RESEARCH REPORT

In-Vitro Indicators of Natural Resistance and Milk-Producing Ability in Dairy Buffaloes (*Bubalus bubalis*)

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The aim of this study was to explore the possibility of detecting novel phenotypes of natural resistance at the molecular level through the in-vitro stimulation of monocyte-derived macrophages (MDMs). This study was conducted with 16 healthy buffaloes who were reared for milk production and for whom data on milk-producing ability were available for several lactations. MDMs from circulating monocytes were activated with interferon-gamma and lipopolysaccharide. The response was evaluated using Western blotting to detect the presence of 2 types of proteins separated by electrophoresis: tyrosine-phosphorylated proteins, which are indicators of the dynamic control of biochemical pathways, and IkB-alpha (Kappa light polipeptide gene enhancer in B-cells Inhibitor, alpha) protein, which controls the activity of nuclear factor kappa-light-chain-enhancer of activated B cells—a transcription factor that is responsible for the expression of proinflammatory cytokines. The results showed that the buffaloes who were positive for IkB-alpha proteins had a significantly higher milk-producing ability than the buffaloes who did not express IkB-alpha. On the contrary, no significant difference was detected between the high and low milk-producing buffaloes with regard to the presence of tyrosine-phosphorylated proteins. This preliminary study indicated that it may be possible to identify the more disease-resistant nonhuman animals on a molecular level. The results, therefore, indicate that an intense selection toward the increase of milk yield could impair natural disease resistance in future dairy buffalo generations.

Keywords: buffalo, welfare, natural resistance, milk

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The water buffalo (*Bubalus bubalis*) is economically important in the livestock industry in many parts of the world, particularly in Southeast Asia, the Middle East, and Italy. The unique properties of the milk produced by buffaloes make it well suited for the production of dairy products and have stimulated the market demand in Western countries, resulting in the introduction of buffalo herds in place of dairy cow herds. Nonhuman animal health and welfare traits as well as functional traits have become increasingly important relative to the traits that have traditionally been the targets of animal-breeding practices, such as milk yield and quality. Functional traits are even more important in buffalo, as this species of livestock is susceptible to endemic diseases in many parts of the world, leading to important economic losses. Because the most dangerous zoonosis is brucellosis, and because infected animals must be killed according to the Italian law (Ministerial Decree N. 84/1991), attempts have been made to detect potentially disease-resistant animals (Capparelli et al., 2007) without infecting them (Taraktsoglou et al., 2011).

The innate immune system provides an immediate and nonspecific defense against infections. Individuals, however, vary in their natural resistance to infectious diseases because immune system activity is determined by genetic background (Turner, Begon, Jackson, Bradley, & Paterson, 2011). Macrophages are key cells in innate immune response. Bacterial components or specific cytokines, recognized by macrophages, trigger the immune cellular activation, which consists of a complex system of intracytoplasmatic protein modifications, ultimately regulating gene transcription for either microbe elimination or the progression to a disease state (Mohamed, 1983). The tyrosine phosphorylation of macrophage proteins is a pivotal mechanism involved in cellular activation. The presence of the tyrosine phosphorylated residues or of the specific transcription factors indicates a highly dynamic cellular state that controls the natural immune response (Kovářová, Hajduch, Livingstone, Dzubak, & Lefkovits, 2003).

Several authors have suggested using in-vitro systems to reproduce many of the intracellular events that occur during in-vivo bacterial exposure (Kovářová et al., 2003; Taraktsoglou et al., 2011).

Farrar and Schreibe (1993) described a trial in which macrophage activation was performed with interferon-gamma (IFN-γ), a key cytokine secreted by T lymphocytes that plays a pivotal role in the modulation of the immune response. Mosser (2003) reported that lipopolysaccharide (LPS), the most important constituent of the bacterial cell wall in gram-negative bacteria, was able to trigger the immune response by binding to specific receptors on monocyte-derived macrophages (MDMs). Tyrosine phosphorylation of macrophage proteins following LPS stimulation was positively correlated with disease resistance, while the presence of the IkB-alpha (Kappa light polipeptide gene enhancer in B-cells Inhibitor, alpha) protein, following IFN-γ stimulation, was correlated with disease susceptibility (Kovářová et al., 2003). Therefore, IkB-alpha traps the nuclear factor kappa-light-chain-enhancer of activated B cells protein in the cytoplasm and prevents its activity as a transcription factor for cytokines and chemokine genes, which are important for natural resistance.

Based on the hypothesis that productive traits, disease resistance, and animal welfare are correlated, we investigated the effect of the presence of key proteins involved in innate immunity on the milk-producing ability of healthy dairy buffaloes. In particular, we reproduced macrophage activation in vitro using cells from healthy buffaloes and assessed the presence of tyrosine-phosphorylated proteins and IkB-alpha.

MATERIALS AND METHODS

Animals

Sixteen healthy buffaloes who were registered in the Italian National Herdbook and whose milk production had been regularly recorded according to the regulations of the International Committee for Animal Recording were selected for this study. The buffaloes had undergone at least three lactations to ensure the reliability of the data regarding their milk-producing ability. The buffaloes were reared on the same experimental farm, and they were maintained under uniform feeding and housing conditions.

The animals were negative for the following infections: brucellosis (*Brucella abortus*), Johne's disease (*Mycobacterium avium* subspecies *paratubercolosis*), leukosis (*Bovine leukemia virus*), and tuberculosis (*Mycobacterium bovis*). The farm on which the buffaloes were raised had adopted a seasonal calving practice with most calvings occurring in the spring so that the highest milk yield was obtained at the end of the spring and during the summer, which corresponded to the months in which the market demand for buffalo milk was highest. All of the in-vitro analyses were performed in May or June to avoid variability due to either the days of lactation or the season.

Monocyte Extraction, MDM Culture, and MDM Activation

Peripheral blood mononuclear cells from each animal were isolated by density gradient, distributed at a density of 1×10^7 cells/well in six-well tissue culture plates with 4 mL of RPMI medium containing 2 mM streptomycin, 2 mM glutamine, 0.05 mg/mL gentamicin, 1% fungizone, 0.05 mM mercaptoethanol, and 10% fetal bovine serum maintained at 37°C in a humidified atmosphere under 5% carbon dioxide. The monocytes were obtained by adherence and were left to differentiate into macrophages for 7 days. Macrophage activation was performed by treatment with IFN-γ (250 ng/mL) and by LPS (100 ng/mL).

Protein Separation

The cell pellets were lysed with NP-40 buffer (1% NP-40, 0.5% sodium deoxycholate, 2 mM EDTA, 150 mM NaCl, 50 mM Tris pH 7.5, 50 mM NaF, 0.1 mM sodium orthovanadate, 1 μg/mL leupeptin and aprotinin, 1 mM PMSF). The total protein concentration in the samples was quantified using the 2-D Quant Kit (GE Healthcare, Niskayuna, NY). Equivalent amounts (50 μg) of proteins were separated by 1D SDS-PAGE on 10% acrylamide minigels at 200 V for 50 min.

Western Blotting

The separated proteins were transferred onto polyvinylidene difluoride membranes at 15 V for 50 min. The membranes were blocked overnight at 4°C in TTBS with 3% BSA. The primary antibodies were mouse monoclonal antibodies against phospho-tyrosine and IkB-alpha (diluted 1:1000, Cell Signaling Technologies). The proteins were detected with a stabilized

goat antimouse horseradish peroxide conjugated antibody (diluted 1:1500, Thermo scientific) and an ECL reagent (Bio-Rad).

Statistical Analysis

The traits selected for comparison among the buffaloes were the milk-, fat-, and protein-producing abilities. These parameters were officially calculated by the Herdbook Society using an algorithm that corrected for the lactation number, the age of the buffaloes, and the season of calving for all milk yields obtained from each buffalo. Milk-producing ability was then expressed as a difference in kilograms of milk yield of the mature-equivalent lactation compared to the recorded buffalo population. Similarly, fat- and protein-producing abilities were expressed for each buffalo as percentages compared to the recorded buffalo population. The cows were divided into two groups on the basis of the evidence of macrophage activation. Due to the low number of animals, the differences between the positive and negative samples were calculated separately for the two indicators (tyrosine phosphorylation and IkB-alpha) using a nonparametric statistic test: the PROC NPAR1WAY in SAS (2007). Within this procedure, the "Wilcoxon" option was applied, allowing for the calculation of the means of the quantitative traits under analysis as well as the probabilities of the differences between the groups. Moreover, with this option, the Wilcoxon rank-sum test allowed for the evaluation of the probability of the differences between the groups also through the Kruskal-Wallis test.

RESULTS

The phospho-tyrosine antibody revealed a 60-kDa protein band in eight buffaloes, and the IkB-alpha antibody revealed a 38-kDa band in seven buffaloes (Table 1).

Figures 1 and 2 show the Western blot for tyrosine-phosphorylated proteins and IkB-alpha of 16 animals.

The buffaloes for whom Western blot revealed the presence of IkB-alpha in MDMs following stimulation with LPS were the most productive with regard to milk yield as well as fat and protein percentage. In this group, milk yield (kg) and fat percentage were significantly different

TABLE 1
Milk Production and Characteristics (Mean ± SE) as Affected by Innate Immune Indicators

Trait/Group	Tyrosine Phosphorylation Positive		Tyrosine Phosphorylation Negative		IkB-α Positive		IkB-α Negative	
n	8		8		7		9	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Milk (kg)	17.1	5.8	2.6	6.3	19.9	5.9	2.0	5.5
Fat (%)	9.1	4.9	6.6	5.3	16.1	4.3	0.8	4.0
Protein (%)	12.4	5.4	5.5	5.8	16.5	5.2	2.7	4.9

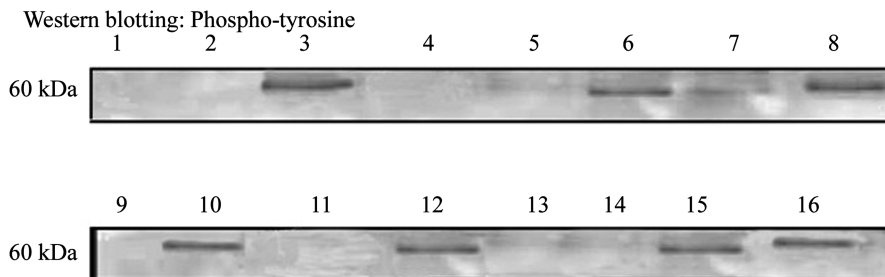Western blotting: Phospho-tyrosine

FIGURE 1   Tyrosine phosphorylation was evaluated by Western blot of total monocyte-derived macrophage proteins following stimulation with interferon-gamma. Animals 3, 6, 7, 8, 10, 12, 15, and 16 were positive and showed a 60-kDa band.

from the IkB-alpha negative group (pF = .04 and .02, respectively). When the two groups were compared using the nonparametric Kruskal-Wallis test, the differences between the groups were only slightly reduced ($p\chi^2$ = .06 and .03, respectively). Protein-producing ability tended toward significance. Additionally, the buffaloes who showed tyrosine-phosphorylated proteins after IFN-γ stimulation had higher milk-, fat-, and protein-producing abilities, although the difference between the two groups was not statistically significant.

DISCUSSION

Natural resistance plays a central role in early disease defense, and it is directly related to animal welfare because good health is essential for welfare. In animal breeding, objectives related to health, functional traits, and welfare have become increasingly important to society and consumers.

In this work, we showed that the macrophages of the low milk-yielding buffaloes, under conditions of simulated infection, demonstrated a better response than the macrophages of the high milk-yielding buffaloes. Based on these results, we can infer that an intense selection
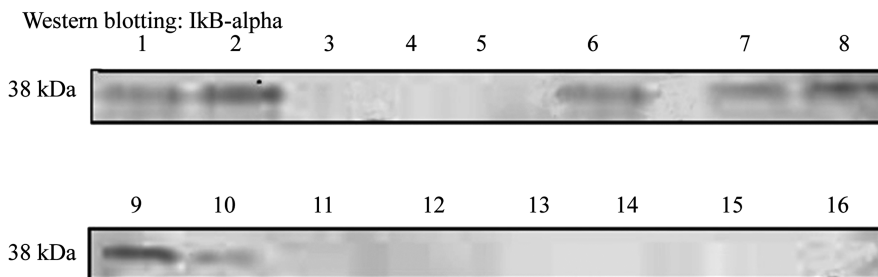
Western blotting: IkB-alpha

FIGURE 2   IkB-alpha presence was evaluated by Western blot of total monocyte-derived macrophage proteins following stimulation with lipopolysaccharide. Animals 1, 2, 6, 7, 8, 9, and 10 were positive and showed a 38-kDa band.

for the increase of milk yield could affect natural disease resistance in future dairy buffalo generations, as we found that buffaloes who produce less milk exhibit the characteristics of higher natural disease resistance. On the other hand, the results of this study indicated that in-vitro stimulation with LPS may be a useful technique for identifying animals who may be more resistant to bacterial diseases. These animals will then be more appropriate for sustainable production systems in which the use of chemicals is forbidden.

The response to the in-vitro stimulation with LPS, assessed based on the presence of IkB-alpha, may be a marker of better individual adaptation, indicating more efficient use of the behavioral and physiological components of the regulatory systems and allowing individual animals to cope with their environmental conditions (Broom, 2006).

The welfare of animals on the farm has become increasingly important and relevant from the societal point of view, and its importance is now recognized by all stakeholders in the farm animal production chain. Selective breeding for productive traits could be successful in combination with selection for better animal welfare. Many breeding companies are working toward developing more balanced breeding goals by incorporating functional traits. In this study, we hypothesized that the in-vitro simulation of an infection and the assessment of the response in healthy buffaloes might provide markers of adaptation to the environment in farm animals under selection. We found that the presence of IkB-alpha following LPS stimulation may be one such marker; however, because the analysis included only 16 buffaloes, our findings can only be regarded as preliminary, and they need to be further confirmed in independent dairy populations. When they are confirmed, this marker may be included in the selection programs for both production and better animal welfare.

CONCLUSION

The present preliminary work explores the possibility of approaching the welfare of buffaloes at a biomolecular level by correlating productive traits that are directly connected to protein phenotypes associated with natural resistance. Further experiments with a higher number of buffaloes may provide a useful and complete experimental model for the analysis of specific welfare markers.

FUNDING

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