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NATRIURETIC PEPTIDES ELEVATIONS AFTER CHRONIC EXPOSURE TO COCAINE

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ABSTRACT

Cocaine is known to produce life-threatening cardiovascular complications. When cocaine abuse is suspected, the investigation of the causes of death may be challenging in forensic medicine. No exhaustive answer can be obtained in most cases, reinforcing the need of further investigational tools to be used in this field. The increasing knowledge in the available biomarkers of cardiac function together with the availability of high sensitive assays can provide new tools in the investigation of sudden cardiac death in chronic abuser. In this work, the assessment of cardiac dysfunction was assessed by measuring troponin I and natriuretic peptides as biomarkers, and considering other standard endpoints used in preclinical toxicology studies. All the procedures and endpoints considered were designed to allow an easy and complete translation from the laboratory animals to human beings. Lister Hooded rats were treated with cocaine in chronic (up to 12-18 weeks) self administration studies. Troponin I (cTnI) and Atrial Natriuretic Peptide (ANP) were evaluated at different timepoints and heart weight and histopathology was assessed at the end of the treatment period. Furthermore, cocaine and its main metabolites were measured in the rat fur, to assess rats' cocaine exposure. The results obtained showed that no morphological changes were present in the rat hearts, despite their chronic exposure to cocaine. Cardiac troponin I values in cocaine treated rats were normal, not different from control rats, further supporting the absence of morphological changes. On the contrary, ANP showed an increasing trend with time in all cocaine treated animals considered. The same approach was also adopted with a group of chronic health human cocaine abusers: 10 healthy cocaine abuser volunteers (with no cardiac pathologies) were considered, and troponin T, NT proBNP serum levels, cocaine and metabolites hair concentrations were measured. As in rats, no changes were observed in troponin serum levels, whereas the natriuretic peptides showed variations that suggest a parallelism with the minimal changes observed in rats. Similar changes are described in subjects considered at risk for hypertension. Natriuretic peptides are normally adopted as diagnostic/prognostic markers in the follow up of a number of cardiac or cardiovascular diseases; in this context, natriuretic peptides may represent a sensitive biomarker of heart dysfunction after cocaine chronic exposure.

In conclusion, in this work we have collected evidences supporting that rats chronically exposed to cocaine show a time-related increasing trend in ANP values. Histopathological examination of the heart did not reveal any morphological changes, in agreement with the absence of modifications in the cardiac troponin values. Comparable results were obtained also in a group of cocaine abuser volunteers, suggesting that natriuretic peptides could represent an early indicator of heart dysfunction in chronic cocaine abusers.

INTRODUCTION

Cocaine as other similar psychostimulant/addictive abused drugs is known to produce life-threatening cardiovascular complications. Cocaine chronic use also happens to be the most frequent cause of drug-related deaths reported by medical examiners; furthermore, over the last few decades cocaine addiction has attained epidemic proportions, imposing a tremendous burden on society and the health care system[1,2]. Cocaine's main cardiovascular complications include myocardial ischemia and necrosis, prothrombotic effects, vasospasms, coronary atherosclerosis, chest pain and a number of electrocardiographic changes. If acute toxicity due to massive drug exposure is excluded (as in couriers and "body packers"), cocaine severe cardiac complications and sudden deaths represent an unforecasted event in chronic abusers[3]. The causes of these adverse effects have not been completely understood, and several mechanisms are described in literature; as a general rule, cocaine causes an increase of ventricular contractility, blood pressure and heart rate, leading to an escalating myocardial oxygen demand[1,2]. An increased cardiac load can be considered one of the main risk factors, which may cause cardiac dysfunction after long term administration.

In forensic medicine, the criteria used to investigate the causes of death when cocaine abuse is suspected are based on a series of evaluations (including autopsy and cocaine's metabolites measurement in blood fluids); however, in most cases no exhaustive answer can be obtained from these evaluations, reinforcing the need of further investigational tools to be used in this field[4,5,6].

The increasing knowledge in the currently available cardiac functional biomarkers together with the introduction in the market of high sensitive assays can provide new tools in the investigation of the causes of death, when sudden cardiac death in chronic abuser is suspected.

The natriuretic peptides (mainly the atrial natriuretic peptide ANP, the brain natriuretic peptide BNP and their N-terminal pro-peptides NT proANP and NT proBNP) are polypeptidic hormones secreted by the heart muscle cells[7,8,9]. They are powerful vasodilator, and they are involved in the homeostatic control of body water, sodium, potassium and fat (adipose tissue) in response to high blood pressure (typically atrial/ventricular distension and stretching of the vessel walls). They are co-secreted

along with their respective inactive pro-peptides, which have longer half lives and provide an indirect but more robust tool for their measurement in blood fluids. For years, both ANP and BNP were considered as a sort of functional biomarkers, as they reflect the general cardiac overload rather than a toxicologic/pathologic dysfunction; however, BNP and NT proBNP are recently used in the diagnosis of acute congestive heart failure, and may be useful to establish prognosis in heart failure, as both markers are typically higher in patients with worse outcome[7]. The plasma concentrations of both BNP and NT proBNP are also typically increased in patients with asymptomatic or symptomatic left ventricular dysfunction[h]. The natriuretic peptides were also recently described to provide an early prognostic value, and they have been used as early biomarkers for the preclinical toxicologic assessment in drug development[10].

In this study, the early cardiovascular effects in cocaine chronically treated rats are investigated by using a toxicological approach and enabling the study of both structural and functional cardiac changes by using an animal model and in fully controlled experimental conditions. The approach used provided a deep evaluation of the cardiac changes induced by cocaine in rats, and it could be easily translated to human cocaine abusers.

Cocaine induced cardiovascular changes have been already observed and investigated in rats [11,12,13]; the response to cocaine in terms of dependence and cardiovascular changes as well as cholinesterase activity pattern (that represent the primary mechanism by which cocaine is metabolized to less active compounds) seem to be comparable in humans and rats[14,15], making rats a suitable model to investigate the cocaine-induced cardiovascular changes. In this study, Lister Hooded rats were chosen because they are particularly suitable for self administration protocols, demonstrating clear drug seeking behaviours after short training periods[16].

Two biomarkers commonly used in preclinical toxicology were considered in this study: the high sensitive Troponin I (cTnI) and the atrial natriuretuc peptide (ANP), monitoring both the structural and the functional rat heart impairment. These biomarkers are non-invasive and easily applicable to human beings: the high sensitivity Cardiac Troponin I (cTnI) is a commonly used cardiac necrosis marker, and it can be a very powerful marker to assess subtle cardiac injuries, as it is

believed to leak from cardiac cells also before cell death in some physiological stressful conditions, leading to a transient, smooth increase in cTnI blood levels[10,17].

The atrial natriuretic peptide ANP can play important roles in monitoring the cardiac functionality and in some cases some pre-necrotic cardiac stressful conditions[10,18,12].

Cocaine exposure in rat fur was also evaluated, to provide information on rat drug exposure with a method that could be fully translable also to humans.

The aim of this study was to investigate the use of the natriuretic peptides as early biomarkers for the evaluation of cardiac dysfunction induced by chronic treatment with cocaine in rats, considering to explore the usefulness of natriuretic peptide biomarkers into chronic cocaine abusers.

MATERIALS AND METHODS

Pre-clinical study in rats

Study Design

A total of 120 Lister Hooded rats were assigned to five chronic (up to 12-18 weeks) self administration studies. Each animal was previously prepared with a surgical implantation of a catheter in the jugular vein, and trained to push a lever to activate the cocaine self administration. When rats became addicted and fully trained to the self administration, they were treated at two different dosing regimen: the low dose regimen (adopted in 4 of the 5 studies) included the self administration of 0.5 mg\kg of cocaine each infusion in the jugular vein for 3 hours\day, whereas in the high dose rats were free to self administer the same amount of cocaine for up to 12 hours\day. Rats followed a controlled diet regimen, in order to standardize the weight increase.

Samples collection

Blood samples were taken at pre-dose, at study start, and at different timepoints throughout the study, generally after 1, 3, 6, 9, 12, 15 and 18 weeks of treatment (depending on the duration of the study). Serum was obtained and stored at -80 C°. Animals were not fasted before sampling.
At the end of each study, animals were euthanized by exsanguination in isofluorane deep anesthesia; blood samples were taken and serum was prepared and stored at -80 C°. During necropsy session, a macroscopic observation of the abdominal/thoracic cavity was performed; then, hearts were collected and put in formalin pots. Both heart and brain were weighed.

Cocaine intake

The cocaine intake was estimated on the basis of the number of administrations the animal was able to activate, by pressing a lever in the cage; at each lever press, each animal received 0.5 mg/kg of cocaine. All these data were then summarized by calculating the mean value for all animals per day, and expressed as mg/kg/day.
Furthermore, cocaine exposure was evaluated by measuring cocaine, benzoylecgonine and ecgonine methylester in rat fur by using a gas chromatography

mass spectrometer GC-MS (Agilent Technologies). Rat fur samples were washed two to three times with Tween 20% and after drying were cut in small fragments. Approximately 50-100 mg were hydrolyzed in 0.1M HCl, overnight at 40°C. Surnatant was neutralized by adding 100 ul NaOH and 1 ml of phosphate buffer (pH 6.8). Samples were purified by SPE (Bond Elut Cerfify, Agilent Technologies). Samples were taken to dryness and before analysis, samples were reconstructed with 50 ul of MSTFA (Sigma-Aldrich). Further details on GC-MS method are reported in Appendix 1.

Separated exposure evaluations for white and black fur were performed, to assess the different cocaine's affinity to melanin-rich fur.

Histopathology examination

Cardiac tissue was selected from hearts collected at necropsy, and all selected samples were processed by an automated processor (Tissue-Tek VIP 5-Sakura, Torrance, CA, USA) and embedded into paraffin wax blocks. From these, 3-μm thin sections were produced using a rotative microtome (Micron HM355S, Haryana, India), stained with hematoxylin and eosin (Tissue-Tek DRS 2000 Slide Stainer, Medical Equipment Source, LLC, Mars, PA, USA), and microscopically examined with a light microscope.

Biomarkers evaluation

The ultra sensitive cardiac Troponin I (cTnI) was measured on rat serum by using a Bayer Advia Centaur CP^{TM} immunochemistry automated analyser. The Atrial Natriuretic Peptide (ANP) evaluations were performed on rat serum samples by using the Bio SPI Rat Atriopeptin enzyme immunoassay. The NT proANP evaluations were performed on rat serum samples by using the Biomedica pro-ANP enzyme immunoassay. Other natriuretic peptides (such as BNP or NT proBNP, the latter widely used for humans) were not used as rat-specific commercial kits were not available.

Cocaine human abusers study

Study design

A panel of 10 chronic cocaine abuser volunteers were selected from the Verona's SERT (Servizio Tossicodipendenze). The selection was made on the basis of their age, absence of cardiac symptoms and cocaine consumption preference. All candidates were young, with ages between 22 and 42 years old (30.8 as average). According to what was reported by each volunteer, they never suffer of cardiac symptoms or past history of medical care due to cardiac affection and they refer to have assumed cocaine for the last time between 4-5 months (for occasional consumers) and 1 day before sampling (5 days as median). Further details on human volunteers panel are reported in Table 6.

Samples collection

From each volunteer, blood and hair was collected; serum was obtained and stored at -20 C° until analyzed.

Cocaine intake

Cocaine, benzoylecgonine, ecgonine methylester and cocaethylene were measured in the hair by using a gas chromatography mass spectrometer GC-MS (Agilent Technologies). Hair samples were washed twice with Tween 20% and after drying were cut in small fragments. One hundred mg were hydrolyzed in 0.1M HCl, overnight at 40°C. Surnatant was neutralized by adding 100 ul NaOH and 1 ml of phosphate buffer (pH 6.8). Samples were purified by SPE (Bond Elut Cerfify, Agilent Technologies). Samples were taken to dryness and before analysis, samples were reconstructed with 50 ul of MSTFA (Sigma-Aldrich). Further details on GC-MS method are reported in Appendix 1.

Biomarkers evaluation

The ultra sensitive cardiac Troponin T (cTnT) and the NT proBNP were measured on human serum by using a Roche immunochemistry automated analyser. It was not possible to measure the same analytes as in rats: even if available, ANP is not used

for human diagnostics. Amongst natriuretic peptides, the NT proBNP was chosen as this is the commonly used biomarker in human diagnostics.

Data handling

Atrial natriuretic peptide's results in rats were not grouped together, and are presented as separated experiments. No statistical analysis was performed, due to the inter assay variability (data were obtained in separated experiments, with different calibration curves) and because different timepoints were collected in different experiments. All data in this work are presented as mean values (one for each timepoint/experiment for rat ANP) and standard deviation (SD) or standard error of means (SEM).

RESULTS

Pre-clinical study in rats

The number of animals available at the end of each chronic study was limited by complications occurred during the study, such as obstructed catheter or infections. Animals that regularly assumed cocaine throughout the study are only presented.

Cocaine Intake

Animals were left to self-administer cocaine for 3 hours/day (low dose group) and for 12 hours/day (high dose group). In the low dose group, the average dose administered was 15.4 mg/kg/day, whereas animals treated at the high dose received 112.6 mg/kg/day (mean value). The estimated doses administered for the low and high dose regimen are reported in Table 1.

Low dose treatment regimen	
Rat number	**Estimated dose (mg/kg/day)**
H206	13.17
I206	16.00
K206	11.16
M206	18.33
R206	18.16
T206	22.50
V206	NM
W206	16.17
U351	20.24
V351	27.98
AC351	14.13
V654	NM
W654	NM
K207	18.90
P214	13.08
A233	12.32
B233	11.07
C233	9.57
D233	13.70
D250	14.50
F250	12.43
F233	NM
A250	11.10
E250	NM
E233	22.50
I250	16.13
C250	14.58
L250	NM
H250	NM
J250	NM
K250	NM
B250	16.75
G250	12.42
M184	11.92
R184	10.42
Mean:	**15.36**

High dose treatment regimen	
Rat number	**Estimated dose (mg/kg/day)**
B426	106.80
D426	102.17
H426	100.15
K426	134.35
L426	136.76
M426	135.26
N426	112.27
O426	118.75
R426	129.37
S426	114.60
V426	105.92
W426	81.00
Y426	112.15
AA426	112.13
AC426	115.61
C426	161.05
I426	65.43
U426	82.32
Mean:	**112.56**

Table 1: estimated doses administered to Lister Hooded rats The low and high dose regimens are reported separately, with the respective mean values. Dose was estimated by assuming 300 g for rat body weight. NM = not measured.

Cocaine exposures by means of cocaine and benzoylecgonine concentrations in rat fur was measured in a subpanel of animals; results are reported in Table 2 and 3.

	Dose [(1)]	Exposure (black fur)		Exposure (white fur)	
Rat number	**mg/kg/day (mean)**	**Cocaine (ng/mg)**	**BE (ng/mg)**	**Cocaine (ng/mg)**	**BE (ng/mg)**
W654	20.66	4.008	1.086	0.231	0.508
V654	26.84	0.474	0.481	Out	Out
K207	18.90	0.982	0.375	0.218	0.360
P214	13.08	0.534	0.287	0.290	0.334
R206	18.16	3.446	0.951	0.160	0.313
T206	22.50	0.833	0.437	0.187	0.480
K206	11.16	1.280	0.380	0.108	0.259
M206	18.33	0.648	0.303	0.152	0.305
H206	13.17	1.139	0.398	0.129	0.671
I206	16.00	0.645	0.232	0.172	0.954
W206	16.17	Out	Out	0.218	0.393
mean	17.72	1.399	0.493	0.186	0.458
SD	4.55	1.261	0.288	0.054	0.213

Table 2: exposures data from rats treated at low dose treatment regimen. Results obtained from black and white fur are reported separately. Ecgonine methylester is not reported as not measurable in most of the samples. Out = Outlier; BE = Benzoylecgonine; [(1)] = Assuming 300 g of rat body weight

As shown above, black fur demonstrated a higher affinity for cocaine, as expected. Some variability was present, maybe due to fur contamination with urine.

Exposures data from rats treated at higher doses - 112.6 mg/kg/day (mean value) – are reported below. In this case, white fur was only collected and a stronger washing protocol was adopted, to minimize the fur external contamination:

	Dose [1]	Exposure (white fur)		
Rat number	**mg/kg/day (mean)**	**Cocaine (ng/mg)**	**BE (ng/mg)**	**EME (ng/mg)**
M426	135.26	2.29	0.87	0.65
C426	161.05	2.93	1.22	1.26
I426	65.43	70.31	10.62	1.72
U426	82.32	2.82	1.32	0.60
O426	118.75	86.62	53.93	2.59
S426	114.60	96.04	21.92	2.65
R426	129.37	72.56	9.01	1.46
AA426	112.13	4.93	1.08	0.88
V426	105.92	1.93	1.27	0.82
W426	81.00	2.83	1.09	0.79
AC426	115.61	2.97	0.98	0.57
Y426	112.15	2.85	1.28	0.90
H426	100.15	28.35	4.63	2.07
B426	106.80	4.06	2.07	0.96
L426	136.76	1.99	0.91	0.64
D426	102.17	8.33	2.73	1.07
N426	112.27	9.56	3.09	0.98
K426	134.35	3.15	0.69	NM
mean	112.56	22.47	6.59	1.21
SD	22.66	33.35	12.96	0.67

Table 3: exposures data from rats treated at high dose treatment regimen. White fur only was considered. Out = Outlier; BE = Benzoylecgonine; EME = Ecgonine methylester; NM = not measured; [1] = Assuming 300 g of rat body weight

In Figure 1, the comparison between dose and exposure measured in rat fur and evaluated at the two different doses is reported.

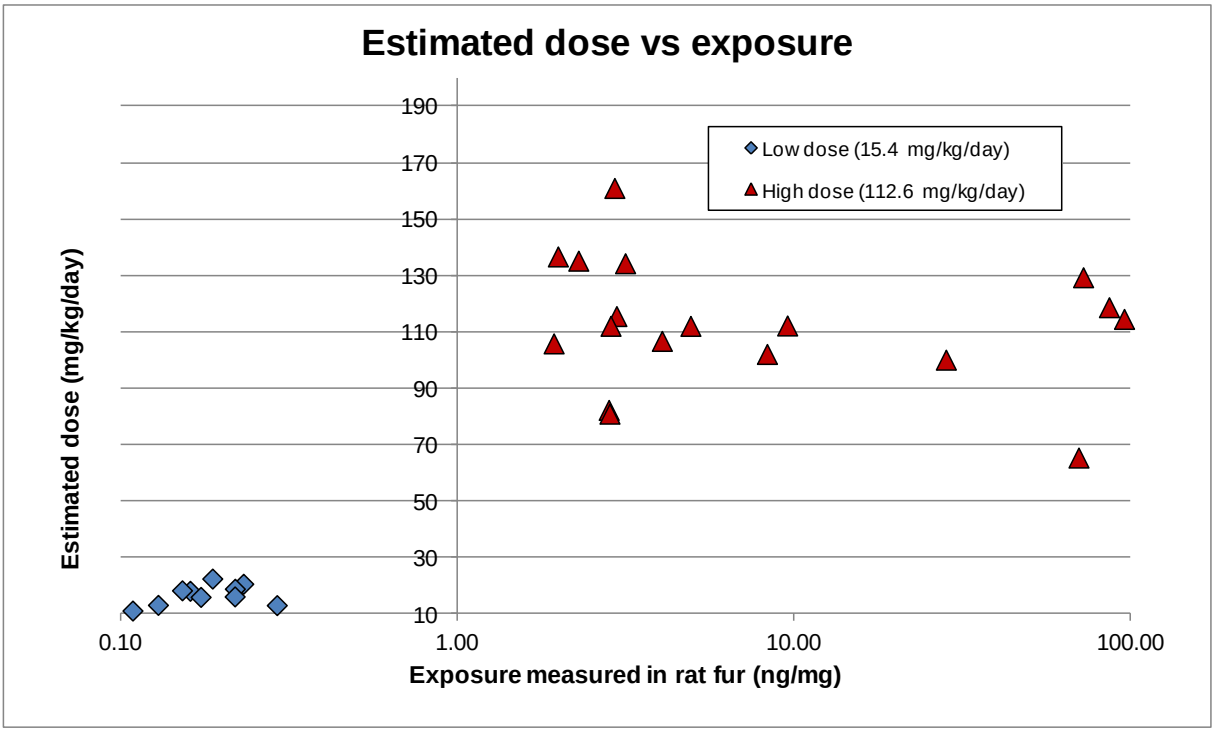


Figure 1: comparison between dose and exposure measured in rat fur at the dose regimen of 15,4 and 112,6 mg/kg/day.

Organ Weights and histopathology examinations

Heart and brain were collected where possible and for animals chronically treated. Tissues were processed in paraffin wax and stored for future evaluations. No macroscopic observations were done at necropsy. A summary of the measured heart weights is reported in Table 4.

Rat code	Treatment duration (self administration)	Treatment type	Body weight (g)	Heart weight (g)	Brain weight	Heart / body ratio	Heart / brain ratio	ANP (pg/mL)
M184	4 months	Cocaine - low dose	320	1.178	NM	0.368	-	9101.50
R184	4 months	Cocaine - low dose	305	1.081	NM	0.354	-	11853.38
A233	3 months	Cocaine - low dose	360	1.085	NM	0.301	-	10175.46
B233	3 months	Cocaine - low dose	320	1.095	NM	0.342	-	10748.12
C233	3 months	Cocaine - low dose	348	1.073	NM	0.308	-	6231.20
D233	3 months	Cocaine - low dose	335	1.260	NM	0.376	-	19779.27
A250	3 months	Cocaine - low dose	340	1.095	NM	0.322	-	NM
D250	3 months	Cocaine - low dose	360	1.050	NM	0.292	-	5046.99
F250	3 months	Cocaine - low dose	355	1.004	NM	0.283	-	4238.72
I184	4 months	Saline control	300	1.086		0.362		NM
V654	35 days	Cocaine - low dose	NM	0.957	NM	-	-	NM
W654	35 days	Cocaine - low dose	NM	0.913	NM	-	-	NM
K207	35 days	Cocaine - low dose	NM	0.937	NM	-	-	NM
P214	35 days	Cocaine - low dose	NM	0.966	NM	-	-	NM
A426	31 days	Saline control	318	0.930	1.66	0.292	56.02	6386.06
C426	2 weeks	Cocaine high dose	315	1.080	1.75	0.343	61.71	18800.68
G426	31 days	Saline control	327	0.930	1.76	0.284	52.84	7243.34
I426	2 weeks	Cocaine high dose	249	0.880	1.71	0.353	51.46	17559.12
L426	31 days	Cocaine high dose	312	0.940	1.66	0.301	56.63	6609.90
M426	31 days	Cocaine high dose	288	1.230	1.5	0.427	82.00	6151.69
O426	31 days	Cocaine high dose	326	0.960	1.7	0.294	56.47	6997.02
U426	2 weeks	Cocaine high dose	302	1.100	1.63	0.364	67.48	25211.15
V426	31 days	Cocaine high dose	307	0.920	1.77	0.300	51.98	7186.62
Mean values per dose:		**0 mg/kg/day**	**315.0**	**0.982**	**1.710**	**0.313**	**54.433**	**6814.70**
		15.4 mg/kg/day	**338.1**	**1.053**		**0.327**		**9646.83**
		112.6 mg/kg/day	**299.9**	**1.016**	**1.674**	**0.340**	**61.105**	**12645.17**

Table 4: summary of the heart weight data available. A comparison with treatment type, dose and ANP values is also included. A colour code was associated to the estimated dose: white for saline controls, light grey for low dose and dark grey for high dose.

Heart weight increases were seen in isolated animals, with no group trend. One animal treated at 112.6 mg/kg/day showed the highest increase in term of heart weight relative to body weight.

A correlation trend between the heart weight and ANP values was seen, even if not fully consistent for all animals considered (Figure 2).

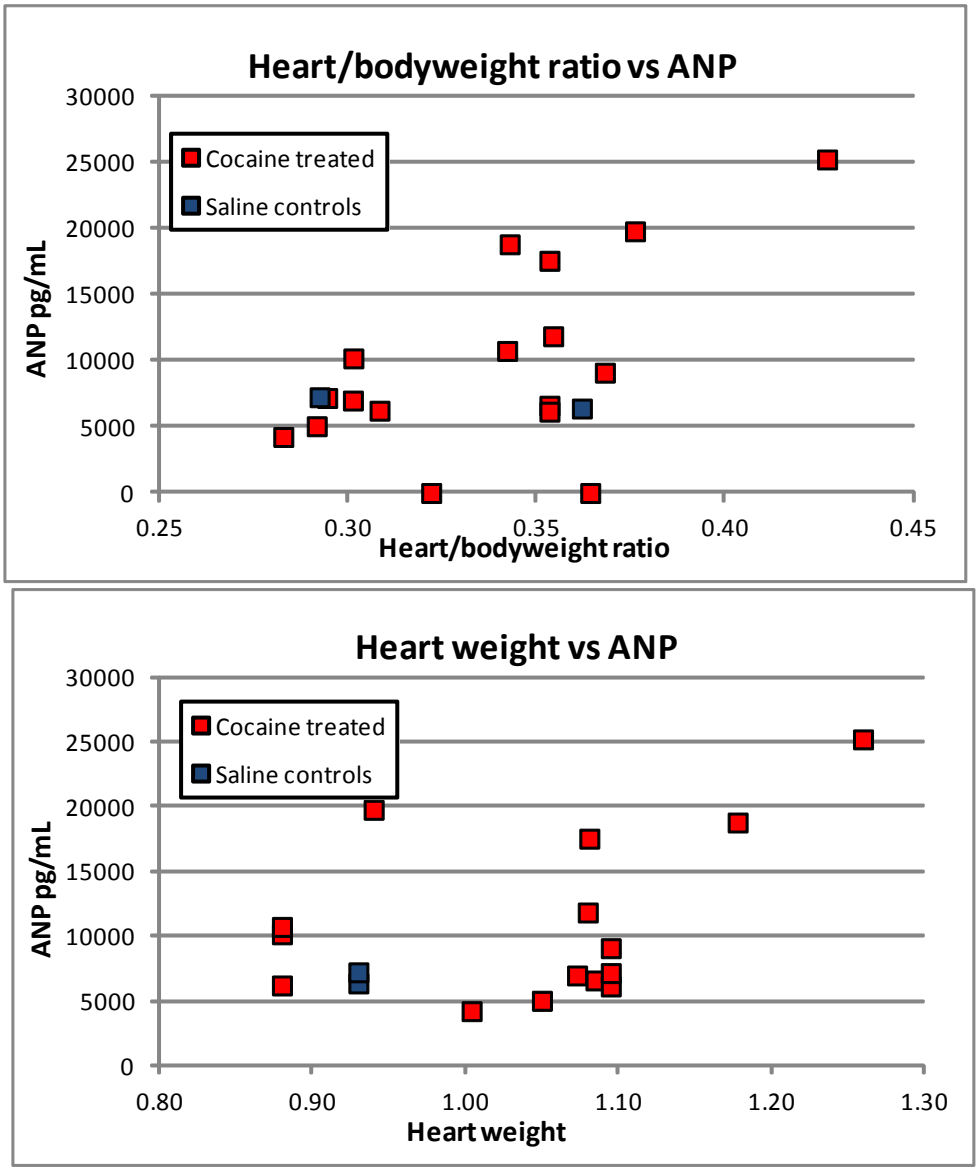
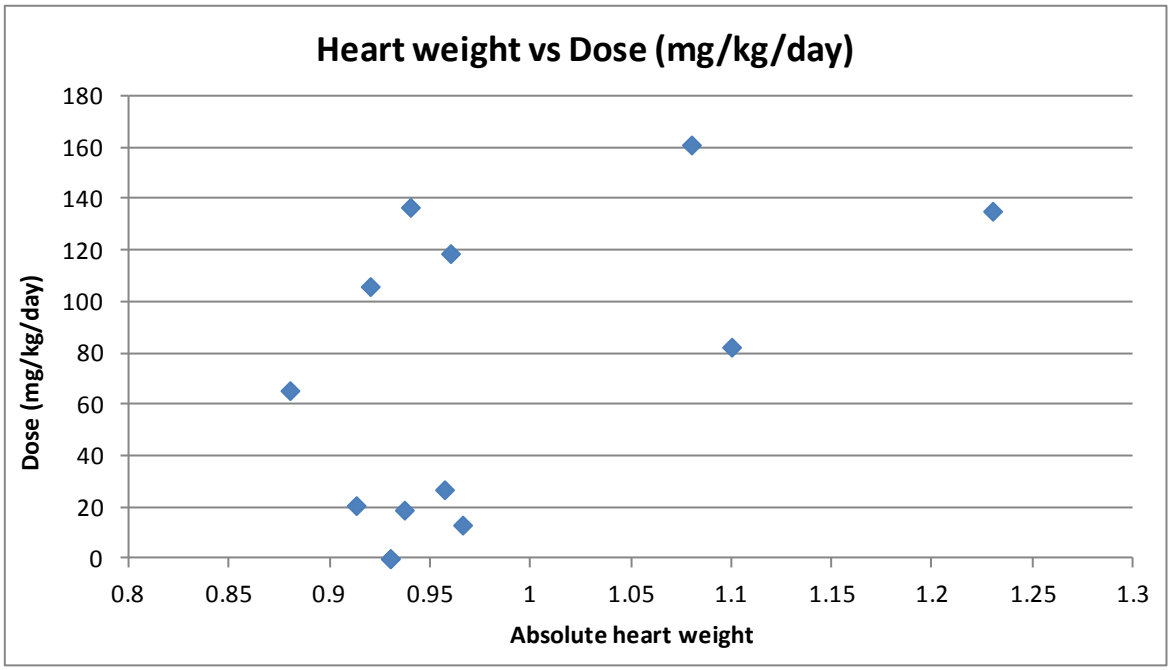


Figure 2: correlation between the heart weight (absolute or normalized by bodyweight) and ANP values.

A similar correlation can be done when the heart weight is compared to the estimated dose, as shown in Figure 3.



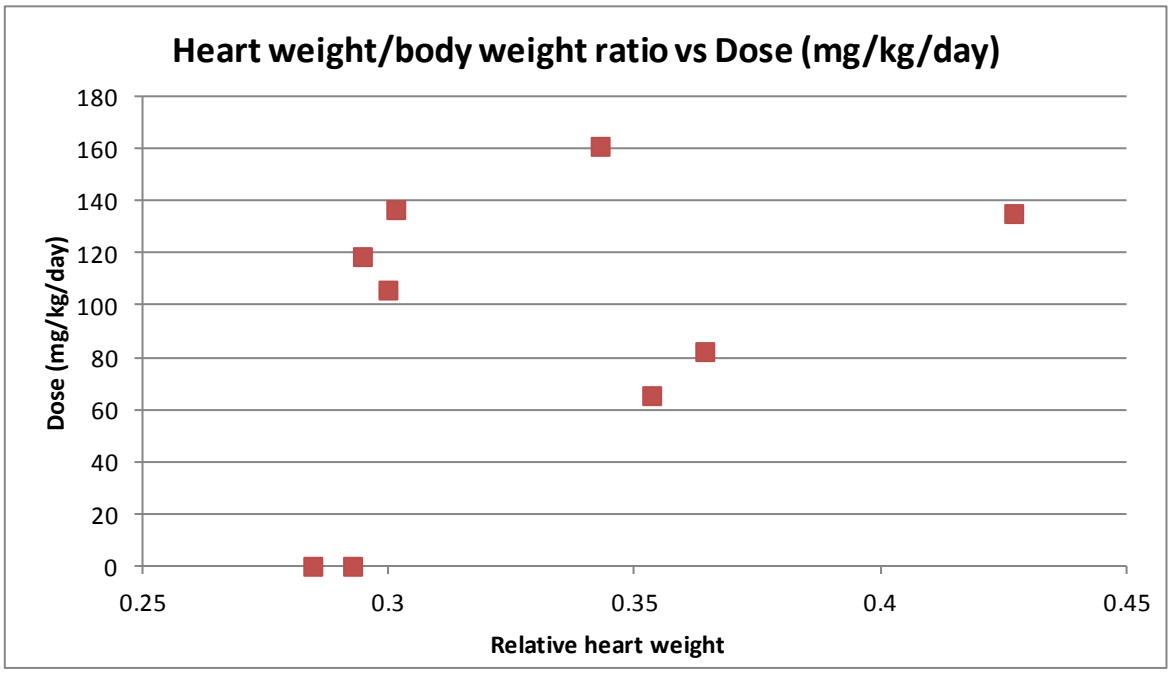


Figure 3: correlation between the Lister Hooded rat heart weight (absolute or normalized by bodyweight) and the estimated dose.

Microscopic examinations of heart from both low (15.4 mg/kg/day) and high (112.6 mg/kg/day) doses did not reveal any morphological alteration.

Biomarkers evaluation

In line with the lack of histopathological findings, no significant increases in Troponin I (cTnI) were found in all animals examined from both low and high doses.
Atrial Natriuretic Peptide (ANP) data are summarised in Figure 4.

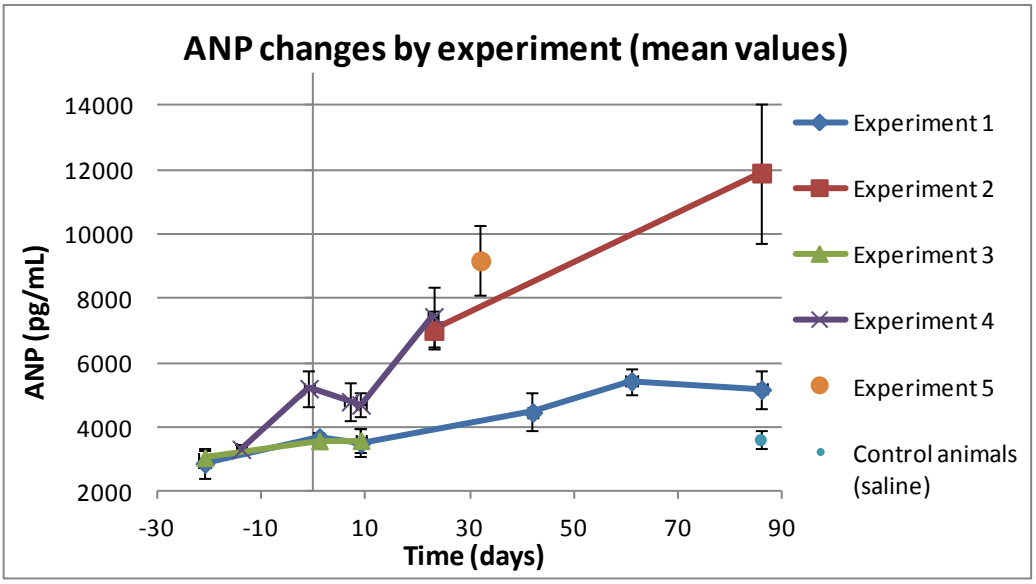


Figure 4: Atrial Natriuretic Peptide (ANP) values by time in chronic cocaine treated rats (mean values ± SEM). To minimise inter assay variability, data are presented by experiment. The first day of treatment is Day 1.

The chart above suggests a clear trend to increase for ANP, generally time dependent. The inter assay variability and the different timepoints evaluated amongst experiments limited the possibility to group together all data from different experiments, but considering every single experiment (as shown above) a constant increasing trend was demonstrated in all the experiments performed. The same results shown in Figure 4 are replicated in Figure 5, where ANP results obtained in the first and last timepoints were group together:

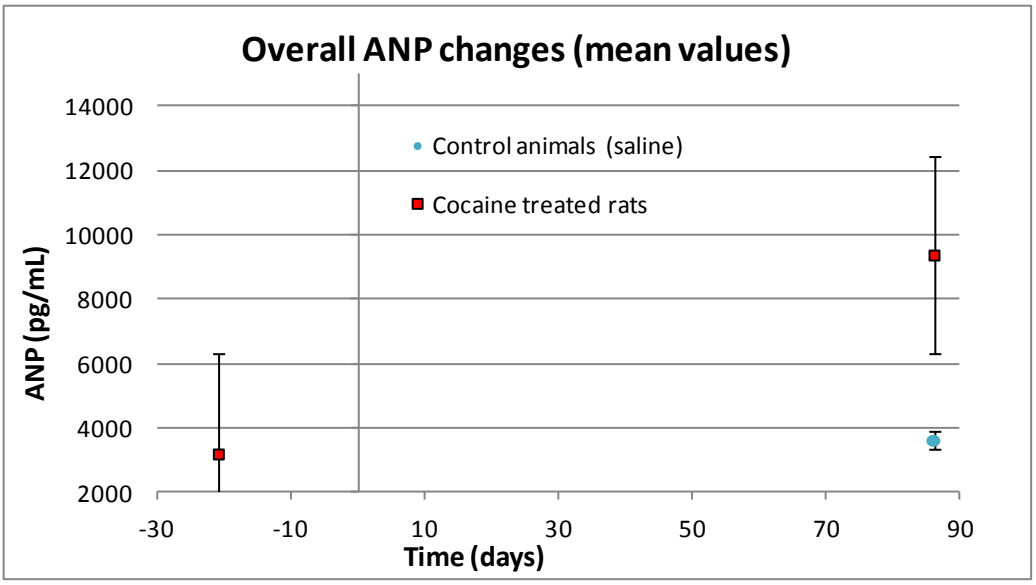


Figure 5: Overall Atrial Natriuretic Peptide (ANP) changes in chronic cocaine treated rats (mean values ± SEM). The first and the latter available timepoints were group together (mean value of data from different experiments). The first day of treatment is Day 1.

All experiments except n. 5 were performed at low (15.4 mg/kg/day mean value) dose; The experiment number 5 was the only performed at higher dose (112.6 mg/kg/day mean value), but a single timepoint (Day 31) for all animals is only available. ANP values seem to be slightly higher in high dosed animals, in comparison to low dosed animals at the same timepoint. However these differences seem to be minimal, as the intra experiment increase (calculated as times increase against control animals, data not shown) is comparable to the ones observed in the other experiments. In conclusion, ANP values seem to increase along time, but further evidences are needed to demonstrate its relationship with dose or exposure.

NT proANP was also measured in a restricted panel of samples, to further confirm the increasing trend observed with ANP. A good correlation between ANP and NT proANP data was demonstrated, with the latter showing slightly higher values (data not shown). The slightly higher values obtained with the NT proANP suggested a lower clearance than ANP, as expected by the longer half life. Similar results were also found in a study conducted on control and hypertensive SHR rats[25]. Furthermore, even if more variable, the NT proANP seems to better discriminate the different effects of cocaine at low and high doses.

Cocaine human abusers study

Cocaine intake

All the chronic abusers were exposed to cocaine and its metabolites. Only 3/10 showed a signal for the cocaethylene metabolite in the hair. The majority of the volunteers (6/10) refer to have assumed cocaine within 5 days the sampling procedures. Massive or regular assumption of cocaine was reported by 4 volunteers. Results are summarized in Table 6.

Biomarkers evaluation

As already observed in rats, no increases in cTnT values were observed in humans. All values except one were below the quantification limit, and the only measured value was within the normal range and very low.

NT proBNP showed values ranging from 5.32 pg/mL to 111.6 pg/mL. All these values were within the normal range defined on the basis of the laboratory's historical data, however some of them are close to the upper limit reference of 125 pg/mL (Table 5 and 6). All the data collected from human volunteers are referred to a single timepoint, and no evaluations across time were done, as performed in the rat study. In this context, data collected from human volunteers can be compared to the ones collected for rats and reported in Figure 5, where at the latest timepoint treated animals showed a higher variability and values approximately 2 fold than the corresponding values measured in the untreated animals. As in rats, the cocaine abusers showed variability in NT proBNP values, with minimal increases observed in few subjects.

Sample number (A=abuser, C=control)	cTnT (pg/mL)	NT ProBNP (pg/mL)
A	<LLoQ	5.32
A	3.76	24.00
A	<LLoQ	27.56
A	<LLoQ	33.83
A	<LLoQ	36.17
A	<LLoQ	67.17
A	<LLoQ	81.02
A	<LLoQ	81.73
A	<LLoQ	102.20
A	<LLoQ	111.60
C	<LLoQ	43.34

Table 5: summary of the cardiac troponin T (cTnT) and NT proBNP values measured in human chronic cocaine abusers. <LLoQ = below the low limit of quantification.

Furthermore, the comparison between NT proBNP increases and cocaine or benzoylecgonine levels measured in the hair seem to suggest a correlation, with the exception of a single outlier sample (Figure 6).

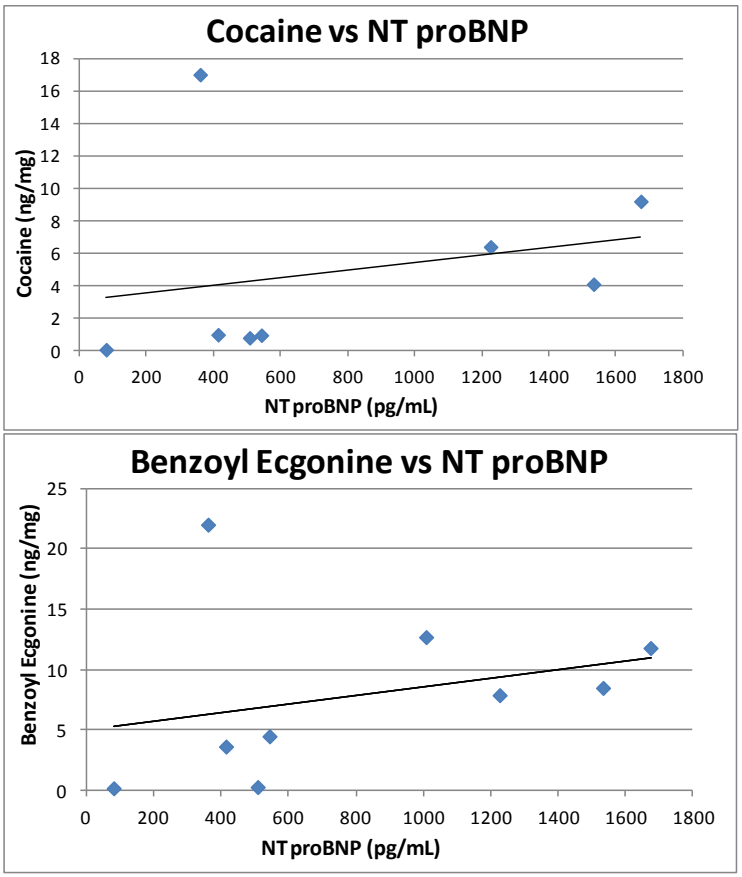


Figure 6: correlation between NT proBNP values and cocaine/benzoylecgonine levels measured in the cocaine abusers' hair.

One sample only (number 6, as reported in Table 6) seems to fall outside the expected trend; the regression coefficient r^2 ranges between 0.27 and 0.30 (for cocaine and benzoylecgonine respectively), but it increases up to 0.82 and 0.88 respectively, if the outlier sample number 6 is excluded.

Again, 3 out of 4 volunteers that refer to have massive or regular cocaine assumption had also the 3 highest values of NT proBNP recorded (Table 6), suggesting another possible correlation between natriuretic peptide increases and cocaine consumption.

Code	Age	Cardiac pathologies	Abused drugs	Frequency	Alcohol assumption	cTnT (pg/mL)	NT Pro-BNP (pg/mL)	Cocaine (ng/mg)	Benzoyl Ecgonine (ng/mg)	Cocaethylene (ng/mg)	Ecgonine methylester (ng/mg)
1	29	None	Cocaine	Occasional since 4/5 months	Y	<LLoQ	1007.6	8.70	12.70	NM	5.80
2	32	Endocarditis 3 years ago	Cocaine and THC	Regular/massive	Y	<LLoQ	1226.0	6.40	7.90	7.00	1.10
3	28	None	Cocaine and heroin	Massive	Y	<LLoQ	1674.0	9.20	11.80	NM	5.60
4	42	None	Cocaine	Regular	Y	<LLoQ	413.4	0.99	3.65	NM	0.67
5	25	None	Cocaine and heroin	Regular (heroin)/ occasional (cocaine)	Y	<LLoQ	542.6	0.96	4.50	NM	NM
6	37	None	Cocaine	Occasional	N	3.76	360.0	17.00	22.00	NM	5.30
7	41	None	Cocaine and heroin	Regular (cocaine)/ occasional	Y (always)	<LLoQ	1533.0	4.10	8.50	3.10	2.10
8	25	None	Cocaine and heroin	Massive (heroin)/ occasional (cocaine)	N	<LLoQ	1215.3	NM (Morphine, Codeine and Methadon)			
9	22	None	Cocaine and heroin	Occasional (both)	Y	<LLoQ	507.5	0.80	0.30	0.50	NM
10	27	None	Cocaine and heroin	Occasional (both)	Y	<LLoQ	79.8	0.07	0.20	NM	NM
11	43	None	None (control)	None	N	<LLoQ	650.1	NM	NM	NM	NM

Table 6: overall comparison of chronic cocaine abusers data. NM = Not measurable; LLoQ = below the low limit of quantification.

DISCUSSION

The main objective of this study was to provide an evaluation of the cocaine cardiac effects using a toxicologic approach and with non-invasive biomarkers. The aim was to start from an animal model to identify some tools that can be immediately translated to human beings for the real-life monitoring of cocaine's cardiac effects. In that context, the rat self administration model was chosen, as it best reproduces the human condition of the chronic abuser: rats were described to have comparable cardiovascular changes as the ones described for humans; furthermore, decreases in cardiac output and heart rate as well as increases in systemic vascular resistance were described in rats treated with cocaine at 5 mg/kg iv for several (3-13) times[15,19].

Several evidences are already available in literature describing the cardiovascular alterations induced by cocaine[1,2,3] in human abusers; starting from this basis, this work focuses its attention on some new features of commonly available biomarkers, to explore its possible application to cocaine abusers monitoring. For example, cTnI was also described to marginally increase in some physiologic conditions such as athletic performances[20,21]. Similar increases were also described to be prognostic of cardiac damage and necrosis in some preclinical toxicology works, showing minimal increases even before any histopathologic evidence of cardiomiocytes necrosis[10]; cTnI demonstrated to be a far more sensitive parameter in measuring cardiac necrosis than H&E histology, as described in several works on preclinical species[10,22,23]. On the other hand, the natriuretic peptides such as ANP provide a more functional set of parameters, more focused on cardiac myofiber stretch and, generally speaking, heart workload. The atrial natriuretic peptides are often used in the asymptomatic or symptomatic left ventricular dysfunction, and with a prognostic value in the diagnosis and follow-up of acute congestive heart failure. Furthermore, BNP and NT proBNP are commonly used as a sensitive diagnostic tool in the patients follow up, and its prognostic value was also explored in some preclinical toxicology studies, where it evidences changes in the cardiac functionalities before any functional or structural evidence of damage[10,24].

All these evidences suggested the exploration of cTnI and ANP in a well controlled animal model study design, for their possible toxicologic use in cocaine chronic abusers. Both the above parameters demonstrated to be a very sensitive tool in

toxicology studies[10,22,23,24], but no evidence is available in literature on their application as prognostic biomarkers or in monitoring the cardiac effects of healthy chronic cocaine abusers; for example, cocaine is also known to produce life-threatening cardiovascular complications in some but not all individuals, and hemodynamic effects of cocaine may vary in humans. These features seem to be confirmed in the rat model, as some authors were able to select some responding individuals from a panel of rats, on the basis of their alterations in cardiovascular parameters after treatment with cocaine[12,16].

In the present study, the cocaine cardiac effects were monitored in healthy rats by using the cTnI and atrial natriuretic peptides as biomarkers. The research was also completed with the histopathologic evaluations and the cocaine exposure assessment. Cocaine and benzoylecgonine (major metabolite) exposures evaluated in rat fur demonstrated that all rats were exposed to cocaine, and rat data were comparable to the ones normally found in humans. Furthermore, black fur showed higher cocaine/metabolite content than white fur, due to the differences in melanin content. Rats treated at higher doses (mean value = 112.6 mg/kg/day) had higher exposures in rat fur, and a good correlation between dose and exposure was demonstrated. The use of fur evaluation made exposure measurements possible in rats as in humans, and allows a direct comparison between the two species.

No significant troponin I and morphological alterations were observed, after chronic (~4 months) low dose (15.4 mg/kg/day mean value) and after 1 month high dose (112.6 mg/kg/day mean value) cocaine administration. This result reflects the absence of any morphological alterations at the histopathological examination of the rat heart.

Heart weight was affected in isolated animals only. A slight and not consistent correlation between heart weight and ANP was seen. Heart weights seem to be slightly correlated to dose, suggesting a trend towards cardiac hypertrophy caused by cocaine, however further data are required to confirm this assumption.

When functional parameters are considered, both ANP and NT proANP confirmed a time related increasing trend in all the studies and at all doses. NT proANP provided apparently higher values, and maybe also higher and long-lasting increases by means of its longer half life[25,26,27]. ANP changes are already reported in literature [11,12,18], and in time-course studies it can reflect changes in cardiovascular parameters

(cardiac output, heart rate and systemic vascular resistance) [15,19]. Furthermore, the findings observed in our study confirmed that the atrial natriuretic peptides can monitor the altered functional state of the heart in random samples, regardless the cocaine intake timings; this makes this biomarker suitable for the cardiac dysfunction assessment in chronic human abusers, as it is often impossible to know when the last administration occurred.

No correlation was seen between ANP increases and dose/exposure: ANP confirmed to be a sensitive marker, but the severity of its alteration cannot be related to the dose/exposure of drug in these experimental conditions. Many hypotheses can be done to explain this: first, the ANP clearance and short half-life can increase the variability, with a negative effect on the alteration/exposure correlation; second, the ANP increases may reflect a cardiac adaptive change that is not directly linked to dose/exposure, but rather depends on the frequency by which cocaine is taken, assuming an acute “peak” effect on heart.

The animal model adopted allowed a deep characterisation of the cardiac dysfunction in controlled experimental conditions as well as insights on the cocaine exposure in the time, providing the basis for the translation of these techniques to human abusers. On the basis of the results obtained, this approach was also successfully applied to human chronic cocaine abusers. NT proBNP was measured in human healthy cocaine abusers, and showed values within the normal range defined on the basis of the laboratory’s historical data. Comparable results were also found by Acquaro *et al* [28], where other determinations such as echocardiography, ECG and cardiovascular magnetic resonance confirmed a high prevalence of cardiac impairment/damage in asymptomatic cocaine addicts. However, the natriuretic peptides reference value ranges widely used in literature were normally designed for human diagnostics and it cannot be used for our purposes: in fact, the cutoff generally available in literature are set on the basis of the risk of heart failure and/or death evaluated in patient’s follow up (often in elderly patients), whereas the healthy young cocaine abusers considered in the present work have a much lower risk, and they should be better compared with the reference values for the healthy population of the same age range. Not many papers reporting the NT proBNP values in normal healthy and young subjects are available: according to McDonagh *et. al.* [29], the medians for the NT proBNP values are below the 20 pg/mL in 549 normal subjects

<40 years of age; similarly, Bernstein et al.[30] found 60.5 pg/mL as median in a mixed population <50 years of age that included normal healthy volunteers (blood donors) and patients seen in acute care for cardiac symptoms. According to these values, at least 5 of the 10 samples evaluated showed values higher than both the medians reported by McDonagh [29] and Bernstein [30]. Furthermore, the NT proBNP values observed in the human cocaine abusers seem to reproduce the same ANP trend observed in rats, where after cocaine chronic treatment animals showed a higher variability and 2 fold increases in comparison to the untreated animals.

Both in rats and humans, the changes observed in the natriuretic peptides measured are comparable to the ones observed in subjects considered at risk for hypertension. Increases in ANP and NT proANP in spontaneous hypertensive (SHR) rats were already described in literature [25,31]. Furthermore, correlations between NT proBNP and blood pressure increases have been found by several authors in humans [32,33], and hypertensive patients seem to have minimal increases (less than doubled) in NT proBNP if compared to normotensive patients [34], in agreement with the results observed in chronic cocaine abusers.

CONCLUSIONS

In this work, the natriuretic peptides demonstrated enough sensitivity in both the animal model and in humans to be used in monitoring the cocaine's cardiac effects in human healthy cocaine abusers (with no history of cardiac findings/symptoms). Even if the natriuretic peptides are known to increase in many physiologic conditions, they are also often described as diagnostic/prognostic markers in the follow up of a number of cardiac or cardiovascular diseases. The adoption of the natriuretic peptides demonstrated to be a promising biomarker in this context, however the use of this biomarker and its relevance in monitoring the cardiac/cardiovascular risk in healthy human cocaine abusers require further and wider follow up studies.

Furthermore, cocaine-alcohol co-administration is another interesting aspect that was not investigated in this study: the major metabolite cocaethilene is assumed to have the most powerful effect on cardiac function [35,36], and specific studies may be set up in both the animal model and the chronic abuser to further explore these aspects.

REFERENCES

1. Crack Whips the Heart: A Review of the Cardiovascular Toxicity of Cocaine. L Afonso, T Mohammad, and D Thatai. Am J Cardiol 2007; 100: 1040-1043.

2. Cardiovascular complications of cocaine use. Lange RA, Hillis LD. N Engl J Med 2001; 345: 351-358.

3. Cocaine-related deaths: An enigma still under investigation. E Bertol, C Trignano, MG Di Milia, M Di Padua, F Mari. Forensic Science International 176 (2008) 121–123.

4. When is cocaine the cause of death? SB Karch and BS Stephens. Am J Forensic Med Pathol 1991; 12(1): 1-2.

5. Value of hair in postmortem toxicology. P Kintz. Forensic Science International 2004; 142: 127-134.

6. Postmortem toxicology of drugs of abuse. OH Drummer. Forensic Science International 2004; 142: 101-113.

7. Widmaier, Eric P.; Hershel Raff, Kevin T. Strang (2008). Vander's Human Physiology, 11th Ed. McGraw-Hill. pp. 291, 509–10.

8. Natriuretic peptides: their structures, receptors, physiologic functions and therapeutic applications. Potter LR, Yoder AR, Flora DR, Antos LK, Dickey DM. Handb Exp Pharmacol 191 (191): 341–66 (2009).. doi:10.1007/978-3-540-68964-5_15. PMID 19089336.

9. De Bold A. Atrial natriuretic factor: a hormone produced by the heart. Science 230 (4727): 767–770 (1985).

10. A novel and Integrated Approach for the Identification and Characterisation of Drug Induced Cardiac Toxicity in the Dog. A Casartelli, A Lanzoni, R Comelli, F Crivellente, R Defazio, R Dorigatti, N Fasdelli, I Faustinelli, S Pagliarusco, M Tontodonati, P Cristofori. Toxicol Pathol 39: 361-371 (2011).

11. Cocaine increases circulating levels of atrial natriuretic peptide and pro atrial natriuretic peptide N-terminal fragment in conscious rats. M Pelkonen, M Luodonpaa, O Vuolteenaho, M Pasanen, H Ruskoaho. European Journal of Pharmacology, vol. 304, 55-62 (1996).

12. Molecular characteristics of cocaine-induced cardiomyopathy in rats. S Besse, P Assayag, C Latour, C Janmot, V Robert, C Delcayre, G Nahas, B Swynghedauw. European Journal of Pharmacology, vol. 338, 123-129 (1997).

13. Cocaine-induced myocardial ultrastructural alterations and cardiac output responses in rats. M Knuepfer, CA Branch, Q Gan, VW Fisher. Experimental and molecular pathology 59 155-168 (1993).

14. Role of cholinergic receptors and cholinesterase activity in hemodynamic responses to cocaine in conscious rats. M Knuepfer and Q. Gan. Am. J. Physiol. 276 (1999): R103-R112.

15. Review of evidence for a novel model of cocaine induced cardiovascular toxicity. Mark Knuepfer and PJ Mueller. Pharmacology Biochemistry and Behaviour, vol. 63 (3) 489-500 (1999).

16. Strain and schedule-dependent differences in the acquisition, maintenance and extinction of intravenous cannabinoid self-administration in rats. S Deiana L Fattore, MS Spano, G Cossu, E Porcu, P Fadda, W Fratta. Neuropharmacology 52 (2007) 646-654.

17. The Influence of Exercise Upon Cardiac Biomarkers: A Practical Guide for Clinicians and Scientists. R Shave, K George and D Gaze. Current Medicinal Chemistry, 2007, 14, 1427-1436.

18. Norcocaine is a potent modulator of haemodynamic responses, plasma catecholamines and cardiac hormone release in conscious rats. J Mahalakaarto, H Ruskoaho, P Huttunen, E MacDonald, M Pasanen. Toxicology, vol. 128, 101-111 (1998).

19. Stress and cocaine elicit similar cardiac output responses in individual rats. M Knuepfer, CA Branch, PJ Mueller, Q Gan. Am. J. Physiol. 265 (34) H779-H782 (1993)

20. Cardiac Troponin T and I, Electrocardiographic Wall Motion Analyses, and Ejection Fractions in Athletes Participating in the Hawaii Ironman Triathlon. N Rifai, PS Douglas, M O'Toole, E Rimm, and GS Ginsburg. Am J Cardiol; 83: 1085-1089 (1999).

21. Cardiac Troponin Increases Among Runners in the Boston Marathon. EB Fortescue, AY Shin, DS Greenes, RC Mannix, S Agarwal, BJ Feldman, MI Shah, N Rifai, MJ Landzberg, JW Newburger, CSD Almond. Ann Emerg Med. 49: 137-143 (2007).

22. Cardiac troponin I is a sensitive, specific biomarker of cardiac injury in laboratory animals. P J O'Brien, DEC Smith, TJ Knechtel, MA Marchak, I Pruimboom-Brees, DJ Brees, DP Spratt, FJ Archer, P Butler, AN Potter, JP Provost, J Richard, PA Snyder and WJ Reagan. Laboratory Animals 40: 153-171 (2006)..

23. Characterization of Troponin Responses in Isoproterenol-Induced Cardiac Injury in the HanoverWistar Rat. M York, C Scudamore, S Brady, C Chen, S Wilson, M Curtis, G Evans, W Griffiths, M Whayman, T Williams, and J Turton. Toxicologic Pathology, 35: 606-617 (2007).

24. Cardiac troponin is the most effective translational safety biomarker for myocardial injury in cardiotoxicity. PJ O'Brien. Toxicology. Mar 20; 245(3): 206-18 (2008).

25. Atrial natriuretic peptides in Han Wistar, Sprague–Dawley and spontaneously Hypertensive rats. F Crivellente, N Bocchini, M Bonato, L Vandin, I Faustinelli, P Cristofori. Journal of Applied Toxicology, in press.

26. NH2-terminal fragment of rat pro-atrial natriuretic factor in the circulation: identification, radioimmunoassay and half-life. G Thibault, KK Murthy, J Gutkowska, NG Seidah, C Lazure, M Chretien and M Cantin. Peptides 9(1): 47–53 (1988).

27. Biochemistry of natriuretic peptides. TG Yandle. J Intern Med 235(6): 561–576 (1994).

28. Silent myocardial damage in cocaine addicts. GD Acquaro, A Gabutti, M Meini, C Prontera, E Pasanisi, C Passino, M Emdin, M Lombardi. Heart, in press.

29. NT proBNP and the diagnosis of heart failure: a pooled analysis of three European epidemiological studies. TA McDonagh, S Holmer, I Raymond, A Luchner, P Hildebrant, HJ Dargie. The European Journal of Heart Failure 6 (2004) 269–273.

30. What is the best approximation of reference normal for NT proBNP? Clinical levels for enhanced assessment of NT proBNP (CLEAN). LH Bernstein, MY Zions, ME Alam, SA Haq, JF Heitner, S Zarich, B Seamonds and S Berger. Journal of Medical Laboratory and Diagnosis Vol. 2(2), pp. 16-21, March 2011.

31. An Initial Characterization of N-Terminal-Proatrial Natriuretic Peptide in Serum of Sprague Dawley Rats. HM Colton, AH Stokes, LW Yoon, MP Quaile, PJ Novak, JG Falls, CL Kimbrough, NF Cariello, HL Jordan and BR Berridge. Toxicol. Sci. (2011) 120 (2): 262-268.

32. Plasma NT proBNP concentration is related to ambulatory pulse pressure in peripheral arterial disease. P Svensson, U de Faire, U Niklasson, Lars-Olof Hansson, and J Östergren. Blood Pressure, 2005, Vol. 14, No. 2 : Pages 99-106.

33. Plasma NT proBNP concentrations are associated with ambulatory blood pressure in black hypertensive patients with normal systolic function on echocardiography. EN Libhaber, H Abbasi, CA Toyin, GR Norton, A Woodiwiss, CD Libhaber, MR Essop and K Sliwa. American Journal of Hypertension 18, 37A (May 2005).

34. NT proBNP Levels and Hypertension. Their Importance in the Diagnosis of Heart Failure. M Rivera, R Taléns-Visconti, A Salvador, V Bertomeu, V Miró, F García de Burgos, V Climent, R Cortés, R Payá, JL Pérez-Boscá, L Mainar, A Jordán, F Sogorb, J Cosín, V Mora, JL Diago, and F Marín. Rev Esp Cardiol 2004;57(5):396-402.

35. Cocaine-Induced Cardiovascular Responses in Rats During Acute Ethanol Withdrawal. RJ Briscoe, TJ Baird, MR Lerner, D Brackett and DV Gauvin. Alcohol, Vol. 19, No. 2, pp. 131–137 (1999).

36. Pharmacodynamic Evaluation of the Cardiovascular Effects after the Coadministration of Cocaine and Ethanol. SC Laizure and RB Parker. Drug Metabolism And Disposition, 37: 310-314 (2009).

APPENDIX 1

GC-MS METHOD

Column: Ultra-2 (5% phenyl, methylsilicone) 12 mX0.2mmX0.33mm.

Injection: 1μL splitless.

Temperature program: 1'@100°C; 30°C/min to 200°C; 15°C/min to 290°C; 5'@290°C.

T interface: 280°C

T source (EI): 250°C

He flow: 1ml/min

SIM mode acquisition: 3 fragments each test substance, single ion quantification (*):

Compound	**ions (m/z)**
Cocaine	82; 182*; 303
BE-TMS (metabolite)	82*; 240; 346
Cocaethylene (metabolite)	82*; 196; 272
EME-TMS (metabolite)	82*; 96; 240; 271

BE-TMS: Benzoylecgonine; EME-TMS: Ecgonine methylester

Results acceptance and validation criteria:

- Internal standards should be present in all samples;
- No analyte should be detected in the negative control;
- All analytes should be measurable in the positive control

Identification parameters: any analyte detected in the sample should have:

- The relative retention time should correspond to the calibrator's one, with an acceptable variability below the 0,5%.
- Areas ratio of the detected fragments should correspond to the ones calculated in the calibrator, with an acceptable variability below the 20%.

Substance quantification limits:

An analyte concentration >0.1 ng/mg in the sample is considered positive and measurable; concentrations between 0.1 and 0.04 ng/mL are reported as "traces".