Seasonal effects on the outcome of reproduction tests with silver nanoparticles, silver nitrate and the Collembola *Folsomia candida*

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Abstract

Background

Toxicity of silver nanoparticles (AgNP) are increasingly studied due to a rise in application in various products. Various studies on AgNP toxicity with terrestrial and aquatic organisms confirmed their negative effects. In our previous experiments, strong variability was observed in the reproduction of Collembola in different seasons. To investigate the effects of silver nanoparticles (AgNP) on the reproduction of Collembola in different seasons, *Folsomia candida* were exposed to AgNP and silver nitrate ($AgNO_3$) at a concentration of 30 mg/kg dry soil for 28 days. The reproduction tests were repeated

during different seasons throughout one year in order to assess if animals' sensitivity varied with the season.

Results

Significantly lower reproduction was found in the control in winter with only 101 (± 7) juveniles per adult, compared to 126-158 individuals in other seasons. Strong toxic effects (inhibition of reproduction by up to 50%) were observed during summer, spring and autumn in both treatments. However, AgNP showed no toxic effects on the reproduction of *F. candida* in winter. The relative toxicity of both substances varied with the seasons: AgNP were more toxic than $AgNO_3$ in spring and summer, and less toxic in autumn and winter.

Conclusion

These findings indicate that seasonal effects on the reproduction of *Folsomia candida* are significant. Moreover, we demonstrated the reproductive toxicity of AgNP in soil at a much lower concentration than reported thus far. These effects can mainly be attributed to soil conditions, which raises concern whether these commonly used test substrates are really protective.

Keywords: Silver nanoparticles; reproduction test; toxicity; Collembola; seasonal effects; *Folsomia candida*

Background

The application of silver nanoparticles (AgNP) is strongly increasing in several areas electrical, medicine, food and textile products. AgNP can be released from these products during washing [1], disposal, and industry wastewater [2,3]to the environment. Up to 90%

of Ag remains in sewage sludge in which the estimated Ag annual increase is 1.6 µg/kg [4,5]. There are concerns about unintended exposure of humans and the environment to AgNP [6], resulting in a large research effort into the hazards and behaviour of AgNP in the environment [7]. Numerous aquatic toxicity tests with AgNP and silver ions using a wide range of species have resulted in classifying AgNP as “particularly toxic” [8-10]. Research studies focusing on terrestrial matrix have shown that soil pH, texture, organic matter, and ionic composition could affect the toxicity of AgNP to soil invertebrates [11,12]. Negative effects of AgNP were shown for reproduction, survival and growth of nematodes [13-15], earthworms [15-19] and enchytraeids [19]. The toxicity of AgNP for Collembola was first reported by Waalewijn-Kool et al. [20], who observed no effect on survival and reproduction for *Folsomia candida* (*F. candida*) exposed to AgNP (3-8 nm coated with paraffin) at a measured concentration of 673 mg Ag/kg dry soil. Mendes et al. (2015) [21] reported a negative effect of AgNP (NM-300K) on the reproduction of *F. candida*, with EC_{20} and EC_{50} values of 173 and 540 mg Ag/kg, respectively.

Collembola are an essential part of soil ecosystems and are among the most abundant arthropods on earth with a long evolutionary history [22]. Their diet is mainly composed of fungi and bacteria. As they are vulnerable to soil contamination, the abundance and diversity of Collembola have been widely used to assess the environmental impact of a range of pollutants in soils [23]. Filser et al. [24] introduced a minaturised reproduction test of *F. candida* according to the OECD 232 (2009) [25] standard reproduction test in which 4 adults and 10 g soil were used instead of 10 adults and 30 g soil.

In previous experiments (X. Zhang, unpublished data), strong variability was observed in the reproduction of *Folsomia candida*: in summer, it was twice as high as during winter. Therefore, in the present study, the miniaturized approach was used to evaluate the toxicity of AgNP and $AgNO_3$ on the reproduction of *F. candida* during four seasons throughout one year. We hypothesized that (a) the reproduction of *F. candida* is different during four seasons and (b) the toxic effects of AgNP and $AgNO_3$ vary between seasons.

Materials and methods

Chemicals

$AgNO_3$ (purity 99.0%, Sigma-Aldrich, Steinheim, Germany) was used to provide a reference for dissolved silver toxicity as negative control. AgNP were NM-300K, a representative manufactured dispersion containing uncoated spherical nanoparticles (diameter: 15 nm), which has been used in a variety of studies and projects including the OECD Working Party on Manufactured Nanomaterials sponsorship programme. As a dispersion in stabilizing agents, NM-300K contains 4% w/w each of polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan monolaurat (Tween 20) with a silver content of 10.16% by weight [26]. It was distributed by the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME) and provided by Joint Research Centre of the European Commission as a part of the UMSICHT project (BMBF 0340091A).

Test soil

RefeSol soils were selected as reference soils by the German Federal Environment Agency, and they matched the properties stated in various OECD terrestrial ecotoxicological guidelines. In this study we used RefeSol 01-A (provided by the

Fraunhofer IME, Schmallenberg, Germany), a loamy sand soil with a pH of 5.67, 0.93% organic carbon, 71% sand, 24% silt, and 5% clay.

Soil preparation and reproduction test

Stock solutions of $AgNO_3$ and AgNP were prepared by diluting both substances with deionized water. The flasks with stock dispersions of AgNP were placed in an ultrasonic bath (Bandelin sonorex RK 100H with an output of 160 W, 35 kHz) and sonicated for 20 min before use. After the dispersions and solutions were prepared, they were added to the soil to obtain a concentration of 30 mg Ag/kg dry soil. In order to obtain a homogeneous distribution, the test substance solution was first added to a small portion of the soil (20 g), which then was mixed to the final test soil thoroughly with a spoon. All soil samples were adjusted to 50% of the maximum water holding capacity and thoroughly mixed. Additionally, a control without any chemicals was included in each study. For each treatment and control, 6 replicate glass vessels (30 mL) were filled with 10 g prepared soil one day before the test began. For each test treatment and control, three replicate soil samples were analysed for pH (Jürgens, WTW, Weiheim, Germany) at the end of the reproduction test. The mean soil pH_{Cacl2} was 5.59 (SD = 0.39) in the control, 5.57 (SD = 0.45) in soil spiked with AgNP and 5.44 in soil spiked with $AgNO_3$ (SD = 0.46). No significant differences were detected during the four seasonal studies between treatments and control. The reproduction test was carried out following a miniaturized reproduction test of OECD 232 [24], i.e., 4 individuals and 10 g soil per test unit. *F. candida* were taken from our lab culture, originally obtained from the working group of Professor Achazi at Freie Universität Berlin in the early 1990s. To synchronize *F. candida*, adults were placed

in a breeding container for 3 days to lay eggs and then were removed. After hatching, four 9-12 day-old juveniles were placed randomly in each test vessel. The vessels were incubated in a climate chamber (Sanyo MLR-350H) at 20 °C with a 12-hour light/12-hour dark cycle with 80% humidity and 500 Lux illumination. During the test, 5 pieces of dried baker's yeast (Dr. Oetker) were added to the animals twice a week, and the old food bunches were removed. The test vessels were aerated twice a week, and moisture content of the soil was kept constant at 50% of the maximum water holding capacity by replenishing the water loss once a week. After 28 days of exposure, 100 mL deionized water was added to each test container, and the soil was transferred to a plastic container. *F. candida* floating on the surface of the dispersion were visible after adding 2 drops of ink to the water. A picture was taken of each container, in order to count the juveniles using Image J 1.46r software package. The same procedure was repeated four times (spring, summer, autumn and winter) during one year to examine seasonal effects.

Statistics

Statistical analyses were performed with SPSS 17.0 and R 3.4.0. For the reproduction test, data were log-transformed to obtain normal distribution (according to Shapiro-Wilk test), and a general linear model (GLM) was used to analyse the main effect and interactions of treatment and season as influencing factors. For comparing the toxicity within each season, additionally one-factorial models were run with R.

Results

Reproduction in different treatments during four seasons

The controls in all reproduction tests met the validity criteria according to OECD guideline 232. Mortality did not exceed 20%, and did not differ between the treatments (p=0.628). The mean number of juveniles per vessel was not lower than 100 for each replicate control (n=6), and the coefficient of variation of reproduction was less than 30% (Table 1).

Table 1: Summary of juveniles per introduced adult and validity criteria in the treatments during four seasons (n=6)

Season	n	AgNO3		AgNP		Control	
		Mean	CV	Mean	CV	Mean	CV
Spring	6	96	0.21	78	0.15	126	0.27
Summer	6	93	0.14	73	0.16	146	0.25
Autumn	6	89	0.21	113	0.04	158	0.08
Winter	6	78	0.09	91	0.12	101	0.07

Collembola reproduction was significantly affected by both season and treatment. The reproduction in the control was lowest during winter, followed by spring, summer and autumn (Figure 1). In the AgNP treatment, the highest reproduction was detected during autumn whereas in soil spiked with $AgNO_3$, *F. candida* reproduction did not differ between the four seasonal tests (Table S1-2 in additional file).

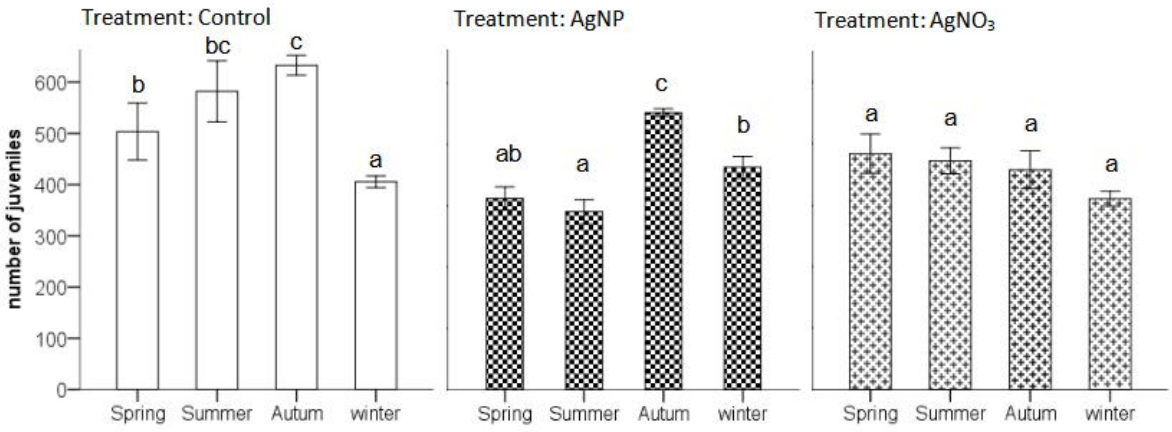

Figure 1: Number of *F. candida* juveniles in the AgNP treatment, $AgNO_3$ and control during four seasons. Mean values ± SE, n=6. Values followed by the same letters do not differ significantly ($p < 0.05$) by using pairwise comparisons of log-transformed original data.

Toxicity of AgNP and AgNO3 during four seasons

The relative inhibition of reproduction in treatment of AgNP and $AgNO_3$ compared to the control was used to graphically display their toxicity (Figure 2). Analysing the log-transformed original data, the reproduction of *F. candida* was significantly reduced during spring, summer and autumn in the AgNP treatment ($p<0.05$), but not during winter ($p=0.269$), and during all seasons in the $AgNO_3$ treatment ($p<0.05$, Figure 2, Table S4 in Appendix). AgNP were significantly (1) less toxic for the reproduction of *F. candida* than $AgNO_3$ in autumn and winter ($p<0.05$), (2) but more toxic in spring and summer ($p<0.05$, Figure 2, Table S3-4 in Appendix).

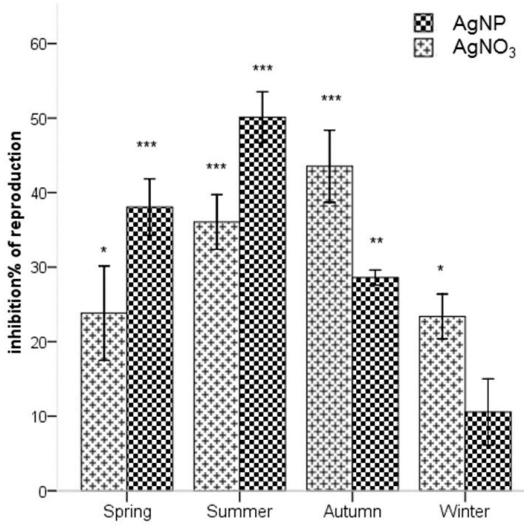

Figure 2. Relative inhibition of *F. candida* reproduction in soil spiked with AgNP and $AgNO_3$ during four seasons. Mean values ± SE, (n=6) are shown. Asterisks indicate

158 statistically significant differences of log-transformed original data to controls (* p≤ 0.05,
159 **<0.01 **P≤ 0.001)
160

161 Discussion

162 We investigated the toxicity of AgNP and $AgNO_3$ to *F. candida* during different seasons.
163 For the first time, strong toxic effects of AgNP at a concentration of 30 mg Ag/kg on the
164 reproduction of *F. candida* were observed. This is in contrast to Waalewijn-Kool et al. [20]
165 who found no effect on survival and reproduction for *F. candida* exposed to AgNP at a
166 measured concentration of 673 mg Ag/kg dry soil, which was more than 20 times higher
167 than the concentration in our study. Mendes et al. (2015) [21] found that NM-300K
168 reduced *F. candida* reproduction by about 50%, yet at a concentration of 640 mg Ag/kg
169 soil. Mainly three factors may explain the differences between these studies: (1)
170 Waalewijn-Kool et al. [20] used paraffin-coated AgNP which were held in a water-only
171 dispersion, while NM-300K are uncoated and dispersed in a suspension that contained
172 three organic agents; (2) The size of AgNP used by Waalewijn-Kool et al. was 3-8 nm
173 AgNP, whereas NM-300K have a diameter of 15 nm.; (3) Loamy sand soil (LUFA- Speyer
174 2.2, Sp 2121, Germany, 2009) with a pH_{cacl2} of 5.5 was used by [20] and [21] whereas
175 we used RefeSol 01A, a loamy, medium-acidic, and lightly humic sand with pH_{cacl2} of 5.67.
176 For NM-300K, an effect of the organic dispersion can be excluded, because tests have
177 been made in advance to ensure that the dispersion showed no toxic effect of on the
178 reproduction of *F. candida* (X. Zhang, unpublished data). McKee et al. (2019) studied the
179 dispersion of NM-300K in OECD soil pore water and found that the dispersion caused

significant immobilization of *F. candida* at 10 mg L^{-1} whereas no toxic effect occurred at 40 mg L^{-1}[27]. This is in line with our findings. Secondly (except for differences in release kinetics, see Engelke et al. 2014), particle size can also be excluded for nanoparticle reactivity increases with decreasing size [28]. Therefore, coating and soil type might the main reasons for the fate and toxicity of the particles found in our studies. The presence of a coating is important, because it can modify the particle structure, the electrostatic surface charge and therefore its potential toxicity over time [29]. Nguyen et al. [30], for instance, found considerable differences in toxicity between AgNP coated with citrate and polyvinylpyrrolidone and uncoated AgNP to macrophages and epithelial cells. They reported that uncoated AgNP, at a concentration of 1 µg/ml, decreased cell viability by 20-40% and that 20 and 40 nm particles were 10% more cytotoxic than the 60 and 80 nm particles. In exposures to coated AgNPs, cell viability dropped at 25 µg Ag/ml or higher concentrations. Similar coating effects were observed in a study with ZnO-NPs and *F. candida* [31] and in studies with iron oxide nanoparticles and mouse fibroblast cells [32]. There is strong support for the assumption that the different soil types were the main reason for the large difference in toxicity between our study and the one by Mendes et al (2015) [21], as various studies in our lab with Collembola (McKee et al. 2019) and enchytraeids (Voua Otomo et al, under revision) have rendered much stronger toxic effects of AgNP in RefeSol 01A than in Lufa 2.2 and artificial OECD soil.

In the present study, the toxicity of AgNP was also season-dependent. Significant toxic effects of AgNP on reproduction were observed in spring, summer and autumn, but not in winter, while $AgNO_3$ caused toxic effects during all seasons. The reproduction of *F.*

candida in the control in winter was 19.5% - 35.9% lower than in summer and autumn. On the other hand, in the soil spiked with AgNP, *F. candida* inhibition in winter was even higher than that in summer. In the following, we discuss three possible explanations: entomopathogenic fungi (EPF), differences in dissolution kinetics of AgNP and AgNO3, and avoidance behaviour.

Fungi observed in our test vessels during winter might account for the reduction of the reproduction of *F. candida*. EPF play an important role in the regulation of insect and Collembola populations [33-36]. Study showed that outbreaks of infection with entomopathogens such as *Entomophthora muscae* tend to occur in spring and autumn and sporulation usually takes place in cool, humid conditions [36]. Although the climatic conditions in the lab were constant during all seasons it is possible that EPF spores were transported inside during autumn and/or winter, compromising reproduction. Interestingly, the situation in the treatment with AgNP was definitely different. There was no significant reduction in reproduction in winter in the soil treated with AgNP (Figure 1), most likely due to their continuous antimicrobial activity. Krishnaraj et al. [37] found strong inhibitory activity on six pathogenic fungi at all concentrations (5 mg, 10 mg, and 15 mg Ag/kg dry soil). A similar study was done with plant-pathogenic fungi *in vitro* [38]. Most fungi were strongly inhibited by AgNP at a concentration of 100 ppm. These results support our assumption that on one hand AgNP are capable of inhibiting entomopathogens, on the other hand, the direct negative effects of silver on the Collembola would partly be masked by the indirect positive effect through its suppressing effect on EPF.

In winter, the reproduction was significantly lower in the treatment of $AgNO_3$, but there was no difference in the treatment of AgNP compared to control (Figure 1). We postulate that the seasonally different performance of both Ag forms is due to their reaction kinetics. $AgNO_3$ dissociates readily in water, newly dissolved but only part of the Ag^+ ions are bioavailable; they will react with anions in the soil solution, forming insoluble precipitates, or complexes with organic acids. In turn, AgNP dissolve slowly, constantly releasing new Ag^+ ions. Therefore, over a longer period it is likely that more Ag^+ is bioavailable from AgNP than from $AgNO_3$.

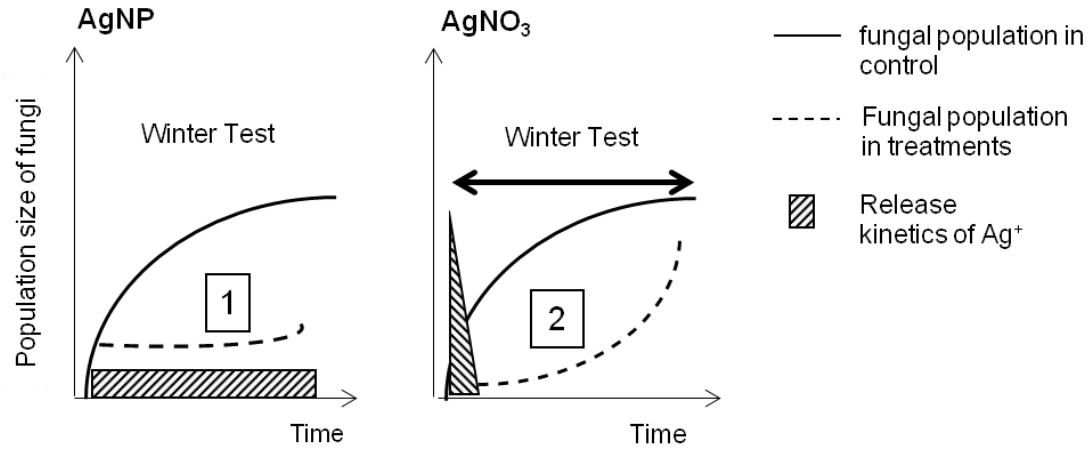

Figure 3: Hypothetical model on the development of EPF in winter and spring in the different treatments as affected by the released Ag^+ ions. There is a slow and continuous ion release from AgNP, whereas $AgNO_3$ ions dissolve at test start. Numbers indicate different phases on EPF populations in the single treatments and tests: (1) Efficient control of the originally small population by continuous Ag^+ ion release; (2) High mortality and exponential recovery due to high growth rate during winter

But what are the reasons for those seasonal differences? The hypothetical model in Figure 3 illustrates why the treatment with $AgNO_3$ had a negative effect on *F. candida* in

240 winter, not the one with AgNP: The presumed contamination with EPF and their spores
241 should have been present in low numbers at the beginning of winter, then increased due
242 to favourable conditions and decreased again in spring due to increasing temperature.
243 The release of dissolved Ag^+ upon adding AgNP to moist soil provides a low, but constant
244 supply of Ag^+ ions. The low Ag^+ concentration should be sufficient to control the small
245 initial EPF population in winter and to prevent their further increase. The negative effect of
246 EPF on the reproduction of *F. candida* was inhibited by AgNP, and a part of the AgNP was
247 attached to the EPF, so that the toxicity of AgNP on the reproduction during winter was
248 decreased. With $AgNO_3$, the sudden release of dissolved Ag^+ upon adding $AgNO_3$ to
249 moist soil will kill most of the present fungi, but the population will quickly recover during
250 winter (Figure 3).
251 On one hand, the likely appearance of EPF made the situation more complicated, and a
252 reduction in toxicity of AgNP may only be due to the interaction between EPF and silver as
253 discussed previously. On the other hand, some studies used the difference in toxicity
254 between AgNP and $AgNO_3$ to demonstrate that the toxic effects of nanoparticles could
255 possibly be explained by a release of Ag^+ from the particles and by a slower assimilation
256 of AgNP, which leads to lower toxic effects on soil fauna compared with $AgNO_3$ [39-41].
257 Such differences in toxicity were also reported in studies with earthworms [16], [17].
258 Similar results were observed in our study during autumn and winter. Stronger toxic
259 effects were found in the treatment with $AgNO_3$ than that with AgNP, which supports the
260 ion release theory. However, the result was totally reversed in spring and summer. We
261 believe this is a combination of Ag^+ release kinetics (see above) and avoidance behaviour.

Avoidance studies in our laboratory gave hints that *F. candida* and enchytraeids avoid high, but not low concentration As of Ag. Assuming that they sense rather the ions than the undissolved metal it is possible that they actively avoided (e.g. by staying mostly at the uncontaminated food patch on the surface) only the $AgNO_3$ treatment but not the one with AgNP in our study. Thus, in the latter treatment the animals were exposed to low concentrations of Ag^+ permanently released by the AgNP, reducing their reproduction. It is possible that the EPF started developing already in autumn (not yet visible in the quickly developing population, but perhaps supported by high population density) so that the hypothetical model described above for the test in winter might partly apply also for autumn.

Circannual biological rhythms might be another explanation for the seasonal toxicity results which have been described in Rozen (2006). Earthworms were collected (*Dendrobaena octaedra*) from the field and cultured in the laboratory under constant conditions. The author found that reproduction was highest in spring and summer, and dropped significantly in the winter months, which indicated that internal regulation of reproduction may exist in the earthworm *D. octaedra* [42]. However, the mechanisms have not yet been understood. Nevertheless, we cannot fully elucidate what exactly caused the toxicity of AgNP in the present study and why it was seasonal. The hypothesis of the EPF theory should be tested in further investigations, to identify the fungi species present during winter. In order to better understand the mechanisms for nanoparticle action, the uptake and elimination kinetics of Ag in F. candida and their avoidance behaviour should be studied as well.

Conclusions

We demonstrated for the first time that AgNP in natural soil can have strong toxic effects on the reproduction of *F. candida* at a concentration of only 30 mg Ag/kg, which is about 20 times lower than reported earlier. As these effects can mainly be attributed to soil conditions (compared to Lufa 2.2 and artificial OECD soil), this raises concern whether these commonly used test substrates are really protective. This is also the first paper reporting a seasonal effect during a reproduction test of *F. candida*. Although no clear explanations for the different performances throughout the year were found, three independent repeats in spring, summer and autumn should be recommended to give comprehensive results in further toxicological tests. To corroborate our hypothetical model on the different outcome in winter and spring, studies specifically addressing ion release kinetics of AgNP and EPF identification are needed. Furthermore, avoidance behaviour should be taken into account as well.

Abbreviations

AgNP: silver nanoparticles;

$AgNO_3$: silver nitrate;

IME: Institute for Molecular Biology and Applied Ecology;

GLM: general linear model;

EPF: entomopathogenic fungi.

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Authors' contributions

Both authors contributed to the design of the experiments in this study. X.Z. initiated and drafted the manuscript. J.F. revised and commented the manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Informed consent was obtained from all individual participants included in the study.

Availability of data and material

The datasets used during this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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