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## A comparative study of iron-related metabolic parameters in the eye of three animal species

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In order to obtain a wider perspective of iron homeostasis in the eye, a comparative study was undertaken of several iron-related parameters (Total Iron, TI; Total Iron Binding Capacity, TIBC; Transferrin, Tf; and saturation of Tf) both in blood serum and in ocular tissues (lens, cornea, iris-ciliary body, retina) and fluids (vitreous body, aqueous humor) of several animal species (pig, cow and rat). The relative degree of oxidative stress of tissues and fluids was evaluated based on the criteria that high values of TI and Tf saturation, and low values of TIBC and Tf, would promote iron-related oxidative stress. The inclusion of both diurnal (pig, cow) and nocturnal (rat) animal species in this comparative work provided the opportunity to explore if iron homeostasis parameters are in some way influenced by the higher oxidative stress level expected in animals with diurnal living habits.

This project involved also the design of new and very sensitive methods of analyses, given the fact that in many cases very small amounts of sample (i.e., aqueous humor), and/or low concentration of analytes (i.e., transferrin) are available.

All results were expressed as concentration relative to mg protein as determined with the Bradford method (microplate assay). When analyzing TI/TIBC it was possible to define a loosely bound iron pool (LBIP) in ocular tissues that was proportional to the degree of vascularization of the tissues analyzed. The comparison of iron related parameters patterns within the eye and among species allowed us to reach the following

conclusions: 1) The aqueous humor and vitreous body of cow and pig exhibited highest concentration of Tf and a very low saturation of Tf, while the lowest value of Tf was detected in all species in the lens; 2) TI showed the tendency to be highest in the vitreous body of cow and pig, and lowest in the lens of all three species. The very low iron concentration in the lens may effectively counteract the risk of having a low Tf level; 3) The pattern of distribution of TIBC is the same for the cow and the pig; 4) Saturation of Tf was very high in the rat aqueous, also high in the rat lens and serum; 5) Highest differences in the pattern of distribution of iron parameters in the eye among the three species studied were found between the rat and the other two species, suggesting that the nocturnal habit of living is influencing iron related parameters in the rat. Lacking the full effect of UV light on the eye, the rat apparently lives exposed to high risk iron parameters without overt pathology, but its antioxidant reserves must be minimal. For example, the combination of high TI aqueous with relatively low Tf, gives a dangerous high saturation of Tf (82%) in the rat; 6) The posterior part of the lens is exposed to high levels of iron and Tf in diurnal animals, with saturation of Tf in the vitreous in the 40% level. This could facilitate postvitrectomy cataracts in addition to the documented increase in vitreous level of oxygen under those conditions.

*Key words: Ocular iron homeostasis, Ocular tissues, Aqueous humor, Vitreous body, Transferrin, Total iron binding capacity, Diurnal/nocturnal animals, Postvitrectomy cataract*

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### Oxidative Stress in the Eye

**A**lthough the main function of light in the eye is initiating the visual process, the transparency of eye tissues allows not only visible light but more harmful wavelengths in the UV and IR range invade the interior of the eye, making intraocular tissues and fluids

vulnerable to radiation from the environment (1). This factor may influence the level of reactive oxygen species (ROS) including free radicals produced in ocular tissues leading to ocular damage. Levels of oxidative stress higher than normal (2) could thus be present in the eye (3-4).

The presence of metal ions available to react with some ROS represents a serious complication, since it opens new avenues for the formation of the most reactive species, the hydroxyl radical ( $\cdot\text{OH}$ ), through the iron-catalyzed Haber-Weiss reaction and the Fenton reaction (5). The hydroxyl radical has a very short half-life and, due to its reactivity, it is near impossible for any antioxidant to have

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the opportunity to neutralize it before the radical damages neighboring molecules (6).

### **Iron and Iron Binding Proteins in the Eye**

In considering iron biochemistry in the eye, the work of McGahan and collaborators represents a fundamental contribution. Their early work (7) brought a needed reevaluation of the levels of iron in ocular compartments, since previously there was a tendency to report inconsistent, very high concentrations of this metal. These authors attributed existing problems to the techniques used for the determination of iron and reported more reliable iron concentration values in the serum and the aqueous humor and vitreous body of several animal species, using an atomic absorption spectroscopic (AAS) method. Total Iron Binding Capacity (TIBC) and saturation of Tf values were also included in the publication.

In spite of these new tools and the growing interest in ocular oxidative stress, information about iron and iron related proteins in ocular tissues remained deficient (8), and was not often provided in a systematic way but mostly as isolated findings. For example, if no simultaneous determinations are made of TI and TIBC, it is not possible in general to estimate the % saturation of Tf (a very informative parameter) in many cases, unless a direct estimation of Tf is also made by an alternative procedure.

The need for a systematic work in the eye was evident in order to be able to establish the potential existence of oxidative stress and to evaluate if the tissues and fluids within the eye were differentially affected.

Another line of reasoning also stresses the fact that a better knowledge of iron homeostasis parameters in ocular tissues and fluids would be worth pursuing. Cortés-Velázquez and García-Castiñeiras (9) found a progressive specific enrichment in Tf in the aqueous humor of patients undergoing cataract surgery, as the cataracts become severe. This finding could provide an interesting link between cataracts and the hypothesis that oxidative stress is in some manner involved. Also, calculations made on the data of Fong, et al. (10) indicate that iron concentration in the aqueous humor of glaucoma patients may be elevated beyond total saturation of Tf in this condition.

This work is directed to start filling that gap and to specifically address the hypothesis that photo-oxidative stress could be differentially reflected in iron related parameters of ocular tissues and fluids. As a first approach, we expect an increase in total iron (TI), a low total iron binding capacity (TIBC), a low transferrin (Tf) concentration, and high % Tf saturation under conditions of iron-related oxidative stress.

Behavioral aspects (diurnal/nocturnal living habits) of the different species studied will be considered in this work since more oxidative stress is expected in animals with diurnal living habits. This project involved also the design of new and very sensitive methods of analyses, given the fact that in many cases very small amounts of samples are available, or very low concentrations of specific analytes. This is particularly true of the aqueous humor, iron and transferrin.

## **Materials and Methods**

### **Sample Collection**

For this study we used normal (control) animals that were euthanized in terminal protocols approved by the IACUC of the Medical Sciences Campus. This was the source of pigs (female) and rats (Sprague Dawley, females). Dogs proceeded from the Municipal Pound, and cows from La Muda Abattoir near the San Juan Medical Center. In the case of pigs and dogs aqueous humor and blood samples were taken under deep anesthesia before death of the animal. After cardiac arrest the eye globes were enucleated, placed in ice and immediately taken to the laboratory for dissection. In the case of cows aqueous and blood samples were first obtained at the site and then the enucleated eyes were transported in ice to the laboratory for dissection. In the case of rats blood was taken just before death, then the eyes were immediately enucleated and taken in ice to the laboratory to obtain the aqueous humor and for further dissection.

The eye globe was divided in an anterior and a posterior half. From the anterior half, the cornea, the iris-ciliary body and the lens were obtained. The vitreous body and the retina were dissected from the posterior half of the eyeball. The wet weight of all samples was recorded. All samples were frozen in polypropylene test tubes and stored at  $-80^{\circ}\text{C}$  until homogenization.

We used three species in this work, including two diurnal (cow and pig) and one nocturnal (rat) animal. Dogs were used only for the specific purpose of determining the highest dilution of aqueous humor convenient for the determination of total protein. Animals other than dogs were of the same sex and of approximately the same weight. Six to twelve animals of each species were used per parameter analyzed. Each determination was performed in triplicate, if possible, depending on the amount of sample available. Pooled samples had to be used in some cases (rat, for example).

### **Sample Processing**

Serum and aqueous humor samples were diluted in phosphate buffered saline (PBS) or water as required by

**Table 1.** Dilutions of Tissue and Fluid Samples for the Different Methods

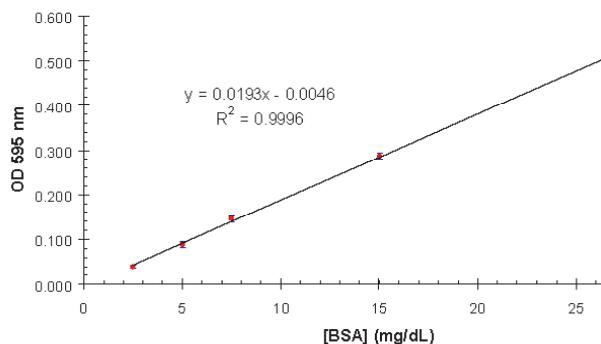
Sample	Total Protein	ELISA	Total Iron	TIBC
Cornea	1:10	1:1,000	Undiluted	1:2
Aqueous	1:4	1:1,000	Undiluted	1:2
Iris-Ciliary				
Body	1:25	1:500	Undiluted or 1:2	1:2
Lens	1:200	1:2,000	Undiluted	1:2
Vitreous	1:4	1:1,000	1:2	1:4
Retina	1:50	1:1,000	Undiluted	1:2
Serum	1:1,000	1:100,000	1:20	1:60

the respective method of analysis (Table 1). Ocular tissues and vitreous body were submitted to homogenization using a whole glass tissue grinder (previously acid washed in a solution 10% HNO<sub>3</sub> + 10% H<sub>2</sub>SO<sub>4</sub>) with the help of an electric drill. Tissues were dispersed in a volume of liquid ten times their wet weight in grams (i.e., 1 g /10 mL 20 mM Tris-HCl, pH 7.4). In order to remove cell debris all homogenates were centrifuged at 3,000g for 10 minutes at 4°C. The supernatant was fractionated in aliquots in siliconized microtubes and frozen at -80° C until analyzed for Total Protein, Transferrin, Total Iron or Total Iron Binding Capacity.

### Total Protein Determination

Total protein was performed following the instructions of the Bio-Rad Protein Assay Kit, Microtiter Plate Standard version, which provided maximal sensitivity with the lowest amount of sample assayed. This assay is based on the Bradford method (11), which involves the addition of an acidic dye (Coomassie Brilliant Blue G-250) to the protein solution. A differential color change of the dye occurs which is quantitatively related to the concentration of protein. Absorbance values were obtained with a Bio-Rad Microplate Reader, model 550. All samples were assayed in triplicate. Dilutions of the Bovine Serum Albumin (BSA) protein standard 2 mg/mL (Pierce, IL) were prepared ranging from 2.5 mg/dL to 30 mg/dL to construct the standard curve (Figure 1). Aliquots of 10 µL of each diluted sample/protein standard were delivered into separate wells. Then 200 µL of Bio-Rad dye reagent were added to each well with a multi-channel pipette (Fisher Scientific, PA). This addition was rapidly made to thoroughly mix the sample with the dye. After five minutes at room temperature the plate was read at 595 nm.

**Aqueous humor.** Using dog aqueous humor it was shown that a convenient working dilution for aqueous humor to be safely used with the microplate protein method would be 1:4 (8). This represents the equivalent



**Figure 1.** Typical BSA Calibration Curve for Total Protein Determination (Range 2.5-30 mg/dL): Each point is the mean ± standard deviation of 6 replicates.

of 7.5 µL of pure aqueous humor per assay, a significant saving of aqueous humor.

**Serum.** In order to determine total protein in serum this fluid was diluted 1:1,000 for the assay to be performed at about the same total protein level as the aqueous humor. The use of only one protein standard curve for both serum and aqueous humor is a protection against potential non-linearity problems with the method if used in two very different ranges of protein concentration. It assures reliable comparisons between the composition of the aqueous humor and serum.

### Tf Determination by Enzyme Linked Immunosorbent Assay (ELISA)

Tf was quantified by ELISA in an indirect configuration (12). Species-specific anti-Tf antibodies were obtained from Bethyl Laboratories (TX) and Sigma (MO). Species-specific apo-transferrins (apo-Tf) were purchased from Sigma (MO). No pig apo-Tf was available at the time these assays were done. All samples were appropriately diluted as indicated in Table 1.

The ELISA procedure was carried out in the following general format (8): adsorption of Tf Ag was done overnight at 4°C into the wells surface of Immulon 2 HB plates (VWR Scientific, PR) with 100 µL/well of an appropriate dilution of the homogenate in 20 mM Tris-HCl (pH 7.4), serum (about 70 ng of protein/well) and aqueous humor (about 20 ng of protein/well) in PBS. The plate was washed 3 times with 125 µL of PBS, and twice with MQ dH<sub>2</sub>O. All plates were blocked with 125 µL of a 3% essentially fatty acid free BSA (Sigma, MO) solution in PBS/0.05 % Tween-20 (PBS-T), for 1 hour at 37°C. Then 100 µL of primary antibody (Ab<sup>1</sup>) were added at an appropriate dilution (Table 2) and, after 1hr incubation at room temperature, 100 µL of secondary antibody (Ab<sup>2</sup>) Alkaline Phosphatase Conjugate (APC) were added (for dilution, see Table 2). Plates were further

**Table 2.** Species Specific Tf-ELISA Conditions.

Species	Ab <sup>1</sup> Dilutions	Ab <sup>2</sup> Dilutions	Linear Range (µg/L)
Cow	1:50 (Bethyl Labs. A10-122)	1:15,210 <sup>1</sup> (Sigma A-8062)	9.77 - 625
Dog	1:800 (Bethyl Labs. A40-112)	1:11,859 <sup>2</sup> (Sigma A-4062)	9.77 - 625
Human	1:800 (Sigma T-6265)	1:11,859 (Sigma A-4062)	4.88 - 625
Pig <sup>3</sup>	1:200 (Bethyl Labs. A100-109)	1:11,859 (Sigma A-4062)	N.D.
Rabbit	1:100 (Bethyl Labs. A120-107)	1:15,210 (Sigma A-8062)	9.77 - 312.5
Rat	1:400 (Bethyl Labs. A110-123)	1:11,859 (Sigma A-4062)	4.88 - 312.5
Mouse	1:100 (Bethyl Labs. A90-129)	1:11,859 (Sigma A-4062)	9.77 - 625

<sup>1</sup> Mouse Monoclonal Anti-Goat/Sheep IgG Clone GT-34 Alkaline Phosphatase Conjugate

<sup>2</sup> Rabbit Anti-Goat IgG (Whole Molecule) Alkaline Phosphatase Conjugate

<sup>3</sup> Although commercial pig Tf was not available, it was possible to establish the Ab<sup>1</sup> dilution in the optimization method with pig serum.

incubated for 1 hr at room temperature, and then washed 5 times with 125 µL of PBS-T. The Alkaline Phosphatase (AP) substrate para-Nitrophenyl Phosphate (pNPP) was added to the wells (100 µL, 1 mg/mL in Alkaline Buffer: 50 mM Na<sub>2</sub>CO<sub>3</sub>, 334 µM MgCl<sub>2</sub> and 0.02% NaN<sub>3</sub>, pH 9.7). The plates were left at room temperature and the yellow color of para-Nitrophenolate (pNP) was read at 15 and 30 minutes after the addition of the substrate with a 405 nm filter on the Bio-Rad Microplate Reader, model 550. In each plate, several controls and blanks were always included: 1) only MQ dH<sub>2</sub>O as a control for the absorption of plastic; 2) no antigen, only Ab<sup>1</sup> + Ab<sup>2</sup>APC, to correct for non specific binding of primary antibody (this value was actually used as the blank to be subtracted from the standards and samples); 3) no Ag, no Ab<sup>1</sup>, only Ab<sup>2</sup> APC, for the non specific binding of the secondary antibody; and 4) only pNPP, to check the spontaneous oxidation of the substrate. Also included were six standard concentrations to construct the calibration curve.

#### ELISA Optimization for Tf Determination

The Tf assay was standardized for quantitative purposes by making it species specific and titrating both the primary and secondary antibodies for optimal response (8). Optimal conditions are summarized in Table 2. The procedure was carried out using as the standard the corresponding species specific apo-Tf, ranging from 30.5 to 1,000,000 ng/dL. The central, linear portion of the curve was identified in every case (Table 2). For each ELISA experiment a calibration curve was constructed using 5 or 6 points. Finally, several serial dilutions of the samples to be tested were always used to identify the best to calculate the results (8).

#### Tf Estimation from TIBC

When indicated, transferrin was estimated from TIBC assuming two moles of iron per mol of the protein, and

a MW for Tf of 77,000 dalton. This procedure overestimates transferrin, since there are iron-binding proteins other than Tf contributing to TIBC.

#### Iron Analysis

The procedure of McGahan and Fleisher (7) was basically followed, with modifications (8).

**Instrument:** Perkin-Elmer Model AAnalyst 800 Atomic Absorption Spectrometer (AAS) equipped with a graphite furnace and a Zeeman background corrector was used.

The AAS was operated with an iron hollow cathode lamp (Fe-HCL) at a wavelength of 248.3 nm and 0.2 nm slit bandwidth.

**Reagents:** All glassware and plasticware was soaked in 10% (v/v) HNO<sub>3</sub> + 10% (v/v) H<sub>2</sub>SO<sub>4</sub> and 20% HCl, respectively, followed by thorough rinsing with MQ dH<sub>2</sub>O, and drying, then stored until needed. All samples were digested with a matrix modifier to a final concentration of 0.5% trichloroacetic acid (TCA) and 0.5 mg/L of Triton X-100. A blank was subtracted from all standards and samples to compensate for the iron present in the matrix modifier. Reagent grade chemicals (MgCO<sub>3</sub>, Iron Standard, TCA) and trace metal grade acids (HNO<sub>3</sub>, H<sub>2</sub>SO<sub>4</sub> and HCl) were obtained from Fisher Scientific (PA). Triton X-100 was obtained from Bio-Rad (CA).

**Total Iron:** Five dilutions of the iron reference standard were prepared ranging from 12.5 ppb to 200 ppb. A typical calibration curve for this procedure is shown in Figure 2. Aliquots of 200 µL of each standard were mixed with 800 µL of matrix modifier. The final dilution was the same for standards and samples. This range was appropriate to measure levels of iron in the samples expected to have the lowest concentrations in this study. Adequate blanks for each sample dilution were included in each experiment and subtracted from the samples.

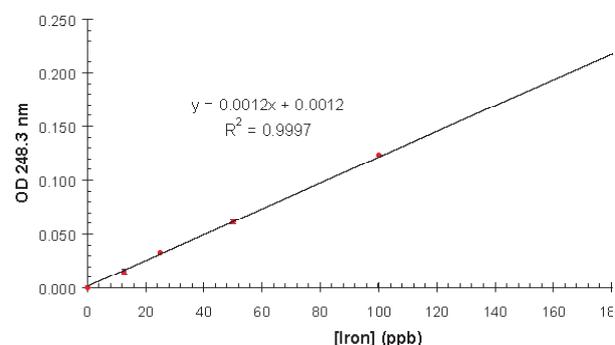
Once thawed, samples to be analyzed were diluted as summarized in Table 1. An aliquot of 200 µL of the sample dilution was mixed thoroughly with 800 µL of matrix modifier in a 1 mL polystyrene microtube (Fisher Scientific, PA). In the case of non-abundant samples, a 50 µL aliquot (or less, keeping the appropriate proportions) of the dilution was mixed with 200 µL of matrix modifier. After an incubation period of 15 minutes at room temperature the samples were centrifuged using a Sorvall Biofuge at 16,060 x g for 10 minutes at 4 °C to remove protein. About 800 µL (for abundant samples), or 200 µL (for scarce samples) of the supernatant were carefully

**Table 3.** Atomic Absorption Program for TI and TIBC.

Step No.	Furnace Temperature	Time (s) Ramp Hold	Internal Gas Flow	Read On
1	110 °C	1 40	250	
2	130 °C	15 30	250	
3	150 °C	15 30	250	
4	1,300 °C	10 20	250	
5	2,100 °C	0 5	50	
6	2,450 °C	1 3	250	
7	2,500 °C	1 3	250	

Steps (1-3) → Drying; Step (4) → Pyrolysis; Step (5) → Atomization; Steps (6-7) → Clean Out Steps

transferred to a 2 mL or 0.25 mL polystyrene sample cup, respectively (Fisher Scientific, PA). Forty microliters of the samples were injected into the AAS for the analysis of iron according to the optimized graphite furnace program in Table 3. In general, the program includes three drying steps, pyrolysis, atomization and two clean out steps. The bell shaped absorbance peak was complete and strong. A typical iron calibration curve is shown in Figure 2.



**Figure 2.** Typical Calibration Curve for Total Iron Determination: Each point is the mean ± standard deviation of two injections.

**Total Iron Binding Capacity (TIBC):** TIBC measures the amount of iron that can potentially be stored in Tf. Equal volumes of the sample to be analyzed and an iron saturating solution prepared from  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  from Sigma (5 mg Fe/L in 0.005 N HCl) were mixed in a 1 mL polystyrene microtube (Fisher Scientific, PA) for a period of 20 minutes at room temperature. This saturates all Tf molecules, to the diferric state. The excess of unbound iron was removed by adding  $\text{MgCO}_3$  (Fisher Scientific, PA) in a proportion of 0.1 mg per  $\mu\text{L}$  of solution (26). Samples were left at room temperature for 30 min and thoroughly mixed by vortex at 5 min intervals. At the end of the incubation period, all samples were centrifuged at 3,000 rpm for 10 minutes to precipitate the  $\text{MgCO}_3$  with adsorbed free iron. The supernatant was diluted as necessary (Table 1) and then mixed and digested with

the matrix modifier. Once digested all samples were centrifuged in a Sorvall Biofuge at 16,060xg, 10 minutes at 4°C. The supernatant was transferred to a sample cup carefully enough not to carry over the precipitated material at the bottom of the microtube and then analyzed in the same way as for TI.

TIBC should be greater than TI because every Tf molecule has all iron sites occupied. This was indeed the case for Serum, Aqueous and Vitreous Humor, but when tissues homogenates (Cornea, Iris-Ciliary Body, Lens and Retina) were analyzed the opposite resulted,  $\text{TIBC} < \text{TI}$ . For tissues, then, Tf saturation cannot be established with the standard methodology in a straightforward way. In order to solve this complication the TI procedure described before was modified for tissues. In the modified procedure, an aliquot of 150  $\mu\text{L}$  of tissue homogenate to be analyzed for TI was submitted to a precipitation cycle with  $\text{MgCO}_3$ , as done with the sample to be analyzed for TIBC (but skipping, of course, the exposure to the iron saturating solution). We could show that this treatment removes from the preparation the form of bound iron that interfered with TI determination, leaving behind the iron that is specifically, strongly bound to Tf (8). The iron fraction that is removed by  $\text{MgCO}_3$  was called loosely bound Iron Pool (LBIP).

**Saturation of Tf:** Per cent saturation of Tf was always calculated as  $(\text{TI}/\text{TIBC})100$ .

### Data Analysis

We have chosen to present the data for TI and TIBC as concentration of iron relative to total protein (TP) since this is probably most meaningful from the point of view of functional significance, given the fact that most iron is necessarily bound to protein.

The rejection of outliers was performed using the Q-Test (13). All data are reported as the mean ± standard deviation. The significance of the interspecies differences in iron parameters among tissues/fluids was evaluated by ANOVA. The criteria for statistical significance was  $P < 0.05$ .

## Results

### Total Protein in Ocular Fluids and Serum

Total Protein (TP) in serum, aqueous humor and vitreous body in the three species reported is shown in table 4. The rat showed high levels of protein in the aqueous humor relative to the other species, but this is in agreement with the results of Stjernschantz (14) using the method of Lowry,  $100.3 \pm 4.3$  mg/dL. Bovine and porcine aqueous humor total protein is in the order expected for many animal species including humans (15). The aqueous to

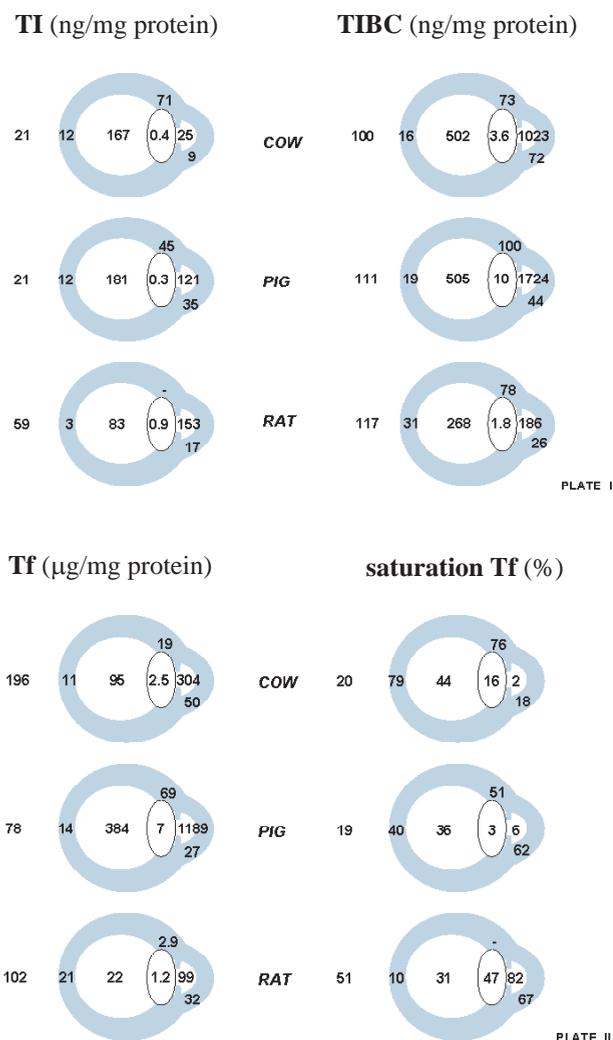
**Table 4.** Total Protein Concentration (mg/dL) in Serum and Intraocular Fluids of Rat, Cow and Pig.

Sample	Rat	Cow	Pig	p-Value
Aqueous	99.8	10.6 ± 1.40	38.7 ± 53.2	0.029
Vitreous	241.3	55.5 ± 9.71	50.2 ± 17.1	4.6 x 10 <sup>-11</sup>
Serum	6810.6 ± 774.91	6502.6 ± 1136.3	5723.6 ± 668.34	0.046

All rat samples were pools except the serum.

serum protein ratios varied from 0.16% in the cow (similar to human, 0.18% (16)) to 1.47% in the rat, and values of 0.68% for the pig. The comparison of aqueous to serum values is especially valid in this work because all were assayed at dilutions giving OD values fitting only one standard curve.

Total protein in the vitreous was very similar in the pig and cow (~50 mg/dl) and over 4-fold higher in the rat.



**Species Specific Distribution of Iron Biochemical Parameters in the Eye**

Results are summarized in Plates I and II, where concentration units are: Tf (µg/mg TP), TI (ng iron/mg TP), TIBC (ng iron/mgTP) and saturation of Tf (%), (TI/TIBC) 100).

**Serum:** Highest concentration of Tf was found in the serum of the cow (196 µg/mg TP), followed by progressively lesser Tf concentrations in rat (102 µg/mg TP) and pig (78 µg/mg TP) serum. Iron concentration (TI) was highest in the serum of the rat (58.8 ng iron/mg TP), followed by cow (21.1 ng iron/mg TP) and pig (20.8 ng iron/mg TP) sera with essentially the same concentration. Iron TIBC ranged from 100 ng iron/mg TP (cow) to 117 ng iron/mg TP in the rat. The percent saturation of Tf was very similar in cow (20.4%) and pig (19.4%) sera, and about 2.5 times higher in the serum of the rat.

**Aqueous humor:** The concentration of Tf was highest in the aqueous humor of pig (1,189 µg/mg TP), lower in the cow (304 µg/mg TP), and lowest in the rat aqueous humor (99 µg/mg TP). TI concentration was relatively low in the cow (25 ng iron/mg TP) and highest in the rat (153 ng iron/mg TP). TIBC was lowest in the rat (186 ng iron/mg TP), more than 5 times higher in the cow (1023 ng iron/mg TP) and even higher in the pig (1724 ng iron/mg TP). The saturation of Tf was very low in cow and pig (2 % and 6%, respectively) and much higher in the rat (82%).

**Vitreous:** Tf concentration kept increasing from rat to cow to pig (22, 95, 384 µg/mg TP, respectively). Total iron also increased from rat (83 ng iron/mg TP) to cow (167 ng iron/mg TP) to pig (181 ng iron/mg TP). TIBC was about 2-fold lower in the rat (268 ng iron/mg TP) and similar in cow and pig (503 and 505 ng iron/mg TP, respectively). Saturation of Tf was about the same in the three species: cow, 44%; pig, 36%; rat, 31%.

**Retina:** Tf was about the same in cow and pig (11 and 14 µg/mg TP, respectively) and almost double in the rat (21 µg/mg TP). TI was the same in cow and pig (12 ng iron/mg TP) and 3 ng iron/mg TP in the rat. TIBC was about the same in cow and pig (16 and 19 ng iron/mg TP) and 31 ng iron/mg TP in the rat. Saturation of Tf was very low in the rat (10%) and increased in the pig (40 %) and cow (79%).

**Lens:** Tf was lowest in the rat (1.2 µg/mg TP), increased to 2.5 µg/mg TP in the cow and to 7 µg/mg TP in the pig. TI was very low and similar in cow (0.35 ng iron/mg TP) and pig (0.31 ng iron/mg TP) but higher in the rat (0.85 ng iron/mg TP). TIBC followed the same trend than Tf, lowest in rat (1.8 ng iron/mg TP), highest in pig (10 ng iron/mg TP). Saturation of Tf increased from pig (3%) to cow (16%) to rat (47%).

**Cornea:** Tf concentration levels were lowest in pig (27 µg/mg TP), similar in rat (32 µg/mg TP) and highest in cow (50 µg/mg TP). TI was very low in cow (9 ng iron/mg TP) and highest in pig (35 ng iron/mg TP), the rat showing intermediate values (17 ng iron/mg TP). TIBC kept increasing from rat (26 ng iron/mg TP) to pig (44 ng iron/mg TP) to cow (72 ng iron/mg TP). Saturation of Tf was lowest in the cow (18%) and similar in pig (62%) and rat (67%).

We now examine the distribution of iron biochemical parameters in each of the species analyzed: the rat, cow and pig.

**Rat:** Tf concentration was highest in the aqueous (99.3 µg/mg TP), followed by cornea (32.2 µg/mg TP), vitreous (21.7 µg/mg TP) and retina (21.4 µg/mg TP). It was lowest in the lens (1.24 µg/mg TP) and Iris-Ciliary Body (2.86 µg/mg TP). Iron concentration was highest in the aqueous (153 ng iron/mg TP) and vitreous (83 ng iron/mg TP), and lowest in the lens (0.85 ng iron/mg TP). TIBC was highest in the vitreous (268 ng iron/mg TP) and aqueous (186 ng iron/mg TP), and lowest in the lens (1.8 ng iron/mg TP). Saturation of Tf was highest in the aqueous (81.98%) and cornea (66.4%), followed by lens (47.28%) and vitreous (30.93%), and lowest in the retina (10.22%).

**Bovine:** Tf concentration was highest in the aqueous (304 µg/mg TP), followed by the vitreous (95.3 µg/mg TP). It was lowest in the lens (2.5 µg/mg TP). Iron concentration was highest in the vitreous (167 ng iron/mg TP); TIBC was highest in the aqueous (1,023 ng iron/mg TP), followed by the vitreous (502 ng iron/mg TP), and lowest in the lens (3.63 ng iron/mg TP). Tf was very little saturated in the aqueous (2.21%), saturated almost to 20% in the cornea and lens, and saturated about 75% in the iris-CB and retina.

**Pig:** Tf concentration was highest in the aqueous (1,189 µg/mg TP) and vitreous (384 µg/mg TP), and very low in the lens (6.92 µg/mg TP). Iron concentration was highest in vitreous (181 ng iron/mg TP), followed by the aqueous (121 ng iron/mg TP), and lowest in the lens (0.31 ng iron/mg TP). TIBC iron was highest in the aqueous (1,724 ng iron/mg TP) and the vitreous (505 ng iron/mg TP), and lowest in the lens (10 ng iron/mg TP) and retina (20.1 ng iron/mg TP). Saturation of Tf was highest in the cornea (62.08 %), followed by iris-ciliary body (51.46%) and retina (40.41%), then by the vitreous (35.55%) and lowest in the lens (2.95%).

## Discussion

Organisms have been forced to develop specific iron-sequestering molecules to maintain iron in the safest

form possible without compromising its availability for cell's demands. The study of these molecules in the eye is of the utmost significance given the vulnerability of the eye to environmental radiation (1). Here the importance of constraining iron is enormous, in order to avoid photooxidative stress and associated pathologies such as cataracts, macular degeneration of the retina, or vitreous degenerations.

A review of the literature (8) reveals lack of information about iron and iron related proteins in ocular tissues and fluids, particularly when one tries to establish comparisons and relationships among them. McGahan and collaborators (7, 17-19) contributed strongly to establish definitive data on iron levels and iron-binding proteins in the eye after adapting atomic absorption spectroscopy (AAS), and other methods, such as a transferrin ELISA (18) of different configuration than that described here, for that purpose.

A more comprehensive and systematic knowledge of iron homeostasis parameters in ocular fluids and tissues still is needed. The present work will partially fill the existing gaps specifically addressing the questions, (i) to what relative extent are the different fluids and tissues of the eye exposed to iron-associated oxidative stress? And, (ii) do the living habits of the species studied (diurnal versus nocturnal) determine differences in iron homeostasis parameters in the eye? The following criteria will direct the discussion of the first question: low TI and low saturation of Tf, and high Tf and high TIBC correspond to antioxidant parameters; on the contrary, high TI and high saturation of Tf, and low Tf and TIBC correspond to parameters favoring oxidative stress. We will also consider that the low or absent LBIP as defined in this work would probably constitute a protective parameter of the avascular tissues of the eye.

This work represents only a partial account of the contents of the doctoral thesis of one of the authors (LVQ). Additional results and biochemical aspects will be published elsewhere. We have also referred the reader to that thesis work (8) for some Results labeled as "not shown here".

## Methodological aspects

There are some methodological considerations worth noting, already described in the Materials and Methods section. As far as the determination of protein with the Bio-Rad protein assay is concerned we were able to extend the linear range to 2.5 mg/ml, allowing to double the sensitivity of the method and thus reducing significantly the amount of aqueous humor needed for this assay (7.5 µl).

The AA spectroscopic method for iron determination was carried out, too, with less volume of sample than

previously used, without compromising the quality of the procedure.

### ELISA Transferrin Analysis Optimization

Due to scarcity of Tf values and methodology in the literature it was necessary to develop a sensitive technique for the measurement of Tf. This was done by establishing species specific Tf ELISAs for the animals that we used in this study and for others like the dog, mouse, rabbit, and humans. In all cases the ELISA technique was carried out in an indirect configuration and using a secondary antibody-alkaline phosphatase conjugate since these were the basic conditions providing best sensitivity and consistency of results previously found in ELISAs designed to measure lens crystallins in our laboratory (12). The procedure was strictly standardized for quantitative purposes and to permit to establish valid comparisons of Tf levels among the different species. A summary of the final conditions is presented in Table 2 for all the species specific Tf -ELISAs.

### Loosely bound iron pool (LBIP)

When studying the ratio TI/TIBC in order to establish transferrin saturation we found in ocular tissues the anomalous result that TI was greater than TIBC. After considering several possibilities (8) experiments led us to the conclusion that this was due to the presence in tissues of a pool of loosely bound iron, i.e., iron behaving as “free iron” in the sense that coprecipitates with magnesium carbonate (26) when the latter is added to the sample analyzed for TIBC, lowering the value of iron in the TIBC assay but not in the TI assay (see section of Methods). Since this form of iron is not present in ocular fluids nor serum, it could be related, in principle, to some form of cell storage of non-transferrin iron.

The method to establish % saturation of Tf was therefore modified when applied to tissues, to remove this pool of iron not only from TIBC but also from TI. This was accomplished by exposing the TI samples to precipitation with magnesium carbonate prior to digestion with the matrix modifier (see Methods section). The iron precipitating with magnesium carbonate was labeled

loosely bound iron pool (LBIP). Table 5 shows LBIP for the different tissues of the eye. It is lowest in the avascular tissues, in particular the lens, and highest in the iris-ciliary body preparation. It is, thus, closely related apparently to the degree of vascularization of the tissue, but no further attempt was done at this stage to identify its nature. This pool, however, could contain labile forms of iron, and could thus be a potential source of oxidative stress when present. Its existence was recognized before (27) since it was a source of variability when determining TIBC in different clinical laboratories.

### Iron metabolism related parameters in ocular tissues and fluids

One of the main objectives of this work was to provide a comparison of several biochemical parameters related to iron metabolism in different ocular tissues and fluids of several animals, looking for patterns that might provide an idea of the relative levels of oxidative stress present in the different compartments of the eye. The corresponding blood serum of each of the animals studied could also provide a systematic frame of reference against which to compare findings in the eye.

**Tf concentration:** Tf levels were highest in the aqueous, followed by the vitreous of the cow and pig. The level in the human aqueous humor (not shown) is similar to that found in the cow. The lowest level of Tf was found in every case in the lens. In the cow and pig the pattern was the same: aqueous>vitreous>cornea>retina>lens. In the rat the pattern was distorted: the highest value was again found in the aqueous, but the cornea ranked second and vitreous third, followed by retina.

It would then appear that as far as iron related oxidative stress the tissue at the highest potential risk could be the lens, having a very low Tf concentration. This could be particularly true in the central region of the lens if Tf is mainly localized in the anterior epithelium and equator. However, the lens has the added characteristic of being able of Tf synthesis (18) and its iron concentration is minimal among eye tissues and fluids.

It could be of significance that there is no difference between serum and aqueous Tf in the rat. However, in the other two species (and in the human eye) the concentration of Tf in the aqueous humor is much higher than in the respective sera, particularly in the pig. This would make these fluids ideally suited to impede the access of iron to the lens, more so when they exhibit a very low saturation of Tf (see below). The fact that pig Tf was estimated from TIBC could explain its highest Tf value.

The rat, from the point of view of Tf levels could be the most prone to develop oxidative stress of the three species evaluated, and it will soon be evident that the rat

**Table 5.** Loosely Bound Iron Pool (LBIP) in Ocular Tissues and Fluids (\*).

	Pig	Cow	Rat	average
Lens	27.8	0.6	32.4	20
Cornea	70.9	27.9	46.4	49
Retina	95.9	56.2	101	84
Iris-Ciliary B.	769	1,589	-	1,179

(\*) All values in ppb

exhibits very high Tf saturation values in serum and in most tissues and fluids of the eye.

**TI:** Highest TI concentration has been found in the vitreous body of the cow and pig, about twice that found in the vitreous of the rat. In the rat, iron was higher in the aqueous humor than in the aqueous humor of the other two species. Also, values in the vitreous of cow and pig are about 9 times higher than in their respective sera. In the rat, the leading compartments are still the aqueous and the vitreous, but in reverse order.

In the three species the lowest iron concentrations were found in the lens. This may counterbalance the risk of lowest Tf concentrations described above in this structure. On the other hand, movements of iron in the lens may also be difficult given their requirement for transporters and enzymes to keep iron in the proper redox state (22) and given the limited extracellular space and high concentration of membranes in the lens (20).

Rat serum is about three times richer in iron than the cow or the pig sera.

The vitreous body, given its high TI values, particularly in the cow and the pig, may represent a potential source of iron related oxidative stress for the posterior part of the lens, and could be a contributing factor to postvitrectomy cataract, in addition to the increased oxygen concentration already known to occur in the vitreous in that condition (21).

**TIBC:** The pattern of distribution of TIBC is identical in cow and pig (aqueous>vitreous>serum>cornea>retina>lens) and coincidental with the Tf pattern described before. The pattern is different in the rat, with the remarkable finding that rat's TIBC in the aqueous humor is much lower (about 7 times as the average) than in the other two species. Serum TIBC is about the same in the three species. If we take TIBC as reflecting antioxidant protection, both the cow and the pig would be better prepared to neutralize the damaging effects of the presence of iron than the rat.

**Saturation of Tf:** Serum % saturation of Tf in the cow and the pig (~20%) was in the low side of that found in many animals (30 %) (7, 22). In the rat we found a value of 51%. Apparently, however, it is not unusual for the rat and other rodents (22-24) to have high saturation of Tf. Highest saturation of Tf was found in the aqueous humor of the rat (82%), which markedly contrasts with that in the aqueous humor of the other two species. Retinas, excepting that of the rat, gave saturations of 79% in the cow and of 40% in the pig. Very low saturation was found for the aqueous humor of the cow (2%) and pig (6%), giving these species an ample reserve against iron-related oxidative damage in the anterior pole. The lens of the pig also showed a very low saturation of Tf (3%).

The vitreous of the cow and pig gave saturations higher than in serum (44% and 36%, respectively).

Rat vitreous saturation was lower relative to rat's own serum, although its absolute value still was 31%. In addition to rat aqueous humor, other components of the anterior pole of rat's eye, the lens and the cornea, gave high saturation values for Tf, 47% and 67%, respectively. This gives the rat a very small margin to fight iron associated oxidative stress as Zigler, et al. (25) commented when comparing the response of the rat and Rhesus monkey lenses in culture to an imposed oxidative stress.

McGahan and Fleisher (7) simultaneously studied plasma, aqueous humor and vitreous of several species (dog, cat, pony, pig and rabbit). Only the pig was common to their work and the present work. They concluded that "iron levels were similar between species and were generally higher in the vitreous than in the aqueous", plasma exhibiting much highest values. Our data, converted to the same units of concentration (mg/L) supported the same conclusion (serum > vitreous > aqueous). We could not conclude, however that values were similar among the species studied since there were very clear differences. Curiously, in the McGahan series (7) the pig showed lowest saturations of Tf in all fluids, in comparison to the other species. In our series this also happened but only in the aqueous humor of the pig and the cow. They also concluded that "TIBC of the aqueous and vitreous humors from the same species is similar", a statement not supported by our data. In the present work we found aqueous TIBC much greater than in the vitreous. Finally, they found the "vitreous TIBC to be more highly saturated in all species". This was also found in the present work (except in the rat) if we consider only data for the plasma and intraocular fluids. We conclude the comparison indicating that in general, and excluding the rat, our iron concentrations in serum are very close to those reported by McGahan and Fleisher (7) in the plasma of their animals; in the vitreous, however, we report values that are, as the average, about 2 times higher than theirs. In the aqueous humor we found only the pig's iron close to that reported by them. To what extent these differences are due to truly species differences or methodological variation is something that only accumulation of data will eventually reveal.

**Nocturnal vs diurnal:** From the patterns discussed in this work there is a clear difference between the pattern of iron related parameters in the rat and the patterns of the cow and the pig. In terms of the living habits of these animals one could interpret these differences in the following way. Being a nocturnal animal, the rat does not need to worry much about the presence of iron, since the catalyzing factor represented by UV light is not present

as it is in the animals of diurnal habits of living. For that reason, iron in the rat is high in the vitreous, blood serum and aqueous; and the concentration of Tf, and TIBC, is low in most rat tissues and fluids relative to the other two species (excepting retina and cornea). In the same line of thought, Tf saturation is very high in the rat, both in blood serum and aqueous, and the lens. The rat, lacking antioxidative reserves, may easily go to overt oxidative stress, and associated pathology, if forced to adopt diurnal habits, or if exposed to additional oxidative risk factors. This is particularly important to take into account in an animal so often used as a model of cataract formation. More animals with nocturnal and diurnal habits should be studied in order to confirm the initial differences and patterns described in this work. It may be significant that the only animal in the McGahan and Fleisher series (7) that can be considered of predominantly nocturnal habit of living, the cat, shows the highest saturation of Tf of all the series, 77%, in the vitreous.

### Resumen

Se realizó un estudio comparativo de varios parámetros metabólicos relacionados con hierro (hierro total, capacidad total para fijar hierro, transferrina y saturación de transferrina) en suero sanguíneo y tejidos (lente cristalino, córnea, iris-cuerpo ciliar, retina) y líquidos oculares (cuerpo vítreo, humor acuoso) de varias especies animales (cerdo, vaca y rata) con el propósito de adquirir una perspectiva amplia de la homeostasis de hierro en el ojo. El grado relativo de estrés oxidativo a que están expuestos estos tejidos y fluidos se evaluó a base del criterio de que valores altos de hierro y de saturación de transferrina, y valores bajos de capacidad de fijación de hierro y transferrina, propiciarían estrés oxidativo relacionado a hierro. La inclusión de animales de hábitos diurnos (cerdo, vaca) y nocturnos (rata) en este estudio nos brindó la oportunidad de explorar si los parámetros metabólicos relacionados con hierro se ven afectados por el nivel de estrés oxidativo mayor esperado en animales con hábitos de vida diurnos.

Este proyecto requirió el desarrollo de métodos analíticos nuevos y muy sensitivos dado que en muchos casos la cantidad de muestras disponibles son muy pequeñas (por ejemplo, humor acuoso) y la concentración de las sustancias a ser analizadas es muy baja (por ejemplo, transferrina). Todos los resultados se expresaron en términos de la concentración de hierro relativa a mg de proteína, según determinada a través del método de Bradford. Al hacer las determinaciones de hierro en las muestras se definió una fracción de hierro débilmente fijada en los tejidos oculares proporcional al grado de

vascularización de los tejidos analizados. La comparación de los patrones de homeostasis de hierro en el ojo y entre especies reveló lo siguiente: 1) el humor acuoso y vítreo de la vaca y el cerdo contiene los niveles más elevados de transferrina y una saturación muy baja de transferrina, mientras que las concentraciones más bajas de transferrina fueron detectadas en el cristalino en todas las especies; 2) hierro total mostró la tendencia a ser más alta en el vítreo de la vaca y el cerdo y más baja en el cristalino de las tres especies. Una concentración tan baja de hierro en el cristalino debe condicionar que su baja concentración de transferrina no constituya una amenaza oxidativa seria; 3) el patrón de distribución ocular de la capacidad total de fijación de hierro es semejante en la vaca y el cerdo; 4) la saturación de transferrina fue muy elevada en el humor acuoso, en el cristalino y en el suero de la rata; 5) la rata se diferencia claramente de las otras dos especies animales en cuanto al patrón ocular de parámetros relacionados con hierro, sugiriendo que el hábito de vida nocturna de este animal está influenciando la homeostasis. Se puede hipotetizar que como la rata está expuesta a un efecto dañino muy limitado de la luz ultravioleta ambiental, esta puede tolerar el riesgo oxidativo relacionado con hierro sin sufrir patología ocular alguna, pero sus reservas antioxidantes deben ser mínimas. Por ejemplo, la combinación de una concentración elevada de hierro en el acuoso con un nivel relativamente bajo de transferrina da una saturación peligrosamente elevada de 82% en la rata; 6) la parte posterior del lente está expuesta a niveles altos de hierro y transferrina en los animales diurnos, con una saturación de transferrina en un nivel de 40%. Esto podría facilitar la aparición de cataratas posvitrectomía, junto con el conocido mecanismo de aumento de los niveles de oxígeno en el vítreo que ocurre en esas condiciones.

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