PHYSIOLOGY

The Detection of Blood Contamination in Human Follicular Fluid

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Purpose: The aim of this study was to compare the reliability of the methods conventionally used to identify low levels of blood contamination in human follicular fluid (hFF) as applicable in the clinical environment.

Methods: Follicular fluid ($n = 339$) and plasma samples ($n = 20$) were collected from patients ($n = 138$) attending the Centre for Fertility Studies, HF Verwoerd Hospital, University of Pretoria, South Africa. hFF blood contamination was assessed by means of (a) visual inspection, (b) hematocrit (Hct), (c) spectrophotometric analysis, (d) spectrophotometric hemoglobin kit, and (e) Combur-9-test urine sticks.

Results: (1) Neither hematocrit nor spectrophotometry provided reliable detection at low levels of blood contamination. (2) Visual inspection presented with a better discriminatory ability than either Hct or spectrophotometry. (3) Combur-9-test sticks identified up to 50% of blood-contaminated fluids. (4) Spectrophotometrically determined hemoglobin levels presented with weak discriminatory abilities for detecting blood-contaminated fluids.

Conclusions: Visual inspection as performed in this study provides a fast and relatively reliable method for the determination of blood-contaminated hFFs. In a laboratory environment, however, it would be recommended that a combination of visual inspection, Hct, and spectrophotometric evaluation be employed for the selection of blood-free fluids.

KEY WORDS: human follicular fluid; blood contamination; spectrophotometry; visual inspection; urine dipsticks.

INTRODUCTION

Human follicular fluid (hFF) has an ever-growing potential for practical application in assisted reproductive programs (ARPs). It has been used in intrauterine insemination, gamete intrafallopian tube transfer (GIFT), and subzonal insemination techniques and as a transfer, preincubation, or acrosome reaction-inducing medium (1–5). hFF has also been used as a successful serum substitute for media supplementation (6). Significantly more oocytes reach maturity and a greater number of embryos develop to the morula and blastocyst stage when medium is supplemented with hFF in comparison to other serum supplementations (7,8). The beneficial effects of hFF addition to media are, however, still questioned by some (9,10).

The introduction of hFF in an ARP demands the selection of fluids which are beneficial for spermatozoa, oocyte development, sperm-oocyte interaction, fertilization, and embryo development. It would be preferable if the collected fluids were free from contaminants, such as blood and medium. Cyst-derived fluids should also be eliminated. Blood and medium contamination leads to altered hFF biochemical profiles with lower steroid concentrations, possibly resulting in observed variations with hFF experimentation (11). The pH of hFF has been shown to decrease significantly with an increasing degree of blood contamination (12). There are also indications that blood contamination has a detrimental effect on two-cell murine embryo development in comparison to blood-free fluids (Huyser *et al.*, unpublished findings).

Although some studies do not appear to screen hFF for blood contamination, it is accepted that such tests should be performed (13). Generally applied methods

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for detecting blood in hFF include visual inspection (14–17), hematocrit (Hct) (18), and haemoglobin (Hb) determination (19,20), as well as spectrophotometric analysis (11,21). The aim of this study was to compare the reliability of the existing methods for the detection of hFF blood contamination (red blood cells as well as hemoglobin) and to investigate the possibility of using simple alternatives such as visual inspection and the Combur-9-test urine sticks.

MATERIALS AND METHODS

Follicular fluid ($n = 356$) and plasma ($n = 20$) were collected from patients ($n = 138$) attending the Centre for Fertility Studies, HF Verwoerd Hospital, University of Pretoria, South Africa. The stimulation protocol and aspiration of hFF have been presented elsewhere (11). Follicular fluids supplemented with heparin or Earle's balanced salt solution medium (spectrophotometric absorbance peak at 562 nm; Phillips Pye Unicam SP800 spectrophotometer), or fluids that presented with a spectrophotometric peak absorbance at 407–411 nm (cyst fluids), were excluded from this study (21). The total number of fluids included in the study was 339, from 138 patients.

Five methods for hFF contamination with red blood cells and/or Hb content were investigated.

- (a) Visual inspection was graded into five categories, namely, clean, lightly cloudy, cloudy, lightly blood contaminated, and blood contaminated.
- (b) Hct levels were classified into three groups, namely, clean, red blood cells detectable but Hct not measurable, and red blood cells present and Hct determinable.
- (c) For spectrophotometric analysis, fluids were scanned from 400 to 600 nm, and absorbances determined at 415, 458, 540, and 576 nm [hemoglobin-contaminated fluids present, with a peak at ≈ 418 and 540 nm and/or 576 nm; relatively hemoglobin-free fluids present, with only one peak, at ≈ 458 nm (11)].
- (d) Hb content was determined with a spectrophotometry Hb test (Total Haemoglobin Analysis Kit, Catalog No. 535, Sigma, Bryanston, RSA).
- (e) Urine sticks (Combur-9-test; Boehringer Mannheim, RSA) were used, grading Hb contamination as $\leq 1+$, $> 1+$ and $\leq 2+$, or $> 2+$.

Once fluids were aspirated and scanned for oocytes, visual inspection was performed by the same skilled person throughout this study, followed by the determination of Hct levels (determined by Hawksley micro-hematocrit centrifuge, with nonheparinized capillary tubes; Medsurge, RSA). Fluids were thereafter centrifuged (800g, 10 min) and analyzed spectrophotometrically (scanned at 400–700 nm) for the presence of hemoglobin peak absorbances. The level of Hb was also determined (using the spectrophotometry Hb test). Finally, fluids which were judged visually clean were tested for Hb content using Combur-9-test sticks (donated by Boehringer Mannheim, RSA). Fluids were only assumed to be blood-free if no red blood cells were present (as determined by Hct) and no Hb peak maximum at the Soret band (412–418 nm) was observed, accounting for hFF red blood cell and/or hemoglobin contamination. Hematocrit determinations were also performed on collected plasma samples.

Data were analyzed by independent t , chi-square, Wilcoxon matched pairs, and Spearman rank correlation tests.

RESULTS

Results are presented in Tables I–IV. When either Hct or spectral detection was used to eliminate blood-contaminated fluids, both visual inspection and the Combur-9-test sticks appear to be more sensitive, in that both indicate blood contamination in a significant percentage of fluids classified as “clean” by the first two methods (Tables I and II). Neither Hct nor spectral analysis alone reliably detected blood-contaminated fluids, as 34% of red blood cell-free fluids (Table I) were spectrophotometrically blood contaminated, while 48% of spectrophotometrically clean fluids had red blood cells present prior to centrifugation.

When fluids were selected by a combination of Hct and spectrophotometric spectra (Table III), visual inspection presented with a stronger discriminatory ability than the Combur-9-test sticks. Visual inspection presented with a 82.26% sensitivity and a 88.81% specificity compared to fluids selected by Hct and spectrophotometry. The Combur-9-test showed a $\approx 90\%$ ability to differentiate correctly clean from blood-contaminated fluids, however, 50% of fluids that presented with a $\leq 1+$ value were blood contaminated.

Approximately 90% of fluids classified as clean by means of visual inspection (Table IV) were red blood cell-free, while $\approx 68\%$ of visually clean fluids were

Table II. The Ability of Follicular Fluid Spectrophotometric Spectral Analysis to Discriminate Between Blood-Contaminated and Blood-Free Fluids in Comparison to Visual Inspection, Hematocrit, Hemoglobin Content, or Urine Dipsticks^{a,*}

Spectrophotometry	Visual inspection (%)	Hematocrit (%)	Combur-9-test sticks (%)	Hemoglobin level (g/dl)
Clean: one peak at 458nm (<i>n</i> = 119)	1-47.06 ^a (<i>n</i> = 56) 2-13.45 (<i>n</i> = 16) 3-15.97 (<i>n</i> = 19) 4-20.17 (<i>n</i> = 24) 5-3.36 (<i>n</i> = 4)	1-52.10 ^a (<i>n</i> = 62) 2-45.38 (<i>n</i> = 54) 3-2.52 (<i>n</i> = 3)	1-81.05 ^a (<i>n</i> = 77) 2-18.95 (<i>n</i> = 18) 3-0.00 (<i>n</i> = 0)	0.0580 ± 0.0616 ^c (<i>n</i> = 119)
Blood contaminated: peak at 415 or 540 or 576 nm (<i>n</i> = 220)	1-11.82 ^b (<i>n</i> = 26) 2-5.45 (<i>n</i> = 12) 3-13.18 (<i>n</i> = 29) 4-25.45 (<i>n</i> = 56) 5-44.09 (<i>n</i> = 97)	1-14.61 ^b (<i>n</i> = 32) 2-53.42 (<i>n</i> = 117) 3-31.97 (<i>n</i> = 70)	1-35.21 ^b (<i>n</i> = 25) 2-54.93 (<i>n</i> = 39) 3-9.86 (<i>n</i> = 7)	0.0874 ± 0.0611 ^d (<i>n</i> = 220)

^a Visual inspection: 1 = clear; 2 = slightly cloudy; 3 = cloudy; 4 = slightly blood contaminated; 5 = blood contaminated. Hematocrit: 1 = no red blood cells present; 2 = red blood cells present but indeterminable; 3 = red blood cells present and determinable. Spectrophotometry: 1 = peak maximum absorbance at 458 nm; 2 = peak maximum at 415 and 540 and/or 576 nm.

* Superscripts a and b, $P < 0.0001$ (χ^2 test); superscripts c and d, $P = 0.000$.

Table I. The Ability of Follicular Fluid Hematocrit to Discriminate Between Blood-Contaminated and Blood-Free Fluids in Comparison to Visual Inspection, Spectrophotometric Spectral Analysis, Combur-9-Test Urine Sticks, and Hemoglobin Levels^{a,*}

Hematocrit	Visual inspection (%)	Spectrophotometry (%)	Combur-9-test sticks (%)	Hemoglobin level (g/dl)
Clean (<i>n</i> = 94)	1-78.72 ^a (<i>n</i> = 74) 2-7.45 (<i>n</i> = 7) 3-5.32 (<i>n</i> = 5) 4-6.38 (<i>n</i> = 6) 5-2.18 (<i>n</i> = 2)	1-65.96 ^a (<i>n</i> = 62) 2-34.04 (<i>n</i> = 32)	1-79.27 ^a (<i>n</i> = 65) 2-17.07 (<i>n</i> = 14) 3-3.66 (<i>n</i> = 3)	0.0664 ± 0.0772 ^c (<i>n</i> = 94)
Present and/or determinable (<i>n</i> = 245)	1-3.27 ^b (<i>n</i> = 8) 2-8.57 (<i>n</i> = 21) 3-17.55 (<i>n</i> = 43) 4-30.20 (<i>n</i> = 74) 5-40.41 (<i>n</i> = 99)	1-23.27 ^b (<i>n</i> = 57) 2-76.73 (<i>n</i> = 188)	1-50.00 ^b (<i>n</i> = 37) 2-44.59 (<i>n</i> = 33) 1-5.41 (<i>n</i> = 4)	0.0813 ± 0.0559 ^d (<i>n</i> = 245)

^a Visual inspection: 1 = clear; 2 = slightly cloudy; 3 = cloudy; 4 = slightly blood contaminated; 5 = blood contaminated. Spectrophotometry: 1 = peak maximum absorbance at 458 nm; 2 = peak maximum at 415 and 540 and/or 576 nm. Combur-9-test stick: 1 = ≤1+ on stick; 2 = >1+ and ≤2+ on stick; 3 = >2+ on stick.

* Superscripts a and b, $P < 0.0001$ (χ^2 test); superscripts c and d, $P = 0.0056$.

Table III. The Ability of Follicular Fluid Hematocrit in Combination with Spectrophotometric Spectral Analysis to Discriminate Between Blood-Contaminated and Blood-Uncontaminated Follicular Fluid in Comparison to Visual Inspection, Combur-9-Test Sticks, and Hemoglobin Levels^{a,*}

Hematocrit and spectrophotometric analysis	Visual inspection (%)	Combur-9-test sticks (%)	Hemoglobin level (g/dl)
Hematocrit and spectrophotometric analysis clean (<i>n</i> = 62)	1-82.26 ^a (<i>n</i> = 51) 2-6.46 (<i>n</i> = 4) 3-4.84 (<i>n</i> = 3) 4-4.84 (<i>n</i> = 4) 5-0.00 (<i>n</i> = 0)	1-92.86 ^a (<i>n</i> = 52) 2-7.14 (<i>n</i> = 4) 3-0.00 (<i>n</i> = 0)	0.0561 ± 0.0740 ^c (<i>n</i> = 62)
Hematocrit and/or spectrophotometric analysis blood contaminated (<i>n</i> = 277)	1-11.19 ^b (<i>n</i> = 31) 2-8.66 (<i>n</i> = 24) 3-16.25 (<i>n</i> = 45) 4-27.44 (<i>n</i> = 76) 5-36.46 (<i>n</i> = 101)	1-50.00 ^b (<i>n</i> = 50) 2-43.00 (<i>n</i> = 43) 3-7.00 (<i>n</i> = 7)	0.0818 ± 0.0591 ^d (<i>n</i> = 296)

^a Visual inspection: 1 = clear; 2 = slightly cloudy; 3 = cloudy; 4 = slightly blood contaminated; 5 = blood contaminated. Combur-9-test stick: 1 = ≤1+ on stick; 2 = >1+ and ≤2+ on stick; 3 = >2+ on stick.

* Superscripts a and b, $P < 0.0001$ (χ^2 test); superscripts c and d, $P = 0.004$.

Table IV. The Ability of Visual Inspection to Discriminate Between Blood-Contaminated and Blood-Free Follicular Fluid in Comparison to Follicular Fluid Hematocrit, Spectrophotometry, and Hemoglobin Content Determined by Spectrophotometric Analysis or Urine Dipsticks^{a,*}

Visual inspection	Hematocrit (%)	Spectrophotometry (%)	Combur-9-test sticks (%)	Hemoglobin level (g/dl)
Clean (<i>n</i> = 82)	1-90.24 (<i>n</i> = 74) 2-9.76 (<i>n</i> = 8) 3-0 (<i>n</i> = 0)	1-68.29 (<i>n</i> = 56) 2-31.71 (<i>n</i> = 26)	1-76.62 (<i>n</i> = 59) 2-20.78 (<i>n</i> = 16) 3-2.60 (<i>n</i> = 2)	0.0648 ± 0.0768 ^a (<i>n</i> = 82)
Lightly cloudy (<i>n</i> = 28)	1-25.00 (<i>n</i> = 7) 2-75.00 (<i>n</i> = 21) 3-0 (<i>n</i> = 0)	1-57.14 (<i>n</i> = 16) 2-42.85 (<i>n</i> = 12)	1-79.17 (<i>n</i> = 19) 2-20.83 (<i>n</i> = 5) 3-0.00 (<i>n</i> = 0)	0.0686 ± 0.0114 ^b (<i>n</i> = 28)
Cloudy (<i>n</i> = 48)	1-10.41 (<i>n</i> = 5) 2-85.42 (<i>n</i> = 41) 3-4.17 (<i>n</i> = 2)	1-39.58 (<i>n</i> = 16) 2-60.42 (<i>n</i> = 27)	1-45.45 (<i>n</i> = 20) 2-45.45 (<i>n</i> = 20) 3-9.09 (<i>n</i> = 4)	0.0751 ± 0.0505 (<i>n</i> = 48)
Lightly blood contaminated (<i>n</i> = 80)	1-7.50 (<i>n</i> = 6) 2-81.25 (<i>n</i> = 65) 3-11.25 (<i>n</i> = 9)	1-30.00 (<i>n</i> = 24) 2-70.00 (<i>n</i> = 56)		0.0765 ± 0.0733 (<i>n</i> = 80)
Blood contaminated (<i>n</i> = 101)	1-2.00 (<i>n</i> = 2) 2-36.00 (<i>n</i> = 36) 3-62.00 (<i>n</i> = 62)	1-3.96 (<i>n</i> = 4) 2-96.04 (<i>n</i> = 97)		0.0909 ± 0.0426 ^c (<i>n</i> = 101)

^a Hematocrit: 1 = no red blood cells present; 2 = red blood cells present but indeterminate; 3 = red blood cells present and determinable. Spectrophotometry: 1 = peak maximum absorbance at 458 nm; 2 = peak maximum at 415 and 540 and/or 576 nm. Combur-9-test stick: 1 = ≤1+ on stick; 2 = >1+ and ≤2+ on stick; 3 = >2+ on stick.

* Superscripts a and c, *P* = 0.005; superscripts b and c, *P* = 0.031.

spectrophotometrically clean. This pattern is reversed as fluids are visually judged to be progressively lightly cloudy to blood contaminated (Table IV). Spectrophotometrically determined Hb levels (g/dl) did not present with good discriminatory abilities, as levels overlap between clean and blood-contaminated groups (Tables I-IV).

Eighty-one fluids presented with determinable Hcts ($\bar{X}$ = 1.04 ± 0.45%; minimum = 0.44%; maximum = 2.44%). The degree of red blood cell contamination [expressed as a percentage of hFF (*n* = 33) to plasma (*n* = 20) Hct levels] never exceeded 5.03%, with a mean ± SD of 2.31 ± 1.02%. None of these fluids were judged visually clean or lightly cloudy (data not shown). This indicates that fluids with a Hct as low as 0.44% can be visually judged as being blood contaminated, being equivalent to <5% blood contamination of hFF as determined by Hct values.

Hemoglobin levels (g/dl) were determined in spectrophotometrically clean fluids (one peak maximum at ≈458 nm) and divided into three peak absorbance levels: absorbance of ≤0.400 AU (*n* = 37), >0.400–≤0.500 AU (*n* = 34), and >0.500 AU (*n* = 43). Hemoglobin levels were $\bar{X}$ = 0.044 ± 0.027, 0.049 ± 0.035, and 0.078 ± 0.089 g/dl, respectively. Levels were significantly lower (*P* = 0.030) in fluids with an absorbance of ≤0.400 AU in comparison to fluids with an absorbance of >0.500 AU.

DISCUSSION

Fluid selection forms the basis for controlled experimental hFF incorporation into ARPs. In view of the growing applications for hFF in ARPs, as well as the possible effects of blood contamination on various aspects of ARPs, it is important to establish the reliability of the methods generally employed for the detection of blood contaminated hFF and to identify inexpensive, rapid, and reliable techniques for this purpose.

Various methods for detecting blood-contaminated fluids have been used in the past, however, none of these techniques consider Hb and red blood cell contamination together. The findings of this study indicate that neither Hct nor spectrophotometry, used on their own, provides reliable information on blood-contaminated fluids, as one does not necessarily account for the other. Visual inspection alone, on the other hand, demonstrated a higher sensitivity for blood-free fluids than either Hct or spectrophotometry, as ≈90% of fluids that are judged visually clean present without red blood cells, and ≈68% are spectrophotometrically clean (Table IV).

When the combination of Hct and spectrophotometric spectral evaluations is taken as the gold standard (assumed to be blood-free fluids in this study), visual inspection presents with a higher discriminatory ability than the other variables tested. Visual inspection, however, led to the elimination of ≈20% of clean fluids

and/or the selection of $\approx 11\%$ mildly blood-contaminated fluids.

Combur-9-test sticks did not present with any advantage over visual inspection for the detection of blood-contaminated fluids, probably due to the intervals used for the Combur-9-test sticks in this study ($\leq 1+$ includes Hb contaminated fluids) and its high sensitivity for Hb. Spectrophotometrically determined Hb levels (g/dl), on the other hand, do not appear to have the required sensitivity for determining blood-contaminated fluids, as large areas of overlapping values for all the intervals tested were noted. Furthermore, clean hFF with higher absorbances at 458 nm (>0.500 AU) present with significantly higher Hb levels in comparison to fluids with lower absorbances (≤ 0.400 AU), indicating that a higher absorbance at 458 nm can conceal absorbance peaks at 418 nm. Spectrophotometric evaluation, however, has been found to be a useful tool in eliminating medium-contaminated (11) and cyst-derived fluids (21).

In summary, it can be said that visual inspection as performed by a capable observer using the criteria of this study, in comparison to the other methodologies investigated, provides a fast and relatively reliable method for the identification of blood-contaminated hFFs. The advantages of visual inspection over the other methods investigated in this study are (a) rapid evaluation, (b) no cost, (c) greater discriminatory ability than either Hct or spectrophotometry alone, (d) visual elimination of all fluids with more than 5% red blood cell contamination, and (e) visual identification of $\approx 80\%$ of clean fluids (red blood cell-free and spectrophotometrically clean). Visual inspection, however, is not as reliable as a combination of Hct and spectrometric evaluation in determining blood-contaminated fluids and is observer dependent. In the laboratory environment it would therefore be recommended that a combination of visual inspection, Hct, and spectrophotometric evaluation be employed for blood-free fluid selection.

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