Different repair kinetic of DSBs induced by mitomycin C in peripheral lymphocytes of obese and normal weight adolescents

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Abstract

In 2013, 42 million children under the age of 5 years were overweight or obese. In the context of obesity, we recently showed that 1) peripheral lymphocytes of obese children/adolescents had an 8-fold increase in double strand breaks (DSBs), expressed as γ-H2AX foci, than normal weight adolescents, and 2) 30% of the damage was retained into chromosome mutations. Thus, we investigated DSBs repair efficiency in a group of obese adolescents assessing the kinetic of H2AX phosphorylation in mitomycin C (MMC)-treated lymphocytes harvested 2 h- or 4 h-post mutagen treatment. According to our previous studies, these harvesting times represent the peak of DSBs induction and the time in which an appreciable DSBs reduction was observed. In addition, we evaluated the expression of the high mobility group box-1 protein (HMGB1), a chromatin remodelling protein involved in DSBs repair and obesity. Compared to normal weight adolescents,

obese subjects 1) showed higher levels of γ-H2AX foci at either 2 h- (0.239 ± 0.041 *vs*. 0.473 ± 0.048, P = 0.0016) or 4 h- (0.150 ± 0.026 *vs*. 0.255 ± 0.030, P = 0.0198) post mutagen treatment, and 2) have repaired a greater amount of the initial lesions (0.088 ± 0.033 *vs*. 0.218 ± 0.045, P = 0.0408). Concordantly, 1) HMGB1 levels of obese individuals increased and decreased at 2h- or 4 h-post mutagen treatment, respectively, and 2) the opposite occurred for the normal weight adolescents where the protein was down-expressed at 2 h and over-expressed at 4 h. In conclusion, lymphocytes of obese and normal weight adolescents showed a distinct temporal kinetic of repairing MMC-induced DSBs, together with a different expression of HMGB1. The finding that obesity may modulate the repair of DNA damage induced in lymphocytes by genotoxic agents should be confirmed by further experiments.

Key words: γ-H2AX; DSBs; peripheral lymphocytes; obesity; HMGB1.

1. **INTRODUCTION**

The worldwide prevalence of obesity is more than doubled between 1980 and 2014. In 2013, 42 million children under the age of 5 were overweight or obese [1]. Obesity is characterized by chronic low-grade inflammation, a series of metabolic changes at the level of the adipose tissue that consist in decreased or increased production of anti-inflammatory molecules or pro-inflammatory cytokines, respectively, hypoxia and oxidative stress [2-6]. The adipose tissue is in fact considered a relevant source of free radicals and ROS, since the redox regulation plays a pivotal role in the adipogenesis [7, 8]. The increase of free radicals in the accumulated fat can induce, not only in the target tissue but also in other districts, DNA lesions such as the double-strand breaks (DSBs) that in turn can initiate and promote carcinogenesis. These lesions can be easily detected in a cell population as nuclear γ-H2AX foci shortly after their induction (within a few minutes) [9], and the

ad hoc developed assay has now become the most widely used method for investigating the presence of early genome damage in the form of DSBs [10-14]. Following a DSBs, modifications to chromatin conformation are necessary; specifically, chromatin should be relaxed to allow and facilitate the access to the repair machinery in the region of the lesion [15, 16]. The dephosphorylation of γ-H2AX indicates the end of the DDR response, *i.e.* that the DSBs have been correctly repaired. Thus, evaluating the kinetic of appearance and disappearance of γ-H2AX foci allows us to investigate the efficiency of the DSBs repair in a given cell population. In addition, the multifunctional high mobility group box-1 protein (HMGB1) was reported to have a key role in DSBs repair participating in the chromatin remodelling at the sites of induced DNA damage [17]. Recent studies have also shown that in response to inflammatory signals, the adipose tissue, in particular adipose-derived stromal cells, of obese adults, was able to produce levels of HMGB1 2-fold higher than those of normal weight subjects [18], and that children had an increase in plasma levels of this protein [19]. In addition, HMGB1 has been reported to be involved in contributing and maintaining a low-grade inflammation in human preadipocytes [20].

With regard to obesity, we have recently reported that 1) peripheral lymphocytes of obese children/adolescents had an eight-fold increase in DSBs as compared to a normal weight group, and 2) a part (about 30%) of this initial genome damage was retained into chromosome mutations [21]. In another work by us we set up the proper experimental conditions to study the time-course of H2AX phosphorylation in human lymphocytes treated *in vitro* with physical or chemical mutagens [22]. Thus, in the light of the above mentioned study carried out on the close association between obesity and DNA damage, the aim of the present work was to investigate whether or not obese adolescents have a different efficiency in repairing the DSBs lesions than normal weight adolescents. To do this, using the γ-H2AX assay, we assessed the kinetic of repair of DSBs induced by mitomycin C (MMC) in peripheral lymphocytes after two and four hours of mutagen treatment.

In addition, we aimed to assess, by western blotting, the levels of the HMGB1 protein and its variation under the same experimental conditions.

2. MATERIALS AND METHODS

2.1. Study populations

The study populations consisted of 38 adolescents (15 normal weight and 23 obese) recruited at the Unit of Pediatric Endocrinology and Diabetes, Department of Clinical and Experimental Medicine, Azienda Ospedaliero-Universitaria Pisana (Pisa, Italy). Only subjects who had not received radiation exposure or drug therapy, free from any pathological conditions, non-smokers and nonalcohol drinkers, were included in the study. Obese adolescents were recruited for the collection of blood during their specialist visit. Controls were selected from subjects who needed to have a blood test examination, *e.g.* in the case of familiar short stature or of planned minor surgery, or who had voluntarily accepted to take part in the study. Adolescents were defined as normal weight or obese on the basis of their body mass index (BMI) Z score, using the International Obesity Task Force BMI cutoffs as a reference [23]. BMI Z scores were then calculated using the least mean square method, reference values of BMI, and the least mean square coefficients [24]. Informed consent was obtained from the parents, or from their legal representatives, before adolescents took part in the study. The protocol was approved by the Azienda Ospedaliero-Universitaria Pisana ethical committee. Table 1 shows the demographic and clinical characteristics of the study population.

2.2. Cell cultures, mitomycin C treatment and cell harvesting

6 ml of blood in lithium heparin tubes were collected by venipuncture from all subjects and cultured on the same day. Culture tube containing 150 μl of whole blood and 2.35 ml of RPMI-1640 medium (Invitrogen, Milano, Italy) was supplemented with 20% FBS (Invitrogen), 1% antibiotic/antimycotic (Invitrogen) and 1.5% phytohaemagglutinin (PHA; Invitrogen). The cells

were incubated at 37°C for 20 h. We treated the stimulated T-lymphocytes *in vitro* with a single dose of a chemical mutagen, the anticancer drug mitomycin C (MMC; Sigma-Aldrich, Milano, Italy), (final concentration: 0.3 μg/mL). The chemical mutagen was dissolved in sterile H_2O and the corresponding negative control cultures received only the solvent used. Treated or untreated cells were then transferred to culture tubes at room temperature and harvested after 2 and 4 h. Cell harvesting was carried out according to the procedure described elsewhere [22]. Briefly, after hypotonic treatment with 0.075 M KCl, lymphocytes were pre-fixed in acetic acid:methanol 5:3, fixed in 100% methanol, washed twice in methanol:acetic acid (3:1) and dropped onto a clean glass slide. The air-dried slides were immediately processed by the immunofluorescence protocol for visualisation of γ-H2AX nuclear foci. For each subject we set up a series of at least two independent cultures per experimental point.

2.3. Immunofluorescence for γ-H2AX analysis

For the detection of γ-H2AX we used a phospho-histone H2AX (Ser-139) polyclonal primary antibody (Cell Signaling, Euroclone, Milan, Italy) and the DyLight 488-conjugated anti-rabbit secondary antibody (Pierce, Euroclone, Milan, Italy). Cells were washed twice in 1X phosphatebuffered
saline (PBS) blocked in 1X PBS/10% FBS/0.3% Triton X-100 (Sigma Aldrich, Milan, Italy) as permeabilization or blocking solution (PS), cells were blocked in PS for 30 min at room temperature and incubated overnight at 4°C with primary antibody diluted 1:50 in PS. The following day, cells were washed 3 times in 1X PBS, incubated at room temperature for 2 h with secondary antibody diluted 1:200 in PS, and, after washing three times in 1X PBS, slides were counterstained with 0.4 μg/ml of 4’,6’-diamidino-2-phenylindole (DAPI) in antifade solution (Fluoroguard, LiStarFish, Milano, Italy). Slides were then analyzed on a Nikon-Optiphot 2 fluorescent microscope properly equipped with filters for DAPI and FITC visualization using a

x100 lens (x1000 final view). The presence of γ-H2AX focus was detected as a green fluorescence spot in the blue counterstained nucleus. For each experimental point, at least 400 cells per culture were scored and the DNA damage induced by MMC is expressed as average number of γ-H2AX foci per nucleus (γ-H2AXF/N) obtained from two independent replicates. To quantify the kinetic of repair, we measured in both obese and normal weight adolescents DNA repair. The number of γ-H2AX foci per nucleus in MMC-treated and untreated cultures at 2 h or 4 h gave the actual level of induced or unrepaired DNA damage, respectively. The difference between these parameters allowed estimating the level of damage which has been repaired from 2 to 4 h (repaired DNA damage).

2.4. Immunoblotting analysis

An aliquot of heparinised whole blood from each adolescent was employed to separate white cells using the following protocol. 3 ml of whole blood and 3 ml of sterile 1X PBS (1: 1 dilution) were mixed in a sterile tube. Then, 3 ml of the mix were gently added to another tube containing 3 ml of Histopaque-1077 (Sigma-Aldrich, Milan, Italy). The mononuclear white cell ring, obtained by a centrifugation at 2100 rpm for 30 minutes, was carefully collected, washed twice in sterile PBS and resuspended in the appropriate amount of PBS to obtain a cell density of approximately 6.5×10^6 cell/ml . 200 μl of the cell suspension were inoculated in each culture tube and then incubated, treated and harvested following the protocol described above. The obtained protein pellet was resuspended in lysis buffer containing protease and phosphatase inhibitors and then total proteins were measured with the Pierce BCA protein assay kit (Thermo Fisher Scientific Inc., Milan, Italy). 20 μg of proteins from each sample were solubilized in Laemmli buffer 1x (BioRad Laboratories, Milan, Italy), boiled for 5 min and sonicated. Protein electrophoresis was conducted on acrylamide/bisacrylamide gel (12% separating gel, 5% stacking gel) in cell Mini-Protean (BioRad Laboratories, Milan, Italy) for about 1 h with constant voltage (120 V). After electrophoresis, the

proteins were transferred to nitrocellulose membrane (BioRad Laboratories, Milan, Italy) for 60 minutes at 200 mA. As primary antibodies we used anti-HMGB1 antibody (Santa Cruz Biotechnology, CA, USA) and anti-GADPH antibody (Santa Cruz Biotechnology, CA, USA); as secondary antibody an anti-mouse IgG (H+L) peroxidase labeled (Santa Cruz Biotechnology, CA, USA) was used for both primary antibodies. Detection was obtained using Clarity Western ECL Substrate (BioRad, Laboratories, Milan, Italy). Acquisition and image analysis were carried out with a Chemi-Doc apparatus (BioRad Laboratories, Milan, Italy); molecular weight of the bands was identified referring to Thermo Scientific Spectra Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific Inc., Milan, Italy). Quantification of protein content was accomplished using ImageJ® software (version 1.46, download at http://imagej.nih.gov/ij/). Values were normalized using the untreated cell and GADPH protein as loading control. Data were calculated as mean ± SEM and reported in arbitrary units.

2.5. Statistical analysis

Statistical analyses were performed with the StatGraphics Plus 5.1 software package (Statistical Graphics Corp., Rockville, MD, USA). To describe and quantify the kinetic of MMC-induced DSBs repair, the box and whisker plots were used that allow to analyze the overall data distribution within each subject class (50% of the data in the box and the remaining 50% distributed between the two whiskers, if no outlier values are present). Median (continuous line within the box) and mean (plus sign) are also displayed. The differences in the average levels of γ-H2AX or of HMGB1 protein between obese and normal weight adolescents were evaluated, at each treatment time, using the ANOVA and T-test, respectively.

3. RESULTS

3.1. Kinetic of repair of MMC-induced DSBs

Following the results obtained in a previous study [22], the time-course of H2AX phosphorylation was investigated 2 h and 4 h post-mutagen treatment, the first time corresponding to the peak of damage, and the second one to the time in which an appreciable reduction of γ-H2AX foci was known to occur. Collectively, Figures 1-4 show the levels of DSBs, expressed as γ-H2AX foci, in normal weight and obese adolescents after MMC treatment. The reported data were substantially homogeneous (no data point was present out the expected distribution). Figures 1 and 2 display MMC-induced and basal levels of the two groups of adolescents at 2 h and 4 h, respectively. In both normal weight and obese subjects, 2 h MMC-treatment produced γ-H2AXF/N average values significantly higher than untreated cultures (normal weight: 0.292±0.045 vs. 0.053±0.006, $P<<0.001$; obese: 0.581±0.053 vs. 0.108±0.012, $P<<0.001$). After 4 h MMC-treatment, the γ-H2AX means begun to reduce although they remain significantly higher than the spontaneous frequencies (normal weight: 0.197 ± 0.028 *vs*. 0.047 ±0.006, $P<<0.001$; obese: 0.405±0.041 *vs*. 0.150±0.022, $P<<0.001$). Figure 3 visualizes the levels of the induced and unrepaired DNA damage parameters described above. The actual induced DNA damage was clearly influenced by the condition of obesity, as the obese group showed, independently of the respective baseline frequency, γ-H2AX values significantly higher ($P = 0.0016$) than normal weight adolescents (0.473 ± 0.048 vs. 0.239 ± 0.041). Also the average levels of DNA damage that peripheral lymphocytes have not yet repaired, resulted, once again, statistically different ($P = 0.0198$) between the obese and the normal weight (0.255 ± 0.030 vs. 0.150 ± 0.026) group. Figure 4 shows the amount of DNA damage repaired (difference between induced and unrepaired DNA damage) by normal weight and obese adolescents, expressed as the levels of phosphorylated histone H2AX (γ-H2AXF/N). Notably, the obese adolescents had repaired a greater amount (about 2.5-fold) of the initial MMC-induced DSBs than controls. In fact, the corresponding values of the repaired DNA damage parameters were significantly different between the two groups (normal weight: 0.088 ± 0.033; obese: 0.218 ± 0.045,

P = 0.0408) (Figure 4A). To further confirm that the observed results are independent of the fact that the obese population started from a higher level of MMC-induced DSBs than the normal weight group, we expressed these data in percentages. Once again, the value of DNA damage repaired by the obese subjects (49.5%) was significantly different (P = 0.045) as compared to that of controls (32.1%) (Figure 4B).

3.2. *Western blot of HMGB1 protein*

As shown in Figure 5, the protein levels differed between normal weight and obese adolescents as a function of the post-treatment harvesting time. We observed, in fact, that 1) in obese individuals the levels of HMGB1 are higher at 2 h than those at 4 h (P=0.0011), 2) on the contrary, in the normal weight group the protein was unregulated at the later time point (P=0.0226). The levels of HMGB1 protein, expressed as relative density normalized to untreated cultures and internal control (GADPH), were significantly different, within both groups, at 2 h and 4 h (obese: 1.72 ± 0.005 vs. 1.12 ± 0.15, normal weight: 1.24 ± 0.05 vs. 1.63 ± 0.08). In addition, when HMGB1 expression was evaluated at 2 h post-treatment, the obese group showed on average significantly increased levels of HMGB1 than the normal weight adolescents (P=0.0379). At variance, 4 h post-MMC treatment, normal weight adolescents expressed the protein at a higher extent than obese subjects (P=0.0092). These results could suggest a different time of HMGB1 protein recruitment between the two groups.

4. DISCUSSION

The use of the phosphorylated variant of histone H2AX allows indirect measurement of damage to DNA, since, immediately after the appearance of DSBs, phosphorylation at Ser-139 of H2AX molecules occurs that subsequently drives the mobilization of different proteins at the site of the lesion. In addition, even low percentages of damage can be detected as there is a 1:1 ratio between

the number of foci and DSBs. The response to DSBs is a complex signalling cascade, in which many components of the DDR are involved and co-localize with γ-H2AX foci to amplify the damage signal and facilitate its repair [25]. In this context, in order to investigate the kinetic of DSB repair between obese and normal weight adolescents, we found it interesting to focus on the HMGB1 protein, as it is involved both in chromatin remodelling associated to DNA repair. To induce the damage we used MMC, a potent antitumor antibiotic acting as a bifunctional alkylating agent that is able to generate DSBs via intrastrand and interstrand DNA crosslinks [26,27]. The experimental conditions of the present work can be considered appropriate, because we chose a MMC dose which is able to induce DSB and, consequently, to initiate and prosecute the DDR response without causing extensive damage. In fact, treatment at MMC doses much higher than those used here by us produced a great amount of DSB that could not be efficiently repaired leading to activation of the apoptotic pathway in the cells [28].

We observed about an 1.8-fold increase in the levels of MMC-induced DNA damage in obese patients as compared to the control group. Because the parameters used by us take into account the spontaneous level of DSBs of each individual, it is reasonable to affirm that the lymphocytes of obese adolescents show an increased sensitivity to the DNA damage induced by this mutagen, and that the unrepaired damage is a consequence of this MMC-sensitivity. An interesting aspect of the present study is that lymphocytes of obese adolescents seemed to have repaired the amount of DSBs induced by 2 h MMC treatment in a more efficient and rapid (*i.e.* per unit of time) manner than normal weight subjects. This means that the higher sensitivity to MMC-induced DNA damage exhibited by obese adolescents did not influence this response. As distinct sensibility to a same mutagen is observed in the case of cells with altered physiology, the different kinetic we found of induced DNA damage repair, is certainly attributable to the pathological condition of the subjects. In this view, some authors were able to distinguish subjects affected by Fanconi anemia (FA) from

other patients with bone marrow failure (BMF) syndrome by using the γ-H2AX assay in fibroblasts, demonstrating a delay in repair kinetic of FA cells compared to cells from BMF and control subjects [29]. Of course, it would be unlikely to attribute to normal weight children a slower kinetic of DSBs repair. The tendency of induced γ-H2AX foci to persist could be due to an alteration of normal cell functions such as synthesis and/or transport of the DDR proteins to the sites of DSBs. In fact, reduced mobilization of repair proteins, probably caused by one of their decreased expression, was effectively found in senescent cells or in cells from elderly individuals exposed to ionizing radiation [30,31].

Thus, a key role in the DSBs repair kinetic could be played by the HMGB1 protein which is involved in many cellular processes, including the ability to remodel chromatin and to bind to DNA regions in which DSBs occurred [32, 33]. Western blot analysis showed that the protein was differentially overexpressed in the peripheral cells of the study population, concurrently with the peak of DNA damage induction in the obese subjects, and 2 h later in normal weight adolescents. This delineated a distinct temporal kinetic between patients and controls that, in the case of the obese patients, would indicate a better efficiency of repairing, in the same time, the MMC-induced DSBs. The over expression of the chromatin remodelling HMGB1 protein by peripheral lymphocytes of obese patients could result in an accelerated access of other and more specific repair proteins at the site of the lesions.

In conclusion, our study showed that lymphocytes of obese adolescents, under the present experimental conditions, were more sensitive to MMC-induced damage, and that they seem to exhibit more efficiency in repair this damage. This does not seem attributable to a different ability in phosphorylating histone H2AX, as we previously demonstrated that the response of unstimulated peripheral cells to bleomycin was similar between obese/overweight and normal weight subjects [21]. A possible explanation of the faster repair observed in obese adolescents could depend on

different recruitment and mobilization of the HMGB1 protein per unit of time. The present findings also suggest that, at least during adolescence, a moderate pathological condition like obesity is able to positively influence the ability to repair DNA damage induced by a genotoxic agent. However, further investigations should be performed, especially to clarify the mechanism by which HMGB1 protein can modulate the DDR response.

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Figure legends

Fig. 1. Box and whisker plots represent the distribution of DSBs detected in MMC treated or untreated cultures (basal) at 2 h expressed as number of γ-H2AX foci per nucleus. For each subject class, the box contains 50% of the values, the whiskers retain the other 50% (if no outlier values are present), and the continuous line within each box or plus sign are the median or the mean of the data, respectively. For each subject, values were obtained from two replicate cultures. In each group, the mean frequency of γ-H2AXF/N induced by MMC was significantly different ($P<<0.001$,

ANOVA test) from that of the respective basal level.

Fig. 2. Box and whisker plots represent the distribution of DSBs detected in MMC treated or untreated cultures (basal) at 4 h expressed as number of γ-H2AX foci per nucleus. For each subject class, the box contains 50% of the values, the whiskers retain the other 50% (if no outlier values are present), and the continuous line within each box or plus sign are the median or the mean of the data, respectively. For each subject, values were obtained from two replicate cultures. In each group, the mean of γ-H2AXF/N induced by MMC was significantly different ($P<<0.001$, ANOVA test) from that of the respective basal level.

Fig. 3. Induced and unrepaired DNA damage (calculated as the difference between MMC-treated or untreated cultures at 2h or 4 h, respectively) in lymphocytes of the adolescent population (normal weight and obese subjects). Box and whisker plots represent the distribution of the number of γ-H2AX foci per nucleus. For each subject class, the box contains 50% of the values, the whiskers retain the other 50% (if no outlier values are present), and the continuous line within each box or plus sign are the median or the mean of the data, respectively. For each subject, values were obtained from two replicate cultures. The average value of both induced and unrepaired DNA damage was significantly higher in the obese subjects ($P = 0.0016$ and $P = 0.0198$, ANOVA test) than in the normal weight group.

Fig. 4. Amount of repaired DNA damage calculated as the difference between the induced DNA damage (at 2 h) and the unrepaired DNA damage (at 4 h) in MMC treated lymphocytes of the adolescent population (normal weight and obese subjects). Box and whisker plots represent the distribution of the number of γ-H2AX foci per nucleus. For each subject class, the box contains 50% of the values, the whiskers retain the other 50% (if no outlier values are present), and the continuous line within each box or plus sign are the median or the mean of the data, respectively. For each subject, values were obtained from two replicate cultures. In **A, v**alues are expressed as the

number of γ-H2AX foci per nucleus: the average level of γ-H2AXF/N of the obese group was significantly higher (P = 0.0408, ANOVA test) than that of the lean counterpart. In **B**, values are expressed in percentage of the amount of DSB repaired with respect to the initial damage induced by MMC: the means differed significantly between obese and normal weight subjects (P = 0.045, ANOVA test).

Fig. 5. Quantitative analysis of the HMGB1 protein in peripheral lymphocytes of the study population under the experimental conditions described in Figure 1. Protein levels were determined by immunoblotting, and equal loading of protein was confirmed by GADPH antibody. Histogram show densitometric analysis of HMGB1 intensity bands after normalizing to untreated cultures and GADPH. Bars represent the mean ± SEM of three experiments. Asterisks denote a significant difference between 2 h and 4 h in the obese or in the normal weight subjects (*P<0.05, **P<0.01). Circles denote a significant difference between the obese and normal weight group at 2 h or at 4 h (°P<0.05, °°P<0.01).

Table 1. Demographic and clinical characteristics of the study population. Data are expressed as mean ± SEM.

	Normal weight	Obese	Total
Subject (n)	15	23	38
Female % (n)	53.33 (8)	52.17 (12)	52.63 (25)
Age (years)	11.07 ± 0.83	12.44 ± 0.42	11.76 ± 0.4
BMI Z-score	0.69 ± 0.13	2.84 ± 0.13	1.99 ± 0.19

Figure 1

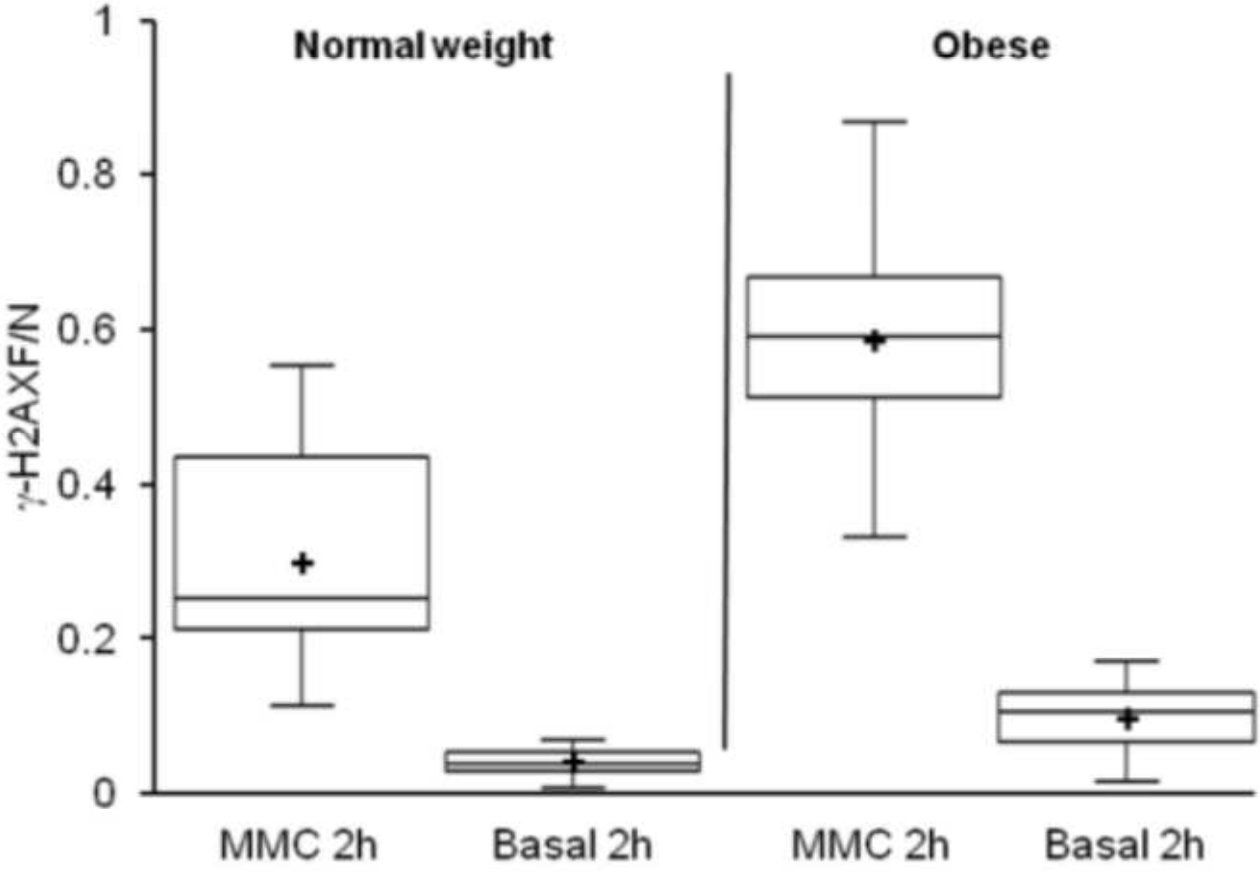

Normal weight
Obese
γ-H2AXF/N
1
0.8
0.6
0.4
0.2
0
MMC 2h
Basal 2h
MMC 2h
Basal 2h

Figure 2

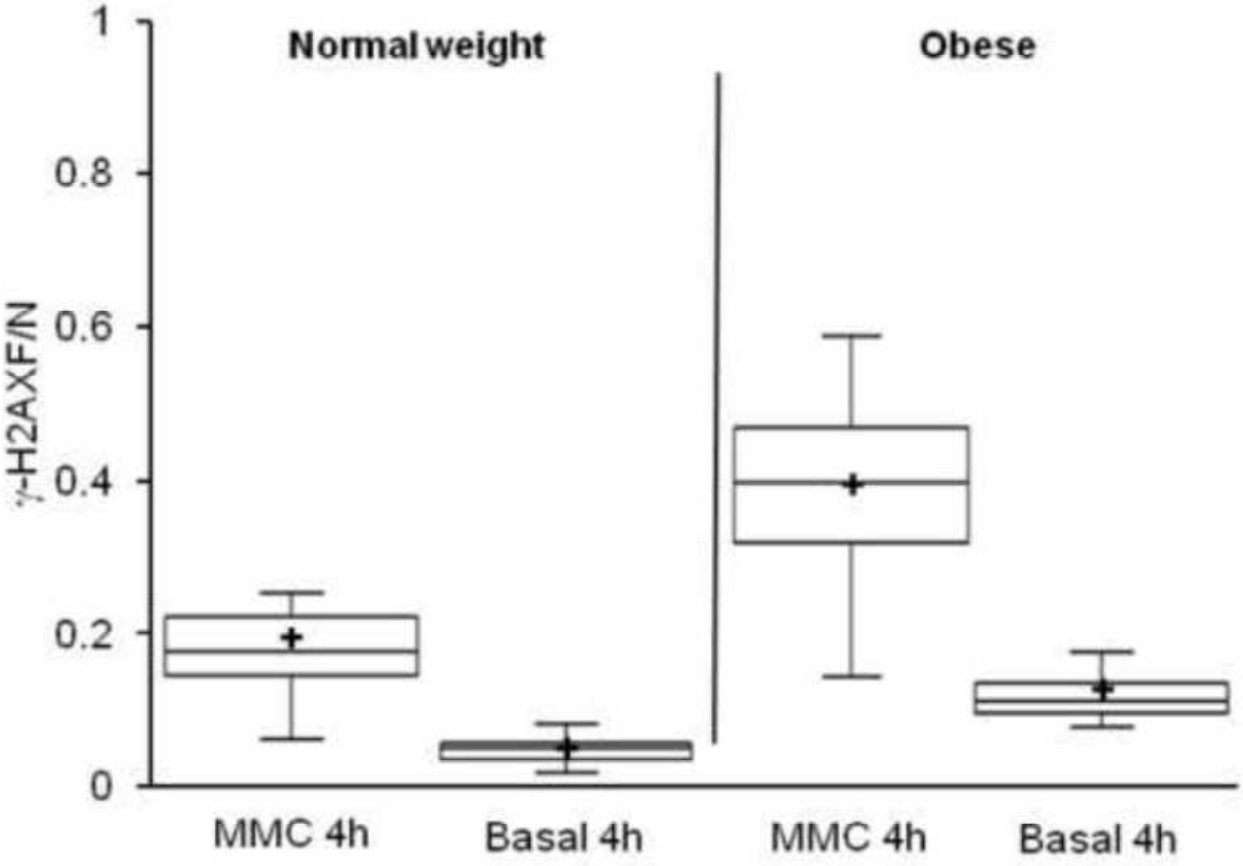

Normal weight
Obese
γ-H2AXF/N
1
0.8
0.6
0.4
0.2
0
MMC 4h
Basal 4h
MMC 4h
Basal 4h

Figure 3

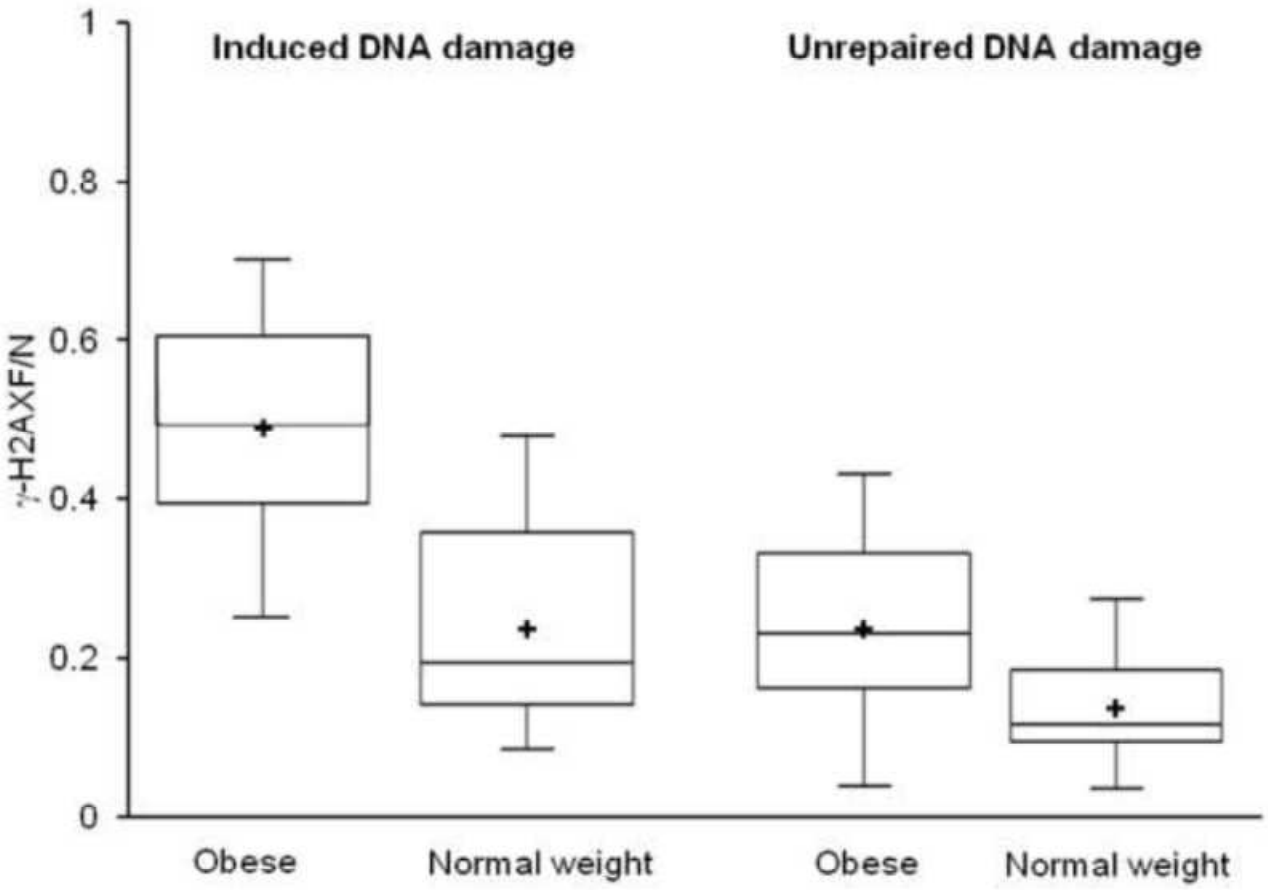

Induced DNA damage
Unrepaired DNA damage
γ-H2AXF/N
1
0.8
0.6
0.4
0.2
0
Obese
Normal weight
Obese
Normal weight

Figure 4

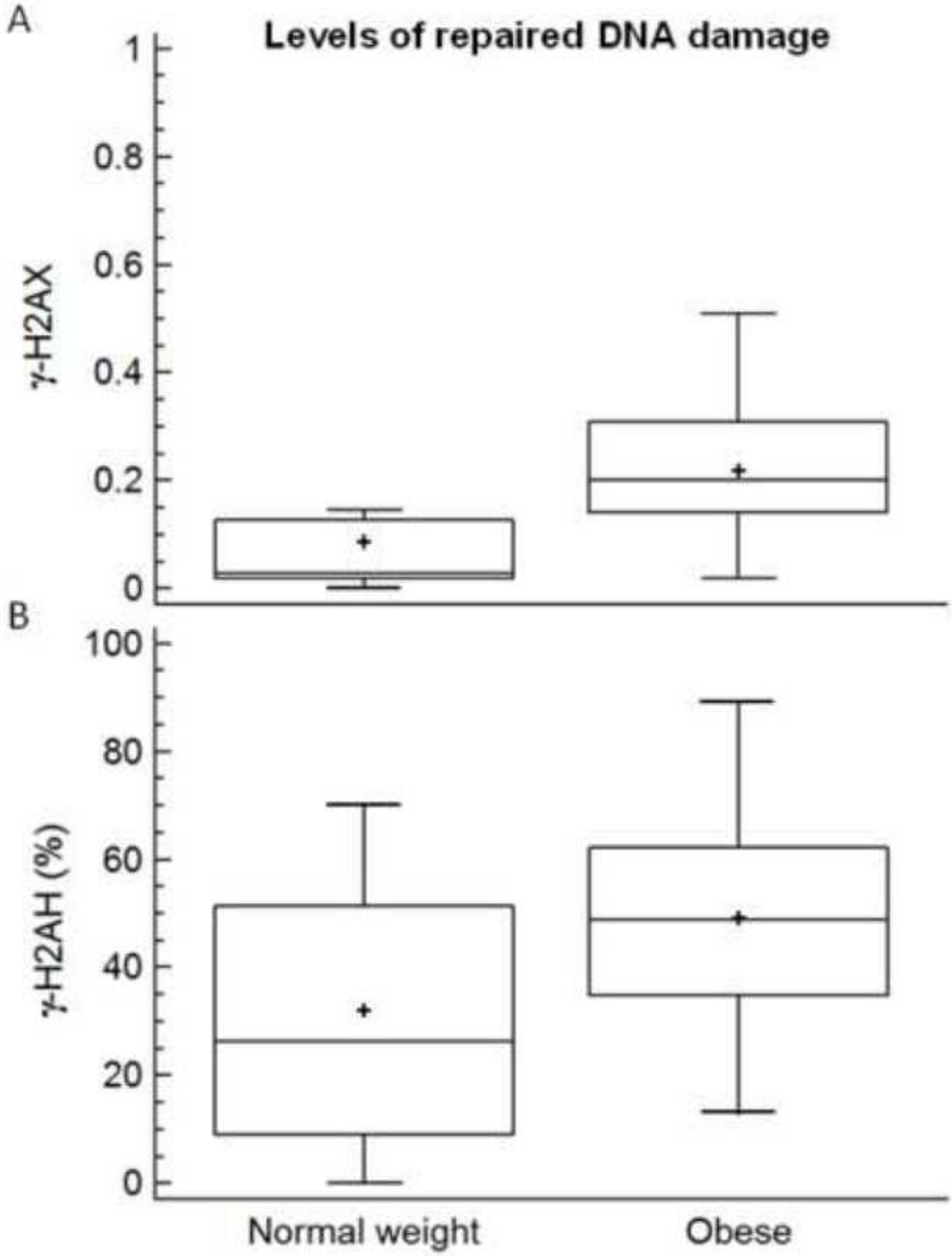
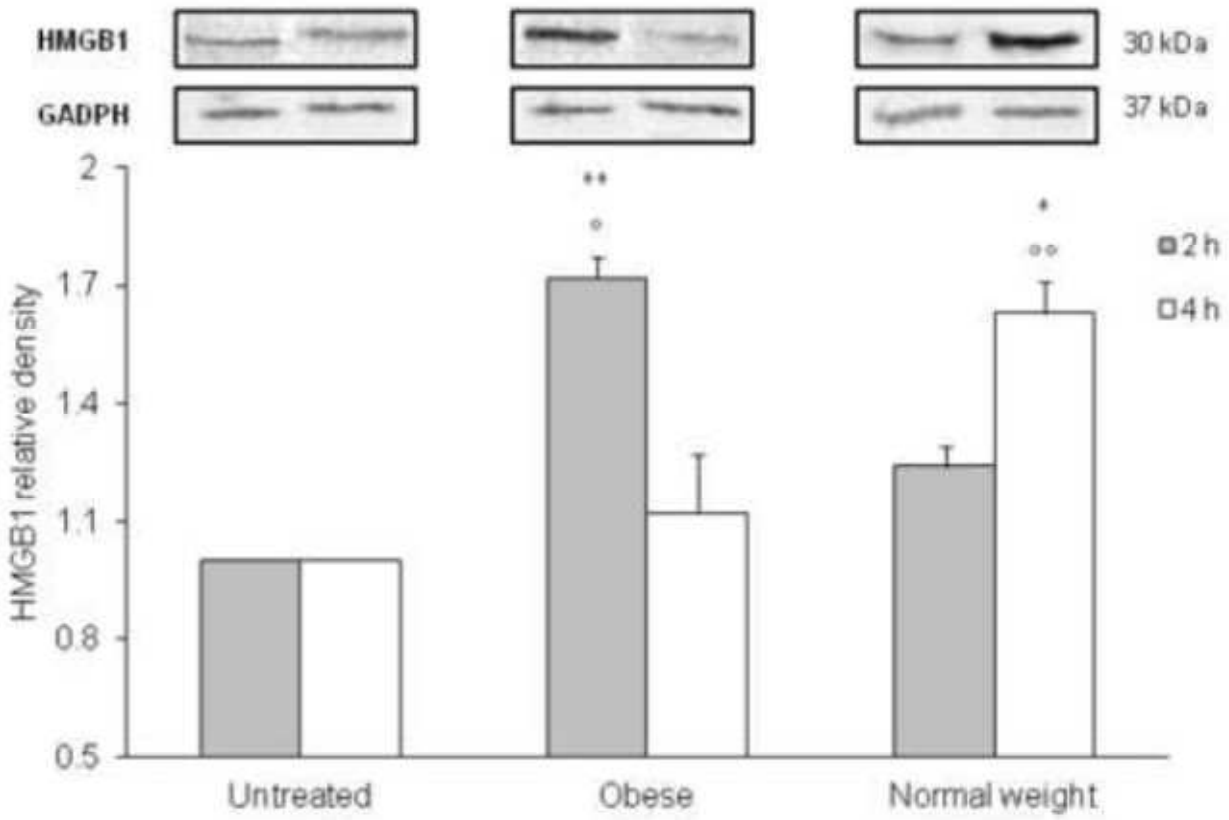

A
Levels of repaired DNA damage
γ-H2AX
1
0.8
0.6
0.4
0.2
0
B
γ-H2AH (%)
100
80
60
40
20
0
Normal weight
Obese

Figure 5


HMGB1
GADPH
30 kDa
37 kDa
2
1.7
1.4
1.1
0.8
0.5
HMGB1 relative density
2 h
4 h
Untreated
Obese
Normal weight