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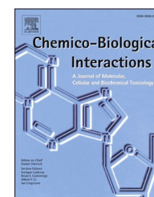

Research paper

Comprehensive characterisation of poly(ADPribosyl)ation in spermatozoa as a novel and early biomarker of sperm health. A preliminary look

Bruno Berman [a,2], Gennaro Lettieri [a,2], Martina Falace [a], Carmela Marinaro [a], Carmen Di Giovanni [b], Anna Rita Bianchi [a,1], Luigi Montano [c,1], Marina Piscopo [a,*], Anna De Maio [a,**]

[a] Department of Biology, University of Naples Federico II, Via Cinthia, 21, 80126, Naples, Italy
[b] Department of Pharmacy, Via Domenico Montesano, 49, 80131, Naples, Italy
[c] Andrology Unit and Service of LifeStyle Medicine in Uro-Andrology, Local Health Authority (ASL) Salerno, Italy

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ABSTRACT

Poly(ADPribose) polymerases (PARPs) are a family of enzymes involved in various biological processes, including DNA repair, maintaining genomic stability, chromatin compaction and cell death induction. PARP activation has been associated with oxidative stress (OS), which causes oxidative DNA damage and negatively affects sperm quality and function. OS plays a fundamental role in the aetiology of male infertility. As nuclear poly(ADPribose) polymerases (PARPs) are markers of DNA damage and their homologues have been found in testicular germ cells, this preliminary study examined the poly(ADPribosyl)ation system in the sperm of men living in the Sele River Valley (an area with low environmental exposure) and in the Land of Fires (an area sadly renowned for its high pollution levels). Additionally, the poly(ADPribosyl)ation of sperm nuclear basic proteins (SNBPs) was investigated for the first time, given that these proteins play a key role in the compaction of sperm chromatin. Our results revealed the presence of a complete and active poly(ADPribosyl)ation system and showed that PARP automodification is linked to the extent of sperm DNA damage. We have also demonstrated the endogenous SNBP heteromodification, which is associated with chromatin decondensation. Finally, we propose a molecular mechanism linking PARP automodification and DNA damage with SNBP heteromodification and the state of DNA compaction.

1. Introduction

Poly(ADPribosyl)ation is a reversible post-translational modification reaction of nuclear proteins that is dependent on DNA damage and catalysed by poly(ADPribose) polymerases (PARPs). PARPs are a family of enzymes consisting of 18 different isoforms [1]. While all PARP proteins share a highly conserved 50-residue catalytic domain known as the 'PARP signature', they differ in terms of their localisation and molecular weight [2]. PARPs use nicotinamide adenine dinucleotide (NAD^+) as substrate to catalyse the transfer of ADPribose (ADPr) to the arginine, lysine, aspartate or glutamate residues of target proteins. These ADPribose units are then linked by glycosidic bonds to form linear or branched polymers known as poly(ADPribose) (PAR). The length of PAR can reach 200–400 monomer units in vitro and in vivo [3,4]. PAR remotion from its substrates is a rapid event which is guaranteed by PAR-degrading enzymes, as poly(ADPribose) glycohydrolase (PARG) and ADPribosyl lyase [5]. While PARPs are encoded by numerous genes, only a single gene coding for PARG was detected in mammalian cells [6]. Human PARG has five isoforms with different molecular weight, localized in different cellular comparts: 55 kDa (in the mitochondria), 60 kDa (in the cytoplasm and mitochondria), 99 kDa (in the cytoplasm), 102 kDa (in the cytoplasm) and 111 kDa (in the nucleus). The 99, 102 and 111 kDa PARGs are yielded through alternative splicing, whereas the 55 and 60 kDa isoforms are thought to derive from degradation of the 111 kDa protein or alternative splicing [7].

The two founding members of the family are the nuclear enzymes

* Corresponding author.
** Corresponding author.
E-mail addresses: marina.piscopo@unina.it (M. Piscopo), andemaio@unina.it (A. De Maio).
[1] These authors are co-last.
[2] These authors contributed equally to this work and are co-first.

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PARP1 and PARP2, which are activated in response to DNA strand breaks [8]. PARP1 (113 kDa) has three main domains: the DNA-binding fragment, which contains two zinc finger motifs and a nuclear location signal; the central auto-modifying domain, rich in glutamate residues and the C-terminal catalytic fragment, that binds NAD^+ [9]. PARP2 (66 kDa) has a C-terminal domain structurally homologous to PARP1, but with significantly different N-terminal regions [10].

Once activated following DNA damage, PARP1 and PARP2 catalyse two types of reactions called automodification and heteromodification: in the first, PAR binds covalently to PARPs (autoPARylation), in the second, it binds to various nuclear protein acceptors (heteroPARylation) [10]. The major PAR covalent acceptor is PARP1 itself, which can bind approximately 90 % of cellular PAR.

Nuclear PARPs play a key role in maintaining genomic integrity and survival in response to genotoxic insults. In fact, PARP1 and PARP2 are considered "sensors" of DNA damage and their activation represents one of the first cellular responses to material genomic damage [9,11].

Both enzymes are also involved in numerous biological processes, including DNA repair, transcriptional regulation, mitotic spindle formation, telomere dynamics, apoptosis and necrosis [2,12]. When DNA damage is mild, PARP1 automodification allows reparative enzymes to intervene, ensuring the cell survival. Conversely, PARP overactivation, resulting from massive genotoxic damage causes depletion of intracellular energy reserves, leading to cell death by necrosis or apoptosis [13]. PARP1 is considered a marker of apoptosis because it is cleaved by caspase-3 into two stable and inactive fragments of 24 kDa and 89 kDa, respectively, during this process of programmed cell death [14].

Poly(ADPribosyl)ation by PARP1 is known to control the nucleosomal unfolding, promoting histone heteromodification. The nucleosomal core histones is poly(ADPribosyl)ated by short oligomers, whereas chain length up to 15 ADPribose units are observed for H1 [15]. H1 heteromodification occurs mainly on arginine, glutamate and lysine residues [16,17].

A large body of evidence suggests the presence of PARPs in gamete differentiation [18,19], in testicular germline cells to guarantee the DNA integrity in spermatogenesis [20] and chromatin remodeling [21]. During the meiotic and post-meiotic phases, spermatozoa gradually replace 85 % of their histones with transition nuclear proteins that interact with DNA, and then with protamines. The interaction between sperm DNA and protamines leads to the coiling of the DNA into toroidal subunits containing 50–100 kb of DNA [22]. The presence of high levels of arginine and cysteine in protamines ensures the paternal genome is optimally compacted. This process is closely linked to sperm head volume, and, consequently, to optimal velocity and successful fertilisation [23,24].

The quality of human spermatozoa, and therefore their fertility status, is closely related to the protamine P1/P2 ratio, which is typically between 0.8 and 1.2, as well as the canonical protamine/histone ratio which is about 85 %–15 % [25]. Defective chromatin compaction is actually indicated by a higher histone/protamine ratio and lower P2 levels. This increases the risk of sperm DNA damage, which is a major cause of male infertility [25,26]. Male infertility is caused by many endogenous and exogenous factors that induce oxidative stress (OS). This results in damage to the sperm membrane and DNA, a phenomenon observed in 30–80 % of infertile men [27].

Recently, changes in the protamine/histone ratio and the P1/P2 ratio have been observed in young men living in the Land of Fires, an area of Campania in southern Italy [28]. This three million m^2 area between the provinces of Naples and Caserta is infamous for its high level of pollution, mainly caused by the burial of toxic waste and the presence of illegal landfill [29]. Changes in the protamine/histone and P1/P2 ratios have been observed in young men living in the Land of Fires, due to the presence of excess of some pollutants in their semen. One of these pollutants is chromium, which inhibits the action of histone acetylases — crucial enzymes involved in replacing histones with protamines during spermatogenesis [30]. Our previous studies have also shown that oxidative DNA damage leads to the hetero-PARylation of protamine-like II (PL-II) in the gonads of *Mytilus galloprovincialis* exposed to chromium. This event seems to be correlated with decondensation of sperm chromatin [31].

Given that PARP homologues have been detected in human semen and chromium has been found in excess in the semen of subjects living in the Land of Fires [32], this study aimed to characterise poly(ADPribosyl) ation in spermatozoa for the first time in men from areas with different environmental exposures. Eight men were recruited from the Sele River Valley, an area with a low level of pollution, located between the provinces of Avellino and Salerno, in Campania (southern Italy), and five from the Land of Fires, which is an area with a high level of pollution, as mentioned above.

The characterization was performed by: 1) analysing PARP and PARG expression, to demonstrate the presence of these enzymes in sperm; 2) measuring both enzyme activities to verify that PARP and PARG were also catalytically active if expressed; and 3) identifying covalent protein acceptors of PAR. SNBPs extracted from the spermatozoa of men from both the Sele River Valley and the Land of Fires were then analysed by electrophoresis on an acid-urea polyacrylamide gel to identify possible alterations in the histone/protamine ratio. Finally, the heteromodification of histones and protamines by PAR was studied for the first time, in order to assess its role in the process of sperm chromatin decondensation, which is essential for DNA damage repair.

The main limitation of this study is its small sample size. However, the aim of this preliminary investigation was to verify whether alterations in the poly(ADPribosyl)ation system in individuals living in polluted areas could represent new, early markers for monitoring sperm DNA health in a larger cohort.

2. Materials and methods

2.1. Recruitment patients and inclusion criteria

The study was carried out, within the Ecofoodfertility project (https://www.ecofoodfertility.it/) in two areas of the Campania region with different levels of environmental impact: the Sele River Valley (VSL) in the province of Salerno, which has a low environmental impact (LEI), and the Land of Fires (LF), which has a high environmental impact (HEI). The Land of Fires is a site recognised by the Campania Regional Environmental Agency as having a high concentration of toxic contaminants, including heavy metals, sewage sludge, battery acid, asbestos and radioactive waste [33]. The men were recruited from two residential areas: the municipalities of Contursi Terme and Oliveto Citra, which have a low environmental pressure index, and the municipality of Acerra, which has a high environmental pressure index [34]. Semen samples were collected from study participants at the Andrology and Lifestyle Medicine Service at Oliveto Citra Hospital which serves the Upper Middle Sele Area (ASL Salerno) and at the Villa dei Fiori Clinic in Acerra. The inclusion criteria for recruitment were the following: age between 18 and 35 years, living in the indicated areas for at least five years, non-smokers, no history of drug and/or alcohol abuse, no occupational exposure to toxic chemicals and no chronic and gastrointestinal diseases (e.g. celiac disease, ulcerative colitis). Partecipants also had to have an abdominal circumference of less than 102 cm at the time of recruitment, and they could not have had any other factors that could interfere with the biological analysis, such as fever, medication, the use of steroids or anabolic hormones, dietary supplements or substances containing plant or animal extracts or trace elements.

2.2. Sperm protein extraction

Sperm protein extracts were prepared from semen as described in Jha et al. [1]. After centrifugation of semen samples, using a microcentrifuge (1–14K, Sigma Laborzentrifugen GmbH, Germany), at 8900×g in tubes (2.0 mL) for 5 min at 4 °C, the supernatant (seminal

plasma) was removed. The pellet was resuspended in 300 μL phosphate buffered saline (PBS) and centrifuged at 8900×g for 5 min at 4 °C to remove the residual seminal plasma. The pellet was then solubilized and homogenised three times at 10 s intervals using an Ultra Turrax T8 (IKA-WERKE) in 200 μL buffer consisting of 187 mM Tris HCl pH 6.8, 2 % SDS, 10 % glycerol, 1 % Triton X-100, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM ethylene glycol tetraacetic acid (EGTA), protease inhibitors. The final homogenate was centrifuged at 12,000×g for 20 min and the supernatant obtained represents the sperm protein extract. The protein concentration was determined by the Bradford assay [35].

2.3. SDS-PAGE and western blotting

Sperm protein extracts (20 μg) in duplicate were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12 % polyacrylamide gel (SDS-PAGE) in 0.025 M Tris-0.192 M glycine-0.1 % SDS buffer, pH 8.3 at 18 mA [36]. A part of the gel was stained with 0.1 % Coomassie G in 10 % acetic acid and 30 % methanol, to detect the protein pattern, while the other part was transferred onto polyvinylidene fluoride (PVDF) filter (0.45 μm; Cat No. IPVH00010, Merck Millipore, Milan, Italy) at 200 mA in buffer containing 0.025 M Tris, 0.192 M glycine and 0.025 % SDS for 2 h at 4 °C. The membrane was blocked with 3 % gelatine in Tris-buffered saline (50 mM Tris-HCl and 150 mM NaCl, pH 8.0) containing 0.05 % Tween-20 (TBS-T) for 2 h at room temperature. After repeated washes in TBS-T the filter was incubated for 2 h with monoclonal anti-poly(ADPribose) polymerase (PARP) (sc-8007, Santa Cruz Biotechnology, Inc., Dallas, TX, USA, 1:500). Horseradish peroxidase (HRP)-conjugated anti-mouse (sc-525409, Santa Cruz Biotechnology, Inc., 1:2000) was used as secondary antibody.

Following Arena et al. [37] two stripping procedures were performed to remove the primary antibodies from the filters. The first stripping step removed the anti-PARP antibody, allowing the filter to be incubated first with a polyclonal anti-poly(ADPribose) polymer (PAR) primary antibody (ab14460, Abcam, 2.5 μg/mL), and then with a horseradish peroxidase (HRP)-conjugated anti-chicken secondary antibody (GTX27118, GeneTex, 1:2000). After the second stripping, the filter was incubated with an anti-β-actin antibody (sc-517582, Santa Cruz Biotechnology, Inc., diluted 1:500). A HRP-conjugated anti-mouse secondary antibody (sc-525409, Santa Cruz Biotechnology, Inc., dilution 1:2000) was then used.

Western blotting was performed to analyse PARG expression using an anti-poly(ADPribose) glycohydrolase (PARG) primary antibody (27808-1-AP, Proteintech Group Inc., Rosemont, IL, USA, diluted 1:2000) and a horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (31460, Thermo Fisher Scientific Inc., diluted 1:2000). After stripping, the filter was incubated with primary antibody against β-actin as described above. Immunodetection by enhanced chemiluminescence (ECL, 32106, Thermo Fisher Scientific Inc., Waltham, MA, USA) and densitometric analysis of immunopositive signals for anti-PARP, anti-PARG and anti-PAR antibodies, were conducted by Image Lab 5.2.1 software in a ChemiDoc system (BioRad), and the results were expressed as optical density (OD; i.e., intensity of a band/ mm^2).

2.4. Poly(ADPribose) polymerase assay activity

Sperm protein extract (20 μg) was incubated in the presence of 0.4 mM [^{14}C]NAD$^+$ (10,000 cpm/nmol) in a reaction mixture (50 μL) containing 500 mM Tris-HCl, pH 8.0; 50 mM $MgCl_2$ and 10 mM dithiothreitol (DTT) [36]. After incubation for 15 min at 25 °C, the reaction was stopped by the addition of ice-cold 20 % trichloroacetic acid (TCA) (w/v). The mixture was then filtered on Millipore filters (HAWPP0001, 0.45 μm pore size, MF Millipore, Milan, Italy) and washed several times with 7 % trichloroacetic acid. The radioactivity of the insoluble acid material associated with the filter was detected using a liquid phase scintillator (LS 1701, Beckman Coulter, Milan, Italy). Enzyme activity, defined as the amount of enzyme required to convert 1 nmol of NAD$^+$ per minute at the optimum of pH and temperature, was expressed in enzymatic milliunits per milligram (mU/mg).

2.5. Poly(ADPribose) glycohydrolase activity

Sperm protein extract (20 μg) was incubated in a reaction mixture (final volume 50 μL) containing 0.5 M Tris-HCl (pH 7.5), 50 mM $MgCl_2$, 10 mM DTT, and 0.4 mM [^{14}C]NAD$^+$ (10,000 cpm/nmol) under PARP assay conditions to generate poly(ADPribose). After incubation for 15 min at 25 °C, the reaction was stopped on ice by the addition of 20 % TCA (w/v). The proteins were then centrifuged at 10,000 rpm for 5 min at 4 °C. After washing in absolute ethanol, the precipitate, containing poly(ADPribosyl)ated and non-(ADPribosyl)ated proteins, was incubated in a reaction mixture (100 mM Tris-HCl pH 8, 10 mM DTT) in the presence of homogenate (20 μg) for 15 min at 37 °C under PARG assay conditions [38]. The reaction was blocked on ice and the precipitates obtained by addition of 20 % TCA were washed with 7 % TCA and filtered on Millipore filters (HAWPP0001, 0.45 μm). PARG activity was measured as acid-insoluble radioactivity by liquid scintillation in a Beckman counter (model LS 1701) and expressed as mU/mg.

2.6. Sperm nuclear basic proteins extraction

Extraction of sperm nuclear basic proteins (SNBPs, i.e., histones and protamines) from spermatozoa was performed as described by Lettieri et al. [28]. Briefly, sperm pellets were washed twice with 500 μL of PMSF, centrifuged at 10,480×g for 5 min at 4 °C, resuspended with 50 μL of 1 mM PMSF and 50 μL of a solution containing 6 M guanidinium chloride and 10 mM DTT and incubated at 20 °C for 30 min. The samples were then added with 5 volumes of cold ethanol 100 % and incubated at −20 °C for 60 min to obtain sperm chromatin precipitation. The samples were centrifuged at 13,680×*g* for 15 min at 4 °C and the pellet obtained was resuspended in 500 μL of 0.5 M HCl to solubilise the SNBPs. The samples were incubated for 5 min at 37 °C and then centrifuged at 1000×*g* for 10 min at 4 °C. At the end of this step, TCA was added to the supernatant to a final concentration of 20 % to precipitate SNBPs. The samples were incubated for 60 min at 4 °C and then centrifuged at 14, 000×g for 10 min at 4 °C. The resulting pellet containing SNBPs was washed twice with 500 μL of acetone containing 1 % β-mercaptoethanol. It was then centrifuged twice at 14,000×g for 10 min at 4 °C, after which the final pellet was dried in a speed vacuum for 10–15 min. The dried proteins were then resuspended in 50 μL of ultrapure water (Milli-Q) and stored at −20 °C in 50 μg aliquots.

2.7. Acid-urea polyacrylamide gel electrophoresis of SNBPs and western blotting by anti-PAR

Acid-urea polyacrylamide gel electrophoresis (AU-PAGE), was carried out to analyse two aliquots of SNBPs (4 μg) extracted from spermatozoa of all tested samples, as described by Lettieri et al. [39]. The gel, with a final volume of 8 mL, consisted of 9.0 % (w/v) acrylamide/N, N′-Methylene-bis-acrylamide (25/0.67), 2.5 M urea, 0.9 M acetic acid, 80 μL TEMED, and 140 μL of 10 % APS. Gel polymerization was performed with a pre-run of 1 h at 150 V using 5 % acetic acid as a running buffer. Each well was loaded with 20 μL of a solution containing 8 M urea and 5 % acetic acid. 4 μg of proteins and 20 μL of a solution consisting of 12.8 M β-mercaptoethanol and 8 M urea were loaded onto the gel. After 1 h, 2 μL of 100 % acetic acid and 2 μL of 0.001 % pyronin were added. Electrophoresis was then performed at 100 V for 1 h

The gel was cut into two parts. The first part was stained with Coomassie Blue R, while the second part was transferred to a PVDF membrane for 2 h at 4 °C at 100 V in 0.7 % acetic acid. The PVDF membrane was blocked with 3 % gelatine in TBS-T as described in the previous paragraph and then incubated for 2 h at room temperature with

polyclonal anti-poly (ADPribose) polymer antibody (ab14460, Abcam, 2.5 μg/mL) and horseradish peroxidase (HRP)-conjugated anti-chicken secondary antibody (GTX27118, GeneTex, Inc., 1:2000).

2.8. SDS -PAGE and western blotting of SNBPs by anti-H1 antibody

SNBPs (20 μg) were subjected to 18 % SDS-PAGE according to Marinaro et al. [35]. The electrophoresis run was stopped when the protamines came out of the gel. After electrophoresis, one part of the gel was stained with 0.1 % Coomassie Blue R in 10 % acetic acid and 30 % methanol, while the other part was transferred to a PVDF membrane at 200 mA for 2 h at 4 °C in buffer containing 0.025 M Tris, 0.192 M glycine and 0.025 % SDS. Immunoblotting was performed using anti-H1 monoclonal antibody (ABclonal A4342). Immunodetection by enhanced chemiluminescence (ECL, 32106, Thermo Fisher Scientific Inc., Waltham, MA, USA) were conducted by Image Lab 5.2.1 software in a ChemiDoc system (BioRad).

2.9. Statistical analysis

Statistically significant differences were assessed by one-way analysis of variance (ANOVA), followed by Holm-Sidak's multiple comparison test using the GraphPad Prism 8.0.1 software. The results were reported in the graph as the mean ± standard deviation (SD), and the minimum level of acceptable significance was $p < 0.05$.

3. Results

3.1. PARP expression and covalent protein acceptors identification

To verify whether and which PARP isoforms were expressed in the sperm of men living in the Sele River Valley (VSL) and the Land of Fires (LF), the sperm homogenates were analysed using 12 % polyacrylamide gels in 0.1 % sodium dodecyl sulfate (Figs. 1a and 2a, respectively) followed by western blotting with anti-PARP antibody, capable of recognising the highly conserved PARP catalytic domain (Figs. 1b and 2b, respectively). Moreover, the covalent protein acceptors of poly (ADPribose) were also identified by western blotting with anti-PAR antibody, which recognises an ADPr chain of at least 2 units (Figs. 1c and 2c, respectively).

Western blot of the VSL and LF sperm homogenates, normalised to β-actin (~43 kDa) are shown in Figs. 1d and 2d, respectively.

The electrophoretic protein patterns of both VSL and LF sperm samples showed significant qualitative and quantitative differences (Figs. 1a and 2a, respectively). An only single immunoreactive signal corresponding to a protein of approximately 75 kDa was recognised by the anti-PARP antibody in all examined sperm samples (Figs. 1b and 2b, respectively). Densitometric analysis of immunoreactive bands revealed no significant difference in the intensity values between the VSL (Fig. 1e) and LF (Fig. 2e) sperm samples.

The 75 kDa band recognised by the anti-PARP, was also immunopositive to the anti-PAR antibody in VSL 1, VSL 2, VSL 3, VSL 4, VSL 5 and VSL 7 homogenates (Fig. 1c). In the latter, the lowest intensity was detected following densitometric analysis (Fig. 1f). In addition, the anti-PAR recognised signals corresponding to proteins with a molecular weight greater than 75 kDa in VSL 3, VSL 4 and VSL 5, while a strong immunopositive signal at the top of filter was only visible in VSL 3, VSL 6 and VSL 8.

Finally, only one immunoreactive signal to anti-PAR at the top of filter was showed in sperm homogenates of LF men (Fig. 2c).

3.2. PARG expression

Western blotting with anti-PARG antibody was carried out on VSL and LF sperm homogenates, to verify the presence of PARG enzymes, responsible of PAR degradation.

Electrophoretic analysis of VSL and LF protein patterns (Fig. 3a) confirmed the qualitative and quantitative differences shown previously in Figs. 1a and 2a, respectively.

Two immunoreactive signals were recognized by anti PARG

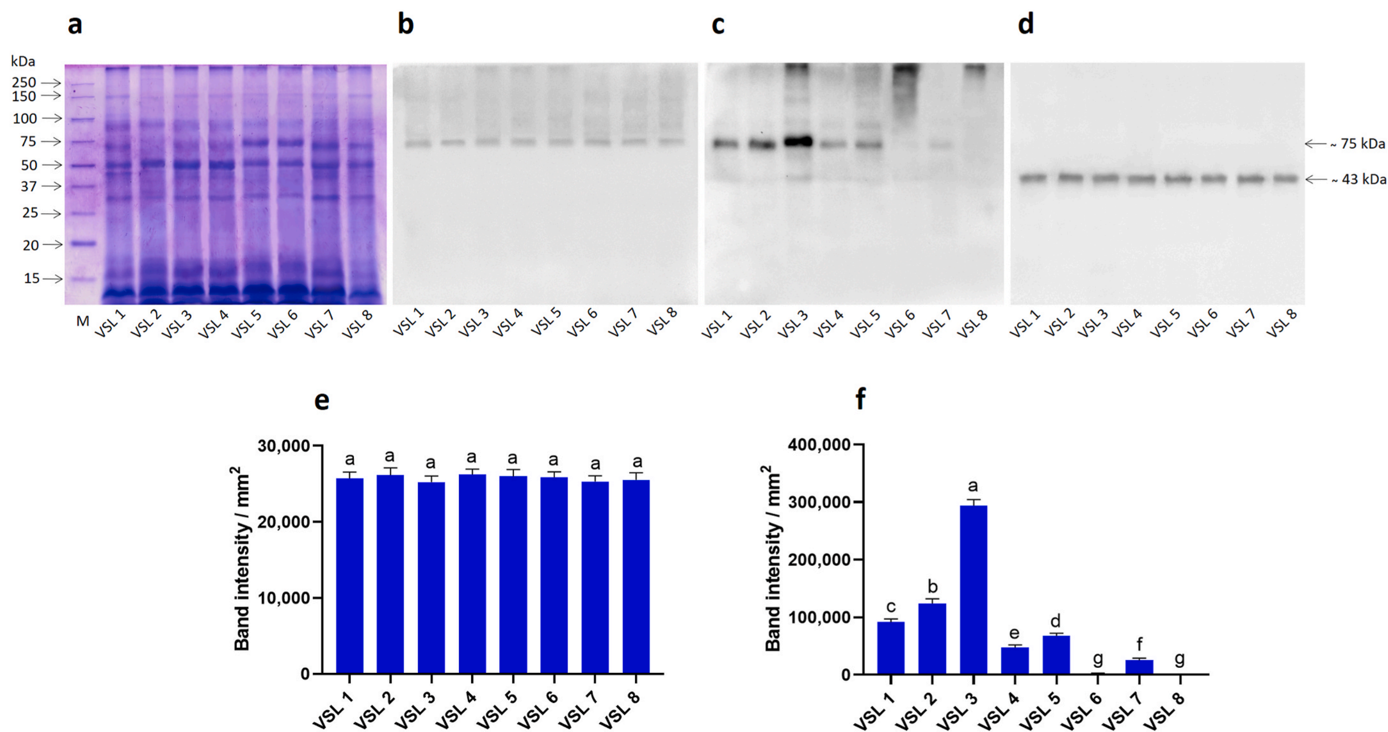

Fig. 1. 12 % SDS-PAGE (a) and immunoblotting with anti-PARP (b), anti-PAR (c) and anti β-actin antibodies (d) in sperm homogenates from VSLs. Densitometric analysis of ~75 kDa bands immunopositive to anti-PARP (e) and anti-PAR (f). The bars represent the mean ± SD. Different letters indicate significant differences for $p < 0.05$.

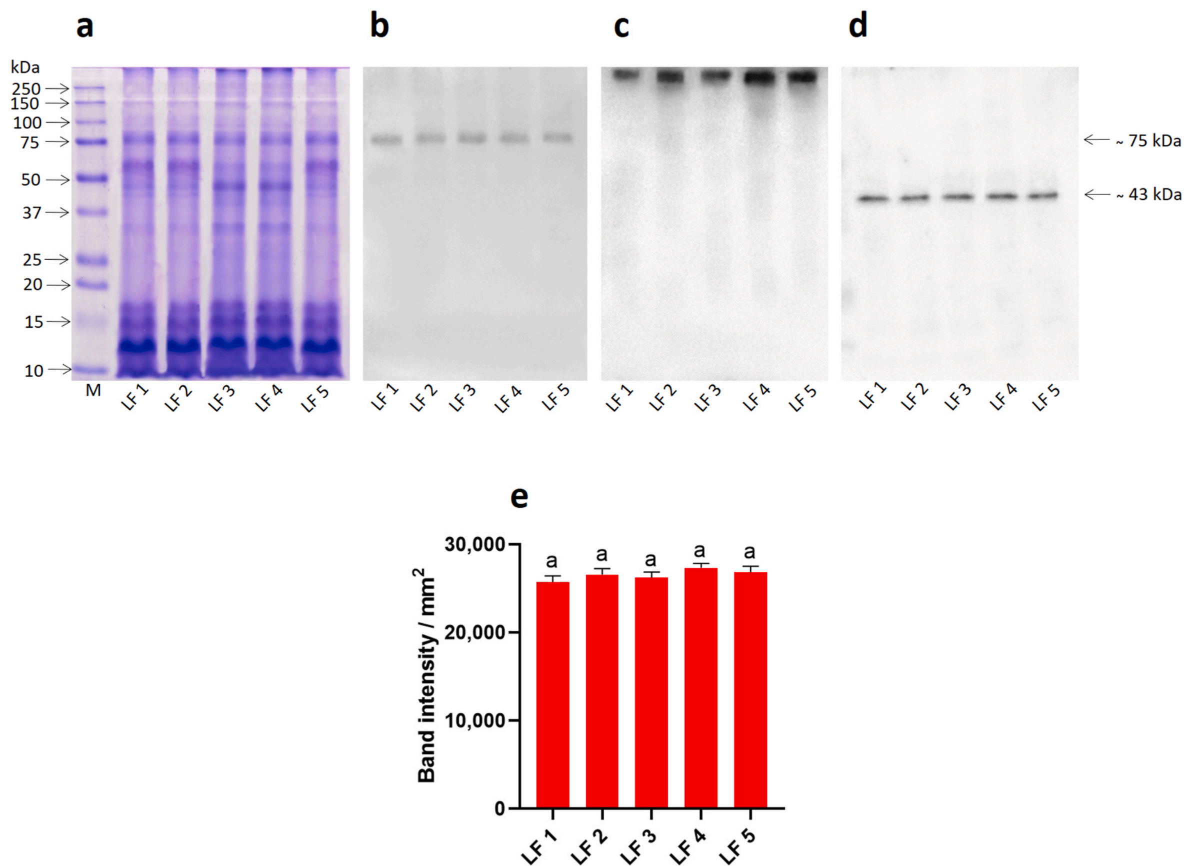

Fig. 2. 12 % SDS-PAGE (a) and immunoblotting with anti-PARP (b) and anti-PAR (c) and anti β-actin antibodies (d) in sperm homogenates from LFs. Densitometric analysis of ~75 kDa bands immunopositive to anti-PARP (e). The bars represent the mean ± SD. Different letters indicate significant differences for $p < 0.05$.

antibody, corresponding to two proteins of molecular weight of about 60 kDa and 110 kDa, respectively (Fig. 3b). Western blot normalized to β-actin (~43 kDa) is shown in Fig. 3c.

No difference in intensity ($p < 0.05$) was measured following densitometric analysis of each signal in VSL and LF sperm samples, demonstrating that both isoforms were equally expressed in each of two examined groups (VSLs and LFs) (Fig. 3d).

3.3. PARP and PARG activity

Having demonstrated the presence of PARP and PARG enzymes in sperm samples from both VSL and LF subjects, we verified whether these enzymes were active. To this end, we measured the activity of both enzymes (Fig. 4a and b, respectively). Additionally, the percentage of PAR degradation was determined (Fig. 4c and d, respectively).

The lowest levels of PARP and PARG activity were found in the sperm homogenate of VSL 7, which was therefore considered the only true control sample. Thus, the activities of both enzymes measured in the other VSLs and LFs were referred to VSL 7 values (Fig. 4a and b). Although, among VSLs, the highest PARP and PARG activity levels were in VSL 6, however, significantly higher levels of both enzyme activities were found in all other VSLs compared to those measured in VSL 7 (Fig. 4a). In addition, the activities of the PARP and PARG enzymes in LFs were consistently higher than those measured in the other VSLs (see supplementary materials, Fig. S1). A percentage of PAR degradation greater than 90 % was calculated in VSL 1, VSL 2, VSL 4, VSL 5 and VSL 7, whereas only 71.2 %, 52.6 % and 57.2 % of PAR was degraded in VSL 3, VSL 6 and VSL 8, respectively (Fig. 4c). Approximately 50 % of PAR was digested in all LF sperm samples compared to degradation percentage determined in VSL 7 (99.6 %) (Fig. 4d).

3.4. Poly(ADPribosyl)ation of SNBPs

The SNBPs extracted from all the tested samples were separated by AU-PAGE, then subjected to immunoblotting with an anti-PAR antibody (Fig. 5a). Electrophoretic analysis revealed higher levels of protamines in VSL 7 and higher levels of histones in VSL 5. Furthermore, the lowest levels of protamines were observed in VSL 6 and LF 1 (Fig. 5a). Western blotting by anti-PAR evidenced very weak immunopositive signals corresponding to PAR covalently bound to protein acceptors (histones and/or protamines) in VSL 7. Conversely, several signals corresponding to electrophoretic migration of both histones and protamines were recognized by antibody in VSL 5. Finally, weak signals in zone of protamines electrophoretic migration together with evident immunoreactive bands in correspondence of histones were detected in VSL 6. In LF 1, instead the anti-PAR recognized PAR bound only to histones (Fig. 5b).

3.5. Identification of H1 histone heteromodified by PAR

The SNBPs were subjected to 18 % polyacrylamide gel electrophoresis (Fig. 6a) followed by western blotting with anti-H1 antibody, to verify the presence of H1 among the histones heteromodified by PAR in VSL 7, VSL 5, VSL 6 and LF 1 (Fig. 6b). Electrophoretic analysis confirmed the previous AU-PAGE findings that the proportion of histones in VSL 7 is lower than in VSL 5, VSL 6 and LF 1. Anti-H1 antibody detected immunopositive signals corresponding to a protein with a molecular weight of approximately 25 kDa in SNBP extracts of VSL 5, VSL 6 and LF 1. Densitometric analysis showed that 25 kDa band in VSL 5, VSL 6 and LF 1 had an intensity (Int/mm^2) significantly higher than that measured in the VSL 7 sample. The highest intensity of 25 kDa signal was detected in LF 1 extract (Fig. 6c).

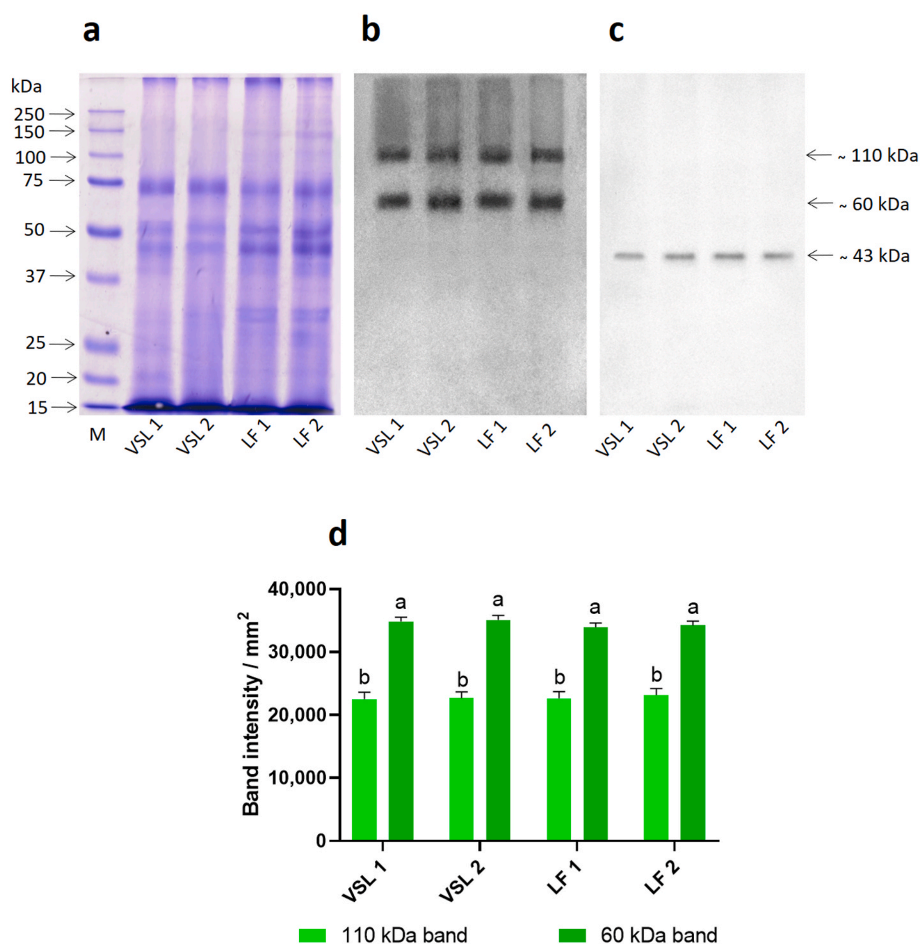

Fig. 3. 12 % SDS-PAGE (a) and immunoblotting with anti-PARG (b) and anti β-actin antibodies (c) in sperm homogenates from VSL 1, VSL 2, LF 1 and LF 2. Densitometric analysis (d). Bars represent mean ± SD. Similar letters indicate no significant differences ($p < 0.05$) in densitometry values between samples for both immunopositive bands.

4. Discussion

In recent decades, environmental pollution has emerged as a major contributor to the increasing prevalence of male infertility worldwide [40]. In this context, a recent meta-analysis published in Human Reproduction Update has further raised the global alarm about the severe decline in sperm count that has occurred not only in Western countries, but also in countries of the Southern hemisphere [41]. The causes of this decline seem to be attributed to improper lifestyles (alcohol and tobacco consumption, reduced night rest, etc.), infections of the reproductive system, and chronic exposure to environmental pollutants. Recent studies have shown that several pollutants accumulate in spermatozoa to a greater extent than in blood and urine matrices [42,43] and contribute to oxidative DNA damage in human sperm [28]. Oxidative damage to sperm DNA was also documented in *Mytilus galloprovincialis* exposed to heavy metals and microplastics [44–48]. Spermatozoa are particularly vulnerable to oxidative damage due to a number of factors. These include their high polyunsaturated fatty acid (PUFA) content in their membranes, limited cytoplasmic volume, reduced antioxidant reserves, and an inability to effectively repair DNA damage. All of these factors make them susceptible to reactive oxygen species (ROS) [49,50].

Moreover, the quality of sperm, and therefore the fertility status of human spermatozoa, was closely related to protamine/histone ratio [25]. During spermatogenesis, histones are extensively replaced by protamines P1 and P2 in the chromatin of human spermatozoa. This replacement has an important role in chromatin condensation, which is necessary for the induction of potent spermatozoa and to protect the sperm from the damaging effects of free radicals [19]. Sperm DNA of young men living in Land of Fires was demonstrated to be more susceptible to oxidative damage when the impairment of the protamination process occurs [28]. In addition, SNBP are involved in the oxidative damage of DNA in that they bind to the pollutants that accumulate in the semen of subjects living in the Land of Fires. These contaminants mainly consist of heavy metals such as chromium and copper, which are known to cause the Fenton reaction. This reaction produces damage to the DNA, which is visible as an increase in the intensity of the relaxed form of plasmid DNA when SNBP are added to circular plasmid DNA in pro-oxidant conditions [32].

The impact of epigenetic modifications on how organisms respond to stress is well understood [51,52]. Poly(ADPribosyl)ation is an epigenetic mechanism where poly(ADPribose) polymerases (PARPs) attach ADPribose chains to proteins, including histones and protamines, regulating DNA damage response, transcription, and chromatin structure.

Since poly(ADPribosyl)ation represents one of the earliest molecular responses to oxidative DNA damage, we characterised a complete poly (ADPribosyl)ation process for the first time in the spermatozoa of men recruited from the Sele River Valley and Land Fires areas. The former is an area with low environmental exposure, while the latter is notoriously polluted [53].

In all VSL and LF sperm homogenates a PARP of about 75 kDa (Figs. 1b and 2b) and two PARG isoforms of 60 kDa and 110 kDa (Fig. 3b) were identified.

Fig. 4. PARP and PARG activity in sperm homogenates from VSLs (a) and LFs (b) and percentage of PAR degradation in VSLs (c) and LFs (d). Bars represent mean ± SD. Different letters indicate a significant difference for $p < 0.05$ for each group.

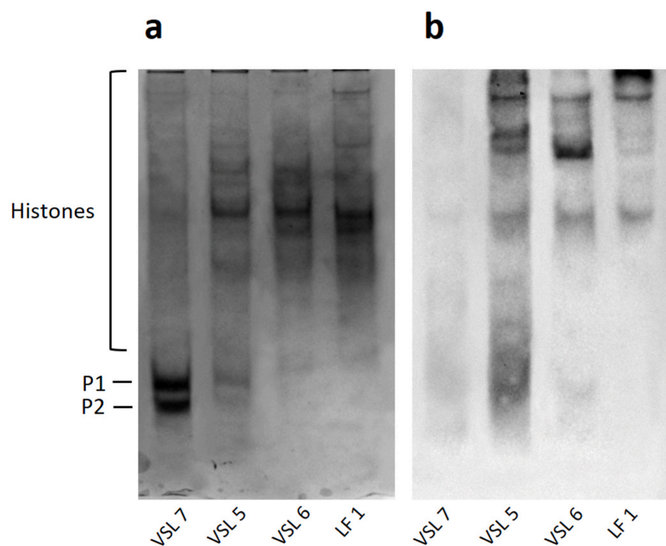

Fig. 5. 11.2 % acetic acid-urea polyacrylamide gel (AU-PAGE) (a) and anti-PAR immunoblotting (b) in sperm homogenates from VSL 7, VSL 5, VSL 6 and LF 1.

The 75 kDa PARP enzyme was already described in semen from 18 healthy and 12 infertile male donors and seems to represent a eukaryotic PARP1 fragment (113 kDa) [1].

The PARG enzyme of 110 kDa is known to be produced by alternative splicing [6], while it is not yet clear whether the 60 kDa PARG is a degradation product of the 110 kDa protein or derives from alternative splicing [7].

In addition, the 75 kDa PARP was a covalent acceptor of PAR and its automodification occurred by polymers with different length and complexity. We hypothesise that PARP automodification is correlated with the extent of sperm DNA damage [54] and we believe that it is "Mild" in sperm samples from VSL 1, VSL 2 and VSL 7. The lack of PARP molecular weight "shift" would indicate that the protein is modified with few ADPr units (Fig. 1c). In addition, the evidence that lowest enzyme automodification levels occurred in VSL 7, seemed to indicate a "physiological" DNA damage (Fig. 1c). The PARP automodification with linear polymers consisting of about 52 ADPr in VSL 4 and VSL 5, led us to define as "Mild/Intermediate" sperm DNA damage. Finally, the presence of anti-PAR immunoreactive signals at the top of the filter indicated a "Massive" damage to genomic material in VSL 3, VSL 6, VSL 8 and in all LFs. The measure of PARP activity in VSLs and LFs confirmed the role of this enzyme as "sensor" of DNA damage and showed that the level of enzyme activity, as well as the PARP automodification, were correlated to extent of material genomic damage. In fact, the lowest levels of enzyme activity and automodification were measured in VSL 7, which we termed 'Mild' DNA damage. We hypothesise that this represents the

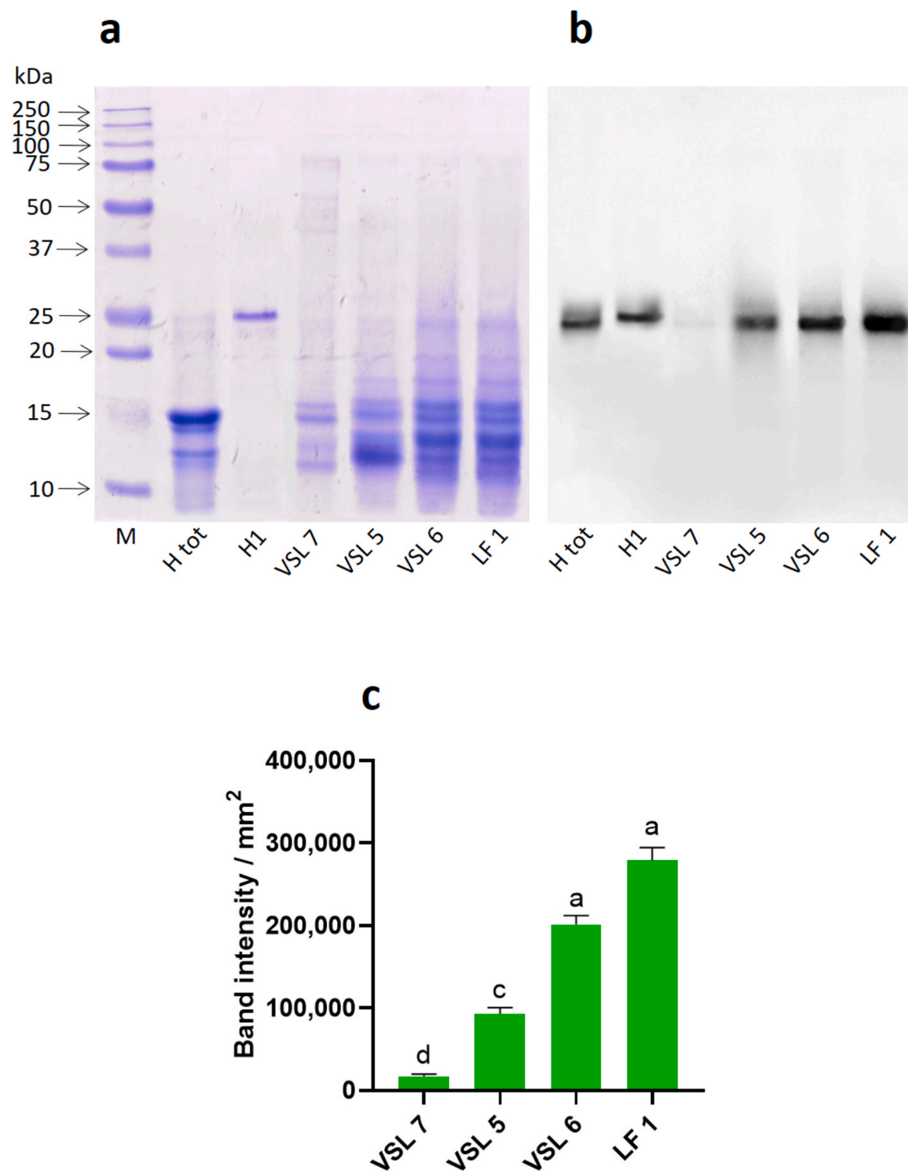

Fig. 6. 18 % SDS-PAGE (a) and anti-H1 immunoblotting (b) in sperm homogenates from VSL 7, VSL 5, VSL 6 and LF 1. Histone H1-like from sperm of the marine annelid worm *Chaetopterus variopedatus* (H1) and the total histone extract from calf thymus (H tot) were used as positive controls. Densitometric analysis of immunopositive bands to anti-H1 (c). The bars represent the mean ± SD. Different letters indicate significant differences for $p < 0.05$.

only true control sample. On the contrary, the highest levels were detected where a “Massive” DNA damage was considered (VSL 3, VSL 6, VSL 8 and LFs) (Fig. 4a and b). In addition, the evidence that a percentage PAR degradation of about 90 % occurs in VSL samples with “Mild” or “Intermediate” DNA damage allows to suppose an equilibrium between PAR synthesis and degradation (Fig. 4c). This equilibrium would confirm the role of PARP in maintaining genomic integrity. In fact, a high percentage of PAR degradation prevents PARP inactivation, promoting cell survival [55].

In contrast, lower percentage of PAR degradation measured in samples with “Massive” sperm DNA damage, could cause PARP inhibition, which would have consequences for DNA repair, or it could stimulate cell death through parthanatos, a form of Apoptosis-Inducing Factor (AIF)-mediated, caspase-independent apoptosis that is involved in many common, important diseases [55,56].

Subsequently, as it is known that histones heteromodification by PAR in mammals regulates the chromatin condensation state [15] we have studied, for the first time, the poly(ADPribosyl)ation of SNBPs and demonstrated a correlation between DNA compaction, alterations in the histone/protamine ratio and their heteromodification by PAR. In detail, in the VSL 7 sample, where the proportion of protamines is greater than that of histones (Fig. 5a) and where these proteins are not significantly poly(ADPribosyl)ated (Fig. 5b), we can assume that the DNA has an optimal compaction compatible with the hypothesis of a “Mild” damage to the genomic material. In contrast, the very low proportion of protamines (Fig. 5a) and the heteromodification of only the histone components in VSL 6 and LF 1 (Fig. 5b) seems to confirm the hypothesis of “Massive” sperm DNA damage and lead to suppose a chromatin decondensation, an event necessary for repair.

Finally, the higher than canonical histone/protamine ratio (between 0.8 and 1.2) [25], together with the heteromodification of both histones and protamines (Fig. 5b), supports the hypothesis that “Intermediate” damage to the genomic material occurs at VSL 5 and corroborates chromatin decondensation in this sample. In VSL 5, as well as in VSL 6 and LF 1, we also demonstrated that H1 histone was heteromodified (Fig. 6b), where intermediate or “Massive” DNA damage was assumed. Finally, we propose a molecular mechanism, reported in graphical abstract, that associates DNA damage, PARP automodification and DNA

damage with SNBP heteromodification and DNA compaction state. Specifically, we believe that the low levels of PARP automodification in the VSL samples with “Mild” DNA damage are related to the absence of SNBP heteromodification. In LF, however, where DNA damage is “massive”, PARP automodifies with long and branched ADPr polymers and simultaneously heteromodifies histone H1. This process promotes chromatin decondensation, which is necessary for repairing oxidative damage to the genomic material of sperm.

We recognize that the number of samples analysed in this study is limited, as they constitute only the preliminary stage of an ongoing research project. Nevertheless, we felt that it was important to share these preliminary data with the scientific community as they demonstrate for the first time that alterations in the poly(ADPribosyl)ation system can be used as new, early markers to monitor sperm DNA health in individuals living in polluted areas (LF), and to identify true “control” samples. For the first time, we discovered that sperm from subjects living in unpolluted areas (VSL) exhibited alterations in the poly(ADPribosyl) ation system. Therefore, studying this system, particularly the automodification of PARPs and the heteromodification of SNBPs, may enable us to distinguish between “true” and “false” controls. potentially be used alongside traditional investigations (spermiograms) in the future to assess male fertility.

5. Conclusions

A complete and active poly(ADPribosyl)ation system was studied for the first time in sperm samples from individuals living in two areas with different environmental impact. Our preliminary data suggest that PARP automodification, as well as the measurement of its activity and heteromodification of SNBPs could to be used as markers of sperm DNA health status. Evidence that sperm DNA damage occurred in individuals recruited from the Land of Fires and in some of those residing in the Sele River Valley suggests that these biochemical tests should be introduced alongside conventional ones to discriminate between “true” and “false” controls. Furthermore, these biochemical tests could be extremely useful in evaluating male infertility. Indeed, the standard sperm analysis, which is currently used to evaluate sperm motility, morphology and concentration, cannot detect damage to genomic material. Consequently, individuals with normal sperm analyses often fail to conceive.

CRediT authorship contribution statement

Bruno Berman: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Gennaro Lettieri:** Writing – review & editing, Writing – original draft, Visualization, Software, Investigation, Data curation. **Martina Falace:** Writing – review & editing, Writing – original draft, Investigation. **Carmela Marinaro:** Writing – review & editing, Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Carmen Di Giovanni:** Writing – review & editing, Writing – original draft. **Anna Rita Bianchi:** Writing – review & editing, Writing – original draft, Visualization, Software, Investigation, Formal analysis, Data curation. **Luigi Montano:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Conceptualization. **Marina Piscopo:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization. **Anna De Maio:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization.

Informed consent statement

All participants provided informed consent before participation.

Institutional review board statement

All experimental protocols were approved by the Ethical Committee of the Local Health Authority Campania Sud-Salerno (Committee code No. 69 of March 19, 2021).

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2025.111747.

Data availability

Data will be made available on request.

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