Perisinusoidal stellate cells of the liver: important roles in retinol metabolism and fibrosis. (1991) FASEB J 5(3): 271–277.

Pubmed | Crossref | Others

14. Zhang, C., Williams, M.A., Sanchez, S.E., et al. Plasma Concentrations of Carotenoids, Retinol, and Tocopherols in Preeclamptic and Normotensive Pregnant Women. (2001) Am J Epidemiol 153(6): 572–580.
Pubmed | Crossref | Others
15. Ross, A.C., Ambalavanan, N., Zolfaghari, R., et al. Vitamin A combined with retinoic acid increases retinol uptake and lung retinyl ester formation in a synergistic manner in neonatal rats. (2006) J Lipid Res 47(8): 1844–1851.
Pubmed | Crossref | Others
16. Bailey, R.L., Fulgoni, V.L., Keast, D.R., et al. Dietary supplement use is associated with higher intakes of minerals from food sources. (2011) Am J Clin Nutr 94(5): 1376–1381.
Pubmed | Crossref | Others
17. Duester, G. Retinoic acid synthesis and signaling during early organogenesis. (2008) Cell 4(6): 921–931.
Pubmed | Crossref | Others
18. Shimeld, S.M. Retinoic acid, HOX genes and the anterior-posterior axis in chordates. (1996) BioEssays 18(8): 613–616.
Pubmed | Crossref | Others
19. Ross, S.A., McCaffery, P.J., Drager, U.C., et al. Retinoids in embryonal development. (2000) Physiol Rev 80(3): 1021–1054.
Pubmed | Crossref | Others
20. Canestro, C., Postlethwait, J.H., Gonzalez-Duarte, R., et al. Is retinoic acid genetic machinery a chordate innovation? (2006) Evol Htmlent Glyphamp Asciiamp Dev. 8(5):394–406.
Pubmed | Crossref | Others
21. Morriss-Kay, G. Retinoic Acid and Development. (1992) Pathobiology 60(5): 264–270.
Pubmed | Crossref | Others
22. Schwartz, E.L., Hallam, S., Gallagher, R.E., et al. Inhibition of all-trans-retinoic acid metabolism by fluconazole in vitro and in patients with acute promyelocytic leukemia. (1995) Biochem Pharmacol 50(7): 923–928.
Pubmed | Crossref | Others
23. Menegola, E., Broccia, M.L., Di Renzo, F., et al. Relationship between hindbrain segmentation, neural crest cell migration and branchial arch abnormalities in rat embryos exposed to fluconazole and retinoic acid in vitro. (2004) Reprod Toxicol 18(1): 121–130.
Pubmed | Crossref | Others
24. Menegola, E., Broccia, M.L., Di Renzo, F., et al. Postulated pathogenic pathway in triazole fungicide induced dysmorphogenic effects. (2006) Reprod Toxicol 22(2): 186–195.
Pubmed | Crossref | Others
25. Menegola, E., Broccia, M.L., Renzo, F.D., et al. Dysmorphogenic effects of some fungicides derived from the imidazole on rat embryos cultured in vitro. (2006) Reprod Toxicol 21(1): 74–82.
Pubmed | Crossref | Others
26. Groppelli, S., Pennati, R., De Bernardi, F., et al. Teratogenic effects of two antifungal triazoles, triadimefon and triadimenol, on Xenopus laevis development: Craniofacial defects. (2005) Aquat Toxicol 73(4): 370–381.
Pubmed | Crossref | Others
27. Morriss, G.M., Steele, C.E. The effect of excess vitamin A on the development of rat embryos in culture. (1974) J Embryol Exp Morphol. 32(2): 505–514.
Pubmed | Crossref | Others
28. Morriss, G.M., Steele, C.E. Comparison of the effects of retinol and retinoic acid on postimplantation rat embryos in vitro. (1977) Teratology 15(1): 109–119.
Pubmed | Crossref | Others
29. Klug, S., Lewandowski, C., Wildi, L., et al. All-trans retinoic acid and 13-cis-retinoic acid in the rat whole-embryo culture: abnormal development due to the all-trans isomer. (1989) Arch Toxicol 63(6): 440–444.
Pubmed | Crossref | Others
30. Fort, D.J., Paul, R.R. Enhancing the predictive validity of Frog Embryo Teratogenesis Assay?Xenopus (FETAX). (2002) J Appl Toxicol 22(3): 185–191.
Pubmed | Crossref | Others
31. Bonfanti, P., Colombo, A., Orsi, F., et al. Comparative teratogenicity of Chlorpyrifos and Malathion on Xenopus laevis development. (2004) Aquat Toxicol 70(3): 189–200.
Pubmed | Crossref | Others
32. Mouche, I., Malesic, L., Gillardeaux, O. FETAX assay for evaluation of developmental toxicity. (2011) Methods Mol Biol 691: 257-269
Pubmed | Crossref | Others
33. Bacchetta, R., Santo, N., Fascio, U., et al. Nano-sized CuO, TiO(2+) and ZnO affect Xenopus laevis development. (2012) Nanotoxicology 6(4): 381–398.
Pubmed | Crossref | Others
34. Ballard, W.W. Morphogenetic Movements and Fate Maps of Vertebrates. (1981) Am Zool 21(2): 391–399.
Pubmed | Crossref | Others
35. Maden, M., Holder, N. Retinoic acid and development of the central nervous system. (1992) Bioessays 14(7): 431–438.
Pubmed | Crossref | Others
36. Durston, A.J., Timmermans, J.P.M., Hage, W.J., et al. Retinoic acid causes an anteroposterior transformation in the developing central nervous system. (1989) Nature 340(6229): 140–144.
Pubmed | Crossref | Others
37. Papalopulu, N., Clarke, J.D., Bradley, L., et al. Retinoic acid causes abnormal development and segmental patterning of the anterior hindbrain in Xenopus embryos. (1991) Dev Camb Engl 113(4): 1145–1158.
Pubmed | Crossref | Others
38. Pennati, R., Manenti, R., Stillitano, A., et al. Teratogenic potential of nanoencapsulated vitamin A evaluated on an alternative model organism, the tunicate Ciona intestinalis. (2018) Int J Food Sci Nutr 69(7): 805-813.
Pubmed | Crossref | Others
39. Nagatomo, K., Ishibashi, T., Satou, Y., et al. Retinoic acid affects gene expression and morphogenesis without upregulating the retinoic acid receptor in the ascidian Ciona intestinalis. (2003) Mech Dev 120(3): 363–372.
Pubmed | Crossref | Others
40. Lee, Y.M., Osumi-Yamashita, N., Ninomiya, Y., et al. Retinoic acid stage-dependently alters the migration pattern and identity of hindbrain neural crest cells. (1995) Dev Camb

Engl 121(3): 825–837.
Pubmed | Crossref | Others

41. Vieux-Rochas, M., Bouhali, K., Baudry, S., et al. Irreversible effects of retinoic acid pulse on Xenopus jaw morphogenesis: new insight into cranial neural crest specification. (2010) Birth Defects Res B Dev Reprod Toxicol 89(6): 493–503.
Pubmed | Crossref | Others
42. New, D.A. Whole-embryo culture and the study of mammalian embryos during organogenesis. (1978) Biol Rev Camb Philos Soc 53(1): 81–122.
Pubmed | Crossref | Others
43. Giavini, E., Broccia, M.L., Prati, M., et al. Effects of ethanol and acetaldehyde on rat embryos developing in vitro. (1992) In Vitro Cell Dev Biol 28(3): 205–210.
Pubmed | Crossref | Others
44. Brown, N.A., Fabro, S. Quantitation of rat embryonic development in vitro: A morphological scoring system. (1981) Teratology 24(1): 65–78.
Pubmed | Crossref | Others
45. Bacchetta, R., Tremolada, P., Di Benedetto, C., et al. Does carbon nanopowder threaten amphibian development? (2012) Carbon. 50(12): 4607–4618.
Pubmed | Crossref | Others
46. Nieuwkoop, P.D., Faber, J., editors. Normal table of Xenopus laevis (Daudin): a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis. (1994) New York: Garland Pub 252.
Pubmed | Crossref | Others
47. Di Renzo, F., Bacchetta, R., Sangiorgio, L., et al. The agrochemical fungicide triadimefon induces abnormalities in Xenopus laevis embryos. (2011) Reprod Toxicol 31(4): 486–493.
Pubmed | Crossref | Others
48. Kanzler, B., Foreman, R.K., Labosky, P.A., et al. BMP signaling is essential for development of skeletogenic and neurogenic cranial neural crest. (2000) Dev Camb Engl 127(5): 1095–1104.
Pubmed | Crossref | Others