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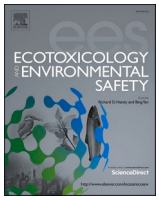

Glyphosate presence in human sperm: First report and positive correlation with oxidative stress in an infertile French population

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ABSTRACT

Environmental exposure to endocrine disruptors, such as pesticides, could contribute to a decline of human fertility. Glyphosate (GLY) is the main component of Glyphosate Based Herbicides (GBHs), which are the most commonly herbicides used in the world. Various animal model studies demonstrated its reprotoxicity. In Europe, GLY authorization in agriculture has been extended until 2034. Meanwhile the toxicity of GLY in humans is still in debate. The aims of our study were firstly to analyse the concentration of GLY and its main metabolite, aminomethyl-phosphonic acid (AMPA) by LC/MS-MS in the seminal and blood plasma in an infertile French men population (n=128). We secondly determined Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) using commercial colorimetric kits and some oxidative stress biomarkers including malondialdehyde (MDA) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) by ELISA assays. We next analysed potential correlations between GLY and oxidative stress biomarkers concentration and sperm parameters (sperm concentration, progressive speed, anormal forms). Here, we detected for the first time GLY in the human seminal plasma in significant proportions and we showed that its concentration was four times higher than those observed in blood plasma. At the opposite, AMPA was undetectable. We also observed a strong positive correlation between plasma blood GLY concentrations and plasma seminal GLY and 8-OHdG concentrations, the latter reflecting DNA impact. In addition, TOS, Oxidative Stress Index (OSI) (TOS/TAS), MDA blood and seminal plasma concentrations were significantly higher in men with glyphosate in blood and seminal plasma, respectively. Taken together, our results suggest a negative impact of GLY on the human reproductive health and possibly on his progeny. A precaution principle should be applied at the time of the actual discussion of GLY and GBHs formulants uses in Europe by the authorities.

1. Introduction

A global decline in human fertility and more precisely in semen quality over the past few decades has been reported in epidemiological studies (Almagor et al., 2003; Carlsen et al., 1992; Cipriani et al., 2023; Levine et al., 2023; Rolland et al., 2013). Environmental exposure to endocrine disruptors, such as pesticides, bisphenol A and heavy metals, is suspected to have contributed to such a decline, in addition to other adverse reproductive outcomes (Gaspari et al., 2011; Skakkebaek et al., 2001). Glyphosate (GLY) is one of the most commonly used herbicide in the world (Duke, 2018). It is frequently used in agriculture, horticulture, forestry, and other fields. GLY is the active ingredient mixed with other chemical adjuvants in commercial formulations of all glyphosate-based herbicides (GBHs). Co-formulants used are most of the time unknown because of trade secret (Mesnage and Antoniou, 2018). However, they seem to be involved in more deleterious effects than GLY alone (Alvarez-Moya and Reynoso-Silva, 2023; Defarge et al., 2016; Mesnage and Antoniou, 2018; Nerozzi et al., 2020).

In plants and animals, GLY is metabolized into CO2 and aminomethylphosphonic acid (AMPA)., It is easily transported to surrounding ecosystems, leading to soil (Pelosi et al., 2022), water, and crop contamination. High concentrations of glyphosate have been detected in the drinking water, as well as in the soils (Lima et al., 2023; Pelosi et al., 2022) and vegetables in agricultural areas (Granby and Vahl, 2001).

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Thus, humans are possibly exposed to GLY through ingestion, inhalation and dermal absorption (Gillezeau et al., 2019). For a long time, GLY was considered to be harmless for mammals and humans because the shikimate pathway is not found in vertebrates (Mesnage et al., 2015). Meanwhile, GLY enters into the bodies of animals and human through food chain, exposing the public to a potential health risks (Lupi et al., 2015; Van Bruggen et al., 2018). Several animal studies have demonstrated that GLY exposure can induce neural, liver, renal, and reproductive toxicity (Ford et al., 2017; Kubsad et al., 2019; Pu et al., 2020; Van Bruggen et al., 2018). Very few studies have been conducted among occupationally exposed workers and the general population and evaluated human exposure to GLY and AMPA (Gillezeau et al., 2019). GLY and AMPA have both already been detected in human in biofluids such as urine (Buekers et al., 2022a, 2022b; Connolly et al., 2022; Grau et al., 2022; Soukup et al., 2020), serum (Serra et al., 2021a; Yoshioka et al., 2011), maternal milk (Camiccia et al., 2022; Gillezeau et al., 2019; Steinborn et al., 2016), umbilical cord (Kongtip et al., 2017), hair (Alvarez-Moya and Reynoso-Silva, 2023), and duodenal fluid (Schusser et al., 2022) but never in human sperm. For this reason, the use of GBHs has become a matter of concern because of their possibly carcinogenic and reprotoxic effects. This point remains still a debate nowadays in Europe. Indeed, Authoritative and regulatory bodies give conflicting opinions about GLY toxicity in humans. For example, the International Agency for research on cancer (IARC) has classified in 2015 GLY as « a probable human carcinogen » (IARC monographs) (IARC, 2016). At the meantime, in 2016, the US Environmental Protection Agency (EPA) (EPA, 2016) and the European Food Safety Authority (EFSA) (European Food Safety Authority (EFSA) (EFSA) (EFSA), 2017) concluded in 2017 that « GLY is not likely to be carcinogenic in human". Recently, on july 6th, 2023, EFSA confirmed that the assessment of GLY's impact of the human and animals health and the environment did not identify critical areas of concerms. These opposite opinions reflect different methodologies in the studies taken into account (Gillezeau et al., 2019). Finally, the European Union (UE) decided to extend the use of GLY in Europe for 10 years until 2034 (Casassus, 2023), although evidence on the carcinogenicity and toxicological effects of GLY has been found and debated (INSERM, 2021). Moreover, toxic effects of GLY on the reproductive system have been demonstrated in *in vivo* and *in vitro* animal studies (Serra et al., 2021a). The main molecular mechanism associated to this toxicity seems to be linked to an increase of oxidative stress (Liu et al., 2023). In human, at the present time, only *in vitro* studies have evaluated the toxicity of GLY on sperm (Ferramosca et al., 2021; Torres-Badia et al., 2022). In this context, the objectives of our study were to investigate the GLY presence *in vivo* in human sperm, among a male human infertile population and to investigate its potential association with the oxidative stress.

2. Materials and methods

2.1. Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee as part of the "INDICA" project (authorisation number 2016_075). All subjects were informed of the study, and they provided an informed written consent before the inclusion on this research. They accepted that their semen and blood samples and the clinical data they supplied might be used for scientific purposes.

2.2. Study design

This study investigated 128 male partners of infertile couples, without physical abnormalities or chronic illnesses, aged 26–57 years, recruited in the course of the consultations between February 2018 and March 2022 for infertility (no conception after one or more years of unprotected intercourse). The study was conducted in the medical center "Pole Santé Leonard de Vinci", settled in the middle of France close to Tours, which is known as both an urban place and also as the attic of France for the grain and wine production. This area reflects the common herbicide exposure in France. Indeed, it is considered as the third french district for the purchase of pesticides (Géniteau and Cherbonnet, 2022). The 128 enrolled men filled in a questionnaire that asked them about the sector of their profession (Sector I: agriculture, fisheries, forestry, mining, deposits. Sector II: industrial activities and construction. Sector III: trade, transport, accommodation and catering; information and communication; finance, insurance, real estate; services mainly to businesses; public administration; education, health; health care, social work; services to households), the environment of their place of residence (town or country), their diet (organic or not), and finally whether they smoked or not. Unfortunately, only 47 questionnaires out of 128 were fully filled (105 with at least one question answered).

2.3. Samples collection

Blood and semen samples were collected the same day. Semen were sampled in a sterile container by self masturbation after 2–7 days of sexual abstinence (without the use of spermicidal lubricants) in the laboratory itself. As sperm quality of an individual can vary widely depending on the duration of abstinence from coitus and febrile illness stress, two samples were collected at different visits within two months for analysis and mean value of both was used, except for one patient. Semen was centrifuged at 250 g (model of centrifuge: Rotina 380 R (Hettich, Tuttlingen, Germany)) for 15 min at room temperature. Aliquots were stored at −80°C until assay analysis.

2.4. Blood and seminal plasma glyphosate and AMPA

Glyphosate and AMPA were measured in blood and seminal plasma of patients by LC/MS-MS after a derivatisation reaction using FMOC-Cl (9-fluorenylmethyl chloroformate) as described in Serra et al., 2021 (Serra et al., 2021a). The values for the limits of quantification (LOQ) were 0.05 ng/mL. All concentrations under the LOQ are set to 0.

2.5. Routine semen analysis

Routine semen analysis was performed microscopically with special interest in the sperm concentration, percent motility, and percent morphology. Based on the sperm concentration/count according to the WHO criteria (World Health Organization (WHO) (WHO) (WHO) and Human reproduction programme (HRP), 2021), the overall samples were considered as normospermia if the concentration is at least 15 million sperm cells/mL semen; the percentage of progressive motility after 1 h is at least 35% and the percentage of abnormal form is less than 95%. Thereafter, samples were centrifuged and the supernatant seminal fluid was separated into another clean and sterile plastic container. The seminal fluid plasma was then stored at −20°C prior to the assay.

2.6. Measurement of total antioxidant status (TAS), total oxidant status (TOS) and the oxidative stress index in blood and seminal plasma

The TAS and the TOS of blood and seminal plasma samples were determined colorimetrically using the commercial Total Antioxidant Status and Total Oxidant Status kits from Abbexa company (Cambridge, UK), respectively. The assays have got very good precision values: within and between precision values were lower than 4%. The results were expressed as μmol H2O2 eq/L for TOS and μmol Trolox eq/L for TAS. The ratio of TOS to TAS comprises the oxidative stress index (OSI), which is used as an indicator for total oxidative stress (Harma and Erel, 2003; Erel, 2005).

2.7. Determination of MDA (malondialdehyde) and 8-OHdG (8-hydroxy-2′-deoxy-guanosine) blood and seminal plasma concentration

We have chosen to determine oxidative stress in blood and seminal plasma by measuring MDA (malondialdehyde), which reflects lipid peroxidation (supplemental Fig. 1) and 8-OHdG (8-hydroxy-2′-deoxy-guanosine) which corresponds to the oxidation of the guanine, one of the four bases of DNA (supplemental Fig. 2). The concentration of 8-hydroxy-2′-deoxy-guanosine and MDA was determined using enzyme-linked immunosorbent assay (ELISA) technique (Elabscience, Texas, USA). For MDA and 8-OHdG measurements, the sensitivity of the assays was 0.25 nmol/mL and 0.94 μg/L, respectively. The intra-assay and inter-assay coefficients of variation (CV) for each assay averaged <10%.

2.8. Statistical analysis

All statistical analyses were performed using R software (version 4.2.2) (R Core Team, https://www.R-project.org/). Data are presented as mean ± SD. Spearman's correlation coefficient was used to associate blood and seminal plasma glyphosate concentration with measured sperm parameters, 8-OHdG and MDA concentrations. As the variables were not normally distributed, permutation tests for ANOVA were carried out using permuco R package with 10 000 permutations (Frossard and Renaud, 2021). A first model was fitted to evaluate the association between glyphosate concentrations or sperm parameters and responses to the questionnaire. This model was adjusted for age and BMI. In a second model, the association between concentrations of MDA and 8-OHdG and those of glyphosate was studied by adjusting for age and BMI. In addition, same models were performed only on patients with non-zero glyphosate concentrations. A p-value < 0.05 was considered as statistically significant.

3. Results

3.1. Charaterization of the patients

We analysed a population of French men with an average age of 36.3 ± 6.2 years (Table 1). The population reflected the general French population as occupational and non occupational workers (76.1% tertiary sector; 2.5% workers in agriculture field including 1.5% farmers; 20% secondary sector (mainly industry) (INSEE, 2020). About two-thirds of patients lived in the countryside, more than half did not smoke and more than half did not eat organic food (Table 1).

3.2. Glyphosate in blood and seminal plasma

Glyphosate was detected in the seminal plasma of 73 out of 128 patients in whom glyphosate was also detected in the blood plasma (except for one with a low concentration of 0.2 in the seminal plasma). For all the other patients (55), levels were below the lower limit of quantification in both seminal plasma and blood plasma. It should be noted that, the metabolite of glyphosate, AMPA, was not detected either blood or seminal plasma. Interestingly, glyphosate levels were nearly four times higher in seminal fluid than in blood samples (0.19 ± 0.23 vs 0.73 ± 0.84, Table 1 **and** Fig. 1**A**). **In addition**, we observed a significant positive correlation between seminal and blood plasma glyphosate ($r = 0.97$, $p<0.001$, Fig. 1**B**).

Patients with glyphosate detected in seminal plasma were significantly younger (35.3 ± 6.0 vs 37.6 ± 6.2, p=0.04) and have a lower BMI (25.2 ± 4.4 vs 27.9 ± 4.8, p=0.002) (Table 2).

As shown in Table 3, blood or sperm glyphosate concentrations were not significantly different between patients living in city and countryside, nor between patients eating organic and non organic food. However, in patients in whom GLY was detected, smokers had blood and seminal plasma concentrations twice as high as non-smokers (0.29 vs 0.18 in blood plasma and 1.22 vs 0.74 in seminal plasma, $p < 0.001$, Fig. 1**C**). Also, we observed that patients with a job in sector I (workers in farm) had higher GLY concentrations in sperm than those with a job in sector III (workers in transport, communication, finance or public administrations). Indeed, the patient with the higher sperm glyphosate concentration was a farmer (4.35 ng/mL). However, we noted that the patients who responded to the questionnaire had lower GLY concentrations than those who did not

Table 1
Characteristic of patients.

	Value	Samples (n)
Patient characteristics		
Age (years)	36.3 ± 6.2 (26–57)	128
BMI	26,8 ± 4.8 (18–44)	127
Sperm parameters		
Sperm concentration (10^6/mL)	60.5 ± 66.0 (0–335)	127
Progressive sperm motility (%)	39.5 ± 17.0 (0–74)	125
Abnormal sperm morphology (%)	91.5 ± 10.1 (9–100)	125
Glyphosate concentrations		
Blood plasma Glyphosate (ng/mL)	0.19 ± 0.23 (0–1.34)	128 but detected only in 72
Seminal plasma Glyphosate (ng/mL)	0.73 ± 0.84 (0–4.35)	128 but detected only in 73
Oxidative status		
Blood MDA (mmol/mL)	0.32 ± 0.29 (0–1.3)	128 but detected only in 100
Seminal MDA (mmol/mL)	0.75 ± 0.64 (0–2.5)	128 but detected only in 103
Blood 8-OHdG (μg/L)	26.6 ± 15.0 (0–70)	128 but detected only in 116
Seminal 8-OHdG (μg/L)	93.7 ± 71.9 (0–445)	128 but detected only in 112
Blood TAS (μmol Trolox eq/L)	0.45 ± 0.12 (0.2–0.7)	128 but detected only in 117
Seminal TAS (μmol Trolox eq/L)	0.51 ± 0.08 (0.3–0.7)	128 but detected only in 104
Blood TOS (μmol H2O2 eq/L)	15.1 ± 9.7 (2–45.3)	128 but detected only in 100
Seminal TOS (μmol H2O2 eq/L)	25 ± 13.4 (3.4–58.6)	128 but detected only in 104
Blood OSI (TOS/TAS)	38.3 ± 34.5 (3.3–176)	128 but detected only in 98
Seminal OSI (TOS/TAS)	49.5 ± 27.4 (7.2–117)	128 but detected only in 104
Questionnaire answers		
Profession	Sector I: 5 (7%) Sector II: 8 (11%) Sector III: 61 (82%)	74
Place of residence	Town: 22 (32%) Countryside: 47 (68%)	69
Organic diet	Yes: 30 (46%) No: 35 (54%)	65
Smoking	Yes: 41 (44%) No: 53 (56%)	94

Mean ± SD and (range) of age, BMI, semen parameters and blood and seminal plasma glyphosate, MDA and 8-OhdG concentrations Number and details of answers to each of the 4 questions.

3.3. Sperm parameters in men with and without glyphosate in seminal plasma

We next compared the two groups of men with and without glyphosate in plasma seminal in term of sperm parameters. As showed in Table 2 **and** Fig. 1**D-F**, we observed no significant difference between these two groups for sperm concentration (56.7± 65.7×10^6/mL vs. 65.5 ±66.5×10^6/mL, p=0.54), progressive sperm motility (38.9 ± 15.4 vs 40.5 ± 19.0, p=0.94) or sperm morphology (anormal forms: 91.4 ± 7.1 vs. 91.5 ± 13.1, p=0.84).

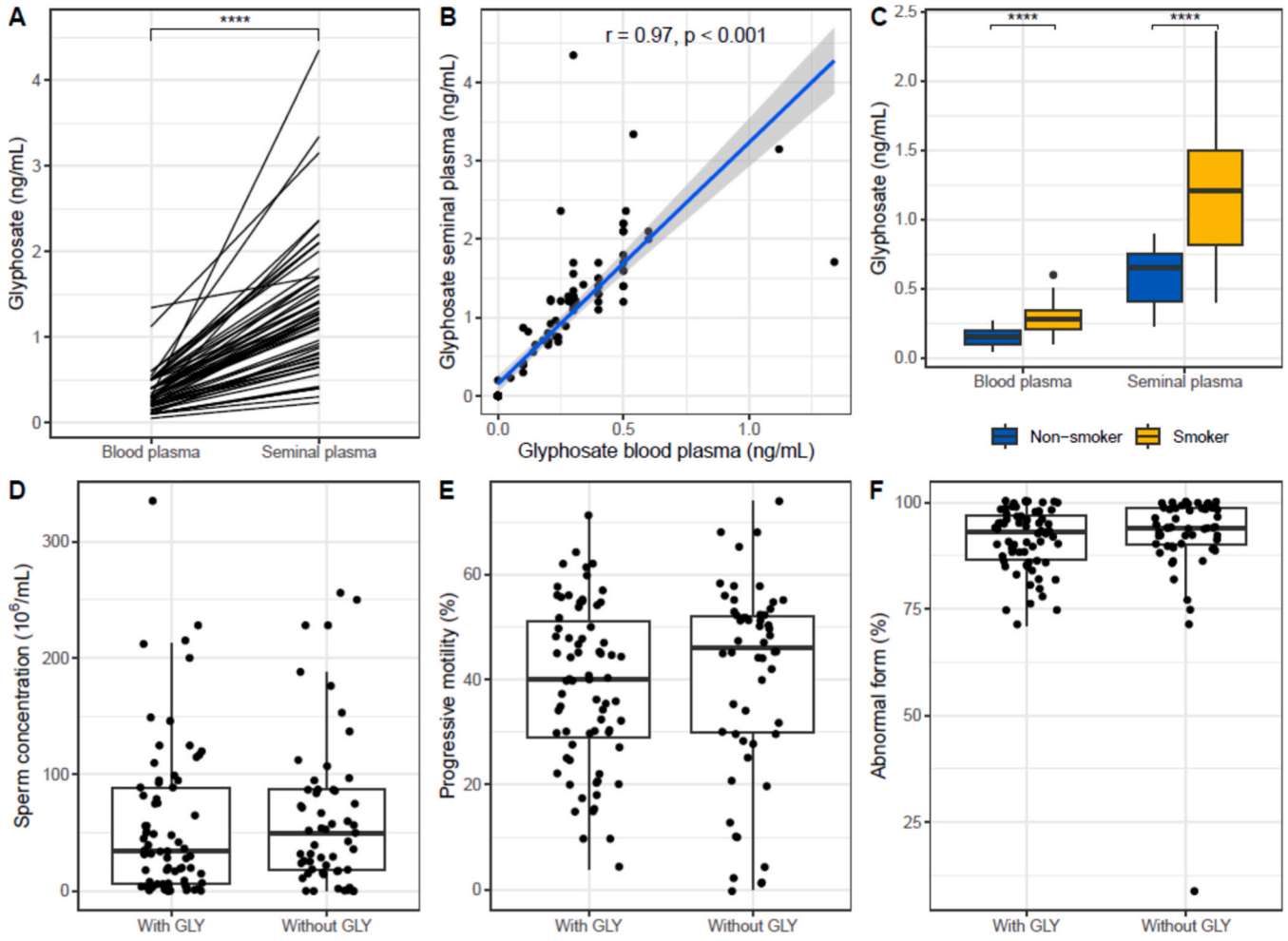

Fig. 1. Glyphosate concentration in blood plasma and seminal plasma of patients, glyphosate concentrations of smoker and non-smoker patients and sperm parameters in patients with or without glyphosate (GLY) in seminal plasma. A. Concentration of GLY (ng/mL) in blood and seminal plasma of 128 patients. B. Correlation between the concentration of GLY (ng/mL) in blood plasma and in seminal plasma. C. Concentration of GLY (ng/mL) in blood and seminal plasma of smoker and non smoker patients. D. Sperm concentration (10^6/mL). E. Progressive motility of sperm (%). F. Abnormal morphology of sperm (%).

3.4. TOS, TAS, OSI, MDA and 8-OHdG concentration in blood and seminal plasma in men with and without glyphosate

As shown in Fig. 2**A and** Table 1**, we found that TOS and OSI,** were significantly higher in men with GLY in blood (TOS: 17.20 ± 10.50 (n=71) vs 9.71 ± 3.00 (n=29) µmol H2O2 eq/L, p=0.002; OSI: 45.6 ± 38.4 vs 20.0 ± 6.4, p<0.001) and seminal plasma (TOS: 28.60 ± 14.0 (n=73) vs 16.40 ± 5.60 (n=31) µmol H2O2 eq/L p<0.001; OSI: 56.3 ± 29.1 vs 33.6 ± 13.5, p<0.001) than in men without GLY, respectively. At the opposite, TAS in both blood and seminal plasma was similar in men with GLY (blood plasma: 0.43 ± 0.12 (n=69) vs 0.48 ± 0.11 (n=48) µmol Trolox eq/L, p=0.051; seminal plasma: 0.52 ± 0.08 (n=73) vs 0.51 ± 0.07 (n=31) µmol Trolox eq/L, p=0.36) and without GLY (Table 1). As shown in Fig. 2**B**, blood and seminal glyphosate concentrations were significantly positively correlated with blood (r=0.65; p<0.001) and seminal TOS (r=0.64; p<0.001) concentrations, respectively. In addition, blood and seminal glyphosate concentration was also positively associated to blood (r=0.64; p<0.001, Fig. 2**D**) and seminal OSI (r=0.59; p<0.0001, Fig. 2**D**).

As shown in Fig. 3**A**, MDA blood and seminal plasma concentrations were significantly higher in men with GLY in blood (0.48 ± 0.30 (n=28) vs 0.28 ± 0.12 (n=72) mmol/mL; p=0.0032) and seminal plasma (1.07 ± 0.62 (n=30) vs 0.57 ± 0.22 (n=73) mmol/mL p<0.0001) than in men without GLY, respectively. Concerning the concentrations of 8-OHdG, we observed significant higher concentrations in men with glyphosate only in seminal plasma (125.09 ± 75.09 (n=39) vs 73.46 ± 23.89 (n=73) µg/L; p<0.001) but not in blood plasma (30.16 ± 11.22 (n=73) vs 27.86 ± 15.53 (n=43) µg/L *;* p=0.36, Fig. 3**C**). As shown in Fig. 3**B**, blood and seminal glyphosate concentration were significantly positively correlated with blood (r=0.71; p<0.001) and seminal MDA (r=0.75; p<0.001) concentrations, respectively. In addition, seminal glyphosate concentration was also positively associated to seminal concentrations of 8-OHdG (r=0.65; p=0.002, Fig. 3**D**).

4. Discussion

GLY and GBHs were authorized in USA in 1974 by EPA for agriculture used. In Europe, European commission gave its approval for GLY's commercialization in 2002. Moreover, at the end of the last century, genetically modified organisms (GMOs) as Glyphosate tolerant crops have been created leading to an increase of GLY use. This phenomena has generated the emergence of tolerant weeds and consequently the rise of GLY utilization in order to control them. In the present study, for the first time we showed the presence of glyphosate in human sperm.

4.1. Detection and accumulation of glyphosate in seminal plasma

Glyphosate was detected or quantified in the blood plasma of 56% of our patients (ranging from 0.05 to 1.34 ng/mL) randomly selected from a cohort of French infertile couples. All the patients resided in the Centre

Table 2
Characteristic of patients with (n=73) and without glyphosate (GLY, n=55) in the seminal plasma (SP).

	Patient with GLY in SP (n=73)	Patient without GLY in SP (n=55)	*p-value*
Patient characteristics			
Age (years)	35.3 ± 6.0 (26–57)	37.6 ± 6.2 (28–53)	**0.04**
BMI	27.9 ± 4,8 (21.9–44.2)	25.2 ± 4.4 (17.8–39)	**0.002**
Glyphosate concentrations			
Glyphosate concentration BP (ng/mL)	0.33 ± 0.21 (0–1.34)	Undetected	
Sperm parameters			
Sperm concentration (10^6/mL)	56.7 ± 65.7 (0–335)	65.5 ± 66.5 (0.1–256)	0.54[a]
Progressive sperm motility (%)	38.9 ± 15.4 (4–71)	40.5 ± 18.9 (0–74)	0.94[a]
Abnormal sperm morphology (%)	91.4 ± 7.1 (71–100)	91.5 ± 13.1 (9–100)	0.84[a]
Oxidative status			
Blood MDA (mmol/mL)	0.44 ± 0.30 (0–1.3)	0.14 ± 0.17 (0–0.6)	**<0.001**[a]
Seminal MDA (mmol/mL)	1.07 ± 0.62 (0.1–2.5)	0.31 ± 0.33 (0–1.2)	**<0.001**[a]
Blood 8-OHdG (µg/L)	30.2 ± 11.2 (12–65)	21.8 ± 18.0 (0–70)	**0.006**[a]
Seminal 8-OHdG (µg/L)	125 ± 75.1 (117–445)	52.1 ± 39.2 (0–135)	**<0.001**[a]
Blood TAS (µmol Trolox eq/L)	0.43 ± 0.12 (0.2–0.6)	0.48 ± 0.11 (0.2–0.7)	0051[a]
Seminal TAS (µmol Trolox eq/L)	0.52 ± 0.08 (0.3–0.7)	0.51 ± 0.07 (0.4–0.6)	0.36[a]
Blood TOS (µmol H2O2 eq/L)	17.2 ± 10.5 (2–45.3)	9.71 ± 3.0 (3.9–16.4)	**0.002**[a]
Seminal TOS (µmol H2O2 eq/L)	28.6 ± 14.1 (3.4–58.6)	16.4 ± 5.6 (4.3–30.6)	**<0.001**[a]
Blood OSI (TOS/TAS)	45.6 ± 38.4 (3.3–176)	20.0 ± 6.4 (9.8–32.8)	**<0.001**[a]
Seminal OSI (TOS/TAS)	56.3 ± 29.1 (8.5–117)	33.6 ± 13.5 (7.2–66.8)	**<0.001**[a]

Mean ± SD and (range) of age, glyphosate concentration in blood plasma (BP), sperm concentration, progressive sperm motility and abnormal sperm morphology.
[a] adjusted by age and BMI.

Table 3
Influence of cigarettes, organic food and employment by sector of activity on the blood and seminal plasma glyphosate (GLY) concentration in patients.

	Blood GLY (ng/mL)		Seminal GLY (ng/mL)	
	All patients	Only patients with GLY	All patients	Only patients with GLY
Without cigarette	**0.04 ± 0.08 (n = 53)**	**0.15 ± 0.06 (n = 15)**	**0.17 ± 0.29 (n = 53)**	**0.56 ± 0.22 (n = 16)**
With cigarette	**0.18 ± 0.17 (n = 41)**	**0.29 ± 0.13 (n = 25)**	**0.74 ± 0.72 (n = 41)**	**1.22 ± 0.51 (n = 25)**
P value (cigarette effect)	***p < 0.001***	***p < 0.001***	***p < 0.001***	***p < 0.001***
Without organic food	0.07 ± 0.10 (n = 35)	0.19 ± 0.08 (n = 14)	0.31 ± 0.42 (n = 35)	0.71 ± 0.33 (n = 15)
With organic food	0.11 ± 0.16 (n = 30)	0.25 ± 0.15 (n = 13)	0.47 ± 0.78 (n = 30)	1.07 ± 0.87 (n = 13)
P value (organic food effect)	*p = 0.30*	*p = 0.41*	*p = 0.33*	*p = 0.51*
Residence in city	0.04 ± 0.08 (n = 22)	0.15 ± 0.06 (n = 6)	0.17 ± 0.30 (n = 22)	0.62 ± 0.19 (n = 6)
Residence in countryside	0.07 ± 0.09 (n = 47)	0.17 ± 0.07 (n = 19)	0.33 ± 0.68 (n = 47)	0.79 ± 0.87 (n = 20)
P value (residence effect)	*p = 0.41*	*p = 0.44*	*p = 0.42*	*p = 0.57*
Employment in sector I	0.15 ± 0.15 (n = 5)	0.25 ± 0.09 (n = 3)	1.34 ± 1.82 (n = 5)	**2.23 ± 1.91 (n = 3)**
Employment in sector II	0.27 ± 0.38 (n = 8)	0.53 ± 0.40 (n = 4)	0.87 ± 1.12 (n = 8)	1.73 ± 0.96 (n = 4)
Employment in sector III	0.15 ± 0.17 (n = 61)	0.29 ± 0.14 (n = 32)	0.62 ± 0.75 (n = 61)	**1.14 ± 0.67 (n = 33)**
P value (employment in sector effect)	*p = 0.29*	*p = 0.06*	*p = 0.15*	***p = 0.04***

Mean ± SD of glyphosate concentration (ng/mL) in blood or semen are reported in subjects (n=128). Permutation tests for ANOVA adjusted for age and BMI were performed.
Sector I: agriculture, fisheries, forestry, mining, deposits. Sector II: industrial activities and construction. Sector III: trade, transport, accommodation and catering; information and communication; finance, insurance, real estate; services mainly to businesses; public administration; education, health; health care, social work; services to households.

Val de Loire region which is considered the breadbasket of France as it is the 4th largest cereal producing region (Tribune Hebdo, 2020) and the 4th largest wine producing region (Géniteau and Cherbonnet, 2022). Most of the patients lived in the countryside. In the literature, most of the studies correspond to GLY urinary detection, which is the main way of GLY elimination. A recent review found that the average reported urinary levels in occupationally exposed individuals vary from 0.26 to 73.5 ng/mL and individuals with environmental exposure had levels ranging from 0.16 to 7.6 ng/mL (Gillezeau et al., 2019). In addition, a recent canadian study showed the presence of GLY and AMPA in urine of nearly 75% of women cohort at the first trimester of pregnancy (n= about 2000 patients (Ashley-Martin et al., 2023). Interestingly, in our study, GLY concentration was also detected in their seminal plasma but at about 4 times the blood plasma concentration. The same ratio between blood and seminal fluid GLY concentration was found in our previous work in roosters dietary exposed to a GBH (Serra et al., 2021b). A plausible mechanism could be a disruption of the blood testis barrier (BTB) sinceGLY exposure is able to disrupt BTB integrity in juvenile rats (Gorga et al., 2021). Furthermore, pure and GLY formulations *in vitro* studies affect localization of claudin-11, an androgen-regulated tight junction protein, crucial for the blood testis barrier (Gorga et al., 2020). Interestingly in animals models, the GLY concentration increased in both compartments (blood and seminal plasma) in a time-dependent manner in response to dietary GBHs exposure, suggesting an accumulation in sperm. There are not yet sufficient studies explaining the pharmacokinetics of GLY in vertebrates and its transport and bioaccumulation in various biological fluids and tissues. However, Panzacchi et al., 2018, showed that the amount of GLY present in urine increases in relation to the duration of exposure suggesting a possible bioaccumulation (Panzacchi et al., 2018). It is known that GLY's half-life in the human body is relatively short (3.5–14.5 hours; (Faniband et al., 2021)). Glyphosate is accumulated in kidney and liver (Faniband et al., 2021) and it is mainly watersoluble (Rodríguez-Gil et al., 2021). Thus, GLY's detection in occupational workers or non occupational exposure (by aerosols, dust ingestion, diet or drinking water) probably reflected to an actual recent exposition and contamination. However, we can not exclude that some genetic, biochemical factors, mix of pesticides, or coformulants of GLY influence GLY metabolism in sperm independently of the total concentration of GLYexposure.

Among the three highest rates of GLY found in both blood and sperm, two were farmers. Surprisingly, the third one who always worked in an office and lived in urbain are, had elevated GLY amount in blood (0.54 ng/mL) and sperm (3.34 ng/mL) detected. He used to practice footing in landscape. Campbell et al., (Campbell et al., 2022) observed that GLY was detected in the urine of 96% of the farmer population analysed. In french population, Grau et al. (Grau et al., 2022) showed that higher occupational exposure in farmers and wine producers. Meanwhile, in this latter study, the urine GLY measurement was performed with a different method as compared to our study (ELISA assay vs mass spectrometry). Connolly et al. (Connolly et al., 2022) reported

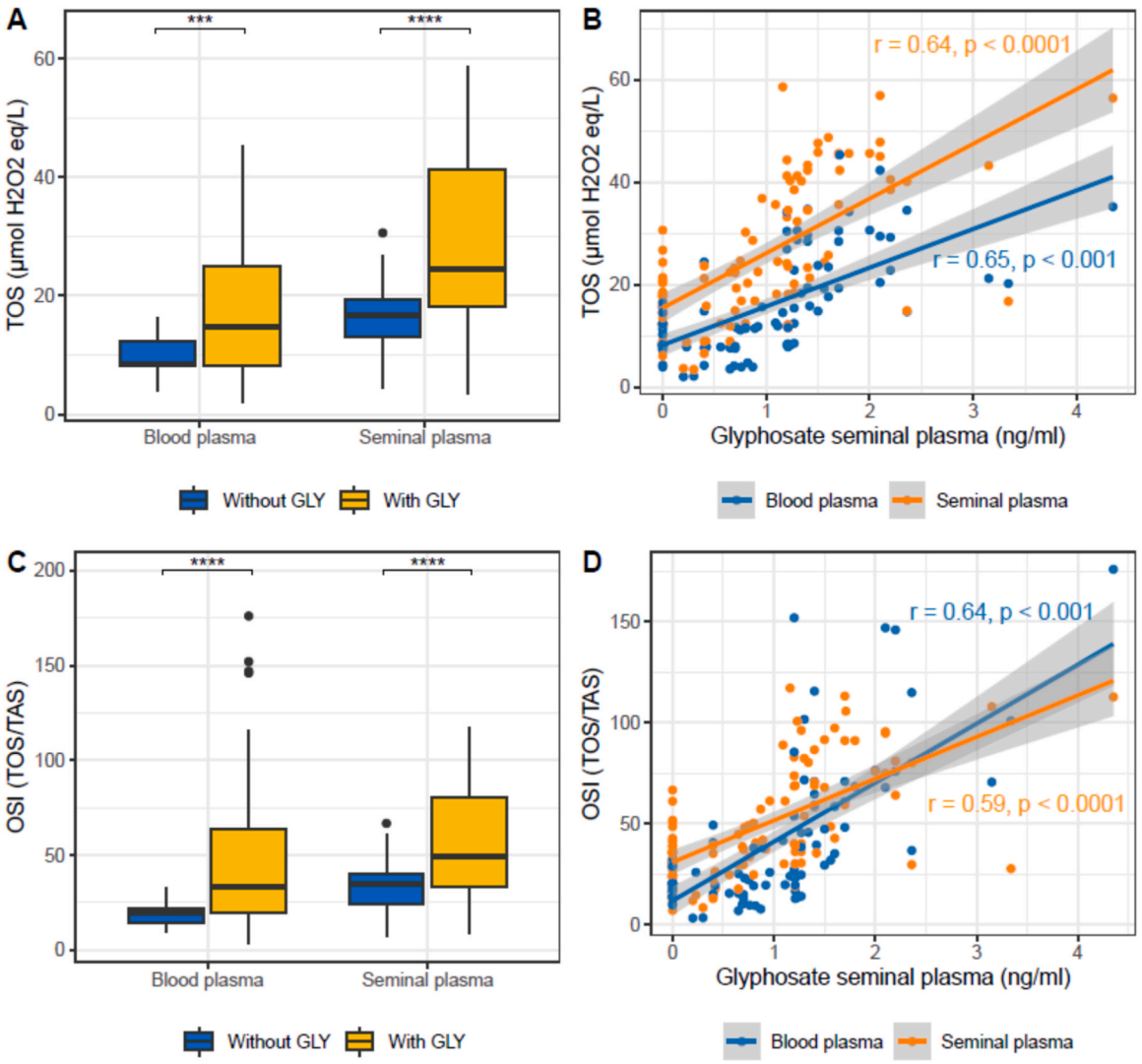

Fig. 2. Total oxidative status (TOS) (A) and oxidative stress index (OSI) (C) of the blood and seminal plasma in patients with or without glyphosate (GLY) and correlations of TOS (B)and OSI (D) with concentration of glyphosate in seminal plasma. A. Total oxidative status (TOS,) (µmol H2O2 eq/L)l/mL) in blood and seminal plasma in patients with or without GLY. B. Correlation between the TOS (µmol H2O2 eq/L) and concentration of glyphosate (ng/mL) in seminal plasma. C. OSI (ratio TOS/TAS (Total Antioxidative Status)) in blood and seminal plasma in patients with or without GLY. D. Correlation between OSI and the concentration of glyphosate (ng/mL) in seminal plasma. *** p<0.001; **** p<0.0001.

that urinary GLY concentrations as determined by mass spectrometry did not statistically differ between farm and non-farm families. At the opposite, similar study in the US also found no statistical differences among the children of non-farm families and the farm children (Curwin et al., 2006). In the present study, we detected higher levels of GLY amounts in smokers (n=44) than non smokers (n=53, ref)). Indeed, the tabacco fields uses high quantity of pesticides including GLY (Kahl et al., 2018).

4.2. A positive correlation between glyphosate content and oxidative stress in seminal plasma

Oxidative stress (OS) is defined as an imbalance between antioxidant mechanisms and reactive oxygen species (ROS) production (Pisoschi and Pop, 2015). Meanwhile, OS is a physiological process in sperm, which enables an appropriate capacitation, acrosomal reaction and spermatozoa-oocyte fusion (Aitken, 2017). ROS are a family of free radicals and non free radicals. The first ones participate mostly to the capacitation. The free radicals are instable molecules with one or more unbound electrons, which are extremely reactive, and interacts with surrounded molecules (Evans et al., 2021) They come mainly from the reduction of O2 and include superoxide O2-, hydroxyl (OH-) and peroxide (H2O2) and the metabolized hydroxyl radical (OH-2). They are hydrophobic, and they have a low membrane permeability, and therefore can be toxic for the cell's membrane. To counteract this phenomena, the Super Oxide Dismutase (SOD) catalyses those reactions in the seminal plasma for example. The seconds ones, the non free radicals are the Nitric oxide (NO-) and peroxinitrite (ONOO-). They interact with surrounded molecules such as lipids, proteins and nucleic acids (Evans, 2021). The two origins of ROS are: 1) exogenous sources represented by inflammation, and the lack of antioxidants and 2) endogenous sources, mostly produced in mitochondria (Cojocaru et al., 2023, Evans et al., 2021) represented by oxidative phosphorylation, peroxisomes and inflammatory cells activation.

It is well known the spermatozoa contains plenty of mitochondria and OS is considered to be one of the most important factors in male fertility by regulating the vitality and functionality of mammalian spermatozoa (Aitken and Curry, 2011; Aitken et al., 2022; Evans et al., 2021). In animal *in vitro* studies, a misbalance between ROS production and antioxidants can produce negative clinical outcomes as damage in

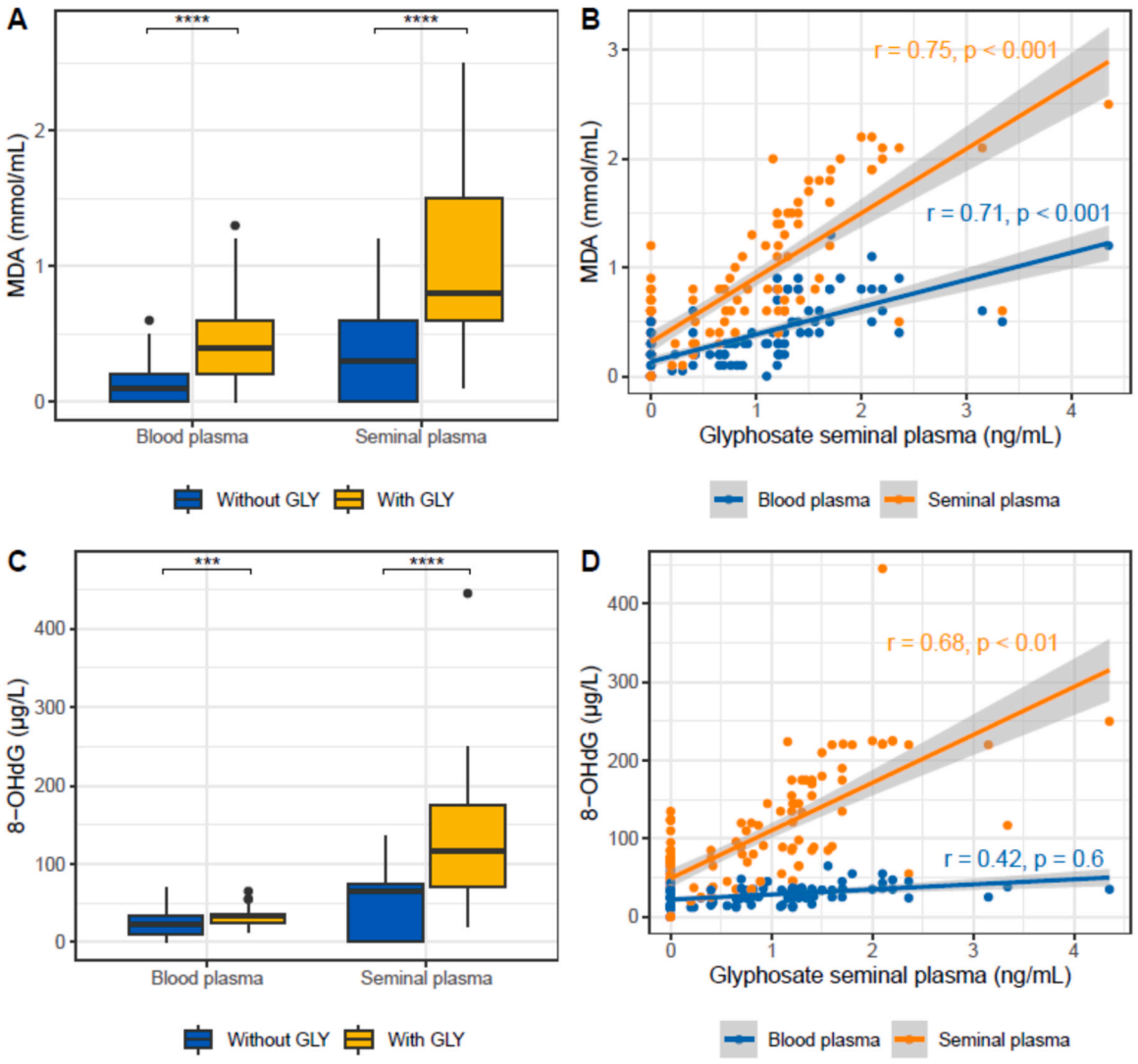

Fig. 3. Concentration of malondialdehyde (MDA) or 8-OHdG (8-hydroxy-2′-deoxy-guanosine) in blood plasma and seminal plasma in patients with or without glyphosate (GLY) and correlations with concentration of glyphosate in seminal plasma. A. Concentration of MDA (mmol/mL) in blood and seminal plasma in patients with or without GLY. B. Correlation between the concentration of glyphosate (ng/mL) in blood and seminal plasma and the concentration of blood MDA (mmol/mL). C. Concentration of 8-OHdG (µg/l) in blood and seminal plasma in patients with or without GLY. D. Correlation between the concentration of glyphosate (ng/mL) in blood and seminal plasma and the concentration of blood 8-OHdG (µg/l). *** p<0.001; **** p<0.0001.

germ cells, impaired fertilization, increased miscarriage and more problematically epigenetics disorders for the next generations (Aitken, 2022; Evans et al., 2021; Opuwari and Henkel, 2016). Thus, high ROS levels in human could be associated to the same disorders with a decreased male fertility (Chen et al., 2012; Evans et al., 2021). Lipid peroxidation is a major cause of the loss of sperm motility and viability (Cerolini et al., 2000). Indeed, human spermatozoa plasma membranes are rich inpolyunsaturated fatty acids(PUFAs), that are composed by double bounds separated by methylene groups, which are sensitive to lipid peroxides and aldehydes compounds (Evans, 2021). The lipid peroxidation generates various electrophilic lipid aldehydes like MDA, 4HNE (4 hydroxynonenal) and ACR (acrolein, **supplemental** Fig. 1). Thus, MDA represents the hallmark of ROS-induced oxidative damage (Dib et al., 2002). DNA damage is usely repaired via the base excision repair pathway and oxidized products are excreted in biofluids such as urine (Graille et al., 2020), sperm (Vorilhon et al., (2018) and plasma seminal (Hologlu et al., 2022) and therefore reflected an oxidative state. Spermatozoa have only got one base excision repair enzyme called 8-oxogananine DNA glycosylase 1 (OGG1). It implies that the DNA repair capacity of spermatozoa is weak (figure supplemental 3, (Evans et al., 2021)). The guanine base in nucleic acids is, among the other bases, the most susceptible to being converted into 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-OHdG) when exposed toROS. Thus, seminal plasma 8-OHdG represents a common biomarker of OS (Gholinezhad et al., 2020). Hosen et al. (Hosen et al., 2015) reported that infertile men have significantly higher levels of 8-OHdG in seminal plasma, as a biomarker of DNA damage, compared with fertile controls. In the present study, we observed a significant positive correlation between GLY concentration and oxidative stress as evaluated by TOS, OSI and MDA and 8-OHdG concentration in seminal plasma. Previous studies have indicated that inducing OSon animal organisms is an important mechanism of GBH toxicity (Tresnakova et al., 2021). In good agreement with our results, an association between OSas determined by the urinary levels of 8-OHdG and GLY exposure has been already demonstrated in human (Chang et al., 2023).

4.3. Potential effect of glyphosate in sperm

In view of the literature, our data on the higher concentration of GLY in seminal fluid than in blood plasma is worrying. Indeed, in rats, GBHs

affect the antioxidant capacities of the testes (Avdatek et al., 2018) and impairs spermatozoa quality by inducing excessive apoptosis of germ cells in mice (Jiang et al., 2018). In our present study, we observed that TAS in both blood and seminal plasma was similar in men with GLY and without GLY. However, TOS and OSI, were significantly higher in men with GLY in blood and seminal plasma than in men without GLY, respectively. GLY is known to negatively affect the mitochondrial respiration efficiency at doses below the NOAEL (Non Observed Adverse Effect Level) (50 mg/kg) and acceptable daily intake (ADI) 0.5 mg/kg bw per day (Ferramosca et al., 2021). I*n vitro* experiments, huge exposure to GLY harms to human germ cells and Leydig cells and therefore results in an obvious decline in progressive motility of human sperms (Anifandis et al., 2018; Gorga et al., 2020). In parallel, *in vivo* animal studies have shown that GLY leads to different alterations of sperm parameters, such as sperm numbers, morphology, motility, and aberration rate (Cai et al., 2017; Lopes et al., 2014; Serra et al., 2021b). These adverse effects might be mediated through mitochondrial impairment (decrease in mitochondrial activity and respiration in human and zebrafish (Ferramosca et al., 2021; Cardona-Maya, 2021; Lopes et al., 2014); decrease in the potential of the mitochondrial membrane and increase in membrane lipoperoxidation in human (Morales Velásquez et al., 2021) (Cardona-Maya, 2021; Ferramosca et al., 2021), OS in rodent (Barikwu et al., 2015) and bird (Serra et al., 2021a) and altered functionality of characteristic testes enzymes in crustaceans (Yang et al., 2018). These effects are in good agreement with omics data performed in mice exposed to GBH showing that GBH impairs the Krebs cycle and respiratory chain (Qi et al., 2023).

Meanwhile, in the present study, no significant reduction of sperm parameters was observed (sperm progressive speed, motility and/or anormal forms) in patients with GLY in sperm. This could be explained by the variability of spermograms or different mixtures of GBH or surfactants used in our country. So, it will be interesting to determine as well the blood and seminal plasma level of surfactants or co-formulants. Indeed, GBH or the commercial GLYformulations are not single ingredient, but contain cocktails of chemicals; with the composition of co-formulants usually undisclosed.Co-formulants could present more deleterious effects than GLY alone because of possible cumulative effects on endocrine and reproductive endpoints (de Araújo-Ramos et al., 2021). It has been underlined in recent animal studies showing that GBH adverse effects on spermatozoa could be mainly due to the surfactant and at less extend to GLY (Torres-Badia et al., 2021; Torres-Badia et al., 2022, Spinaci et al., 2022)).

4.4. Oxidative stress, glyphosate concentration in sperm and epigenetics effects on the progeny

Recent researches on the etiologies of male infertility have stressed the importance of two major actors including genetic factors and OSleading to epigenetic events (Aitken and Gibb, 2022). It has been already demonstrated in animals studies the negative impact of GLY exposure in the offspring (Serra et al., 2021b). Indeed, chronic dietary GBH exposure in roosters induces a strong increase in ROS content and a global DNA hypomethylation in spermatozoa in offspring (Serra et al., 2021b). GLY has been shown to promote the epigenetic transgenerational inheritance of diseases in subsequent offspring (until F3 generation) (Beck et al., 2022; Maamar et al., 2020). Moreover, Teleken et al., (Teleken et al., 2020) showed that maternal exposure to GBH in mice during pregnancy and lactation may lead to decreased spermatogenesis and disruptions in hypothalamus-pituitary-testicular axis regulation in F1 offspring. Thus, all these animal data reinforce the idea of measuring OS and pollutant concentration in sperm from exposed infertile patients in order to better understand the risks in the offspring.

Our data attesting the presence of OS in human sperm with GLY in a proportional manner is a matter of concern. Indeed, oxidized guanine suppresses the methylation of an adjacent cytosine that could lead to aberrant DNA methylation in the embryo, as remethylation of the paternal nucleus is known to occur after fertilization (Drevet and Aitken, 2020). Abnormal methylation of embryonic DNA could lead to altered gene expression and the susceptibility of diseases in the offspring. Our study suggests that it could be relevant to measure systematically oxidative stress in sperm in infertile patient and this for two reasons. The first one is to encourage the patient to improve his environment by reducing psychologic stress, by stopping the toxics (tobacco, cannabis and alcohol…), by eating organic food and by drinking filtered water and trying to decrease endocrine disruptors exposure (Evans et al., 2021). The second one is to give antioxidants to try to counter act the oxidative state. Nevertheless, this remains to be demonstrated in humans. Indeed, it would be interesting to systematically test OS in sperm before and after antioxidant supplementation to evaluate the benefit of this therapy in infertile patient whatever the sperm parameters are.

One of the limitation of our study is the lack of data concerning plasma testosterone. Indeed, several reports showed that GLY or/and GBH were able to impair testosterone synthesis in mammals and birds including pubertal and adult rats (Manservisi et al., 2019; Zhao et al., 2021) and quails (Ruuskanen et al., 2020). Overall, GLY could disrupt the transcription and activity of some components of the steroidogenic machinery such as StAR and CYP17A1 in TM3 Leydig cells (Xia et al., 2020). In addition, recent data showed that GLY induced testosterone synthesis inhibition via ferroptosis (Lu et al., 2024). However, some studies reported an increase (Serra et al., 2021a in chicken; Lorenz et al., 2020 in rat) or no changes in testosterone or oestradiol (Johansson et al., 2018 in rat) in response to GLY or GBH exposure. In human, a recent study observed no significant associations between urinay glyphosate concentration and testosterone levels (Glover et al., 2023).

5. Conclusion

We have reported for the first time in human, the presence of GLY in human sperm in nearly 60% of male patients in a French infertile cohort in our infertility clinic. We found GLY concentrations four time higher in sperm than in blood, corresponding probably to an hemato-testicular barrier alteration. We detected a strong positive correlation between OSin lipids and DNA and GLY's amount in blood and sperm. Meanwhile, we can't exclude the toxicity of the co- formulants of GBHs or other pesticides associated as well. Even if we did not find in our study a correlation between sperm parameters and oxidative stress markers and GLY, it's a matter of concern for the future generations to have detected a such elevated proportion of GLY associated with OS in sperm in our infertile population. It could be interesting to test systematically OS markers in sperm in infertile man in order to improve the lifestyle factors and give antioxidant supplementation to try to counteract oxidative status. Indeed, OS is one of the main mechanisms implicated in the occurrence of pathologies such as obesity, cancer and epigenetic effects in the offspring. It would be wise to apply the precautionary principle for GBHs formulants use despite the extension of the GLY use in Europe.

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CRediT authorship contribution statement

Claudine Vasseur: Writing – review & editing, Writing – original draft, Validation, Formal analysis, Data curation, Conceptualization. **Loïse Serra:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Souleiman El Balkhi:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Gaëlle Lefort:** Writing – review & editing, Validation, Methodology, Formal analysis, Conceptualization. **Christelle Rame:** Writing – review & editing,

Methodology, Investigation, Formal analysis, Conceptualization. **Pascal Froment:** Writing – review & editing, Validation, Investigation, Formal analysis. **joelle dupont:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116410.

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