Nano-Encapsulation of Arsenic Trioxide Enhances Efficacy against Murine Lymphoma Model while Minimizing Its Impact on Ovarian Reserve In Vitro and In Vivo

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Abstract

Advances in cancer therapy have increased the rate of survival of young cancer patients; however, female lymphoma patients frequently face a temporary or permanent loss of fertility when treated with traditional cytotoxic agents. The potential loss of fertility is an important concern that can influence treatment decisions for many premenopausal cancer patients. The negative effect of chemotherapeutic agents and treatment protocols to patients’ fertility—referred to as fertotoxicity—arises thus as an increasingly important cancer survivorship issue. We have developed a novel nanoscale formulation of arsenic trioxide, a potent drug for treatment of hematological malignancies, and demonstrate that it has significantly better activity in a murine lymphoma model than the free drug. In parallel, we have developed a novel in vitro assay of ovarian follicle function that predicts in vivo ovarian toxicity of therapeutic agents. Our results reveal that the nanotherapeutic agent is not only more active against lymphoma, but is fertoprotective, i.e., it is much less deleterious to ovarian function than the parent drug. Thus, our in vitro assay allows rapid evaluation of both established and experimental anticancer drugs on ovarian reserve and can inform the selection of efficacious and fertility-sparing treatment regimens for reproductive-age women diagnosed with cancer.

Introduction

According to the National Cancer Institute, 72,000 adolescents and young adults (ages 15–39 years) are diagnosed with cancer each year [1]. The most prevalent types of cancer in this patient population include lymphoma, leukemia, germ cell tumors (including testicular cancer), melanoma, breast, and cervical cancers [1]. Due to advances in anticancer therapy, many of these young people will survive their cancer. Yet many of these life-saving, potent therapies also threaten the future fertility of young cancer patients [2]. Post-treatment fertility is a major concern of young breast cancer patients; in one survey, 29% of these women made cancer treatment decisions based on the fertotoxicity of therapy, yet only 51% felt their concerns were adequately addressed [3]. Many chemotherapeutic agents can damage ovarian tissue and impair follicle function, causing temporary or permanent infertility in female children, adolescents, and young adults [2,4,5]. In most clinical studies, amenorrhea is used as a measure of the fertotoxicity of chemotherapeutic agents; however, amenorrhea may not be the

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best marker of ovarian damage or the risk of future infertility. The most damaging agents to future fertility are those that reduce the ovarian reserve, or the number of ovarian follicles (each of which encloses a single oocyte) that are capable of supporting oocyte growth, maturation, and fertilization. Alkylating agents are known to damage growing oocytes as well as early-stage follicles, causing temporary and sometimes permanent amenorrhea and reduced uterine receptivity [6–9]. By comparison, we have limited knowledge about the fertotoxic effects of emerging chemotherapeutics, a gap that presents a major obstacle to the informed selection of fertility-sparing treatment regimens for reproductive-age women or the discussion of options for fertility preservation prior to therapy.

Arsenic trioxide ($\text{As}_2\text{O}_3$) is an FDA-approved therapeutic agent that has been highly successful in treating acute promyelocytic leukemia [10–14] and has shown promise in adult T-cell leukemia/lymphoma [15]. The mechanism of action of $\text{As}_2\text{O}_3$ is complex, and includes induction of apoptosis by reactive oxygen species, promotion of cellular differentiation, and inhibition of angiogenesis [16,17]. While $\text{As}_2\text{O}_3$ has shown promising efficacy in preclinical models of solid tumors, this success has not been replicated in clinical trials due to its rapid renal clearance and dose-limiting toxicities [18,19]. Recent reports have shown promising efficacy of $\text{As}_2\text{O}_3$ in both clinical lymphoma specimens and lymphoma cell lines [20]. However, activity of $\text{As}_2\text{O}_3$ in clinical trials has not shown benefit in most subtypes of lymphoma [20]. In order to improve the antitumor activity of $\text{As}_2\text{O}_3$, a nanoparticulate formulation of $\text{As}_2\text{O}_3$ was recently developed [21,22]. In this delivery system, transition metals (e.g., Ni$^{2+}$, Co$^{2+}$, Pd$^{2+}$) are used to stably encapsulate $\text{As}_2\text{O}_3$ as a nanoprecipitate inside a liposomal vesicle that we termed “nanobins” [NB(Ni,As)]; so named because each vesicle contains many precipitated arsenic-nickel particles. This formulation of $\text{As}_2\text{O}_3$ has been shown to decrease the plasma clearance of arsenic, improve tumor delivery of arsenic, inhibit triple-negative breast cancer growth and attenuate toxicity in vitro [23]. We hypothesized that the nanoparticulate formulation of $\text{As}_2\text{O}_3$ in NB(Ni,As) would have antitumor activity at lower doses and be less toxic to female reproductive function than free $\text{As}_2\text{O}_3$.

Despite the clinical use of $\text{As}_2\text{O}_3$, studies on its effects on reproductive function are focused on ingestion of environmental arsenic and developmental toxicology in rodents [24–27]. In vitro methods for evaluating the reproductive effects of drugs involve time-consuming testing in animals and are not required for FDA approval of cytotoxic cancer chemotherapeutics [28]. We sought to develop a rapid in vitro assay to measure the impact of chemotherapeutic agents on ovarian reserve that could guide the selection of therapies based on the potential for reproductive toxicity. Three assay design criteria were important to meet our goal: 1) the assay must quantify follicle health with low operator expertise; 2) the assay must have a short development time; and 3) the assay method must be scalable to enable high-throughput screening.

We hypothesized that the alginate hydrogel system, originally developed for the three-dimensional culture of isolated ovarian follicles and fertility preservation, provided an assay platform that met these criteria [29–31]. Culture of single ovarian follicles within alginate hydrogel essentially recapitulates in vitro the follicle growth that occurs in vivo within the ovary. Using the alginate hydrogel system, primordial follicles from mice, nonhuman primates [32] and humans [31] have been successfully cultured to produce fully mature oocytes. We have also demonstrated that the mature oocytes from in vitro cultured mouse follicles are of good quality, can be fertilized, and result in live births [30]. Importantly, the system is easy to implement and provides rapid assessment of follicle development after treatment with chemotherapeutic agents.

In this study, we first demonstrated that the antitumor efficacy of NB(Ni,As) was superior to free $\text{As}_2\text{O}_3$ in a murine model of lymphoma, and that NB(Ni,As) was less fertotoxic than free $\text{As}_2\text{O}_3$ in vitro. As reported in previous studies, the increased efficacy and reduced fertotoxicity are likely related to differences in plasma pharmacokinetics, tumor uptake, and systemic biodistribution of the encapsulated and free $\text{As}_2\text{O}_3$ agents [23]. We then correlated these in vivo observations with the results of our novel in vitro follicle growth assay. We have now developed an in vitro assay that can be scaled up and utilized to evaluate the potential for reproductive side effects, both in early-stage drug development and of existing agents and their combinations. The issue of fertotoxicity of chemotherapeutic agents is important, not only to the research community, but also to pharmaceutical development teams, the oncologists who treat reproductive-age patients, and patients who wish to preserve their fertility and ensure a high quality of life as cancer survivors. Knowledge of the fertotoxicity of chemotherapy regimens is also critical given that several interventions of fertility preservation, such as oocyte retrieval and ovarian tissue banking, are more effective in chemotheraphy naive patients.

### Materials and Methods

#### Preparation of $\text{As}_2\text{O}_3$-loaded Nanobins

Arsenic trioxide-loaded nanobins [NB(Ni,As)] and NB(NaCl) were prepared as described previously [23]. Briefly, a dry-lipid film consisting of DSPC (1,2-distearoyl-glycerol-3-phosphocholine (Avanti Polar Lipids; Alabaster, AL), DSPE-PEG2000 (1,2-Dis-}

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**Cell Culture and Annexin-V Assays**

L540 [33] and RAMOS [34] cells were obtained from the Tumor Biology Core Facility of the Northwestern University Robert H. Lurie Cancer Center and Z138 cells [35] were a gift from Dr. Steven Rosen. L540 and RAMOS cells were maintained in Roswell Park Memorial Institute medium (RPMI 1640, Invitrogen Corporation, Carlsbad, CA) and Z138 cells were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM, Invitrogen), and all media was supplemented with 2 mM L-glutamine and 10% fetal bovine serum (FBS, Invitrogen). The cells were grown in an incubator at 37°C.
Tumors were allowed to grow to a maximum of 1000 mm$^3$. Animals were also weighed at the time of tumor measurement. With the length (mm) being the longest axis of the tumor.

Cells were resuspended in cold growth medium at 50%–90% confluence and harvested for implantation. The Z138C human B-cell lymphoma mantle cell line was grown to 80%–90% confluence and harvested for implantation.

Six-week-old female CD1 mice (N = 36) were housed in groups of 3 and allowed to acclimate for 1.5 weeks. After confirming normal cyclicity for 2 weeks by vaginal lavage, the mice were randomized into 2 treatment groups (n = 18 in each group) and injected with 4 mg/kg of As$_2$O$_3$ or NB(NaCl), As$_2$O$_3$, or NB(Ni,As). All animals were in either metestrus or diestrus at the time of injection. After injection, As$_2$O$_3$-treated mice were sacrificed at 2, 4, 6, 12, 24, and 48 hours (n = 3 for each time point) and NB(Ni,As)-treated mice were sacrificed at 2, 6, 12, 24, 36, and 48 hours (n = 3 for each time point). The liver, kidneys, ovaries, uterus, and plasma were immediately collected and stored at −80°C. Arsenic uptake in each tissue was measured by inductively coupled plasma-mass spectrometry (ICP-MS), and pharmacokinetic analysis was performed using the SAAM II software system (SAAM Institute, Seattle, Washington).

**Z138C Xenograft Experiment**

The Z138C human B-cell lymphoma mantle cell line was obtained from the Martin Dyer lab (University of Leicester, UK) and tested negative for mycoplasma. Cells were grown in RPMI 1640 supplemented with 2 mM L-glutamine and 10% FBS at 37°C in 5% CO$_2$. Cells from passages 3 to 10 were used in in vitro toxicity assays. Mice were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and protocols were approved by the IACUC at Northwestern University (protocol #A05-135), and were performed in accordance with the Canadian Council on Animal Care Guidelines.

Female CD1 mice (7.5-week-old; Harlan, Indianapolis, IN) were used for in vivo toxicity studies, and ovaries and immature follicles isolated from prepubertal, 12- to 14-day-old female CD1 mice were used in in vitro toxicity assays. Mice were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and protocols were approved by the IACUC at Northwestern University (protocol #A05-135), and were performed in accordance with the Canadian Council on Animal Care Guidelines.

**Inductively Coupled Plasma-mass Spectrometry**

Tissue arsenic levels were determined by ICP-MS with a Thermo X Series II inductively coupled plasma-mass spectrometer (Thermo-Fisher, Waltham, MA). Samples were prepared by digesting tumors in 500 µL concentrated trace metal-free grade nitric acid (69%) in capped, metal-free falcon tubes for 2 hours at 60°C. At 20-minute intervals during the digestion, the sample tubes were vortexed and vented in a fume hood. After 2 hours, the digests were filtered through a 0.45-µm polytetrafluoroethylene (PTFE) filter into a fresh metal-free falcion tube. For ICP-MS analysis, a portion of the filtered digest was diluted with ultrapure laboratory grade water (18 Ω) and an internal standard mixture of Sc, Tb, Y, In, and Bi (CPI International, Santa Rosa, CA) was added. Standards between 0 and 90 ppb were made using a custom mixed element solution (CPI International). The final ICP-MS samples and elemental standards were prepared in a matrix of 2% nitric acid containing 0.1 Triton X-100 and 5% acetic acid.

**In Vitro Follicle Culture and Toxicity Assay**

Ovaries were isolated from 12- to 14-day-old CD1 mice into prewarmed collection media (Løebovitz L-15, Invitrogen) containing 1 mg/ml bovine serum albumin (BSA) and 50 IU/ml penicillin/streptomycin (Invitrogen). Two ovaries were incubated per culture plate. A total of 45 ovaries were used for ICP-MS and 20 ovaries were used for follicle isolation. Early secondary follicles (oocytes surrounded by 2–3 granulosa cell layers) were isolated from the ovaries, and whole ovaries and isolated follicles
were transferred to α-MEM containing 1 mg/ml BSA, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite, and incubated at 37°C at 5% CO₂ on a horizontal shaker (Figure S1). After 3 hours, 250 μl of PBS or 3, 30, or 90 μM As₂O₃, NB(NaCl), or NB(Ni,As) was added to the cultures; after 3 hours, the ovaries and follicles (25 per treatment group and 4 repeats) were washed in media 3×10 minutes. Isolated follicles were then encapsulated into sterile 0.5% (w/v) alginate beads as described previously [30]. Encapsulated follicles were grown for 10 days in α-MEM containing 1 mg/ml bovine fetuin (Sigma-Aldrich, St. Louis, MO), 5 μg/ml insulin, 5 μg/ml transferrin, 5 mg/ml sodium selenite (Sigma-Aldrich), 3 mg/ml BSA, and 10 mIU rhFSH (gift from Organon, Roseland, NJ).

Statistics
Tumor volume, arsenic concentration, and follicle growth and survival data were subjected to one-way analysis of variance followed by Dunnett’s or Bonferroni’s multiple comparison post-hoc test to determine the significance of differences between each treatment group using Prism 4 (GraphPad Software, Inc.). Calculated values are shown as mean ± SEM with a significance level of *P*<0.01 being considered statistically significant, unless otherwise noted.

Results
As₂O₃ is Cytotoxic to Lymphoma Cell Lines in vitro and NB(Ni,As) Inhibits Z138C Lymphoma Xenograft Tumor Growth in vivo
Hodgkin’s lymphoma and Burkitt’s lymphoma are highly prevalent in patients of reproductive age [36]. We evaluated the in vitro induction of apoptosis of As₂O₃ and NB(Ni,As) in L540 Hodgkin’s lymphoma, RAMOS Burkitt lymphoma, and Z138C mantle cell lymphoma cell lines. We determined that free As₂O₃ induces apoptotic cell death in all 3 cell lines, while free NB(Ni,As) and NB(NaCl) (a vehicle control nanobin) have minimal cytotoxicity in this 18 hour assay (Figure S2). We have observed a similar effect in breast, ovarian, SUDHL-4 lymphoma cells and all of the other cancer cell lines that we have evaluated to date [21–23]. The attenuation of cytotoxicity is based on the fact that encapsulated arsenic is not bioactive until it is released, which occurs over a 48 h period [23].

In order to evaluate the in vivo activity of NB(Ni,As) we chose to utilize the Z138C mantle cell lymphoma subcutaneous xenograft model in Rag2M mice. We chose to use the Z138C cells since the Z138C and L540 cell lines had similar cytotoxicity in vitro and our previous experience with the Z138C model [35]. Mice bearing Z138C lymphoma xenograft tumors were treated weekly with either NB(NaCl), As₂O₃ (4, 6, or 8 mg/kg), or NB(Ni,As) (4, 6, or 8 mg As₂O₃/kg) for 1 month. Free As₂O₃ at 4 mg/kg was not effective in slowing tumor growth on this schedule and was equivalent to the control NB(NaCl), whereas the 6 and 8 mg/kg doses of As₂O₃ were acutely toxic to the mice (data not shown). By contrast, NB(Ni,As) effectively inhibited tumor growth at all 3 doses tested compared with NB(NaCl) and free As₂O₃ (Figure 1A). The 4 mg/kg dose of NB(Ni,As) and As₂O₃ were well tolerated by the mice, with no signs of toxicity (<15% weight loss). Treatment with 6 mg/kg and 8 mg/kg of NB(Ni,As) resulted in weight loss (Figure 1B). Thus, NB(Ni,As) at 4 mg/kg showed antitumor efficacy with minimal effect on weight in an animal model of lymphoma.

Figure 1. NB(Ni,As) inhibits mantle cell lymphoma growth. 18 days after inoculation with Z138C lymphoma cells, Rag2M mice were randomized and treated with weekly injections of NB(NaCl), As₂O₃ (4, 6, or 8 mg/kg), or NB(Ni,As) (4, 6, or 8 mg/kg). (A) Tumors treated with NB(Ni,As) were significantly smaller than those treated with NB(NaCl). **, *P*<0.01, ***, *P*<0.001. Arrows indicate treatment. (B) Weight was monitored daily during the treatment period. Injection of As₂O₃ was acutely toxic, whereas mice injected with NB(NaCl) showed normal weight gain. Mice injected with any dose of NB(Ni,As) lost weight, though mice treated with 4 mg/kg showed the least amount of weight loss during the treatment period.

NB(Ni,As) Limits Systemic Exposure of Arsenic Compared with Free As₂O₃
We next examined the plasma pharmacokinetics and biodistribution of NB(Ni,As) and As₂O₃ in vivo. A single 4 mg/kg dose of As₂O₃ or NB(Ni,As) was administered to female CD1 mice by intraperitoneal injection. Mice were sacrificed at various time points up to 48 hours after injection, and the liver, kidneys, ovaries, uterus, and plasma were collected. Total arsenic concentration was measured by ICP-MS (Figure 2). Pharmacokinetic analysis of plasma arsenic levels revealed that plasma elimination clearance and the steady-state volume of distribution (Vₚ) of As₂O₃ were much higher than those of NB(Ni,As). The elimination clearance of NB(Ni,As) was
0.18 ml/hr while that of As₂O₃ was 15.2 ml/hr. Furthermore, the \( Vₚ \) of NB[NiAs] (3.5 ml) was similar to the predicted plasma volume of a mouse, suggesting that NB[NiAs] largely confines arsenic to the intravascular space. By contrast, the \( Vₚ \) of As₂O₃ (139.2 ml) indicated rapid, extensive, nonselective tissue distribution. These pharmacokinetic parameters are consistent with single-dose plasma pharmacokinetic analysis of free As₂O₃ and NB[NiAs] in rats [23].

The peak concentration of arsenic in the ovary was higher in the As₂O₃ group than in the NB[NiAs] group (Figure 2B), which is consistent with rapid and extensive distribution of free As₂O₃. However, the actual peak concentration of arsenic in the ovary was likely missed in As₂O₃-treated animals because distributional equilibrium had been reached by the time the first tissue samples were collected. The arsenic level in mice treated with NB[NiAs] persisted longer in the ovary; however, this measurement likely included arsenic in the vascular space of the ovary. The concentration of arsenic in the uterus paralleled that of the ovary, although total arsenic exposure in the uterus was higher than in the ovary, which is consistent with greater uterine vascularity compared with the ovary (Figure 2B).

Peak arsenic concentrations were higher in the liver and kidneys of As₂O₃-treated mice compared with NB[NiAs]-treated mice, but the arsenic concentration dropped rapidly over the course of 48 hours in these organs, consistent with the rapid plasma clearance of free As₂O₃ (Figure 2C). Levels of arsenic in the liver and kidneys of mice treated with NB[NiAs] fell slowly during the 48 hour time period due to the extended plasma half-life and reduced clearance of NB[NiAs] compared with As₂O₃ (Figure 2C).

Impact of As₂O₃ and NB[NiAs] on Ovarian Reserve in Mice

To assess the effect of repeat treatments of As₂O₃ and NB[NiAs] on female reproductive function in vivo, mice were treated with PBS or 4 mg/kg As₂O₃, NB[NaCl], or NB[NiAs] twice a week for 3.5 weeks. The dose and schedule were chosen based on demonstrated both efficacy and minimal toxicity in both the breast cancer and lymphoma models [23]. Prior to treatment, all mice were cycling regularly (3- to 4-day cycles) for 2 full weeks as determined by vaginal lavage and endometrial cytology examination. During the course of treatment, the estrus cycle was determined daily as a measure of ovarian function. All of the mice injected with PBS showed normal estrus cyclicity (Figure 3A). By contrast, 40% of mice injected with As₂O₃ stopped cycling within 10 days of the initiation of treatment (Figure 3A, B). Mice injected with NB[NaCl] showed 90% normal cyclicity (Figure 3A); 1 mouse missed a single cycle but resumed cycling and continued to do so normally until the end of the study. Notably, mice treated with NB[NiAs] displayed normal cyclicity throughout treatment (Figure 3A, B). Thus, NB[NiAs] had a lower fertotoxic effect than free As₂O₃ in vivo.

At the termination of the in vivo cyclicity study, the arsenic concentration in 2 sets of ovaries from each treatment group was measured. The ovaries of mice exposed to As₂O₃ contained 0.05 nmol/mg arsenic, whereas ovaries exposed to NB[NiAs] contained 0.02 nmol/mg arsenic (Figure S3). The remaining ovaries were fixed and processed for histologic analysis. Mice treated for 3.5 weeks with PBS or NB[NaCl] had normal ovaries that contained follicles at all stages of development, from the primordial through the antral follicle stage, as well as the presence of corpora lutea (Figure 4A, B). Ovaries from mice treated with 4 mg/kg As₂O₃ showed blood filled cysts (Figure 4C, E) and areas of hemorrhage (Figure 4F). By contrast, mice treated with 4 mg/
kg NB(Ni,As) showed normal ovarian histology (Figure 4D). These histologic data confirmed the observed physiologic effects of NB(Ni,As) and As$_2$O$_3$ on reproductive cyclicity in mice.

In vitro Assay of As$_2$O$_3$ and NB(Ni,As) on Follicle Development

To validate our novel in vitro follicle-based assay, individual follicles were isolated from the ovaries of 12- to 14-day-old mice and treated in culture for 3 hours with PBS, NB(NaCl), As$_2$O$_3$ (3, 30, or 90 μM As) or NB(Ni,As) (3, 30, or 90 μM As). Each follicle was then encapsulated in alginate hydrogel matrix and cultured for 10 days to assess the effect of arsenic exposure on follicle survival and growth. Survival of follicles treated with PBS or NB(NaCl) vehicle was similar for all 3 treatment concentrations (Figure 5A-C). As$_2$O$_3$ treatment at doses as low as 3 μM had a detrimental effect on follicle survival by day 6 (Figure 5A), and the majority of follicles treated with 90 μM As$_2$O$_3$ died by day 4 (Figure 5C). Follicles treated with 3 μM NB(Ni,As) initially survived at rates similar to those of follicles treated with either PBS or NB(Ni,As) (Figure 5A), but survival of follicles treated with either 30 μM or 90 μM NB(Ni,As) was only 70% on day 6 (Figure 5B, C). Arsenic content in whole ovaries cultured for 3 hours in PBS or 3, 30, or 90 μM As$_2$O$_3$, NB(NaCl), or NB(Ni,As) was also determined by ICP-MS (Figure 5D). Ovaries treated with NB(Ni,As) contained significantly lower amounts of arsenic compared with ovaries treated with As$_2$O$_3$.

Follicles treated with any dose of NB(NaCl) grew to a mean diameter of approximately 250 μm (Figure 6A–C). By contrast, follicles treated with 3 or 30 μM As$_2$O$_3$ did not grow larger than 200 μm (Figure 6A, B), and those exposed to 90 μM As$_2$O$_3$ actually decreased in diameter from baseline to 100 μm, indicating follicle death (Figure 6C). Follicles exposed to any concentration of NB(Ni,As), however, showed growth similar to that seen with NB(NaCl), to approximately 250 μm (Figure 6A–C).

Discussion

Using traditional in vivo assays and a novel in vitro assay of reproductive (ovarian) function to assess the reproductive toxicity of chemotherapeutic agents, we have determined that nanoscale encapsulation of a potent cytotoxic drug, As$_2$O$_3$, can provide significant protection of fertility while maintaining or improving the efficacy of the free drug. Using these assays, we demonstrated that arsenic administered as NB(Ni,As) has a lower effective dose and is less toxic than free arsenic to ovarian and follicle function. The efficacy of NB(Ni,As) has been demonstrated in murine models of both triple negative breast cancer and a model of lymphoma, two common cancers in females of reproductive age [23]. In addition, our data showed a dose response to As$_2$O$_3$, consistent with that reported by Griffin et al in syngeneic murine tumor models [37]. In these studies, As$_2$O$_3$ as low as 2 mg/kg, induced significant disruption of tumor vasculature, with or without hyperthermia. These studies and our data suggests that delivery of cancer drugs via nanoscale carriers has the potential to increase efficacy and mitigate the impact of cytotoxins on the female reproductive tract by redistributing the agent to the intravascular space. Until the nanoparticle reaches the tumor or is cleared by the reticuloendothelial system, the drug is mostly sequestered inside the vesicle and is not bioactive. This reduces drug exposure to healthy tissues and helps limit systemic toxicities.

Currently, there are several FDA approved nanoformulations approved for cancer therapy [nab-paclitaxel, American BioScience, Inc. [38]; and liposomal pegylated doxorubicin, Ortho Biotech Products L.P. [39]]. Recent reports suggest that liposomal pegylated doxorubicin does not affect the estrus cycle in mice, while it is widely recognized that the parent doxorubicin is highly detrimental to the ovary [40]. Thus, screening of new cancer

![Figure 3. Effect of As$_2$O$_3$ and NB(Ni,As) on ovarian cyclicity.](image-url)
therapies and new formulations for their potential as fertility sparing therapies is urgently indicated.

Though arsenic accumulated in the ovary in vitro and in vivo upon treatment with NB(Ni,As), the nanobin encapsulation sequesters the arsenic, thereby limiting its tissue distribution and

Figure 4. Effect of As$_2$O$_3$ and NB(Ni,As) on ovarian histology. Hematoxylin and eosin staining of ovarian sections from mice following 3.5-weeks of treatment with (A) PBS (4× magnification); (B) 4 mg/kg NB(NaCl) (4× magnification); (C, E, F) 4 mg/kg As$_2$O$_3$ (4× and 10× magnification); or (D) 4 mg/kg NB(Ni,As) (4× magnification). (A, B, D) Ovaries from PBS-, NB(NaCl)-, and NB(Ni,As)-treated mice show normal ovarian histology and contain follicles of all stages as well as corpora lutea. (C, E, F) Ovaries isolated from As$_2$O$_3$-treated mice contained blood filled cysts and leaky vasculature. Measurement bars represent 100 μm (A–D) and 200 μm (E, F). Follicles are indicated with arrowheads and corpora lutea are labeled “CL.” Blood-filled cysts are indicated with arrows and areas of leaky vasculature are labeled “Bl” in panels C, E, and F.

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Figure 5. Follicle survival after in vitro arsenic exposure. Isolated ovarian follicles were incubated in PBS or (A) 3, (B) 30, or (C) 90 μM As$_2$O$_3$, NB(NaCl), or NB(Ni,As) for 3 hours. Individual follicles were then encapsulated in alginate and cultured for 10 days to analyze survival rate. (A) At 3 μM As$_2$O$_3$, follicle survival was not statistically significantly different compared with PBS, NB(NaCl), or NB(Ni,As). (B) At 30 μM As$_2$O$_3$, follicle survival was significantly less starting at day 4. C, At 90 μM As$_2$O$_3$, follicle survival dropped to 30% by day 4. At all concentrations, NB(Ni,As)-treated follicle survival was not significantly different than that of PBS or NB(Ni,As). (D) Ovaries were incubated in PBS or 3, 30, or 90 μM As$_2$O$_3$, NB(NaCl), or NB(Ni,As) for 3 hours. Arsenic content in the cultured ovaries was examined by ICP-MS. Arsenic content was significantly higher in As$_2$O$_3$-treated ovaries than in PBS-, NB(NaCl)-, or NB(Ni,As)-treated ovaries at 30 and 90 μM. Arsenic content in NB(NaCl)-treated ovaries was only significantly less than in ovaries treated with the highest dose of NB(Ni,As) (90 μM). (a) is $P<0.01$ compared with PBS, (b) is $P<0.01$ compared with NB(Ni,As) at the same concentration, (c) is $P<0.01$ compared with NB(NaCl) at the same concentration. Error bars represent ±SEM.
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Figure 6. Follicle growth after in vitro arsenic exposure. Isolated ovarian follicles were incubated in PBS or (A) 3, (B) 30, or (C) 90 μM As$_2$O$_3$, NB(NaCl), or NB(Ni,As) for 3 hours. Individual follicles were then encapsulated in alginate and cultured for 10 days to analyze follicle growth. (A, B) At 3 and 30 μM, all surviving follicles grew to approximately the same size, between 200 and 250 μm. (C) At 90 μM, As$_2$O$_3$-treated follicles showed a decrease in follicle diameter over 10 days of culture, producing significantly smaller follicles than those treated with NB(NaCl) or NB(Ni,As). Error bars represent ± SEM. Asterisk represents significance of $P<0.05$.
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lessening its impact on ovarian and follicular function. Toxic effects are often correlated to the peak drug levels, and we found higher peak arsenic levels in the ovaries and follicles of animals that were treated with As$_2$O$_3$ than in those treated with NB(Ni,As). This finding is consistent with the higher toxicity of As$_2$O$_3$ compared with NB(Ni,As). Systemic treatment with As$_2$O$_3$ resulted in the formation of bloody ovarian cysts and disrupted the estrus cycle in mice, effects that were not seen with NB(Ni,As) treatment. Arsenic has been shown to regulate steroid receptors such as the androgen receptor, which has been linked to ovarian dysfunction, manifested by the development of polycystic ovaries [36]. aberrant regulation of steroid receptors in the ovary may have contributed to the observed cyst formation. Future studies will investigate the mechanism of action of arsenic trioxide on ovarian function in order to better understand its effects and develop new approaches to reducing its fertotoxicity when used as an anticancer agent.

Most importantly, our findings concerning the impact of As$_2$O$_3$ and NB(Ni,As) on ovarian and reproductive function in vitro were corroborated in our novel in vitro follicle toxicity assay. The follicle is considered to be the functional unit of the ovary; its growth and development are strictly regulated by various growth factors, hormones, and cellular interactions to permit the cyclical production of mature oocytes that are competent to undergo ovulation and fertilization [1]. The availability of three-dimensional follicle culture systems allowed us to evaluate specifically the toxicity of As$_2$O$_3$ and NB(Ni,As) on ovarian follicle viability and development in vitro. Compared with As$_2$O$_3$, NB(Ni,As)-treated follicles had higher survival and growth rates in our follicle-based assay system. This is the first report to demonstrate the capacity of an in vitro assay to assess the effect of chemotherapeutic agents on ovarian follicle function, and may be useful for estimating the potential impact of chemotherapies or combination regimens on the future fertility of young female cancer patients. More information about the fertotoxicity of agents used to treat cancer is needed as a functional unit of the ovary; its growth and development are strictly regulated by various growth factors, hormones, and cellular interactions to permit the cyclical production of mature oocytes that are competent to undergo ovulation and fertilization [1]. The availability of three-dimensional follicle culture systems allowed us to evaluate specifically the toxicity of As$_2$O$_3$ and NB(Ni,As) on ovarian follicle viability and development in vitro. Compared with As$_2$O$_3$, NB(Ni,As)-treated follicles had higher survival and growth rates in our follicle-based assay system. 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