Hydroxyurea-induced synchronisation of bloodstream stage *Trypanosoma brucei*

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Abbreviations:

HU: hydroxyurea; *T. brucei*: *Trypanosoma brucei*; N: nucleus; K: kinetoplast; DNA: deoxyribonucleic acid; dNTP: deoxyribonucleotide triphosphate; DAPI: 4, 6-diamidino-2-phenylindole; PBS: phosphate buffered saline.

Abstract:

Synchronisation of the *Trypanosoma brucei* cell cycle proved elusive for many years. A recent report demonstrated that synchronisation of procyclic form cells was possible following treatment with hydroxyurea. Here, that work is extended to the disease-relevant, mammalian-infective bloodstream stage trypanosome. Treatment of bloodstream stage Lister 427 *T. brucei* cells growing *in vitro* with 10 μgml^{-1} hydroxyurea for 6 hours led to an enrichment of cells in S phase. Following removal of the drug, cells proceeded uniformly through one round of the cell cycle, providing a much needed tool to enrich for specific cell cycle stages, in a manner similar to hydroxyurea treatment of procyclic form *T. brucei*.

Key Words: *Trypanosoma brucei*, hydroxyurea, cell cycle, synchronisation, S phase

Introduction

Trypanosoma brucei is a single-celled eukaryotic protozoan parasite that infects mammals, causing the diseases African sleeping sickness in humans and Nagana in cattle. A number of aspects of the biology of trypanosomes (and related kinetoplastids) appear to have diverged from that of 'model' eukaryotes. It may be, therefore, that *T. brucei* and the other Trypanosomatids diverged early in eukaryotic evolution [1] and can provide a different perspective on eukaryotic biology. One example of this evolutionary divergence is found in the single large mitochondrion of trypanosomes, which contains DNA organised into a highly unusual array of concatenated circles, termed the kinetoplast [2]. This organelle, like the nucleus, undergoes replication and division phases during the cell cycle, although the timing of replication and segregation of the nucleus and kinetoplast differs, with the kinetoplast entering S phase just before the nucleus, and dividing before the nucleus. This difference in replication and division timing provides a tool to classify the cell cycle stage of individual cells in an unsynchronised population [3,4]. For example, a cell that possesses 1 nucleus and 1 kinetoplast (1N1K), is in either G_1 phase or at the beginning of S phase, while a 1N2K cell is in (nuclear) S phase or G_2 phase. Finally, a 2N2K cell is either exiting mitosis or in the process of undergoing cytokinesis. Other N-K configurations are abnormal and indicate disruption to cell cycle progression [5].

The ability to generate a population of synchronised cells in a given cell cycle stage is a useful tool for examining the cell cycle of any organism, and is particularly useful when studying proteins which control the cell cycle and may only be present or active during certain phases. Previous attempts to cell cycle synchronise *T. brucei* have largely failed, in part because of toxicity issues (see below) and in part due to unique features of its biology. For example, inhibition of mitosis does not prevent bloodstream stage parasites from re-replicating their nuclei and kinetoplasts, while procyclic form

trypanosomes continue with cytokinesis in the absence of mitosis, producing zoids (0N1K cells) [6-8]. Additionally, methods based on nutrient deprivation were only able to achieve partial synchronisation in procyclic [9] and bloodstream [10] stage *T. brucei.*

The lack of a tool for cell cycle synchronisation has been a hindrance to research into the cell cycle of *T. brucei,* and has necessitated alternative, time-consuming strategies not needed in other model organisms, such as manually identifying within an asynchronous population individual cells of the desired cell cycle stage for further study [11] or using fluorescence activated cell sorting to select cells with the required DNA content [4].

Hydroxyurea (HU) treatment has been addressed previously in several synchronisation studies of *T. brucei.* One study was able to achieve partial cell cycle synchronisation of procyclic *T. brucei* [12], while the others were unable to synchronise either culturable life cycle stage, with authors reporting, under the conditions used, toxicity of HU at lower concentrations than were necessary to disrupt cell cycle progression [13,14]. Recently, however, Chowdhury *et al.* revisited HU-mediated cell cycle synchronisation and, by modifying the experimental conditions, were able to demonstrate a high degree of synchronisation of procyclic form *T. brucei* in S phase using HU [15]. HU synchronisation is hypothesised to function through inhibition of ribonucleotide reductase, which depletes dNTP synthesis and thereby causes DNA replication to stall. This initiates an S-phase checkpoint, albeit a leaky one in higher eukaryotes [16], and apparently in *T. brucei* too [15] and see results].

In the light of the findings of Chowdhury *et al.* [15] we decided to re-examine the issue of bloodstream stage HU synchronisation. Flow cytometry was used to measure relative DNA content of cells, while DAPI staining enabled visualisation of nuclei and kinetoplasts. Using these methods we show that by modifying the concentration of, and length of incubation with, HU, it is possible to cause the accumulation of cells in S phase,

similar to HU synchronisation of the procyclic form [15]. Following release from HU, bloodstream stage cells progressed through the cell cycle synchronously for at least one population doubling time, although synchrony began to be lost after cells underwent cytokinesis. This expands the use of HU synchronisation in the study of the *T. brucei* cell cycle to the mammalian infective stage of the parasite and, in particular, will allow enhanced dissection of the timing and order of events during the cell cycle at the molecular level in this disease-relevant life cycle stage.

Materials and Methods

Culturing of *T. brucei*

Bloodstream stage Lister 427 *T. brucei brucei* were cultured *in vitro* in HMI9 medium [17] containing 10% Serum Plus (SAFC Biosciences) and 10% foetal calf serum, at 37ºC in the presence of 5% CO_2. Cell density was determined using an Improved Neubauer haemocytometer.

Hydroxyurea treatment

HU was dissolved in distilled H_2O and freshly prepared for each experiment. Removal of HU from the culture medium was achieved by centrifuging cells at 1500 x *g* for 5 minutes, washing twice with fresh (drug free) medium and resuspending cells in medium lacking HU.

Cell cycle analysis

4,6-Diamidino-2-phenylindole (DAPI) staining and flow cytometry analysis were performed as described previously [8]. At least 200 DAPI stained cells were counted for each time point while 10^4 events were counted by flow cytometry. Kinetoplast length measurements were performed on images of DAPI-stained 1N1K cells ($n \geq 100$ per time point) using OpenLab v 5.5 software.

Immunofluorescence

Immunofluorescence was performed as described in [18]. Briefly, 5 x 10^5 cells were washed in Trypanosome Dilution Buffer (TDB: 20 mM Na_2HPO_4, 2 mM NaH_2PO_4, 80 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 20 mM glucose, pH 7.4) and fixed in 1% formaldehyde. They were then permeabilised by the addition of 0.1% Triton X-100 and incubated for 10 minutes at room temperature. 1 M glycine in phosphate buffered saline

(PBS) was then added to a final concentration of 0.1 M and the cells were incubated for 10 minutes at room temperature before being settled onto poly-L-lysine coated slides. 20 µl of KMX antibody [19] were added and the slides incubated overnight at 4^{o}C in a humid chamber. The slides were then washed with PBS and incubated with a 1/1000 dilution of Alexa Fluor 488 - conjugated anti-mouse IgG secondary antibody in PBS for 1 hour in the dark. After another PBS wash, VectaShield mounting medium containing DAPI (Vector Laboratories Inc.) was added and a coverslip applied. Fluorescence was observed using a DeltaVision RT microscope system (Applied Precision) and image stacks in the z plane were captured with a Roper CoolSnap-HQ 12-bit CCD camera controlled by SoftWoRx software and deconvolved. Twenty cells were examined for each cell type at each time point measured.

Results

Treatment of bloodstream stage *T. brucei* with hydroxyurea inhibits growth

In order to be a useful tool, any synchronising agent must pause the growth of its target population without being immediately lethal or incurring longer term toxic effects. To investigate whether this was achievable using HU in bloodstream stage *T. brucei*, parasites were incubated with a range of HU concentrations and cell density was measured over time to determine the lowest concentration of drug capable of inhibiting population growth (Fig. 1A). At a concentration of 5 μgml^{-1}, HU had only a modest effect on growth. This was more pronounced when using 7.5 μgml^{-1} HU, but parasites continued to replicate until 18 hours after drug addition. HU concentrations of 10 μgml^{-1} or above arrested population growth after around 9 hours incubation and longer incubations with these concentrations of drug were lethal. Hence, a concentration of 10 μgml^{-1} HU was examined more closely in subsequent experiments, as it was the lowest of the concentrations used that rapidly inhibited growth.

10 μgml^{-1} hydroxyurea inhibits cell cycle progression

To evaluate whether HU arrested growth by causing a cell cycle block, as well as the incubation time required for cells to synchronise at any such block, cell cycle progression was measured by flow cytometry and DAPI staining every 2 hours following exposure of cells to 10 μgml^{-1} HU (Fig. 1 B and C). Before the addition of HU, the asynchronous cell population gave a characteristic flow cytometry profile comprising two peaks representing cells with 2C (G_1 phase) and 4C (cells in G_2 phase, M phase or cytokinesis) DNA contents, respectively. S phase cells with intermediate DNA contents were detected between the two peaks. After just 2 hours incubation with HU, the flow cytometry profile showed a single peak of cells with slightly greater than 2C DNA content (Figure 1C). Indeed, DAPI staining at this time point showed the population to

be comprised almost entirely of 1N1K and 1N2K cells, with virtually no 2N2K cells present. This is consistent with HU causing an S phase block; cells that were post-S-phase at the time of HU addition would have continued to divide as normal and re-entered G_1. At this time, the cell population was not synchronised since it contained a mixed population comprising 1N1K cells (likely to be a mixture of cells in G_1 phase and at the start of S phase) and 1N2K cells in S phase. At 4 hours following HU addition, the proportion of 1N2K cells increased to nearly 40% and, by flow cytometry, the single peak correspondingly progressed further into S phase. However, many (60%) 1N1K cells remained, suggesting that some cells were likely to be still in G_1 phase. By 6 hours after HU addition, the flow cytometry peak was at the centre of S phase and ~75% of the population were 1N2K cells. After 8 and 10 hours' HU treatment, the flow cytometry peak continued to slowly advance through S phase, with the peak centre reaching 4C DNA content after 10 hours. Correspondingly, the proportion of 1N1K cells in the population reduced to ~15%. At these time points, however, 2N2K cells reappeared in the population, suggesting that some cells managed to escape the S phase block and progress all the way through the cell cycle.

From these studies, 6 hours of HU treatment appeared to be the optimal incubation time to obtain the purest S phase population, and cells synchronised under these conditions were examined further. A 1N1K cell population potentially contains a mixture of cells in G_1 phase and cells that are just beginning S-phase. To investigate the proportion of cells in S-phase after HU treatment, the sizes of kinetoplasts of DAPI-stained 1N1K cells were measured at 0 and 6 hours after HU addition (Fig. 1D). The kinetoplast is known to elongate and change morphology as it replicates and divides, and hence the presence of an elongated and v- or bone-shaped kinetoplast in a 1N1K cell indicates it has entered S phase [20]. It was hypothesised that the majority of untreated 1N1K cells would be in G_1 phase, whereas after 6 hours of HU treatment, a greater

proportion of these cells would be in S phase. Kinetoplast length distributions fell into three peaks, as observed previously [21], with the first peak (~0.3-0.89 μm) representing kinetoplasts yet to divide and still round or oval in shape, the second peak (~0.9-1.19 μm) comprising dividing kinetoplasts that were v-shaped and the third (>1.2 μm) comprising bone-shaped kinetoplasts (Figure 1D). In untreated populations the majority of 1N1K cells had round or oval kinetoplasts, while the kinetoplasts in HU-treated 1N1K cells separated more clearly into the 3 peaks, with the two peaks representing dividing kinetoplasts being somewhat more abundant. This indicated that although some cells remain in G_1, many 1N1K cells present at 6 hours after HU addition were beginning S-phase (72/102 cells measured, although this was probably an underestimate since kinetoplasts that had just started DNA replication may not have increased in size sufficiently to be detected by this assay). Taken together with the DAPI profile of cells at this time point, this indicated that at least 90% cells in the population are in S-phase.

Following hydroxyurea removal, bloodstream stage cells emerge synchronously from the cell cycle block

To determine whether HU-treated bloodstream stage cells enriched in S phase could emerge synchronously from the block upon removal of the drug, cells were exposed to 10 $\mu g ml^{-1}$ HU for 6 hours before being washed and resuspended in fresh medium. Growth curves (Fig. 2A) indicated that following HU removal, population growth rapidly returned to a pre-treatment rate with a minimum of lag (< 6 hours), suggesting that this regimen of HU treatment was not unduly toxic to the cells.

Cell cycle progression following HU removal was monitored by DAPI staining and flow cytometry (Figure 2B and C). At the point of HU removal, cells were predominantly in S phase, as expected. At 1 hour after HU removal, little change was observed in the nuclei and kinetoplast configurations of cells, although cells had

progressed further through S phase, as shown in the flow cytometry profile. By 2 hours after HU removal, flow cytometry revealed that most cells had acquired a 4C DNA content, indicating that they had reached the end of S phase, although some cells were still in S phase. A few cells had also exited S phase, as evidenced by the appearance of 2N2K cells as observed by DAPI staining. After 3 hours, significant numbers (~25%) of 2N2K cells appeared, indicating that the population had begun to undergo mitosis. Flow cytometry correspondingly showed a large and tight peak at 4C DNA content, indicating the majority of cells were in G_2 or M phase. A smaller peak at 2C DNA content indicated that a small proportion of cells had undergone cytokinesis and re-entered G_1 phase. At 4 hours after HU removal, approximately one third of the population were 2N2K cells, indicating more cells had undergone mitosis. The 2C peak also increased in size at this time point, and DAPI staining revealed the presence of over 50% 1N1K cells in the population, showing the progression of more cells through cytokinesis and into G_1 phase. Very few cells remained in S phase, as evidenced by 1N2K cells comprising less than 10% of the population, and very few cells with DNA contents between 2C and 4C were present in the flow cytometry profile (compare t=4 profile in Figure 2C with t=0 profile in Figure 1B). Hence, the cell population at this time was almost entirely in G_1 or G_2/M phases. At 5 hours after HU removal, flow cytometry showed the return of cells with DNA contents between 2C and 4C, indicating that a proportion of the 1N1K cells present at 4 hours had now entered S phase. The DAPI profile at 5 hours was not greatly different from that at 4 hours, but there was a decrease of 2N2K cells and a corresponding increase in 1N1K cells, showing the progression of more 2N2K cells through cytokinesis and back into G_1 phase. Finally, at 6 hours following HU removal, all cell cycle stages were represented in the population. Many cells had progressed far enough to re-enter S-phase, but there was still a strong 2C peak and some cells remained with a 4C DNA content. This is likely to be due to synchrony breaking down, causing a

broadening of the flow cytometry peak. Examination of the cell cycle progress of populations past this point confirmed the gradual breakdown of synchrony (data not shown). The data shown here were highly reproducible across three independent experiments (data not shown), with cell populations showing the same profile at the same time points.

As a further control to confirm that the 1N2K cells that accumulated following incubation with HU for 6 hours were in S phase, in accordance with their DNA content, and had not attempted to prematurely enter mitosis without having completed DNA replication, cells were analysed by immunofluorescence using the KMX antibody, which recognises β tubulin [19]. 1N2K and 2N2K cells were also examined for the presence of a mitotic spindle 3 hours after HU removal, when it was predicted, based on flow cytometry data that mitosis would have begun after the S phase block. Following 6 hours of HU treatment, no 1N2K cells contained a mitotic spindle, providing further evidence that these cells had arrested in S phase, but had not yet entered mitosis (Figure 3A and B). However, 3 hours after HU removal, 40% of 1N2K and 60% of 2N2K cells contained spindles (Figure 3A and B). This equated to 34% of the total cell population undergoing mitosis. Hence, the remainder of the 1N2K cells were likely to be still in G_2 phase (approximately 30% of the population), and the remaining 2N2K cells were about to, or were in the process of, undergoing cytokinesis.

Discussion

Here we have shown that incubation of bloodstream stage *T. brucei* Lister 427 strain cells with 10 μgml^{-1} HU for 6 hours induces an S phase block, which can be released upon removal of the HU, resulting in cells advancing through the cell cycle in a relatively synchronous manner. The accumulation of cells in S phase appears to occur by a mechanism highly similar to that described by Chowdhury *et al*. [15] in procyclic form cells, in that HU treatment slows but ultimately does not halt progression of the parasite cells through S phase. Although nuclear S-phase was slowed, kinetoplast replication was apparently unaffected. As Chowdhury *et al*. speculate, this may be due to different K_m values between nuclear and kinetoplast polymerases, or perhaps the smaller amount of DNA in the kinetoplast compared to the nucleus enables it to finish replication before the pool of dNTPs is exhausted. Kinetoplast division, driven by basal body segregation, is an essential pre-requisite for cytokinesis [22], but under the conditions of this study is not sufficient to drive cytokinesis since 1N2K cells persist for several hours without division, and do not divide until they have reached a 2N2K configuration. The 6 hour incubation period found to be optimal for HU-induced synchronisation in this study concurs with the mode of action generally proposed for HU, as it is roughly equal to the doubling time of the cell culture (calculated as 5.9 – 6.1 hours in our experiments). This would allow almost all cells to begin DNA replication and therefore be affected by their depleted dNTP pools. It is worth noting that this is half the exposure time used in a previous study of the effects of HU on bloodstream form trypanosomes, which concluded that HU was too toxic to be an effective synchronisation tool [14]. It should also be considered that although nuclear S phase progression is dramatically slowed, this may not be the case for other organelles and cell cycle processes. Only the fate of the kinetoplast and the nuclear spindle were assessed here following HU treatment, and it is not known whether other organelles continue to divide during HU treatment. Similarly, as little is known about the

processes and signalling networks that monitor DNA replication in *T. brucei*, we cannot rule out downstream effects from HU treatment. These issues are likely to be common to any synchronisation procedure, but the short incubation time and minimal dose of HU used here should minimise non-specific effects.

Using the protocol we have developed, we were also able to significantly enrich for cells in G_2 and M phases of the cell cycle (as determined by DNA content analysis). It is anticipated that by modifying the sampling time, the population enrichment could be fine-tuned more precisely. However, this is likely to depend closely on the strain used and its precise growth rate. Additionally, as the flow cytometry peak at any one time point covers a range of the cell cycle (as indeed was the case in [15]), it is questionable whether any population of cells obtained using this method is truly synchronised, although it is possible to dramatically enrich for a desired cell cycle stage. The only cell cycle stage this procedure could have difficulty enriching for is G_1. As cells start to pass through cytokinesis the population seems to lose much of its uniformity, which also limits the useful period of synchronisation to one cell cycle. This may be due to lesions caused by collapsed replication forks formed when replication stalls. The number of these per cell will be largely random, which will give a range of times before cells can fully repair their DNA and pass any checkpoint the damage initiates. This could perhaps be minimised by further fine-tuning the incubation time and concentration of HU used or perhaps exploring the use of sequential applications of HU, in a manner analogous to the double thymidine block employed to arrest mammalian cells at the G_1/S boundary [23].

This procedure provides obvious benefits for those studying the cell cycle of *T. brucei*, in particular for studying the differential expression, activity or localisation of regulatory proteins through the cell cycle. The ability to simply obtain a population of cells enriched for any one of several cell cycle stages makes HU synchronisation of the

bloodstream stage a valuable addition to the arsenal of molecular tools available for the study of *T. brucei*.

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Figure legends

Fig 1. Analysis of growth and cell cycle progression of bloodstream stage cells following HU treatment.

A: Cumulative growth curves of cells incubated with 0 (♦), 5 (■), 7.5 (▲), 10 (◊), 20 (□) or 50 (x) μgml^{-1} HU. B: Flow cytometry profiles of cells incubated with 10 μgml^{-1} HU over time (as indicated, in hours). The DNA content of peaks is given and the vertical lines show the positions of 2C and 4C DNA content in all profiles. C: Nuclei (N) and kinetoplast (K) configurations of cells sampled at the same times as those shown in B, as revealed by DAPI staining. D: Analysis of kinetoplast size in 1N1K cells before (▲) and after (□) a 6 hour incubation with 10 μgml^{-1} HU. N=100 for each sample. Example images of 1N1K cells with round/oval, v- and bone-shaped kinetoplasts are shown (scale bar represents 1 μm).

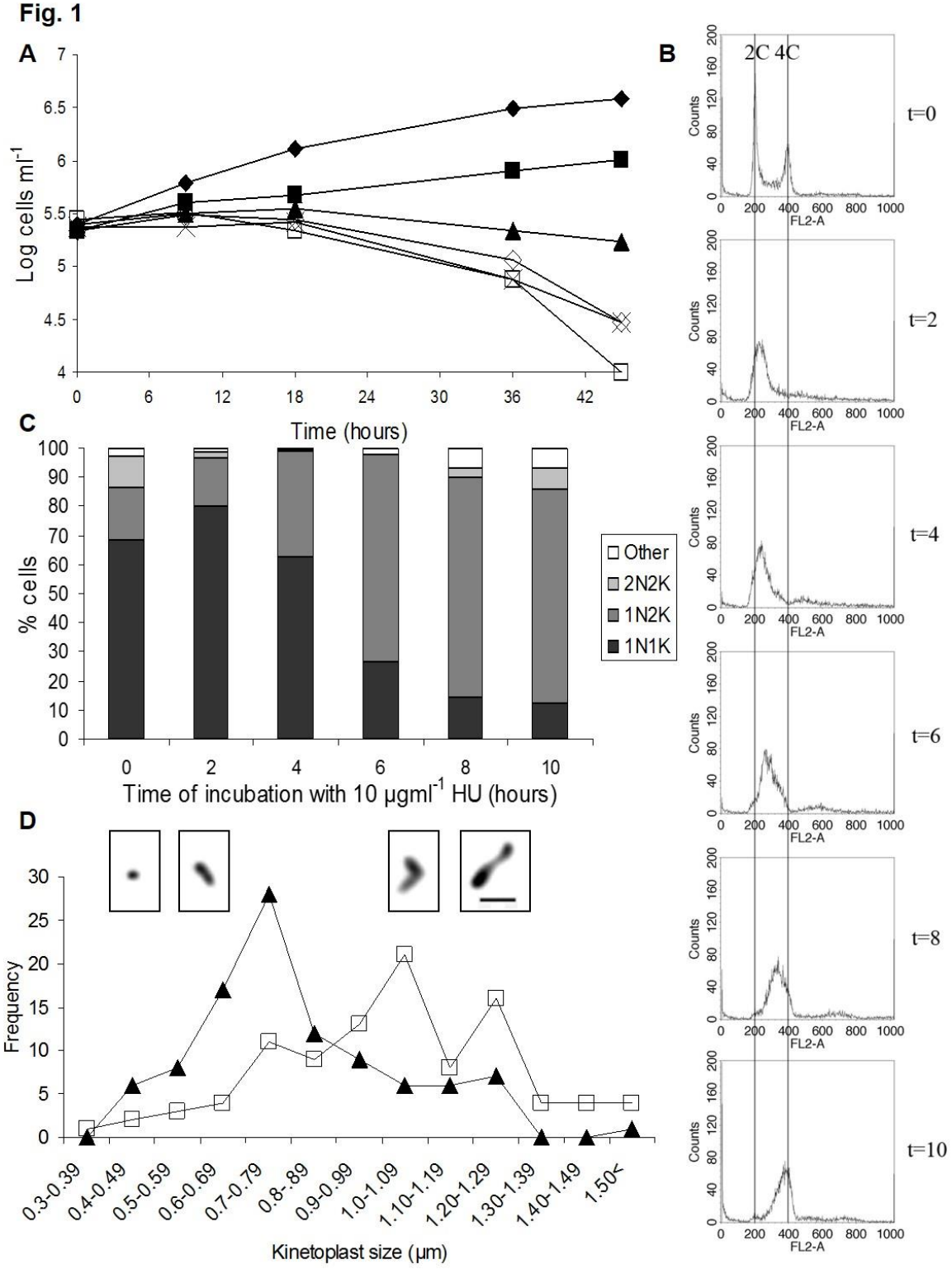
Fig 2. Recovery of bloodstream stage cells from HU treatment

A: Cumulative growth curves of cells during and after HU treatment. ♦: cells grown throughout in the absence of HU; ■: cells incubated with 10 μgml^{-1} HU for 6 hours before its removal. B: Flow cytometry profiles of cells following HU removal at the time points indicated (in hours). The DNA content of peaks is given and the vertical lines show the positions of 2C and 4C DNA content in all profiles. C: Nuclei (N) and kinetoplast (K) configurations of cells sampled at the same times as those shown in B, as revealed by DAPI staining.

Fig 3. Mitotic spindle analysis. A: Presence or absence of mitotic spindles in 1N2K and 2N2K cells at 0 and 3 hours following HU removal, as revealed by immunofluorescence with the KMX antibody. B: Example images of cells classified in (A). Left panels: DAPI

staining; middle panels: KMX staining; right panels: merged image. White arrows point to mitotic spindles.

Fig. 1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Log cells ml-1
Time (hours)
B
2C 4C
Counts
FL2-A
t=0
t=2
t=4
t=6
t=8
t=10
C
% cells
Other
2N2K
1N2K
1N1K
Time of incubation with 10 µgml-1 HU (hours)
D
Frequency
0.3-0.39
0.4-0.49
0.5-0.59
0.6-0.69
0.7-0.79
0.8-.89
0.9-0.99
1.0-1.09
1.10-1.19
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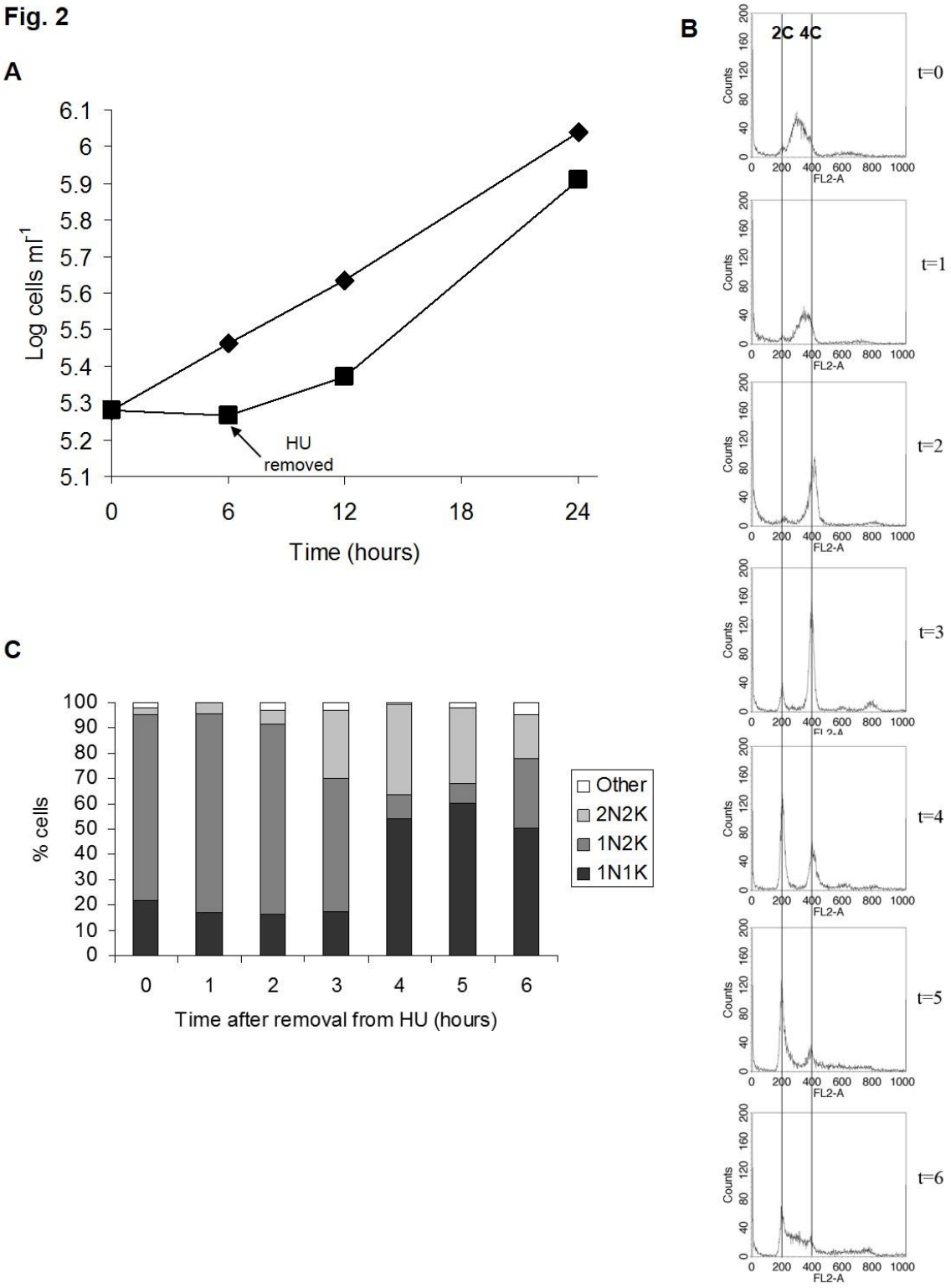
1.30-1.39
1.40-1.49
1.50<
Kinetoplast size (µm)

1

Fig. 2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Log cells ml-1
HU removed
Time (hours)
B
2C 4C
Counts
FL2-A
t=0
t=1
t=2
t=3
t=4
t=5
t=6
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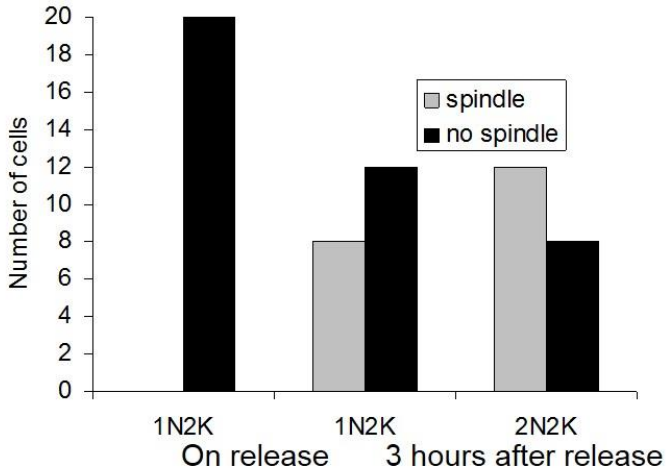
% cells
Other
2N2K
1N2K
1N1K
Time after removal from HU (hours)

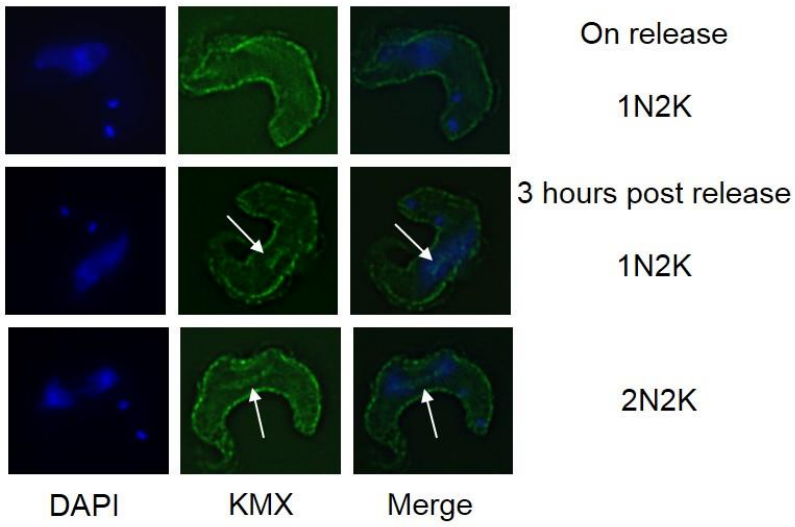
1

Fig. 3

A


20
18
16
14
12
10
8
6
4
2
0
Number of cells
spindle
no spindle
1N2K
1N2K
2N2K
On release
3 hours after release

B


On release
1N2K
3 hours post release
1N2K
2N2K
DAPI
KMX
Merge

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