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Nitric Oxide Donor S-Nitroso-N-Acetyl Penicillamine (SNAP) Alters Meiotic Spindle Morphogenesis in *Xenopus* Oocytes

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ABSTRACT

Nitric Oxide (NO) has been involved in both intra- and extra-cellular signaling pathways in a wide range of organisms, and can be detected in some reproductive tissues. Based upon previous results reporting that NO-donor SNAP (S-nitroso-N-acetyl penicillamine) promoted the release from the metaphase II-anaphase II block in amphibian eggs, the aim of the present study was to assess the influence of SNAP on the activation of the molecular mechanisms triggering meiotic resumption of *Xenopus* oocytes, analogous to G2/M transition of the cell cycle. A high concentration of SNAP (2.5 mM) was found to inhibit the appearance of the white spot (meiotic resumption) and promoted alteration of spindle morphogenesis leading to atypical structures lacking bipolarity and correct chromosomes equatorial alignment. The medium acidification (pH = 4) promoted by SNAP specifically impacted the white spot occurrence. However, even when pH was restored to 7.4 in SNAP medium, observed spindles remained atypical (microtubule disorganization), suggesting SNAP impacted spindle assembly regardless of the pH. N-Acetyl-D,L-penicillamine disulfide, a degradation product of SNAP with the same molecular characteristics, albeit without release of NO, yielded spindle assemblies typical of metaphase II suggesting the specificity of NO action on meiotic spindle morphogenesis in *Xenopus* oocytes. J. Cell. Biochem. 116: 2445–2454, 2015.

KEY WORDS: NITRIC OXIDE; MEIOSIS; *XENOPUS*; SPINDLE; OOCYTE

Together with the gasotransmitters carbon monoxide (CO) and hydrogene sulfide (H_2S), the short-lived endogenously produced nitric oxide (NO) is a key mediator in signaling pathways. Initially identified as an endothelium-derived relaxing factor [Palmer et al., 1987], NO is generated by nitric oxide synthases (NOS) and has been involved in the mediation of both intra- and extra-cellular signaling pathways in a wide range of organisms, including yeasts, plants, and vertebrates [Hess and Stamler, 2012; Majumdar et al., 2012]. It is involved in numerous physiological processes in immune, cardiovascular, and nervous systems [Gross and Wolin, 1995; Kröncke et al., 1995; Napoli and Ignarro, 2009]. In contrast to other signaling molecules, NO acts through a variety of chemical reactions, which depend on contexts and subtle changes in both intra- and extra-cellular media [Moncada et al., 1991]. Thus, NO effects remain complex to untangle, which is a major issue for compounds being included into therapeutic strategies.

Noticeably, NOS has been detected and isolated within mammalian reproductive tissues such as testis, epididymis, ovaries, and uterus, thereby highlighting its potential physiological role in reproductive processes: follicular growth and ovulation in mice [Sengoku et al., 2001], meiosis progression in mice, pigs, and *Xenopus* [Sengoku et al., 2001; Chmelíková et al., 2010; Jeseta et al., 2012], embryo implantation in rats [Biswas et al., 1998], and spermatogenesis in human. Although NO was suggested to exert its effects through evolutionary conserved mechanisms [Zini et al., 1996], the reported data in the above-mentioned species supported

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the hypothesis that requirements for NO might differ from a species to another, or depend on the nature of the NO donor.

In addition to the reported role of NO during cell cycle transition throughout meiosis [Sengoku et al., 2001; Chmelíková et al., 2010; Jeseta et al., 2012], the role of NO and its derivative during the cell cycle progression has been of particular interest. Cell cycle control, such as angiogenesis, invasion, and metastasis, is a cancer-related event reported to be modulated by NO [Ying and Hofseth, 2007]. However, NO has been shown to act either as a friend or a foe towards cell cycle, in a dual or antagonistic manner as it does for cancer, exerting tumor promoting effects as well as anti-tumoral effects [Choudhari et al., 2013]. For example, the NO-donor S-nitroso-N-acetyl penicillamine (SNAP) has previously been reported to prevent cell cycle progression in many cell types: cerebellar glial cells [Garg et al. 1992], BALB/C 3T3 fibroblasts [Garg and Hassid, 1990], endothelial cells [Sarkar et al., 1995], human airway smooth muscle cells [Hamad et al., 1999], lymphocytes [Kosonen et al., 1998], chick embryo retina glial cells [Magalhães et al., 2006], GH3 cells [Tian et al., 2010] and breast cancer cell lines MDA-MB-231 and MCF7 [Laudański et al., 2001]. By contrast, myoblasts were either stimulated or prevented in their proliferation, emphasizing the duality of NO towards cell proliferation [Ulibarri et al., 1999]. Molecular studies on these effects have focused on the G1-S phase transition as NO is more likely to arrest cells at the border of S phase, through the inhibition of the Cdk2/Cyclin A complex: Cdk2 expression was not altered while Cyclin A expression was repressed at the promotor level in vascular smooth cells [Guo et al., 1998; Sharma et al., 1999]. Furthermore, Cdk2 itself was inhibited by the expression of the Cdk Inhibitor $p21^{sdi1/Cip/Waf1}$ [Tanner et al., 2000]. Finally, in breast cancer cell lines MDA-MB-231, increasing NO levels by the use of NO donors was found to block cell cycle in G1, through the inhibition of Cyclin D1 expression, and the maintenance of pRb hyperphosphorylation [Pervin et al., 2001]. Therefore, strategies promoting either NO release or tempering and/or scavenging NO have become of special interest in cancer cell lines.

On the other hand, less interest has been focused on the role of NO during cell division itself, at a non-genomic level. Based on previous research reporting that NO-donor SNAP (S-nitroso-N-acetyl penicillamine) promoted the transgression of the metaphase–anaphase block in amphibian eggs [Jeseta et al., 2012], its effects during meiosis was investigated. In amphibian oocytes, meiosis is a cell division process through which a diploid cell undergoes two successive divisions without replication or depending on transcription, to produce haploid cells, namely oocytes and polar bodies. Vertebrates oocytes are arrested in prophase of the first meiotic division (PI), resume meiosis in response to hormonal stimulation, in a process called maturation, and are stopped at metaphase of the second division, in anticipation to fertilization [Bodart et al., 2002]. Release from prophase I, which is analogous to a G2/M transition, is characterized by the organization of a microtubules array pushing the germinal vesicle towards the apex of the animal cortex. The breakdown of the nuclear envelope (GVBD, Germinal Vesicle Break Down) is followed by chromosomes condensation and organization of a bipolar spindle at the plasma membrane. Anchoring of this spindle at the cortex is accompanied by the appearance of a white spot at the apex of the oocyte. Then, a second meiotic division begins without replication, after the extrusion of the first polar body, but immediately stops at metaphase [Hausen and Riebesell, 1991]. These conditions enable the analysis of the effect of compounds independently of their genotoxicity, since the onset of meiosis and the progression through the maturation process are independent of transcription [Bodart et al., 2002]. Therefore, such cellular context offers the opportunity to evaluate morphological alteration of spindle [Bodart et al., 2005], a structure crucial to genomic material segregation, that directly impacts on the molecular mechanisms driving meiosis. At the molecular level, MPF (Meiotic or M-Phase Promoting Factor), being an universal factor, promotes M-phase entry during mitosis or meiosis, and is made up of a catalytic subunit, Cdk1, and a regulatory sub-unit, Cyclin B [Norbury and Nurse, 1990]. The activity of this heterodimer is regulated by inhibitory phosphorylation on Thr14 and Tyr15, achieved by Wee1 and Myt1 kinases [Fattaey and Booher, 1997] and by regulating the level of Cyclin B, which can be degraded through the ubiquitin pathway [Rolfe et al., 1997]. MAPK xp42Mpk1 [Ferrell et al., 1991] is activated at the same time as MPF, and plays a crucial role in the establishment of the meiotic spindle [Bodart et al., 2005] and the inhibition of replication between the two meiosis divisions [Dupré et al., 2002].

The aim of the present study was to assess the ability of NO-donor SNAP in preventing the activation of the molecular mechanisms triggering meiotic resumption. In addition, the potential effect SNAP on spindle morphogenesis was investigated, having in mind that high levels could be harmless for oocyte integrity. Here, we showed that SNAP-induced effect on M-phase entry in amphibian oocytes are related to pH lowering whereas NO release appeared to alter meiotic spindle morphogenesis.

MATERIAL AND METHODS

REAGENTS AND TEST SUBSTANCES

All reagents were obtained from Sigma–Aldrich Chimie (Saint-Quentin Fallavier, France), except MS222 (Sandoz®) and collagenase (Roche Applied Science®). All tested solutions and media (ND96: 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES-NaOH, pH 7.5) were prepared daily or obtained by appropriate dilutions from stock solutions in ND96 medium.

HANDLING OF FROGS AND OOCYTES

After anesthetizing *Xenopus* females (purchased from the University of Rennes I, France) by immersion in 1 g/L MS222 solution (tricaine methane sulfonate; Sandoz), ovarian lobes were surgically removed and placed in ND96 medium (in mM: 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes–NaOH, pH 7.5). Fully grown stage VI oocytes were isolated and defolliculated by partial collagenase treatment for 30 min (1 mg/ml collagenase A, Roche Applied Science) followed by a manual microdissection. Oocytes were stored at 14°C in ND96 medium until experiments were performed. All animal experiments were performed at the animal facility of Lille University according to the rules of the European Community Council guidelines (86/609/EEC) for laboratory animal experimentation. The animal protocol was approved by the local institutional review board (Comité d'Ethique en Expérimentation Animale Nord-Pas-De-Calais, CEEA 07/2010).

TREATMENT AND ANALYSIS OF MEIOTIC RESUMPTION

Meiotic resumption was induced by incubating oocytes at 19°C in ND96 medium containing 10 μM of progesterone (4 μg/ml; Sigma–Aldrich). Maturation process (M-Phase entry) was scored by the appearance of a white spot at the animal pole of the oocyte.

Oocytes were incubated with SNAP at different concentrations (0.5, 1, 2.5, and 5 mM; Enzo lab science), N-Acetyl-D,L-penicillamine disulfide (NAP; 1.25 mM; Santa Cruz biotechnology), NaOH (2 M), and/or HCl (6 M). Oocytes were placed at 23°C. An individual observation of oocytes was performed each hour during 10 h under binocular microscope and the number of oocytes with a white spot was scored.

MICRO-INJECTION OF CYTOPLASM OF MATURE OOCYTES

Defolliculated oocytes were incubated overnight in the presence of progesterone (4 μg/ml) at 19°C. Oocytes with a white spot were selected and rinsed to remove all trace of progesterone. 40 nl of cytoplasm were withdrawn from donor oocyte, using a positive displacement digital micropipette (Nichiryo) and 10 nl were injected at the equator of recipient immature oocytes.

Before micro-injection, immature oocytes were placed during 1 h in presence of SNAP (0.5, 1, 2.5, and 5 mM; Enzo lab science), NAP (1.25 mM; Enzo lab science), NaOH (2 M), and/or HCl (6 M). Micro-injected oocytes were placed at 23°C for maturation and the white spot appearance was monitored hourly.

ELECTROPHORESIS AND WESTERN-BLOTTING

Eggs were lysed in homogenization buffer and centrifuged for 5 min at 10,000*g* (4°C) to eliminate yolk platelets. Supernatants were added with one volume of Laemmli 2X buffer 4% beta-mercaptoethanol, heated at 100°C for 3 min and stored at −20°C until analysis. Proteins from oocytes were separated by SDS–PAGE (15–17%) and transferred onto a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech, United Kingdom). Blots were blocked with 10% low fat dry milk and incubated with specific antibody. $p90^{Rsk}$ was detected using the polyclonal rabbit antibodies (p90Rsk1 C-21 sc231, Santa Cruz Biotechnology), ERK2 was detected using mouse monoclonal antibodies (Erk2 D-2 sc1647, Santa Cruz Biotechnology), anti-P-H3 antibodies (S10, Cell Signaling), and P-Tyr15 Cdk1 using overnight incubation with mouse monoclonal antibody (tyr15, Cell signaling). Nitrocellulose membranes with bounded primary antibody were then incubated with appropriate secondary antibodies (Sigma–Aldrich). The signals were detected *via* a chemiluminescent assay (Clarity™ Western ECL Substrate, Bio-Rad).

CYTOLOGICAL ANALYSIS

Oocytes were fixed in Smith reagent (Smith A: Potassium Bichromate 17 mM ; Smith B: formol and acetic acid 80/20%) for a minimum period of 12 h. They were then dehydrated and embedded in paraffin. Section of 7 microns were cut and stained with the nuclear red (0.1 g of nuclear red QSP 100 ml aluminium sulfate 5%) to reveal nuclear structures and chromosomes, and with picroindigo carmine (0.25 g of picroindigo carmine QSP 100 ml saturated picric acid) for cytoplasmic structures. Such staining enable spindles and condensed chromosomes detection, even if ectopic or atypic [Flament et al., 1996,1997].

STATISTICAL ANALYSIS

All results are shown as mean +/− standard error of the mean (SEM); N refers to the number of separate experiments performed (number of females) while n is the number of treated oocytes. Experiments were at least performed in triplicates. Significant differences were assessed with SigmaStat 3.1 software (SysStat, Erkrath, Germany) by means of one-way analyses of variance followed by *post-hoc* Tukey's tests. Statistical significance was accepted for $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

RESULTS

NO-DONOR SNAP ALTERS M-PHASE ENTRY AND MEIOTIC SPINDLE MORPHOGENESIS INDUCED BY PROGESTERONE

PI oocytes can be characterized at the histological level by the presence of a germinal vesicle (VG). After addition of progesterone in the medium, the oocyte undergoes progression through meiosis: the first sign is the migration of the germinal vesicle at the animal pole pushing aside the subcorticals pigments, thereby leading to the appearance of a white spot. This phenomenon characterizes the first step of maturation, with the breaking of Germinal Vesicle BreakDown (GVBD). Oocyte maturation is referred to as the set of physiological, biochemical, and electrophysiological events leading from prophase I to metaphase II. Prophase I (PI) and metaphase II (MII) oocytes exhibit different profiles both at the histological and biochemical levels.

Low concentrations of SNAP (500 μM and 1 mM) had no effect on the meiotic resumption process rate induced by progesterone in *Xenopus* oocyte. There was no delay in white spot appearance between the lowest concentrations of SNAP and the control progesterone-treated oocytes (Fig. 1A): 90 ± 3.85, 88.19 ± 5.70, and 82.80 ± 7.22% were obtained in progesterone alone or in association with 500 μM and 1 mM of SNAP, respectively (Fig. 1B). In contrast, at higher concentrations of SNAP (2.5 and 5 mM), meiotic resumption (or M-phase entry) was delayed, and the percentage of white spot dropped to 32.12% and 11.70% in the presence of 2.5 and 5 mM SNAP, respectively (Fig. 1B). As previously reported [Jeseta et al., 2012], SNAP releases high levels of NO after 1 h. This release was dose-dependent, accordingly to the literature.

Following GVBD, a bipolar microtubule spindle is formed at the vicinity of the plasma membrane and the chromosomes are aligned into an equatorial plate. Cytological analysis revealed that the higher the concentration of SNAP, the earlier the maturation is inhibited progression. The control oocytes exposed, or not, to progesterone exhibited respectively metaphase II and prophase I (PI) cytological profiles. In the presence of 500 μM SNAP, 50% of oocytes were in MII, 33% were blocked at metaphase I (MI) and 17% at GVBD, without exhibiting any normal organization. With 1 mM SNAP, 57% of oocytes were blocked in MI phase whereas with 2.5 mM SNAP 43% were blocked in MI and 41% of oocytes were arrested at GVBD stage (Fig. 1C). Similarly, high concentrations of SNAP were correlated with high percentages of atypical spindles observed (disorganization of microtubules). At 2.5 mM SNAP, more than half of the spindles were atypical, lacking distinct poles, and failing to establish equatorial alignment of chromosomes on a plate (Table I and Fig. 1D).

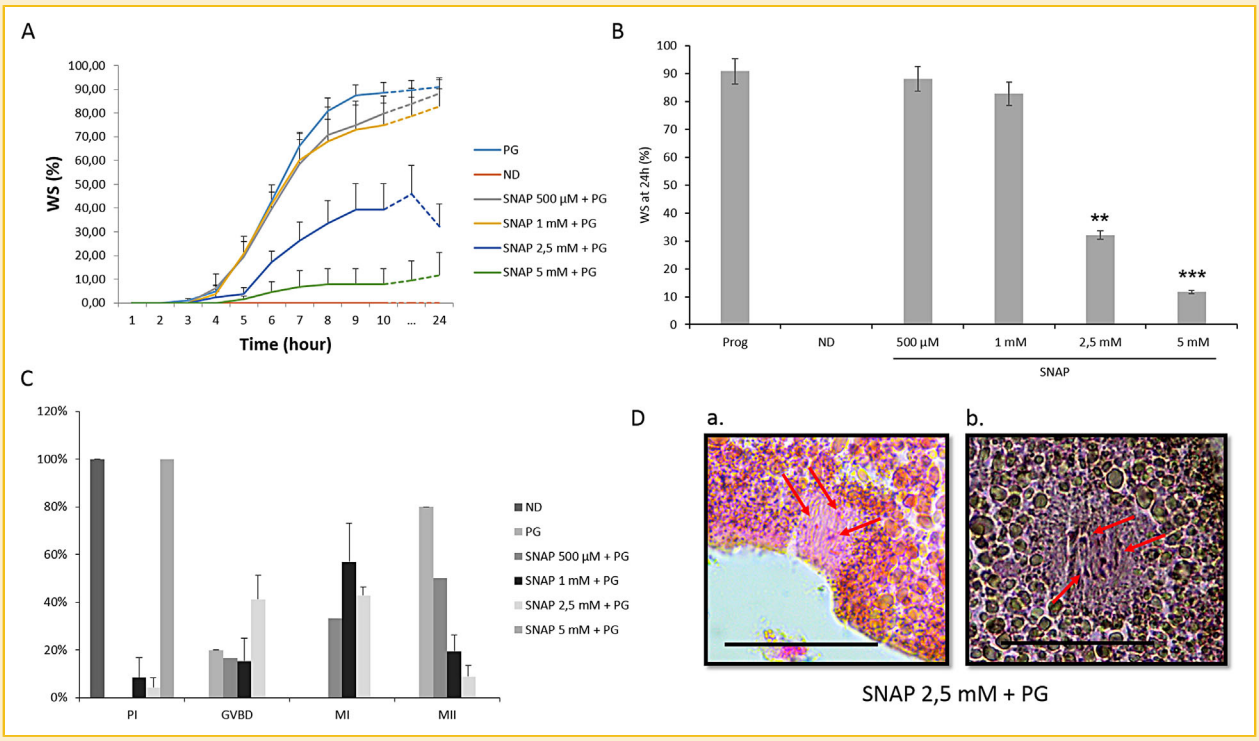

Fig. 1. NO donor SNAP alters M-phase entry and meiotic spindle morphogenesis induced by progesterone. (A) Oocytes were incubated without or with different concentrations SNAP and progesterone (10 µM). Oocytes were either maintained in culture medium without treatment (ND) or in progesterone alone (PG). Oocytes exhibiting white spots were recorded every hour until 10 h and at 24 h; (B) percentage of white spot observed 24 h after addition of progesterone and SNAP at different concentrations; (C) percentage of oocytes found in different phases of meiosis: Prophase I (PI), Germinal Vesicle Break Down (GVBD), Metaphase I (MI), and Metaphase II (MII). Oocyte showing only a spindle anchored to the membrane in cytological analysis were considered as metaphase I (MI) oocytes. First meiosis division leads to the appearance of a polar body and the second meiotic division begins. MII oocytes can be defined by the presence of the first polar body and the presence of a spindle anchored to the membrane. Here, oocytes were treated with progesterone alone or in association with SNAP at different concentrations. The experiment was performed three times on three different females. (D) Atypical spindles in oocyte treated with 2.5 mM SNAP. a. Atypical spindle with unaligned chromosomes. b. Ectopic spindle in deep cytoplasm with unaligned chromosomes. Bar = 20 µm. N = 7; n = 20. Statistical significance was accepted for $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Arrows indicate chromosomes. Please refer to Figure 5A for comparison with normal spindle.

In order to check if oocytes exhibiting no white spot were still blocked in prophase I, the phosphorylation status of Erk and Rsk (MAPK cascade), Cdc2 (MPF), and Histone H3 (downstream MPF) were analyzed by western blot on individual cells. While prophase I oocytes exhibited phosphorylation of Cdc2 on tyrosine 15 and absence of Histone H3, Erk, and Rsk phosphorylation, mature oocytes showed phosphorylation of Histone H3, Erk, and Rsk and dephosphorylation of Cdc2 on tyrosine 15. Analysis of oocytes treated with SNAP did not reveal any homogenous response at the biochemical level. Indeed, 67.6% of oocytes exhibited a phosphorylation of Histone H3 with an active MAPK cascade (phosphorylated Erk and Rsk) but an inactive MPF profile (Cdc2 was phosphorylated on Tyrosine 15) (Fig. 2, supplementary Fig. 1 and supplementary Tables I and II). This heterogeneous response might reflect antagonistic effects of NO within the different signaling pathways.

TABLE I. SNAP Increased the Atypical Spindle Rate in a Dose-Dependant Manner Upon Progesterone Stimulation

	Spindle	Atypical spindle	Total
ND	0	0	15
PG	12	0	15
SNAP 500 µM + PG	11	0	15
SNAP 1 mM + PG	19	4 (21.1%)	25
SNAP 2,5 mM + PG	14	8 (57.14%)	26

This table shows the number of oocytes with a spindle and the number of oocytes with an atypical spindle (disorganization of microtubules and chromosomes dispersion). Oocytes were incubated during 24 h with progesterone alone or with SNAP at different concentrations.

NO-DONOR SNAP ALTERS M-PHASE ENTRY AND MEIOTIC SPINDLE MORPHOGENESIS INDUCED BY MATURE OOCYTE CYTOPLASM INJECTION

Micro-injection of mature oocyte cytoplasm, containing active MPF, triggers Cdc25 activation in prophase I oocytes (Masui and Markert, 1971; Rime et al., 1994). In turn, Cdc25 activates an inactive stock of MPF by Cdc2 dephosphorylation and enables oocyte meiotic resumption in absence of any hormonal stimulation (MPF auto-amplification loop). Low concentrations of SNAP (500 µM and 1 mM) showed no significant effect on the meiotic resumption of *Xenopus* induced by oocyte micro-injected with cytoplasm of MII oocytes. Indeed, 7 h after the micro-injection, 83% of control oocytes exhibited a white spot against 67 and 55% in the presence of 500 µM

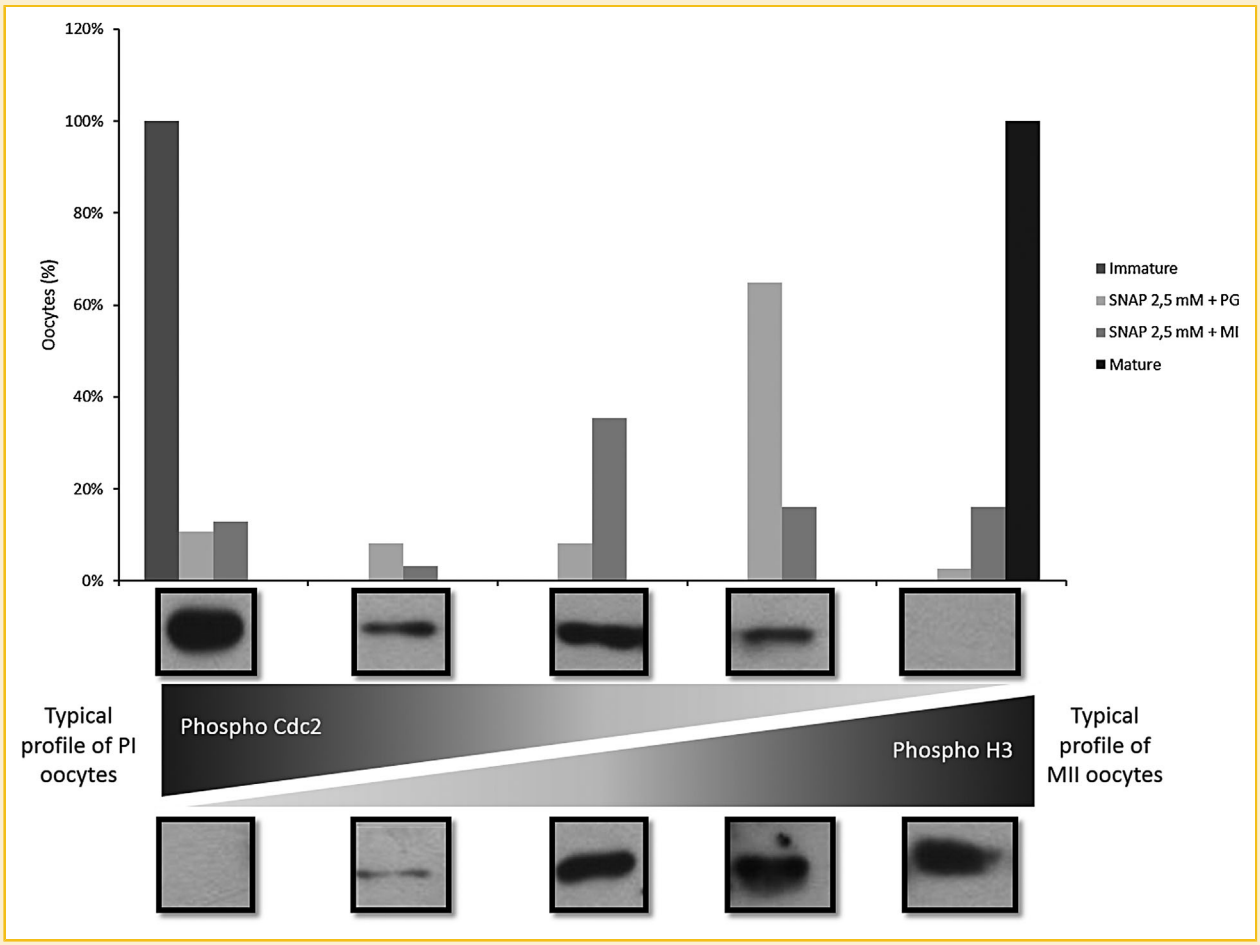

Fig. 2. Biochemical analysis of MPF activation in presence of SNAP 2.5 mM (Cdc2 tyr15 and Histone H3 phosphorylations patterns in oocytes). Immature oocytes were non-treated oocyte and, presented no white spot. Mature oocytes were treated with progesterone only (or micro-injected with cytoplasm of mature oocyte) and exhibited white spots. SNAP 2.5 mM + PG and SNAP 2.5 mM + MI are oocyte treated with SNAP 2.5 mM during 15 min and exposed to progesterone or injected with cytoplasm of mature oocyte, respectively. In these conditions, no oocyte exhibited white spot at the apex of the cell. Typical western blot patterns are shown.

and 1 mM of SNAP, respectively (Fig. 3A). A 2.5 mM concentration of SNAP inhibited the appearance of a white spot in micro-injected oocytes, underliying a potential sensitivity of the MPF auto-amplification loop towards NO (Fig. 3A).

Morphological analysis was completed by histological study. Control oocytes, micro-injected (micro-inj) or not (ND) were mainly blocked in MII and PI phases, respectively. In the presence of SNAP at 500 μM, oocytes stopped at MI and GVBD phases (43 and 39%, respectively). Then, in the presence of 1 mM or 2.5 mM of SNAP, 37 and 61% of the oocytes were respectively blocked at GVBD (Fig. 3B). As with progesterone stimulation, spindles became atypical when the highest concentration of SNAP was used. With 2.5 mM SNAP, all spindles observed were found to be atypical (Table II).

Oocytes without external morphological sign of meiotic resumption were individually analyzed at the biochemical level. Only 12% of oocytes (4/31) showed typical profiles. Heterogeneity was noticed in the detected profiles (Fig. 2 and Supp. Tables III and IV). Nevertheless, 68.1% of oocytes exhibited phosphorylation of histone H3, relevant for MPF activity.

These results therefore suggest that signaling pathways were able to activate in the presence of NO-donor. However, morphological events associated to meiotic resumption were altered within these conditions.

ACID PH PROMOTED BY SNAP SPECIFICALLY INHIBITS THE WHITE SPOT APPEARANCE

SNAP is a NO-donor that acidifies culture medium. Therefore, the respective role of the SNAP-induced NO release and/or pH decrease on M-phase entry and meiotic spindle morphogenesis was determined. The highest concentration of SNAP induced a pH decrease close to 4 (data not shown). In previous studies, intracellular acidification was shown to delay hormonal-induced meiotic resumption and alter spindle morphogenesis in *Xenopus* oocytes [Sellier et al., 2006]. Twenty-four hours after addition of progesterone, 89.19% of the control oocytes exhibited a white spot while only 1.11% oocyte showed white spot occurrence in acidic media. When pH was buffered with NaOH to 7.4, GV migration and then white spot occurrence were restored (79.89%). The percentage

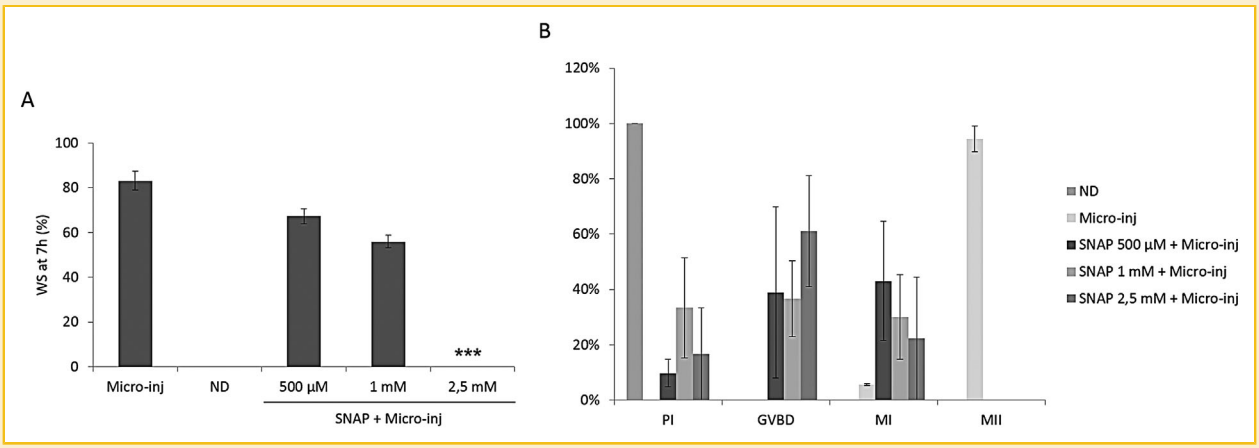

Fig. 3. NO donor SNAP alters meiotic resumption (M-phase entry) and meiotic spindle morphogenesis induced by mature oocyte cytoplasm injection. (A) Oocytes were incubated without or with increasing concentrations of SNAP during 15 min and micro-injected with cytoplasm from mature oocyte. Oocytes showing white spot were recorded after 7 h. Oocytes were also maintained in culture medium without treatment (ND) or micro-injected with cytoplasm from mature oocyte alone (micro-inj). All experiments were performed in triplicate, using oocytes from three different females (15 oocytes per condition). (B) Percentage of oocytes in the different steps of meiosis: Prophase I (PI), Germinal Vesicle Break Down (GVBD), Metaphase I (MI), and Metaphase II (MII). The oocytes micro-injected with cytoplasm from mature oocyte were cultured without further treatment or in the presence of SNAP at different concentrations. N = 3, N = 15. Statistical significance was accepted for $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

of oocytes showing a white spot, which dropped to 0% in the presence of 2.5 mM SNAP alone, was also restored up to 87.78% in buffered solution (Fig. 4A). Nevertheless, more than half of the spindles remained atypical and ectopic—not located at the plasma membrane—under the latter conditions.

Similarly, when maturation was induced by cytoplasm micro-injection, we observed that an acidic medium significantly impaired meiotic resumption and that NaOH-buffering media restored GV migration and morphological signs of meiotic resumption (Fig. 4B). Surprisingly, the effects of 2.5 mM SNAP were reversed by buffering pH: 75.67% of white spots were detected. Nevertheless, 93.33% of these oocytes still exhibited ectopic and atypical spindles.

ATYPICAL SPINDLE ASSEMBLY IS NO RELEASE DEPENDENT

The latter observations suggest the effect of SNAP on the spindle assembly is independent of any pH variation and only dependent of the NO release. To validate this hypothesis, N-Acetyl-D,L-penicillamine disulfide (NAP), a degradation product of SNAP, was employed. Essentially, NAP exhibits the same molecular characteristics as SNAP, but does not release NO and is commonly used as a negative control when studying SNAP [Babich et al., 1999; Bourdon et al., 2003; Chen et al., 2005]. Oocytes treated with progesterone showed typical characteristics of MII oocytes with no alteration of spindle morphogenesis (Fig. 5A). Oocytes exposed to SNAP showed atypical spindles (disorganized microtubules) with no aligned chromosomes (70%), which were maintained even in restored pH NaOH-buffered media (81.25%, Fig. 5B/C). By contrast, when SNAP was replaced by NAP and pH was buffered, abnormal spindles were scarcely observed: 8.88% of the total spindles remained atypical. This result suggests that without NO release, there is no effect of SNAP on microtubule organization and spindle bipolarity (Fig. 5D). Therefore, SNAP-induced effect on spindle assembly appears to be NO-dependent.

TABLE II. SNAP Increased the Atypical Spindle Rate in a Dose-Dependant Manner Upon Micro-Injection From Mature Oocyte Stimulation

	Spindle	Atypical spindle (%)	Total
Micro-inj	17	0	17
ND	0	0	18
SNAP 500 µM + Micro-inj	9	2 (22.22%)	18
SNAP 1 mM + Micro-inj	6	4 (66.67%)	21
SNAP 2,5 mM + Micro-inj	4	4 (100%)	16

This table shows the number of oocytes with a spindle and the number of oocytes with an atypical spindle. Oocytes were micro-injected with cytoplasm of mature oocytes alone (micro-inj) or in the presence of SNAP at different concentrations.

DISCUSSION

Gasotransmitters such as nitric oxide (NO) have been involved in many physiological and pathological processes, making them also of interest for pharmacopeia. Indeed, NO-Donor therapies, including SNAP, are considered as treatment hypoxic respiratory failure in neonates, soft tissues healing and regeneration. NO-delivering polymers are currently developed to increase NO's ability to reach more tissues. Such efforts are performed due to the efficiency of these therapies [VanWagner et al., 2013]. Nevertheless, the innocuousness of high level of SNAP remains to be fully established. Among the variety of functions impacted by NO level changes, there have been several reports in the field of reproductive biology regarding gametes and early embryos [Sengoku et al., 2001; Chmelíková et al., 2010; Jeseta et al., 2012]. Many experiments have been carried out on cancer pathology (for review please see Ying and Hofseth, 2007; Choudhari et al., 2013). Studies perfomed in cancer cell lines focused on the effects at the transcription level, which revealed inhibition of

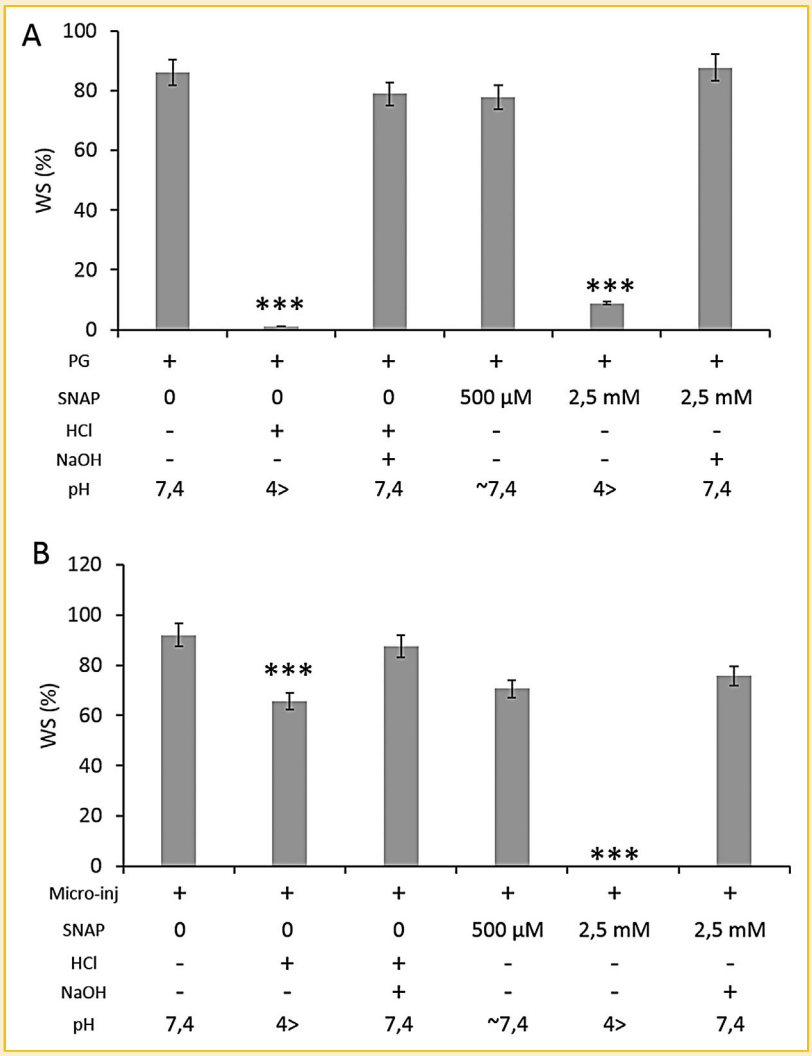

Fig. 4. Acid pH promoted by SNAP inhibits the white spot appearance. Oocytes were incubated (or not) in SNAP at different concentrations, the culture medium (ND96) being buffered or not, during 15 min. Then oocytes were treated with progesterone (A) or injected with cytoplasm of mature oocytes (B). Oocytes with white spot were recorded after 7 h. N = 5, N = 15. Statistical significance was accepted for $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Cdk-Cyclin complexes responsible for the G1/S transition, either through the down-regulation of Cyclin expression levels or up-regulation of Cdk inhibitor expression [Ishida et al., 1997; Guo et al., 1998; Sharma et al., 1999; Tanner et al., 2000; Pervin et al., 2001). In the present study, the role of NO during the early steps of cell division during meiosis was addressed, taking advantage of the *Xenopus* oocytes release from prophase being considered as analogous to G2/M (or M-Phase entry), in a non-genomic context [Bodart et al., 2002]. The aim of this study was also to assess the harmlessness of SNAP on physiological transitions of the cell cycle at both molecular and cytological levels. Either triggered by hormonal stimulation (progesterone) or by mature oocyte cytoplasm injection, M-phase entry was delayed by NO-donor SNAP: occurrence of white spots was delayed and spindle organization was disturbed: microtubules were disorganized and failed to establish a bipolar axis and chromosomes appeared dispersed in the bulk of microtubules.

As decomposition of SNAP, and then NO release, was accompanied by a pH decrease, the effects relative to pH in comparison of those of NO itself were discriminated. In *Xenopus* oocytes, the resumption of meiosis is accompanied by a transient alkalization [Lee and Steinhardt, 1981], which, when buffered by MOPS pH 6.9, hinders the migration of germinal vesicle towards the animal pole and promotes ectopic spindle formation in deep cytoplasm in 30% of cases [Flament et al., 1996]. Similarly, acidification induced by procaine alters spindle positioning, which remain in the center of the cells [Flament et al., 1997]. Based on the latter studies, it appeared that microtubules apparatus involved in germinal vesicle migration was affected by pH decrease, but that the spindle morphology *per se* was not affected. Furthermore, the authors of the present study previously reported that decreasing pH with NH_4Cl delayed progesterone-induced GVBD in a dose-dependent manner. Under the conditions used herein, pH buffering in SNAP solutions

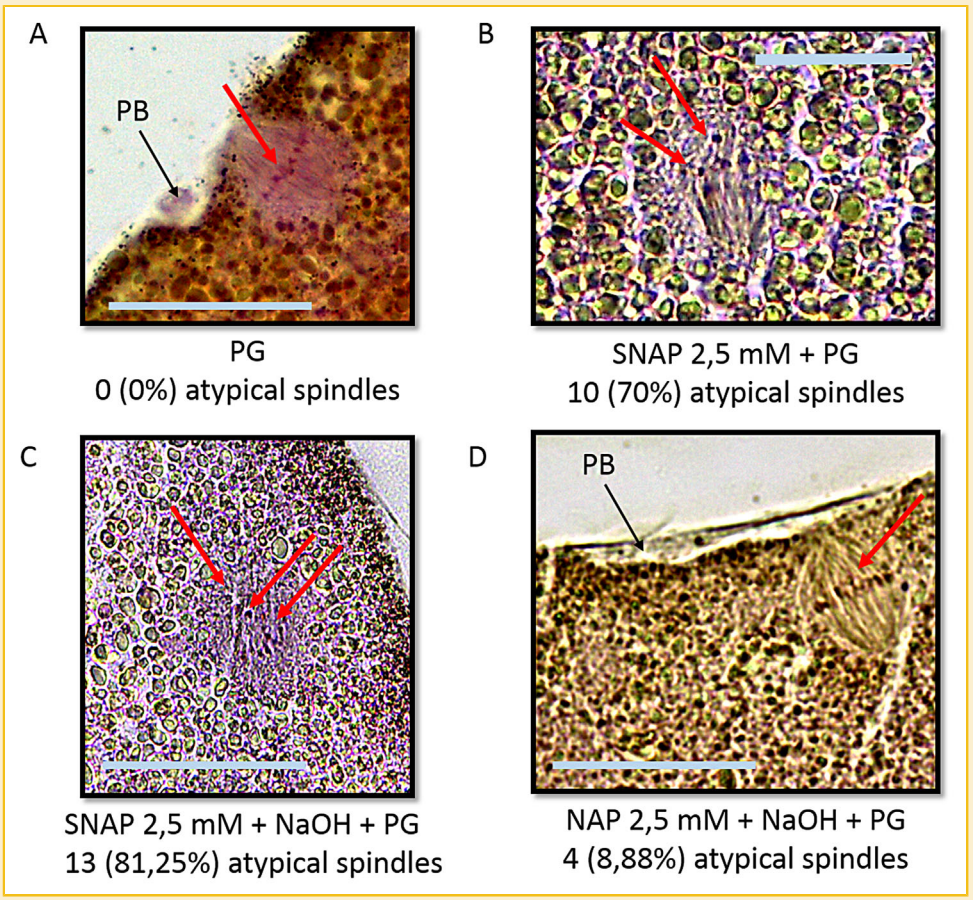

Fig. 5. Atypical spindle assembly is NO-released dependent. Representative spindles observed in the different experimental conditions. Red arrows indicate the chromosomes. The total number of atypical spindles is indicated for each condition. (A) Mature oocyte (MII), treated with progesterone (PG), showing spindle membrane anchored with aligned chromosomes and the first polar body (PB). (B) Oocytes exposed to SNAP 2.5 mM and progesterone, showing atypical spindle (disorganization of microtubules) with unaligned chromosomes. (C) Oocytes exposed to SNAP 2.5 mM and progesterone in buffered pH with NaOH. These oocytes show atypical spindle with unaligned chromosomes. (D) Oocytes exposed to NAP 2.5 mM and progesterone, in NaOH-buffered medium. These oocytes typically exhibit spindle membrane anchored with aligned chromosomes and a first polar body. Bar = 20 μm. All experiments were performed in triplicate, using oocytes from three different females.

prevented SNAP-induced inhibition of M-phase entry but not meiotic spindle morphogenesis alteration, regardless to the type of M-phase entry stimulation. Therefore, the effects on meiosis kinetics were more likely to be attributed to pH changes than to NO release itself.

A differential sensitivity towards SNAP was observed between progesterone and cytoplasm micro-injection-induced mechanisms of meiotic resumption or M-phase entry. The two mechanisms are quite different since progesterone requests protein synthesis and activation of both MPF and MAPK pathways for meiosis completion until metaphase II, while injection of active MPF within the mature oocyte cytoplasm triggers MPF auto-activation loop, which involves Cdc25. In order to investigate the difference of sensitivity between progesterone and microinjected oocytes, western blots were performed with individual oocytes treated with 2.5 mM SNAP followed by mature oocyte cytoplasm injection. The observations made at the molecular level suggested that the majority of oocytes exhibited a biochemical activation profile, similar to those of positive control oocytes that resumed meiosis.

Proteins S-nitrosylation formally occurs through an oxidative reaction of NO and Cys thiol, and have been involved in several pathologies [Foster et al., 2009b]. In the case of the molecular network herein described as responsible for M-phase entry, Cdc25 isoforms were identified in other studies as Cys-dependent phosphatases, exhibiting a Cys-containing catalytic loop that is recognized for S-nitrosylation target [Foster et al., 2009a]. Different studies showed that nitrosative stress, that is, a dramatic increase in NO levels, resulted in Cdc25 inhibition [Tomko and Lazo, 2008; Majumdar et al., 2012]. If one considers that Cdc25 is impacted by S-nitrosylation [Foster et al., 2009b; Tomko et al., 2009], one might have expected a predominant presence of an inactive profile of MPF, with phosphorylated tyrosine 15. On one hand, it might be hypothesized that *Xenopus* Cdc25C is not sensitive towards NO as much as the human Cdc25 isoforms. On the other hand, the MPF injected within the cytoplasm might be sufficient to promote a partial activation of the auto-amplification loop or direct phosphorylation of Histone H3. A pH effect on protein phosphatases was ruled

out at this stage as it was previously demonstrated that the auto-amplification loop of MPF was not affected by pH changes [Sellier et al., 2006].

Once established that delay and inhibition of M-phase entry were attributed to pH-related effects of NO-donor SNAP, the alteration of spindle morphology was assessed in relation to NO effects. Two different strategies were employed. NO-chelator CPTIO was first used but the latter failed to hinder the alteration of the spindle induced by SNAP (data not shown). As NO-chelation failure could not be ruled out, as well as persistence of microdomains of NO-increased levels, NAP was subsequently used, and provided the most reliable negative control. When oocytes were stimulated for meiotic resumption in the presence of a pH 7.4 buffered solution of NAP, no alteration of spindle morphogenesis was recorded, suggesting that NO released by SNAP was responsible for the lack of bipolarity establishment and chromosome misalignment. Furthermore, due to the heterogeneity of treated oocytes with SNAP for MPF and MAPK pathways, no correlation could be established between these proteins and spindle phenotype.

Thus, microtubules assembled as a disorganized bulk and failed to establish a bipolar axis in the presence of high levels of nitric oxide. In this structure, the chromosomes were dispersed and never exhibited a correct alignment. Interestingly, it was reported that NO inhibits mitosis through Tyrosine residue nitrosylation in plant models, and alters cross walls orientation, presumably by impairing microtubule organization [Jovanović et al., 2010; Blume et al., 2013]. In other eukaryotic models such as neuronal cells, spermatozoa and red blood cells, both cytoskeleton elements themselves (microtubules, actin, [Jaffrey et al., 2001; Lefièvre et al., 2007]), or microtubule-associated proteins [Lefièvre et al., 2007; Stroissnigg et al., 2007; Grau et al., 2013] are affected by nitric oxide changes. Therefore, S-nitrosylation may alter cytoskeleton at several levels. The present study demonstrated that high concentrations of NO donor SNAP resulted in premature maturation inhibition in *Xenopus* oocytes and alteration of meiotic spindle morphogenesis. Nitric oxide was found to impair spindle morphogenesis in several aspects mentioned above. Further work is required to identify the proteins targeted by NO, which are involved in the formation of the spindle and subjected to S-nytrosilation. It is important to notice that these atypical spindles mostly presented the same formation defect: nonaligned chromosomes, defect in the microtubule assembly and mainly ectopic spindles.

Together, these observations suggest that NO would more specifically affect cytoskeletal proteins. Studies in animals and plants models have already suggested that S-nytrosilation can impact proteins such as the Microtubule-associated protein 4 [Greco et al., 2006], Tubulin α 6 chain, Tubulin α 4 chain, and Actin 2/7 [Lindermayr et al., 2005]. It must also be noted that spindle morphogenesis is a self-organized process, depending on microtubule dynamic, microtubule associated proteins, and motor proteins interactions. As a result, any disorganization of the spindle is said to be the consequence of the S-nitrosylation of one of these components. Further work is therefore required to identify which of these components is affected when using *Xenopus* oocytes as a model.

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