

# Impression Cytology for Detection of Vitamin A Deficiency

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• Vitamin A (retinol) deficiency causes blindness, increased morbidity, and mortality among preschool children in many developing nations. Previous studies suggest that impression cytology may represent the first simple, reliable test to detect mild xerophthalmia in young children. We used impression cytology to evaluate and follow up 75 Indonesian preschool children with mild xerophthalmia and an equal number of age-matched, clinically normal neighborhood controls. Results of impression cytology, which were closely correlated with baseline serum vitamin A levels, documented histologic improvement following treatment with vitamin A. Furthermore, results of impression cytology, where abnormal, improved to normal following vitamin A treatment in a significant percentage (23%) of otherwise clinically normal children. Impression cytology appears to detect clinical and physiologically significant preclinical vitamin A deficiency.

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Xerophthalmia, or vitamin A (retinol) deficiency, is the major cause of childhood blindness in many developing countries.<sup>1,2</sup> Mild xerophthalmia (manifested by night blindness, conjunctival xerosis, and/or Bitot's spots) is associated with increased morbidity and mortality.<sup>3\*</sup> Vitamin A supplementation of nonxerophthalmic children reduces mortality, suggesting that subclinical vitamin A deficiency (ie, vitamin A deficiency without ocular manifestations of xerophthalmia) is also associated with increased mortality.<sup>7</sup> The need exists for a relatively simple, objective test of vitamin A status to identify communities in which vitamin A deficiency is prevalent and constitutes a public health problem. Previous studies demonstrated the ability of conjunctival impression cytology to distinguish between a small number of seemingly normal children and those with mild xerophthalmia.<sup>8</sup> We report herein the preliminary results of a much larger prospective, clinical trial correlating the results of conjunctival impression cytology with indexes of clinical and subclinical vitamin A deficiency.

## SUBJECTS AND METHODS

The study was performed in central Java at the Cicendo Eye Hospital, Bandung, Indonesia. Children with mild xerophthalmia, ie, a history of night blindness or the presence of conjunctival xerosis with Bitot's spots, were identified in surrounding villages by a trained nurse. Whenever a case was identified, an age-matched control from the same village was also identified. All subjects (patients and controls) were brought to the hospital where they were examined by one of two ophthalmologists to confirm the presence of Bitot's

spots in patients and normal eyes in controls. Participation in the study was limited to children aged 36 to 72 months. Seventy-five patients and 74 age-matched controls were identified.

A dietary and disease history was obtained from the parent or guardian of each child. After obtaining parental consent, each child underwent baseline evaluation consisting of an ocular examination, anthropometry, biochemical studies, and impression cytology. All ocular findings were drawn and photographed. Venous blood was obtained and promptly separated, and the serum frozen. Serum vitamin A was analyzed by high-performance liquid chromatography.<sup>9</sup>

Conjunctival impression cytology was performed on each patient using a previously described technique.<sup>8</sup> In summary, this technique consists of applying 5 × 5-mm pieces of cellulose acetate filter paper (HAWP 304F0, Millipore Corp, Bedford, Mass) to the nasal and temporal bulbar conjunctiva of each eye after application of topical 0.5% proparacaine hydrochloride. The filter paper was gently applied to the eye for 3 to 5 s and then removed with a peeling motion. The filter paper with the adherent epithelial cells was immediately placed in a fixative solution prepared by mixing 70% ethyl alcohol, 37% formaldehyde, and glacial acetic acid in a 20:1:1 volume ratio.

Specimens were collected separately from the nasal and temporal quadrants of each eye. After fixation, the specimens were stained with periodic acid-Schiff (PAS) and modified Papanicolaou's stain as described previously.<sup>10</sup> All impression cytologic specimens were examined in masked fashion and staged according to the degree of squamous metaplasia as previously described (Table 1).<sup>8,10</sup> Each child was assigned to the lowest stage (ie, the most normal) found among the four specimens (Fig 1).

All patients received at least 200 000 IU



oral vitamin A within one week of collection of baseline specimens, as part of a concurrent therapeutic trial.

All children were reexamined one week after their baseline examination. All children then received a second capsule containing 200 000 IU of vitamin A. Follow-up ocular examinations with impression cytology were performed one week, two months, and six months after receiving the second capsule. One hundred twenty-one children (81% of the original sample) remained throughout the six-month follow-up period. Examiners were masked at each examination as to treatment group, previous diagnosis, impression cytologic results, and baseline serum vitamin A level.

For statistical analyses, we used Student's *t* test for the difference between means and a nonparametric test for monotonic trends.

### RESULTS

#### Baseline Serum Vitamin A Levels

Serum vitamin A levels were obtained at baseline in all but one of the 149 subjects. Levels of serum vitamin A decreased monotonically with increased severity of cytologic abnormality ( $P < .002$ ) (Table 2). Stages 0 and 1, representing normal conjunctival impressions, were associated with mean serum vitamin A levels greater than  $0.70 \mu\text{mol/L}$  ( $20 \mu\text{g/dL}$ ). Each abnormal stage, ie, stages 2 through 5, was associated with a mean serum vitamin A level significantly less than  $0.70 \mu\text{mol/L}$  ( $20 \mu\text{g/dL}$ ) ( $P < .025$ ). This trend persisted even after separating the subjects, according to clinical criteria, into patients and controls ( $P < .002$ ) (Tables 3 and 4). All subjects were reclassified into two cytologic groups, normal (stage 0 or 1) and abnormal (stages 2 through 5). These two groups showed the same highly significant correlation with mean serum vitamin A levels ( $P < .0001$ ) (Table 5).

#### Response to Vitamin A

One hundred twenty-one children were followed up for six months after receiving orally at least 200 000 IU of vitamin A. An equal number (14) of patients and controls were unavailable for follow-up evaluation during this period.

Almost all cytologic scores improved following treatment. Fifty-six subjects (42 patients, 14 controls) entered the study with abnormal results of conjunctival impression cytology (stages 2 through 5) and were followed up for the full six months. Fifty-three subjects (95%) (40 patients, 13 controls) returned to normal (stage 0 or 1) following vitamin A therapy. Three subjects had abnormal

Table 1.—Staging of Conjunctival Squamous Metaplasia\*

Stage	Criteria
0	Abundant goblet cells and mucin spots, small epithelial cells
1	Fewer goblet cells and mucin spots, small epithelial cells
2	Loss of goblet cells and mucin spots, enlarging epithelial cells
3	Enlarging and separating epithelial cells
4	Large, separate epithelial cells with scattered keratinization and pyknotic nuclei
5	Large keratinized epithelial cells with pyknotic nuclei or loss of nuclei

\* Adopted from Tseng.<sup>10</sup>

Table 2.—Comparison of Mean Serum Vitamin A Levels and Results of Conjunctival Impression Cytology in All Subjects

Stage	No. of Subjects	Serum Vitamin A Level, $\mu\text{mol/L}$ ( $\mu\text{g/dL}$ )		
		Mean*	SD	Range
0	39	0.83 (23.7)	0.16 (4.6)	0.56-1.19 (16-34)
1	44	0.73 (20.6)	0.21 (5.9)	0.35-1.26 (10-36)
2	21	0.56 (15.9)	0.17 (5.0)	0.10-0.87 (3-25)
3	29	0.54 (15.4)	0.15 (4.2)	0.31-0.80 (9-23)
4	11	0.52 (14.9)	0.14 (4.1)	0.28-0.84 (8-24)
5	4	0.39 (11.2)	0.13 (3.6)	0.28-0.56 (8-16)

\*  $P < .002$  for monotonically decreasing trend.

Table 3.—Comparison of Mean Serum Vitamin A Levels and Results of Conjunctival Impression Cytology in Patients With Mild Xerophthalmia

Stage	No. of Patients	Serum Vitamin A Level, $\mu\text{mol/L}$ ( $\mu\text{g/dL}$ )		
		Mean*	SD	Range
0	11	0.82 (23.6)	0.21 (6.1)	0.56-1.19 (16-34)
1	15	0.66 (18.9)	0.17 (5.0)	0.35-0.91 (10-26)
2	13	0.52 (15.0)	0.19 (5.3)	0.10-0.84 (3-24)
3	24	0.54 (15.4)	0.14 (3.9)	0.31-0.80 (9-23)
4	8	0.49 (13.9)	0.12 (3.3)	0.28-0.70 (8-20)
5	4	0.39 (11.2)	0.13 (3.6)	0.28-0.56 (8-16)

\*  $P < .002$  for monotonically decreasing trend.

Table 4.—Comparison of Mean Serum Vitamin A Levels and Results of Conjunctival Impression Cytology in Controls

Stage	No. of Controls	Serum Vitamin A Level, $\mu\text{mol/L}$ ( $\mu\text{g/dL}$ )		
		Mean*	SD	Range
0	28	0.83 (23.7)	0.14 (4.0)	0.59-1.15 (17-33)
1	29	0.76 (21.9)	0.22 (6.2)	0.35-1.26 (10-36)
2	8	0.61 (17.5)	0.15 (4.2)	0.45-0.87 (13-25)
3	5	0.54 (15.4)	0.21 (5.9)	0.31-0.77 (9-22)
4	3	0.62 (17.7)	0.19 (5.5)	0.49-0.84 (14-24)

\*  $P < .002$  for monotonically decreasing trend.

Table 5.—Mean Serum Vitamin A Levels of All Subjects With Normal Results vs All Subjects With Abnormal Results of Conjunctival Impression Cytology

Status	No. of Subjects	Mean ( $\pm$ SEM) Serum Vitamin A Level, $\mu\text{mol/L}$ ( $\mu\text{g/dL}$ )
Normal (stage 0 or 1)	83	$0.78 \pm 0.19$ ( $22.2 \pm 5.5$ )
Abnormal (stages 2-5)	65	$0.53 \pm 0.16$ ( $15.2 \pm 4.5$ )

\*  $P < .0001$  for difference between means.



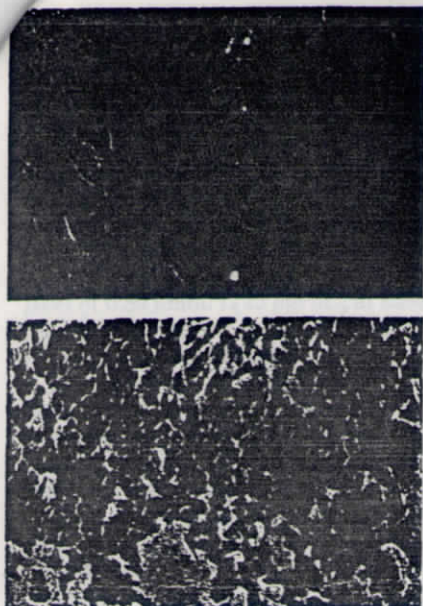


Fig 1.—Top, Specimen from nasal quadrant of right eye is typical of stage 4, exhibiting loss of all goblet cells and enlarged, separated epithelial cells (periodic acid-Schiff and modified Papanicolaou's stain, X100). Bottom, Specimen from nasal quadrant of left eye of same patient. Specimen is typical of stage 1, exhibiting goblet cells and normal epithelial cells (periodic acid-Schiff and modified Papanicolaou's stain, X100). Child was therefore graded stage 1 (normal) overall.

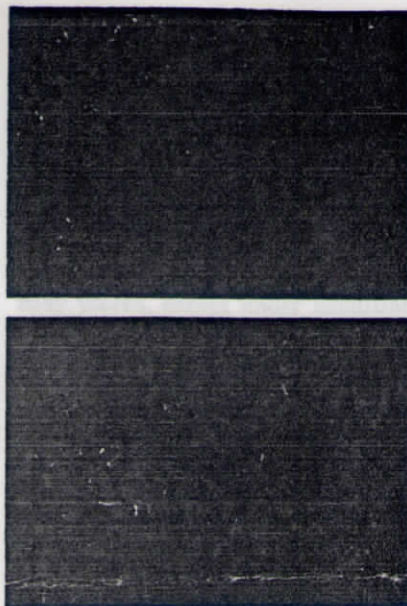
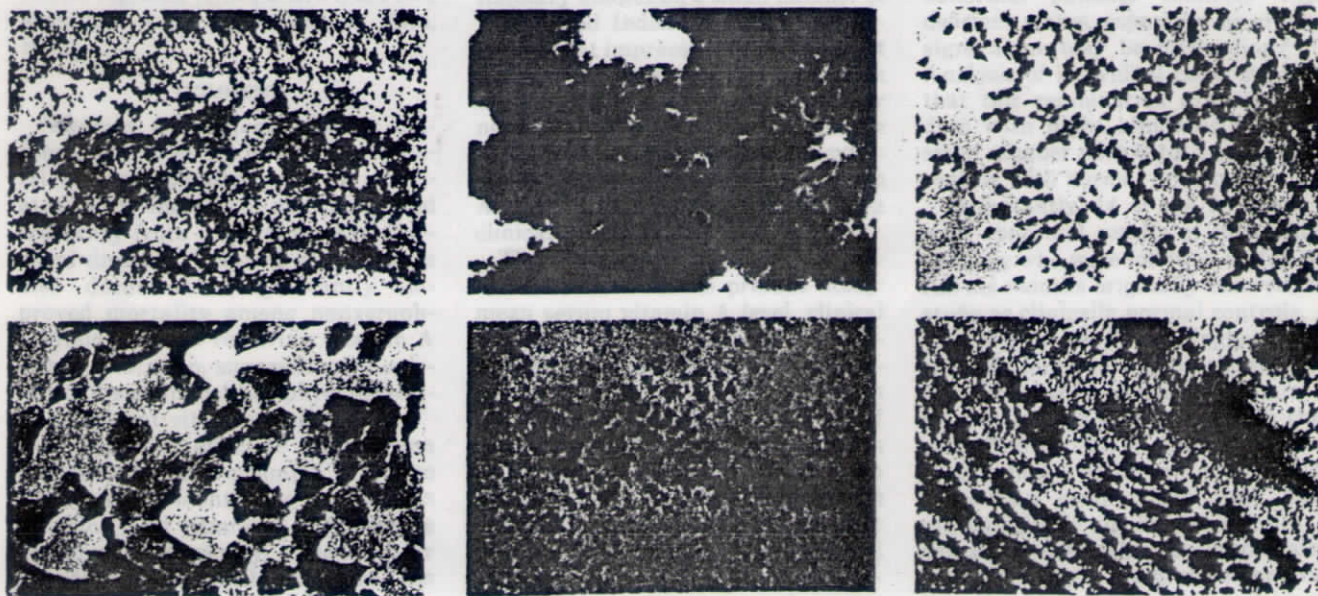


Fig 2.—Top left, 42-month-old child with night blindness and Bitot's spots entered study at stage 5 with marked epithelial cell enlargement, loss of nuclei, and keratinization (periodic acid-Schiff [PAS] and modified Papanicolaou's stain, X100). Top right, Two weeks after receiving vitamin A therapy, results of impression cytology had improved to stage 4, characterized by enlarged, nucleated epithelial cells and patchy keratinization (PAS and modified Papanicolaou's stain, X100). Bottom left, Two months after treatment, results of impression cytology continued to improve, showing mixed picture with some persistent, enlarged epithelial cells, some smaller, more normal epithelial cells, and, most importantly, PAS-positive smears representing mucin from returning goblet cells (PAS and modified Papanicolaou's stain, X100). Bottom right, Six months after treatment, impression cytologic specimen is typical of stage 0, exhibiting small, normal epithelial cells, goblet cells, and abundant mucin spots indicating abundant goblet cells (PAS and modified Papanicolaou's stain, X100).

Fig 3.—Top left, Normal conjunctival impression with abundant goblet cells, sheets of small epithelial cells, and mucin spots (periodic-acid Schiff [PAS] and Harris' hematoxylin, X160). Top center, Higher power of normal conjunctiva, showing contrast between PAS-positive goblet cells and epithelial cells (PAS and Harris' hematoxylin, X400). Top right, Abnormal conjunctival impression with complete loss of goblet cells and mucin spots, along with appearance of enlarged epithelial cells (PAS and Harris' hematoxylin, X100). Bottom left, Higher power of abnormal, enlarged conjunctival cells (PAS and Harris' hematoxylin, X400). Bottom center, PAS-positive mucin spots representing "impressions" of goblet cells on conjunctival surface (PAS and Harris' hematoxylin, X400). Bottom right, Results of impression cytology from normal child showing transition from abundant normal epithelium (lower left) to abnormal epithelium (upper right). Specimen was graded as normal (PAS and Harris' hematoxylin, X100).





results at all examinations, but two of these subjects improved from stage 5 to stage 2 at two months and from stage 4 to stage 2 at two months, respectively. Both subjects had low baseline serum vitamin A levels (0.52 and 0.56  $\mu\text{mol/L}$  [15 and 16  $\mu\text{g/dL}$ ], respectively), and both had dropped to stage 3 by six months. The third subject (control with a serum vitamin A level of 0.80  $\mu\text{mol/L}$  [23  $\mu\text{g/dL}$ ] at baseline) was at stage 2 initially, dropping to stage 3 at six months.

The rapidity of response to oral vitamin A treatment varied, with improvement requiring from as little as two weeks to more than two months (Fig 2). Seventeen of the 53 vitamin A-responsive patients were still at stage 2 at the two-month examination. Fourteen of these 17 patients subsequently improved to stage 0 or 1 by six months. Therefore, 95% of the patients who started with abnormal results of conjunctival impression cytology reverted to normal at some point during the six months following large-dose vitamin A treatment. Interestingly, ten of these 53 responsive subjects relapsed and were again abnormal by six months despite having received vitamin A. An additional eight of 83 subjects who entered the study with normal results of impression cytology became abnormal by six months. This finding is consistent with other observations that a single large dose of vitamin A is inadequate to protect all individuals in a deficient population for a full six months.<sup>11</sup> In fact, in this series the serum vitamin A levels had returned to baseline between two and six months regardless of the therapeutic regimen (G.N., J.R.W., K.P.W., Muhilal, A.S., unpublished data, 1987).

#### COMMENT

Vitamin A deficiency among preschool children in developing countries causes blindness and is associated with increased morbidity and mortality from respiratory and diarrheal diseases.<sup>16</sup> Recent reports have also documented significantly improved mortality among nonxerophthalmic children receiving vitamin A supplementation, suggesting the existence of subclinical but physiologically significant vitamin A deficiency.<sup>7</sup>

Initiation of effective intervention programs requires a simple, objective technique for identifying populations in which mild vitamin A deficiency is prevalent. Presently, the ocular changes of xerophthalmia are the most accessible physiologic indicator

of vitamin A status. Measurement of serum vitamin A levels requires invasive sampling procedures, sophisticated equipment, highly trained personnel, and methods for preparing, storing, and transporting delicate samples that are impractical for most field surveys in developing regions of the world. Serum vitamin A levels also suffer from poor correlation with body stores, except under conditions of severe depletion, and are not a direct indicator of individual physiologic status.<sup>12</sup> Clinical surveys for xerophthalmia assess physiologic status but require large sample sizes because of the low prevalence of detectable disease. Furthermore, they miss individuals with subclinical deficiency. Impression cytology provides a means for partially overcoming many of these limitations.

Vitamin A is essential for the proper differentiation and maintenance of mucosal epithelium.<sup>13,14</sup> Absence of vitamin A causes loss of goblet cells and keratinizing metaplasia of the epithelium.<sup>13,14</sup> The process occurs on mucosal surfaces of the respiratory, urinary, and gastrointestinal tracts as well as diffusely throughout the bulbar conjunctiva.<sup>15-18</sup> Furthermore, biopsy specimens have already shown that conjunctiva undergoing squamous metaplasia may appear normal clinically.<sup>18</sup>

Impression cytology permits atraumatic sampling of superficial conjunctival epithelial cells for histologic examination.<sup>19</sup> Impression cytology detected the early disappearance of goblet cells and the appearance of enlarged epithelial cells in the vitamin A-deficient rabbit model.<sup>20</sup> Preliminary studies on a small number of Indian and Indonesian children suggested that impression cytology might distinguish between children with mild xerophthalmia and seemingly normal controls.<sup>8</sup> This large-scale study confirms that impression cytology is closely correlated with vitamin A status and is more sensitive than a clinical ocular examination.

Three indicators of vitamin A status were used in the present study: mean serum vitamin A level, clinical xerophthalmia, and response to vitamin A treatment. Staging by impression cytology correlated directly with the mean serum vitamin A level of subjects in each stage. The change in mean serum vitamin A level from greater than 0.70  $\mu\text{mol/L}$  (20  $\mu\text{g/dL}$ ) to less than 0.70  $\mu\text{mol/L}$  (20  $\mu\text{g/dL}$ ) at the transition between stages 1 and 2 suggests that we can collapse our earlier six-stage classification into two

stages: normal (0 to 1) and abnormal (2 through 5), based primarily on the presence or absence of goblet cells or evidence of their presence (mucin droplets).

An individual was considered *normal* if any area of any specimen obtained from either eye demonstrated a substantial proportion of normal epithelium with evidence of goblet cells. This criteria is meant to compensate for well-documented variations of goblet cell density across the conjunctival surface.<sup>21</sup> (Fig 3, bottom right, demonstrates one such area of transition from normal to abnormal epithelium in a normal, vitamin A-sufficient child.)

Collapsing the staging into *normal* and *abnormal* based on the presence or absence of goblet cells also facilitates specimen processing and evaluation. The modified Papanicolaou's stain needed for detailed staging of the degree of keratinization is no longer necessary, and personnel need only be trained to recognize the presence or absence of goblet cells. Furthermore, specimens with poor epithelial cell adherence may still reveal the presence of goblet cells by the presence of multiple, discrete mucin droplets staining PAS-positive (Fig 2, bottom left, and Fig 3, bottom center). These droplets correspond to goblet cells on the conjunctival surface.<sup>22</sup>

Following vitamin A treatment, impression cytology detected improvement in vitamin A status in 95% of the subjects who had abnormal results at baseline examination. This response to vitamin A coupled with the significantly lower mean serum vitamin A level of the cytologically abnormal patients confirms their deficient status, especially as clinical signs alone may be misleading.<sup>2,3,18,23</sup> Analysis of individual cases suggests that this combination explains children with night blindness (and the small number with Bitot's spots) presenting with normal imprints.

Importantly, 14 of the 56 subjects with abnormal results of impression cytology who were followed up for the full six months originally entered the study as clinically normal controls. A total of 14 of the 60 seemingly normal controls followed up for six months therefore had evidence of vitamin A deficiency. This finding suggests that up to 23% (14/60) of clinically nonxerophthalmic children in similar Indonesian communities may have physiologically significant vitamin A deficiency detectable by impression cytology. This is the first demonstration that a significant proportion of



seemingly normal children are suffering metabolic consequences of vitamin A deficiency and may be at increased risk of ocular and systemic consequences of vitamin A deficiency. This finding may well explain the marked reduction in mortality among non-xerophthalmic children receiving vitamin A supplementation.<sup>7</sup> It is consistent with earlier observations that neighborhood controls of xerophthalmic patients are likely to be vitamin A deficient.<sup>23</sup>

The rate of response to oral vitamin A, as measured by impression cytology, varied from two weeks to more than two months. This variation is consistent with that noted in previous clinical studies describing patients

with conjunctival xerosis or Bitot's spots requiring two weeks to more than two months to return to normal.<sup>24</sup>

Thus, impression cytology detects early, physiologically significant vitamin A deficiency. The technique is well suited for population surveys to determine a community's vitamin A status. Specimens are obtained easily and atraumatically, can remain in fixative indefinitely, and require only an ordinary microscope for interpretation. Prevalence rates of abnormal cytologic findings are likely to be manifold that of clinical disease, drastically reducing sample size requirements. Simpler staining techniques to determine the presence or

absence of goblet cells will facilitate objective determinations of vitamin A status. Staining with PAS and Harris' hematoxylin is simpler to perform and improves recognition of PAS-positive goblet cells and mucin spots (Fig 3).<sup>20</sup> A large-scale field evaluation utilizing this modified staining process is presently in progress.

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