Hemin pre-conditioning upregulates heme-oxygenase 1 in deceased donor renal transplant recipients and may offer protection: a randomised, controlled, phase IIB trial

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Registration of clinical trial

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Disclosure

The authors declare no conflicts of interest.

Footnotes

Author's specific contributions

[1]RT, DK and LM designed the trial with scientific research input from CB. RT was Chief Investigator for the HOT study and ran the day-to-day research and lab analysis. DK, SM and LM were Principle Investigators guiding the trial and approving changes to protocols and analysis. CB performed the histological scoring of renal tissue and provided laboratory advice. AC provided lab advice and assisted with specimen analysis. RT, DK and LM participated in data analysis.

RT wrote the first draft of this paper with significant input by DK and LM. All authors reviewed the manuscripts, made changes and approved it.

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Abbreviations:

CI	Chief Investigator
CIT	Cold ischemic time
CO	Carbon monoxide
DGF	Delayed graft function
ERI	Edinburgh Royal Infirmary
ESRD	End stage renal disease
HA	Heme arginate
HO-1	Heme-oxygenase 1
IQR	Interquartile range
IRI	Ischaemia reperfusion injury
KIM-1	Kidney injury marker 1
NGAL	Neutrophil gelatinase-associated lipocalin
MHRA	Medicines and Healthcare products Regulatory Agency (UK drug safety agency)
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
WIT	Warm ischaemic time

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Abstract

Background:

The enzyme heme oxygenase-1 (HO-1) degrades heme and protects against ischaemia-reperfusion injury (IRI), which is inevitable in transplantation. Monocytes/macrophages (MΦ) are the major source of HO-1 and higher levels improve renal transplant outcomes. Heme Arginate (HA) can safely induce HO-1 in humans. This randomised controlled trial evaluated the effect of HA on HO-1 upregulation and renal function in recipients of deceased donor kidneys.

Methods:

40 recipients were randomised to either active (3mg kg^{-1} HA: pre-operatively and day 2) or placebo (NaCl: same schedule). Recipient blood was taken daily for peripheral blood mononuclear cells (PBMC) extraction. Urine was also collected. Graft biopsies were taken pre-op and day 5. Immunosuppression was standard.

Results:

HA upregulated PBMC HO-1 protein at 24 hours more than placebo: HA 11.1ng/ml vs. placebo 0.14ng/ml ($p=<0.0001$). PBMC HO-1 mRNA was also increased: HA 2.73 fold vs. placebo 1.41 fold ($p=0.02$). HA increased day 5 tissue HO-1 protein immunopositivity compared with placebo: HA 0.21 vs. placebo -0.03 ($p=0.02$) and % HO-1 positive renal MΦ also increased: HA 50.8 cells per hpf vs. placebo 22.3 ($p=0.012$). Urinary biomarkers were reduced after HA but not significantly so. Histological injury and renal function were similar but the study was not powered to these endpoints. Adverse events were equivalent between groups.

Discussion:

The primary outcome was achieved and demonstrated for the first time that HA safely induces HO-1 in renal transplant recipients. Larger studies are planned to determine the impact of HO-1 upregulation on clinical outcomes and evaluate the benefit to patients at risk of IRI.

Introduction

Renal transplantation is the optimal management for end-stage renal disease (ESRD) because it improves quality of life and survival (1). Deceased donor renal transplantation inevitably results in ischemia-reperfusion injury (IRI), which is characterised by the production of reactive oxygen free radicals, alterations in blood flow, leucocyte infiltration and significant cell dysfunction (2). IRI increases the risk of delayed graft function (DGF), necessitating post-transplant dialysis and leading to an increased risk of rejection and reduced long-term graft survival (3). Apart from limiting cold ischemic time, few methods exist to minimise the effects of IRI. One potential technique is preconditioning, using either ischemia or pharmacology (4).

The enzyme heme-oxygenase 1 (HO-1) has emerged as a potential approach to attenuate IRI (5). HO-1 is the rate-limiting enzyme in heme catabolism and degrades the pro-inflammatory, oxidative free heme molecules into biliverdin (further degraded to bilirubin), carbon monoxide (CO) and free iron(6). Bilirubin is an anti-oxidant. CO has vasodilatory and anti-apoptotic properties and inhibits a number of pro-inflammatory signalling pathways and platelet aggregation. Free iron is sequestered by ferritin to limit its toxic effect (7).

Experimental models have shown that following renal IRI, macrophages have roles in both injury and repair. They are also the primary HO-1 expressing leucocyte (8). Macrophage infiltration into the kidney occurs within 24 hours of IRI and these initial cells have a pro-inflammatory phenotype (M1), contributing to various injury processes. As a result, depletion of macrophages at early time-points post-IRI protects renal function (9). By contrast, renal macrophages present on days 3-5 following IRI have an alternative activation phenotype (M2) and contribute to tissue repair and recovery of renal function (9). In a renal IRI model, increased macrophage HO-1 expression was associated with reduced injury (10). Improved renal function was also seen after infusion of HO-1 expressing macrophages in a similar model (11). Our laboratory has demonstrated in a mouse IRI model that heme arginate (HA) pre-conditioning upregulates renal HO-1, principally in interstitial macrophages, and results in a significant reduction in subsequent renal injury. This protective effect was lost following macrophage depletion (12). Thus, it was hypothesised that amplified HO-1 expression in recipient macrophages and renal grafts might reduce IRI and improve outcomes after clinical

transplantation. HA, a form of hemin, is suitable for clinical studies, as it has been used safely in the treatment of acute porphyria for over 30 years with few reported side effects. In healthy volunteers, both hemin and HA have been shown to increase HO-1 concentration in peripheral blood mononuclear cells (PBMC) and plasma (13, 14).

The HOT study was a randomised, placebo controlled, blinded, single centre study with the primary objective of determining whether pre-treatment of renal transplant recipients with HA upregulated HO-1 in PBMCs. Secondary objectives explored the effect of HA on graft tissue, urinary biomarkers and renal function.

Materials and methods

All patients on the East of Scotland renal transplant waiting list were informed about the study by post before recruitment started. When patients were admitted to Edinburgh Royal Infirmary (ERI) for potential deceased donor renal transplant, they were assessed for eligibility.

A patient was ineligible for inclusion if:

1. they were unable to receive the standard immunosuppressive regimen
2. they were unable to give informed consent
3. they had hypersensitivity reactions to HA
4. it was a combined transplant or their 3rd or subsequent kidney transplant
5. they were to be anticoagulated post-operatively or on combined anti-platelet agents due to biopsy risks

Study protocol

This was a single-centre, randomised, blinded, placebo-controlled trial sponsored by ACCORD (a joint company from University of Edinburgh and NHS Lothian) and funded by NHS Blood and Transplant. The trial protocol was registered at the European Clinical Trials database (EudraCT no: 2011-004311-23) and ClinicalTrials.gov (NCT01430156) and adhered to the Declaration of Helsinki and CONSORT guidelines. The Scottish Regional Ethics Committee and the Medicines and Healthcare products Regulatory Agency (MHRA) approved the study (approval number 2011/R/TR/03 and protocol number HOTstudy_Thomas11).

The sample size calculation was performed on the observed heme oxygenase-1 (HO-1) upregulation in cultured human macrophages following HA treatment. Using a two-sided two-sample test with a 5% level of significance and 80% power, assuming a mean baseline to 24-hour change of 7.47 and a common standard deviation of 3.87, the minimum detectable difference in means would be 3.8 with a sample size of 17 per group. The final sample size of 20 per group allowed for dropouts.

The randomisation was done by random, random block, with stratification by donor type. The trial statistician generated the random allocation sequence and an independent party produced sequentially numbered, sealed, opaque envelopes, which were stored securely and only opened by the Chief

Investigator (CI) after consent had been given and participant number had been assigned. Subjects were randomised to receive either 100ml 0.9% NaCl (placebo) or 3mg kg^{-1} HA (Normosang, Orphan Europe, France) diluted in 100ml 0.9% NaCl. In both groups, participants received the first infusion pre-operatively (D0) and this was repeated on day 2 (D2). To ensure maximum post-conditioning exposure, the D0 infusion was given as early as possible post-dialysis (if required). All staff (except the CI giving the infusion) and participants were blinded.

Prior to infusion, venous blood was taken for PBMC extraction and analysis of baseline HO-1 expression (D0). Venepuncture was repeated 24 hours after the first infusion (D1) and also on days 2 (D2, pre-2^{nd} infusion), D3 and D5. Urine was collected on the same days for analysis of KIM-1 and NGAL. A needle-biopsy of the graft was taken at the back table (D0) for routine pathology and study analysis. A repeat biopsy was requested for all participants on D5. Laboratory samples were analysed by the CI after blinding by an independent lab member. All samples were stored in accordance with guidelines.

Otherwise all pre-operative and post-operative care was typical, including standard immunosuppression, which comprised of induction therapy with basiliximab, tacrolimus, mycophenolate mofetil (MMF) and prednisolone.

HO-1 mRNA and protein levels in PBMCs

PBMCs were isolated using the previously described Dextran-Percoll method and stored in appropriate buffers for subsequent batch analysis of mRNA and protein expression (15). PBMC RNA was extracted using the ISOLATE kit (Bioline, UK) and cDNA was synthesised using TaqMan, RT-PCR core kit (Invitrogen, USA). The cDNA was analysed in duplicate by quantitative real-time PCR using fluorogenic probes for HO-1 and 18s (housekeeping gene) on an ABI Prism 7900 PCR machine (Applied Biosystems, USA). For quantification, the threshold cycle of HO-1 (C_T) was correlated to the constant expression of the housekeeping gene; ΔC_T was defined as C_T (HO-1)- C_T (18s). The differences in expression pre and post infusion were plotted as $\Delta\Delta C_T$ and $2^{-\Delta\Delta CT}$ calculation was performed to determine the fold increase and the two groups were compared.

Each sample protein concentration was determined using protein assay (Biorad, USA). All samples were standardised to 1mg/ml of total protein and this was analysed in duplicate by the HO-1 ELISA kit to determine the amount of HO-1 protein at each time point (ENZO, USA).

Analysis of renal tissue

The D0 biopsy and D5 biopsy samples were handled and analysed as below:

- RNA extracted from fresh tissue and analysed in the same manner as PBMC RNA
- a blinded expert (CB) examined a fixed sample to assess for renal injury using a 13 point system.
- a fixed section of all the tissue samples underwent immunohistological staining for HO-1 (ENZO, USA). ImageJ (NIH) was used to quantify the amount of HO-1 protein as percentage of total protein.
- another section of tissue underwent dual immunofluorescence staining for HO-1 (ENZO, USA) and CD68 (macrophage marker (Abcam, UK)) and the numbers of dual positive cells were counted per high power field.

Urinary biomarkers

Commercially available ELISA kits from R&D Systems measured urinary KIM-1 and NGAL. KIM-1 and NGAL values are recorded as a ratio to urinary creatinine to allow for variations in urine concentrations.

Clinical outcomes

Clinical blood results were recorded daily. Need for dialysis, adverse events and urine volumes were also recorded. In this study, DGF was determined by change in creatinine in the first week after transplantation. Renal function was recorded at day 30 and day 90.

Data and statistical analysis

The anonymised data was held in a secure, auditable database. PBMC and renal HO-1 expression, urine biomarkers and renal macrophage data were not normally distributed; a non-parametric Mann-Whitney statistical test was used. A comparison of proportions test was used to compare rates of DGF between groups. Results are presented as median [IQR] and % values (95% confidence

intervals) as appropriate. Statistical analysis was performed on SDSS and graphs created in Prism. A p-value of <0.05 was considered statistically significant.

Results

Trial participation

Patient recruitment began in January 2012 and continued until 40 patients had been randomised in May 2013 (Figure 1). Baseline characteristics were similar between the groups (Table 1). No adverse reactions and no deaths occurred during the seven-day trial period. There was one graft loss due to technical reasons on day seven (placebo group). Acute rejection occurred in one participant in placebo group and two in HA group. Five serious adverse events were reported including one ITU admission for pulmonary oedema (placebo group), and one further procedure was required in each group (placebo: radiological embolization for renal arterio-venous fistula, HA group: nephrectomy for graft bleeding). All participants were followed up to 90 days post transplant.

Three potential recipients received the infusion but were not transplanted following back-table examination of the kidney at day 0 (D0). All 37 participants who were transplanted provided a blood sample at 24 hours (D1) post infusion for primary analysis. One participant in each group refused the second infusion and subsequent venepuncture but consented to clinical follow-up. Thus 17 in the placebo group and 18 in HA group received both infusions. Five participants in the placebo group and three in the HA group declined the day 5 (D5) biopsy and two in each group were not considered fit for biopsy. Therefore, there were 25 paired renal tissue samples available for analysis. All participants with a transplant *in situ* at day seven were followed-up. Results are expressed as median values with interquartile range [IQR].

HA upregulated HO-1 in peripheral blood monocytes

Expressed as the difference in HO-1 expression on D1 compared with D0, HA upregulated HO-1 concentration by 11.1ng/ml [1.0-37.0], compared with placebo -0.14ng/ml [-0.7-0.3] ($p<0.0001$, Figure 2a). This effect was confirmed with increased expression of HO-1 mRNA: HA treatment upregulated HO-1 mRNA expression 2.73 fold [1.8-3.2] compared with placebo 1.41 fold [1.2-2.2] ($p=0.02$, Figure 2b). There was a peak in HO-1 PBMC protein concentration after each HA infusion (Figure 2c) but mRNA upregulation did not significantly follow this pattern (Figure 2d).

HA upregulated HO-1 in renal macrophages

The number of CD68-positive macrophages per high power field (hpf) was similar between the treatment groups at D0 (HA: 3.50 cells per hpf [1.50- 5.25], placebo: 3.00 [2.25- 4.25]) (p=0.956, Figure 3a). At D5 there were more CD68-positive macrophages in HA group (HA: 7.38 cells per hpf [4.80- 9.13], placebo: 11.0 [4.5- 25.0]) but this was not significant (p=0.13, Figure 3a). However, there was a significant increase in the number of HO-1-positive CD68 macrophages following HA treatment at D5 (HA: 50.8 cells per hpf [40.0- 59.8], placebo: 22.3 [0.0- 34.8]) (p=0.012, Figure 3b and images Figure 3c-f).

HA upregulated HO-1 in renal tissue

There was significant increased expression of renal HO-1 protein in the HA-treated group over time, which was not seen with placebo (change in protein expression [D5-D0]: HA: 0.21 [0.1- 0.6], placebo: -0.03 [-1.3- 0.1]) (p=0.03, Figure 4a and images 4c-f). HO-1 mRNA expression was not significantly affected by HA treatment: HA: 1.68 [0.20- 4.03], placebo 2.02 [0.75- 10.39] (p=0.45, Figure 4b).

HA had limited effect on urinary biomarkers

There was no significant difference in urinary KIM-1 and NGAL levels between patients treated with HA versus placebo at each time point (Figures 5a and 5b).

HA did not alter histological injury or renal function

There were no significant differences in the severity of tubular injury or necrosis between HA and placebo (data not shown).

There were 12 cases (67%) of DGF in the placebo group and 10 cases (53%) in HA group, a difference of 14% (95% CI; -17.2 – 45.3), (p=0.38). When DGF was redefined as dialysis within the first seven days post-transplant except for hyperkalaemia, the results were the same. At day 90, there was one participant on dialysis in placebo group and none in HA group. There was no improvement in creatinine after HA treatment at day 30 and day 90 (data not shown).

Discussion

The study achieved its principal objective: pre-treatment of deceased donor renal transplant recipients with HA was feasible, safe and well tolerated in immunosuppressed ESRD patients. It also led to significant upregulation of HO-1 protein and mRNA in recipient PBMCs. HO-1 is induced in response to multiple stimuli including hypoxia and ischaemic cytokines so it was anticipated that all participants would experience an upregulation in HO-1 after transplantation. Nevertheless, HA treatment increased macrophage HO-1 more than placebo treatment and reiterated results from cellular, pre-clinical and healthy volunteer studies (12-14). Figure 2c confirmed that, as expected, there is a distinct peak in protein concentration after each HA infusion with a return to near baseline expression 48 hours later. This replicates the findings of previous healthy volunteer studies (14). The pattern of mRNA upregulation did not mirror this because although there was a significant peak at 24 hours, this was not seen again after the second infusion. This anomaly may be due to the $\Delta\Delta C_T$ method used because all samples were referenced to baseline (D0) and it may be more appropriate to normalise D3 to D2 to identify a second upregulation.

This is the first clinical study to show that HA treatment increases HO-1 protein immunopositivity and the number of HO-1 positive macrophages in renal graft tissue. Renal HO-1 mRNA did not have the same response which may be related to timing of the biopsy. Earlier research has shown that the half-life of HO-1 mRNA *in vitro* renal cells after hemin treatment is only four hours (16) and therefore the D5 biopsy may be too late to detect a difference in the more transient mRNA.

The present study was powered for a cellular outcome but given its importance, the study attempted to establish whether pre-treatment with HA was associated with a reduction in renal injury or function post-transplant. Serum creatinine is an unreliable marker of acute kidney injury and does not accurately reflect the degree of damage until it reaches a nadir, which may be several days post-op. The urinary biomarkers KIM-1 and NGAL have been validated as independent predictors of graft survival after renal transplantation (17, 18). In this study, the maximum NGAL and KIM-1 levels were lower after HA treatment on all days indicating reduced renal injury but the differences were not statistically significant. There was also no evidence of histological protection after biopsy analysis.

One of the challenges in clinical trials of renal transplantation is the lack of a single clear definition of DGF (19). In the present study the 'functional' definition was used, defined as increased or stable serum creatinine, or a decrease of <10% per day for three consecutive days in the first week after transplantation (20). This was preferred over the definition of DGF as the requirement for dialysis within the first seven days following transplantation, because the decision to dialyse a patient may be subjective with variation in practice between clinicians. Also a recent study found functional DGF but not dialysis-defined DGF to be associated independently with subsequent transplant failure (20).

Despite clear evidence that HA upregulated HO-1 in this study, it did not translate into the structural or functional cytoprotection seen in pre-clinical and observational studies (12, 21-24). In laboratory studies, induction of HO-1 in macrophages (10), and renal tissue (12, 23) offers protection against renal IRI and the presence of HO-1 expressing renal macrophages has been shown to safeguard renal function in the face of significant structural injury (11). There are a few possible explanations why HO-1 upregulation did not confer protection; firstly, there was a modest protection but the sample size was too small to detect it. This provisional study was not powered to this endpoint and larger studies are required. This is a common issue in renal transplant research and few interventions have impacted on DGF rates despite promising pre-clinical results.

Secondly, two doses of HA were insufficient. Human studies have shown that HO-1 expression increases with additional HA doses and more may be required to maximise and sustain the effect on macrophages and renal tissue for clinical benefits (14).

Thirdly, it may be that the equivocal structural and functional results are genuine because HA has other effects in clinical IRI that inhibits the anticipated positive consequences of HO-1 upregulation.

Fourthly, it has also been proposed that the protective response of HO-1 may be limited and susceptible to being overwhelmed. It may be that once a level of injury has been reached, further HO-1 induction cannot prevent the damage (25). Clinical renal transplantation delivers sizable, diverse insults to the kidney, which are not fully modelled in animal experimentation, and may explain why preclinical findings do not equate to human studies.

Given the unique nature of this study, further research is required to determine whether the amount of HO-1 upregulation seen here is sufficient for protection. This study establishes a sound basis for undertaking a clinical trial of HA vs. placebo powered to detect clinical differences in graft function and this is planned. There is also scope to increase the number of HA infusions within the UK prescribing licence and this is also currently under investigation in our centre. HA is a safe alternative to hemin because the effects of ferric heme are reduced when hemin is liganded to arginine as in HA (26). HA may offer an alternative to other renal transplant conditioning strategies that have not fulfilled their potential when translated to clinical studies such as erythropoietin (27, 28), statins (29) and ischaemic pre-conditioning (30). HA is well suited for such a trial in renal transplant recipients because it was well tolerated and has demonstrated induction of HO-1 in PBMCs and renal tissue when administered pre and post-renal transplant surgery.

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Table

Tables for HOT document

Table 1. Baseline characteristics of the 40 randomised participants by treatment group

Characteristic	**Placebo (n=19)**	**HA (n=21)**
Mean age (range)	52.4 (25- 79)	52.1 (21- 69)
Male (%)	11 (58)	12 (57)
1st transplant (%)	19 (100)	20 (95)
2nd transplant (%)	0	1 (5)
Dialysis patient (%)	17 (89)	20 (95)
Drug history; Statin (%)	11 (58)	13 (62)
Drug history; any type antiplatelet agent (%)	8 (39)	6 (29)
Time from infusion to reperfusion in minutes (range)	420.6 (39.0- 1586.0)	249.0 (48.0- 803.0)
Donor characteristics		
Mean age (range)	45.2 (21- 73)	46.8 (14- 69)
Male (%)	13 (72)	10 (53)
DBD (%)	9 (50)	9 (47)
Mode of organ perfusion- hypothermic (%)	3 (17)	2 (11)
Mode of organ perfusion- cold static (%)	15 (83)	17 (89)
HLA match 0 HLA-DRB1 mismatch 1 HLA- DRB1 mismatch 2 HLA-DRB1 mismatch	 3 (17) 9 (50) 6 (33)	 1 (5) 13 (69) 5 (27)
Number with virtual crossmatch[1]	12 (67)	15 (79)
Mean CIT in minutes[2] (range)	691.7 (351- 1287)	731.5 (450- 1213)
Mean WIT in minutes[3] (range)	36.2 (28- 61)	37.6 (23- 59)

Mean values are given for continuous variables, while numbers of patients (percentages) are given otherwise.

[1]Virtual crossmatch= crossmatch that is performed before the organ arrives at RIE. This reduces the ischaemic time and is possible when the recipient's antibody specificities have been identified and the donor HLA type is known.

[2]CIT= Cold ischaemic time= time from storage in ice at recovery to removal from ice during transplant operation

[3]WIT= Warm ischaemic time (2nd)= time from removal from ice for transplant to reperfusion

Figure legends for HOT_article document

Figure 1. Flow chart showing recruitment and study characteristics

Figure 2: Treatment with HA upregulated HO-1 protein and mRNA expression in peripheral blood mononuclear cells (PBMCs).

a) HO-1 protein upregulation: expressed as the difference in HO-1 expression on D1 compared with D0. HA upregulated HO-1 expression by 11.1ng/ml [1.0- 37.0], compared with placebo -0.14ng/ml [-0.7- 0.3]; p<0.0001(n=36, 1 sample could not be analysed). b) HA upregulated HO-1 mRNA expression 2.73 fold [1.8- 3.2] compared with placebo 1.41 fold [1.2- 2.2]; p=0.02 (n=37). c) Change in PBMC HO-1 protein expression over 5 days, demonstrating significantly increased HO-1 expression on D1 and D3 after HA treatment (p<0.005). d) Change in HO-1 mRNA expression over 5 days, expressed as fold increase compared with D0. No significant difference at D5.

Figure 3: There was significant upregulation of HO-1 expression in CD68-positive macrophages after HA treatment compared with placebo at day 5.

a) Number of CD68-positive macrophages at D0 and D5 with HA treatment compared with placebo: D0 p=0.956, D5 p=0.13 (n=21, some tissue not suitable for staining). b) Percentage CD68-positive macrophages expressing HO-1 at D0 and D5 with HA compared with placebo. At D5, HA: 50.81 cells per hpf [40.0- 59.8], placebo: 22.3 [0- 34.8]; p=0.012 (n=21) c) Dual stain immunofluorescent images of renal tissue (green: CD68, red: HO-1, blue: nuclear marker), white arrows highlight dual positive cells from D0 HA d) D0 placebo e) D5 HA and f) D5 placebo (x20 magnification)

Figure 4: HO-1 protein but not mRNA was upregulated in renal tissue.

a) HO-1 protein upregulation: expressed as the difference in % HO-1 expression on D5 compared with D0, HA upregulated protein by 0.21% [0.1- 0.6] compared to placebo of -0.03% [-1.3- 0.1], p= 0.017 (n=22, three samples insufficient for protein analysis).

b) HO-1 mRNA upregulation at D5 expressed as fold increase from D0 showed no significant difference between the groups p=0.45 (n=25). Images of immunohistological staining for HO-1 (DAB) in renal tissue at c) D0 HA, d) D0 placebo, e) D5 HA and f) D5 placebo (x20 magnification)

Figure 5: Change in urinary biomarkers over 5 days after HA treatment and placebo. There was no significant difference between the HA and placebo treatment at any time point in a) KIM-1 and b) NGAL-1. All p-values = >0.05.

Figure 1
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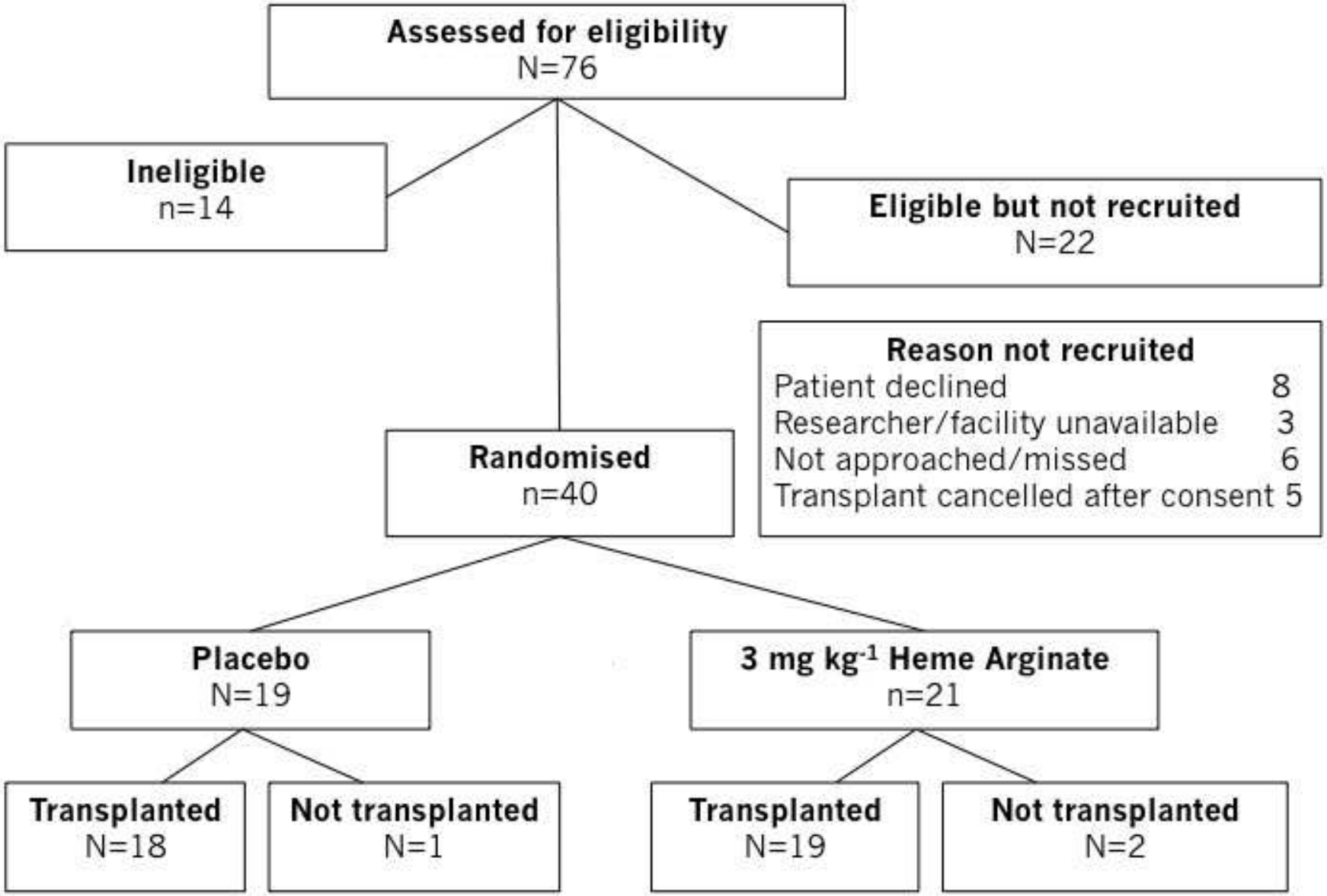

Figure 2
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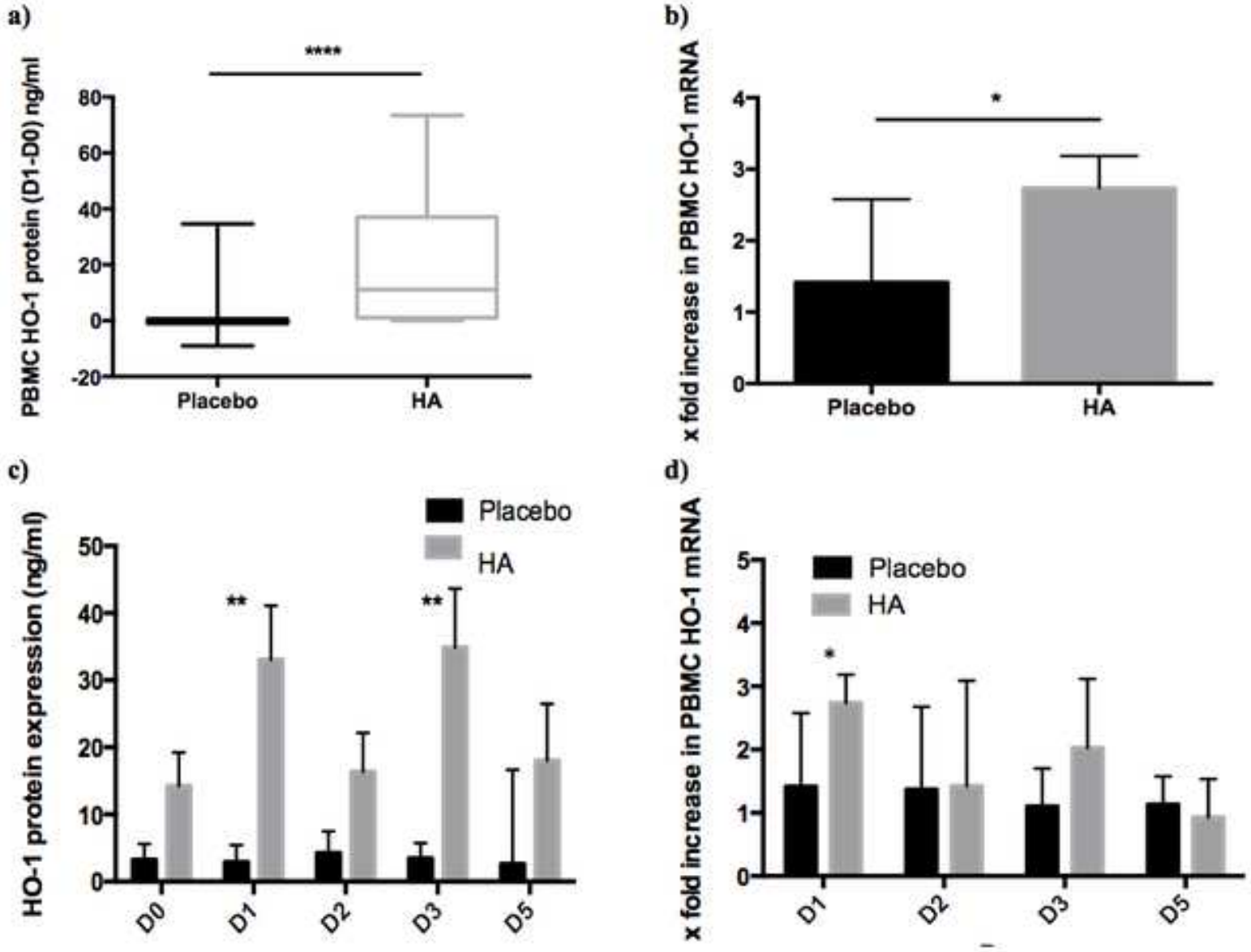

Figure 2: Treatment with HA upregulated HO-1 protein and mRNA expression in peripheral blood mononuclear cells (PBMCs).

Figure 3
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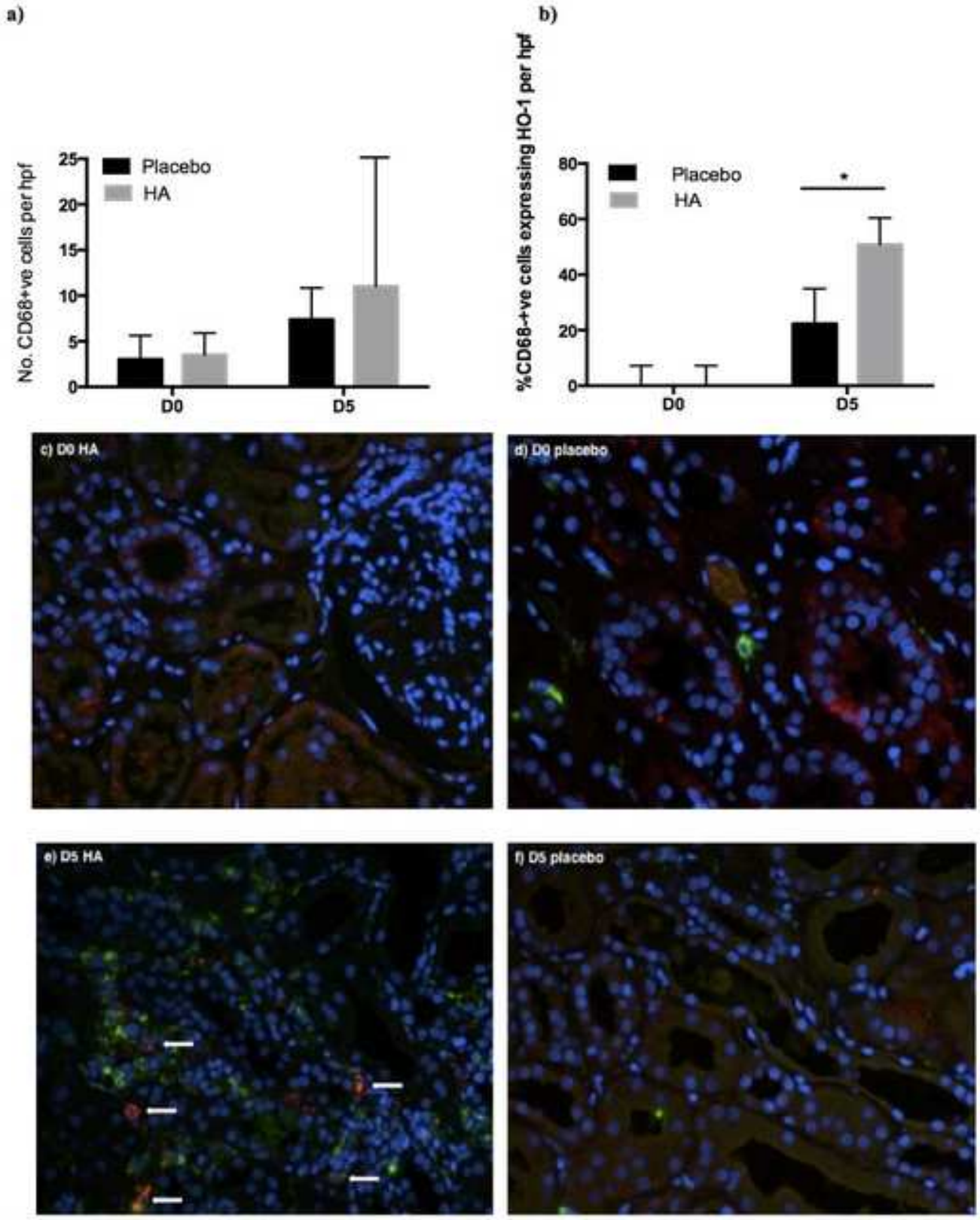

Figure 3: There was significant upregulation of HO-1 expression in CD68-positive macrophages after HA treatment compared with placebo at day 5.

Figure 4
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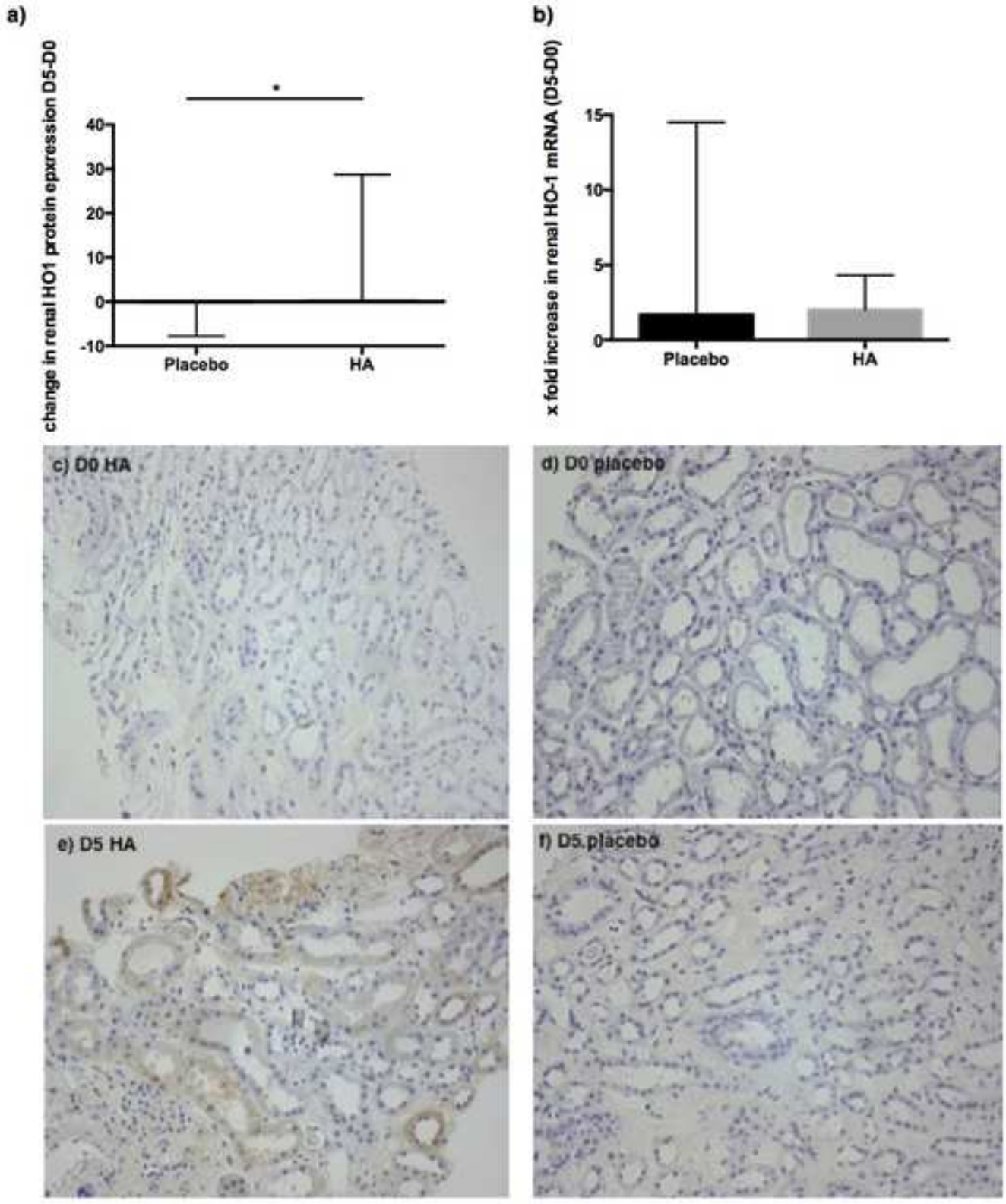

Figure 4: HO-1 protein but not mRNA was upregulated in renal tissue

Figure 5
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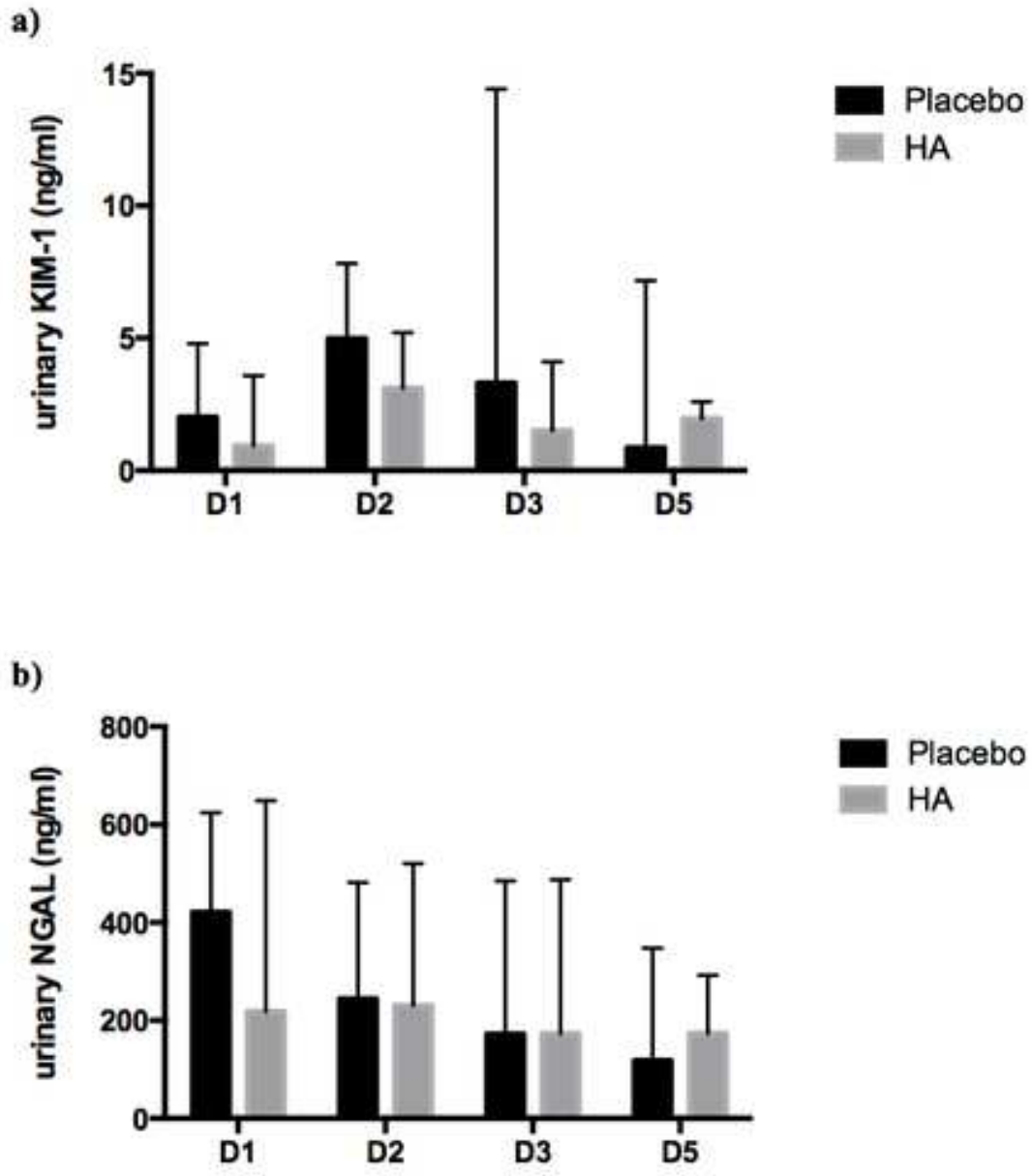

Figure 5: Change in urinary biomarkers over 5 days after HA treatment and placebo.

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	4
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	5/6
	2b	Specific objectives or hypotheses	5/6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	7/8
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	--
Participants	4a	Eligibility criteria for participants	7
	4b	Settings and locations where the data were collected	7
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	7/8
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	6/8/9
	6b	Any changes to trial outcomes after the trial commenced, with reasons	--
Sample size	7a	How sample size was determined	7
	7b	When applicable, explanation of any interim analyses and stopping guidelines	--
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	7
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	7
Allocation concealment mechanism	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	7/8
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	7/8
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those	8

		assessing outcomes) and how	
	11b	If relevant, description of the similarity of interventions	8
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	9/10
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	—
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	11/ figure 1
	13b	For each group, losses and exclusions after randomisation, together with reasons	11/ figure 1
Recruitment	14a	Dates defining the periods of recruitment and follow-up	11
	14b	Why the trial ended or was stopped	11
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	table 1
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	11/ figure 1
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	11/12
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	—
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	—
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	11
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	14/15
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	14
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	13/14/15
Other information			
Registration	23	Registration number and name of trial registry	1/7
Protocol	24	Where the full trial protocol can be accessed, if available	1/7
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	1/7

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

CONSORT flow diagram

CONSORT
TRANSPARENT REPORTING of TRIALS

CONSORT 2010 Flow Diagram for HOT study