Redox state as assessed using the measurement of human non-mercaptalbumin in embryo culture media is associated with successful embryo development in human *in vitro* fertilization

Yoshika Kusumoto,[1,2] Masaya Sato,[1,3,]* Hitoshi Ikeda,[1] Keiko Yasukawa,[1] Xiaohui Tang,[2] and Yutaka Yatomi[1]

[1]Department of Clinical Laboratory Medicine and [3]Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
[2]Akihabara ART Clinic, 1-1-12 Ueno, Taito-ku, Tokyo 110-0005, Japan

The role of oxidative stress in the pathogenesis of various diseases has been attracting attention. We speculated as to whether the redox state of treatment solutions used for various diseases may play a role in treatment success. In the current study, we focused on the human embryo culture medium used for *in vitro* fertilization (IVF). A total of 173 oocytes from a total of 91 patients treated with IVF were enrolled. The redox state was assessed by measuring the levels of human non-mercaptalbumin (HNA). We analyzed factors related to blastocyst formation on day 5 or 6 after insemination. We also developed a random forest (RF) model for the prediction of blastocyst formation. The variable importance in the predictive model was assessed using the mean decrease in the Gini impurity. Blastocyst formation was observed in 41.04% (71/173) of the oocytes and was associated with a lower %HNA in the culture medium, a younger patient age, and the fertilization method (standard IVF or intracytoplasmic sperm injection). The RF model developed using these factors and 70% of the samples (training set, $n = 121$) was validated in the remaining testing set ($n = 52$) and produced an area under the curve of 0.761, where the %HNA in the culture medium was the most important variable for predicting blastocyst formation. In conclusion, lower levels of oxidative stress in embryo culture media were associated with the success of IVF treatment. The redox state of treatment solutions should be considered to support treatment success.

Key Words: human non-mercaptalbumin, assisted reproductive technology, blastocyst, random forest, machine learning

Oxidative stress is known to play important roles in the pathogenesis of various diseases, such as kidney diseases,[1,2] diabetes,[3] liver diseases,[4,5] and Parkinson disease.[6] Thus, evaluations of the redox state in patients with these diseases could be useful in clinical settings. Human non-mercaptalbumin (HNA), an oxidized form of albumin was originally measured using HPLC.[7] Recently, novel colorimetric method with bromocresol purple was also reported.[8] To this end, we recently reported that HNA is a useful marker for evaluating the redox state in humans,[9] and we established a reliable and less time-consuming method for measuring HNA.[10–12] At present, many studies on oxidative stress have sought its potential roles in the pathogenesis of various diseases. We speculated as to whether

the redox state of solutions used for the treatment of various diseases might be important for treatment success. Few reports have evaluated the redox state of treatment solutions from this viewpoint.

In the current study, we examined the human embryo culture medium that is used for *in vitro* fertilization (IVF) treatment for infertility. Recently, increasing numbers of patients with infertility have been treated using assisted reproductive technology (ART), where a culture medium is routinely used for embryo development. We aimed to examine the potential association between the redox state as assessed by measuring HNA in embryo culture media and successful human embryo development.

Materials and Methods

Subjects. We enrolled a total of 91 patients who received ART treatment at the Akihabara ART Clinic between February 2018 and July 2018. From these patients, we extracted and cultured a total of 173 oocytes for which the redox ratio of HNA to total albumin (%HNA) was measured in the culture medium. We analyzed the association between the HNA levels in the culture medium and blastocyst formation on day 5 or 6 after insemination. This research project was approved by the Ethics Committee of the University of Tokyo Hospital (approval number, 10964) and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained in the form of an opt-out on the institution's website. Patients who declined to participate in our study were excluded.

Sample collection. Culture dishes containing droplets of the culture medium covered with mineral oil (Reproline Medical GmbH, Rheinbach, German) were prepared on the day before oocyte retrieval; at this time, a sample of the G-TL medium (Vitrolife AB, Göteborg, Sweden) was collected and stored at −80°C until the HNA measurement. Because of ethical concerns, we adopted an observational study design. Consequently, the culture medium was sampled before the addition of the oocyte. Thus, the study's procedure had no chance of altering the viability of any of the oocytes.

G-TL is a ready-to-use commercial medium that is supple-

*To whom correspondence should be addressed.
E-mail: masayasato0407@gmail.com

doi: 10.3164/jcbn.21-114
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J. Clin. Biochem. Nutr. | July 2022 | vol. 71 | no. 1 | 69–72

mented with 5 mg/ml of human serum albumin. Upon arrival and after use, the bottles were immediately stored in a refrigerator at 4°C. As instructed by the manufacturer, the content of each culture bottle was used within a period of two weeks after the opening of the bottle, and the entire content of each bottle was used before the expiration date. The lot numbers of the G-TL media used in this study were recorded.

IVF procedure. A clomiphene citrate-based mild stimulation or a drug-free natural cycle IVF treatment was performed using previously described clinical and laboratory methods.[13–15] After oocyte retrieval, oocyte maturity was immediately checked. Immature (metaphase I or germinal vesicle) oocytes were observed for up to a maximum of 12 h, at which time most of them had spontaneously matured and were suitable for intra-cytoplasmic sperm injection (ICSI). Mature (metaphase II) oocytes were inseminated using a standard IVF or ICSI proce-dure and were then cultured in G-TL medium. Fertilization was assessed 16–20 h after insemination. Normally, fertilized zygotes containing two pronuclei were continuously cultured until the blastocyst stage without changing the culture medium. Embryonic development was recorded every day. Blastocyst development was assessed by blastocyst formation on day 5 or 6 after insemination.

Measurement of HNA and human mercaptalbumin (HMA). %HNA measurements were performed according to a previously described procedure.[10] Briefly, after sample collection, the sample tubes were kept at −80°C until use in the assay. After defrosting, oxidative albumin was measured using a basic HPLC system (LabSolutions System; Shimadzu Co. Ltd, Kyoto, Japan) with an anion-exchange column (50 × 7.6 mm I.D.). The HPLC conditions were as follows: eluent A consisted of a solution of 25 mM phosphoric acid buffer containing 60 mM sulfuric acid and sodium salt (pH 6.0), and eluent B consisted of a 250 mM magnesium chloride solution. The linear gradient time from eluent A (100%) to eluent B (100%) was programmed at 7.5 min. The total measurement time was 15 min per sample.[8,9]

Statistical analysis. Continuous variables were expressed as the mean and SD, while categorical variables were expressed as the percentage (%). Categorical data were analyzed using the chi-square test and the Fisher exact test, as appropriate. For continuous data, the univariate associations were evaluated using the Mann–Whitney U test. Stepwise logistic regression model analyses were used to calculate the adjusted odds ratios (OR) and the 95% confidence intervals of various factors. The correlations between the biomarkers and the clinical states were assessed using the Spearman rank correlation test. A random forest (RF) algorithm was applied to construct a model for predicting the successful formation of a blastocyst. RF algorithms are powerful machine learning tools that have been successfully used to analyze high-dimensional biomedical datasets.[16] A split-sample method was used for the development and evaluation of the predictive model. The samples were randomly assigned to either a training set (70%) or a testing set (30%). The clinical data, including the %HNA in the culture medium, were then assessed as predictors of blastocyst formation by applying the RF algo-rithm to the training set. The developed model was then validated in the testing set using a receiver operating characteristic (ROC) curve analysis. The area under the curve (AUC) was evaluated as the ability to predict the successful formation of a blastocyst. The variable importance for class discrimination in the predictive model was assessed using the mean decrease in the Gini impu-rity.[17] All the statistical analyses were two-sided, and a p value of less than 0.05 was considered to indicate statistical signifi-cance. All the statistical analyses were performed using R 3.6.3 software (http://www.r-project.org).

Table 1. Patient characteristics ($n = 91$)

Characteristics	Values
Maternal age (years)	37.46 ± 4.33
Gestation	0.57 ± 0.82
Parturition	0.11 ± 0.31
Body mass index	22.00 ± 3.61
Paternal age (years)	39.54 ± 5.93
Causes of infertility	
Tubal factors	7.69%
Male factors	15.38%
Endometriosis	3.30%
Combination	1.10%
Others	16.48%
Unexplained	56.04%

Continuous variables were represented as the mean ± SD and cate-gorical variables were represented as the number and percentage (%).

Results

Patient characteristics. The patient characteristics are shown in Table 1. The mean patient age at the time of oocyte retrieval was 37.46 years. The causes of infertility were tubal factors in 7.69%, male factors in 15.38%, endometriosis in 3.30%, a combination of factors in 1.10%, other causes in 16.48%, and unexplained in 56.04% of the patients.

HNA levels in the culture media. The median %HNA in the supplemented G-TL media from 4 lots was 89.36% (range, 80.12% to 94.84%; data not shown). The median %HNAs of each of the 4 lots of G-TL media were 84.51%, 89.28%, 89.39%, and 91.88%, respectively (data not shown).

Relationships between blastocyst development and %HNA in embryo culture media, patient age, insemination methods, and oocyte stage. Successful blastocyst develop-ment was observed in 41.04% (71/173) of the embryos. Table 2 shows the relationships between blastocyst development and clinical background factors. In univariate and multivariate analyses, successful blastocyst development was associated with a lower %HNA in the culture medium ($p = 0.001$), a younger patient age ($p<0.001$), and the use of standard IVF ($p = 0.007$).

RF model for the prediction of successful blastocyst formation. Finally, we developed a model for predicting successful blastocyst formation using an RF algorithm with four factors (%HNA, patient age, method of fertilization, and stage of oocyte maturation). To develop the RF model, we randomly divided the samples into two groups: a training set ($n = 121$) and a test set ($n = 52$). The ROC curve for the prediction of blastocyst formation was plotted for the RF model (Fig. 1). The AUC for the prediction of blastocyst formation was 0.761. We also investigated the variable importance of the developed RF model. Figure 2 shows the mean decrease in the Gini impurity of the current model. The %HNA in the culture medium was the most important variable for the prediction of successful blastocyst formation, followed by patient age.

Discussion

Oxidative stress is known to play a role in the pathogenesis of various diseases.[1–6] We speculated as to whether oxidative stress might play some role in the treatment of various other diseases. To examine this point, we thought that an investigation of treatment procedures, including cell culture systems, would Consequently, we focused on IVF treatment for infertility as an ART in which culture medium is used for embryo development,

Table 2. Associations between blastocyst formation and clinical findings (*n* = 173)

Variable	Mean/Proportion		*p* values	
	Success	Failure	Univariate	Multivariate
Mean age (years)	36.87 ± 3.93	38.77 ± 4.33	0.003†	<0.001* (OR 0.872)
%HNA	88.86 ± 3.15	90.11 ± 2.21	0.010†	0.001* (OR 0.791)
Method of fertilization			0.007†	
Standard IVF	49	48		—
ICSI	22	54		0.007* (OR 0.396)
Stage of oocyte maturation at time of oocyte retrieval			0.042†	
Metaphase II	70	91		—
Metaphase I	1	8		—
Germinal vesicle	0	3		—

†*p* value calculated using the Mann–Whitney *U* test, chi-square test or Fisher exact test.
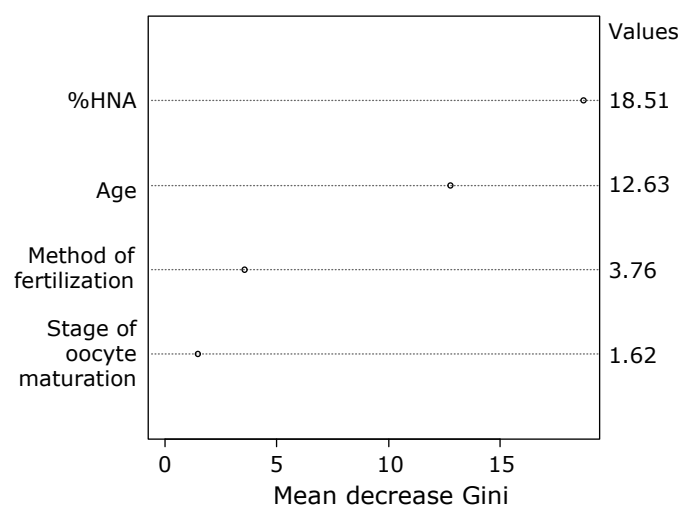*p value calculated using a stepwise logistic regression analysis.
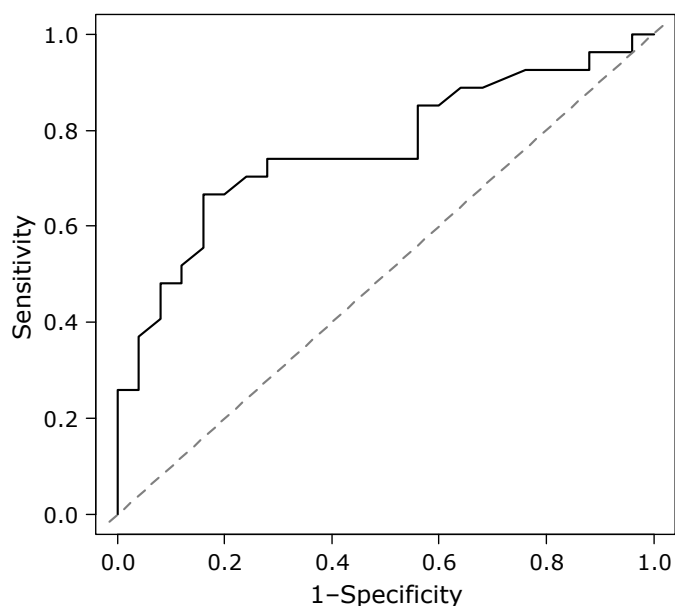
Fig. 1. Receiver operating characteristic (ROC) curve for predicting successful blastocyst formation based on a random forest model.

Fig. 2. Order of importance of variables for random forest classification as measured by the mean decrease in the Gini impurity index.

since an increasing number of patients with infertility are being treated.

As a result, an association between the %HNA in the embryo culture medium and successful blastocyst development, which is a necessary step in successful ART treatment, was observed. This finding suggests that the redox state in the treatment solutions used for the embryo culture medium may contribute to successful ART treatment. To the best of our knowledge, this is the first report to show that the redox state in treatment solutions evaluated using an oxidative stress marker, %HNA, may be associated with treatment success.

Patient age is well known to be an important clinical factor influencing blastocyst development.[18,19] Of note, an association between %HNA and blastocyst development in age-corrected results was observed in the current study. Consequently, the contribution of each factor to blastocyst development was further assessed by creating an RF algorithm to develop a machine learning model for predicting successful blastocyst formation.

According to this model, the %HNA in the culture medium was found to be the most important variable among patient age, HNA, insemination method, and the stage of oocyte maturation for predicting successful blastocyst formation. Machine learning models may provide a personalized assessment of the probability of a clinical event using patient-specific characteristics; they have been increasingly incorporated into medical practice in the field of cancer,[20,21] and they offer potential for the prediction of successful ART treatment.

In IVF treatment, blastocyst transfer is increasingly used to enhance implantation and clinical pregnancy rates. Therefore, we have adopted blastocyst cultures as the main treatment strategy for performing blastocyst transfer and for obtaining blastocyst development as a necessary step in achieving successful results.

To examine the potential influence of oxidative stress in embryo culture media on the results of ART treatment, the ingredients of the medium samples other than HNA and HMA should be the same. For this reason, only one kind of culture medium for embryos, G-TL medium, was used in the current study. Thus, further studies are needed to confirm the current results regarding oxidative stress in different culture media and its influence on treatment success.

The current evidence suggests that the measurement of HNA may enable culture media to be optimized, ensuring an oxidative environment that is suitable for human embryos. If the measurement of %HNA allows for the selection of an appropriate culture medium, this could lead to a higher efficiency of securing implantable embryos by increasing the incidence of blastocyst formation.

The %HNA in the G-TL medium was 89.36% (range, 80.12% to 94.84%; data not shown), which was much higher than the value for blood in healthy humans, (approximately 25%).[12] The higher levels of oxidative stress in embryo culture media suggest that little attention has been paid to the redox state of solutions used for infertility treatments.

In conclusion, lower levels of oxidative stress, as evaluated using %HNA, in embryo culture media, were associated with successful infertility treatment using ART. In general, the redox state of treatment solutions should be considered to support treatment success.

Author Contributions

The seven authors are justifiably credited with authorship, according to the authorship criteria.

In detail: YK–conception, design, analysis and interpretation of data, data collection, drafting of the manuscript, final approval given; MS–design, analysis and interpretation of data, drafting of the manuscript, final approval given; HI–supervision of research, critical revision of manuscript, final approval given; KY–HNA measurement, interpretation of data, critical revision of manuscript, final approval given; XT–data collection, critical revision of manuscript, final approval given; YY–supervision of research, critical revision of manuscript, final approval given.

Abbreviations

ART	assisted reproductive technology
AUC	area under the curve
HMA	human mercaptalbumin
HNA	human non merccaptalbumin
HPLC	liquid chromatography
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilization
OR	odds ratio
RF	random forest
ROC	receiver operating characteristics

Conflict of Interest

No potential conflicts of interest were disclosed.

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