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MANUSCRIPTS OF THE II INTERNATIONAL CAPARICA CONFERENCE IN TRANSLATIONAL FORENSICS 2019

ORIGINAL ARTICLES

SPECIAL ISSUE

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SPECIAL ISSUE: SELECTED ABSTRACTS OF THE II INTERNATIONAL CAPARICA CONFERENCE IN TRANSLATIONAL FORENSICS (FORENSICS 2019)

RNA markers analysis and post-mortem interval: a review of the evidence

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ABSTRACT

One of the most important problems in forensic sciences is the post-mortem interval (PMI) determination. Forensic scientists always provided different methods (physical, chemical, and entomological) to estimate a correct PMI, without success. However, the improvements of last two decades in the field of molecular biology, allowed us to evaluate the time-dependent degradation of biological markers (e.g. proteins, DNA and RNA). Thus, we want to present a review of the recent progress in the estimation of PMI, mainly focusing on the potential usefulness of RNA markers. Therefore, we reviewed 29 studies, each one chosen according to specific inclusion criteria. These studies evaluated the role of endogenous reference genes in different biological samples in order to determine the PMI based on post-mortem RNA degradation in relation with other influencing factors such as time, cause of death and environmental conditions.

Acknowledgments:

We wish to thank Prof. Caenazzo for critical discussion and comments on the different drafts of the present contribution.

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SPECIAL ISSUE: SELECTED ABSTRACTS OF THE II INTERNATIONAL CAPARICA CONFERENCE IN TRANSLATIONAL FORENSICS (FORENSICS 2019)

DNA-based victim identification in mass disasters: ethical aspects.

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ABSTRACT

Even if every mass disaster is an unexpected event with specific characteristics that depend on the nature of the event, it always causes human, material, economic and/or environmental losses. Each disaster arises a chain of events in which victims' identification, independently from the absolute number of them, has a wide relevance. DNA analysis is often applied to the identification of mass disasters' victims. In fact, DNA profiling plays a prominent role in the identification of human remains, becoming sometimes indispensable, especially when they are badly damaged, commingled, or decayed. Since everyone involved in forensic work is also involved in an activity characterized by ethical obligations towards the deceased and the bereaved, forensic geneticists can be asked to face some ethical issues concerning DNA profiling. The DNA techniques, in fact, allow identification of an individual, distinction between fragments, and enable fragments to be reassembled and assigned to an already identified body, but raise specific problems, including ethical issues. Even if several guidelines and standards have been published in order to improve identification process and technical aspects of data collection and analysis, the complex implications of DNA typing for the purpose of identifying victims of mass disasters need to be further explored. Authors seek to delineate the principal ethical aspects of individual identification based on DNA technology, ranging from humanitarian importance of identification, through resource allocation in emergency contests with resource limitations, to the appropriateness of using samples collected for identification purposes for the secondary purpose of research.

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Methodological approach to detect HLA-B27 allele in case of degraded human bone remains

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ABSTRACT

Paleopathology is a subdiscipline of Forensic Anthropology, which is specialized in the study and application of methods and techniques for investigating diseases from skeletal remains [1]. Paleopathology offers a wide range of forensic applications, such as skeletal identification to reconstruct the ante-mortem biological biography, and providing key information to solve criminal investigations [2].

Spondyloarthropathies (e.g. Ankylosing Spondylitis, Reactive Arthritis, Psoriatic Arthritis) are a group of arthropathies (diseases that affects the joints in the body), whose most remarkable feature is the axial involvement of the skeleton [3]. In some cases, it is possible to establish a morphological diagnosis based on the presence or absence of some macroscopic change criteria, which may help to establish the differential diagnosis [4]. However, paleopathological studies face several problems such as the absence, in many cases, of important parts of the skeleton or the presence of some taphonomic alterations. In these cases, the morphological diagnosis is limited and sometimes impossible.

Considering the genetic background of Spondyloarthropathies [5], we are trying to investigate the use of molecular technology in forensic genetics to help to establish the diagnosis of these diseases. This is particular relevant when analyzing degraded human bone remains, in which morphological information is quite limited. Traditionally, the presence/absence of HLA-B27 allele by conventional PCR has been used as a genetic marker associated with Spondyloarthropathies [6]. However, this technique has some important limitations, especially in the case of degraded human bone remains. A negative result for HLA-B27 may be determined as an absence of the allele in that individual, while this result can be a consequence of the scarcity of DNA in the sample to obtain a result.

In the present study, we propose a new methodological approach to detect the presence or absence of HLA-B27 allele by genotyping 2 SNPs of this allele (rs_116488202 / rs_4349859) using quantitative-PCR (qPCR). In the case of detecting these 2 SNPs in the DNA sample, we can conclude that HLA-B27 allele is present in the individual. In comparison to conventional PCR, qPCR reduces negative results. This is because by using qPCR, the two alleles of each SNP are determined, obtaining always a result, even from a DNA extracted from degraded human remains.

In conclusion, a new methodological approach is proposed to detect specifically HLA-B27 allele, through the analysis of 2 SNPs by quantitative PCR (rs_116488202 / rs_4349859). This methodological tool could help to improve the diagnosis of Spondyloarthropathies, precisely, in degraded human remains, in which morphological diagnosis is quite limited.

Keywords: Forensic Anthropology, Forensic Genetics, Paleopathology, Spondyloarthropathies, HLA-B27, SNPs

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Human DNA extraction from larvae: a brief review of the literature

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ABSTRACT

The analysis of the insects present on the corpses is a new frontier of Forensic Entomology Sciences useful for medico-legal evaluation, in order to extract human DNA and facilitate the estimation of the post-mortem interval.

Starting from a case of an unidentified and mummified body, colonized by insects at different developmental stages, we searched in the Literature the procedures of extraction and analysis of human DNA from the larvae. Our analysis found no trace of human genetic material in larvae's puparia and crops.

This case report adds to the scarce literature available on the human DNA extraction from insects and highlights the analytical challenge of genetic analysis related to post-mortem tissue degradation.

Keywords: *Forensic entomology, larvae extraction, mummified corpse, genetic analysis*

1. Introduction

It is reported in literature that the genetic analysis of human DNA extracted from larvae, puparia and adult insects, found on human corpses, could provide important medico-legal information about the estimation of the post-mortem interval (PMI), in particular when the time of death is beyond 72 h [1-2].

Holometabolous insects, such as Diptera, perform a complete metamorphosis based on three stages: larvae, pupas and adult flies. During the first phase, after the hatching of the eggs, the maggots eat decomposing organic material and mature through a series of changes (feeding stage), but, before starting the formation of the pupal-cage, the maggots reduce their metabolism, stop eat and, in some cases, move away from the corpse (post-feeding stage). For this reason, it is common to find insects on the corpse, at any stage of development, always considering the many factors that influence the rate of colonization and the composition of the insects, such as for example temperature, environment, clothes and cause of death [3].

In many cases, morphological and environmental analysis is the first approach of entomologic evaluation [4].

Recent studies have shown that the analysis of intestinal contents of insects and flies that feed carrion has a genetic potential to be used in forensic sciences [1-2, 5]. In particular, they demonstrated that, after ingestion of human tissue, during the digestion process, the hydrolysed host tissues are normally stored in the maggot's crop. Therefore, it is possible to sample the host tissue residues from the crop, subject it to STR analysis and generate a genetic profile for the identification of an unknown body.

After a forensic investigation performed on a "mummified" human body and the subsequent genetic analysis performed on larvae puparia and crop, without success [6], we decided to perform a review of the literature about the forensic genetic entomology in order to highlight the conclusions of other researches.

2. Material and Methods

We have searched articles in Pubmed and Scopus databases, with the keywords "Human DNA extraction" matched at "Larvae", "Puparia", "Crop", "Human DNA" matched at "Larvae", "Puparia", "Crop" and "Human DNA" AND "Extraction" matched at "Larvae", "Puparia", "Crop".

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We included those studies that evaluated the use of the intestinal contents of insects and flies at different stages as carrier of human genetic material. We excluded studies regarding other use of insects for genetic purposes different from human DNA identification or non-English language articles.

Our research was not limited by chronological parameters. The articles were selected based on the review of their titles and abstracts. In addition to a critical review of each abstract, an evaluation of the full text was made in the case of articles whose summary was not conclusive.

We identified 25 articles, of which 12 were finally included in the review and summarized in Table 1 distinguishing them by: author, year of publication, type of study (case-work vs experimental study), utilized sources, remarks.

3. Discussion

Entomology has become a promising part of forensic sciences able to provide essential informations and, in future, through a more in-depth study of insects, it will be a useful tool to obtain new crucial elements in the global forensic evaluation of real caseworks.

In particular, the study of the insects is important for toxicological and genetic researches.

Indeed, the material contained in the digestive system of larvae and flies can be a source from which to derive genetic profiles and or the presence of drugs [1-2, 7].

After ingestion of human tissue, the digestion process causes the hydrolysis of the host tissues stored in maggots' crop. Therefore, it is possible to sample the host tissue residues from the crop and perform a short tandem repeats (STR) analysis to generate a genetic profile, comparable with the profile of the corpse or hypothetic relatives.

In our knowledge, there are just few studies about human DNA analysis sampled from larvae's puparia and crop.

Zehner et al. first tried to perform a STR typing and HVR amplifications using the crop contents of maggots collected from 13 corpses after various postmortem intervals. In seven cases, a complete STR profile was established, in two cases, an incomplete set of alleles was obtained, and in four cases, STR typing was not successful. The obtained human STR profiles supported the association of a maggot to a specific corpse. The time of storage of the maggots and the length of the post-mortem interval up to 16 weeks appeared to have no particular influence on the quality of the results. [8].

Similarly, Linville et al. dissected maggots after 2 weeks, 8 weeks and 6 months of preservation. They were able to amplify mtDNA (mitochondrial DNA) and STRs from maggots stored in ethanol or without any preservation fluid. Each control maggot produced a complete HVII haplotype and STR profile. Both the mtDNA haplotype and STR genotype matched those of the maggot's food source (human spleen). [9].

Differently, Carvalho et al. considered the potential to detect the ingested human DNA from immature stages of Calliphora dubia which had fed on sheep liver. They detected the host DNA by day 2, even if the crop was visually empty, while from day 3 the material was no longer detectable as it was eliminated, reduced to pieces below 87 bp, or was perhaps present in such a low number of copies that it couldn't be detected by PCR [10].

An Italian group headed by Di Luise made a comparison between different specimen preservation and DNA extraction strategies from the crop of third instar maggots (larvae of Calliphoridae) recovered from a cadaver in decay stage of decomposition with the aim of obtaining autosomal and Y-STR profiles. They observed that ethanol-based preservation dramatically decreased the quantity of typeable human DNA whereas preservation by simple refrigeration produced the best results. None of the batches conserve at room temperature, both in ethanol and dry condition, yielded useful results. Furthermore, extraction methods based on the use of silica columns (i.e. QiagenTM DNA MicroKit) showed the higher DNA yield and purity. DNA IQTM system resulted in useful profiles although a great degree of variation between samples. ChelexTM system followed by filter purification resulted in useful profile only for specimen stored in dry condition [11].

Similarly, Gulden Onur Kondakci et al. tried to identify human DNA from gut contents of third instar maggot (larvae of Lucilia sericata) placed on diabetic patient's wound for treatment purpose. In three samples complete STR profiles were obtained. In three cases incomplete STR profiles were observed. In two samples STR typing failed may be due to highly degradation of DNA within the gut of the maggot. SNP typing was performed and genotypes were obtained successfully after amplification from all third instar maggots extracts and from reference sample, so they concluded that if STR profiles are not obtained, because of crop-content DNA degradation, SNP analysis should be recommended [12].

Also Xi Li et al. showed that the mtDNA and STR analysis of maggot crop contents may potentially be used to associate the maggots with human corpse, even if physical contact between the maggots and corpse is not observed [13].

Afterwards, De Lourdes Chávez-Briones et al. obtained complete STR profiles from groups of 20 third-instar larvae of Calliphoridae albiceps, left in bovine ground meat and human blood for a period of 48 h, even after 2 months of storage in 70% ethanol. They concluded confirming that ethanol is a useful preservative for tissue that has to be analysed for DNA [14].

Oliveira at al. left a group of 20 third-instar larvae of Calliphoridae albiceps in bovine ground meat and human blood for a period of 48 h to ensure higher levels of larval activity with the same diet. Their results showed complete profiles of human STRs for a short period during degradation of the material, concluding that within the first 48 h of death, full-DNA profiles can be obtained from larvae [15].

Similarly, Njau et al. studied the period in which it is

possible to obtain successfully human DNA, using STR analysis, from third instar maggots of Protophormia terraenovae present on decomposing human corpses. In particular, they investigated the degradation and disappearance times of human DNA in the larvae's crop after their removal from the corpse and/or a feeding phase with different food source (for example beef meat). Results showed that the amount of human DNA recovered from maggots decreased with time in all cases. For maggots fed on beef, the human DNA could only be recovered up to day two and up to day four for the starved maggots [16].

Powers et al. observed that human DNA profile could be obtained from second and third instar life stages, as well as pupal and casing samples, of the forensically relevant blowfly species Calliphora augur and Calliphora stygia that have consumed human semen. In particular, the results of this study indicate that the second and third instar, as well as the pupal life stages, would be most pertinent samples to collect at a crime scene where a sexual assault is suspected, and conventional sources of genetic material are not suitable [17].

Mukherjee et al. identified two different preservation techniques (preservation by freezing at -20 °C and preservation in Ethanol (98%)) as optimal to extract non-insect DNA from the gut contents of III instars Megaselia scalaris larvae as they not only aid the process of dissection but do not interfere with the molecular analysis. Despite these fixing methods have been proven to be better in terms of ease of dissection and in the amount of DNA yield per crop, the preservation of some morphological features useful for PMI estimation (e.g. length) is not guaranteed, so the authors strongly recommend collecting enough specimens in order to avoid the risk to lack of sufficient material to perform both the analyses as above mentioned if requested by the Court [18].

Finally, our group has recently published a study on DNA extraction from corps and puparia of Diptera and Hymenoptera's larvae recovered on a mummified unidentified human body in order to obtain a valid genetic profile. We used two different methods: the first one was the procedure reported in Marchetti et al.[19] and in Skowronek et al.[5], the second one is the one suggested by Campos et al. [20]. None of the two techniques used gave a genetic profile, not even a pattern attributable to a degraded DNA. The hypothesis of those negative results is that the process of digestion and degradation of ingested host tissues, already very compromised by the processes of putrefaction-mummification, occurs more quickly within the digestive path of the larva, reducing the time in which it is possible to derive human DNA from the larvae's crops [6].

4. Concluding Remarks

Forensic entomology could have a key-role in pursuing justice. It could provide a huge amount of information that can be helpful for the investigators to place someone at the scene of a crime by a more accurately determination of the time of death, the location, how long a body has been in a specific area, if it has been moved, and other important factors.

In homicides with entomological evidence, it may be important to prove the presumed association of fly larvae to a corpse, especially if it is in doubt whether all maggots used for entomological expertise developed and fed on it.

Most recently, casework and simulated studies based on short tandem repeat (STR) analysis of DNA extracted from the gut contents of larval blow flies have demonstrated that blow flies can provide molecular evidence for the identification of both victims and criminals.

The most significant limitation of the majority of the studies we presented is that most of them are conducted under experimental conditions, placing in contact specific insects with specific biological tissues in standardized experiments and only few of them focused on the evaluation of larvae directly taken from a human corpse in a forensic context. The consequence is that these protocols are often difficult to apply to real cases.

Furthermore, few studies are systematic in evaluating results over time: it seems that the effect of time is a very critical parameter in the validity of the results obtained. In the medical-legal field we often confront with unknown body badly decomposed exposed for a long time (more than the 6 weeks studied in the experimental studies presented) and to different environmental agents. In these cases, any further information that can be obtained from the body, the scene or the fauna that colonizes the body may be crucial.

Therefore in future, for the reasons explained above, further studies should necessary focus on the times of the digestive phase of the larvae in order to: characterize a ratio of time vs quantity of ingested tissue and identify the time necessary for complete DNA degradation within the larval digestive pathway, related to different special post mortal conditions in order to improve our knowledge in this context.

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AUTHOR	YEAR	TYPE OF STUDY	SOURCES OF STUDIES	REMARKS
Zehner et al. [8]	2004	Case-work	Maggots of Calliphoridae were collected from 13 corpses after various post-mortem intervals.	In seven cases, a complete STR profile was established, in two cases, an incomplete set of alleles was obtained, and in four cases, STR typing was not successful. HVR analysis was successful in all cases except one. The time of storage of the maggots and the length of the post-mortem interval up to 16 weeks appeared to have no influence on the quality of the results.
Linville et al. [9]	2004	Experimental study	Maggots of Calliphora vicina collected from a human spleen at room temperature under a 24 h light source were dissected following 2 weeks, 8 weeks and 6 months of preservation.	Each control maggot produced a complete HVII haplotype and STR profile. Both the mtDNA haplotype and STR genotype matched those of the maggot's food source (human spleen).
Carvalho et al [10]	2005	Experimental study	Immature stages of the bluebodied blowfly Calliphora dubia (Macquart) that had fed on sheep liver.	This study suggests that the crop, although visually empty, still contains food materials. However, by day 3, the material has either been eliminated, degraded to pieces smaller than 87 bp, or is perhaps present in such low copy number by this stage that the PCR is not sensitive enough to detect it. This study shows that ingested DNA can be detected in the immature stages of C. dubia until the second day of pupal development
Di Luise et al. [11]	2008	Case-work	Comparison between different specimen preservation and DNA extraction strategies from the crop of third instar maggots (larvae of Calliphoridae) recovered from a cadaver in decay stage of decomposition.	Ethanol based preservation dramatically decreased the quantity of typeable human DNA whereas preservation by simple refrigeration produced the best results. Boiling did not seem to affect DNA recovery while the presence of thawed water into the tube increased spoiling and degradation of the crops. None of the batches conserve at room temperature, both in ethanol and dry condition, yielded useful results.
Kondakci et al. [12]	2009	Experimental study	Identification of human DNA from gut contents of third instar maggot of Lucilia sericata placed on diabetic patient's wound for treatment purpose.	In three samples complete STR profiles were obtained. In three cases incomplete STR profiles (amplification was poor and the peaks were low and/or allelic drop-out) were observed. In two samples STR typing failed may be due to highly degradation of DNA within the gut of the maggot. SNP typing was performed and genotypes were obtained successfully after amplification from all third instar maggots extracts and from reference sample. STR and SNP profiles obtained from the gut content matched the profile of the corresponding volunteers in all samples.
Xi Li et al. [13]	2011	Case-work	Third instar maggots of Aldrichina grahami were collected from a male headless corpse and a skull.	This study showed that the mtDNA and STR analysis of maggot crop contents may potentially be used to associate the maggots with human corpse, even if physical contact between the maggots and corpse (or even two different parts of corpse) is not observed.
de Lourdes Chávez -Briones et al. [14]	2013	Case-work	Three maggots of Calliphoridae and Sarcophagidae were collected from a badly burned body Several attempts to obtain a genetic profile from the fragment of liver recovered at autopsy were unsuccessful.	STR profiles obtained from the maggots were incomplete. However, the number of loci successfully amplified was sufficient to perform a comparative DNA test against the alleged father, which was adequate for conclusive identification of their mains. However, complete STR profiles could be obtained from maggots Even after 2 months of storage in 70% ethanol confirming the fact that ethanol is a useful preservative for tissue that has to be analysed for DNA. Thus, it is possible that the quality of DNA extracted from maggots was in function of the state of decomposition of their mains.
Oliveira et al. [15]	2016	Experimental study	Groups of 20 third-instar larvae of Chrysomya albicenps left in bovine ground meat and human blood for a period of 48 h.	Extraction techniques were successful in obtaining human autosomal DNA from the larvae that was compatible with a reference sample, generating full profiles that matched the reference buccal swab mouth sample. The results show complete profiles of human STRs and this only occurs for a short period during degradation of the material, typically within 48 h. This means that, within the first 48 h of death, full-DNA profiles can be obtained from larvae.
Njau et al. [16]	2016	Case-work	Third instar maggots of Protophormia terraenovae obtained from three different decomposing human corpses	Results showed that the amount of human DNA recovered from maggots decreased with time in all cases. For maggots fed on beef, the human DNA could only be recovered up to day two and up to day four for the starved maggots.
Powers et al. [17]	2019	Experimental study	Samples were taken from adult and juvenile blowflies of Calliphora augur and Calliphora stygia that had consumed human semen.	Samples taken from adult and juvenile blowflies that had consumed semen were able to generate functional profiles from second and third instar life stages, as well as pupal and casing samples. The results of this study indicate that the second and third instar, as well as the pupal life stages, would be most pertinent to collect at a crime scene where a sexual assault is suspected, and conventional sources of genetic material are not available.
Mukherjee et al. [18]	2019	Experimental study	Gut contents of III instars M. scalaris larvae fed on Sus scrofa Linneaus 1758 and Bos Taurus Linneaus 1758 tissues.	This study identifies 2 preservation techniques (preservation by freezing at -20 °C and preservation in EtOH (98%)) as optimal for this kind of analysis as they not only aid the process of dissection but do not interfere with the molecular analysis. The preservation of some morphological features useful for PMI estimation (e.g. length) is not guaranteed.
Sanavio et at. [6]	2019	Case-work	DNA extraction from corps and puparia of Diptera and Hymenoptera's larvae recovered on a mummified human body in order to obtain a genetic profile and identify the unknown body.	None of the two techniques gave a genetic profile, not even a pattern attributable to a degraded DNA. The hypothesis of those negative results is that the process of digestion and degradation of ingested host tissues, already very compromised by the processes of putrefaction-mummification, occurs more quickly within the digestive path of the larva, reducing the time in which it is possible to derive human DNA from the larvae's crops.

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Alcohol and drug use in drug-related deaths in Campania (Italy): a snapshot study over the years 2008-2018

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ABSTRACT

The aim of the study is the evaluation of incidence of mortality, directly or indirectly linked to drug abuse in one of the most populated Italian region (Campania). Trends on psychotropic substances intake and prevalence of drug-related deaths (DRDs) are assessed over an eleven-year observation period from 2008 to 2018. Data from toxicological investigations applied, for forensic purpose, on post-mortem blood sampled from 640 suspected DRDs (267 in the years 2008– 2012 and 373 in the years 2013 – 2018) has been revised. A Systematic Toxicology Analysis (STA) by validated GC/MS or LC-MS/MS methods for licit drugs (benzodiazepines, antidepressants/antipsychotics, hypnotics) and illicit drugs (amphetamine and analogous, cocaine, opiate, methadone, barbiturate, buprenorphine, cannabis and new psychoactive substances) was applied. A conventional methodology (GC/HS-FID) was used to test the blood alcohol concentration (BAC). Toxicological results were positive in 403 cases out of 640 autopsies totally performed: 202 DRDs out of 267 deaths were identified during the years 2008-12 and 201 DRDs out of 373 during the 2013-2018 period. Among the 403 DRDs, more than 90% of cases were males aged between 41-50 years. A remarkable increase in the number of alcohol related deaths (42 cases) was observed in the years from 2013 to 2018 compared with the previous one. Most of these cases showed a BAC in the range 1.5 – 4 g/L, compared to the only 3 cases occurred in 2008-2012 years. During the eleven-year observation period, the poly-drug use has been recognized as responsible for 258 deaths (64%) out of 403 cases in total. The association of cocaine and morphine was predominant in the years 2008-2012 while in the years 2013-2018 pharmaceuticals (benzodiazepines, antidepressants etc.), alcohol and illicit drugs (cocaine and morphine), alone or in combination, were the substances mainly detected. Methadone was detected more frequently in associations with other drugs (mainly pharmaceuticals) or ethyl alcohol but it was also found alone in 3 DRDs only. Surprisingly in few DRDs cannabis metabolites were found neither a single fatal poisoning by amphetamines and new psychoactive substances (NPS) was recorded. During the eleven-year observation period, an increase in DRDs involving ethyl alcohol intake has been observed. A relevant variability in the prevalence among the licit/illicit drug use has been also found, mainly represented by the poly-drug intake respect to the abuse of a single drug. Data collected demonstrate that it is crucial a continuous updating about the territorial trends of the drug abuse in order to develop a Community Data Bank, for planning strategies of prevention.

Keywords: *Drug-related deaths; GC-LC/MS analysis; alcohol/drugs abuse; blood alcohol concentration; poly-drug abusers; pharmaceuticals abuse.*

1. Introduction

Drug-related mortality is a complex phenomenon, which accounts for a considerable percentage of deaths in many Countries. Odds of dying of drug abusers have been estimated to be from three to seven times more likely than those of the general population [1]. According to the system for categorizing mortality data involving drug abuse deaths developed by the Drug Abuse Warning Network (DAWN), drug-related deaths (DRDs) are "deaths where there is an evidence of voluntary and recent use of drugs under circumstances that reveal a causal relationship between these and do not suggest other causes of death". There are several types of DRDs: a) deaths caused by overdose both

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intentional or accidental events (including body packers); b) diseases directly caused by the drug use or chronic abuse, as infections due to subsequent non-sterile needles use (HIV-AIDS, hepatitis), myocarditis, and respiratory deficit; c) drugs abuse related suicides and homicides, and d) other violent deaths attributed to traumatic injuries due to the behavior's alterations under drug use (traffic accidents for drivers under the influence, work-related accident, etc.) [2].

The pattern of drug abuse usually differs among Countries and even territories of the same Country. It changes also over the time. Aim of the study is the evaluation of incidence and prevalence of mortality due to DRDs in one of the most populated Italian region (Campania). It is the third-most-populous region of Italy with a population of around 5.800.000 people located on the Southern west coast. In Campania the "Forensic Toxicological Unit" of the University of Campania "L. Vanvitelli" is the "Forensic Reference Laboratory" (FRL) for the entire territory. The FRL performs toxicological analyses for medico-legal purpose on biological samples collected from people alive but also on post-mortem specimens.

Goal of the forensic approach to DRDs is to support the diagnosis of fatal poisoning through the analytical detection of xenobiotic substances (alcohol, illicit drugs, pharmaceuticals or other poisons) in traditional body fluids and tissues (blood, urine, bile and samples of internal organs) or alternatives matrices (humor vitreous, hairs and saliva) according to the information available from the medical history and the circumstances of the fatal event. In this retrospective study, only toxicological results obtained in post mortem blood samples were reviewed in order:

a) to assess the trends of alcohol and drugs abuse among DRDs during the years from 2013 to 2018;

b) to compare the results observed during the years 2013 to 2018 with those collected during the previous years from 2008 to 2012 already assessed and published in a previous study [3];

c) to provide data useful for the development of preventive strategies with regional relevance.

2. Material and Methods

During the eleven-year observation period (from 2008 to 2018), post-mortem toxicological analyses have been performed by FRL on 640 suspected DRDs. The distribution was the following: 267 autopsies during the years 2008– 2012 and 373 fatalities in total during the years 2013 – 2018. The analytical procedures on post-mortem blood include an immunoassay screening techniques, addressed to the most common drugs of abuse such as amphetamines, barbiturates, cannabis, cocaine, methadone, opiates, 6-AM (6-acetylmorphine, heroin metabolite) and buprenorphine. A qualitative Systematic Toxicology Analysis (STA) [4] was also applied using a mass spectrometry methodology coupled with gas or liquid chromatography (GC/MS or LC-MS/MS) for licit drugs (benzodiazepines, antidepressants/antipsychotics, hypnotics) and illicit drugs (amphetamine and analogous, cocaine, opiate, methadone, barbiturate, buprenorphine, cannabis) and the new psychoactive substances (NPS). Each positive results obtained from the STA was confirmed by quantitative methods, validated in accordance with both National and International Guidelines and Recommendations [5,6], using specific analytical cut-offs established in National Guidelines. A conventional GC/HS-FID methodology was used to test the blood alcohol concentration (BAC). Finally, the interpretation of toxicological data was achieved in accordance with circumstantial information and medical history.

3. Results and Discussion

During the observation period from 2013 to 2018, toxicological investigations were requested in 373 judicial autopsies in which drug toxicity was suspected to be involved. Positive toxicological results were achieved only in 202 cases (54%) finally assessed as DRDs. No great differences in the incidence of DRDs were observed between the two considered period. Although in the recent observation period from 2013 to 2018 there were more medico-legal autopsies (373 in total) than the previous observation period 2008-2012 (267 only), the total amount of positive toxicological analyses performed on postmortem samples was quite the same: 201 in the year 2008-2012 vs 202 in the years 2013-2018.

3.1 Age and gender distribution among DRDs

During the years 2013-2018 the 202 DRDs involved mostly males in 191 fatalities (94.5%). Females were in only 11 cases (5.5%). Victims aged between 41 and 50 years occurred in the majority of cases (29%). The mortality rate among younger people (less than 25 years of age) was significantly low (6.4%) Age and gender distribution among DRDs occurred during the years from 2013 to 2018 is shown in Table 1.

These results were consistent with data observed in the years 2008-2012 [3]. In fact, a very low mortality rate was also found among young males (< 25 years old) accounted in

Table 1 | Age and gender distribution among the 202 drug-related deaths (DRDs) occurred in Campania (Italy) during the years 2013-2018.

Age group (years)	Number M (male) / F	Frequency
< 25	12 M / 1 F	6.4 %
25-30	21 M / 2 F	11.4 %
31-40	37 M / 2 F	19.3 %
41-50	**56 M / 2 F**	**28.7 %**
> 50	35 M / 4 F	19.3 %
N.D.	30 M / 0 F	14.9 %

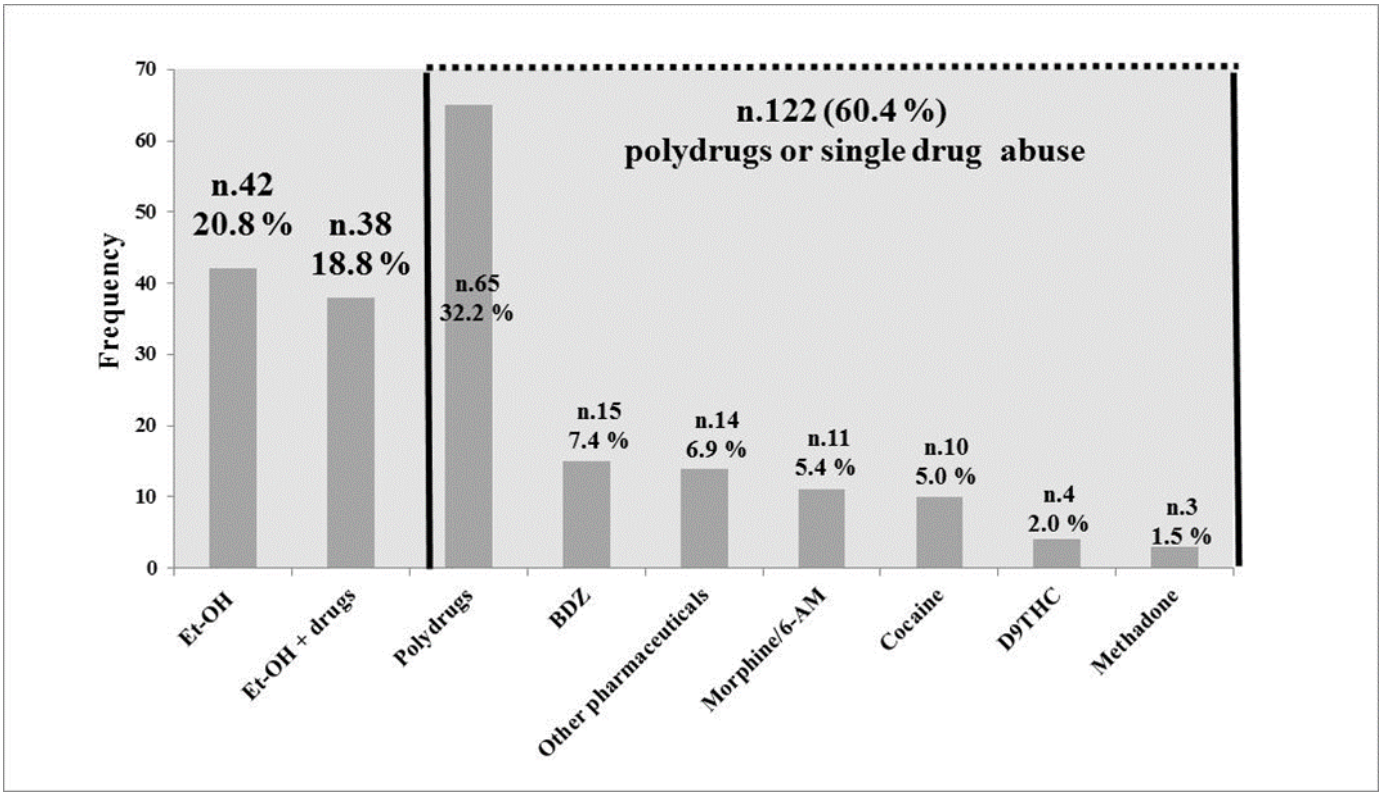

Figure 1 | Distribution of ethyl alcohol, licit and illicit drugs among 202 drug-related deaths (DRDs) occurred in Campania (Italy) during the years 2013-2018.

the years 2008-2012. During the overall eleven year observation period more than 90 % of the 403 victims were males, mostly with an age range of 41-50 years. It is quite interesting that in DRDs the mean age of victims seems to vary greatly between Countries. The lowest values have been reported in Slovenia (20-24 years old) [7] and Eastern Germany (20-24 years old) [8] followed soon after by Finland (29 years old) [9], Romania (31 years old) [10], Norway (34 years old), Sweden (36 years old) and Denmark (38 years old) [9]. In all these Countries, so far the mean age victims by DRDs is lower than that observed in our Southern Italian territory.

3.2 Typology of drug involved in DRDs

The distribution of alcohol and drugs among the 202 DRDs accounted during the observation period from 2013 to 2018 is shown in Figure 1. The overall toxicological results demonstrate that the highest mortality rate (60.4%) is related to the intake of psychoactive substances (illicit and licit drugs) and that the multiple drug abuse is a critical issue (32.2%). Among these 202 cases, the ethyl alcohol alone was the most frequently detected xenobiotic substance in 80 fatalities (39.6%) out of 202 totally. Among these fatalities, ethyl alcohol was the only poison detected in 42 cases (20.8%) while it was in association with one or more drugs in 38 DRDs (18.8%). The intake of illicit drugs and pharmaceuticals, as single or in combination with multiple drugs was detected in 122 DRDs (60.4%), more than half of the total fatalities occurred during the observation period.

In DRDs involving ethanol, a BAC greater than 1.5 and up to 4 g/L was observed in most of the cases, 28 fatalities (66.6%) out of the 42 in total where ethyl alcohol was detected. A BAC between 0.8 -1.5 g/L and a BAC less than 0.8 g/L were detected in 9 (21%) and in 5 (12%) deaths involving ethanol, respectively.

The distribution of blood alcohol levels found in combination with one single drug or with a poly-drug intake among 38 DRDs is summarized in Table 2.

In 18 out of 38 DRDs involving ethanol in association with a single drug, cocaine or therapeutic agents were the xenobiotic substances more represented. Among these 18 cases involving ethanol in combination with a single drug, in 9 DRDs BAC level was > 1.5 g / L. In 20 out of 38 DRDs involving ethanol in association with poly-drugs, the intake of cocaine/morphine and pharmaceuticals/cocaine was well represented in all four BAC concentration ranges (Table 2).

Among the 202 DRDs, 57 fatalities were positive to a single substance (Figure 1) mostly represented by pharmaceuticals: benzodiazepines (BDZ) occurred in 15 cases and antidepressants in 14 cases. Among the illicit drugs, morphine was detected only in 11 DRDs (5.4%) and cocaine in 10 cases (5%). Surprisingly the metabolites of cannabis and methadone have been identified alone respectively in 4 and 3 cases out of 202 DRDs in total. None case was positive only to amphetamines or to NPS.

Over the years 2013-2018, deaths due to poly-drug abuse have been assessed in 65 cases out of 202 DRDs in total

Table 2 | Distribution of single/multiple drug intake versus the blood alcohol concentration (BAC) levels among 38 drug-related deaths (DRDs) occurred in Campania (Italy) during the years 2013-2018.

Range of BAC (g/	Single Drug (18)	Polydrugs (20)
< 0.5	Δ^9THC (1)	Cocaine + Morphine/6-AM (1)
0.5 – 0.8		Cocaine + Morphine/6-AM (2)
		Cocaine + Δ^9THC (1)
		Cocaine + Methadone + Pharmaceuticals (1)
		Cocaine + Morphine/6-AM + Methadone (1)
0.8 – 1.5	Pharmaceuticals (2)	Cocaine + Morphine/6-AM (3)
	Cocaine (1)	Cocaine + Pharmaceuticals (2)
	Δ^9THC (1)	Morphine/6-AM + Pharmaceuticals (1)
	Methadone (1)	Morphine/6-AM + Methadone (1)
		Methadone + Pharmaceuticals (1)
> 1.5	Δ^9THC (1)	Cocaine + Δ^9THC (1)
	Cocaine (3)	Cocaine + Morphine/6-AM (2)
	Pharmaceuticals (6)	Cocaine + Pharmaceuticals (1)
	Morphine/6-AM (1)	Δ^9THC + Pharmaceuticals (1)
	Methadone (1)	Cocaine + Morphine/6-AM + Methadone (1)

Table 3 | Distribution of poly-drug abuse among the 65 drug-related deaths (DRDs) occurred in Campania (Italy) during the years 2013-2018.

Poly-drugs	Total number of DRDs 65
Pharmaceuticals (BDZ + other medicinal drugs)	10
Pharmaceuticals + Methadone	8
Pharmaceuticals + Morphine	6
Pharmaceuticals + Cocaine + Methadone	5
Pharmaceuticals + Morphine + Methadone	4
Pharmaceuticals + Cocaine + Morphine	4
Pharmaceuticals + Cocaine + Morphine + Methadone	2
Pharmaceuticals + Cocaine	2
Pharmaceuticals + Cocaine + Morphine + THC + Methadone	1
Pharmaceuticals + Morphine + Methadone + THC	1
Pharmaceuticals + THC	1
Cocaine + THC	5
Cocaine + Morphine	4
Cocaine + Methadone	3
Cocaine + THC + Morphine	2
Cocaine + Morphine + Methadone	2
Cocaine + THC + Morphine + Methadone	1
Cocaine + Morphine + Cocaethilene	1
Methadone + THC	2
Methadone + Morphine	1

(Table 3). Among these, in 44 DRDs, pharmaceuticals were found in 11 different associations with other substances. Cocaine was detected in 12 different associations in 32 cases totally. Methadone and morphine in combination with other substances, were detected each in 29 cases. Metabolites of cannabis were found to a lesser extent in only 13 DRDs but in seven different associations. It is worth mentioning that methadone was the only one drug abused in 3 cases while it was more frequently detected in combination with other drugs (mainly pharmaceuticals) or ethyl alcohol.

Finally, toxicological results reviewed during the eleven-year observation period from 2008 to 2018 have been sub-grouped in two different data sets: the first data set goes from 2008 to 2012 with details already discussed in a previous article [3], the second data set goes from 2013 to 2018 as illustrated above. A comparison of the two sub-groups is shown in Table 4.

Table 4 | Distribution of single/multiple drug intake versus the blood alcohol concentration (BAC) levels among 38 drug-related deaths (DRDs) occurred in Campania (Italy) during the years 2013-2018.

	years 2008-2012 (201 DRDs / 267 autopsies)	years 2013-2018 (202 DRDs / 373 autopsies)
Substances	n. cases (frequency)	n. cases (frequency)
Et-OH	3 (1.5%)	42 (20.8 %)
Et-OH + Drugs	33 (16.4%)	38 (18.8 %)
Drugs or pharmaceuticals	165 (82.1%)	122 (60.4 %)
(single drug / polydrug)	(43 / 122)	(57 / 65)

The comparison of data sets show a significant increase in DRDs due to the intake of ethyl alcohol occurred in the years 2013-2018 with 80 cases in total versus only 36 in the years 2008-2012. The 42 DRDs involving ethanol alone in the years 2013-2018 are fourteen times more than the 3 only cases observed during the years 2008-2012. During the years 2008-2012, the multiple drug abuse was recognized as responsible for most of the DRDs with 122 cases out of 201 (60.6%). In the recent observation period 2013-2018, this result was not confirmed with only 65 cases out of 202 (32%) probably due to a descending trend of heroin or cocaine intake in the general population.

In fact, although the association of heroin (morphine and/or 6-AM) and cocaine was the combination of drugs more frequently observed with 60 cases out of 122 DRDs during the years 2008-2012 [3], in the more recent observation period 2013-2018, the xenobiotic substances detected mostly were the pharmaceuticals (mainly BDZ, such as clonazepam, diazepam, lorazepam) and alcohol, alone or in combination with cocaine and morphine.

BDZ are commonly prescribed psychoactive drugs because considered safe and effective against anxiety disorders and panic attacks [11-14]. The large use of BDZ around the world is consistent with the high detection rate of BDZ among the DRDs occurred in the recent observation period 2013-2018. It seems that in Campania region also, a relevant shift to pharmaceuticals abuse in association with traditional illicit drugs, is taking place. Such trend in the behavior of drug abusers has been already reported in many European countries. In fact, BDZ, barbiturates, antipsychotics and anti-epileptics, were also identified as the most common agents in 35% DRDs in Romania [10]. Half of the reported DRDs in 2017 in Scotland (UK) involved new BDZ such as etizolam [15]. A combination of BDZ (mainly diazepam) and methadone has been recorded in 12% of DRDs in the Republic of Macedonia, with a clear increase since 2011 [16]. Finally, during the eleven-year observation period, the poly-drug use has been recognized as responsible for 258 deaths (64%) out of 403 cases in total.

4. Concluding Remarks

Despite several recommendations and always more deterrent laws suggest caution in the use of drugs and alcohol, in Campania region (Italy), toxicological data on DRDs demonstrate a constant number of fatalities but characterized by a great variability in the prevalence of licit/illicit substances.

During the eleven-years observation period from 2008 to 2018, there was an increasing trend of the ethanol intake, alone or in combination with other agents. Moreover, over the time, a shift to pharmaceuticals abuse in association with traditional illicit drugs was highlighted. The fast growing of the poly-drug intake, compared to single drug abuse and a descending trend of overdose rate due to heroin or cocaine abuse were also recorded. Data revised demonstrate that it is crucial the continuous updating of the local trends of the drug abuse. Providing this information to the Italian Department for the Antidrug Policies and to the European Monitoring Center for Drugs and Drug Addictions is the main goal of forensic toxicologists in order to develop a Community Data Bank, for planning strategies and preventing the consumption of all substances harmful to the population.

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The importance of a specific medico-legal training for health care professionals in the management of sexual assault victims

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ABSTRACT

The World International Organization (WHO) defines sexual violence as "any sexual act, attempt to obtain a sexual act, unwanted sexual comments or advances, or acts to traffic, or otherwise directed, against a person's sexuality using coercion, by any person regardless of their relationship to the victim, in any setting, including but not limited to home and work". This definition includes a very wide range of behaviors. According to the Italian National Statistical Institute (ISTAT), the 31.5% of Italian women are estimated to have been victims of physical or sexual violence during their lifetime. Given the acute nature of sexual assault, emergency medicine providers are the first clinicians to take care of the victim, and care of such patients differs from care of those presenting other kind of trauma and injuries. Healthcare professionals treating victims of sexual assault admitted to Emergency Departments (ED) need to deal not only with clinical priorities, but also with the emotional suffering and anguish characterizing the experience of this type of patients. Furthermore, they can effectively assist the victims in their medico-legal proceedings by documenting injuries and by collecting biological evidence for forensic purposes. In order to avoid discrepancies between the medical report and the reconstruction of the event, it is essential to set up strategies which focus on the technical aspect of evidence collection and on the way the victim's story shall be recorded. Sometimes, indeed, information collected from the victim in the ED are still inadequate or incomplete to determine how the case event should be reconstructed. Such efforts could lead to a better management of sexual assault victims and to a strengthened legal impact of forensic evidence and of the crime reconstruction. For this reason, it is necessary for health structures: to define specific pathways for the victim's management; to adopt homogeneous operational protocols which allow a standardization in the methods of collection and preservation of biological material for forensic-genetic analyses; finally, to provide an adequate forensic training for health personnel in order to ensure that they are competent in the medical reporting and in documenting evidences of the sexual assault. From a forensic point of view this could be crucial, as medical documentation may be used in Court.

Keywords: *Sexual violence, sexual assault, sexual victims, medico-legal training, emergency department*

1. Introduction

The World International Organization (WHO) defines sexual violence as "any sexual act, attempt to obtain a sexual act, unwanted sexual comments or advance, or acts to traffic, or otherwise directed, against a person's sexuality using coercion, by any person regardless of their relationship to the victim, in any setting, including but not limited to home and work" [1]. According to this definition, sexual violence involves a wide range of non-consensual sexual activities, which includes acts with and without vaginal and/or anal and/or oral penetration and acts that involve the use of physical strength or psychological coercion.

According to the Italian National Statistical Institute (ISTAT), the 21% of Italian women, between 16 and 70 years of age, are estimated to have been victims of sexual violence during their lifetime and the 5.4% are estimated to have been victims of more severe forms of sexual violence as rapes or attempted rapes [2]. Sexual assault has important negative consequences on the victim's health, both in the short and long term, representing one of the main causes of morbidity,

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disability and mortality among female subjects. Furthermore, it has negative economic impacts. Despite all this, the phenomenon is still overall underestimated.

Sexual violence is a complex traumatic event that requires a multidisciplinary approach to produce the most efficient evaluation and management of the victim, whose needs are not limited to the clinical and psychological sphere; care should be addressed also to the forensic aspects of sexual assault in order to effectively aid the administration of justice and to guarantee the rights of the woman. Documentation about injuries, medical reports and other problems arising from violence can be used as evidence in Court by the abused woman, should she choose to take a legal action, and lead to an improvement of the prosecution process. Therefore, it is necessary to provide the intervention of different professional figures: clinicians, gynaecologists, psychologists, social workers but also forensic medicine experts.

Given the acute nature of the phenomenon, emergency medicine (ED) providers are the first clinicians to take care of the victim and they have a dual responsibility in the management of this kind of patients. The first one is to provide the victim with the required treatment care, both physical and psychological. The second one is to assist the victim in the management of medico-legal proceedings, through episode's recording, clinical examination, injuries documentation and biological evidence collection for forensic purposes.

2. Undeclared sexual violence

First, the ED staff involved in triage should have the consciousness and the ability to recognize sexual violence even when it is not explicitly declared by the victim. Some studies have shown that women who have experienced violence are more likely than non-abused women to seek health care, even if they do not disclose the violence [3].

There are two different strategies: that of "universal screening", according to which all women accessing ED are routinely asked a question about sexual violence (even if some authors stress the that there is no evidence that routine screening significantly improve the outcomes), and that of "case-finding", according to which this question is asked only to women who present risk factors or symptoms suggestive of violence.

Possible physical indicators that should lead healthcare professionals to suspect sexual violence are for example: skin signs (bruises, scratches, bites, grasping marks) if the abuse was carried out with the help of physical violence; physical symptoms or itching in the genital area; pelvic pain, dysmenorrhoea, sexual disfunction; walking difficulties; torn underwear, sperm or blood traces on clothes or in the vagina and/or rectum; presence of urethral, vaginal and/or rectal foreign bodies; genital and/or anorectal injuries or unjustified bleeding. There are also some behavioural indicators such as: passivity, fear, distrust of people, history of sexual abuse, attention difficulties and anxiety.

In order to recognize these signs and, consequently, to identify undeclared sexual violence, all the ED staff – and especially triage staff –must be adequately trained, but it is just as important to improve their sensitization towards this issue. medical history.

3. Clinical-forensic examination

The competence of ED health personnel in conducting the history taking, the clinical examination and the evidence collection is crucial in order to guarantee a correct management of a victim of sexual assault.

Before collecting the samples for clinical and forensic reasons, it is necessary to subject the patient to a complete clinical examination, that should be performed as soon as possible and anyway within 24 h after the sexual assault. A delay may result in lost therapeutic opportunities (e.g. medical treatment of injuries, prophylactic treatment for pregnancy prevention, empiric treatment for sexually transmitted diseases, vaccination for hepatitis B, preventive treatment for HIV), changes in physical evidence (e.g. healing of injuries), loss of forensic material (e.g. evidence of contact with the assailant including blood and semen).

Healthcare professionals must then record and classify the injuries by photographing or drowning them, because the aim of an objective medical and forensic examination is to describe the health status of the woman and to record the injuries and their consequences.

Several studies suggested that sexual victims benefit from a single health care setting strategy since it results in a lesser psychological and also physical impact: the provision of necessary care (clinical, psychological and forensic) in one location that brings together all the relevant elements would guarantee a minimal mobilization of the victim, the avoidance of unnecessary stress and the minimalization of testimonial mistake and discrepancies [4]. Unfortunately, nowadays the victim often must file a police report on the assault at the police station, seek medical treatment in a hospital ED and, finally, obtain psychological support in an appropriate health care setting.

Different studies [5,6] show that there is sometimes an important discrepancy between the case history (i.e. information derived from the victim's report at the ED) and the laboratory findings (i.e. the detection of the presence of male DNA on collected swabs – vaginal, vulvar and/or rectal). In particular, the study by Tozzo et al. considered processed samples from 122 sexual assault cases: of the 103 cases in which the victim reported penetration and ejaculation, only 67 (55% of all the samples) correlated with a positive feedback match from the laboratory. In the remaining 36 cases (29%) the victim's report was not supported by laboratory data, because no male DNA was found in the samples.

Nowadays the ability to ascertain the presence of male DNA in collected samples is not a problem for forensic

laboratories and it does not depend on the methods of analysis used. It is influenced by other factors, such as the fact that the woman did or did not perform actions that could reduce the probability of finding the aggressor's DNA (like washing her body or changing clothes), and the time elapsing between the sexual assault and the medical examination (as stated before, the victim's clinical examination should be performed as soon as possible, and anyway within 24 h, after the assault in order to avoid the loss of important trace evidence). For the cases in which laboratory results are negative even though the medical examination has been performed within 24 h after the assault, it may be hypothesized that either the victim and/or the physician was inaccurate in reporting the assault. On one hand, the victim's story might be inaccurate or untrue because of the trauma associated with the sexual violence: these victims have suffered a major trauma and an important emotional stress, so it may be that during the interview with the ED staff they fail to mention or alter some details concerning the aggression, either because they are ashamed or because they forgot. It must be considered that the temporary memory loss may also be due to the involvement of rape drugs or to alcohol consumption shortly before the assault. On the other hand, medical reports may be superficial, inadequate or incomplete: despite a large number of international recommendations give indications on these aspects (how to record a complete medical history, how to properly document any injuries, and how to collect evidences), there is still today a lack of consistency in how these guidelines are applied in some hospital. This may be for several reasons, for example healthcare professionals may not be adequately trained or they are often struggle with excessive workload.

These findings suggest that hospital health professional which deal with victims of sexual assault should pay more attention to the method used to interview patients about the episode and also to the method used to record the material collected during the medical examination (i.e. documentation of physical injuries and samples of biological evidences).

In order to avoid discrepancies between the medical reporting and the laboratory findings it is crucial to apply standardized record both for the technical aspects of evidence collection and for the way the victim's story is recorded. Such efforts would lead to a better management of sexual assault victims who entered the ED and have an important legal impact in the crime reconstruction, as they could help to confirm the victim's record about the violence circumstances and sometimes also identify the aggressor. There are two possible strategies to aim this goal: (1) the first one is to involve healthcare professionals specialized in the forensic field, which would be desirable for supporting clinical heath personnel involved in the assistance to the victims in the ED. In case such a specialist cannot be present, especially in smaller and/or peripheric hospitals, (2) it is important that the ED staff components have the competence to respond appropriately to all the victim's need, included the medico-legal aspects. In many countries, health professionals are already adequately trained in collecting medical and forensic evidence to corroborate the victim's report. Unfortunately, in most countries there is still a gap between the needs of the victims and the existing level of health services provided, first the victims are not examined by a forensic expert neither by a specifically trained health care professional. This leads to a misapplication (or non-application) of existing protocols and guidelines and, consequently, to an inappropriate management of the victim in the ED (for example, the victim is subjected to multiple examination by different professionals, instead of one single examination both for medical care and forensic investigation) and to an inadequate or incomplete record of evidence for forensic purpose.

The healthcare personnel's training and increased awareness are therefore essential in order to collect all relevant information to understand what happened and to identify the perpetrator. This specific training needs to be addressed to all the ED staff, including psychiatrists and counsellors.

Once biological samples and clinical documentation have been collected, it is fundamental to establish a chain of custody that guarantees their safety and traceability from the moment they are collected to the moment they are analyzed, protected from any type of manipulation.

Last, but not least, it is also important to improve healthcare staff's sensitization toward the victim's emotional needs, towards the suffering and anguish characterizing this type of patient. To ensure an approach based on empathy, understanding and willingness to listen would maximize the victim's confidence in the healthcare providers and enable to obtain more detailed information, even when the woman is uncooperative.

Unfortunately, the number of publications related to this topic is very low today. Increasing it would improve healthcare professional's sensitization towards these issues and lead to a better management of sexual assault victims and to a strengthened legal impact of forensic evidence and of crime reconstruction. .

4. Concluding Remarks

The management of sexual assault victims in the ED required a multidisciplinary approach that comprises various care providers –clinicians, gynaecologists, psychologists, social workers, forensic medicine experts, police officers.

All ED health care professionals should be provided with a specific training concerning sexual violence, and its medico-legal aspects. Such a training would:

a) give health care workers greater acknowledge and awareness of sexual violence; b) make them more able to detect victims of sexual violence even when undeclared; c)

allow health care operators to provide the most efficient and inclusive evaluation and management of the victim, to produce temporally adequate responses to her needs and to perform intervention and treatments of proven effectiveness; d) guarantee the victims a more gentle and empathic approach.

Heath personnel's training and increased awareness are essential in order to: (1) contribute to the welfare of the victim, significantly improving her future recovery; (2) collect all relevant information required to effectively aid the administration of justice and to guarantee the rights of the woman: documentation about injuries, medical reports and other problems arising from violence may be useful to identify the perpetrator and can be used as evidence in Court by the abused woman, should she choose to take a legal action.

Acknowledgments:

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mRNA profiling in casework analyses

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ABSTRACT

Our work involved the application of mRNA profiling to three bloodstains previously processed by the laboratory, in which routinely methods (immunochromatography) used for specific blood identification showed negative results.
Analyses were accomplished on a sweater worn by the suspect. No bloodstains were found during the routine inspection. In order to identify possible latent bloodstains, luminol was applied. After the reagent vaporization, three distinct areas of luminescence appeared. In order to confirm the presence of the human hemoglobin, luminescent surfaces were collected using 4N6FLOQSwabs® and tested using HemDirect Hemoglobin test (SERATEC®). All samples analysed showed negative results.
Due to the merging blood-specific markers mRNA profiling, the areas previously identified were sampled newly and tested using the three blood specific markers HBB, ALAS2 and CD93, together with two housekeeping genes represented by ACTB and 18S-rRNA.
All samples showed positive results for all three blood specific mRNA markers.

Keywords: *Body fluid identification; mRNA profiling; STR*

1. Introduction

Body fluids are one of the main evidence recovered from a crime scene, and increasingly in low quantities and with poor quality [1]. Identify a particular body fluid is a crucial step since the nature of the fluid is itself highly informative to the investigation [2]. Current and commercial tests use chemoluminescence and the detection of specific proteins (immunochromatography). These commercial kits commonly used in forensic laboratories are mostly presumptive, are carried out for only one body fluid at a time, and generally are limited in specificity or sensitivity [3]. The main limit showed is represented by the destruction of the sample tested. Many times a case can be solved only by using a limited amount of biological evidence, so these must be examined as possible by non-destructive methods. The most relevant reason for these tests to be non-destructive is represented by the preservation of DNA [4].

Bloodstains, as well as other biological traces, could benefit from the development of novel and more sensitive methods for their identification.

As an emerging technique for body fluid identification, mRNA typing has seen remarkable progress and wide application in forensic genetics in recent years [5].

Research showed that messenger RNA is much more persistent than it was previously thought on the grounds of inevitable RNA enzyme-catalyzed degradation.

Despite the degradation, caused by high temperature and/or humidity, our previous work suggests that under ideal preservation conditions, mRNA can last for long and be detected in 50 plus-year-old samples [6].

Due to this novel method, our work shows the application of the mRNA typing to an old murder occurred in Ferrara in 1998. The victim died from numerous stab wounds, after a prolonged struggle and with a large amount of blood had been spattered in the room in which corpse was found.

Some witnesses claimed to have seen the husband leave home with a light-colored sweater, spotted on the front, and walking toward his mother's home.

Investigators inspected the house of the suspect's mother finding a white sweater inside a washbasin located in the laundry. During the environmental examination, no bloodstains were found. After the luminol application, three areas of luminescence were identified on the front side.

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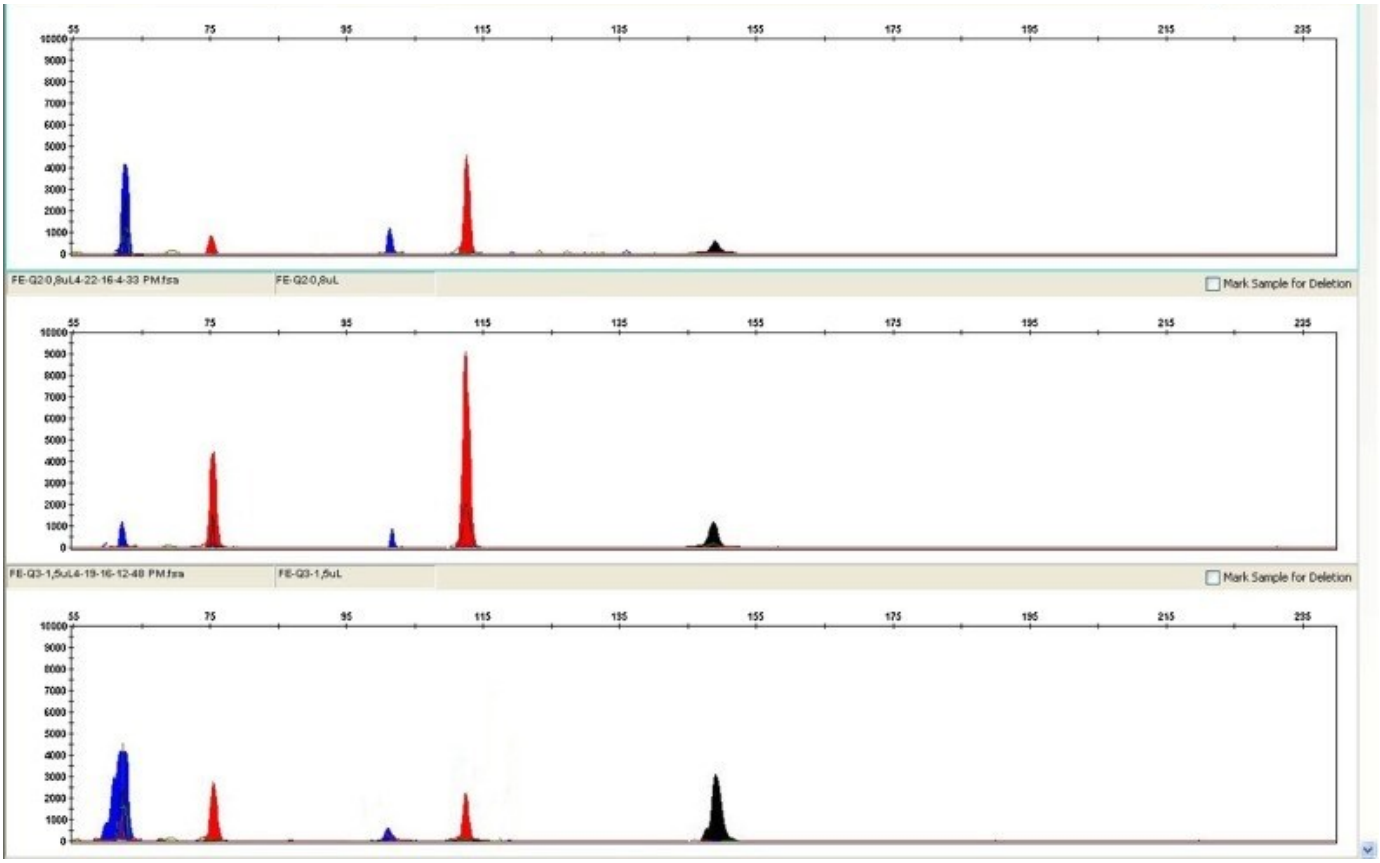
Figure 1 | mRNA profiles achieved from the analysed samples



To confirm the presence of human blood, these spots were sampled using 4N6FLOQSwabs® and tested with the HemDirect Hemoglobin test (SERATEC®). All samples showed negative results. Genetic analysis, accomplished with a conventional STRs kit (AmpFlSTR® NGM amplification kit (Thermo Scientific®)), revealed the presence of the victim's profile.

Although the negative results achieved after body fluid identification, it has been still possible to convict the suspect only by one witness evidence collected by investigators.

2. Material and Methods

Samples were collected from the luminescent areas previously isolated and labeled using luminol by three distinct 4N6FLOQSwabs®.

A DNA/RNA co-isolation protocol was developed incorporating the widely used AllPrep DNA/RNA Mini Kit (QIAGEN®), following a modified protocol developed in the laboratory.

cDNA was synthesized using the RETROscript (Ambion®). After cDNA quantification, samples were amplified using Multiplex PCR Mastermix (QIAGEN®) according to the manufacturer's instructions, in a total volume of 25 µL.

Markers, primer sequences, and concentrations were adopted from Van den Berge et al. [7]. This molecular system was previously tested in an ISFG Italian Working Group - GEFI collaborative work [8]. All thermal cycling steps were accomplished in a Veriti® 96-Well Thermal Cycler (Thermo Scientific®). DNA isolate from the co-extraction process was typed using a conventional STR kit, represented by the AmpFlSTR® NGM PCR Amplification kit (Thermo Scientific®). Detection of all amplified fragments was performed using an ABI PRISM 310 Genetic Analyzer (Thermo Scientific®) and allele calling was accomplished using GeneMapper ID-X V1.0 software (Thermo Scientific®). Allele designation was carried out in comparison to control DNA 007 and allelic ladder provided by the manufacturer. The detection threshold chosen for both DNA and mRNA profiling was set at 70RFU.

3. Results

As reported in figure 1, all samples collected revealed all three blood specific mRNA markers ALAS2, CD93, and HBB, specific for identification of blood, together with the simultaneous presence of the two housekeeping genes (ACTB; 18S-rRNA) used as internal reaction control.

Typing of co-extracted genomic DNA provided the same full STR profile from all the samples tested. As previously achieved, the profile was fully compatible with the victim's profile.

4. Discussion

Our results prove how this merging and novel

methodology can be helpful in forensic science for the identification of body fluid stains, such as bloodstains.

RNA and DNA co-extraction also represents the most important goal since the amount of sample is highly limited. The quantity and quality of DNA isolated using the co-extraction process seem to be enough in environmentally exposed samples, even though the results were slightly poorer than other conventional and commercial DNA isolation methods.

5. Concluding Remarks:

This study showed how without mRNA profiling, it would not have been possible to confirm the presence of human blood. Due to this lack, crucial evidence would be lost, endangering the entire investigation.

Gene expression analysis represents therefore a robust and alternative approach to conventional protein-based methods applied to body fluid identification, particularly when only low starting material or micro traces are available for laboratory analysis.

In conclusion, mRNA profiling is likely to play a major role in the future of forensic genetics, not only for the identification of body fluids and tissues, which was the topic of the proposed work, but also in the prediction and determination of the age of an individual, and as well as the age of a stain [9-10].

As suggested from our work, mRNA profiling will be the body fluid identification method of the future, supporting or replacing existing immunochromatographic methods.

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Original Articles

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Pre and post analytical pitfalls of neutrophil gelatinase-associated lipocalin and cell cycle arrest biomarkers predicting acute kidney injury

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ABSTRACT

AKI disorder is one of most serious clinical conditions recognized worldwide. In recent years, many improvements were made both for renal biomarkers and to know better the total testing process (TTP) in laboratory medicine including pre and post analytical phases. However, TTP for new renal biomarkers remains an issue. We conducted the present study to determine pre/post-analytical pitfalls of neutrophil gelatinase-associated lipocalin (NGAL) and tissue inhibitor of metalloproteinase 2-insulin-like growth factor binding protein 7 [TIMP-2]-[IGFBP-7] called Nephrocheck test. Material and Methods. We analyzed urinary samples collected in two previous studies at intensive care unit (ICU) from an adult population undergoing cardiac surgery. We performed measurements using a chemiluminescent method on an automatic analytical platform and a point of care testing Nephrocheck (Astute-Meter). Biochemical results were analyzed without and with the urine creatinine (uCrea) correction. Furthermore, a method to calculate the mean variation rate was applied for different biomarker concentrations using differential equations. Results. A received operating curve for predicting AKI was obtained with AUC value= 0.66 corresponding to the better NGAL cut-off= 29 ng/mL (p= 0.016). A significant association between the NGAL increase and AKI severity was achieved (p= 0.02). NGAL increases showed: NGAL pre= 18.3 (7.7-23.7) ng/mL, NGAL post= 19.2 (3.8-86) ng/mL median (interquartile range) statistically not significant. Analyzing the corresponded urinary creatinines, we found many uCreas with very low concentrations suggesting a possible dilution of patient samples collected. Correction with the urinary creatinine did not have added significant outcomes. It seemed that [TIMP-2]-[IGFBP-7] expressed as Nephrocheck AKI score $(ng/mL)^2/1000$ introduces a variation due to the amplification of TTP errors. Model of differential equations applied to Nephrocheck test results demonstrated a mean variation rate that rises as the biomarker concentration increases. Conclusions: NGAL results on urinary testing had a modest diagnostic accuracy probably due to pre-analytical variability in ICU critical patients. Pre and Post-analytical variability affected Nephrocheck results as observed by the expression of measurements $(ng/mL)^2/1000$. In addition, the too low numerical measurements as fraction of 1 and high variability around the AKI risk score cut-off= 0,3 might determine possible overlap between different patient groups. We suggested an analytical expression for the cell cycle arrest biomarkers as cumulative concentration in linear form (ng/mL).

Keywords: *Pre/post analytical variability, NGAL, Nephrocheck AKI score, acute kidney injury, differential equation*

1. Introduction

Acute kidney injury (AKI) is a common complication in several diseases as well as hematologic and no hematologic cancer, infection diseases, cardiovascular diseases especially following cardiac surgery. Laboratory diagnostics is more and more a growing field with decisive contribution to modern medicine by supporting the prevention, diagnosis and therapeutic monitoring of human disorders [1]. It is now well established that pre-analytical phase affects the stability of human biological tissue (urine or plasma) with consequences for both basic proteomic experiments and biomedical testing [1,2,3,4].

Furthermore, in laboratory medicine it is important to consider the complete diagnostic process called total testing process (TTP) including the post-analytical phase too. Actually, standard procedures and diagnostic common

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protocols for pre and post-analytical phase applied accurately to clinical practice remain an issue. Biomedical research suggests that many so-called "biomarker candidates" fail to progress beyond the discovery phase, and much emphasis is placed on pre and post-analytical variability [3]. In our diagnostic assay experience, we though that better understanding of the overall process from the biomarker discovery to assay validation are need. In fact, pre -analytical phase should be added to the diagnostic appropriateness of clinical testing, in instance for diagnosis or early diagnosis. To date, the diagnostic tools on the acute kidney injury (AKI) diagnosis have been disappointing, even if new biomarkers are extensively suggested [5,6], as well as, the neutrophil gelatinase-associated lipocalin (NGAL) and particularly the cell cycle arrest proteins: tissue inhibitor of metalloproteinase 2-insulin-like growth factor binding protein 7 (TIMP-2)-(IGFBP-7). Combination of the two proteins was called Nephrocheck test performing measurements in urinary samples. Both biomarkers are frequently recognized as useful for AKI detection after cardiac surgery in a timely manner to prevent adverse outcomes in clinical practice. In fact, Nephrocheck test was considered superior in early AKI detection in critically ill patients. However, diagnostic tests are affected by different biological and analytical factors producing a significant variability and uncertainty. Despite a huge of literature supported the new biomarkers for assessing AKI risk, many doubts remain in regard of their applicability in clinical practice. We conducted the present study to determine diagnostic pitfalls of NGAL and [TIMP-2]-[IGFBP-7] expressed as Nephrocheck score in the early AKI diagnosis after cardiac surgery (CSA-AKI). We focused on pre-analytical phase as sample collection and sampling time then on post-analytical phase as result expression. Hence, evaluating experimental data, the investigation concerning pre and post-analytical features of renal biomarkers, aimed to detect the causes of our diagnostic outcomes in urinary samples belonging to an intensive care unit (ICU) patient population.

2. Material and Methods

A subset of thirty-two patients (females=nine, males= twenty-three) with complete data, belonging to a previous study on the CSA-AKI [see reference 7] was analyzed for urinary NGAL (uNGAL) measurements (ARCHITECT i1000SR® analyzer, Abbott Diagnostics GmbH, Wiesbaden, Germany) to evaluate the diagnostic accuracy parameters: sensitivity, specificity, received operating characteristic (ROC) curve. Patients were undergoing cardiac and cardiovascular surgery, provided a written consensus as stated by the local Ethic Committee. Urinary samples were collected in ICU according to the collection procedure of manufacturer. Patients were selected following the AKI risk inclusion criteria with two or more conditions: 1) age > 70 years, 2) eGFR < 60mL/min/1.73m2 (estimated by MDRD formula), 3) ejection fraction (EF) < 41%, 4) redo operation, 5) combined surgery. Consecutively, we performed a similar study, always through the same conditions and working team (laboratorians and clinicians), measuring the [TIMP-2] -[IGFBP-7] Nephrocheck score (Astute Medical, San Diego, CA, USA) on sixty-eight patients [see reference 8]. An expert clinician team in ICU following the KDIGO criteria formulated the AKI diagnosis. For the AKI definition, all the renal injury stages were considered equally, from AKI stage 1 (low severity) to AKI stage 3 (high severity). Sampling time was the same for the two studies: before surgery (baseline, pre-biomarker) and within 4 hours from the patient arrival in ICU (post-biomarker), to obtain an early detection of kidney impairment. We performed data analysis considering urinary creatinine (uCrea) as dilution marker and the expression of AKI biomarkers corrected by its concentration. A critical evaluation of experimental data in regard of pre and post-analytical variability was made using new methodological tools: in particular interpreting consecutive results for the uNGAL and applying a mathematical model for the Nephrocheck test. Statistics have been carried out on continuous variables using the t-test for independent samples. Variables not normally distributed were presented as median and interquartile ranges and compared with the Wilcoxon rank-sum test. Categorical data were compared using the chi-square test or the Fisher exact test, as appropriate. Receiver operating characteristic (ROC) curves were calculated and the area under curve (AUC) with 95% confidence interval (CI) was used to measure the NGAL prediction for AKI. Analysis were performed by SAS version 9.4 (SAS Institute Inc., Cary, NC). Correlation study was performed through Pearson correlation with a statistical significance of 0,05.

We also evaluated the uNGAL increases (pre-post surgery), considering a clinically significant increase in urinary samples, uNGAL (positive test), if the second value post-surgery was ≥50 ng/mL [7,9]. Post-analytical phase was investigated focusing on the expression of biological measurements, in particular the [TIMP-2]-[IGFBP-7] measure, Nephrocheck AKI score expressed as (ng/mL) 2/1000. We applied a mathematical model (differential equation) to estimate the variation rate of the assay [10] (see below).

Furthermore, we simulated a systematic error by dilution of the samples: 1 control sample and 2 biological samples from 1/2 to 1/32 in NaCl 0.9% solution and in urine pool of 5 healthy subjects, respectively. Control sample was Nephrocheck liquid control (high level) with concentration equal to 30.5 $(ng/mL)^2/1000$. Biological samples belonged to two ICU patients with AKI associated to cardiac surgery, had a Nephrocheck AKI score as follows: patient A= 1.87 $(ng/mL)^2/1000$, patient B= 0.41 $(ng/mL)^2/1000$. Then, we compared each point of dilution to its mathematical derivative (see figures 5-8), estimating the propagation of the systematic error simulated. Nephrocheck AKI score reference range associated to No AKI risk (0.002-0.3) (ng/

mL)^2/1000 and from a healthy and pathological population was analyzed; hence, analytical features of Nephrocheck test kit was critically taken into consideration.

2.1 Model of differential equation and Nephrocheck variation rate

It is well known that the differential equation model can be applied for the study of the biological system [10]. Differential equation is a relationship between a function and one of its derivatives. We calculated the first derivative of the function to investigate the rate of change of Nephrocheck AKI score calculation formula: (ng/mL) 2/1000. This is the measure unit of each Nephrocheck test as product of concentration of the two biomarkers: [TIMP-2]⊠ [IGFBP-7].

Hence, we investigated the influence of measure unit on a systematic error propagation. We observed clearly that (ng/mL)^2/1000 is a power function then if Y= X^2 = (ng/mL)^2/1000, then the differential equation will be: $dY/dX= dX^2/dX= D\ (ng/mL)^2/1000= DX^2 = 2X^{2-1}= 2X$. This means that the propagation of any error will follow a linear relationship (dY/dX= 2X), that is, the measure unit will contribute to the error propagation then to the analytical variability.

3. Results

For clinical characteristics and type of cardiac surgery of patients from both NGAL and Nephrocheck studies, we referred to the previous works on plasma consecutive NGAL measurements [7] and Nephrocheck after cardiac surgery [8] respectively. Here, we described results of urinary biomarkers to take into account pre and post-analytical pitfalls that can occur in detecting early AKI after cardiac surgery.

3.1 Urinary NGAL diagnostic accuracy and pre-analytical variability

Urinary NGAL results (pre-surgery) that are referred to the sampling time before operation (pre-uNGAL) as well as for plasma NGAL [7] results, did not show any association to AKI (p= 0.79). Instead, post-uNGALs showed a significant association to AKI with a cut-off= 29 ng/mL even if with modest sensitivity= 0.64 and specificity = 0.67 (p=0.016). Receiver operating characteristic curve revealed an AUC= 0.66 (Fig 1). We tried to correct post-NGAL in urinary samples through the corresponded urinary creatinine (uCrea) but we did not achieve significant results (p=0.25). Morever, we studied the uNGAL increases to detect potential acute damage according to an expert opinion [9], considering two consecutive measurements (pre -post uNGALs). Considering a biomarker increase of at least 50 ng/mL, we did not reach any sensitivity improvement for AKI detection. Instead, a significant association between the uNGAL increases and AKI severity was achieved (p=0.02).

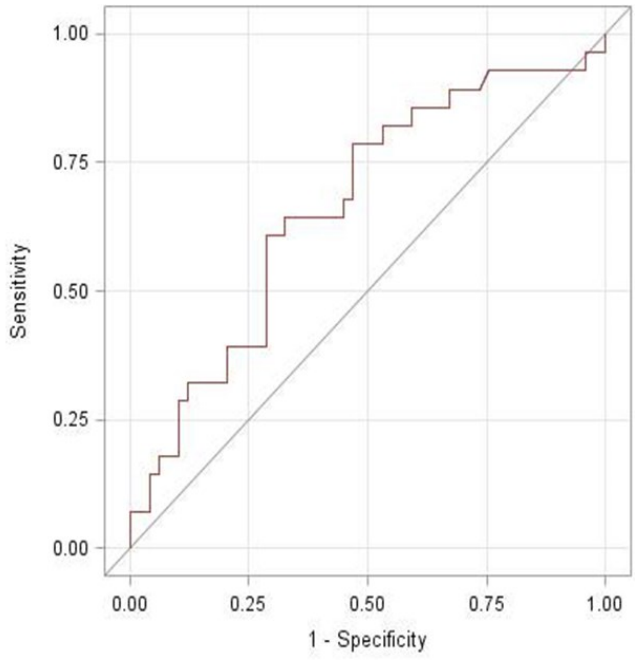

Figure 1 | ROC curve of urinary NGAL test (post-surgery)

In summary, for the urine NGAL and creatinine determinations (N=32) we obtained the follow results (median and interquartile range): pre-uNGAL= 18.3 ng/ml (7.7-23.7), pre-uCrea= 95.5 mg/dL (51.75-142.25); post-uNGAL= 19.2 ng/mL (3.8-86), post-uCrea= 12.4 mg/dL (7.8 -26). The first outcome illustrated that there was not any increase of uNGAL (pre-post surgery) with low uCrea concentration post-surgery, considering critically ill patients in intensive care unit (ICU). Urinary determinations were retrospectively analyzed to investigate possible causes of test inaccuracy. We plotted uNGAL versus uCrea concentrations (Fig 2) showing that 93% of results have fallen under 50 mg/dl of urine creatinine. Many uCreas had very low concentrations under 50 mg/dL, considered as graphical limit, close to the lower reference limit of a healthy population equal to 40 mg/dL. This finding suggests a possible dilution of urinary samples with a probable

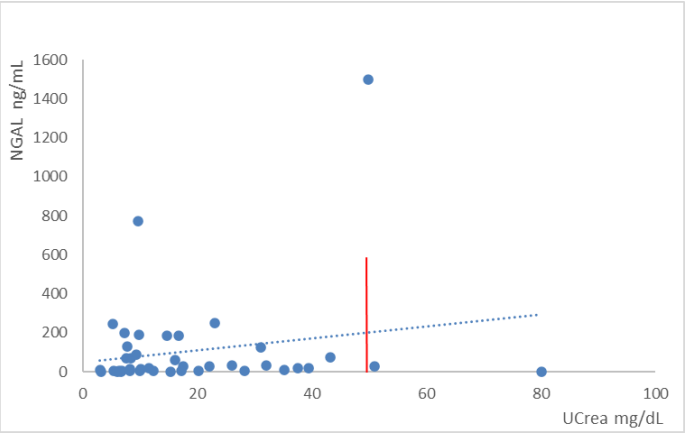

Figure 2 | Analysis of uNGAL and uCrea concentrations. Red line indicates uCrea concentration= 50mg/dL. uCrea as dilution marker showed that many biomarker concentrations falled under this line.

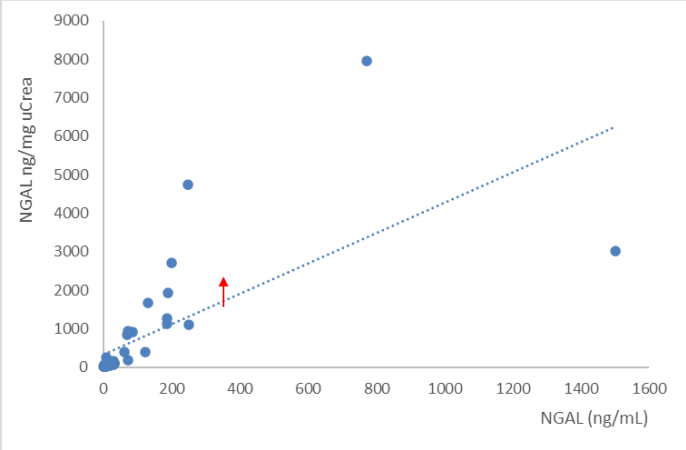

Figure 3 | uNGAL and uNGAL/uCrea correction results. Red arrow indicates the uNGALs trend to fall over the interpolation line with very high uNGAl values.

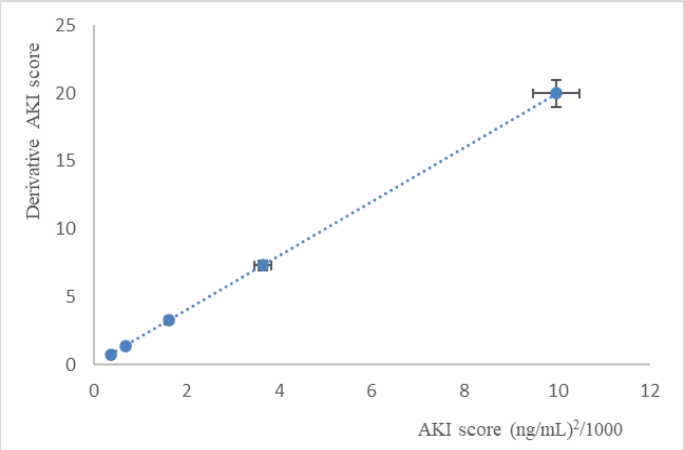

Figure 5 | Comparison between [TIMP-2]-[IGFBP-7] level and its derivative from control sample. (Error bars expressed as percentage)

interference or high pre-analytical variability. In fact, we tried to analyze all urine data by uCrea correction (Fig 3). Results showed a non-linear relation and mostly dispersion of data as uNGAL concentration increases. However, three patients reached a clinical significance for AKI diagnosis considering uNGAL increases >50 ng/mL. Otherwise, many uNGALs corrected by uCrea showed uNGAL values (N=10) not-specifically raised for AKI (Fig 3). These results showed that the NGAL correction by uCrea generates uNGAL ng/mg uCrea values too high (see Y-axis, Fig 3) for a useful clinical interpretation.

3.2 Nephrocheck AKI score results and pre/post-analytical variability

3.2.1 Pre-analytical variability

Results published in the previous study, illustrated the follow ROC curve parameters: AUC= 0.64, (confidence interval= 0.5- 0.77) (p= 0.048). Although, it was reach a statistical significance, diagnostic accuracy did not reach clinical significance to justify Nephrocheck test for AKI prevention, considering our study design and patient population [8].

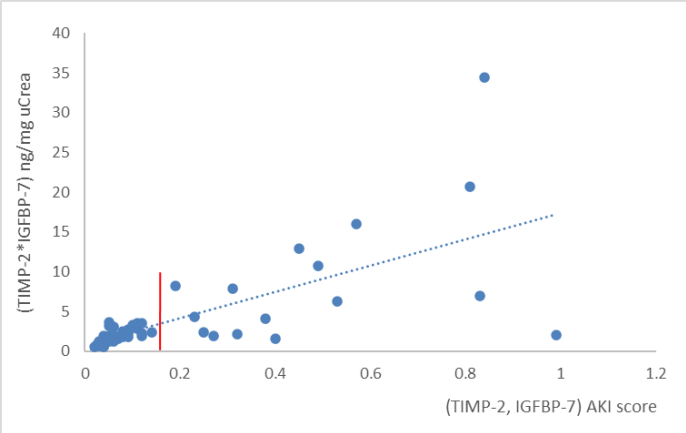

Figure 4 | Interpolation of [TIMP-2]-[IGFBP-7] AKI score with the same values corrected by uCrea.
Better correlation was achieved for AKI score under 0.18 (red line).

Morever, analysis of [TIMP-2]-[IGFBP-7] AKI scores corrected by uCrea revealed the following results:

Y= 16.589X+0.8134, R^2= 0.46, R= 0.67 (Fig. 4). The present equation suggested an overestimation of biomarker values corrected, (see high [TIMP-2]-[IGFBP-7]ng/mg uCrea values) with a possible systematic error. Similarly to the uNGAL results, Nephrocheck AKI score seemed to be affected by pre-analytical variability as well as sample dilution (results published by l'Acqua et al. [8]). Correction of [TIMP-2]-[IGFBP-7] concentration through uCrea did not add significant improvements for AKI detection, instead generated uninterpretable results. We observed that only for low Nephrocheck AKI scores referred to physiological conditions (Nephrochek AKI score < 0.2) could be obtained a good correlation (Fig 4).

3.2.2 Post-analytical variability

Model of differential equation allowed us to know better post-analytical variability and the behavior of result expression to prevent any error propagation. We supposed that the measure unit $(ng/mL)^2/1000$ of [TIMP-2]-[IGFBP-7] concentrations affects clinical data then introduces a post-analytical variability due to the amplification of analytical and pre-analytical errors. Experimental (Nephrochek AKI score) and mathematical (Derivative Nephrocheck AKI score) data obtained from liquid control and biological samples were compared. As expected, for the three different evaluations, mathematical derivative of biomarker measurements was significantly associated to the biomarker concentration. In fact, D $(ng/mL)^2/1000$= 2X, then the variation rate follows a linear function (see Fig 5, Fig 6, Fig 7) with coefficient of correlation R=1. Thereby, the error propagation, as mean variation rate, increases as [TIMP-2]-[IGFBP-7] measure increases, linearly. This finding underlined that the measure unit of Nephrocheck test: $(ng/mL)^2/1000$ is a power function. In addition, we plotted the

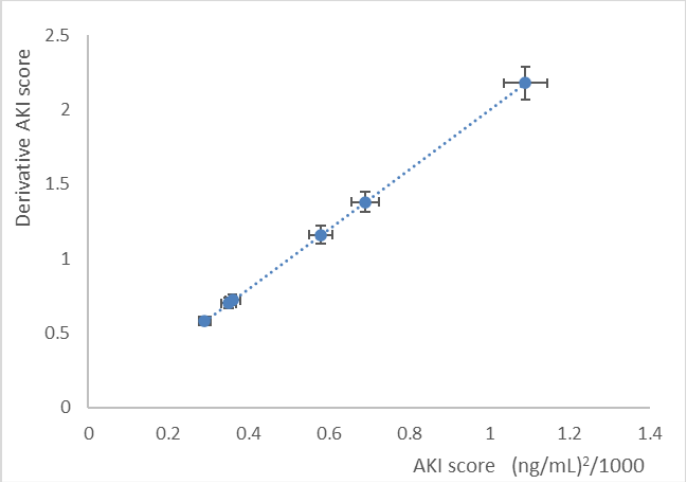

Figure 6 | Comparison between [TIMP-2]-[IGFBP-7] level and its derivative from Patient A. (Error bars expressed as percentage)

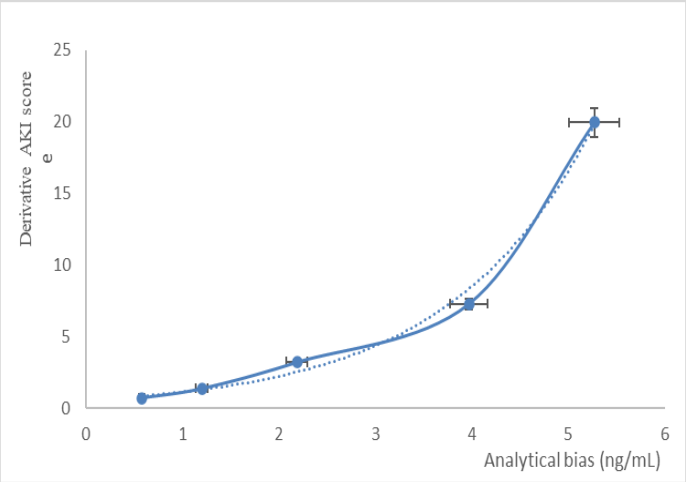

Figure 8 | Graphic from control sample illustrates systematic error (absolute bias values) and relative derivatives. Note that for analytical bias higher than almost 4 ng/mL, trend becomes exponential. (Error bars expressed as percentage)

systematic error calculated from the experiment of pathological control sample considering the analytical bias (Fig 8.) Results showed: Y= 0.5954 (e) exp 0.665X with R^2= 0.98 and R= 0.99. Data demonstrated an exponential relation between the Derivative AKI score and the analytical bias calculated as difference (expected value – observed value). Analysis of curve suggests, however, that the exponential trend occurs only for high bias values (Fig 8.)

3.2.2.1 Comparison of Nephrocheck score versus NGAL for the mathematical derivative

NGAL biomarker with measure unit equal to (ng/mL) follows a linear function like most of clinical measurements in laboratory medicine based on a single concentration. In fact, if we applied the differential equation to the NGAL with measure unit (ng/ml) we obtain as follows: Y= ng/mL=X, then the differential equation dY/dX=D (ng/mL)= X^{1-1} =X^0 =1, which is a constant. In summary, dY/dX of Nephrocheck is equal to 2X and dY/dX of NGAL is 1. This means that uNGAL testing, is not affected by the error propagation or variation change due to the result calculation or measurement expression.

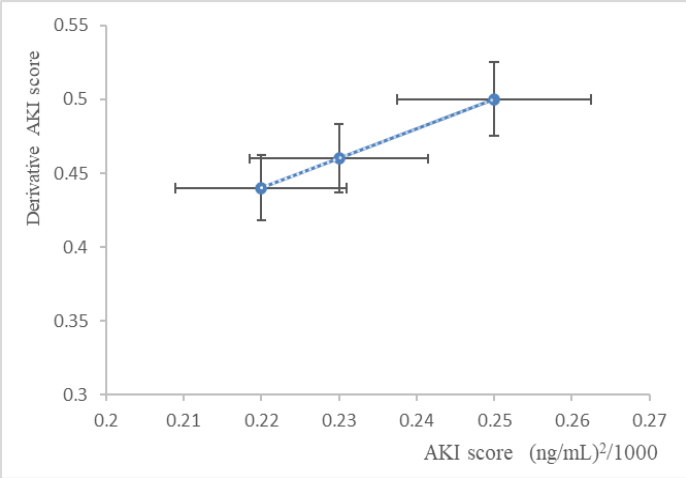

Figure 7 | Comparison between [TIMP-2]-[IGFBP-7] level and its derivative from Patient B. (Error bars expressed as percentage). (See the high dispersion of measures due to very low biomarker levels)

3.2.2.2 Analytical features of Nephrocheck AKI score and possible improvements

About the performance characteristics of Nephrocheck test, we observed that normalization of biomarker concentrations $(ng/mL)^2$ divided for 1000 provides a numerical reduction of biomarker levels as fraction of 1. For this reason, Nephrocheck AKI score cut-off equal to 0.3, extensively used in clinical trials, may be easily misinterpreted among different groups of patients. In fact, we considered the measure unit or fraction: $(ng/mL)^2/1000$ as an attempt to show both [TIMP-2]-[IGFBP-7] concentrations and not a true and necessary analytical normalization. The effect of this transformation is a detection and quantification limit of Nephrocheck score equal to 0.002. This means a quantification limit unsuitable for clinical use as a very low fraction of unit 1. Our observations noted that, to reduce the analytical variability should be used a concentration product of the two biomarkers [TIMP-2]-[IGFBP-7] in linear form as (ng/mL). Then, we could assume a transformation through a square root of the concentration product [TIMP-2]-[IGFBP-7] $(ng/mL)^2$, as follows:

$[(ng/mL)^2]^{1/2}$ =ng/mL. In instance, if we have a [TIMP-2]-[IGFBP-7] concentration product equal to 300 $(ng/mL)^2$, we can obtain a cumulative concentration equal to 17.32 ng/mL through its square root, corresponded to Nephrocheck score equal to 0,3 $(ng/mL)^2/1000$. This could allow a numerical result suitable for laboratory practice and not affected by the error propagation. However, we highlighted as a declared post-analytical variability of Nephrocheck AKI score the reference range expressed as $(ng/mL)^2/1000$: (0,04–2.25) and

(0,05–2.20) for apparently healthy subjects and for subjects with stable chronic morbidities respectively.(data published from the Nephrocheck test kit package insert). If we consider the reference range (0,04–2.25) expressed as (ng/mL)^2/1000, we can obtain, through a square root and removing the division of a 1000, the expression (ng/mL) equal to (6.32-47.73). This reference range should determine a reduction of measure dispersion, supporting the use of a linear unit (ng/mL).

4. Discussion

AKI diagnosis is currently defined by the serum creatinine and urine output results, according to KDIGO guidelines. Nevertheless, both serum creatinine and urine output are tardive for an appropriate early AKI diagnosis and to start therapy. In the last years, in this scenario, much attention has focused on novel renal biomarkers as tools to detect kidney injury, or as recently described, on acute tubular damage or biochemical stress detection. At the same time, the extra-analytical phase in laboratory medicine (pre and post-analytical variability) (TTP) became really an issue to be also solve for new biomarkers, with researches and scientific interest worldwide. Our study has pointed out the pre and post-analytical phases of new renal biomarkers: urinary NGAL and [TIMP-2]-[IGFBP-7] proteins called Nephrocheck test. Indeed, we evaluated urinary biomarker results of two previous studies [7,8] conducted in our Center in predicting CSA-AKI, on an adult ICU population with high risk of renal impairment. Recently, it seemed that [TIMP-2]-[IGFBP-7] biomarkers provide better diagnostic outcomes to detect kidney stress than NGAL or other new promising tests [11,12]; nevertheless biomarker levels become significant 12-24 hours after clinical insult only. In addition, studies with different patient settings gave controversial information for biomarker application in medical practice; in summary requiring more evidences [13]. Our results on urinary biomarkers were obtained with an experimental protocol based on a sampling time within 4 hours from arrival of the patients in ICU (post-biomarker), then 6 hours after clinical insult, approximately. Reason of an early biomarker sampling was to achieve timely information to predict CSA-AKI. In fact, we reached good outcomes in a first study conducted on plasma samples, considering two consecutive NGAL measurements [7]. Instead, considering urinary samples of a subset of patients, both NGAL and Nephrocheck test were poor predictors of AKI (modest AUC) then not useful for clinical application. Analyzing urinary creatinine, as dilution marker, we showed that 93% of patients had urine creatinine concentration under 50 mg/dL, according to previous study [8] in the same clinical setting and study design. Indeed, in accordance with other works, we suggested the possibility of a sample dilution, with the advice that the results should be corrected for the fluid variations [8,14,15]. As suggested by RG Hahn [14], Nephrocheck AKI score might increase when urine was concentrated and likewise decrease when urine spot was diluted, generating an overlap of results among patient RIFLE groups. Accordingly, in the present investigation we sought to correct urinary biomarkers by the uCrea, because of a urine spot could contain the effects of hemodilution or concentration, in instance during the diuretic therapy. Although these considerations, our results showed that both uNGAL and Nephrocheck AKI score had not any significant correction and useful result by uCrea. Furthermore, the graphics (Fig. 3-4) illustrated a data dispersion as biomarker concentration increases. In fact, Waikar S Sushrut et al. [16,17] demonstrated that biomarker normalization by uCrea may introduce a bias with an underestimation or overestimation of biomarker excretion rate depending on clinical context. In addition to pre-analytical variability, we evaluated post-analytical phase using a mathematical model and observations on the analytical features of Nephrocheck test. Firstly, differential equations revealed an error propagation due to the measure unit of Nephrocheck AKI score. Notably, Nephrocheck AKI score is an arithmetic product of two biomarkers: [TIMP-2]-[IGFBP-7] with generation of concentration square (ng/mL)^2. Then, every small error (pre-analytical or analytical) could be amplified by result expression (ng/mL)^2/1000, according to its mathematical derivative. We supposed that this finding might contribute to the inaccuracy of Nephrocheck test. Secondly, it is possible that the transformation of TIMP-2, IGFBP-7 concentrations to Nephrocheck AKI score, could introduce a high analytical variability around the AKI risk cut-off=0.3, causing an overlap of different patient groups with suspect of AKI. Analyzing the measure unit (ng/mL) 2/1000, we suggested that an expression of the TIMP-2, IGFBP-7 concentrations as cumulative concentration in linear form (ng/mL) may reduce the post-analytical variability. Remains to plan further studies to verify whether [TIMP-2]-[IGFBP-7] cumulative concentration may improve diagnostic outcomes for the AKI risk assessment. In conclusion, we thought that a standardized sample collection and a better expression of Nephrocheck test could represent a worthy step to AKI prediction in ICU critical patients.

5. Concluding Remarks

The urgent need to have new renal biomarkers inspires the effort of researchers in proteomic basic studies as well as in translational and clinical trials. Recent works by expert nephrologists suggest the AKI biomarker application in clinical practice through algorithms or consecutives measurements (AKI biomarker curves) [11,18]. In the last months, notably, another cause of AKI was described: Covid -19 associated AKI, requiring important insights to prevent organ failure [19]. Furthermore, a reproducible diagnostic accuracy and validation of new biomarkers is crucial in own laboratory and clinical practice. In the present study we evaluated urinary NGAL and [TIMP-2]-[IGFBP-7] with an early timing (4-6 hours after renal damage) and

consequently the outcomes have not been good as expected. According to other authors, we indicated that, in critically ill patients underwent cardiac surgery, pre-analytical conditions were characterized by concentration/dilution of urinary samples. Advances in timed urine collections [16] and standardization of pre-analytical procedures may add new findings. Probably, our sampling time too early, determined sample dilution, because of high hemodynamic changes in critical post-surgical patients. Another issue was the analytical expression of Nephrocheck test or measure unit. We demonstrated that error propagation and Nephrochek test uncertainty may be reduced using a linear measure unit as ng/mL. Finally, new urinary biomarkers might be improved for clinical use whether pre and post-analytical variabilities will achieve a meaningful reduction, allowing a medical help for AKI prevention.

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Bioradical homeostasis as new complex parameters of different biological systems

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ABSTRACT

The current state of free radical biomedicine allows us to formulate a fundamentally new parameter of homeostasis – bioradical homeostasis, understood as a state of free radical processes, reflecting the optimal intensity of the body metabolism of oxygen, nitrogen and halogens reactive species, carbonyls and other radical molecules. This term allows us to formulate another term – "bioradical stress". It is proposed to understand the effective violation of the physiological level of free-radical processes associated with the formation of active forms of oxygen, nitrogen or halogens and/or a decrease in the activity of systems that limit their damaging effect. It combines all known syndromes associated with bioradical level shifts (oxidative, nitrosative, halogenating and carbonyl stress). The introduction of the concept of bioradical stress involves the study of the effectiveness of various options for specific pathogenetic correction of bioradical stress (the administration of antioxidants, directed stimulation of antioxidant capacity by active oxygen forms, the use of regulatory properties of NO, etc.).

Keywords: *Free radicals, bioradical homeostasis, bioradical stress, oxidative stress, nitrosative stress, halogenic stress*

1. Introduction

Research in the field of free radical biology has been conducted in all countries of the world for several decades. During this time, indicated discipline has formed its methodology, methodological apparatus, as well as terminology base [1-3]. Ideas about the significant role of free radical processes in the functioning of living systems in normal and pathological conditions have not been questioned. Thus, the participation of free radicals in cell membrane renewal and intercellular signaling has been proved [4, 5]. They serve as an essential element of phagocytosis, being a key agent of the phenomenon of "respiratory explosion" of leukocytes [6]. They also realize other physiological functions [3-6].

On the other hand, in recent years, the concept of "oxidative stress" has become widely known in biomedicine [4, 6, 7], interpreted as a significant increase in the concentration of free radicals, occurring in conjunction with a sharp decrease in antioxidant reserves. At the same time, ideas about oxidative stress went far beyond free radical biology, finding application in almost all areas of medicine [4-7]. Knowledge of other possible dysmetabolic disorders, in particular, associated with changes in the level of nitrogen -and chlorine-containing radicals toxic to biomolecules, is also being developed. These conditions received the name as nitrosative and halogenic stress, respectively [8-10].

On the other hand, the above effective (causing disease or pathological conditions) shifts in the concentrations of oxygen, nitrogen and halogen radicals are considered in isolation. At the same time, in the Russian and world literature there is a significant amount of data indicating the relationship of metabolism of these molecules [4, 5, 9]. Therefore, it seems appropriate to analyze the processes occurring with the participation of oxygen, nitrogen and halogen-containing radicals within a single fragment of metabolism, which is the purpose of this work.

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2. Bioradical homeostasis and bioradical stress: the terms

It is known that one of the basic concepts of biomedicine is homeostasis, which is a set of indicators supported by the body at a constant level and limiting its functioning as an independent unit, as well as mechanisms that provide this state [5, 6]. The range of homeostasis indicators includes a wide range of parameters (body temperature, blood pressure, pH of blood plasma, acid-base balance, etc.). The up-to-date achievements of free radical medicine allow us to hypothesize about a new component of homeostasis linked with maintaining a constant concentration of bioradicals. Accordingly, the term "bioradical homeostasis" is interpreted by us as a state of free radical processes, reflecting the optimal intensity of metabolism of oxygen, nitrogen, halogens, carbonyls and other radical molecules. From this concept and on the basis of a critical analysis of information retrieved from literature, as well as the results of our own experimental studies, we hypothesize the concept of "bioradical stress", understood by us as an effective (leading to a negative change in the functional-metabolic status of the organism) violation of the physiological level of free-radical processes associated with the formation of active forms of oxygen, nitrogen or halogens and/or a decrease in the activity of systems that limit their damaging effect. With this in mind, bioradical stress combines all known syndromes associated with shifts in the level of bioradicals – oxidative, nitrosative and halogenating stress. In addition, the advantage of the proposed integrative concept of simultaneous presence of more than one component of bioradical stress in the same patient or animal is substantiated.

Diagnosis of the presence of bioradical stress should consist of the definition of its individual components:

1) intensification of lipoperoxidation on the background of inhibition of antioxidant reserves (component of oxidative stress);

2) improve markers nitrosative stress (in particular, 3-nitrotyrosine);

3) increase of myeloperoxidase activity and other parameters visualizing halogenating stress.

The presence of at least two of these components indicates the presence of bioradical stress.

At the same time, both their specific markers and nonspecific functional-metabolic criteria are used to obtain a complex characteristic of the body's response to the formation of bioradical stress. Given that the concept of the study involves consideration of bioradical stress as a set of three main interacting components – oxidative, nitrosative and halogenating, the study will include the definition of their specific markers:

- diagnosis of oxidative stress is based on the study of the intensity of the processes of lipoperoxidation in the blood plasma in conjunction with the total antioxidant activity of the plasma;
- identifying nitrosative stress based on the definition of level 3-nitrotyrosine plasma and halogensilver activity of myeloperoxidase.

3. Possibilities of the correction of bioradical stress

The formation of the concept under consideration creates the prerequisites for its directional correction, which is logical to carry out in accordance with the diagnostic approach, i.e. component by component. At the same time, the possibilities of elimination of individual components of bioradical stress were studied to varying degrees. Thus, the widest range of methods of correction of oxidative stress. First of all, it is represented by ozone therapy and antioxidant therapy. These trends in recent decades have taken a strong place in all areas of practical medicine. In particular, the possibilities of ozone therapy are actively revealed in obstetric and gynecological practice, which uses antibacterial, antiviral, antihypoxic and bioregulatory properties of ozone and the resulting active forms of oxygen [11, 12]. In addition, there is extensive evidence of their use in surgery based on similar effects (mainly on antibacterial action and oxidative detoxification), as well as in traumatology and combustiology, for which the entire range of biological effects of ozone is significant [13, 14].

A similar situation occurs with regard to antioxidant therapy, which is included in the algorithms and treatment schemes for many diseases, both surgical and therapeutic profile [4, 6, 15-17]. The effectiveness of this approach is verified by numerous experimental and clinical studies [15-18], as a result of which several hundred drugs with antioxidant activity are currently known, and their list continues to increase [4, 6].

For other components of bioradical stress, the possibilities of specific correction are significantly more limited. This is partly due to the lack of diagnostic verification [9, 19, 20]. On the other hand, for nitrosative, halogenic and carbonyl stress, antioxidant therapy, acting in this case as a non-specific remedy, is practically the unique way of treatment [3, 4, 21]. Thus, an additional important research task is the development of technologies for the directed correction of these variants of bioradical stress [1, 22, 23].

4. Our data on research for possibilities of the correction of some componentes of bioradical stress

Our team has been working over the past decades to discover new options and opportunities for the use of innovative methods of correction of various components of bioradical stress. Thus, historically, Nizhny Novgorod is one of the leading centers for the study of biological effects and methods of medical use of ozone and ozonated media (in particular, ozonated saline). For example, our studies have established the optimal therapeutic effect of "low therapeutic doses" (less than 1000 μg/l solvent), confirmed by a registered discovery [14, 24]. The features of the effects of ozone in the system subchronic administration (for 30 days

or more) were studied [25]. The variants of local and systemic ozone therapy in burn disease, purulent inflammatory diseases of bones and soft tissues (in particular, in osteomyelitis) are disclosed in detail [14].

The therapeutic potential of "alternative" reactive oxygen species is also being actively studied. In particular, we describe the biological and sanogenetic effects of the special high-energy state of molecular oxygen – singlet oxygen. Our researches have allowed to establish that, in contrast to ozone, the gas stream from the generator of singlet oxygen is moderately stimulate the pro- and antioxidant mechanisms, but it has a strong positive effect on the antioxidant system, including its enzymatic component [26]. In addition, the favourable effect of the considered factor on a number of components of energy metabolism (for example, the activity of lactate dehydrogenase in direct and reverse reactions in combination with the level of one of the reaction products - lactate), as well as enzyme detoxification systems in the blood (alcohol dehydrogenase, aldehyde dehydrogenase) was demonstrated. These metabolic changes are reflected in the systemic reactions of the body. In particular, when inhaled singlet oxygen was used, stimulation of microcirculation was observed, mainly carried out at the expense of intravascular NO-dependent mechanisms [27]. These changes ensure the effectiveness of singlet oxygen inhalations in the correction of oxidative stress and hypoxia phenomena arising as a result of experimental thermal trauma.

The second object of our research was NO metabolism and the possibility of its directed correction by exogenous sources of the compound. As the latter, nitrogen monoxide generators and physiological donor of this compound – dinitrosyl iron complexes with glutathione ligands were used [24, 28]. It is established that they have a different effect on the parameters of biological systems [28, 29]. Thus, NO-containing gas stream demonstrates a moderate pro-oxidant effect, but it acts as an antioxidant under the experimental pathological state associated with hypoxia and bioradical stress (for example, severe thermal injury) [30]. On the contrary, dinitrosyl-iron complexes have antioxidant properties in all cases, while encouraging intermediate and energy metabolism [28, 29]. These features of biological effects of NO sources allow us to consider them as a way to correction of bioradical homeostasis (by components of oxidative and nitrosative stress).

In whole, the data obtained by us allow to reveal new methods of specific correction of not only oxidative, but also nitrosative stress.

5. Concluding Remarks

Thus, the current state of free radical biomedicine allows us to formulate a new parameter of homeostasis – bioradical homeostasis, understood as a state of free radical processes, reflecting the optimal intensity of the body metabolism of oxygen compounds, nitrogen, halogens, carbonyls and other radical molecules. This term allows us to formulate conception about bioradical stress. We propose to understand it as the effective violation of the physiological level of free-radical processes associated with the generation of active forms of oxygen, nitrogen or halogens and/or a decrease in the activity of antioxidant systems. It combines all known syndromes associated with bioradical level shifts (oxidative, nitrosative, halogenic and carbonyl stress). The introduction of the concept of bioradical stress involves the study of the effectiveness of various options for specific pathogenetic correction of bioradical stress (the introduction of antioxidants, directed stimulation of antioxidant capacity by active oxygen forms, the use of regulatory properties of NO, etc.).

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We would thank the ICU and laboratory technician staff for their contribution in the patient recruitment and samples processing.

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Multiparametric analysis of crystallogenic properties of blood serum of healthy people and patients with burn disease

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ABSTRACT

The aim of the study was to assess some of the biophysical properties of dehydrated biological fluids in patients with thermal trauma. Own and initiated by the basic substance (0.9% sodium chloride solution) crystallization of blood serum of 32 patients with burn disease (contact burn, the area of skin lesions - 15-45% of the body surface; acute toxemia phase; age 21-40 years), as well as 30 healthy people of comparable age were studied. It is shown that in thermal injury undergo significant changes all outcome measures of biocrystalloscopic and impedance parameters in relation to their own and initiated the crystallogenesis of the blood serum. These changes are manifested in both time and quality parameters. Thus, burn disease in the stage of acute toxemia is characterized by slowing and inhibition of dehydration structuring of samples, increased degree of destruction of the formed elements, relative narrowing of the boundary zone, etc., which is confirmed by the data of registration of acoustic impedance. Based on these results, it is assumed that the visualization and study of the impedance characteristics of the dynamics of drying of serum droplets can be a convenient tool for diagnosing the patient's condition in combustiology.

Keywords: *Blood serum, crystallization, acoustic mechanical impedance, thermal trauma*

1. Introduction

Burn disease is a complex and multifaceted set of pathological reactions, prevalent among which at the organizational level are metabolic changes [1]. This is due to the leading role in its development of burn endotoxemia, provided by the massive flow of organic toxic substances into the systemic bloodstream [1-3]. The spectrum of these toxins includes both intermediate (interpreted and detectable as "medium-mass molecules") and terminal (urea, creatinine) metabolites [2, 4]. Their presence can significantly transform not only the component composition of biological substrates, but also to ensure the transformation of their biophysical properties. The latter circumstance can be useful in terms of assessing the depth, severity and degree of reversibility of metabolic changes of the body in thermal injury.

On the other hand, the choice of conditions (or model effects) on the biological medium in which the study of their physical and chemical properties will be carried out is of fundamental importance. One of the most closely studied options is dehydration self-organization of biological substrates [5-8]. However, it is important to emphasize that currently the common approach to the interpretation of crystallogenic properties of a biological material is given by a qualitative description provided with photography of the bounds [8, 9], whereas quantitative methods study of biocrystalloscopy are underutilized [5-7]. Other chemical and physical methods in biocrystallomics are used only by a limited number of specialists [10, 11].

It is important to emphasize that the character of crystallogenic roperties of biosubstrates in patients with burn disease has not been studied [6, 12]. Therefore, the aim of this study was evaluation of some biophysical properties by dehydration (drying) of biological media in patients with thermal lesions.

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2. Material and Methods

We have investigated own and initiated crystallization of the blood serum of 32 patients with burn disease (the contact burn, the affected area of the skin - 15-45% of the body surface; acute phase toxemia; age 21-40 years) and 30 healthy individuals of comparable age. Drugs of biological media were prepared using our own methodology [5-7, 13]. Own crystallization of blood serum (crystalloscopic test) was tested by drying of biological fluid on horizontal plane (standard microscopic glass slide) without any physical and chemical stimulators. Initiated crystallization was studied in teziographic test by co-drying of biological fluid and basic substance (stimulator of crystallization) in proportion 1 : 1 on one slide. We used 0.9% sodium chloride solution as the basic substance for teziographic test.

During the whole time of drying of micro-preparations the dynamics of their biophysical parameters were recorded. This complex included the study of the acoustic-mechanical impedance of the drying droplet, as well as a minute-by-minute assessment of the main quantitative parameters of teziocrystalloscopy. Drying was performed in the laboratory conditions (temperature - 23-25°C, humidity - 45-60%) without thermal stimulation of crystal formation.

Measurements of acoustic mechanical impedance (AMI) were performed using a special hardware and software complex provided by Institute of Applied Physics of Russian Academy of Sciences [11, 13, 14]. The volume of samples subjected to AMI analysis was 5 μl. In order to minimize errors, the AMI curve was registered three times. The duration of the signal registration was fixed (30 minutes).

The range of indicators of visualestimation of microspecimens of blood serum were included: for crystalloscopic test – index structure, crystallizability, facia destruction degree and clearity of marginal belt. For teziography we used main teziographic coefficient Q, belt coefficient P, facia destruction degree and clearity of marginal belt [5-7].

Statistical data processing and correlation analysis were performed using Microsoft Excel 2007 spreadsheets, as well as the program Primer of biostatistics 4.03.

3. Results and discussion

The study of the features of free and initiated crystal formation of biosubstrates in thermal trauma allowed us to establish that in the early period of burn disease, the crystallogenic properties and the initiatory potential of blood serum undergo significant transformations that, from our point of view, are destabilizing (Fig. 1 and 2). Thus, and in accordance with the data of figure 1, the General trend found with respect to the crystal formation of the considered biological environment of patients with thermal trauma is a significant slowdown in the rate of dehydration self-organization of the biomaterial. Thus, the stage of the visible beginning of structure formation in drying samples of healthy people begins with 7-8 minutes (without thermal stimulation), whereas in burn disease the signs of crystallization are found only on 17-19 minutes from the moment of applying the biofluid on the glass.

The qualitative and quantitative characteristics of the crystalloscopic sample also differ significantly. In particular, during the whole period of observation, significantly lower level of the index of structure, crystallizability and clarity of the marginal protein zone in comparison with the "pattern" revealed for the control group ($p<0.05$) was revealed in serum micro-preparations.

A slightly different dynamic was obtained in assessing the degree of destruction of facies: in patients with burn disease, despite the much later time of crystallization, the rate of growth of destructive changes is incomparably higher than the control figures, and from the 25th minute the level of this indicator exceeds the values established for healthy people.

The revealed changes were reflected in the analysis of the AMI curve of the control group and patients with thermal lesions (Fig. 2).

Taking into account the information shown in figure 2, we can confirm the thesis of slowing the rate of crystallization of biological fluid in burn disease, as well as the transformation of its dynamics.

The next stage of the study was the evaluation of the initiating properties of the biological substrate in respect of isotonic and pH-free medium of the crystal-0.9% sodium chloride solution.

Considering the nature of the initiatory process of blood serum inert crystal, it is possible, as well as for its crystallogenic properties, to note the significant transformation of the dynamics of dehydration of the biomaterial for all major performance indicators (Fig. 3) caused by the accumulation of toxic substances into the biological medium [2, 4]. The inhibitory effect of these substances led to decreasing of studied parameters of the facias of patients with burn disease is compared to levels of control subjects. The potential mechanism of this phenomenon, from our point of view, is due to the fact that in endotoxicosis of moderate severity the concentration of medium - and low-molecular organic compounds-metabolites stabilizes the biological medium in non-crystalline form (Sol, gel). This explains the low level of the main teziographic coefficient throughout the observation period. Existing patients reporting severe hypoproteinemia reduces the values of coefficient zones and width of the regional protein zone, and can also indirectly (because of dysproteinemia and relative hypoproteinemia) to reduce the triggering properties of the biosubstrate in thermal injury. All of the above explains the high rate and severity of destructive (destabilizing) processes in the drying drops of blood serum.

Study of the impedance metric of the biological environment confirmed the General trend in the depression of crystallogenic and initiating properties of biofluids in

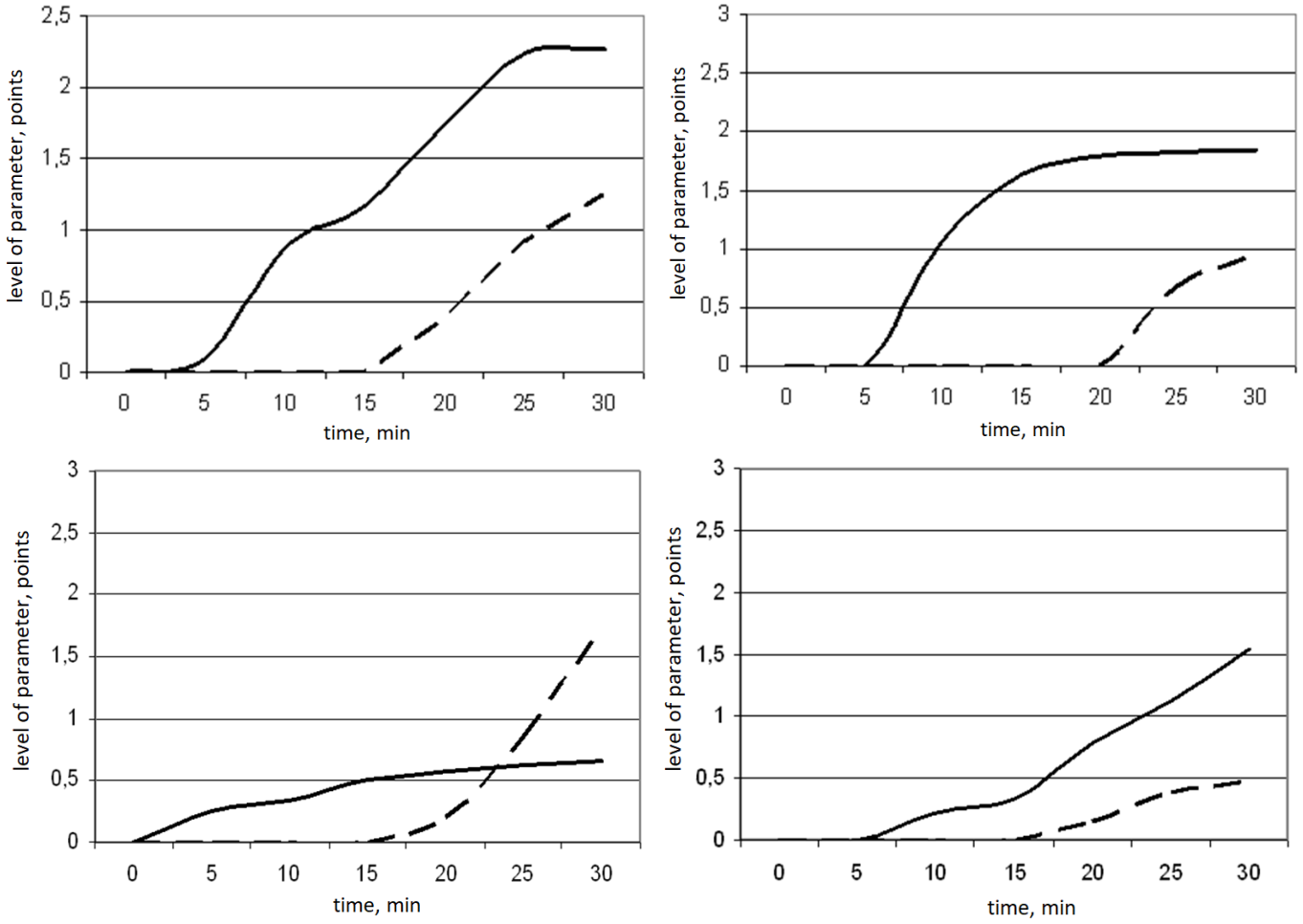

Figure 1 | Dynamics of main parameters of crystalloscopic facias of healthy people (n=30) and burned (n=32) persons (solid line indicates healthy people; punture line indicate patients with burn disease)

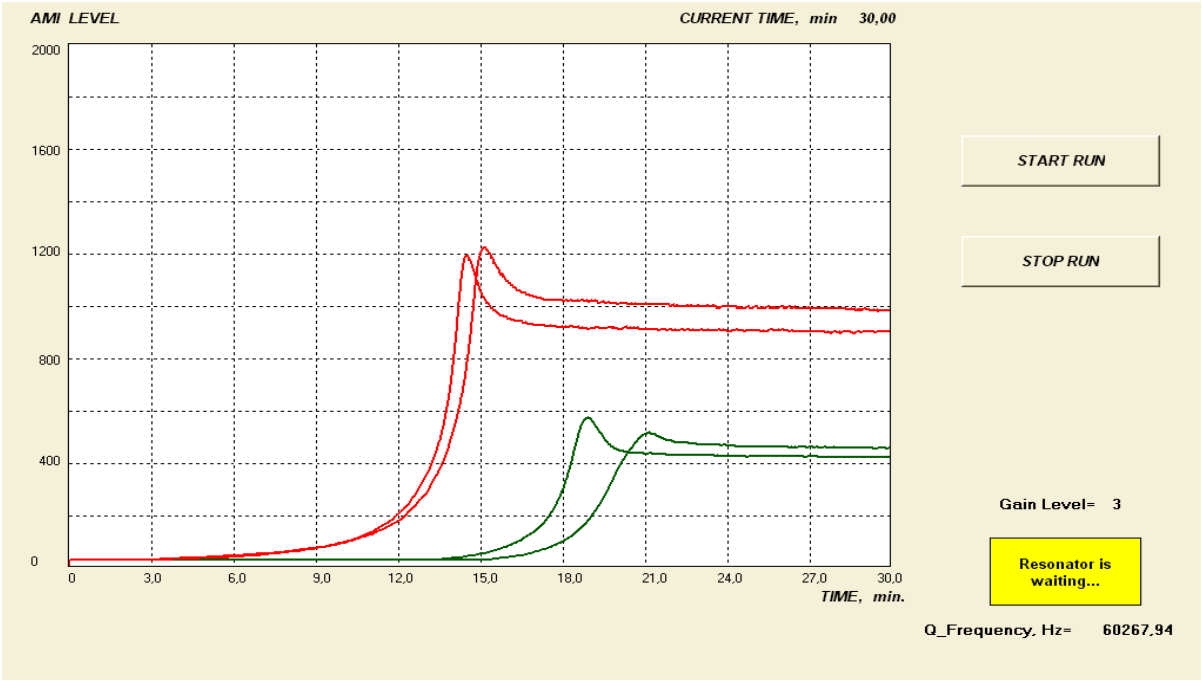

Figure 2 | Example of curves of acoustic and mechanical impedance of drying drops of blood serum from healthy people (1) and burned persons (2)

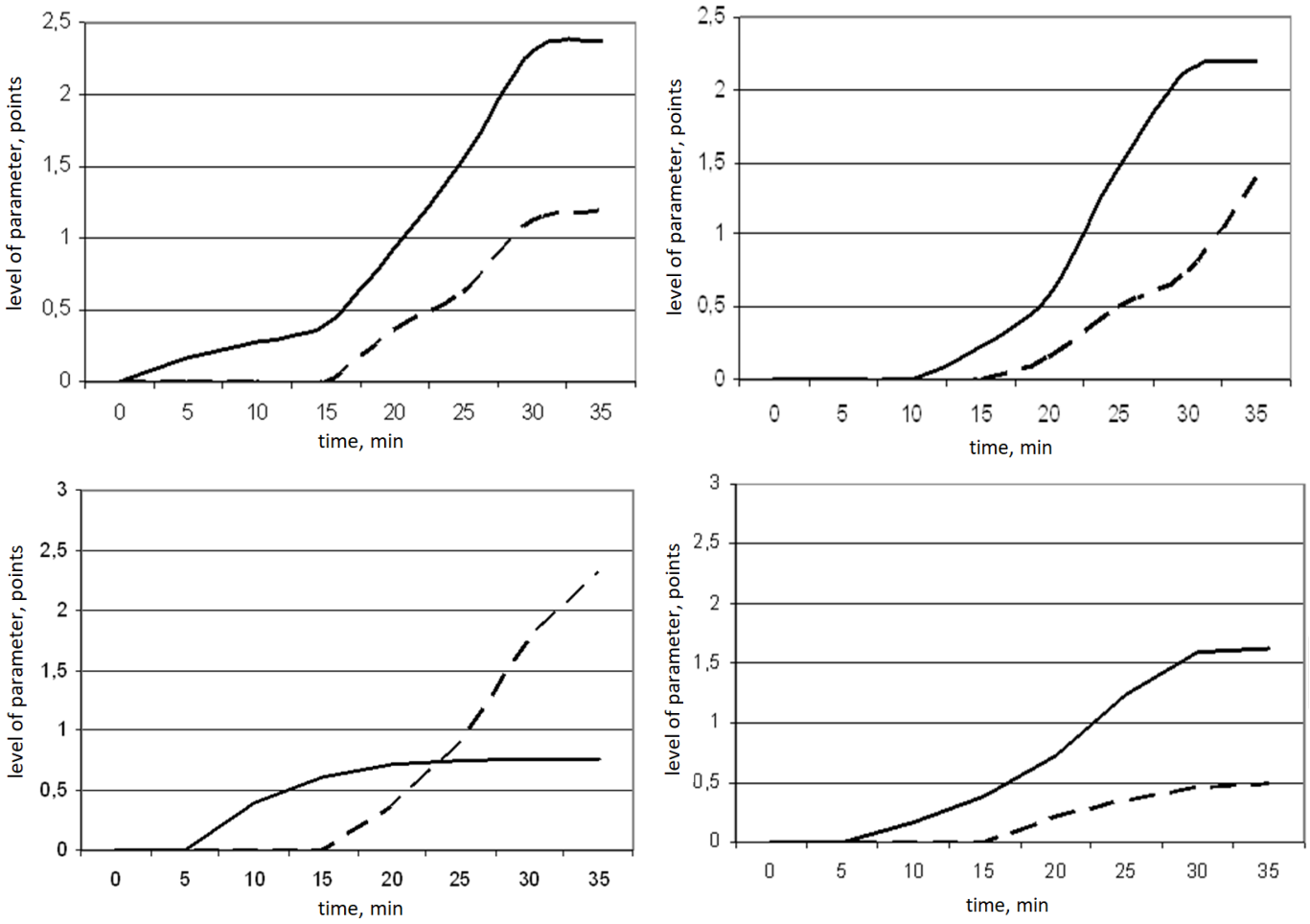

Figure 3 | Dynamics of main parameters of crystalloscopic facias of healthy people (n=30) and burned (n=32) persons (solid line indicates healthy people; punture line indicate patients with burn disease)

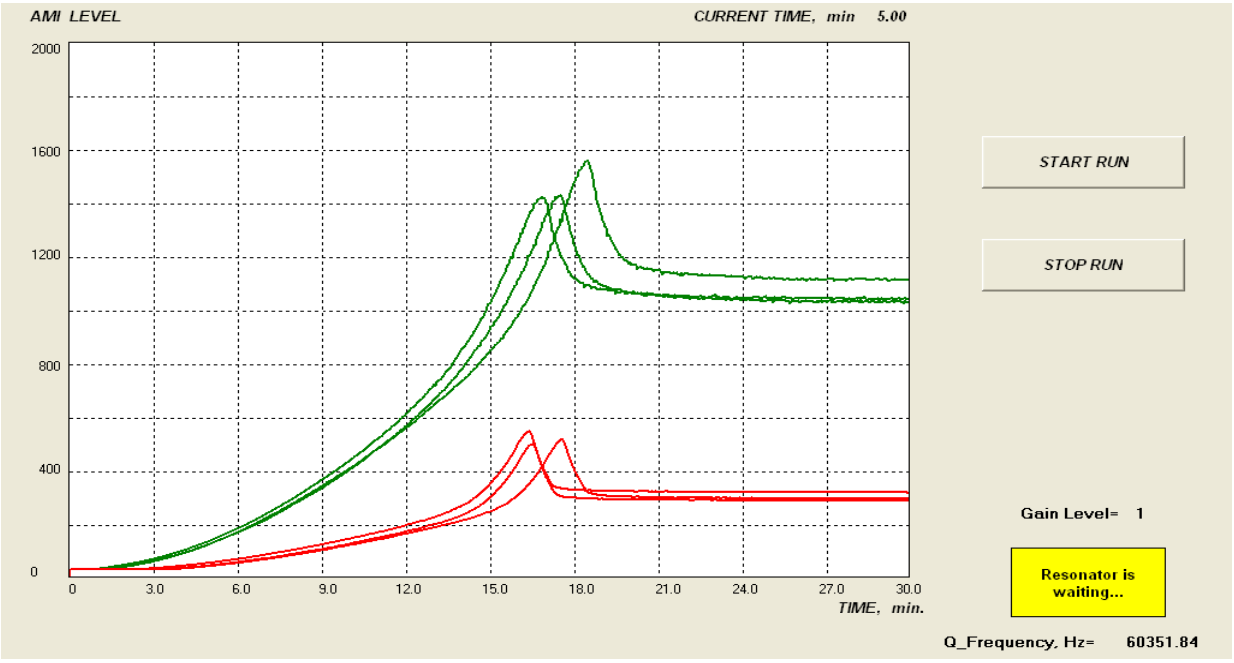

Figure 4 | Example of curves of acoustic and mechanical impedance of drying drops of blood serum from healthy people (1) and burned persons (2)

thermal injury. With respect to this method, this is reflected in the time and amplitude parameters (Fig. 4). It can be assumed that the appearance in the systemic circulation of substrates of burn intoxication (intermediate and terminal metabolites) in conjunction with changes in other components of the biofluid change its biophysical parameters, which can be reflected in the form of crystallodiagnostics of this pathological condition in the dynamics of management of patients of the profile under consideration.

The conducted correlation analysis within and between groups of indicators (visualization and acoustic-mechanical impedance) allowed to establish the presence of multiple correlations of average [0.3≤r≤0.7)] and high (r≥0.7) force (Fig. 5 and 6), which further confirms the reliability of the selected trends and consistency of changes in the biophysical properties of blood serum in thermal injury. These co-directional shifts give reason to conclude that in burn disease there are pronounced endotoxicosis-dependent rearrangements of the composition and properties of biofluids [1, 2].

Separately, it is necessary to emphasize the possibility of further transformation of physical and chemical characteristics of the studied biomaterial in the later postthermal period, as well as the importance of clarifying the contribution of the severity of metabolic disorders in the change of free and initiated crystal formation of body fluids in thermal lesions. All of the above is the subject of a new biomedical synthetic science – metabonomics, the essence of which is to study the possibility of monitoring the functional and metabolic status of a person on the basis of an integral or partial assessment of the characteristics of the biological medium. Thus, the biophysical metabonomics of burn disease through the methods of biocrystallomics can provide a large amount of information about the current state of the patient and its dynamics, which is valuable in terms of primary diagnosis of the severity of metabolic disorders and further metabolic control.

4. Conclusion

On the basis of the conducted studies it was found that the dynamics of dehydration self-organization of blood serum of healthy people and patients with thermal trauma varies significantly. In this case, we have shown the presence of relationships between the parameters of crystalloscopic test and registration of the acoustic impedance of drying droplets of this biofluid in burn disease. These facts allow to conclude that crystalloscopy of dried samples of biological fluids and study of its acoustic mechanical impedance can serve as informative tools biophysical metabonomic of thermal injury.

Acknowledgments:

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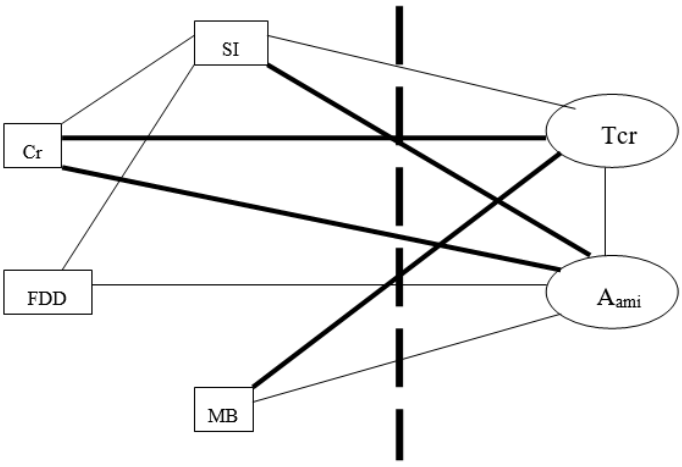

Figure 5 | Correlations between crystalloscopic (indicate as rectangles) and AMI-parameters (indicate as ovals) in facias of blood serum from healthy (n=30) people and burned (n=32) persons (SI – structure index, Cr - crystallizability, FDD – facia destructiob degree, MB – clearity of marginal belt, Tcr – time of crystallization beginning, Aami – maximal amplitude of the level of acoustic mechanical impedance; thin lines indicate moderate correlations, fat lines are strong correlations).

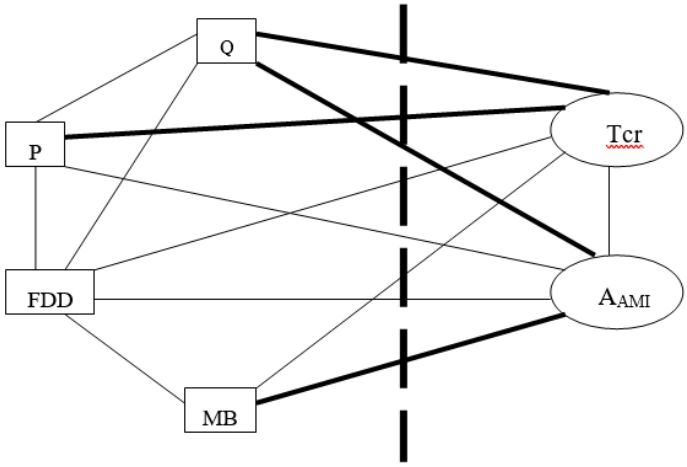

Figure 6 | Correlations between teziographic (indicate as rectangles) and AMI-parameters (indicate as ovals) in facias of blood serum from healthy (n=30) people and burned (n=32) persons (basic substance – 0.9% NaCl solution; Q – main teziographic coefficient, P – belt coefficient, FDD – facia destructiob degree, MB – clearity of marginal belt, Tcr – time of crystallization beginning, Aami – maximal amplitude of the level of acoustic mechanical impedance; thin lines indicate moderate correlations, fat lines are strong correlations)

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The influence of reactive oxygen species and NO on oxidative metabolism and dielectric properties of living tissue

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ABSTRACT

The aim of this work was a comprehensive assessment of the action of reactive oxygen species and nitric oxide on the scar tissue fragment ex-vivo. The study was performed using fragments of scar tissue (n=10) removed intraoperatively in patients with Dupuytren's contracture and preserved in an isotonic solution of sodium chloride until the beginning of the experiment (for saving of living state of cells in specimens). Each fragment was divided into 3 equal parts. No manipulations were performed to the first part, the second one was treated with singlet oxygen, the third piece was processed with nitrogen oxide (20 ppm). The duration of the tissue treatment period was 5 minutes. Upon completion of the experiment in all samples, the methods of near-field resonance microwave sensing evaluated the dielectric properties of tissue using a software package developed at the Institute of Applied Physics of the Russian Academy of Sciences. Further, each portion of the tissue was homogenized using the certified apparatus "UltraTurrax" according to the standard procedure. The parameters of oxidative metabolism (intensity of free radical oxidation and total antioxidant activity) were studied in the homogenates by Fe - induced biochemiluminescence. It was found that the treatment of scar tissue fragments by gas flow from singlet oxygen and nitric oxide generators leads to a change in the dielectric properties of the tissue and the intensity of free radical processes in it, and the nature of the response is specific to the influencing factor (NO or singlet oxygen). It is shown that the singlet oxygen effect is a moderate increase in the dielectric permeability of the tissue and a balanced stimulating effect on the pro - and antioxidant systems. The NO effect at a concentration of 20 ppm is associated with a marked increase in dielectric permittivity and conductivity, as well as a significant increase in the antioxidant potential of the tissue.

Keywords: *Reactive oxygen species, nitric oxide, oxidative metabolism, tissue dielectric properties, microwave probing*

1. Introduction

Modern physiotherapy has a wide range of diverse therapeutic application. At the same time, their type is constantly expanding. Thus, since some decades ago, ozone therapy, has found application in various fields of practical medicine [1]. Our previous works and also from other teams have shown that not only ozone, but also other exogenous forms of oxygen and nitrogen can have positive effects on living systems [2-7]. In particular, positiveshifts for low concentrations of nitrogen monoxide (NO; 20-100 ppm) [8] and gas flow from the singlet oxygen generator were demonstrated for blood samples [3, 9, 10]. It was found that at certain levels these aforementioned applications increase the antioxidant potential of blood plasma and moderately stimulate the activity of one of the main antioxidant enzymes – superoxide dismutase [9, 10]. Also, the beneficial effect of singlet oxygen on the parameters of energy metabolism was revealed [8], however, all these results related to the treatment of blood in vitro. It should be noted that there are no data on the nature of modification of tissue parameters in the literature.

In this regard, the aim of the work was a comparative assessment of the action of reactive oxygen species and nitric oxide on a fragments of scar tissue.

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2. Material and Methods

For the experiment, a special installation was assembled (fig. 1), which allows to create a gas environment around a fragment of tissue under a glass dome, into which a gas stream was pumped from a singlet oxygen generator or NO-generator. The duration of the tissue treatment period was 5 minutes for all factors. The NO concentration in the gas stream was 20 ppm, singlet oxygen was created in the mode of 100% of the power of the generator.

The study was performed using fragments of scar tissue (n=10) removed intraoperatively in patients with Dupuytren's contracture and preserved in an isotonic solution of sodium chloride until the beginning of the experiment (for saving of living state of cells in specimens). Each fragment was divided into 3 equal parts, the first was intact (did not carry out any manipulations with it), the second one was treated with singlet oxygen, the third one was treated with nitrogen oxide.

Upon completion of the experiment in all samples, we evaluated the dielectric properties of the tissue with near-field resonance microwave sensing method using a software package developed at the Institute of Applied Physics of the Russian Academy of Sciences (Nizhny Novgorod) [11-13]. This complex allows to calculate dielectric permittivity (ε) and conductivity (σ) of the biomaterial. The depth of sounding of the biological sample is 5 mm.

Further, each portion of the tissue was homogenized using certified "UltraTurrax" apparatus according to the standard procedure is not saturated the tissue specimen by side substances. The parameters of oxidative metabolism were studied in the obtained homogenates by Fe-induced biochemiluminescence: the intensity of free radical oxidation processes (by the level of the maximum flash, I max) and the total antioxidant activity (as the value of the inverse light sum of biochemiluminescence in 30 seconds, 1/S). Biochemiluminescence analysis was performed with certified biochemiluminometer "BHL-06" (NizhnyNovgorod, Russia). It automatically registers chemiluminescence, which can be spontaneous or induced by different substances, such as Fe, luminol etc. [2, 4, 15, 16].

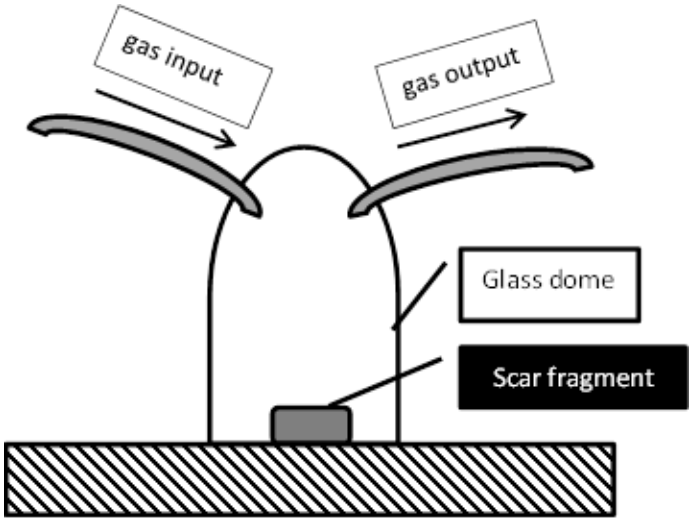

Figure 1 | Installation scheme for the experiment.

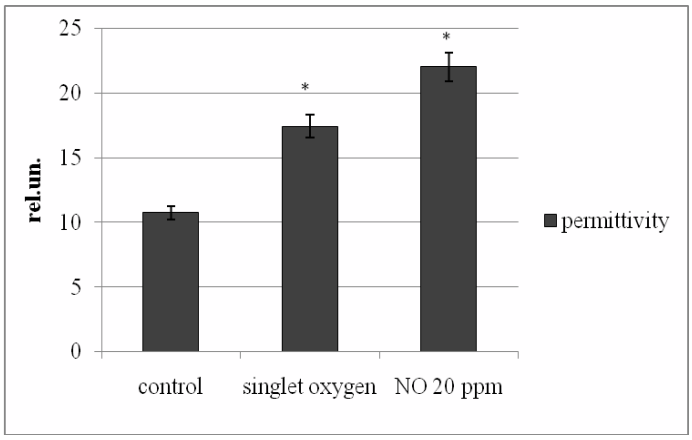

Figure 2 | The level of dielectric permittivity of intact tissues and specimens processed with singlet oxygen and NO («*» - p<0,05).

Statistical processing of the results was performed using Statistica 6.1 for Windows. The normality of the distribution of parameter values was evaluated using the Shapiro-Wilk criterion. Taking into account the nature of the distribution of the trait, the Kruskal-Wallace H-criterion was used to assess the statistical significance of the differences. The critical level of significance in testing statistical hypotheses in this study was taken to be 0.05.

3. Results

It was found that the treatment of a fragment of scar tissue by radical sources for 5 min leads to significant shifts in their dielectric properties and the state of free radical processes. Thus, both factors under consideration provide an increase in the dielectric permeability of the tissue, but the severity of this permeability depends directly on the nature of the impact (Fig. 2). In particular, the treatment of the biomaterial with a gas stream initially containing singlet oxygen leads to an increase in the parameter value by 1.63 times ($p<0.05$ relative to the intact fragment), while the effect of NO induces a more significant increase in the dielectric constant (2 times $p<0.01$ compared to the untreated piece of tissue).

The variability of the response was also found for conductivity (Fig. 3). At the same time, the effect of singlet oxygen did not contribute to the formation of shifts in this criterion, while the treatment of no biotissue at a concentration of 20 ppm led to an increase in the value of the indicator by 1.57 times ($p<0.01$ relative to the control).

Changes in the dielectric characteristics of the samples were accompanied by shifts in free-radical processes in them.

Thus, the activity of the studied factors was opposite (Fig. 4). Treatment of tissue fragments with singlet oxygen provided moderate stimulation of radical reactions is estimated by dynamics of level of the maximum flash (in

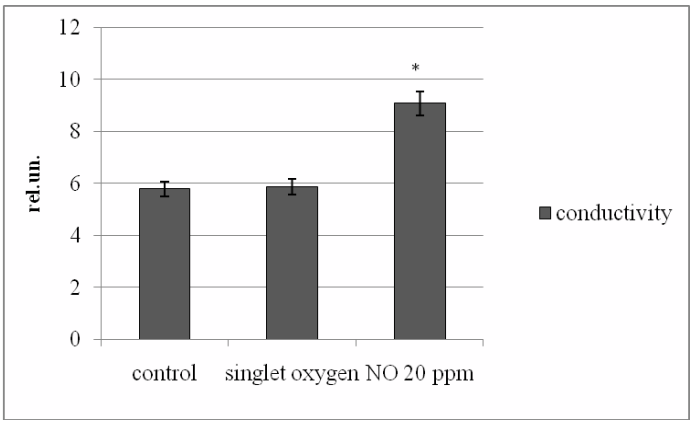

Figure 3 | The level of conductivity of intact tissues and specimens processed with singlet oxygen and NO («*» - p<0,05).

1.21 times; p<0.001 compared to the control sample, with which no manipulation was performed). On the contrary, when using a low concentration of nitrogen monoxide (20 ppm), a decrease in the studied parameter by 12.4% (p<0.05 relative to the intact tissue fragment) was found.

According to the effect on the total antioxidant activity of biomaterial, both estimated factors show a tendency to increase the value of the parameter (Fig. 5).

It was found that nitric oxide to a greater extent increases the antioxidant potential of the biological sample is measured by value of the inverse light sum of biochemiluminescence in 30 seconds (by 28.9%; p<0.05 compared with intact tissue fragment). Coupled with a decrease in the intensity of free radical processes, this indicates a pronounced antioxidant effect of nitric oxide. The impact of singlet oxygen also leads to increase of antioxidant activity of homogenates of the tissue, but these changes are expressed to a lesser extent (+15,7%; p<0.05 relative to the fragment of the biomaterial, which has not carried out impacts). In this case, it is possible to assume the balance of the factor influence on the state of Pro - and anti-oxidant systems of the biological sample.

4. Discussion

Our experimental studies were aimed at verification of the modulating effect of gaseous sources of reactive oxygen species and nitric oxide on a fragment of biological tissue. Confirmation of this effect is fully consistent with the previously obtained data on human blood samples (in vitro) and animal organism (in vivo) [8-10, 14]. It is important to emphasize that the effect is directly determined by the nature of the factor. At the same time, the features of the applied research methods allow us to conclude that in the treated tissue fragments not only metabolic reactions (primarily shifts in oxidative metabolism as a direct target of the action of reactive oxygen species and NO by dynamics of biochemiluminescence test [1, 15, 16]), but also other processes due to changes in the degree of tissue hydration (in the dynamics of dielectric characteristics of the analyzed objects depending on the content of water and aqueous solutions [17, 18]) are triggered. The given variants of the reaction can be both parallel manifestations of molecular-cellular tissue response to the action of reactive oxygen species and nitrogen monoxide, and dependent processes mediated through ROS-dependent regulatory cascades that determine the permeability of cell membranes for water molecules [15-19].

The obtained data allow to justify the expediency of local therapy carried out and the use of singlet oxygen generators and nitric oxide, but to clarify the nature of the processes involved, it is necessary to decipher the mechanisms for the implementation of the identified effects. This task is expected to be solved in further studies

5. Conclusions:

The experiment made it possible to establish that the treatment of scar tissue fragments by gas flow from singlet oxygen generators and nitrogen monoxide leads to a change in the dielectric properties of the tissue and the intensity of free radical processes in it , and the nature of the response is

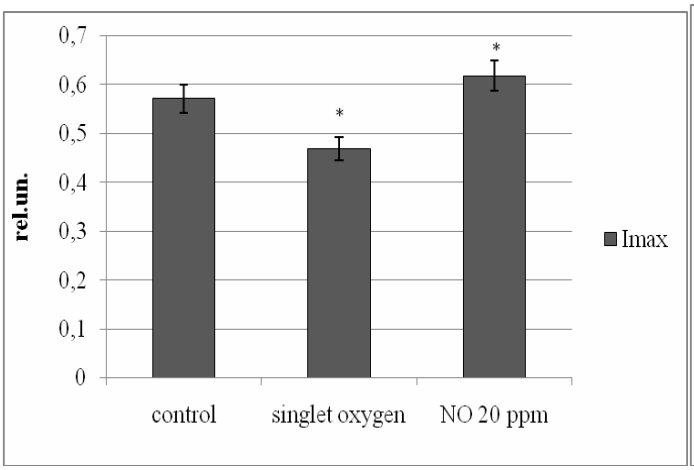

Figure 4 | Intensity of free radical processes of intact tissues and specimens processed with singlet oxygen and NO («*» - p<0,05).

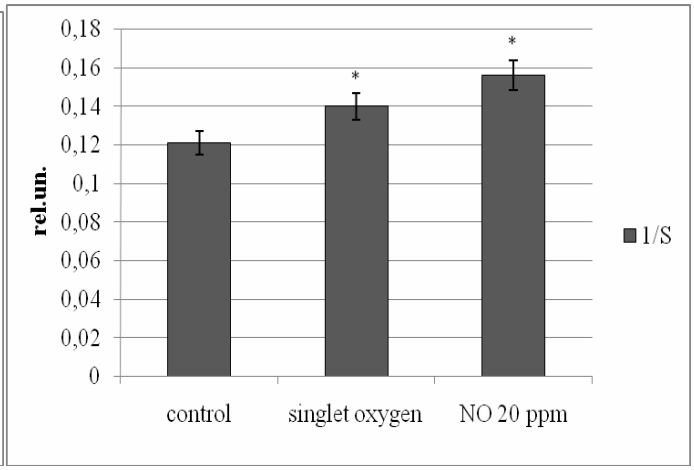

Figure 6 | Total antioxidant activity of intact tissues and specimens processed with singlet oxygen and NO («*» - p<0,05).

specific with respect to the nature of the influencing factor. It is shown that the peculiarity of the singlet oxygen action is a moderate increase in the dielectric permeability of the tissue and a balanced stimulating effect on pro- and antioxidant systems. The effect of NO at a concentration of 20 ppm is associated with a marked increase in dielectric permeability and conductivity, as well as a significant increase in the antioxidant potential of the tissue.

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